

FY2006 Final Report

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

SERDP Project ER-1235

August 2008

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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:

Todd A. Anderson
Co-Principal Investigator

Date

- 1.0 DESCRIPTIVE STUDY TITLE:**
Exposure to RDX: Plant Uptake and micro-RNAs Biomarkers
- 2.0 STUDY NUMBER:**
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Termination: 08/2008
- 6.0 KEY PERSONNEL:**
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Dr. Andrew Jackson, Co-Investigator
Mr. Baohong Zhang, Co-Investigator
Ms. Sameera Sanka, Co-Investigator
Mr. Brian Birdwell, Quality Assurance Manager
Dr. Ron Kendall, Principal Investigator
- 7.0 STUDY OBJECTIVES / PURPOSE:**
Little information is available regarding the uptake kinetics of RDX or HMX by typical wetland (capable of root penetration into anaerobic zones) plants in constructed or real wetland systems. RDX uptake in aquatic and wetland plants has been studied previously (e.g. Best et al., 1997) but has not been rigorously explored. Wetlands are a key interface between non-point source runoff (e.g. firing ranges) and surface water or groundwater. Uptake kinetics are critical for an overall understanding of exposure as real systems are typically transiently loaded and concentration profiles are variable with depth due to microbial degradation. Leaching of RDX from simulated rain events and from simulated seasonal or event flooding will also play an important role in overall fate and exposure.

In addition, we proposed to evaluate the potential for microRNAs (miRNAs) to serve as biomarkers of contaminant exposure in earthworms and plants. miRNAs are an abundant new class of non-coding endogenous small RNAs (~20-24 nucleotides) that regulate gene expression in plants and animals (Lim et al., 2005), controlling multiple biological processes from carcinogenesis to development. We hypothesized that miRNAs also play important roles in responses of organisms to toxicant stress. To address this, we identified miRNAs involved in earthworm stress response to energetic materials using both computational and genetic screening approaches.

8.0 STUDY SUMMARY:

Uptake kinetics of RDX were determined using existing continuous flow mesocosms and smaller individual microcosms containing typical wetland media. Emphasis was placed on determining the uptake kinetics as a function of evapotranspiration, and specific depth of RDX exposure. Significant variables addressed included steady state versus unsteady state exposure and plant transformation rate. Leaching of live tissue to mimic rain events and seasonal or transient flooding were examined in separate systems.

We initially identified miRNAs in earthworms based on the currently available sequence data using a computational approach (Zhang et al., 2005). Available earthworm sequences include ca. 4,000 sequences for *E. fetida*, ca. 17,000 sequences for *Lumbricus rubellus* and ca. 1,000 sequences for *Eisenia andrei* (www.earthworms.org for Lumbribase and Eandreibase databases). Using quantitative PCR, we were able to experimentally verify several candidate miRNAs initially identified computationally. Conservatively, we identified 5 miRNAs from the 24 candidates. This study represents the first time that miRNAs have been determined in earthworms.

9.0 TEST MATERIALS:

Test Chemical: RDX (1,3,5-trinitroperhydro-1,3,5-triazine)
CAS Number: 121-82-4
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 (18M Ω)
CAS Number: NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

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/surface water and the rate of plant uptake in relation to bioavailability is required. Ongoing work indicates that in saturated sediments characteristic of areas which receive periodic run-off and or groundwater discharge, RDX is rapidly transformed in anaerobic wetland media regardless of electron acceptor with substantial plant uptake (Jackson et al., 2005). Continuing work investigated the RDX profile at cm resolution in order to understand the interactions of plant, microbes, and sorption on the fate and persistence of RDX in these systems including major breakdown products (Jackson et al., 2005).

Ecotoxicogenomics is a relatively new discipline that has grown rapidly in the past few years thanks to the explosive development of genomic technologies. Recent studies in human toxicogenomics indicate that it is no longer sufficient to focus on the 25,000 or so protein-coding genes that make up roughly 2% of the human genome because new insights will not be gained simply by acquiring more and more gene expression data (Gershon, 2005). According to a computational analysis, 30% of human genes may be regulated by microRNAs (Lewis et al., 2005). Because energetics contamination in soil is principally a problem of the Army and some civilian activities, there is little incentive for research organizations outside of the Army to pursue a fundamental understanding of how energetics move in the environment and potentially affect ecological receptors like earthworms. However, we believe that the concept of miRNA biomarkers is one that is applicable to multiple contaminants. Genetic tests, methodologies, and information developed here could directly support and permit interpretation of ongoing toxicity benchmarking studies for a variety of chemical contaminants. miRNAs are currently the focus of much research as indicated by the flood of papers appearing in print. New information on miRNAs is appearing on a weekly (if not daily) basis. In addition, the prediction of risks of harmful effects due to contaminant exposure, and the development of biomarkers of contaminant exposure are also areas of high research interest.

11.0 TEST ANIMALS:

Species: *Lumbricus rubellus* (Earthworm)

Strain: N/A

Age: adult

Number: 12

Source: Advanced Biotechnology, Inc. (Elliott, IL)

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All test systems (earthworms) were placed in glass 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:

Wetlands are a key interface between non-point source runoff (e.g. firing ranges) and surface water or groundwater. Uptake kinetics are critical for an overall understanding of exposure as real systems are typically transiently loaded and concentration profiles are variable with depth due to microbial degradation. Leaching of RDX from simulated rain events and from simulated seasonal or event flooding will also play an important role in overall fate and exposure. Uptake kinetics were determined using existing continuous flow mesocosms and smaller individual microcosms containing typical wetland media. Emphasis was placed on determining the uptake kinetics as a function of evapotranspiration, and specific depth of RDX exposure. Significant variables addressed included steady state versus unsteady state exposure and plant transformation rate. Leaching of live tissue to mimic rain events and seasonal or transient flooding were examined in separate systems.

In addition, we evaluated the potential for microRNAs (miRNAs) to serve as biomarkers of contaminant exposure in earthworms by first identifying computationally the presence of miRNAs from a database of earthworm genetic sequences. Next, we verified the miRNAs experimentally using quantitative PCR.

14.0 METHODS:

14.1 Plant Uptake Experiments

14.1.1 RDX Uptake in Actively Growing Bull Rush

RDX uptake was evaluated by exposing juvenile bull rush plants to varying concentrations (0.5, 1, 3, mg/l) of RDX over a 16 week period. Dark green bulrush plants (*Scirpus atrovirens*), 4 to 6 inches in height, were procured from Environmental Concern, a wetland nursery. Tree pots with slotted bottom drainage, were procured from Hummert International. The pots were filled with gravel at the bottom, coarse sand in the middle and fine sand at the top at a ratio (by weight) of 2:2:1 respectively. As the pots were slotted at the bottom, a fine filter fabric was placed at the bottom of each pot before filling with gravel in order to prevent the sand from seeping through the slotted bottom. The bulrush plants were planted with a density of one plant per pot. As the plants were analyzed in triplicate, a set of three pots were placed in individual plastic saucers. All plants were grown in a green house at the horticulture department of Texas Tech University under sunny conditions. Initially the plants were watered with tap water, in

order to allow them to acclimate to the local conditions. The plants were divided into three groups with 9 sets of triplicate plants in each group. After 10 days of acclimatization each group of plants were watered with one of 3 different concentrations of RDX solution (0.5, 1, 3 mg/l). Simultaneously, 5 sets of control plants (15 total) were watered with plain tap water. RDX stock solution (10 mg/l) was made weekly from powdered RDX using DDI water. Watering solutions were made daily from the stock solution. All plants were watered daily with the same total volume irrespective of RDX concentration. Each day the effluent water was collected from each plastic saucer and weighed. Sub-samples of influent water and effluent water were collected and stored in a refrigerator and analyzed weekly.

One set of plants from each group were sacrificed at 9 different time points and were analyzed for RDX, MNX, DNX, and TNX. The control plants were sacrificed in 1st, 6th and 12th week. These plant samples and corresponding soil samples from respective containers were also analyzed for RDX and RDX metabolite concentrations. Sacrificed containers were inverted over a container placed on the floor. The plant along with the root was carefully removed from the soil, cleaned and weighed. Subsequently the plant is stored and sealed in a zip lock cover and placed in a refrigerator until the time of extraction. Pebble rocks and gravel are removed from the soil and subsequently the soil is mixed homogeneously and weighed. Later a sub sample of soil is taken from this homogeneous mixture and is stored and sealed in a zip lock cover and placed in a refrigerator until the time of extraction.

14.1.2 RDX loss from Exposed Plants

After 16 weeks of exposure and sacrificing 7 of the 9 sets of plants, exposure to RDX was discontinued and the remaining plants were watered with tap water. The remaining two sets of plants were sacrificed at weeks 19 and 22 weeks. The control was sacrificed at the end of the 22nd week. The same experimental and analytical procedures as in the first experiment were carried out in this experiment as well.

14.1.3 Uptake of RDX by Mature Bull Rush

For this study, another lot of mature Dark Green Bulrush plants 15 to 18 inches height were procured from the same vendor cited in the first experiment. The plants were acclimatized for the local conditions for two weeks. No plant growth was observed during this period. These plants were arranged in six sets and two control sets in the green house in a similar fashion detailed in the earlier experiment. The plants were watered with 1 mg/l RDX contaminated water. At each time point a set of pots were sacrificed. The controls were sacrificed at the 3rd and 6th weeks. The same experimental and analytical procedures as in the first experiment were carried out in this experiment as well.

14.2 Earthworm micro-RNA Biomarkers

14.2.1 Earthworm Genetic Sequences

We obtained genetic sequences from earthworms.org (<http://earthworms.org>) for *Lumbricus rubellus* and *Eisenia andrei*. BLAST searches of those sequences were

performed using known microRNAs from the nematode, *Caenorhabditis elegans* obtained from a miRNA database {The miRNA Registry (<http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml>)}. The BLAST software used was modified slightly through a collaboration with the bioinformatics research group at Miami University.

The secondary structure of genomic sequences, with no more than four mismatches with previously known *C. elegans* mature miRNAs, were predicted using the web-based computational software MFOLD. In previous studies, we found that miRNA precursor sequences have significantly higher negative minimal folding free energies (MFEs) and minimal folding free energy indexes (MFEIs) than other non-coding RNAs or mRNAs (Zhang et al., 2006). To avoid designating other RNAs as miRNA candidates, these two characteristics and GC content were considered when predicting secondary structures. RNA sequences were considered miRNA candidates only if they fit the following criteria: (1) a RNA sequence can fold into an appropriate stem-loop hairpin secondary structure; (2) a mature miRNA sequence site in one arm of the hairpin structure; (3) miRNAs had less than six mismatches with the opposite miRNA sequence in the other arm; (4) no loop or break in the opposite miRNA sequences; (5) predicted secondary structures had higher MFEIs, negative MFEs, and 30–70% A + U contents; (6) predicted mature miRNAs had no more than four nucleotide substitutions compared with *C. elegans* mature miRNAs. These criteria significantly reduced false positives and required that the predicted miRNAs fit the criteria proposed by Ambros and co-workers (2003).

14.2.2 Experimental Verification of Earthworm micro-RNA

Following computational identification of possible miRNAs in earthworms, we attempted to verify those miRNAs using quantitative PCR. First, we designed and synthesized one forward primer for each of the potential miRNA sequences. Next, we isolated total RNA from *L. rubellus* and then performed a polyadenylation reaction and first strand cDNA synthesis on the total RNA using the NCode™ miRNA First-Strand cDNA Synthesis Kit (MIRC-10) from Invitrogen (Carlsbad, CA).

We performed six identical qPCR reactions with the miRNA primer sets and primer sets to the housekeeping genes (18S and actin) using the NCode™ SYBR® GreenER™ miRNA qRT-PCR Kit (MIRQER-100) from Invitrogen (Carlsbad, CA) and an ABI 7900HT qPCR machine. We calculated the average threshold cycle values for all sample sets and determined the standard deviation. Finally, we compared the most abundant miRNA to a housekeeping gene and calculated the relative expression of the other miRNAs in reference to the first.

15.0 RESULTS:

15.1 Plant Uptake Experiments

15.1.1 RDX Uptake in Actively Growing Bulrush

RDX uptake in actively growing bulrush was evaluated over a sixteen week period at different RDX exposure levels (0.5, 1, and 3 mg/l). At each time point, a set of three

plants was sacrificed. The plant samples were divided into leaf and root, with leaf samples further subdivided into top, middle and bottom thirds. These samples along with influent water, effluent water and final soil samples were analyzed for RDX, MNX, TNX and DNX.

15.1.2 Plant Growth

Leaf length and weight progressively increased over the first 10-12 weeks for all treatments, but then declined over the final 4 weeks of the study (**Figure 15.1** and **15.2**). The decrease in the last weeks of the study appeared to be caused by a pathogen. For this reason, the discussion is largely focused on the initial 12 week period even though the results were available for sixteen weeks. Over the course of the experiment, plants grew from a mean length of 12 cm to a mean length of 40 cm and weight increased from a mean of 2 g to 50 g (wet weight). In plants exposed to RDX mean leaf length reached 44 ± 5.8 , 40 ± 4.5 and 36.8 ± 4.5 cm and leaf weight 41 ± 11 , 66 ± 30 , 40 ± 8 g in the 12th week of exposure to RDX concentrations of 0.5, 1, and 3 mg/l, respectively (**Figure 15.3** and **15.4**). Length and weight of sacrificed leaf samples exposed to the highest RDX concentration (3 mg/l) were consistently lower than plants exposed to lower RDX concentrations (0.5 and 1 mg/l) at all time points, except for 1st and 3rd time points. However, plants with no exposure to RDX generally grew at comparable levels to exposed plants for the first 6 weeks but by the 12th week were lower in mass and length than all RDX exposed treatments.

Root weight progressively increased over the first fourteen weeks with only a small decline in the last two weeks for all treatments. The lower root loss may be related to the type of pathogen that apparently impacted above ground tissue. No pattern was observed in the root length, with reference to time or level of RDX exposure (**Figure 15.5** and **15.6**).

15.1.3 Plant Uptake of RDX

Mass of RDX in plants, at a given time point and for a given level of RDX exposure increased with plant weight. Plant weight as well as RDX accumulation peaked in the 12th week. At this time point mass of RDX in the leaf tissues of plants exposed to 3, 1 and 0.5 mg/l RDX was 2.21 ± 1 , 1.55 ± 0.8 , 0.8 ± 0.27 mg, respectively at a concentration of 53 ± 16.7 , 23 ± 1.7 , and 20 ± 8.5 mg/kg. Amount of RDX in the leaf tissues increased with an increase in the influent RDX concentrations and consistently increased with time (**Figure 15.7**). RDX concentrations in leaf material progressively increased from 1st week to the 16th week and these concentrations increased with increase of influent RDX concentration, even though the mass of RDX accumulated peaked in the 12th week, along with the weight of the plant (**Figure 15.8**). RDX concentrations peaked in the 16th week at 60, 44 and 28 mg/kg respectively for 3, 1 and 0.5 mg/l exposure levels. RDX concentrations in leaf material of plants exposed to 3 mg/l almost stabilized from the 9th week onwards at around 55 mg/kg. RDX concentrations in leaf material of plants exposed 0.5 and 1 mg/l did not stabilize in the experimental period. RDX concentrations in leaf tissue of plants exposed to 3 mg/l were around 3.2 times of those exposed to 0.5 mg/l and 1.7 times of those exposed to 1 mg/l water concentrations.

Mass of RDX and RDX concentrations in root samples of plants increased with increase in influent RDX concentration and consistently increased with time until the 9th week. From the 9th week, RDX concentration and mass of RDX in root samples generally decreased until the 16th week (**Figure 15.9** and **15.10**).

15.1.4 Distribution of RDX in Plant

RDX was detected at higher concentrations in the top portion of the leaf when compared to middle and bottom portions. Previous studies have also shown that the top portions of the leaf accumulate RDX to a greater extent (Vila et al., 2007a; 2007b; Price et al., 2002). The RDX concentration in the top portion of the leaf was 2 to 6 times greater than the concentrations detected in middle and bottom portions of the leaf. RDX concentration in the top portion of the leaf gradually increased over the 16 week period for all exposure levels. RDX concentrations gradually increased in the middle section of plants exposed to 0.5 mg/l loading rate but did not follow any pattern for the 1 and 3 mg/l loading rates. RDX concentrations in the bottom portions of the leaf for 0.5 and 1 mg/l exposure levels consistently increased with time and peaked in the 9th week; a similar peak occurred in the 12th week for plants exposed to 3 mg/l concentration (**Figure 15.11, 15.12, 15.13**). For exposure levels of 3 mg/l, 1 mg/l, and 0.5 mg/l, respectively, the highest RDX concentrations (350 mg/kg, 216 mg/kg, and 201 mg/kg) occurred during the 16th week in the top portion of the leaf. RDX concentrations in root samples of plants increased with an increase in influent RDX concentration and consistently increased with time until the 9th week. From the 9th week, RDX concentration in root samples consistently decreased.

15.1.5 RDX Metabolites

MNX was detected in almost 99% of the plant samples regardless of exposure concentration but none was detected in the no exposure control treatment. MNX concentrations in plant samples accounted for up to 2 to 3 % of the RDX concentrations in the plant samples for all treatments (**Figure 15.14, 15.15, 15.16**). MNX concentration in leaf material of the plant samples ranged from 0.12 mg/kg to 2.6 mg/kg. MNX concentrations in leaf followed the same pattern as RDX concentrations in leaf material of plants for all treatments (**Figure 15.17**). A correlation can be observed between RDX and MNX concentrations from (**Figure 15.18**) where MNX concentrations corresponding to RDX concentrations in leaf tissues of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at a given time point were presented. MNX concentrations were highest in top portion of the plant where RDX concentrations were also detected to be highest. MNX concentrations in the top portion of the leaf ranged from 0.22 mg/kg in the first week in the 0.5 mg/l exposure treatment to 13.77 mg/kg in 16th week for the 3 mg/l exposure treatments. MNX concentration in root samples ranged from 0.01 to 0.1 mg/kg. MNX was found in soil samples at very low concentrations, 0.001, 0.0027, and 0.004 mg/kg for 0.5, 1, and 3 mg/l exposure treatments, respectively in soil samples when compared to RDX concentrations of 0.13, 0.2, and 0.4 mg/kg.

DNX and TNX were detected only in plant samples and were not detected in soil samples. They were detected only from 12th week onwards, in 13 samples and 8 samples respectively out of a total corresponding 108 samples of root, top middle and bottom portions of the leaf. DNX was randomly distributed in top, middle, bottom and root

portions of the plant in a concentration range of 0.01 to 0.02 mg/kg. DNX concentrations in plant samples showed no relationship with RDX concentration or time. TNX was mostly observed in root samples of plants exposed to 0.5 mg/l concentration, in a concentration range of 0.01 to 0.1 mg/kg.

15.1.6 Mass Balance of RDX

A mass balance was conducted by calculating the total RDX introduced into the system (influent water), compared to the RDX in the effluent water, the plants, and the soil at each time point. The majority of the RDX introduced into the system was recovered in the effluent water. For plants exposed to 0.5 and 1 mg/l concentrations, on average about 69 % of the RDX introduced was found in the effluent water, and for the 3ppm exposure treatment ~56 % of the RDX introduced was observed in the effluent water. Approximately 2.5, 2, and 1% of the total RDX introduced was found in plant samples and approximately 11, 8, and 6% was found in soil samples for the 0.5, 1, and 3 mg/l RDX exposure treatments, respectively. RDX accumulation in soil appeared to be higher in the initial 6 weeks when compared to the subsequent weeks. This can be attributed to an increase in the plant growth and uptake with time.

Approximately 19, 23 and 38 % of the total RDX added to 0.5, 1, 3 mg/l RDX exposure treatment systems was not recovered. No pattern was observed for 0.5 and 1 mg/l loading rates in the variation of unaccounted RDX with respect to time. However for the 3 mg/l loading, unaccounted RDX stabilized at around 39% from the 6th week onwards. As discussed earlier RDX concentration in plants exposed 3 mg/l loading stabilized around the 9th week. This phenomenon was not observed in other exposure levels. The unaccounted RDX mass as a percentage of introduced RDX increased with the exposure concentration. Earlier studies have indicated phytodegradation and direct photolysis as a feasible fate of RDX in plants (Yoon et al., 2005). The results of this experiment also suggest that phytodegradation and direct photolysis of RDX could be taking place in bulrush plants and these phenomenon could be responsible for the unaccounted RDX. Direct photolysis of the RDX from the open ended saucer in which effluent RDX water is collected might have reduced the RDX concentration in effluent water and could be responsible for some of the unaccounted RDX.

Throughout the experiment we have been comparing plant growth, plant uptake and distribution of RDX of different sets of plants exposed to different RDX concentrations and sacrificed at different time points. RDX in effluent water is the only reading which was possible for us to measure for all time points until the plant was sacrificed, as effluent water samples were collected daily and analyzed. When the mass of RDX in effluent water was measured for different sets (at a given time point and for a given treatment concentration) the amount of RDX followed a similar pattern with very little deviation. This observation supports the assumptions that uptake, distribution, and weight followed a similar pattern in all sets of plants for a given concentration.

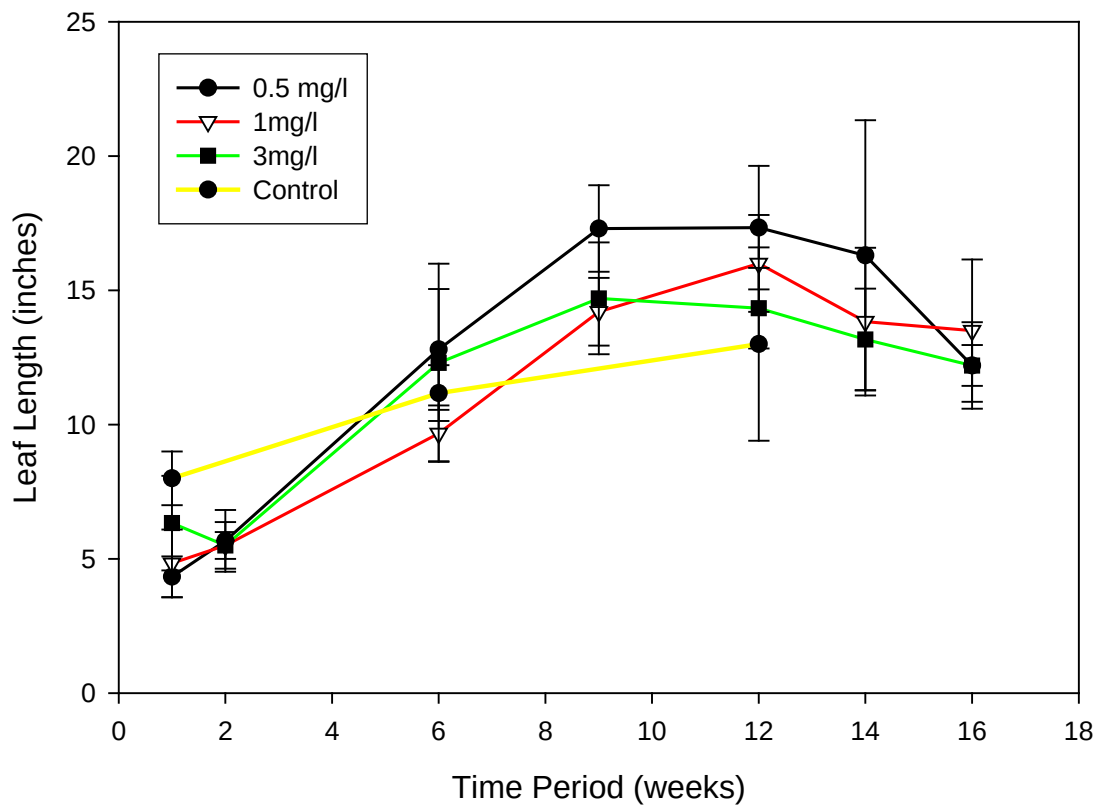


Figure 15.1 Leaf length of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water, at time point where they were sacrificed. Also included is the mean leaf length of bulrush plants with no RDX exposure. Error bars represent sample standard deviations between triplicate plant samples.

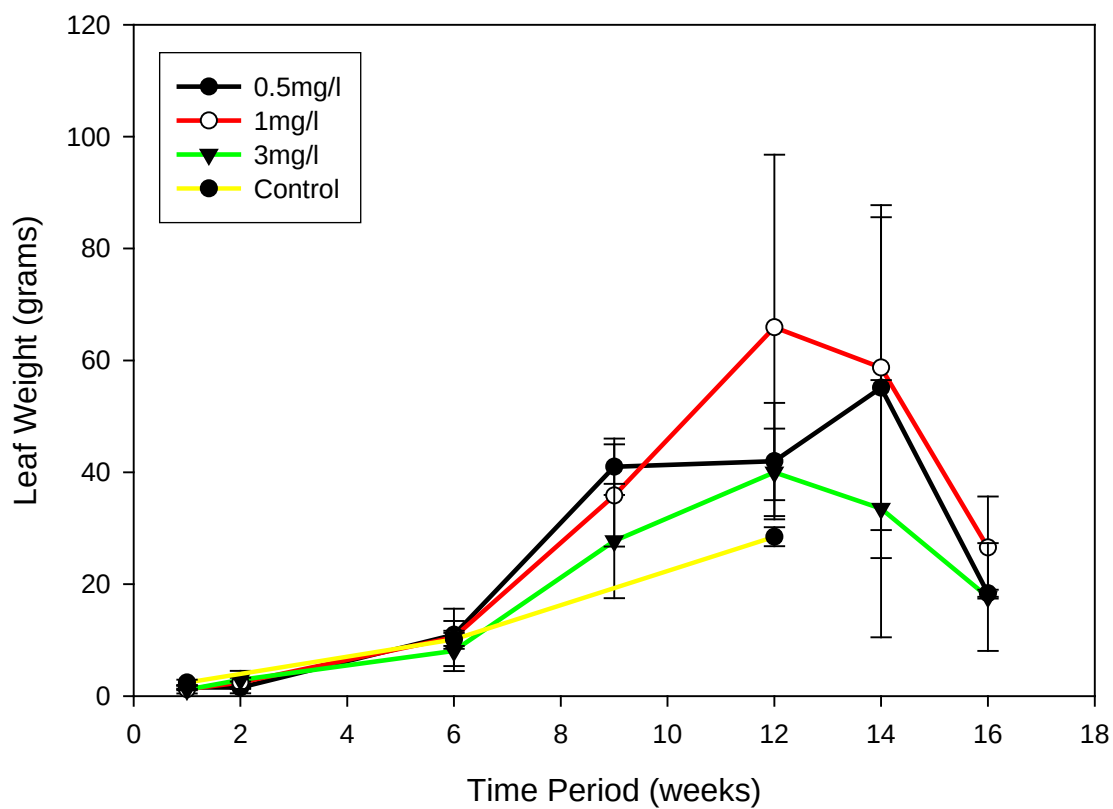


Figure 15.2 Leaf weight of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water, at time point where they were sacrificed. Also included is the mean leaf weight of bulrush plants with no RDX exposure. Error bars represent sample standard deviations between triplicate plant samples.

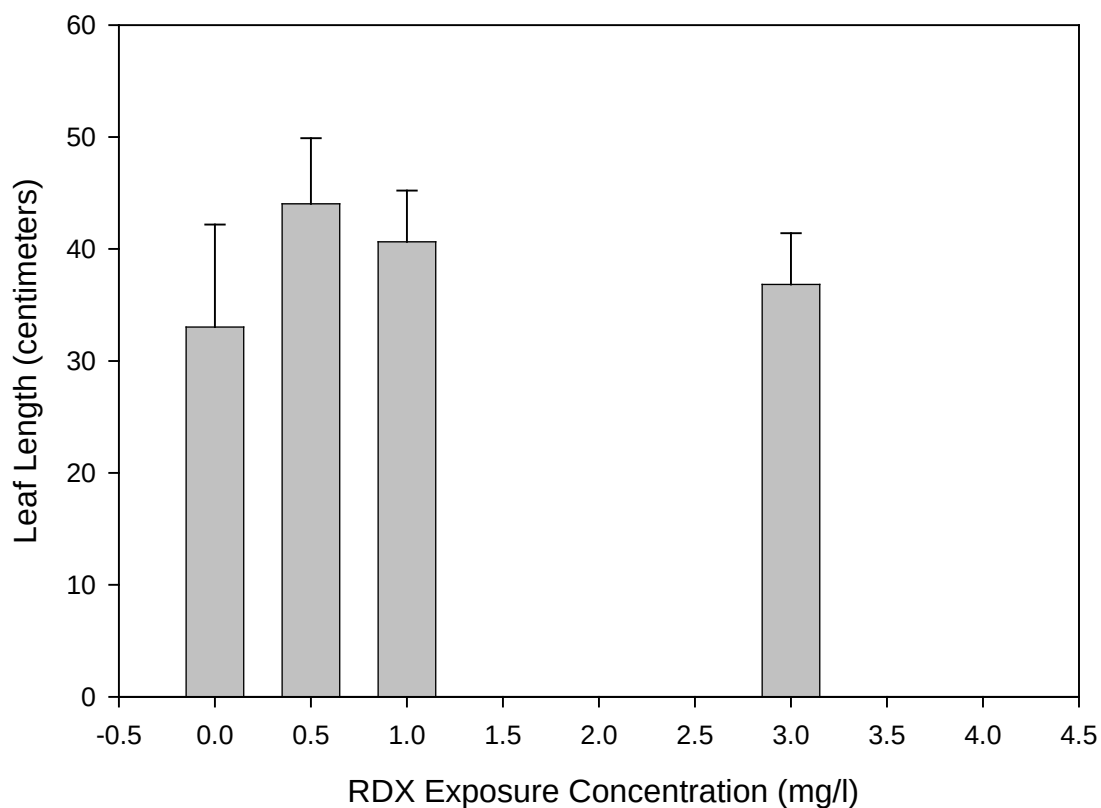


Figure 15.3 Leaf length of Bulrush Plants exposed to 0.5, 1, and 3 mg/l RDX in water in the 12th week, where the leaf length peaked for all the three treatments. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean leaf length of bulrush plants with no RDX exposure in the 12th week.

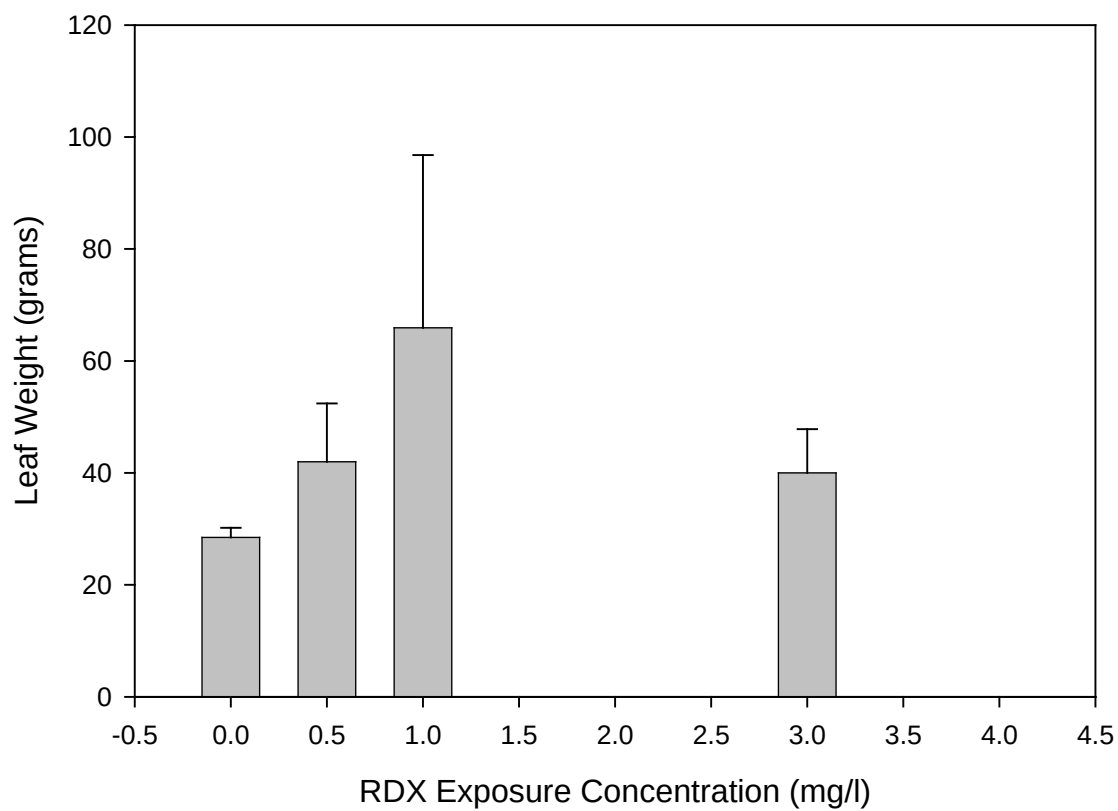


Figure 15.4 Leaf weight of Bulrush Plants exposed to 0.5, 1, 3 mg/l RDX in water in the 12th week, where the leaf weight peaked for all the three treatments. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean leaf weight of bulrush plants with no RDX exposure in the 12th week.

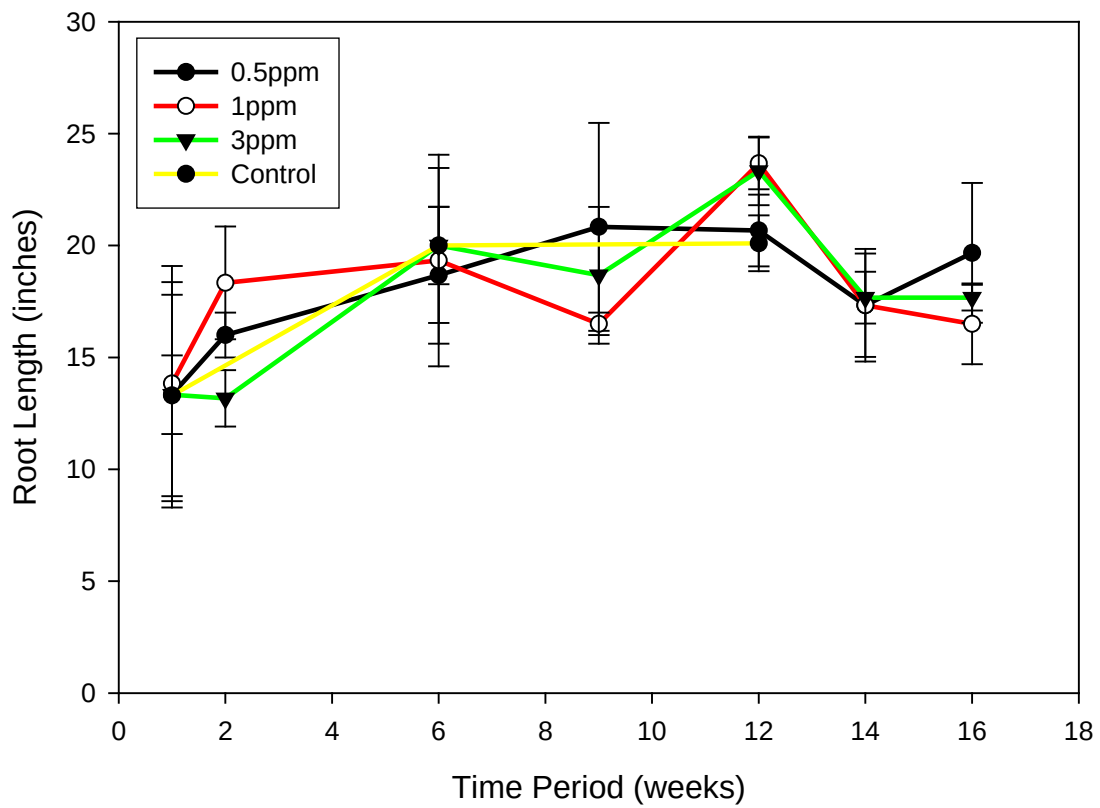


Figure 15.5 Root length of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water, at time point where they were sacrificed. Also included is the mean root length of bulrush plants with no RDX exposure. Error bars represent sample standard deviations between triplicate plant samples.

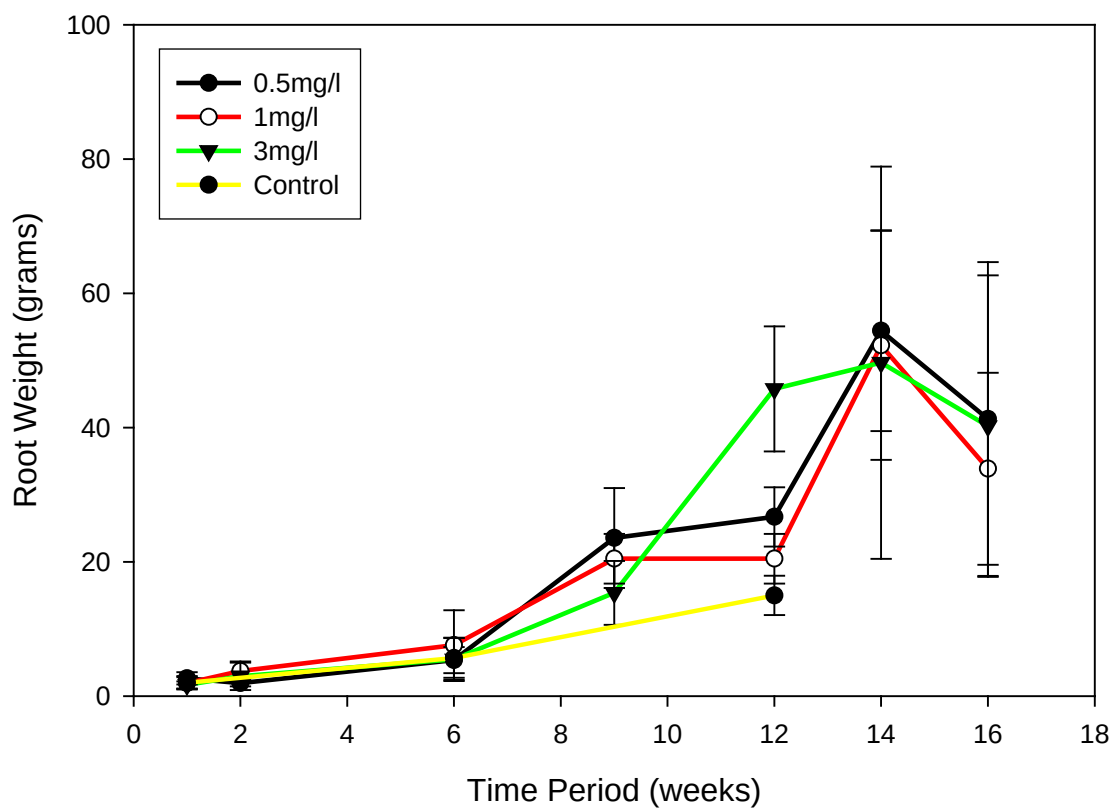


Figure 15.6 Root weight of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water, at time point where they were sacrificed. Also included is the mean root weight of bulrush plants with no RDX exposure. Error bars represent sample standard deviations between triplicate plant samples.

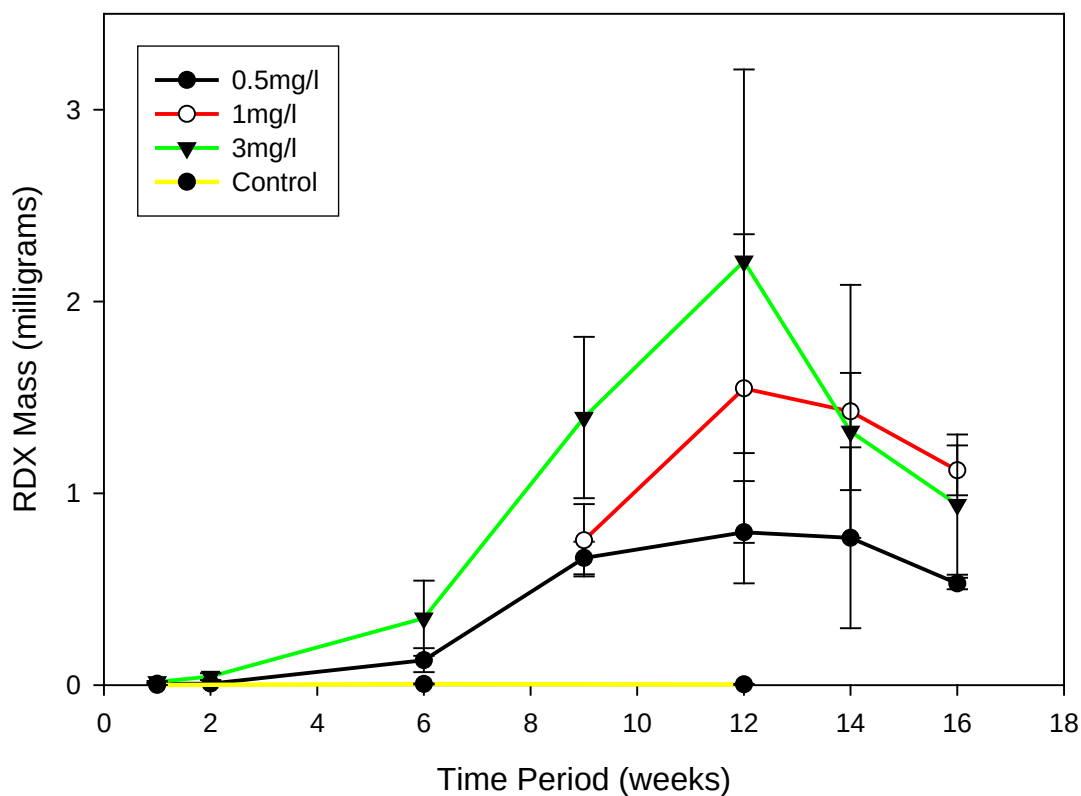


Figure 15.7 Mean mass of RDX accumulated in leaf tissues of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean mass of RDX accumulated in leaf tissues of bulrush plants with no RDX exposure.

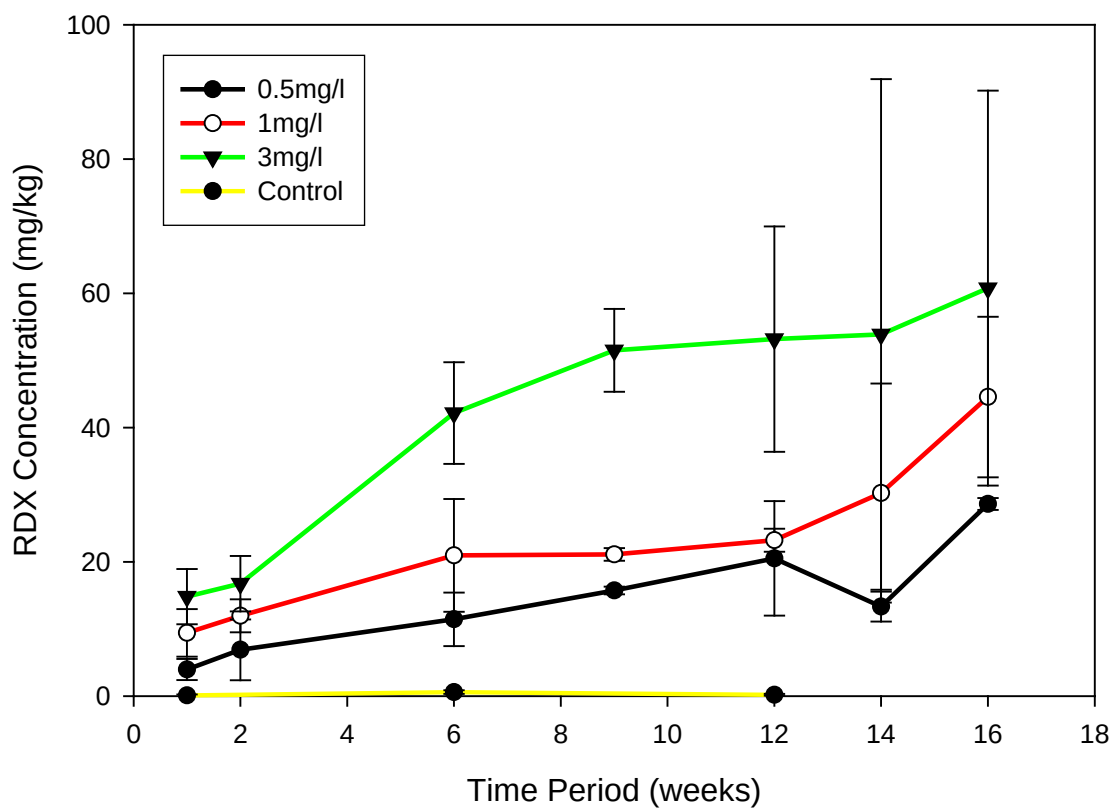


Figure 15.8 Mean concentration of RDX in leaf tissues of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean concentration of RDX in leaf tissues of bulrush plants with no RDX exposure.

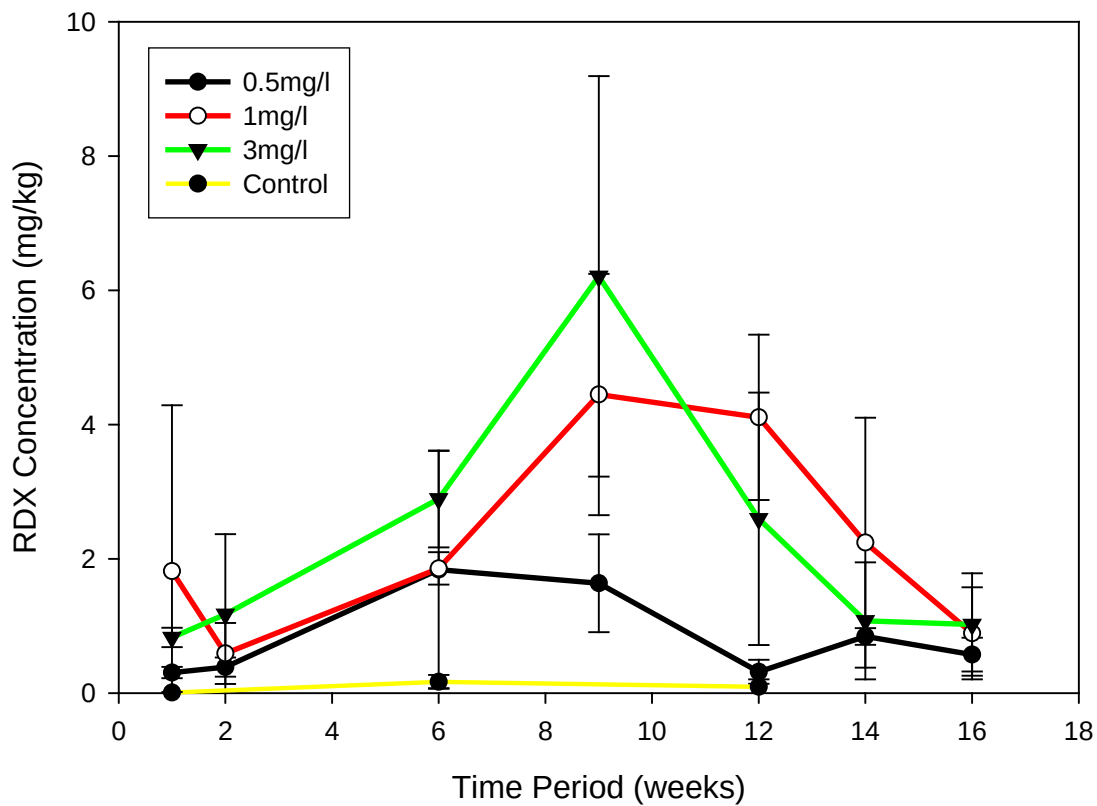


Figure 15.9 Mean concentration of RDX in root samples of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean concentration of RDX in root samples of bulrush plants with no RDX exposure.

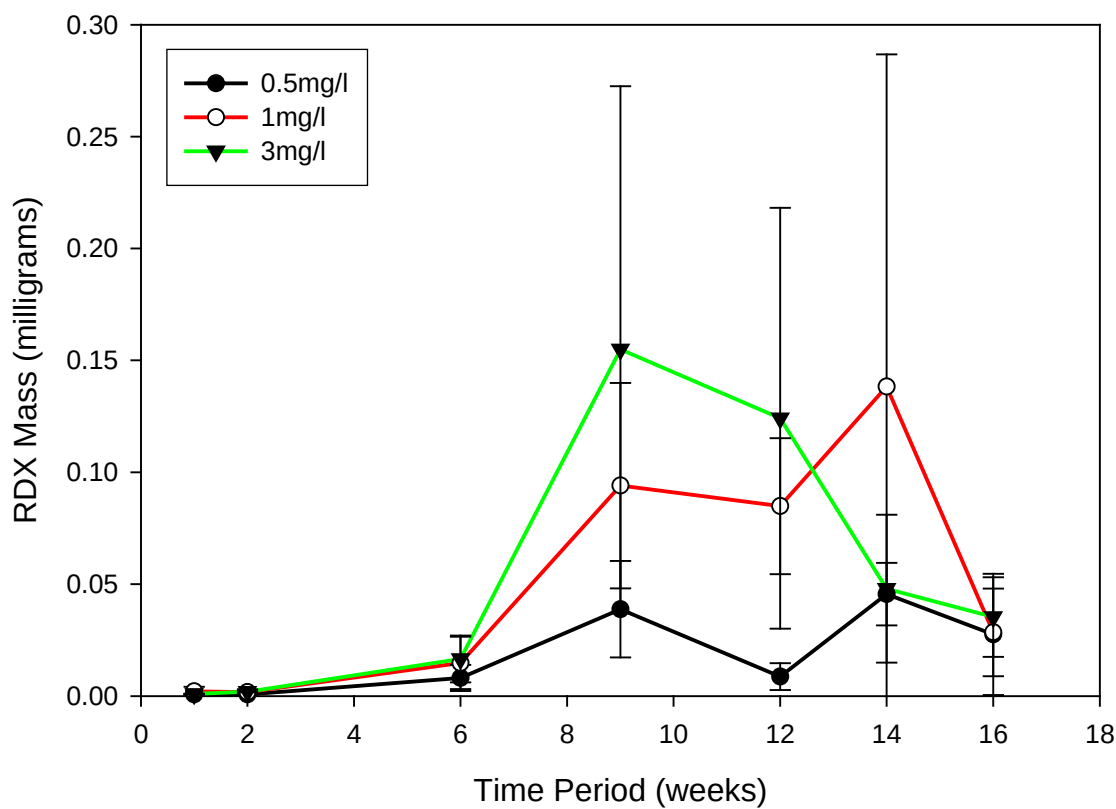


Figure 15.10 Mean mass of RDX accumulated in the root samples of Bulrush plants exposed daily to 3 concentrations (0.5, 1, 3 mg/l) of RDX in water at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples.

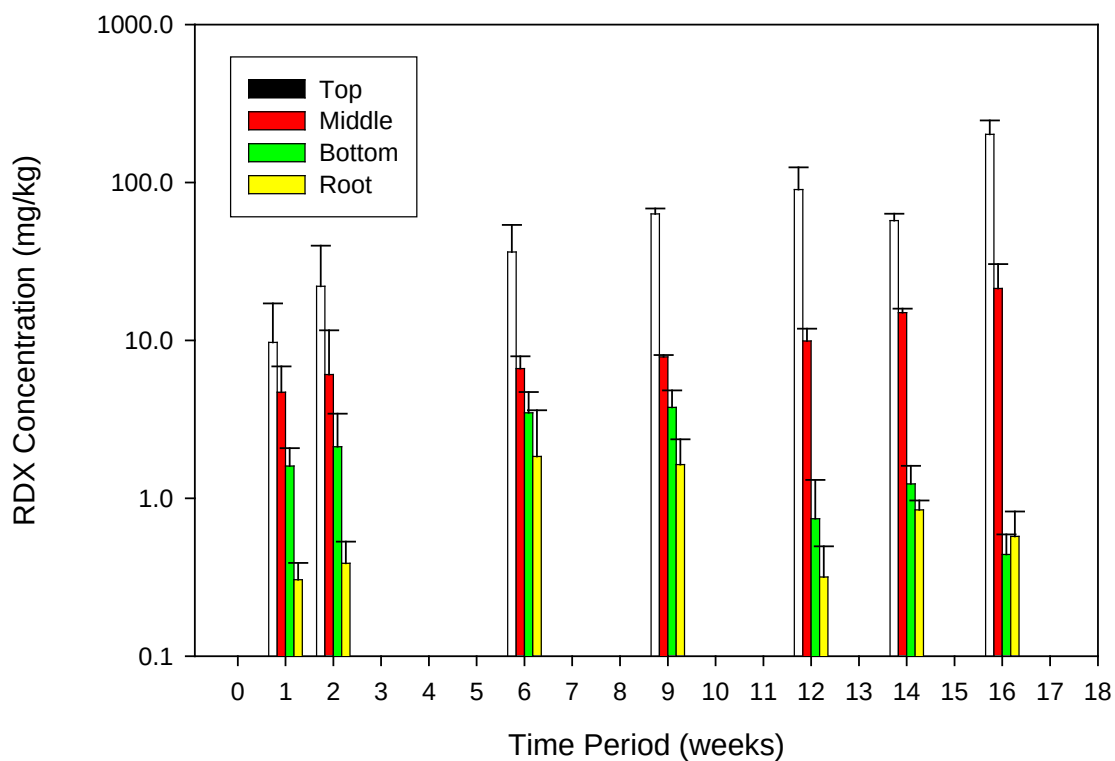


Figure 15.11 Mean concentration of RDX in top, middle, bottom and root portions of Bulrush plants exposed daily to 0.5 mg/l RDX concentration in water at the time point where the plant was sacrificed. Error bars represent sample standard deviations between triplicate plant samples.

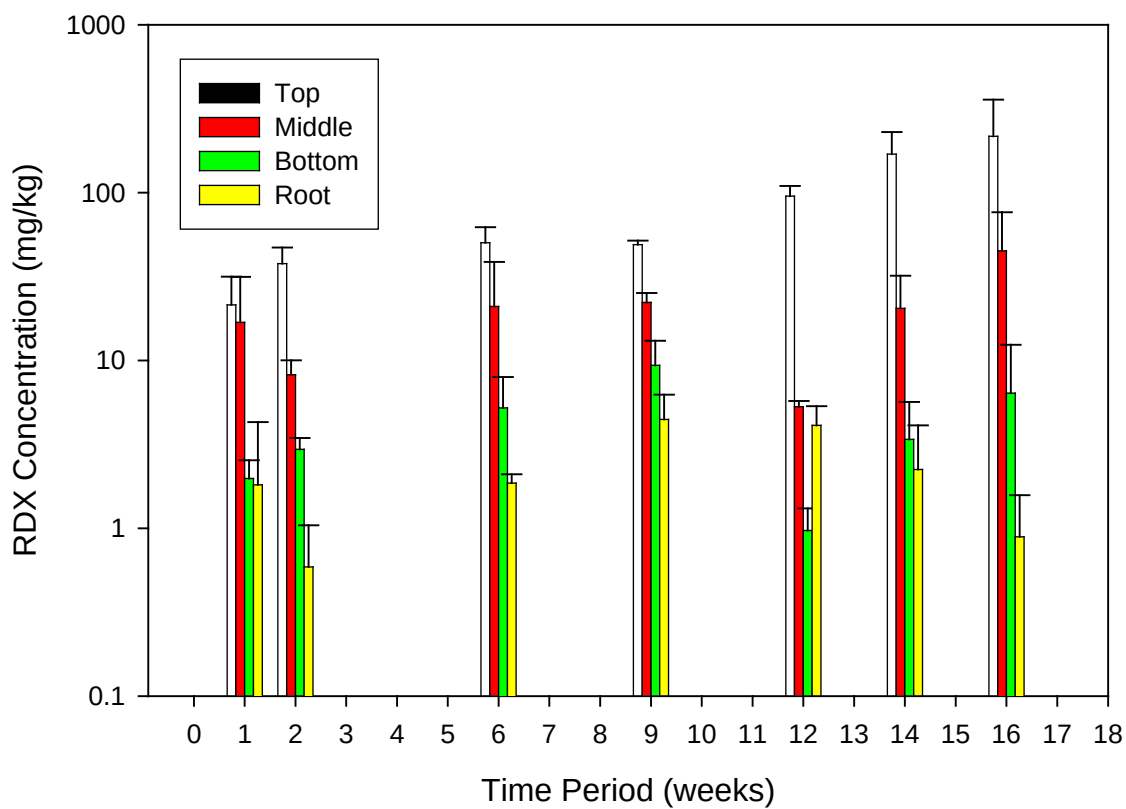


Figure 15.12 Mean concentration of RDX in top, middle, bottom and root portions of Bulrush plants exposed daily to 1 mg/l RDX concentration in water at the time point where the plant was sacrificed. Error bars represent sample standard deviations between triplicate plant samples.

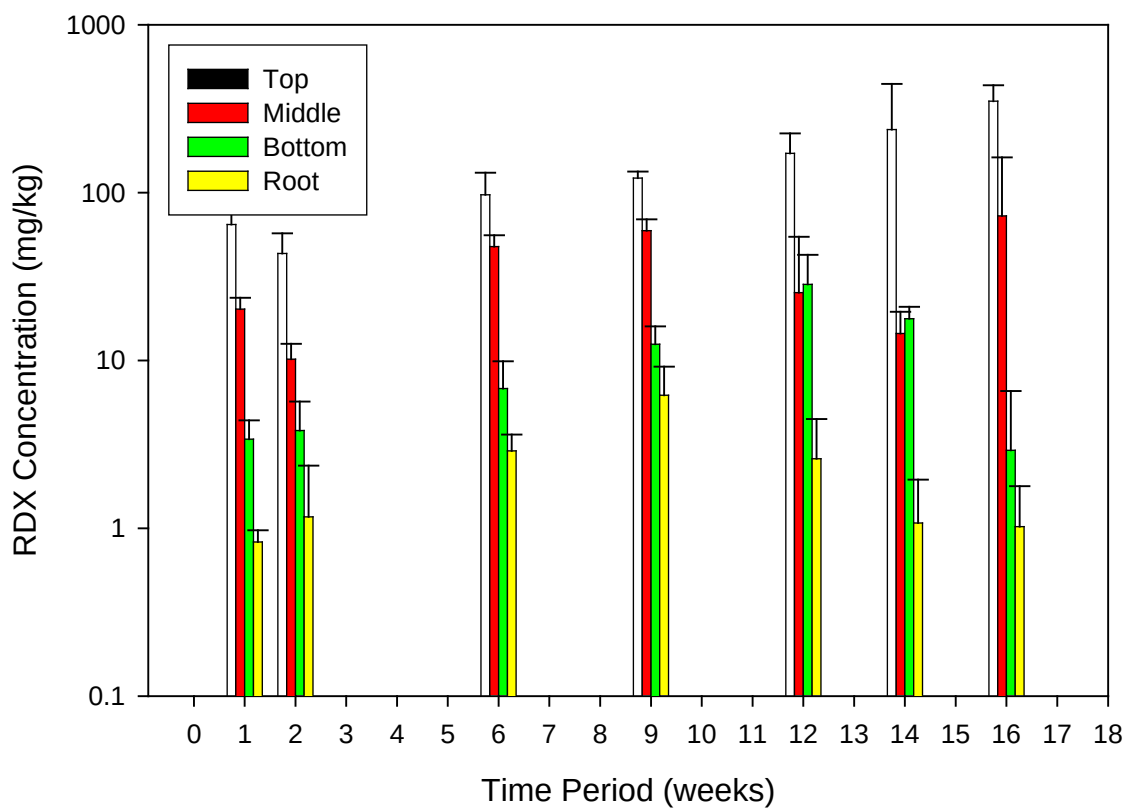


Figure 15.13 Mean concentration of RDX in top, middle, bottom and root portions of Bulrush plants exposed daily to 3 mg/l RDX concentration in water at the time point where the plant was sacrificed. Error bars represent sample standard deviations between triplicate plant samples.

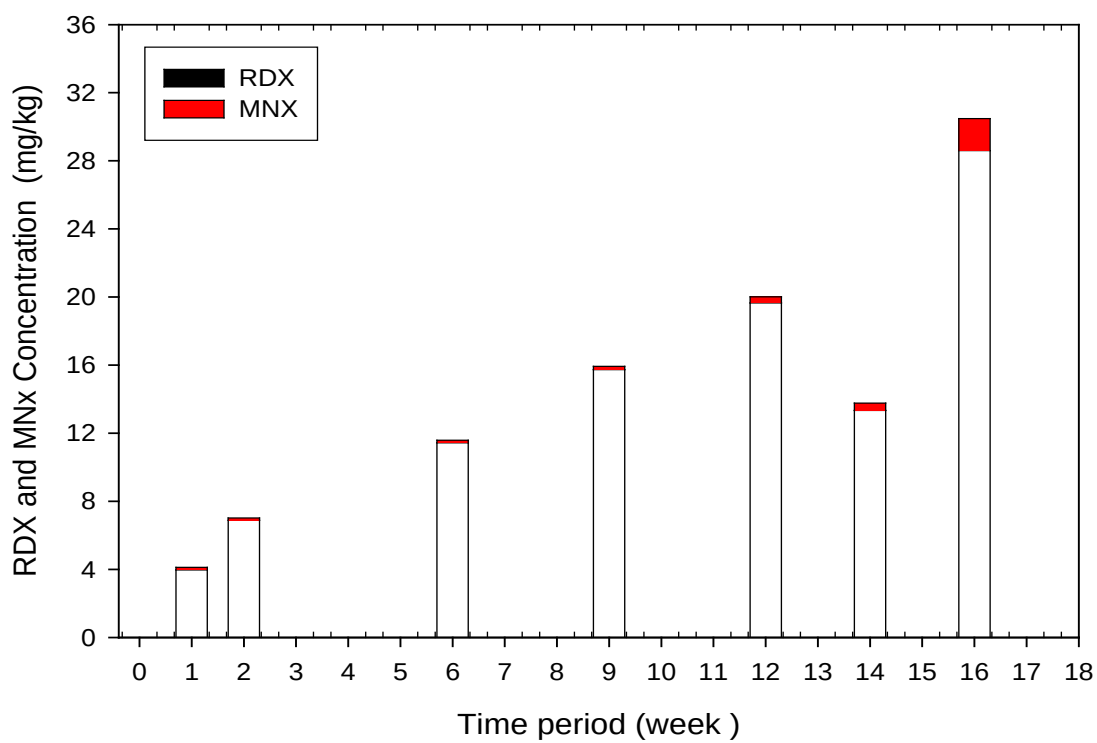


Figure 15.14 RDX and MNX concentrations in same leaf tissues of bulrush plants exposed to 0.5 mg/l RDX concentration in water at the time point where they were sacrificed.

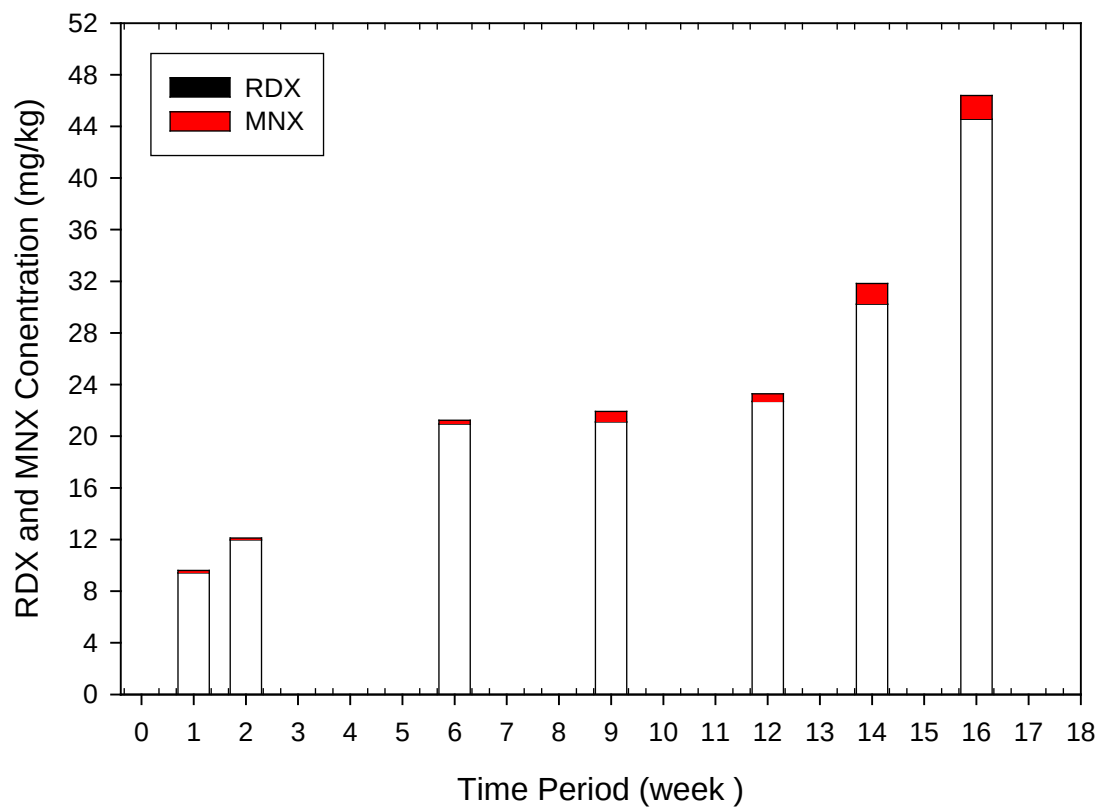


Figure 15.15 RDX and MNX concentrations in leaf tissues of bulrush plants exposed to 1 mg/l RDX concentration at the time point where they were sacrificed.

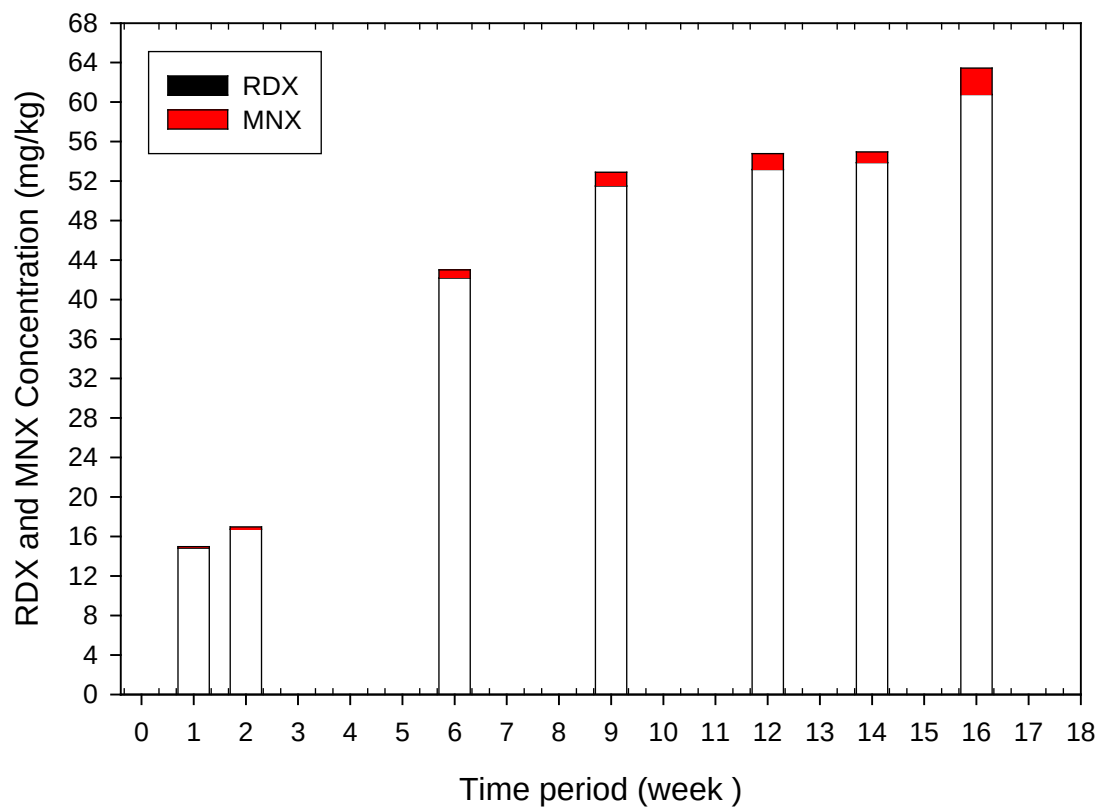


Figure 15.16 RDX and MNX concentrations in leaf tissues of bulrush plants exposed to 3 mg/l RDX concentration at the time point where they were sacrificed.

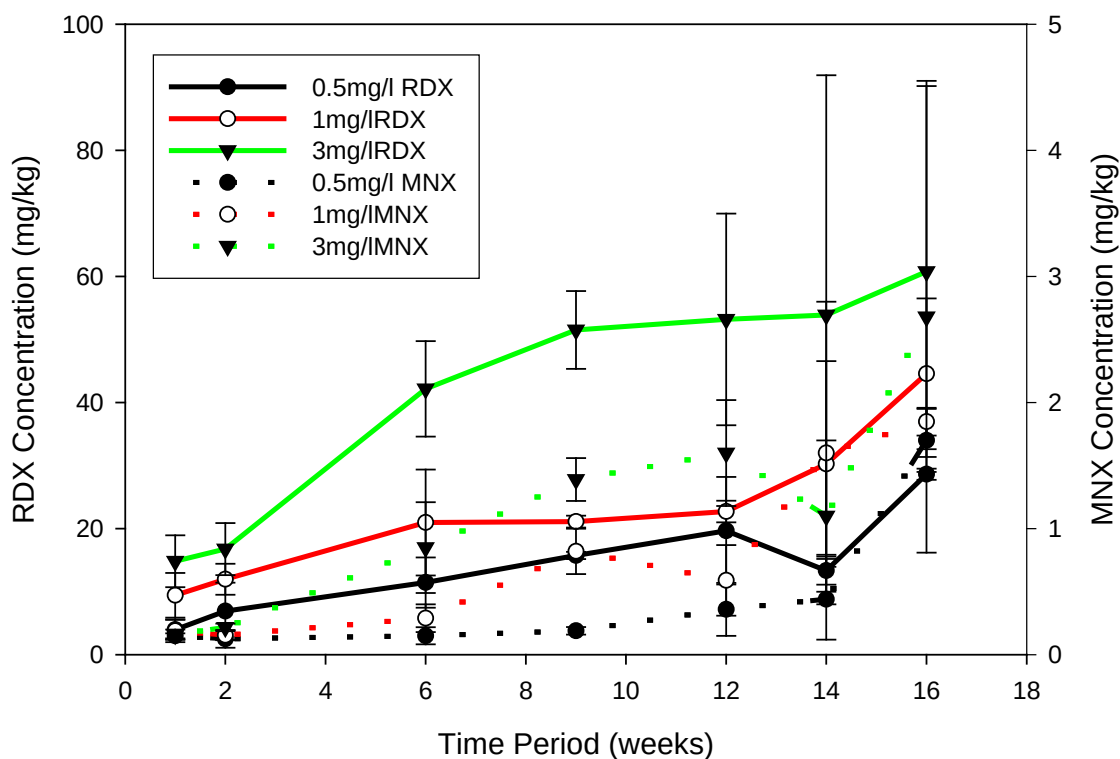


Figure 15.17 Mean concentration of RDX and MNX in leaf tissues of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples.

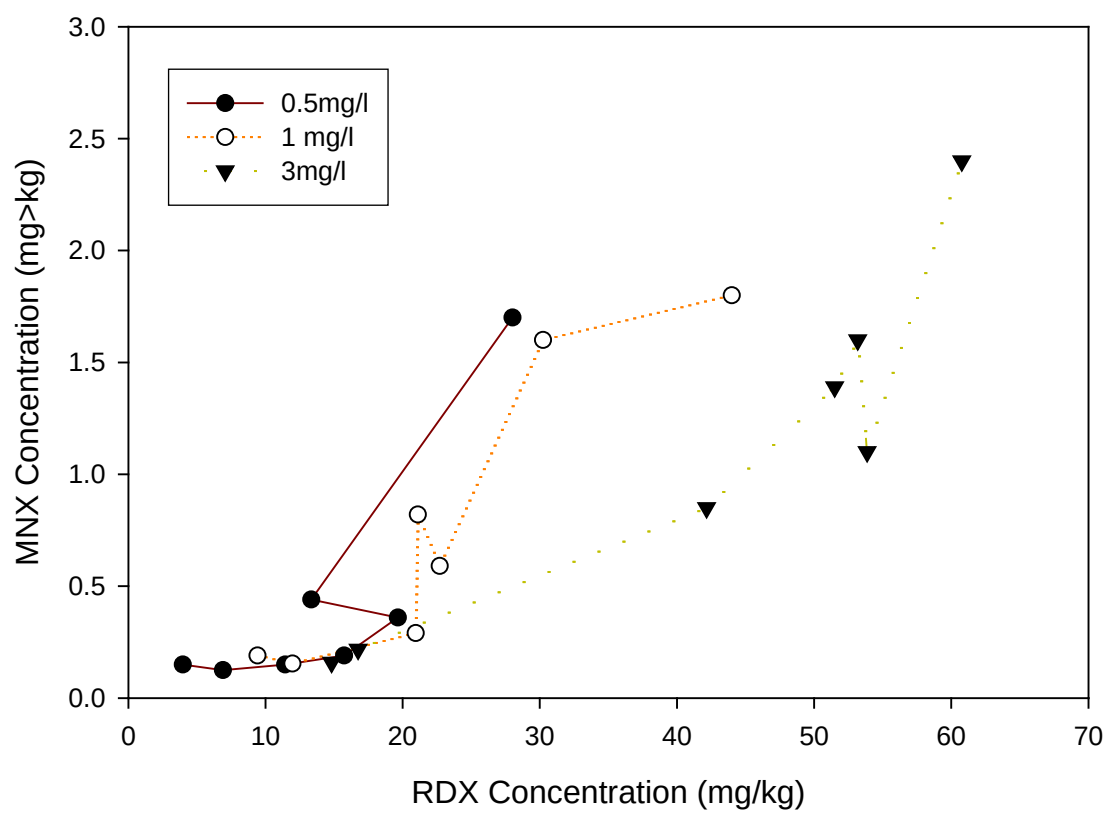


Figure 15.18 RDX and MNX concentrations in leaf tissues of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at a given time point.

15.1.7 Loss of RDX from Exposed Plants

Loss of RDX from bulrush plants was evaluated over a 6 week period after exposing them for 16 weeks. In the first sixteen weeks different sets of bulrush plants were exposed to different RDX exposure levels (0.5, 1, and 3 mg/l), followed by a 6 week period of no exposure. Similar sampling, extraction and analytical procedures as in the first experiment were adopted.

15.1.8 Loss of Accumulated RDX from Plants

Mass of RDX in the plants decreased drastically in all the three treatments, during the period of no exposure. Mass of RDX in the leaf tissues of plants exposed to 3, 1 and 0.5 mg/l RDX exposure level was 0.1 ± 0.047 , 0.07 ± 0.027 , 0.035 ± 0.022 mg respectively in the 19th week and 0.09 ± 0.05 , 0.02 ± 0.008 , 0.02 ± 0.014 mg in the 22nd week, compared to 1 ± 0.37 , 1 ± 0.13 , 0.5 ± 0.03 mg in the 16th week the end of the exposure period (**Figure 15.19**). RDX concentrations in leaf material also decreased drastically from the 16th week in all the three treatments. RDX concentrations in leaf material dropped from 60 ± 30 , 44 ± 12 and 29 ± 0.9 mg/kg in the 16th week to 4 ± 0.7 , 1.86 ± 0.3 , and 1.6 ± 0.7 mg/kg respectively in the 19th week and 3.1 ± 2 , 1.86 ± 1.3 and 0.55 ± 0.07 mg/kg in the 22nd week for 3, 1 and 0.5 mg/l exposure level, respectively (**Figure 15.20**).

Mass of RDX and RDX concentrations in root samples of plants continued to decrease, in the last six weeks as was observed from the 9th week. Mass of RDX in root material of plants dropped from 0.027 ± 0.02 , 0.03 ± 0.02 , 0.035 ± 0.018 mg/kg in the 16th week to 0.008 ± 0.001 , 0.011 ± 0.002 , and 0.015 ± 0.0122 mg/kg respectively in the 19th week and 0.003 ± 0.002 , 0.008 ± 0.004 and 0.12 ± 0.009 mg/kg in the 22nd week for 0.5, 1 and 3 mg/l exposure level (**Figure 15.21**). RDX concentrations in root material of plants dropped from 0.573 ± 0.252 , 0.889 ± 0.686 , 1.021 ± 0.762 mg/kg in the 16th week to 0.22 ± 0.019 , 0.25 ± 0.06 , and 0.596 ± 0.417 mg/kg respectively in the 19th week and 0.07 ± 0.043 , 0.28 ± 0.018 and 0.307 ± 0.081 mg/kg in the 22nd week for 0.5, 1, and 3 mg/l exposure level (**Figure 15.22**).

15.1.9 Distribution of RDX in Plants

RDX was detected at higher concentration in the top portion of the leaf when compared to middle and bottom portions. The RDX concentration in the top portion of the leaf was 6 to 14 times greater than the concentrations detected in middle and bottom portions of the leaf even 6 weeks after exposure was terminated. For exposure levels of 3 mg/l, 1 mg/l, and 0.5 mg/l, RDX concentrations of 12 ± 8 , 6.5 ± 4 , 2 ± 1 mg/kg occurred in the 22nd week when compared to 350 ± 85.8 mg/kg, 216 ± 141 mg/kg and 201 ± 45 mg/kg in the 16th week in the top portion of the leaf. RDX concentrations in root samples continued to decrease consistently till the 22nd week (**Table 15.1** and **Figure 15.23**, **15.24**, **15.25**).

15.1.10 RDX Metabolites

In plants sacrificed after 6 weeks of no exposure, MNX was detected in almost 90% of the plant samples regardless of exposure concentration but none was detected in the no exposure control treatment. The 10% of the samples in which MNX was not detected

constituted root samples. MNX concentrations were about 3 to 6% of the RDX concentration in plant samples for all treatments (**Figure 15.26, 15.27, 15.28**). MNX concentration in leaf material of the plant samples ranged from 0.02 to 0.18 in the last six weeks, when compared to 0.12 mg/kg to 2.6 mg/kg in the first 16 weeks. MNX concentrations in leaves followed the same pattern as RDX concentrations in leaf material of plants for all treatments (**Figure 15.29**). MNX concentrations were highest in top portions of the plant where RDX concentrations were also detected to be highest as was observed for previous time points. MNX was found in soil samples at very low concentrations with a mean of 0.003, 0.003 and 0.004 mg/kg in soil samples for 0.5, 1, and 3 mg/l exposure treatments when compared to RDX concentrations of 0.06, 0.18 and 0.25 mg/kg.

DNX and TNX were detected only in plant samples and were not detected in soil samples. DNX was detected in 12 samples and TNX was observed in 13 samples (out of 72 samples of top, middle, bottom and root samples of plant). About 95% of the time DNX was observed in plant samples exposed to 0.5 mg/l exposure level. DNX was randomly distributed in middle, bottom and root portions of the plant at a concentration range of 0.003 to 0.03 mg/kg. DNX concentrations in plant samples showed no relationship with RDX concentration or time. TNX was mostly observed in root samples of plants exposed to all treatment concentrations, in a concentration range of 0.001 to 0.01 mg/kg.

15.1.11 Mass Balance of RDX:

A mass balance was conducted by calculating the total RDX introduced into the system (influent water), compared to RDX present in the effluent water, plants and soil at each time point. In these mass balance studies, the amount of RDX recovered in the effluent water increased compared to the previous time point in all the treatments, even though no additional RDX was introduced into the system. For a 0.5 ppm exposure, RDX released through effluent increased from 72 % (as a percent of influent weight) in the 16th week to 79% and 82 % in the 19th and 22nd week. Similar increase from 70 to 82% and 58 to 67% from the 16th to 22nd week was observed in the 1 and 3ppm treatment systems. Mass of RDX accumulated in soil samples as percentage of RDX introduced, (on a mean) in the first 16 weeks was 11, 8, and 6%, and in the last 6 weeks was 2.2, 3.3, 1.6 % for 0.5, 1, 3 mg/l exposure levels. Mass of RDX remaining in plants as percentage of RDX introduced (on a mean) in the first 16 weeks was 2.2, 2 , and 1 % and in the last 6 weeks was 0.1, 0.04, and 0.05% for 0.5, 1, 3 mg/l exposure levels (**Figure 15.30, 15.31, 15.32**).

Table 15.1 RDX concentrations in mg/kg in root, bottom, middle and top portions of the Bulrush plant in 16th, 19th, 22nd week for 0.5, 1, 3 mg/l exposure level.

For 0.5 mg/l RDX Exposure Concentration in water				
RDX Concentration (mg/kg)				
Week	Root	Bottom	Middle	Top
16	0.57	0.448	21.3	201
19	0.22	0.918	0.89	5.71
22	0.074	0.189	0.75	1.91
For 1 mg/l RDX Exposure Concentration in water				
RDX Concentration(mg/kg)				
Week	Root	Bottom	Middle	Top
16	0.89	6.39	44.9	216
19	0.25	0.44	1.13	8.98
22	0.28	0.75	1.11	6.4
For 3 mg/l RDX Exposure Concentration in water				
RDX Concentration (mg/kg)				
Week	Root	Bottom	Middle	Top
16	1.02	2.92	72.7	350
19	0.60	0.63	1.95	18.4
22	0.307	0.95	2.13	11.7

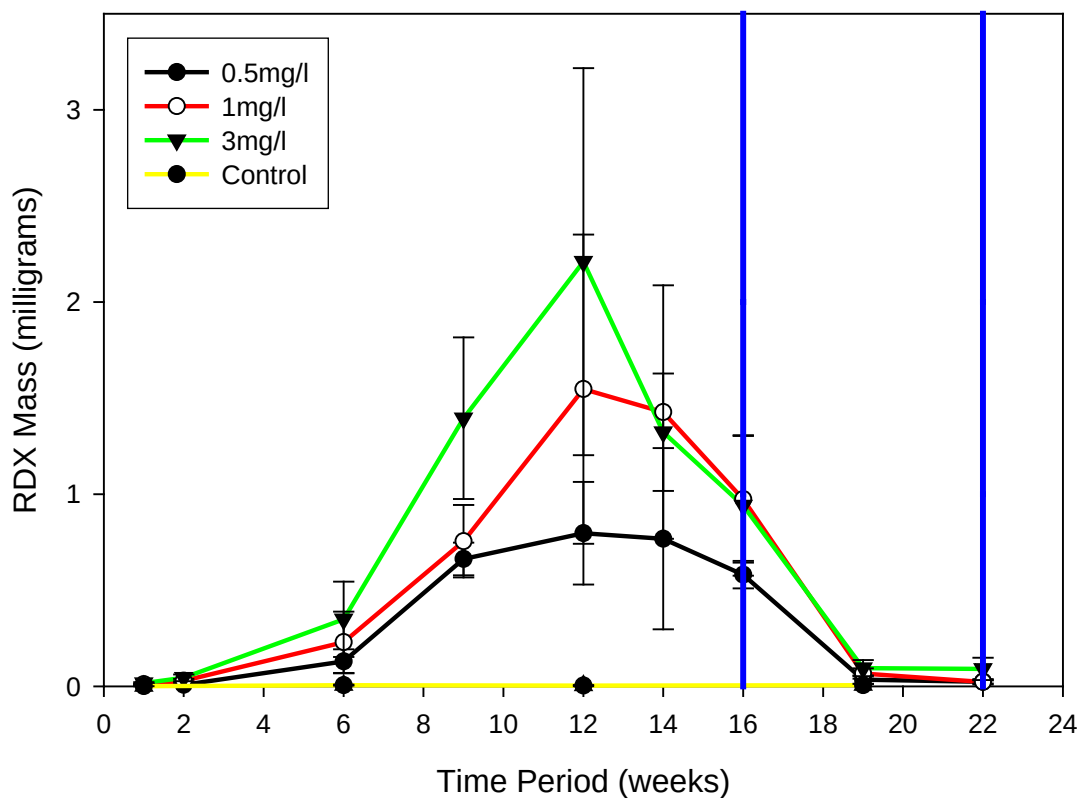


Figure 15.19 Mean mass of RDX accumulated in leaf tissues of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean mass of RDX accumulated in leaf tissues of bulrush plants with no RDX exposure. Plants were exposed to different levels of RDX in the first 16 weeks followed by a no exposure treatment for the next 6 weeks.

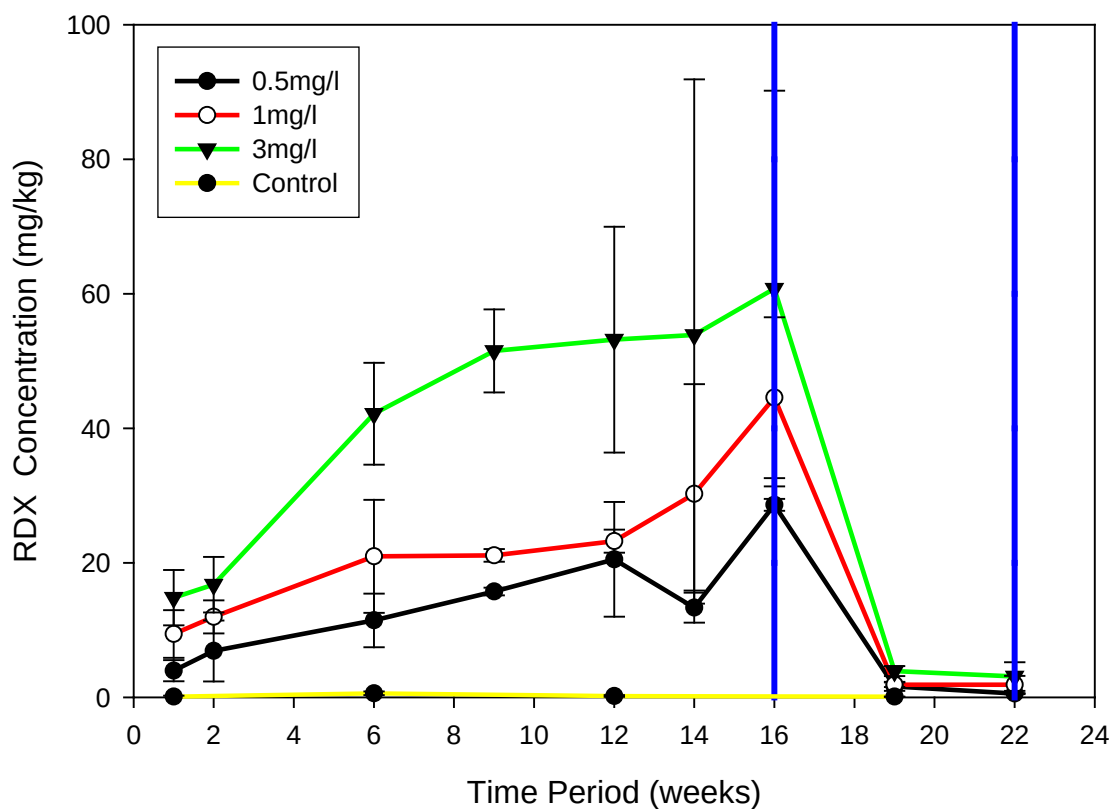


Figure 15.20 Mean concentration of RDX in leaf tissues of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean concentration of RDX in leaf tissues of bulrush plants with no RDX exposure. Plants were exposed to different levels of RDX in the first 16 weeks followed by a no exposure treatment for the next 6 weeks.

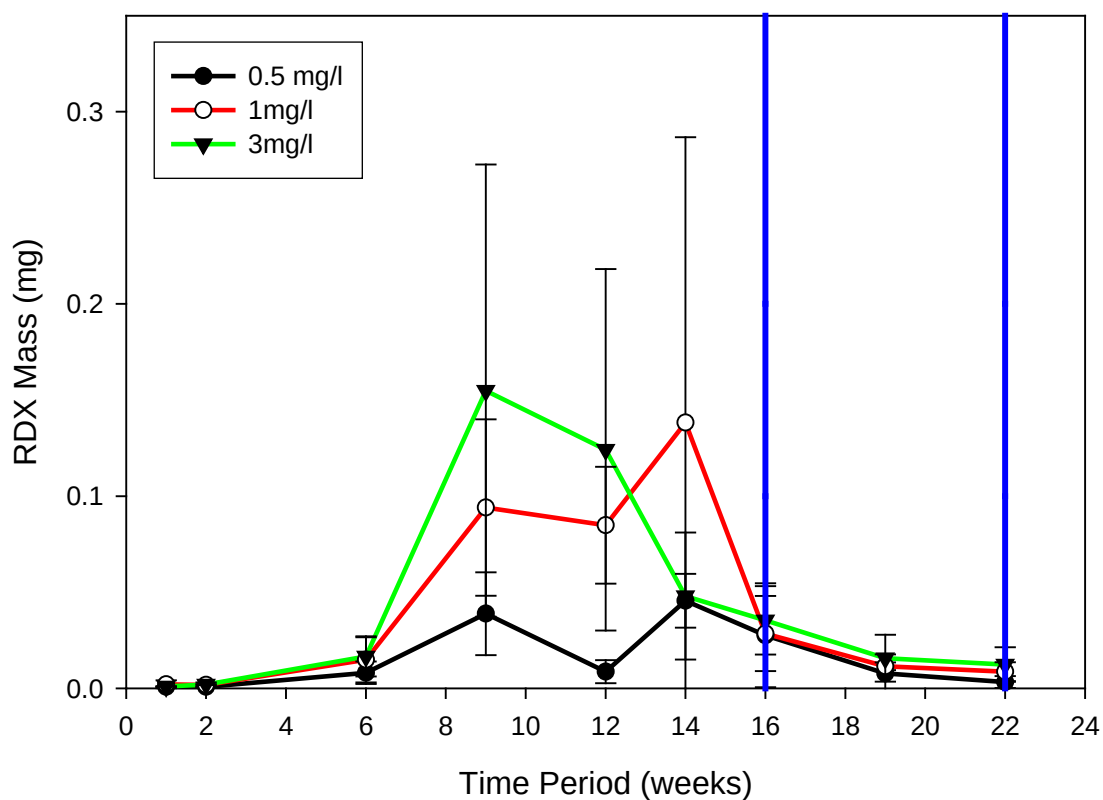


Figure 15.21 Mean mass of RDX accumulated in the root samples of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Plants were exposed to different levels of RDX in the first 16 weeks followed by a no exposure treatment for the next 6 weeks.

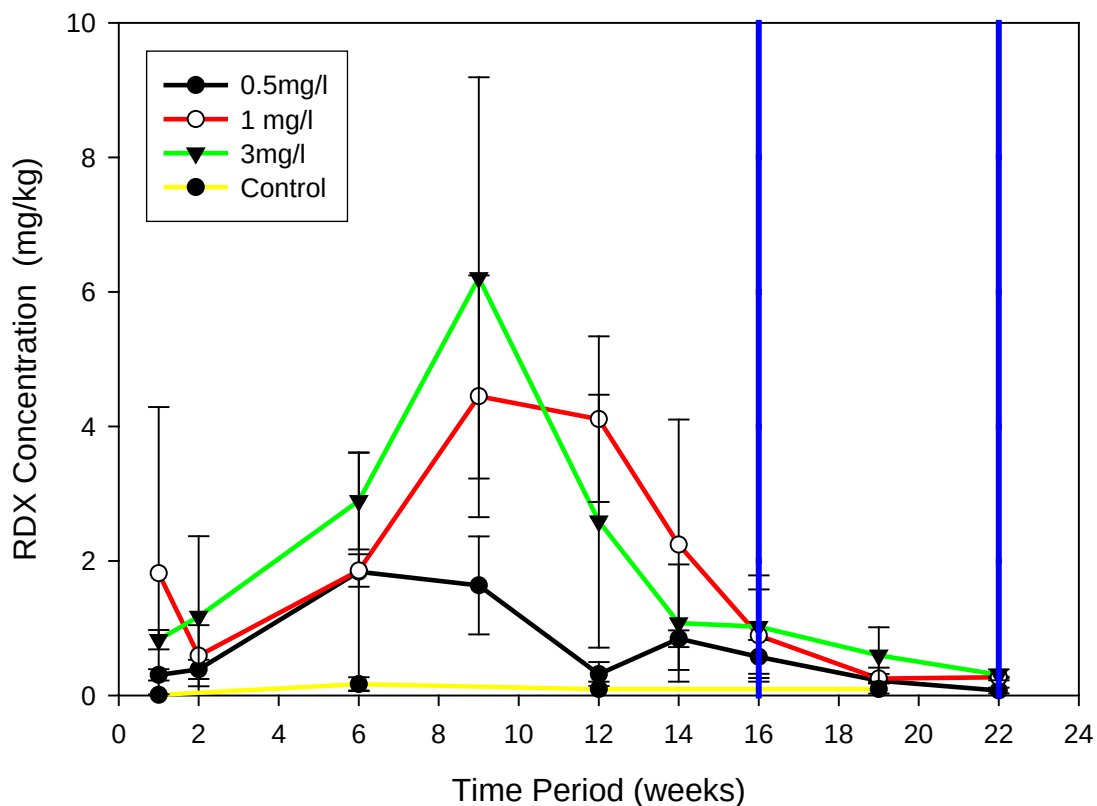


Figure 15.22 Mean concentration of RDX in root samples of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Also included is the mean concentration of RDX in root samples of bulrush plants with no RDX exposure. Plants were exposed to different levels of RDX in the first 16 weeks followed by a no exposure treatment for the next 6 weeks.

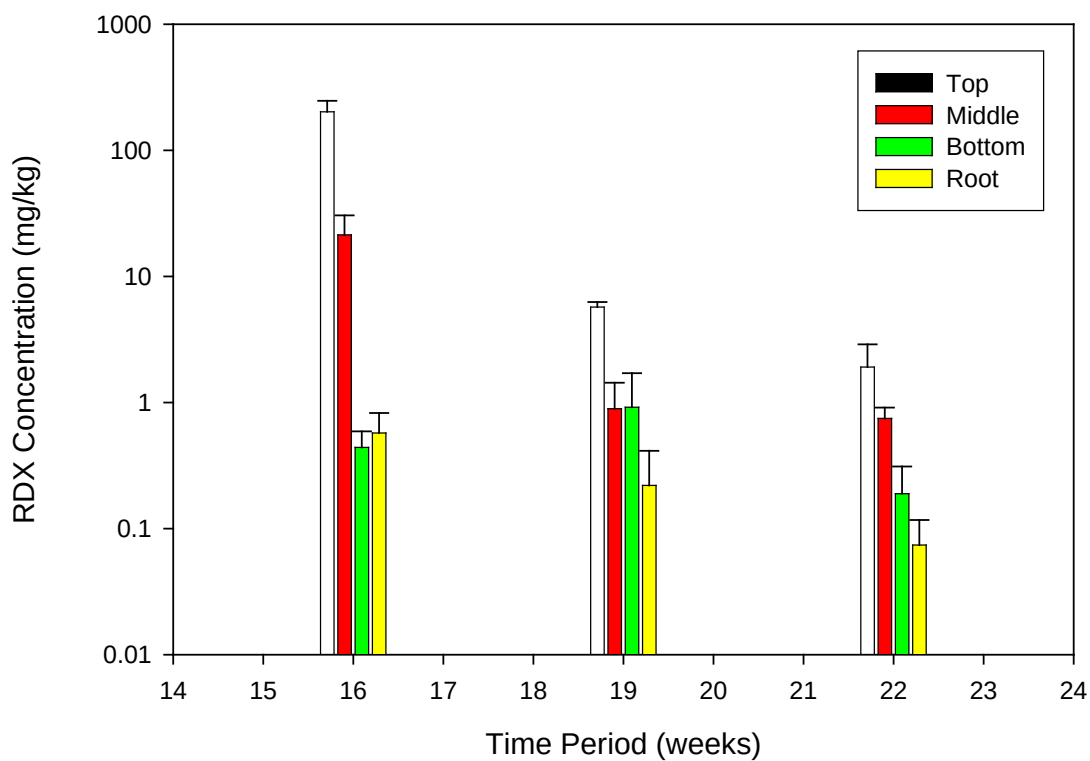


Figure 15.23 Mean concentration of RDX in top, middle, and bottom portions of the leaf exposed to 0.5 mg/l RDX concentration at 16th, 19th, 22nd time point where the plant was sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Here, 19th and 22nd time point represent period of no exposure.

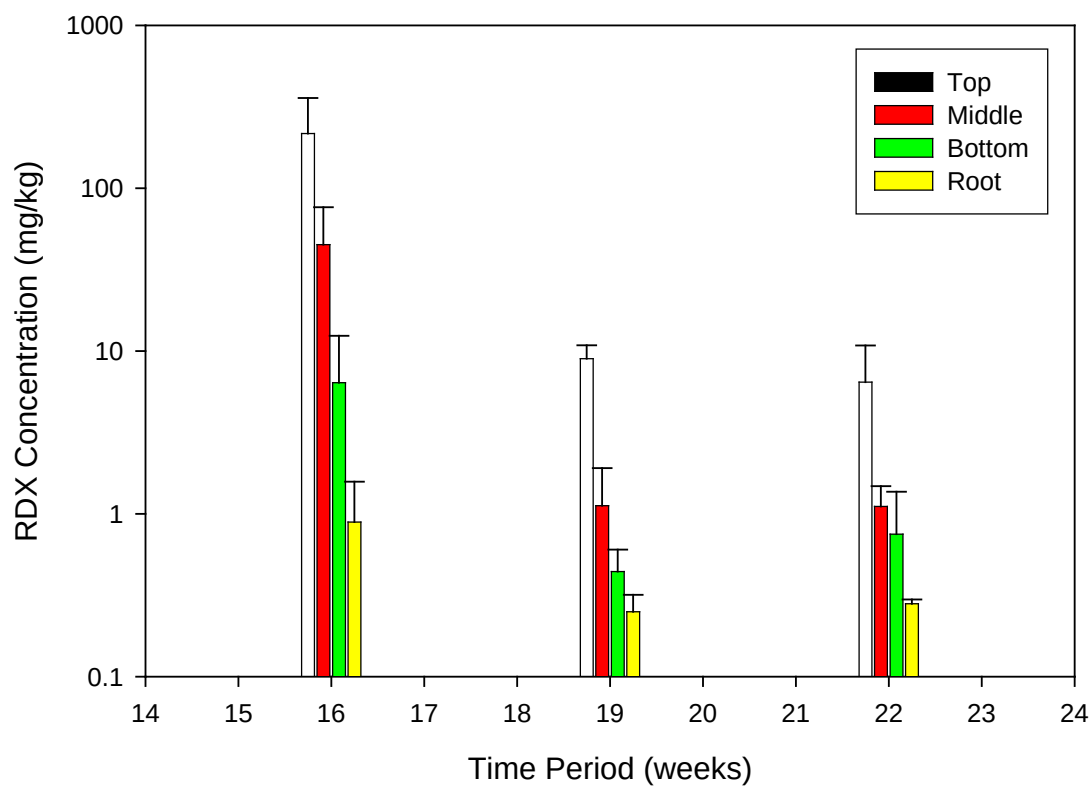


Figure 15.24 Mean concentration of RDX in top, middle, and bottom portions of the leaf exposed to 1 mg/l RDX concentration at 16th, 19th, 22nd time point where the plant was sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Here, 19th and 22nd time point represent period of no exposure.

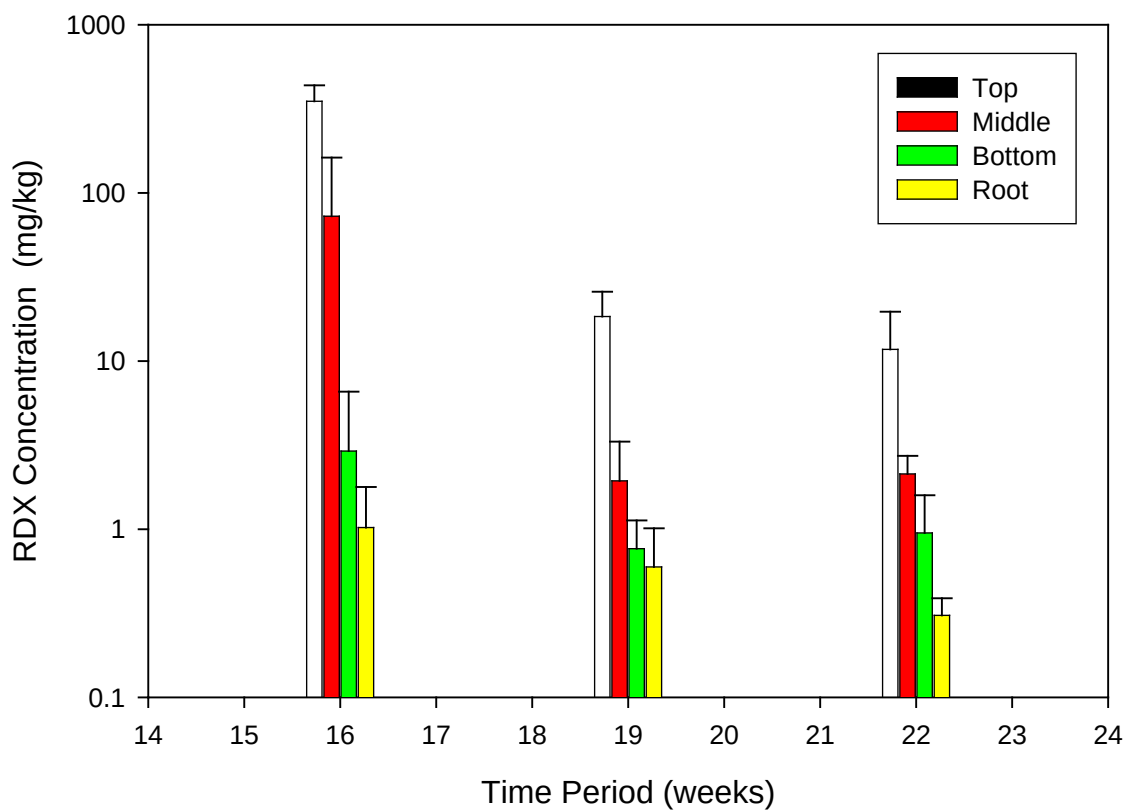


Figure 15.25 Mean concentration of RDX in top, middle, and bottom portions of the leaf exposed to 3 mg/l RDX concentration at 16th, 19th, 22nd time point where the plant was sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Here, 19th and 22nd time point represent period of no exposure.

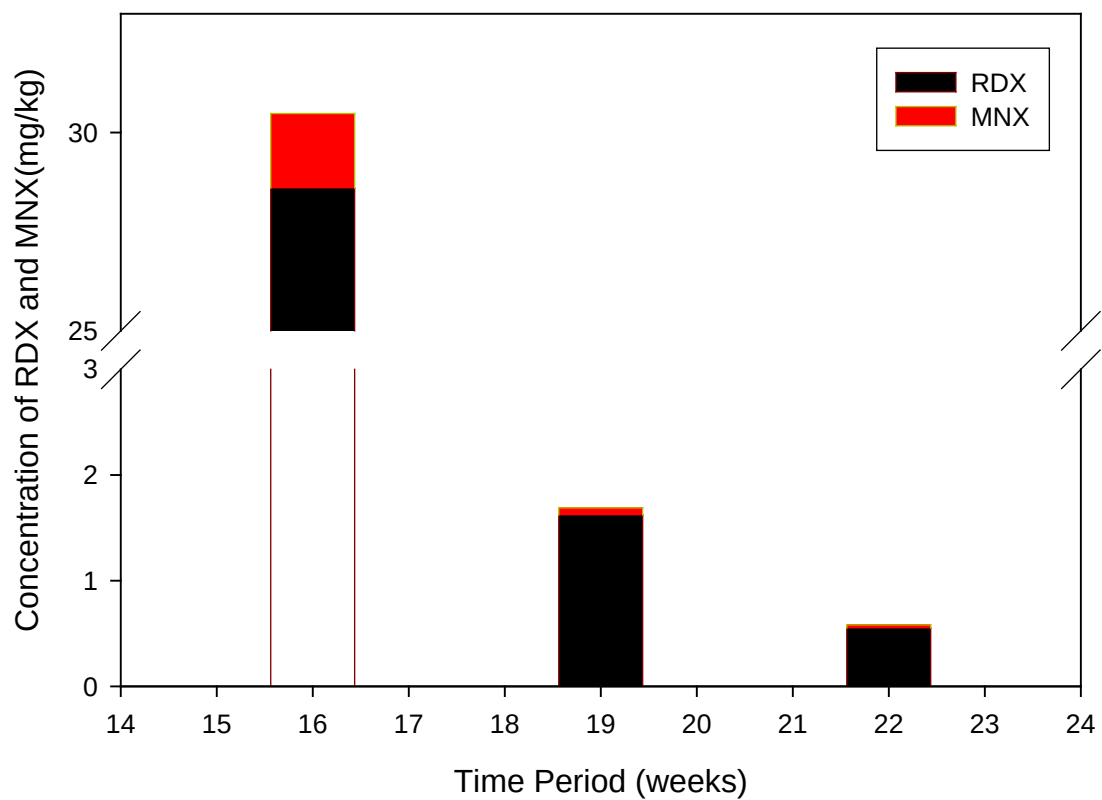


Figure 15.26 RDX and MNX concentrations in leaf tissues of bulrush plants exposed to 0.5 mg/l RDX concentration at the time point where they were sacrificed. Here, 19th and 22nd time point represent period of no exposure.

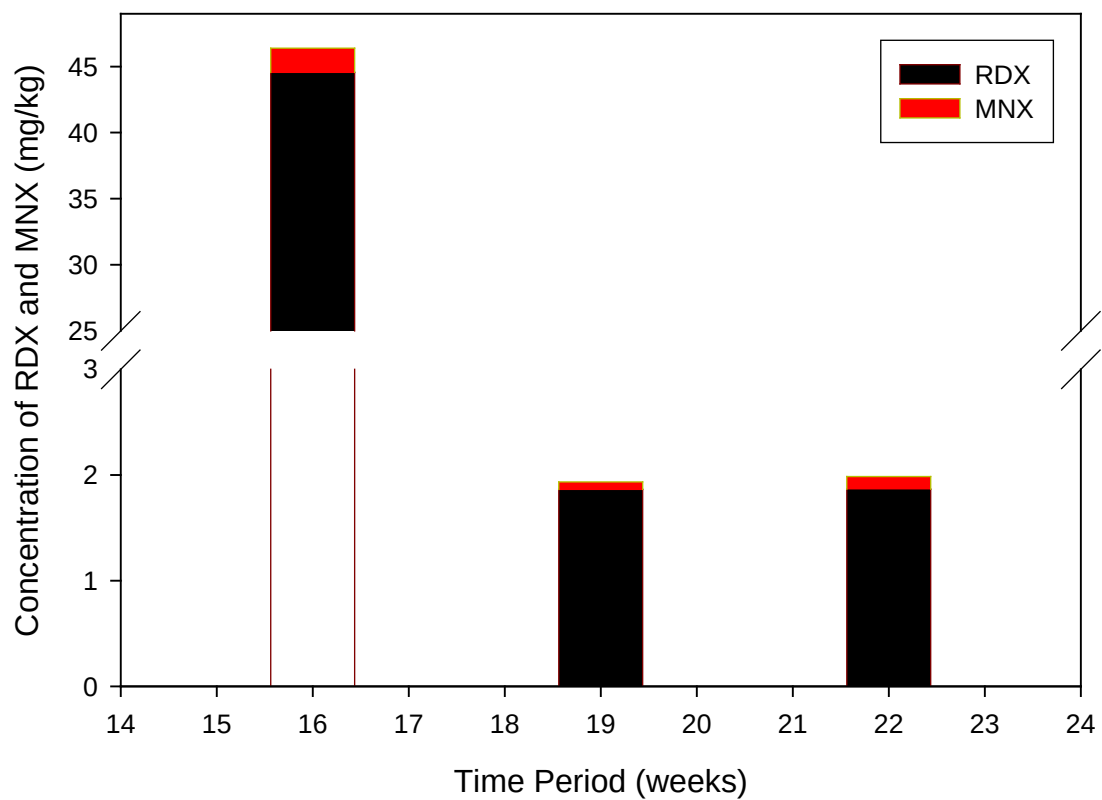


Figure 15.27 RDX and MNX concentrations in leaf tissues of bulrush plants exposed to 1 mg/l RDX concentration at the time point where they were sacrificed. Here, 19th and 22nd time point represent period of no exposure.

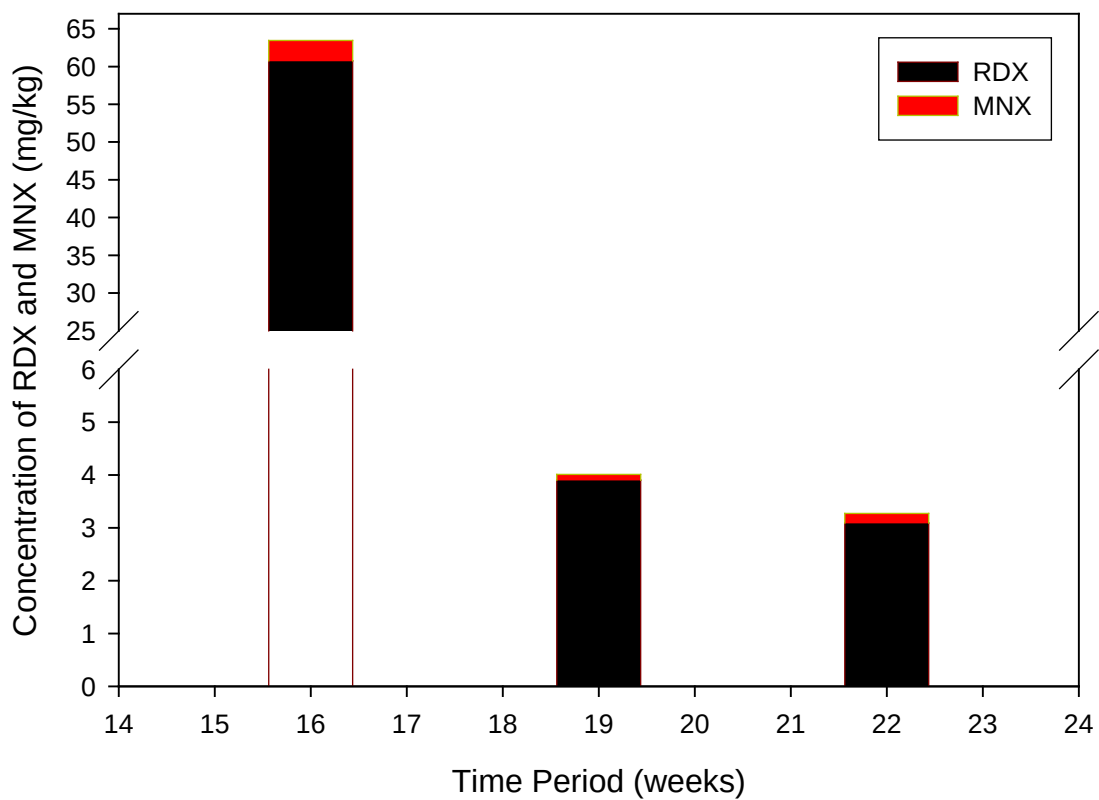


Figure 15.28 RDX and MNX concentrations in leaf tissues of bulrush plants exposed to 3 mg/l RDX concentration at the time point where they were sacrificed. Here, 19th and 22nd time point represent period of no exposure.

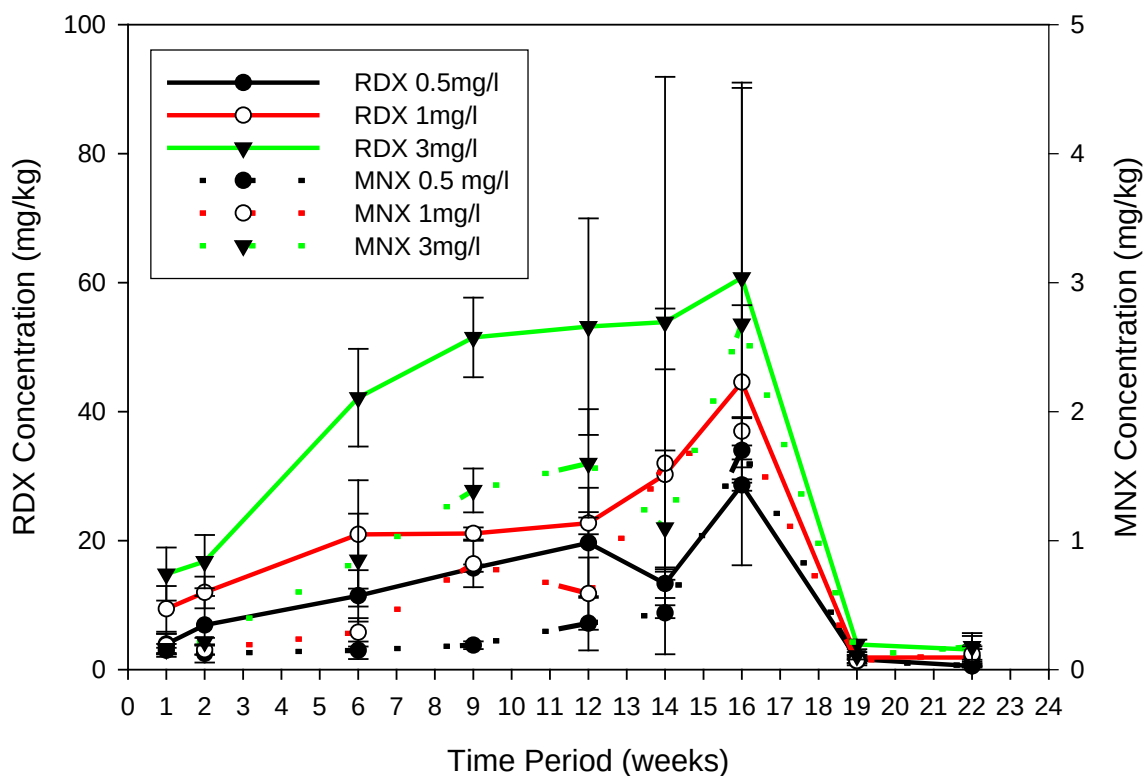


Figure 15.29 Mean concentration of RDX and MNX in leaf tissues of bulrush plants exposed to 0.5, 1, 3 mg/l RDX concentrations at the time point where they were sacrificed. Error bars represent sample standard deviations between triplicate plant samples. Plants were exposed to different levels of RDX in the first 16 weeks followed by a no exposure treatment for the next 6 weeks.

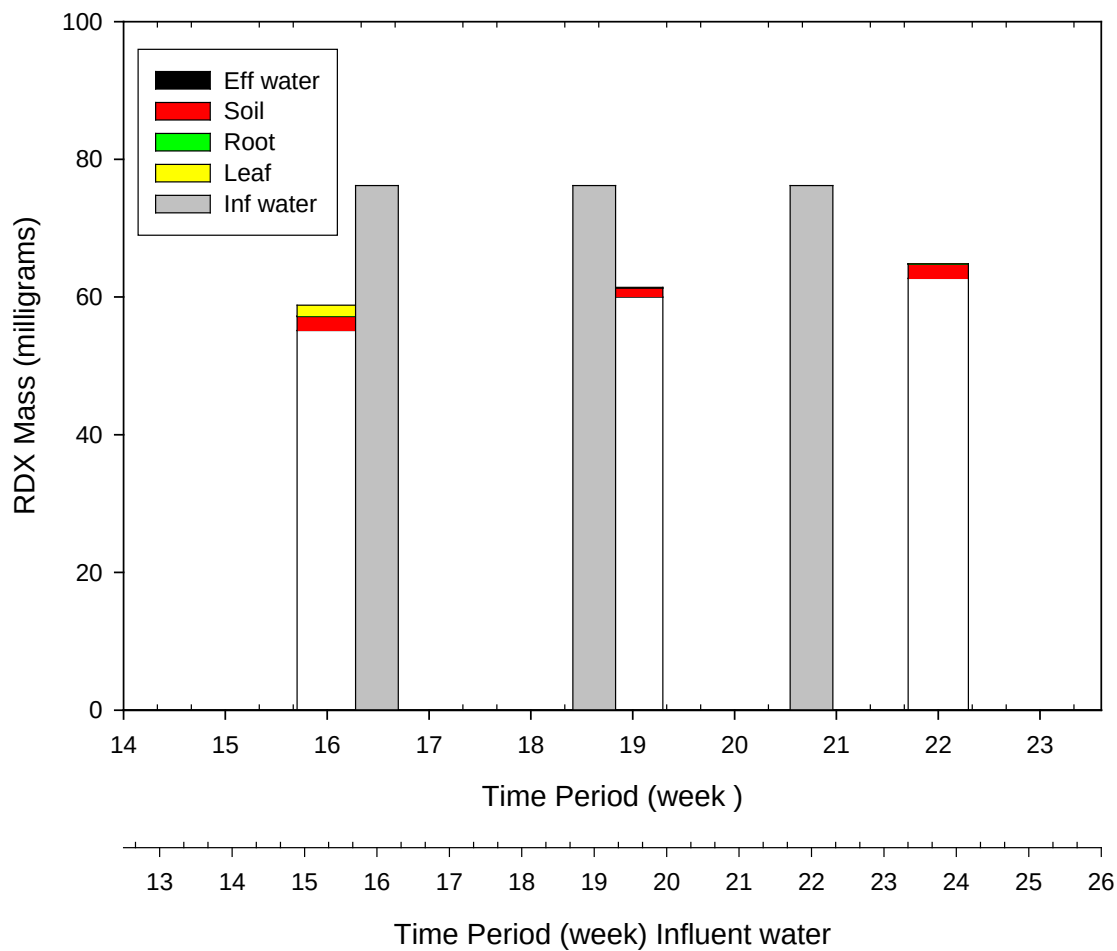


Figure 15.30 Amount of RDX in influent water, effluent water, soil, root and leaf of the plant exposed to 0.5 mg/l RDX up to the time point where the plant was sacrificed. Here, 19th and 22nd time point represent period of no exposure.

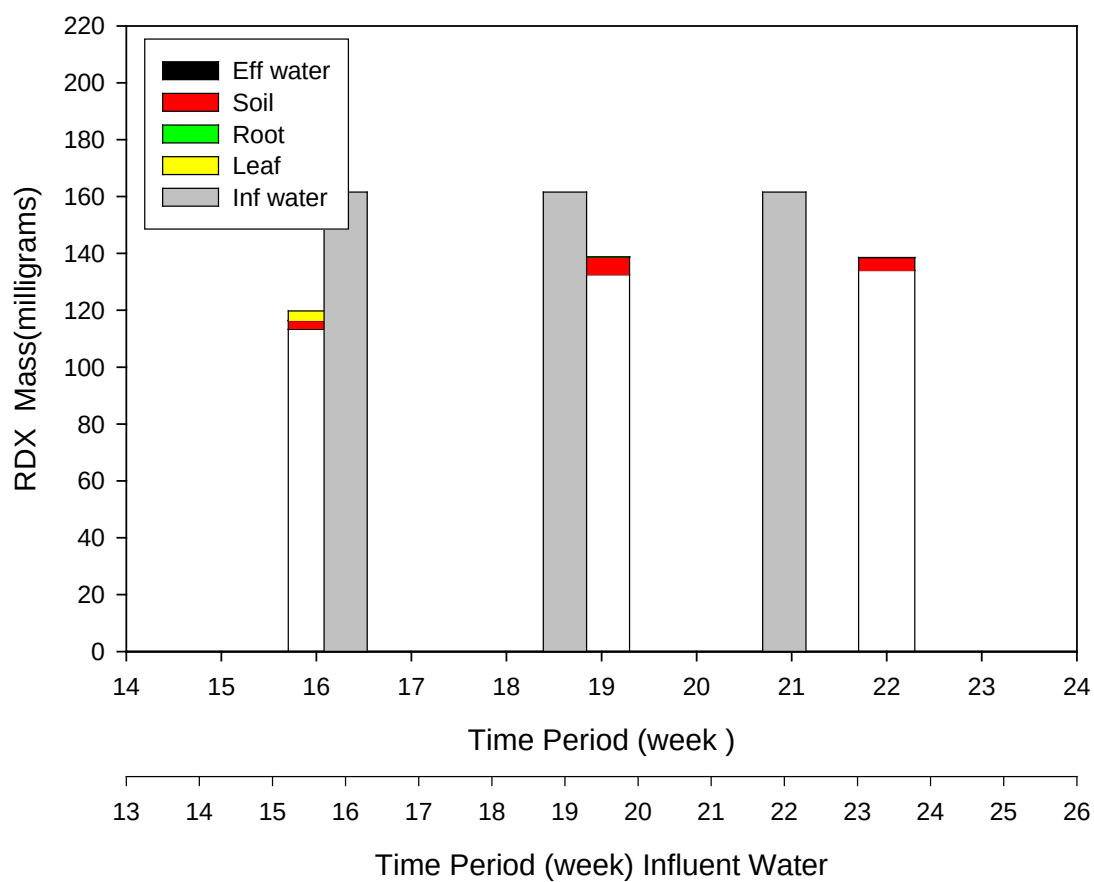


Figure 15.31 Amount of RDX in influent water, effluent water, soil, root and leaf of the plant exposed to 1 mg/l RDX up to the time point where the plant was sacrificed. Here 19th and 22nd time point represent period of no exposure.

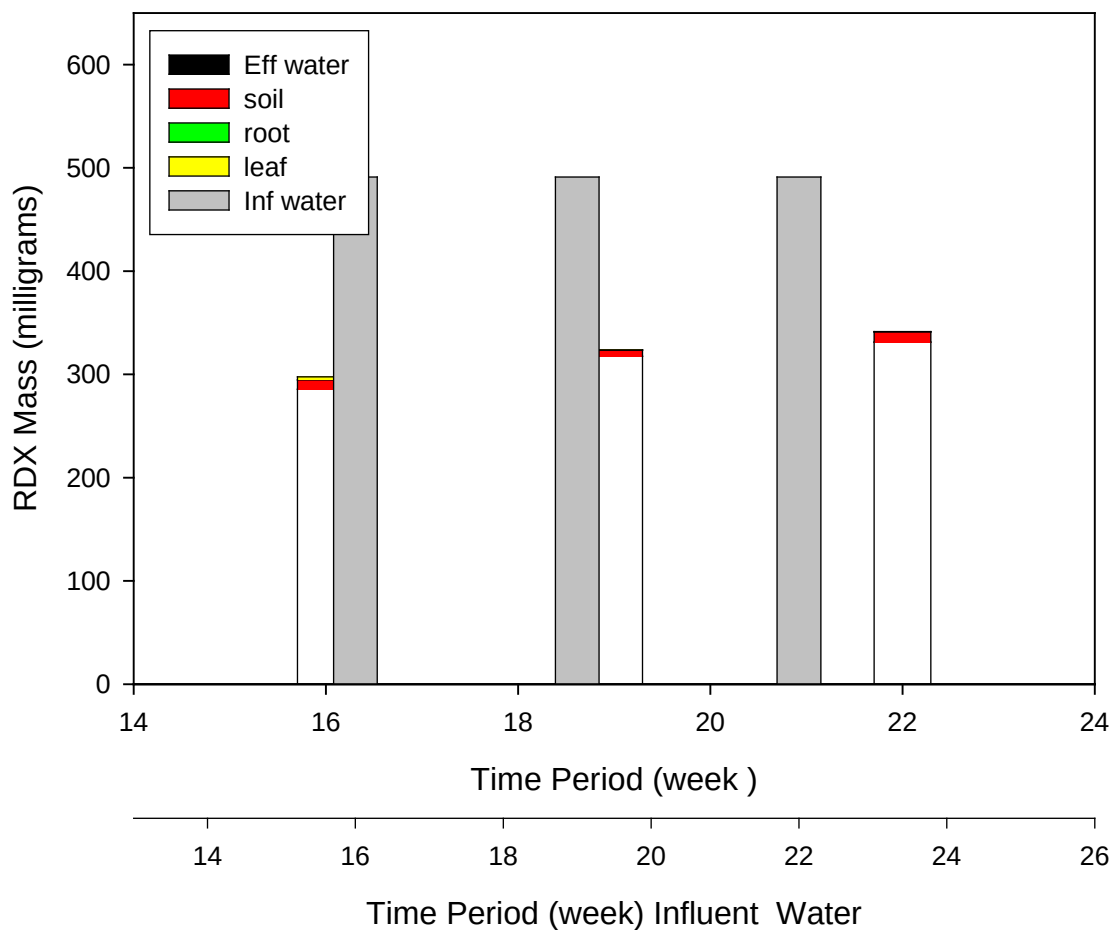


Figure 15.32 Amount of RDX in influent water, effluent water, soil, root and leaf of the plant exposed to 3 mg/l RDX up to the time point where the plant was sacrificed. Here 19th and 22nd time point represent period of no exposure.

15.2 Earthworm micro-RNA Biomarkers

15.2.1 Computational Identification of Earthworm miRNAs

Using the modified BLAST search, we identified 24 candidate microRNAs with homology to *C. elegans* miRNAs. The predicted secondary structures also fit the criteria outlined earlier (Ambros et al., 2003). Of these candidate miRNAs (**Table 15.2**), 21 of 24 were from *Lumbricus rubellus* genetic sequences, while 3 were from *Eisenia andrei* sequences contained in the earthworms.org database.

15.2.2 Experimental Verification of Earthworm miRNAs

Using quantitative PCR, we were able to experimentally verify several candidate miRNAs initially identified computationally. Conservatively, we identified 5 miRNAs from the 24 candidates (**Table 15.3**).

The relative expression of miRNAs less abundant than miR-11 was determined (**Figure 15.33**). The expression level of miR-11 was 260,000 times less than that of the housekeeping gene 18S and 54 times less than that of actin. miR-4, miR-7, miR-8, miR-9, miR-13, miR-15, miR-16, miR-17, miR-18 and miR-22 are either not expressed or expressed at levels too low to detect. miR-15, miR-19, miR-20, and miR-23 have high levels of background noise that make the accuracy of quantification uncertain. miR-5 is expressed at levels too low to detect.

Further confirmation of the identified miRNAs was conducted through an analysis of the specific amplification plots and dissociation curves, an example of which is shown in **Figure 15.34** for miR-11.

Table 15.2 Candidate miRNA sequences from *Lumbricus rubellus* or *Eisenia andrei* with homology to *Caenorhabditis elegans* miRNAs.

miRNA	Homologue	Sequence	Species
miR-1	cel-miR-45	ugacuagaaaacacauu <u>gaugc</u>	<i>E. andrei</i>
miR-2	cel-miR-262	guuucucogauguuu <u>acugau</u>	<i>L. rubellus</i>
miR-3	cel-miR-269	ggcaagacu-ug <u>acaagacu</u>	<i>E. andrei</i>
miR-4	cel-miR-353	c-auug <u>cu</u> cauguguu <u>uguauu</u>	<i>L. rubellus</i>
miR-5	cel-miR-60	uauuag <u>ucca</u> cauuuuc <u>uaguuua</u>	<i>L. rubellus</i>
miR-6	cel-miR-241	ugagguag <u>uugc</u> -agaaa <u>cuga</u>	<i>L. rubellus</i>
miR-7	cel-miR-234	ucauugcuogagaa <u>aaucuu</u>	<i>L. rubellus</i>
miR-8	cel-miR-359	cacugggucuuuc <u>ugagca</u>	<i>L. rubellus</i>
miR-9	cel-lsy-6	uuuugcaugag <u>cag</u> cauuuog	<i>E. andrei</i>
miR-10	cel-miR-272	<u>cugugu</u> -ggcaugggguuuuug	<i>L. rubellus</i>
miR-11	cel-miR-264	guog <u>ug</u> guuguuguu <u>gaug</u>	<i>L. rubellus</i>
miR-12	cel-miR-272	ugu <u>gu</u> ggcagggguuuuug	<i>L. rubellus</i>
miR-13	cel-miR-1	uggaauug <u>caa</u> agaagua <u>auua</u>	<i>L. rubellus</i>
miR-14	cel-lsy-6	uuuugcaugag <u>cag</u> cauuuog	<i>L. rubellus</i>
miR-15	cel-miR-262	guuucuc-a-guuuuc <u>ugagu</u>	<i>L. rubellus</i>
miR-16	cel-miR-262	<u>aa</u> uucucogauguuuu <u>cu</u> gau	<i>L. rubellus</i>
miR-17	cel-miR-78	ug <u>aa</u> ggcucgguguuuu-ug <u>ac</u>	<i>L. rubellus</i>
miR-18	cel-miR-60	uauu <u>gugc</u> acauuuu <u>au</u> -guuca	<i>L. rubellus</i>
miR-19	cel-miR-72	aggcacagauug <u>ggcu</u> agu	<i>L. rubellus</i>
miR-20	cel-miR-270	ggcaugaugu <u>cagcu</u> guuag	<i>L. rubellus</i>
miR-21	cel-miR-72	aggcacagauug <u>ggcu</u> agu	<i>L. rubellus</i>
miR-22	cel-miR-265	ugacggaggaaggg <u>augg</u> aa	<i>L. rubellus</i>
miR-23	cel-miR-77	uucaucaggcaca <u>g</u> -u-uoca	<i>L. rubellus</i>
miR-24	cel-miR-72	aggcacagauug <u>ggcu</u> agu	<i>L. rubellus</i>

Table 15.3 **Summary of miRNA determinations.**

Confirmed miRNAs	Expressed miRNAs (low background)	Expressed miRNAs (high background)	Expressed miRNAs (not quantifiable)	Undetermined miRNAs
mir-1, mir-2, mir-3, mir-10, mir-11	mir-6, mir-12	mir-14, mir-19, mir-20, mir-23	mir-5	mir-4, mir-7, mir-8, mir-9, mir-13, mir-15, mir-16, mir-17, mir-18, mir-22

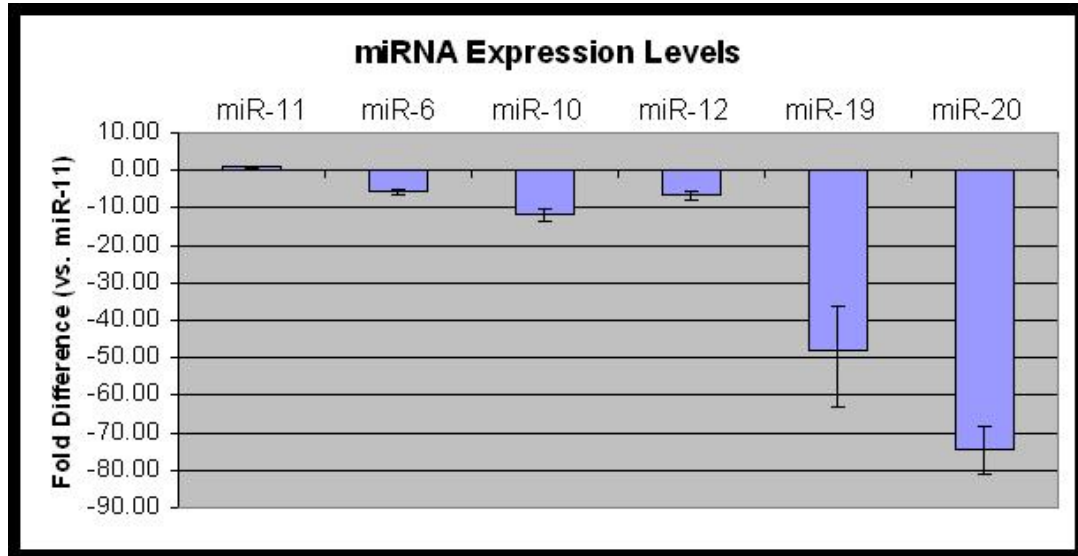


Figure 15.33 Relative expression of some of the identified miRNAs less abundant than miR-11.

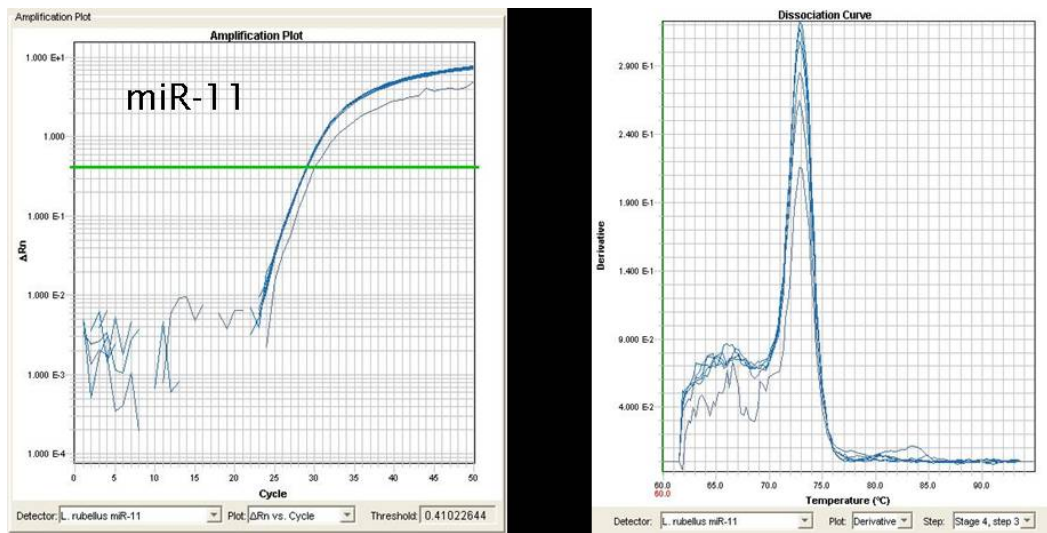


Figure 15.34 Amplification plot and dissociation curve for miR-11. Specific amplification is indicated by the presence of a single peak in the dissociation curve.

16.0 DISCUSSION

16.1 Plant Uptake Experiments

Plant growth was observed in the plants exposed to all the three concentrations of RDX and control plants until the 12th week. Growth rate of plants exposed to 3 mg/l of RDX was not as large compared to plants exposed to 0.5 and 1.0 mg/l RDX concentrations at all time points. Although no toxic symptoms like bleaching and necrosis were observed during the course of the experiment in any of the plants. In an earlier study adverse effects on growth in rice plant were observed only at high concentrations (20 mg/g DW) of RDX exposure (Vila et al., 2007b). However in another study no adverse effects of RDX to terrestrial plants were noticed even at 10,000 mg/kg RDX concentration in plant (Rocheleau et al., 2005). Phytotoxicity to plants varies with plant species, RDX accumulation and time of culture (Vila et al., 2007a; 2007b). Plant growth in control plants was less compared to the growth in plants exposed to RDX, irrespective of the exposure concentration. Similar phenomenon was observed in tomato plants probably because plants might be receiving nutrients like nitrogen from RDX resulting in a better growth in plants exposed to RDX compared to control plants (Price et al., 2002). It may be summarized that RDX exposure of around 1.0 mg/l does not have any adverse effects on bulrush and may even be supporting plant growth but exposure to 3.0 mg/l may adversely effect the growth of the plant.

In actively growing bulrush, it was observed that RDX accumulation increased with increase in exposure concentration during the given time frame. This is in agreement with previous studies (Low et al., 2008; Vila et al., 2007b). RDX accumulation in actively growing bulrush plants increased with time but in mature plants RDX accumulation remained more or less constant, suggesting that RDX accumulation also depends on plant growth. The amount of RDX accumulation was less in mature plants. This could be due to the fact that water uptake was as the experiment was conducted in the winter. RDX concentration in plants exposed to 3 mg/l stabilized from the 9th week at around 55 mg/kg. Leaf weight and accumulated RDX have increased beyond the 9th week till the experiment period of 12 weeks suggesting plant growth. From these results it appears that plant growth supports RDX accumulation in plants.

RDX concentrations occurred in the top portion of the leaf were substantially higher compared to RDX concentrations in all other portions of the plant in actively growing as well as mature bulrush. Previous studies have also shown that the top portions of the leaf accumulate RDX in larger quantities compared to other portions of the plant (Low et al., 2008; Vila et al., 2007a; 2007b; Price et al., 2002).

RDX concentration in plants drastically decreased during the period of no exposure (For RDX exposure of 3 mg/l, from 60 mg/kg to 3 mg/kg). About 97% of the RDX concentration in the top portion and 75% in bottom and middle portions of the plant was lost during the blank weeks of growth following cessation of RDX exposure. This is consistent with a previous study where it was observed that RDX concentrations in plant material substantially declined during the period of no exposure from 500 mg/kg to 80 mg/kg (Low et al., 2008). Photolysis of RDX from leaf tissues of the plant, permanent

sequestration of RDX into leaf tissues or translocation to the leaf tissues and subsequent photolysis could be some of the factors responsible for the drastic reduction in the RDX concentrations (Low et al., 2008).

16.2 Earthworm micro-RNA Biomarkers

Invitrogen's NCode™ miRNA Array system was successfully used to identify miRNAs expressed in *L. rubellus*, given conservation of miRNAs across similar species (*C. elegans*). Earthworms are an important group of organisms in assessing toxicity of chemicals in soil. However, the presence and function of miRNAs in earthworms to this point has not been studied. This study represents the first time that miRNAs have been determined in earthworms.

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:

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Rocheleau S., Martel M., Bardai G., Sarrazin M., Dodard S., Paquet L., Corriveau A., Kuperman R.G., Checkai R.T., Simini M., 2005. Toxicity of Nitro- Heterocyclic and Nitroaromatic Energetic Materials to Terrestrial Plants in a Natural Sandy Loam Soil. DTIC Accession Number: ADA437894

TITLE: Analytical Core

STUDY NUMBER: AC-07-01

SPONSOR: Strategic Environmental and Research
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RESEARCH INITIATION: September 2006

RESEARCH COMPLETION: August 2008

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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 Cobb
Principal Investigator

Date

- 1.0 DESCRIPTIVE STUDY TITLE:** Analytical Core
- 2.0 STUDY NUMBER:**
AC-07-01
- 3.0 SPONSOR:**
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- 4.0 TESTING FACILITY NAME AND ADDRESS:**
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- 5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start: 09/2006
Termination: 08/2008
- 6.0 KEY PERSONNEL:**
George P Cobb Principal Investigator
Todd A Anderson Co-Principal Investigator
Dr. Ronald Kendall Testing Facility Management
- 7.0 STUDY OBJECTIVES / PURPOSE:**
Provide sensitive and consistent analyses for researchers within our explosives research program.
- 8.0 STUDY SUMMARY:**
Residues of high explosives were quantified in biotic and abiotic samples using GC-ECD, GC-MS, LC-UV, LC-MS, and LC-MS-MS.
- 9.0 TEST MATERIALS:**
RDX
MNX
DNX
TNX
HMX

10.0 JUSTIFICATION OF TEST SYSTEM:

Detailed in the studies wherein these methods were applied.

11.0 TEST ANIMALS:

Detailed in the studies where these methods were applied.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Samples were analyzed as submitted from other studies within this research program. Samples were identified by project number, sample type and date.

13.0 METHODS:

We developed, validated, and implemented sensitive analyses that could be used in a wide array of situations. The methods began normally with extraction with appropriate polar organic solvents like methanol or aceto-nitrile, often accompanied by sonication or mechanical agitation. Interferences were then removed with solid phase extraction methods. Extracts were normally concentrated, although some matrices required dilution to avoid matrix interferences with LC-MS analyses.

14.0 RESULTS:

We have employed methods developed within previous phases to determine HMX, TNT, RDX, and RDX transformation products in tissues, water, sediment and dosing media. We used LC-MS and GC-ECD techniques for trace quantities, and we used HPLC-UV for dosing media.

We processed, prepared and verified dosing solutions for aquatic mesocosm studies and for vertebrate toxicity tests. We have also analyzed several hundred other samples for other vertebrate dosing studies.

15.0 DISCUSSION:

Our analytical efforts have been published in top analytically oriented Journals indicating the novelty and importance of this aspect of the TIEHH research effort (1-8). These techniques were also utilized in the performance of the vast majority of studies within the TIEHH explosives research program.

16.0 REFERENCES:

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TITLE: Mammalian Response To Ingestion Of High Explosives

STUDY NUMBER: MAM-07-01

SPONSOR: Strategic Environmental and Research
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RESEARCH INITIATION: September 2006

RESEARCH COMPLETION: August 2008

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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 Cobb
Principal Investigator

Date

Phil N Smith
Co-Principal Investigator

Date

- 1.0 **DESCRIPTIVE STUDY TITLE:** Mammalian Response To Ingestion Of High Explosives
- 2.0 **STUDY NUMBER:**
MAM-07-01
- 3.0 **SPONSOR:**
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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: 09/2006
Termination: 08/2008
- 6.0 **KEY PERSONNEL:**
- | | |
|------------------------------|---------------------------|
| George P. Cobb, Ph.D. | Principal Investigator |
| Philip N. Smith, Ph.D. | Co-Principal Investigator |
| Michael San Francisco, Ph.D. | Co-Principal Investigator |
| Dr. Ronald Kendall | Testing Facility Manager |
- 7.0 **STUDY OBJECTIVES / PURPOSE:**
- Determine the extent of reductive RDX transformations in different GI tract regions (stomach, small intestine and large intestine) of B6C3F1 mice.
 - Evaluate the *in vitro* transformation of the explosive, RDX, to N-nitroso metabolites by bacteria isolated from each of the: stomach, small intestine and large intestine of B6C3F1 mice.
 - Determine the LD₅₀ and associated slope factors for HMX exposure to prairie voles.
 - Determine if prairie voles are capable of converting RDX into N-nitroso metabolites.
- 8.0 **STUDY SUMMARY:**
Mice and voles were dosed with high explosives to determine toxicity and metabolism of these materials.

9.0 TEST MATERIALS:

RDX
MNX
DNX
TNX
HMX

10.0 JUSTIFICATION OF TEST SYSTEM:

Microbial transformation of RDX in the GI Tracts of B6C3F1 mice

In the natural environment, bacteria can sequentially reduce RDX into a series of N-nitroso metabolites: 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) (Lee et al., 2004; Beller, 2002; Hawari et al., 2000; Hawari et al., 2000a; Adrian and Arnett, 2004). A recent study in our lab also demonstrated RDX transformation into MNX and DNX in the gastrointestinal tracts of deer mice (Pan et al., 2007). Several studies demonstrated that MNX and TNX were more toxic than the parent RDX (Zhang et al., 2006, 2006a) and are more mutagenic than RDX in the Ames assay (Zhang et al., 2006, 2006a). Both types of study suggest that more data are needed on the toxicity of the N-nitroso metabolites of RDX. Also, when performing ecological and human health risk assessments of RDX, the biotransformation of RDX to its N-nitroso metabolites should also be considered.

Following a preliminary study to explore the transformation of RDX to its N-nitroso metabolites in the GI tract conducted in our lab (Pan et al., 2007), we designed this more comprehensive study with B6C3F1 mice intending to quantify the absorption, distribution, and biotransformation of RDX to its N-nitroso metabolites in several tissues including stomach, intestine, plasma, brain, and liver.

HMX Toxicity in Hindgut Fermenters

Background:

Numerous military training exercises require detonation of live or training munitions which can release residual chemicals into the environment. Energetic compounds such as octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are commonly found in soils and other environmental matrices at military training installations (Talmage et al., 1999). Heretofore, the ecotoxicological issues involving HMX contamination on military training installations have not been well defined or studied (see USACHPPM 2001). In this sub-project, we intend to fill ecotoxicological data gaps identified by the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) for HMX specifically related to mammals.

There appears to be little information on the toxicity of HMX to wildlife species in the open, peer-reviewed literature. Published studies have described the effects of HMX in

laboratory mammalian models, including rats and mice. Results of these studies indicate variable responses in lethal and sublethal effects. HMX is toxic to both mice and rats in an apparent sex-and species dependant manner. Death was noted in rats during a 14 day study at 9,000 ppm in males and 1,000 ppm in females (Army 1985a). Mice were reversed in the sensitivity of males and females, and responded at lower doses. Death was observed in male mice at 300 ppm and females at 800 ppm (Army 1985b). Other toxic responses in rats and mice were noted in longer studies, including reduced weight gain and food consumption, hematological alterations, liver and kidney pathology (Army 1985c and d). These observations varied among doses and rodent species, but overall, HMX is typically considered of low toxicity in mammals. However, rabbits have been found to be considerably more sensitive to HMX than rodents (Army 1985h). Mortality occurred following single doses of 100 mg/kg, but small sample sizes, lack of control animals, and potential confounding factors limit the utility of these data. Although these data are not considered definitive, they suggest that the digestive processes of hindgut fermenters (and perhaps ruminants) may increase the absorption and/or toxicity of HMX.

Results of HMX studies in rodents demonstrate the rapid nature of elimination of the compound. HMX in rodent plasma after 13 weeks of exposure via food was negligible and did not change with dose levels (Army 1985e). Single dose studies with ¹⁴C-HMX demonstrate this nature (Army 1986). Specifically, 85% of a single dose of HMX at 500 mg/kg in rats was eliminated in feces in 4 days (70% in mice). Similarly, 61% of HMX administered IV to rats was eliminated in urine in 4 days. HMX was rapidly metabolized to very polar metabolites and appears poor at accumulating in tissue after oral dosing. Concentrations of HMX were highest in liver, kidney, and brain.

Yet no data are available on the absorption or elimination of HMX in hindgut fermenting mammals. A number of factors can alter the absorption of xenobiotics including the presence of other chemicals, intestinal motility, intestinal residence time, age, and species differences (Rozman and Klaasen, 1996). In addition, interactions between food and toxicants can affect absorption across gastro-intestinal epithelia (Riviere, 1994). Therefore, we propose to examine the acute and sub chronic lethality of HMX in a hindgut fermenting mammal, the prairie vole (*Microtus ochrogaster*).

The stomachs of voles consist of two compartments, the esophageal pouch and the cecum. Although some limited fiber fermentation may occur in the esophageal pouch, the majority occurs in the cecum and the colon (Kudo and Oki, 1984). Voles are also coprophagous, extracting significant energy, vitamins, and minerals from second-pass digestion (Cranford and Johnson, 1989). Hindgut digestive strategies together with coprophagy may increase absorption and toxicity of HMX in voles.

11.0 TEST ANIMALS:

105 adult, female B6C3F1 mice (*Mus* variant),
45 Prairie voles (*Microtus ochrogaster*) adults, males.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Color coded cards on the fronts of animal cages within the Animal Care Facilities. Cards contained protocol number, investigator, test species, toxicant, and dose.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

See methods

14.0 METHODS:

Mouse GI Tracts

Chemicals

RDX used for dosing was obtained from Accurate Energetics (McEwen, TN, USA). The chemical was 99% pure and supplied in a desensitized form containing about 15%–20% water by weight. For use in instrumental analyses, a 1000 mg/L RDX (> 99% pure) stock solution in acetonitrile was purchased from Supelco (Bellefonte, PA, USA). Standards of MNX (> 99% pure), DNX (59% pure), and TNX (> 99% pure) were purchased as solids from SRI International (Menlo Park, CA, USA).

Dose preparation

RDX was applied to finely ground Purina Certified Rodent Chow® No. 5002 (Purina Mills, St. Louis, MO, USA), which was used as food for B6C3F1 mice. Five RDX doses were prepared including 0, 0.5, 5, 50, and 500 mg/kg in powdered mice chow. RDX was dissolved into acetone and then sprayed onto the mice chow to produce appropriate concentrations. For control groups, an equal amount of acetone was sprayed onto chow to serve as a vehicle control. Intensive manual mixing was performed using a trowel for at least 30 min per batch. Spiked chow was spread in a fume hood, where acetone was evaporated for 4 days before use. The actual RDX concentration in the chow for each exposure group was analyzed using accelerated solvent extraction (ASE) followed by gas chromatography with electron-capture detection (GC-ECD) as described previously (Pan et al., 2007).

Animal treatments and sample pretreatment

Female, virgin, B6C3F1 mice (9 -11 weeks of age) were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA). Mice were acclimated for 5 days and then randomly assigned to different treatment groups with twenty-one mice in each treatment. Mice were housed three per cage, and cages were located in an animal room with temperature ranging from 68 to 72 °F, 25-75% relative humidity, and 16:8-h light:dark cycle. Mice were provided RDX-spiked chow and tap water ad libitum. Mice, food, and drinking water were monitored daily. Animal use and handling protocols were in compliance with Texas Tech University Animal Care and Use Committee guidelines under protocol number 3006.

Exposure lasted for a full twenty eight days, and mice were euthanized on Day 29; mice were euthanized by CO₂ anesthesia and heart puncture. The stomach, intestinal tract,

liver, and brain were removed, weighed, transported to our analytical lab on dry ice, and stored at -80 °C pending analysis. Plasma was collected immediately after necropsy by centrifuging heparinized whole blood. The stomach (tissue and contents), intestine (tissue and contents), plasma, liver, and brain were extracted separately for RDX, MNX, DNx, and TNx determination.

Chemical extraction and cleanup

Pressurized liquid extraction (PLE) was employed to remove RDX and its metabolites from food, liver, brain, stomach, and intestine tissues according to methods reported previously (Zhang et al., 2005; Pan et al., 2005). Briefly, 2 g of food or 0.5-2.0 g of various tissue samples were mixed with ten times the sample weight of anhydrous sodium sulfate (Na₂SO₄). Then, the sample-Na₂SO₄ mixture was loaded into a 22-mL cell and extracted using a Dionex Accelerated Solvent Extractor (Model 200, Salt Lake City, UT). Static extraction was performed at constant temperature and pressure (100°C and 1500 psi). Each extraction began with a 5-min preheating step, followed by a single 5-min static extraction with acetonitrile. The extract (15-20 mL) was then purged from the cell and collected in a 60-mL glass vial. Extract volumes were reduced using rotary evaporation. Concentrated extracts were cleaned using preconditioned styrene-divinylbenzene (SDB) cartridges, filtered (0.2 µm) into a GC vial, and stored (- 20° C) prior to GC analysis.

For plasma, liquid extraction coupled with sonication was employed (Zhang et al., 2007). Briefly, 1.2 mL of acetonitrile was added to the 150 µL plasma sample, followed by rigorous mixing using a vortex-mixer for 1 min. Samples were sonicated using an ultrasonic water bath (Branson, Danbury, CT). During sonication, samples were mixed periodically with a vortex-mixer for 1 min every half hour. After liquid extraction for 2 hours, blood samples were centrifuged (3000 rpm) for 10 min. Supernatants were filtered (0.2 µm) before GC analysis.

Chemical analysis

Analysis of RDX and its N-nitroso metabolites was performed using an Agilent 6890 gas chromatograph (GC) equipped with an autosampler and an electron capture detector (ECD) (Agilent, Palo Alto, CA) according to previously reported methods (Zhang et al., 2005; Pan et al., 2005). Separation was performed with a 30-m × 0.25-mm id × 0.25 µm film thickness DB-5 column. Helium (99.999% purity) served as carrier gas at a constant linear velocity of 80 cm/sec. Argon:methane served as make-up gas for the detector. The oven temperature program began at 90 °C, held for 3 min, increased to 200 °C at a rate of 10 °C/min, then ramped to 250 °C at a rate of 25 °C/min. The injection port temperature was 170 °C, while the detector was 260 °C. A 2 µL standard or sample was injected in splitless mode. The ECD was operated in constant current mode.

Statistical analysis

Concentration data for RDX and its N-Nitroso metabolites in dosed diet, stomach, intestine, liver, brain, and plasma were processed using standard statistical software (SigmaPlot Version 8.0, and SigmaStat Version 2.03, SPSS, Chicago, Illinois, USA). Analysis of variance (ANOVA) and least significant difference (LSD) multiple

comparisons were conducted to compare the means of each treatment group. Data that were at or below the detection limit were assigned the concentrations reported by the GC analysis. Thus blank samples show concentrations in the tables and graphs.

Prairie Vole Studies

HMX LD50 Determination:

Adult male prairie voles were acquired from a colony at Texas Tech University. Vole masses ranged from 32-64g on the exposure dates. We conducted an acute lethality study by dosing four voles with HMX at increasing concentrations (0, 10, 50, 100, 250, 500, 1000, 1500, 2000, and 3000 mg/kg body mass). Dosing solutions of HMX were prepared by carefully dissolving HMX in polyethylene glycol on a warm hot plate. Voles were dosed via oral gavage using a blunt-tipped metal gavage needle attached to a Hamilton syringe. Vole survival was monitored for 2 days post-exposure to develop an estimate of the LD50. 48 hours post-exposure voles were euthanized and necropsied.

Prairie vole Conversion of RDX to N-nitroso metabolites:

Fifteen prairie voles were placed in individual cages and acclimated for two weeks. RDX-treated rabbit food was prepared at a low dose (10 mg/kg), and high dose (100 mg/kg) formulation. RDX-treated food was prepared by dissolving RDX in acetone and spraying the solution over a pre-measured amount (3kg for each group) of food followed by thorough mixing. Food intended for a control group was sprayed with acetone only. Treated food was spread over bench paper in a thin layer for three days to allow the acetone to evaporate. Concentrations of RDX in the food were determined prior to initiation of the study. Actual concentrations of the food were 0 mg/kg for the control group, 8.75 ± 0.8 mg/kg for the low dose, and 89.5 ± 7.6 mg/kg for the high dose formulation. Voles were randomly assigned to one of three treatment groups: control, low-dose, or high-dose. Voles were provided control or RDX-treated food for 14 days. Vole mass was recorded to monitor food consumption and weight gain (or loss). Mass of food consumed was recorded daily. Behavior of the voles was monitored at least every eight hours during the exposure to identify signs of toxicity or stress. Following fourteen days of exposure, voles were euthanized and necropsied. During necropsy, the upper stomach, lower stomach, small intestine, large intestine, lower bowel (large intestine to anus), liver, kidneys, brain, and blood sample were collected, placed in individual containers, labeled, and frozen immediately.

Tissue samples were homogenized with Na_2SO_4 (sodium sulfate), 10gm Na_2SO_4 per 1gm sample, to remove water. Homogenized samples were extracted using acetonitrile in an Accelerated Solvent Extractor. Quality control samples (ASE cells with Na_2SO_4 spiked with RDX 10ppm mixture) were included with each batch of samples extracted. Extracts were evaporated using either a roto-evaporator or pressurized air as required, and run through a clean-up procedure using either floricil, SDP, or CAT cartridges as required. After clean-up, samples were placed in 2mL amber vials and analyzed via GC-ECD. RDX and its breakdown products of MNX, DNX, and TNX were quantified.

15.0 RESULTS:

Mouse GI tracts:

RDX and metabolite residues in stomach and intestine

Stomach and intestine are the first organs that encounter RDX from contaminated food. Thus, the concentration of RDX and its N-nitroso metabolites: MNX, DNX, and TNX are high in those tissues (Table 1). Average RDX concentrations reached 35,900 $\mu\text{g/kg}$ in the stomachs of mice from the 500 mg/kg dose group. A linear trend line ($r^2 = 0.89$) described the relationship between doses and RDX concentrations in the stomach (Figure 1). MNX concentrations were not observed in the stomachs of mice consuming food with RDX concentrations below 5 mg/kg, but at higher exposures, MNX in stomachs increased from 21.0 to 489.9 $\mu\text{g/kg}$ in a dose-dependent fashion. MNX was also found at about 0.1 to 0.2% of RDX in the contaminated food. MNX/RDX ratios in stomachs were 0, 0, 1.1%, 1.5%, and 1.4% for 0, 0.5, 5, 50, and 500 mg/kg dose groups, respectively. This suggested that RDX was transformed to MNX in the stomach. This phenomenon is similar to our experimental results in deer mice (Pan et al., 2007). We also found DNX and TNX in the stomach but not in the dosed food. DNX concentrations were detected in the stomachs of mice from each non-control RDX dose group. TNX concentrations in stomachs were quantifiable in all doses containing 5 mg/kg or more.

Concentrations of RDX and its N-nitroso metabolites were largely reduced in the intestine. Average RDX concentrations were 86.2, 57.6, 108.6, and 2709 $\mu\text{g/kg}$ in the intestine for the 0.5, 5, 50, and 500 mg/kg dose groups, respectively. An exponential trend ($r^2 = 0.55$) described the relationship between doses and RDX concentrations in the intestine (Figure 2). MNX, DNX, and TNX were only found in the intestines of the 500 mg/kg group with concentrations equal to 13.9, 18.0, and 8.5 $\mu\text{g/kg}$, respectively.

RDX and metabolite residues in plasma

Nutrients and toxic compounds in the food are absorbed first into blood and then carried by blood to other tissues and/or organs. After 28 days of exposure, RDX was detected in plasma at average concentrations of 14.5, 16.0, 27.7, and 186.1 $\mu\text{g/L}$ for the 0.5, 5, 50, and 500 mg/kg groups, respectively. An exponential function ($r^2 = 0.69$) described the relationship between the doses and RDX concentrations in the plasma (Figure 3). MNX was detected in blood from mice in the 500 mg/kg group with an average concentration of 1.2 $\mu\text{g/L}$. Small amounts of DNX and TNX were also detected in the plasma and followed a dose-dependent pattern (Table 1).

RDX and metabolite residues in liver

RDX was detected in liver following a dose-dependent pattern with average RDX concentrations ranging from 123.0 to 233.0 $\mu\text{g/kg}$ after 28 days of exposure. Small amounts of MNX and DNX also were detected in liver. A linear trend line ($r^2 = 0.38$) was fitted to describe the relationship between doses and RDX concentrations in the liver (Figure 4).

RDX residue in the brain

RDX and its N-nitroso metabolites were detected in the brain of B6C3F1 mice (Table 1)

at concentrations ranging from 29.6 to 15,350 µg/kg. An exponential function ($r^2 = 0.85$) described the relationship between doses and RDX concentrations in the brain (Figure 5). MNX concentrations ranging from 5.4 to 165.1 µg/kg were observed in brain tissue of mice consuming food containing 5 mg/kg RDX or more. MNX concentrations in the brain were marginally described by MNX in the intestine ($p=0.062$). Brains of mice in all RDX dose groups contained average DNX concentrations of 4.5 to 38.3 µg/kg and average TNX concentrations of 6.3 to 9.7 µg/kg. DNX in brain was described by DNX in plasma ($p= 0.045$), but not by DNX concentrations in the intestine. TNX concentrations in brain were not correlated with TNX concentrations in plasma or intestine.

Prairie Vole Toxicity Tests:

A total of 44 voles were used in an attempt to identify the approximate LD50 for HMX in a hindgut fermenting species. Since other hindgut fermenters were thought to be quite sensitive to HMX, (mortality occurred in rabbits following single doses of 100 mg/kg, Army 1985h), we began our vole study with low doses. Although a single control, and a 10 mg/kg treated vole died, neither death was attributable to HMX, but rather difficulties associated with the gavage method. No further mortalities were observed at doses below 1000 mg/kg. At 100 mg/kg, one vole died, two at 1500 mg/kg, and three at 2500 mg/kg. There were no mortalities in voles treated with 3000 mg/kg HMX. No treatment groups experienced 100% mortality up to 3000 mg/kg, at which point our supply of male prairie voles was exhausted. Therefore, we were unable to generate, or estimate a median lethal dosage. In general, signs of HMX intoxication among voles were lethargy, extreme thirst, dispnea, limited response to stimuli, and partial paralysis.

RDX did occur in the gastrointestinal tracts of Prairie voles that ingested RDX (Tables 4&5). High concentrations occurred in the stomachs, but there was no obvious production of reductive transformation products in the GI tracts. There was also limited accumulation of RDX in the kidney and blood of the voles with slightly higher amounts of transformation products in these matrices (Table 6).

16.0 DISCUSSION

Mouse GI Tracts

Absorption and distribution of RDX in mice

Although many studies have been performed on the toxicity of RDX and its N-nitroso metabolites (Levine et al., 1990; Levine et al., 1981; Schneider et al., 1977; Meyer et al., 2005; Smith et al., 2006), RDX uptake and distribution data for mammals following continuous dietary exposure have been lacking. The average food consumption during our study was 7.1, 6.6, 6.2, 5.6, and 6.4 g/mice/day for 0, 0.5, 5, 50, and 500 mg/kg groups, respectively. The average daily RDX dose was 0, 3.25, 31, 280, and 3200 µg for 0, 0.5, 5, 50, and 500 mg/kg groups, respectively.

RDX in the stomachs of mice represented up to 25% of the average daily dose. It is possible that the highest concentrations found in the stomachs in each dose group reflect a short time between last food consumption and euthanasia. This possibility is quite likely since no clearance time was allowed between removal of food and euthanasia.

RDX was absorbed into the blood at ppb concentrations for all five dose groups. Schneider *et al* reported RDX concentrations in plasma of Sprague-Dawley (SD) rats (Schneider et al., 1977) at 1.1 and 13.8 $\mu\text{g/g}$ following intraperitoneal administration of 50 mg/kg and 500 mg/kg of RDX. In a parallel study where RDX was administered by gavage, RDX concentrations in plasma varied from 1.5 to 3 $\mu\text{g/g}$ during the 24 hr time period following 100 mg/kg dosing (Schneider et al., 1977). This suggests that more RDX was absorbed into the blood if RDX were administered via gavage or intraperitoneal injections. Thus, care must be taken when comparing data from different routes of administration.

In our study, RDX was also detected in liver, but only at ppb concentrations for all five treatment groups of mice and with tissue/plasma ratio ranges from 1.2 to 11.1. Low concentrations of RDX in the liver suggest that RDX is unlikely to accumulate there. This may occur because RDX enters the liver continuously but slowly through blood circulation, and the abundant hepatic enzymes are capable of degrading RDX rapidly. Water has also been used to administer a chronic RDX exposure to SD rats (Schneider et al., 1978). In that study, RDX was also found in liver at 0.80, 0.09, and 0.20 $\mu\text{g/g}$ after 30, 60, and 90 days of exposure. Their findings are comparable to those reported in our study.

We found considerable RDX concentrations in brain tissue, especially in the 500 mg/kg dose group. Ratios of RDX in tissue/plasma were 2.7, 2.9, 4.5, and 83.0 for 0.5, 5, 50, and 500 mg/kg, respectively. The increase in the tissue/plasma ratio of RDX concentrations suggests that RDX may progressively damage the blood-brain barrier as dose increases, allowing more RDX transfer into and accumulation in the brain. Relatively high RDX concentrations were also found in SD rat brain following gavage or intraperitoneal administration of RDX (Schneider et al., 1977). When SD rats received 100 mg/kg RDX by gavage, RDX concentrations in brain were 5.6 – 11.3 $\mu\text{g/g}$. RDX in brain was 3.7 and 29.5 $\mu\text{g/g}$ for 50 mg/kg and 500 mg/kg intraperitoneal administration of RDX (Schneider et al., 1977). Chronic exposure to RDX-saturated drinking water, produced RDX in the brain at concentrations of 0.59, 0.40, and 0.65 $\mu\text{g/g}$ after 30, 60, and 90 days of exposure (Schneider et al., 1977); data at the 30 d time point were comparable with uptake found in our study.

Biotransformation of RDX in mice

It is well understood that RDX can be transformed into a series of N-nitroso metabolites (MNX, DNX, and TNX) under anaerobic environmental conditions by several bacteria (Lee et al., 2004; Beller, 2002; Hawari et al., 2000; Hawari et al., 2000a; Adrian and Arnett, 2004). However, few reports describe the possible *in vivo* biotransformation of RDX to its N-nitroso metabolites in mammals. Recently, we found that RDX can be transformed into MNX, DNX, and TNX in deer mouse stomach and GI tract although this is not the major biotransformation pathway (Pan et al., 2007). In our current study, we also found significant amounts of the N-nitroso metabolites in the stomach and in other tissues. This confirmed that RDX can be transformed to its N-nitroso metabolites in mammals *in vivo*, mainly in the stomach. The extent of transfer for N-nitroso metabolites of RDX from the stomach to plasma and other organ tissues is important to understand

the toxic effects of RDX and the possible role played by reductive transformation products in overall toxicity. Occurrence of RDX and reductive transformation products in the brain is a critical finding, given the overt neurotoxicity caused by these compounds at high doses (Meyer, 2005; Smith et al., 2007). The existing data do not allow us to conclusively determine if MNX, DNX, and TNX are transferred into the brain directly, produced once RDX is in the brain, or some combination of the two. Further investigations to evaluate nitramine detoxification and transformation processes in the brain would allow better understanding of the presence and effects of RDX and its reductive transformation products.

It should also be noted that when RDX in the brain exceeds approximately 40 µg/kg, MNX and DNX concentrations in the brain increase in a dose-dependent fashion, while TNX concentrations remain constant. This implies that detoxification mechanisms cannot remove RDX fast enough to prevent accumulation of its reductive transformation product, MNX, at concentrations that are dose-dependent. Similarly MNX removal is sufficiently slow to allow dose-dependent DNX accumulation. However TNX concentrations appear once a threshold of RDX is present and TNX concentrations do not increase at increasing doses. This indicates detoxification mechanisms of TNX or its precursors that are not overwhelmed by the concentrations of TNX or precursors found in our study.

These data raise new questions regarding the nature of RDX toxicokinetics and the role that RDX, MNX, DNX, and TNX play in neurotoxicity. Given the fact that many military, demolition, and mining personnel are exposed to such explosive residues, and given the emerging information regarding neuropathies manifesting from chronic low-level exposures to neurotoxicants, these processes should be evaluated to determine the effects of RDX exposure on humans.

Prairie Vole Toxicity

Our results do not indicate that prairie voles, a hindgut fermenting species, are sensitive to HMX exposure. We observed no mortalities (associated with HMX treatment) at concentrations up to 500 mg/kg.

Metabolism data indicate that voles do not produce large amounts of RDX reductive transformation products in the GI tract, but may produce them in other parts of the body.

TABLES and FIGURES

Table 1. Concentrations of RDX and its N-nitroso metabolites in mouse tissues. Different capital letters represent significant differences among treatment groups for the same compounds in the same tissue.

Tissue	Dose mg/kg	N	RDX µg/kg	MNX µg/kg	DNX µg/kg	TNX µg/kg
			Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
Plasma	0	6	13.9 ± 1.1 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	0.5	6	14.5 ± 2.0 A	0.0 ± 0.0 A	0.9 ± 0.9 A	2.1 ± 1.0 AB
	5	6	16.0 ± 2.1 A	0.0 ± 0.0 A	3.9 ± 2.1 AB	0.7 ± 0.7 AB
	50	6	27.7 ± 3.6 B	0.0 ± 0.0 A	14.3 ± 6.8 B	1.5 ± 0.9 AB
	500	4	186.1 ± 71.5 C	1.2 ± 1.2 A	25.9 ± 8.6 B	3.6 ± 2.1 B
Brain	0	5	36.0 ± 2.2 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	0.5	5	29.6 ± 3.0 A	0.0 ± 0.0 A	4.5 ± 2.8 A	7.1 ± 1.8 B
	5	3	39.4 ± 2.2 A	5.4 ± 3.6 A	2.6 ± 3.1 A	7.4 ± 2.4 B
	50	4	113.3 ± 14.2 B	10.0 ± 3.4 A	7.4 ± 4.3 A	9.7 ± 0.8 B
	500	3	15350 ± 10950 C	165.1 ± 128.4 B	38.3 ± 21.1 B	6.3 ± 3.2 B
Liver	0	5	104.5 ± 6.5 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	0.5	5	123.0 ± 12.3 A	0.0 ± 0.0 A	23.1 ± 14.2 A	0.0 ± 0.0 A
	5	5	124.1 ± 4.5 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	50	5	133.4 ± 10.3 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	500	5	223.0 ± 73.0 B	19.4 ± 12.7 B	22.0 ± 13.5 A	0.0 ± 0.0 A
Stomach	0	5	83.4 ± 11.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	0.5	5	144.1 ± 9.3 A	0.0 ± 0.0 A	11.3 ± 10.1 A	0.0 ± 0.0 A
	5	5	1907 ± 1306 B	21.0 ± 14.9 A	8.8 ± 8.8 A	6.4 ± 6.4 A
	50	5	7682 ± 3879 C	118.1 ± 29.1 AB	50.9 ± 21.8 AB	18.7 ± 8.3 AB
	500	4	35900 ± 20820 D	489.9 ± 343.7 B	119.0 ± 64.7 B	36.1 ± 14.6 B
Intestine	0	3	49.2 ± 11.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	0.5	2	86.2 ± 33.6 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	5	5	57.6 ± 7.9 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	50	5	108.6 ± 42.5 A	0.0 ± 0.0 A	0.0 ± 0.0 A	0.0 ± 0.0 A
	500	4	2709 ± 970 B	13.9 ± 9.2 B	18.0 ± 6.9 B	8.5 ± 4.3 B

Table 2. Relationship of toxicant concentration in plasma and intestine versus toxicant concentrations in brain.

Toxicants	Regression equation^a	R²	P
RDX	$\log[\text{RDX}]_b = 2.4471 \times \log[\text{RDX}]_p - 1.4022$	0.9953	<0.0001***
	$\log[\text{RDX}]_b = 1.5751 \times \log[\text{RDX}]_i - 1.2457$	0.9733	0.002**
MNX	NA	NA	NA
	$\log[\text{MNX}]_b = 1.4989 \times \log[\text{MNX}]_i + 0.4619$	0.7374	0.062
DNX	$\log[\text{DNX}]_b = 0.8571 \times \log[\text{DNX}]_p + 0.1483$	0.7858	0.045*
	$\log[\text{DNX}]_b = 0.8126 \times \log[\text{DNX}]_i + 0.5552$	0.6434	0.102
TNX	$\log[\text{TNX}]_b = 1.2197 \times \log[\text{TNX}]_p + 0.3103$	0.5387	0.158
	$\log[\text{TNX}]_b = 0.1512 \times \log[\text{TNX}]_i + 0.7155$	0.0247	0.802

a- subscripts are defined as follows b=brain, p=plasma, i=intestine

* - P<0.05

** - P<0.01

*** - P<0.001

NA: Not applicable, because MNX concentrations were non-detectable in plasma

Table 3. Results of acute lethality test of HMX in Prairie voles (*Microtus ochrogaster*).

Vole #	Group	Sex	Dose Group (mg/kg)	Volume PEG (ml)	Vole Mass (g)	Outcome
1	A	M	0 (Control)	0.56	56	Died
2	A	M	0 (Control)	0.63	63	Survived
3	A	M	0 (Control)	0.45	45	Survived
4	A	M	0 (Control)	0.37	39	Survived
1	B	M	10	0.4	40	Survived
2	B	M	10	0.43	45	Survived
3	B	M	10	0.33	32	Survived
4	B	M	10	0.59	59	Died
1	C	M	50	0.41	41	Survived
2	C	M	50	0.39	39	Survived
3	C	M	50	0.46	46	Survived
4	C	M	50	0.51	51	Survived
1	D	M	100	0.48	48	Survived
2	D	M	100	0.48	48	Survived
3	D	M	100	0.64	64	Survived
4	D	M	100	0.49	49	Survived
1	E	M	250	0.58	58	Survived
2	E	M	250	0.39	39	Survived
3	E	M	250	0.4	40	Survived
4	E	M	250	0.58	58	Survived
1	F	M	500	0.49	49	Survived
2	F	M	500	0.44	44	Survived
3	F	M	500	0.37	37	Survived
4	F	M	500	0.33	33	Survived
1	G	M	1000	0.35	35	Survived
2	G	M	1000	0.38	38	Died
3	G	M	1000	0.33	33	Survived
4	G	M	1000	0.38	38	Survived
1	H	M	2000		34	Died
2	H	M	2000	0.31	31	Survived
3	H	M	2000	0.5	50	Died
4	H	M	2000	0.46	46	Died
1	I	M	1500	0.47	47	Survived
2	I	M	1500	0.39	39	Died
3	I	M	1500	0.4	40	Survived
4	I	M	1500	0.46	46	Died
1	J	M	2500	0.43	43	Died
2	J	M	2500	0.42	42	Died
3	J	M	2500	0.37	37	Survived
4	J	M	2500	0.37	37	Survived
1	K	M	3000	0.51	51	Survived
2	K	M	3000	0.62	62	Survived
3	K	M	3000	0.38	38	Survived
4	K	M	0 (Control)	0.4	40	Survived

Table 4. Occurrence (ng/g) of RDX and its transformation products in the stomachs of prairie voles ingesting RDX contaminated food.

Control	Upper Stomach		Lower Stomach	
	Mean	SD	Mean	SD
TNX	130.01	165.08	105.00	60.01
DNX	15.43	34.51	49.51	68.54
MNX	91.35	160.71	24.92	55.73
RDX	215.87	227.94	116.33	118.63
Low				
TNX	91.82	112.60	86.83	50.73
DNX	0.00	0.00	47.54	66.88
MNX	0.00	0.00	92.80	113.18
RDX	322.61	170.83	284.07	160.83
High				
TNX	52.46	39.27	97.05	61.73
DNX	52.31	39.03	73.12	68.63
MNX	165.79	77.99	257.34	144.88
RDX	1668.33	2049.24	50471.80	107717.85

Table 5. Occurrence (ng/g) of RDX and its transformation products in the intestines and colon of prairie voles ingesting RDX contaminated food.

Control	Upper Intestine		Lower Intestine		Large Colon	
	Mean	SD	Mean	SD	Mean	SD
TNX	62.29	36.57	22.88	51.15	70.52	17.74
DNX	68.91	10.60	0.00	0.00	16.91	10.89
MNX	54.43	30.95	22.82	51.03	37.90	17.41
RDX	27.95	38.35	199.00	202.59	42.46	26.81
Low						
TNX	80.04	35.66	78.58	46.59	40.66	14.30
DNX	46.72	31.75	0.00	0.00	15.25	6.16
MNX	14.28	28.57	15.68	35.07	15.16	22.22
RDX	67.26	11.79	210.57	262.43	31.42	17.02
High						
TNX	41.67	39.19	36.95	55.31	65.08	39.03
DNX	51.17	30.14	41.28	64.79	12.53	8.77
MNX	9.60	21.48	44.78	69.53	19.74	20.53
RDX	269.99	143.52	176.81	77.99	25.88	12.30

Table 6. Occurrence (ng/g) of RDX and its transformation products in the blood and kidney of prairie voles ingesting RDX contaminated food.

Control	Blood		Kidney	
	Mean	SD	Mean	SD
TNX	4.47	6.12	5.94	8.14
DNX	13.01	8.58	0.00	0.00
MNX	23.45	8.74	5.82	7.97
RDX	3.20	7.16	7.99	7.30
Low				
TNX	0.00	0.00	0.00	0.00
DNX	40.34	44.44	0.00	0.00
MNX	62.87	58.01	5.71	7.82
RDX	9.97	15.07	8.53	7.83
High				
TNX	0.00	0.00	2.84	6.35
DNX	12.63	3.38	3.00	6.70
MNX	18.61	5.69	9.10	8.31
RDX	6.83	7.01	18.85	4.29

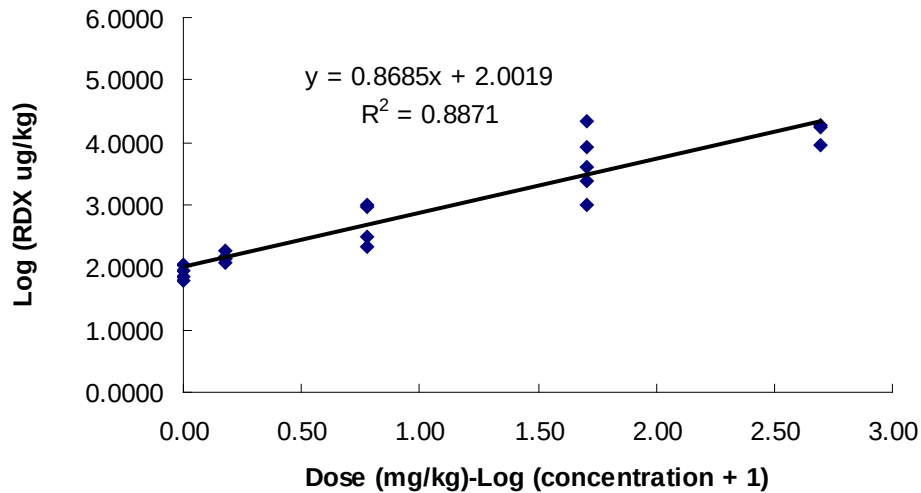


Figure 1. The relationship of log (dose (mg/kg) + 1) and log (RDX μ g/kg) in the stomach of B6C3F1 mice after 28 days of RDX dietary exposure.

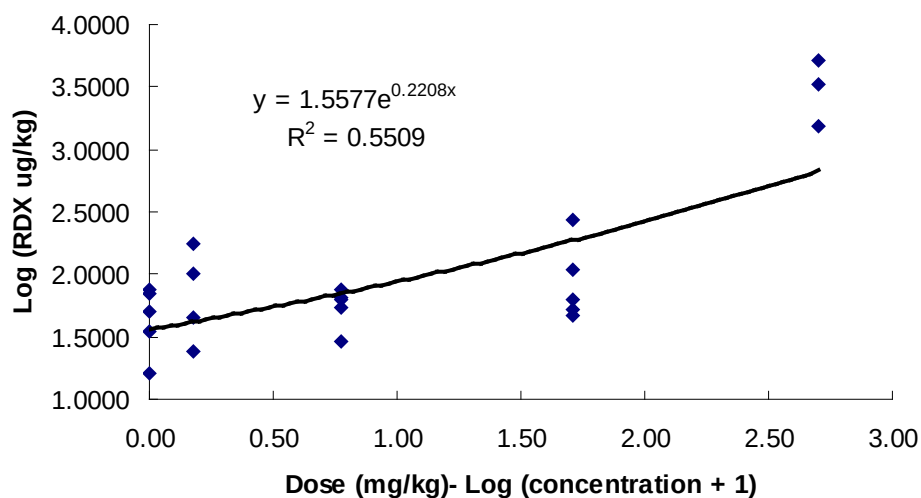


Figure 2. The relationship of log (dose (mg/kg) + 1) and log (RDX μ g/kg) in the intestine of B6C3F1 mice after 28 days of RDX exposure in diet.

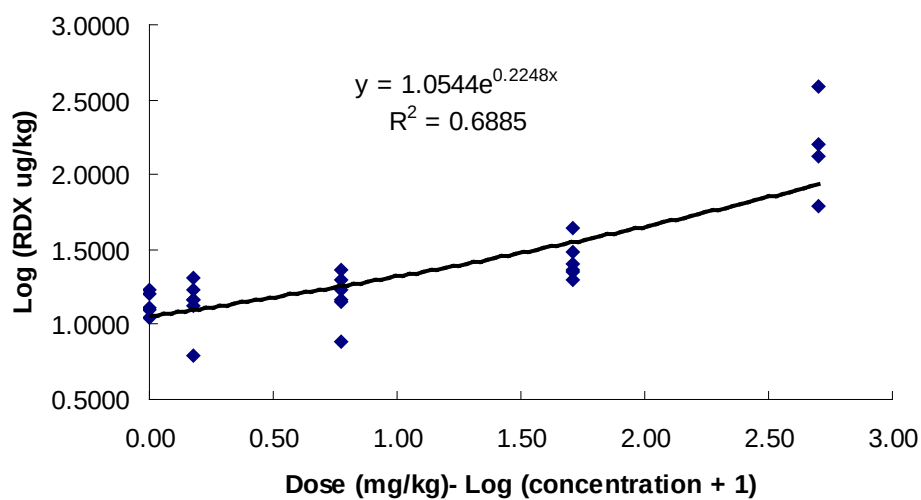


Figure 3. The relationship of log (dose (mg/kg) + 1) and log (RDX μ g/kg) in the plasma of B6C3F1 mice after 28 days of RDX exposure in diet.

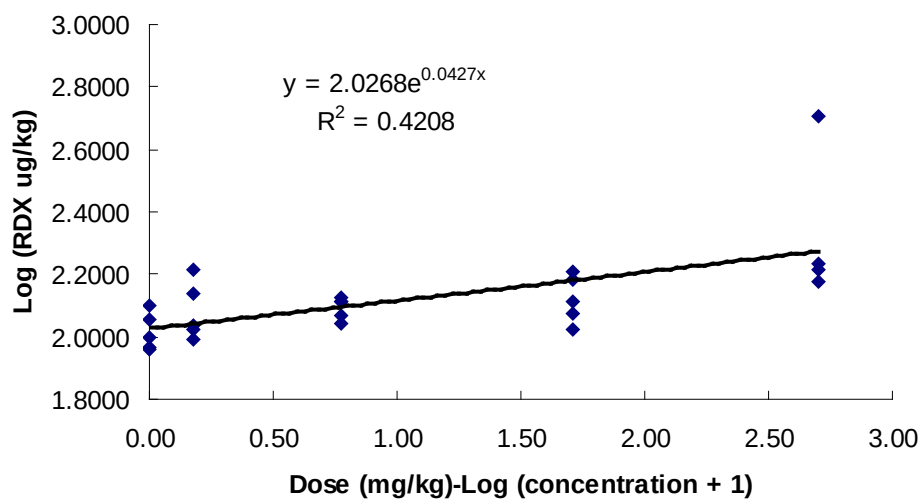


Figure 4. The relationship of log (dose (mg/kg) + 1) and log (RDX μ g/kg) in the liver of B6C3F1 mice after 28 days of RDX exposure in diet.

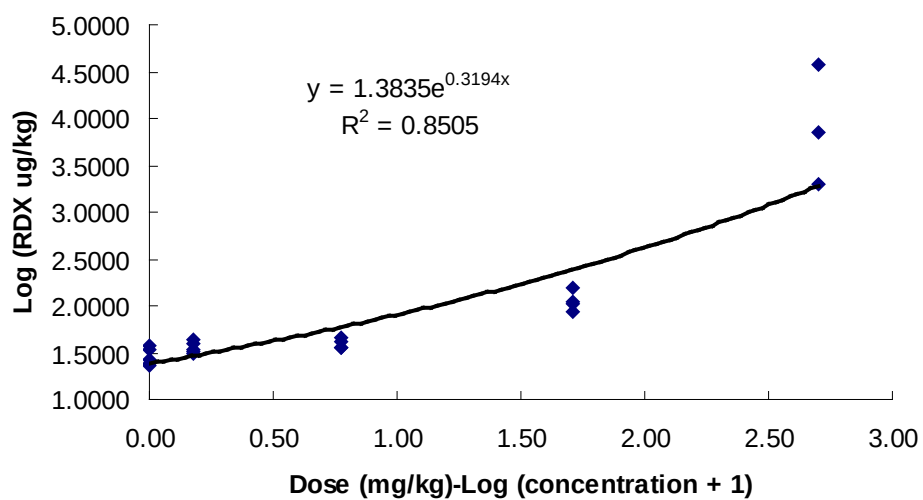


Figure 5. The relationship of log (dose (mg/kg) + 1) and log (RDX μ g/kg) in the brain of B6C3F1 mice after 28 days of RDX exposure in diet.

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TITLE: Effects of 2,4-DNT and 2,6-DNT on *Xenopus laevis* and *Rana catesbeiana*

STUDY NUMBER: TNT-07-01

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ANIMAL TEST SITE: The Institute of Environmental and Human Health
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RESEARCH INITIATION: January 2006

RESEARCH COMPLETION: May 2008

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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
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:

Brian Birdwell
Quality Assurance Manager

Date

1.0 DESCRIPTIVE STUDY TITLE: Effects of 2,4-DNT and 2,6-DNT on *Xenopus laevis* and *Rana catesbeiana*.

2.0 STUDY NUMBER: TNT-07-01

3.0 SPONSOR:

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SERDP Program Office
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4.0 TESTING FACILITY NAME AND ADDRESS:

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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: January 2006
Termination Date: May 2008

6.0 KEY PERSONNEL:

Ernest Smith, Principal Investigator
Mike Wages, Study Director
Ronald Kendall, Testing Facility Management
Brian Birdwell, Quality Assurance Manager

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the acute toxicity and effects of 2,4-DNT and 2,6-DNT on growth and development using *Xenopus laevis* and *Rana catesbeiana*.

8.0 STUDY SUMMARY:

Acute toxicity study

Xenopus laevis

Xenopus laevis larvae were exposed in the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) (ASTM E1439-98) to 2,4-DNT or 2,6-DNT in separate exposures. Each exposure consisted of five replicates of one control group, and 6 concentrations of 2,4-DNT or 2,6-DNT. *Xenopus* larvae were exposed to these contaminants starting at Nieuwkoop-Faber (NF) stages 8-10. Exposure was terminated at stage 46 (between 72 and 96 hours) for 2,4-DNT and 2,6-DNT. During the exposure and at termination, the number of dead and malformed larva was counted. Larva found dead before termination of the experiment were removed and placed in 10% buffered formalin. Developmental retardation and other abnormalities were associated with the exposure to these contaminants. A 96-hour LC₅₀ of 17.5 mg/L for 2,4-DNT and a 72-hour LC₅₀ of 22.2 mg/L for 2,6-DNT was calculated for *Xenopus laevis* based on observed lethality.

Rana catesbeiana

Rana catesbeiana larvae were exposed using the approach of the FETAX assay with instant ocean as the media with 2,4-DNT or 2,6-DNT in separate experiments. The exposure consisted of five replicates for the control group and 6 concentrations of each chemical. *Rana* larvae were exposed to these contaminants starting at Gosner stage 24-26 (Gosner, 1960). Exposure was terminated at 96 hours. During the exposure and at termination, the number of dead and malformed larva was counted. Larvae found dead before termination of the experiment were removed and placed in 10% buffered formalin. Data from the range finding indicated that 2,4-DNT was lethal above 20 mg/L and 2,6-DNT had similar effects at 40 mg/L. It is clear that our results suggest that 2,4-DNT and 2,6-DNT, at the concentrations used in this study, appear to induce developmental toxicity to *Rana catesbeiana* larvae; during the early window of development. Toxic effects seen in response to exposure to these chemicals appear to perturb biochemical homeostasis that might be related to critical regulatory pathways in the early developmental stage.

Up and Down Procedure:

Xenopus laevis-Adults

Adult male and female *Xenopus laevis* were exposed via a single intraperitoneal injection using the OECD Up and Down Procedure (OECD, 2001). An LD₅₀ of 620.4 µg/g was calculated for 2,4-DNT for *Xenopus*. The LD₅₀ for 2,6-DNT was 350 µg/g for male *Xenopus* and 1320 µg/g female *Xenopus*.

Rana catesbeiana –Adult Males:

Adult male *Rana catesbeiana* were administered a single dose by oral gavage using the OECD Up and Down Procedure (OECD 2001). An LD₅₀ of 1,098 µg/g was calculated for 2,4-DNT and 2,6-DNT in the adult male bullfrog. Both compounds elicited similar symptoms of toxicity including changes of skin color, body weight, development of seizures, liver and kidney necrosis, and lung cyanosis. Relative organ weights did not show significant change (Paden et al. 2008).

9.0 TEST MATERIALS:

Test Chemical name: 2,4-dinitrotoluene (2,4DNT)

CAS number: 121-14-2

Characterization: Determination of concentration in water samples.

Source: Alfa Aesar

Test Chemical name: 2,6-dinitrotoluene (2,6DNT)

CAS number: 606-20-2

Characterization: Determination of concentration in water samples.

Source: Alfa Aesar

Xenopus Reference Chemical:

Reference Chemical name: FETAX medium was prepared using distilled, carbon filtered water and reagent grade salts (NaCl, 10.7 mM; NaHCO₃, 1.14 mM, KCl, 0.4 mM; CaCl₂, 0.14 mM; CaSO₄, 0.35 mM; MgSO₄, 0.62 mM).

CAS Number: Not applicable

Characterization: Determination of pH and conductivity.

Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water, FETAX salts were added.

Rana Reference Chemical:

Reference Chemical name: Instant Ocean Sea Salt medium was prepared using distilled, carbon filtered water and 0.36g/L Instant Ocean Sea Salt.

CAS Number: Not applicable

Characterization: Determination of pH and conductivity.

Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water, Instant Ocean Sea Salt was added.

10.0 JUSTIFICATION OF TEST SYSTEM:

2,4 DNT and 2,6 DNT are metabolites of TNT that are often found in the environment. Characterization of their toxicity is limited and currently, there is no benchmark data for these chemicals in amphibians. Amphibians were used in this study because they are particularly sensitive to contaminants, the effects of which may be manifested as developmental abnormalities, lethality, or other toxic responses that may occur (ASTM, 1998). Recent evidence has also suggested that exposure to contaminants increases amphibian's susceptibility to effects of other environmental agents (Burkhart et al., 1998). Also there has been a worldwide decline in population and a high rate of occurrence of deformities in various species of amphibians (Pechmann et al. 1991, Kavlock 1998).

11.0 TEST ANIMALS:

Species: *Xenopus laevis* (African clawed frog)

Strain: Outbred

Age: embryo/Larvae/Adults

Source: All of *Xenopus* used in this proposal were bred from captive stocks currently maintained in our laboratory.

Species: *Rana catesbeiana* (American bullfrog)

Strain: wildtype

Age: embryo/Larvae/Adults

Source: Embryo and larvae were obtained from in-house breeding of adults purchased from Rana Ranch. Adults were purchased from the same source.

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 and aquaria were labeled with species name, animal use protocol number, project number, test system, and date of hatch. Glass aquaria for the Up and Down Procedure were similarly labeled.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Acute toxicity study-Xenopus laevis

Xenopus larvae were exposed to seven concentrations of 2,4DNT and 2,6DNT in separate experiments, including absolute control. Nieuwkoop -Faber [NF] stage 8-10 embryos were placed into clean Petri-dishes containing 10 ml FETAX (control) or treatment solutions. They were allowed to hatch and develop for 96 hours (or until reaching stage 46) while being exposed to toxicants. For exposures, dishes were placed in a 30-gal. aquarium containing plain tap water 4 inches deep. Plastic grids were used to create a platform on which the Petri dishes were seated. This prevented them from sitting directly in the water. The aquarium was covered with a light cover and placed in a controlled-temperature room. The arrangement of the dishes in the aquarium was randomized in order to avoid effects due to microenvironment. Media was changed each day. Larvae were monitored daily. Dead or moribund individuals were removed and stored in 10% buffered formalin. At the end of the exposures all tadpoles were euthanized in 0.5 g/L MS-222 and stored in 10% buffered formalin for gross morphology observations including axial, gut, optic, heart, and head malformations, as well as edema.

Acute toxicity study-Rana catesbeiana

The test system consisted of six treatment groups and one control for each chemical. Each treatment group and the control were replicated twice in the range finding studies and five times in the definitive studies and once in the chronic study. Larvae were maintained in a 112 L tank with 84 L of the medium. On posthatch day-8, tadpoles were transferred to 9L tanks (30 per tank) containing 6L of the medium. Seven treatments and three replicates per treatment were performed for each chemical (2,4 DNT and 2,6 DNT) during 90 days. The exposure concentrations were determined based on a previous 96-h range finding study on larvae. Developing tadpoles were monitored daily for changes in health conditions (number of abnormal individuals). Animals found dead or moribund were recorded and removed from the study and preserved in 10 % buffered formalin for gross morphology observations including dorsal flexure, curved tail, incomplete coiling, deformed head, as well as optic and abdominal edema. Abnormal swimming was also recorded daily and forelimb emergence was recorded every day. Food was provided to tadpoles after each water change. Aeration system and water bath temperature was also checked every day.

In the chronic study a total of 60 tanks of 9L tanks were set up in living streams containing water with a temperature ranging from 25 to 29° C. Contents of the test tanks were aerated continuously throughout the study period in order to provide adequate amount of oxygen for the tadpoles. Fifty percent water changes were performed every 72 hours. All test tanks, nets, and glassware to prepare the stock solutions were color coded in order to properly identify the treatment tanks and avoid cross contamination of test compounds during water changes. After 90 days of exposure survivors were removed from the tanks and euthanized by immersion in MS-222 (3-amino benzoic acid ethyl ester), rinsed in distilled water, and immediately weighed, and measured. Animals were given an identification number that included the study number, tank number, and animal

number. A middle incision was made in big specimens to allow the penetration of the fixative. Animals were divided into groups for respective analysis. For the histological analyses tadpoles were placed in plastic cassettes and placed in Bouin's fixative for 48 hours followed by storage in 70 % ethanol. For chemical analyses, tissues were stored on dry ice and then placed at -80° C until analyzed.

Up-and-Down Procedure

Adult *Xenopus laevis* (male and female) and male bullfrogs were exposed in covered 2.5 gallon glass tanks.

Test animals were dosed in a sequential manner starting with a preliminary dose in the range of anticipated toxicity. Based on the initial response, the dose was increased or decreased at half log intervals (a factor of 3.2-fold) to reach a dose that resulted in a reversal of response – i.e., if initial doses were non-lethal, the dose was increased until a lethal dose is reached. Alternatively, if the initial dose proved lethal, decreasing doses were administered until a non-lethal dose occurred. In either case, once the reversal (change in survival) occurred, the dose reverted back to the previous dose until another reversal occurs. This continued until one of 5 different criteria were met, a process that was guided by software provided by the EPA (AOT 425). These tests allowed the collecting of information such as LD50s, survival (percentage of mortality) and toxicity symptoms before death (gasping, loss of righting reflex, erratic swimming, and disorientation).

Dosing for the bullfrogs was by oral gavage using polyethylene glycol (CAS # 25322-68-3) as a carrier. Controls were dosed with PEG only.

Adult *Xenopus laevis* were exposed to a single intraperitoneal injection of 2,4DNT or 2,6DNT dissolved in DMSO (Dimethyl sulfoxide). Due to the stomach capacity of the *Xenopus* (.39ml/40g frog) and to accommodate the required higher concentration and volume during exposure, we utilized IP injections. In addition, DMSO was used instead of PEG since both 2,4-DNT and 2,6-DNT are more soluble in DMSO and thus facilitated the exposure at the required concentrations at a lower volume. Controls were exposed to DMSO and IP injection.

14.0 METHODS:

14.1 Animal Selection

Adult *Xenopus* frogs were selected from in-house breeding colonies and adult bull frogs were purchased from Ranch Bullfrog farm, Idaho. Mating pairs from our in-house stock were used as the source for larvae.

Assignment of Animals to Study Group and Identification

Larvae were placed into Petri dishes labeled with the name and test chemical concentration, the study protocol number, the animal use protocol number, and a unique identification for each Petri dish. Identification was by test group since identification of individual animals is not possible at this stage of development. Tanks for the Up and

Down procedure were each labeled with the individual frog ID number.

14.2 Acclimation

No acclimation was necessary for the adult *Xenopus* and bullfrogs used in the Up and Down Procedure since they were maintained in aged tap water before and during the Procedure.

Xenopus breeders for the FETAX assay were acclimated from aged tap water to FETAX over a week period.

Rana catesbeiana breeders were maintained and bred in deionized water with .46g/L Instant Ocean Sea Salt (Aquarium Systems, Inc).

14.3 Animal Husbandry and Test Material Application

Acute toxicity study- Xenopus laevis

Xenopus larvae were kept in FETAX solution, specifically formulated for the *Xenopus* larvae at this stage of development, according to ASTM (1998) or FETAX containing the dissolved test chemical. The FETAX or test solution was changed every 24 hr. The Petri dishes were kept in 30 gal aquarium with water at a level of 4 inches. Plastic grids were used to create a platform on which the Petri dishes were seated. This prevented them from sitting directly in the water. A water heater was kept in the water bath. This maintained the ambient temperature at 23° C for the 2,4-DNT. Ambient room temperature was higher for the 2,6-DNT, resulting in tank temperatures of 24-26° C and faster growth rates. The 2,6-DNT control larvae reached stage 46 in 72 hours.

Test solutions consisted of 2,4-DNT and 2,6-DNT at concentration from 0-60 mg/L dissolved individually in control medium (FETAX). Sufficient amount of each test solution was prepared to last the duration of the experiment. Embryos (Nieuwkoop - Faber [NF] stages 8-10, (Nieuwkoop and Faber 1998) were placed into pre-cleaned Petri dishes containing the appropriate test solution or FETAX. These were allowed to develop for 96 hours while being exposed to the 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. In addition to the chronic study described below, the overall experimental design consisted of range finding and definitive tests, in which the larvae were exposed to 2,4 DNT and 2,6 DNT according to the following scheme:

Range finding tests

There were 10 larvae/replicate x 2 replicates per treatment x 7 treatments per chemical. Treatment groups consisted of non-treated controls (FETAX) and 6 concentrations 2,4 DNT and 2,6 DNT.

Definitive tests

There were 10 larvae/replicate x 5 replicates per treatment x 8 treatments for 2,4 DNT

and 2,6 DNT. Treatment groups consisted of non-treated controls (FETAX) and 6 or 7 concentrations of 2,4 DNT or 2,6 DNT. The Petri dishes were kept in 30 gal aquarium with water at a level of 4 inches. Plastic grids were used to create a platform on which the Petri dishes were seated. This prevented them from sitting directly in the water. A water heater was kept in the water bath. This maintained the ambient temperature at 23°C.

Up and Down Procedure

Xenopus were kept in 2.5 gallon aquaria in 4L of aged tap water. A 100% water change was done every other day.

Acute toxicity study-Rana catesbeiana

Test solutions consisted of 2,4-DNT (0-35 mg/L), 2,6 -DNT (0-60 mg/L) dissolved individually in control medium (Instant Ocean solution). Sufficient amount of each test solution was prepared to last the duration of the experiment. Embryos at Gosner stages 24-26 (Gosner, 1960) were placed into pre-cleaned Petri dishes containing the appropriate test solution or Instant Ocean solution. These were allowed to develop for 96 hours while being exposed to the 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. The overall experimental design consisted of range finding tests, in which the larvae were exposed to 2,4-DNT and 2,6-DNT according to the following scheme:

Range finding tests

There were 10 larvae/replicate x 2 replicates per treatment x 7 treatments per chemical. Treatment groups consisted of non-treated controls (Instant Ocean solution) and 6 concentrations 2,4-DNT and 2,6-DNT. Eggs were kept in 20ml Instant Ocean solution (0.36gm/L). The Instant Ocean solution or test solution was changed every 24 hr. The larvae used for 2,4-DNT and 2,6-DNT were kept in 100ml solution because of their size.

Definitive tests

There were no definitive tests. We were unable to obtain more eggs or larvae.

Chronic study: Adult male and female bullfrogs were purchased from Ranch Bullfrog Farm, Twin Falls, ID, USA. Animals were maintained in an 888 L tank containing 118 L of medium and acclimatized for one week on a 12:12 h light:dark regime at 20-22 °C. Four animals were transferred to 112 L glass tanks containing 28 L of the same type of medium. Frogs were fed large live crickets three times a week immediately after a water change. Naturally fertilized eggs were obtained from 2 pairs of adults and transferred to 112 L glass tanks containing 84 L of the medium and maintained at 20-22° C on a 12:12 h light: dark regime. Starting on post hatch day 8, larvae were fed rabbit pellets LabDiet, St. Louis, MO, USA every 72 h immediately after 50 % water change. Lettuce was also provided as an additional source of food in order to avoid predation. Animal care and maintenance followed the animal protocol approved by the Institutional Animal Care and

Use Committee of Texas Tech University (ACUC # 05049-09). Water temperature and conductivity in the tanks averaged 22.19 °C (ranging from 21.02 - 23.52 °C) and 0.68 mS/cm (ranging from 0.65 - 0.73 mS/cm) respectively. Salinity and dissolved oxygen values averaged 0.33 mg/L (ranging from 0.32 - 0.35 mg/L) and 7.5 mg/L (6.08 - 8.4 mg/L), respectively, while pH and ammonia averaged 6.8 (ranging from 6.54 - 7.06) and 0.58 mg/L (ranging from 0.15 - 1.29 mg/L).

The stock solution (100 mg/L) for each chemical was prepared every 72 hours. The actual concentrations of the stock solutions were determined using high performance liquid chromatography with ultraviolet light (HPLC-UV). Similarly, concentrations of aliquots from tanks were monitored throughout the study. Actual concentrations of the stock solutions for each chemical were measured in duplicate and averaged (standard error, n=40) for 2,4-DNT was 92.2 ± 3.1 and 94.4 ± 2.9 mg/L for 2,6 DNT. Water samples were collected once a week with one sample per treatment for each compound during the twelve weeks of the study in order to determine the actual concentrations. Nominal concentrations of 2,4- DNT were 0.125, 0.25, 0.5, 1, 2, and 4 mg/L. While the nominal concentrations for 2,6-DNT were 0.25, 0.5, 1, 2, 4 and 8 mg/L.

Up and Down procedure

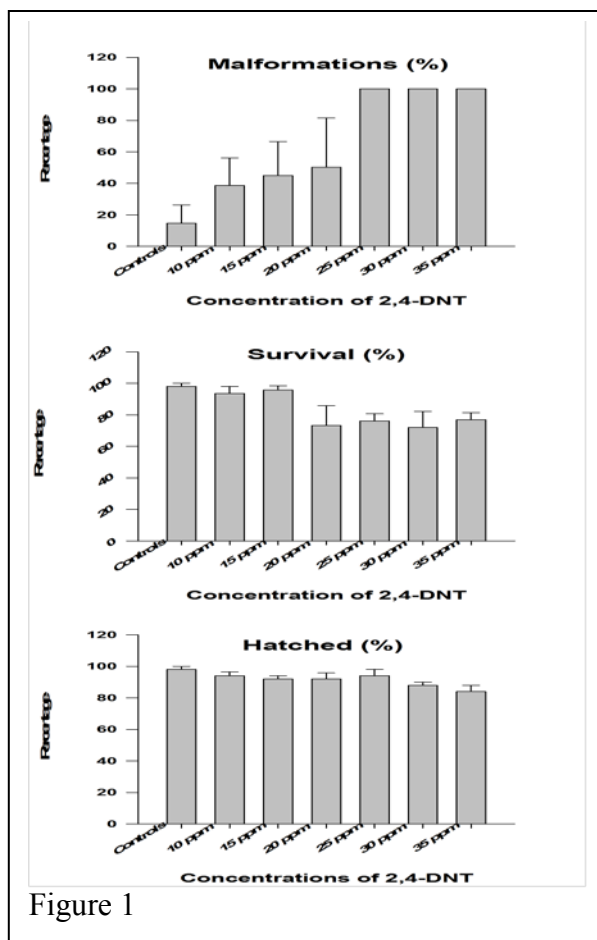


Figure 1

Bullfrogs were kept in 2.5 gallon aquaria in 4L of aged tap water. A 100% water change was done every other day.

14.4 Daily Observations

All Petri dishes and aquaria were examined for dead and malformed embryos each day.

14.5 Euthanasia

At the end of the exposure, all larvae and adults were euthanized by immersion in 1.5 g/L or 3.0 g/L MS222.

14.6 Sample Collection

Tadpoles were collected at the end of exposure. Endpoints collected were mortality, stage, snout-vent length, and deformities.

15.0 RESULTS:

Acute toxicity of 2,4-DNT and 2,6-DNT in Xenopus and Bullfrog
Using the Up-down method we

calculated an estimated LD₅₀ of 620.5 mg/Kg for 2,4-DNT. Based on an assumed sigma of 0.5 the testing range is 350-1100 mg/Kg with an approximate 95% confidence interval.

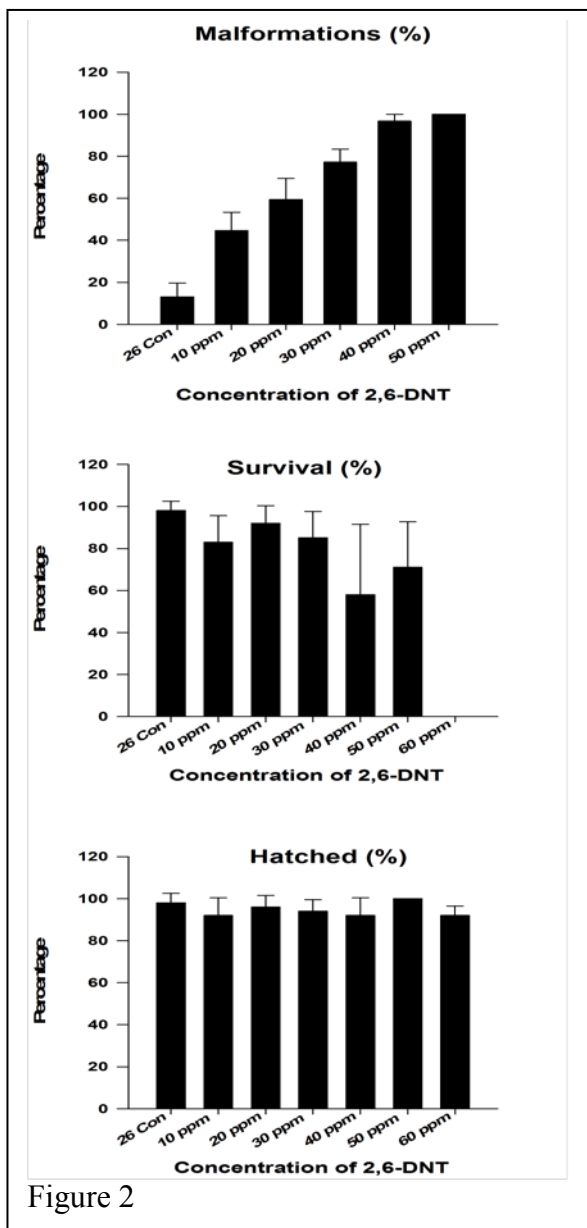


Figure 2

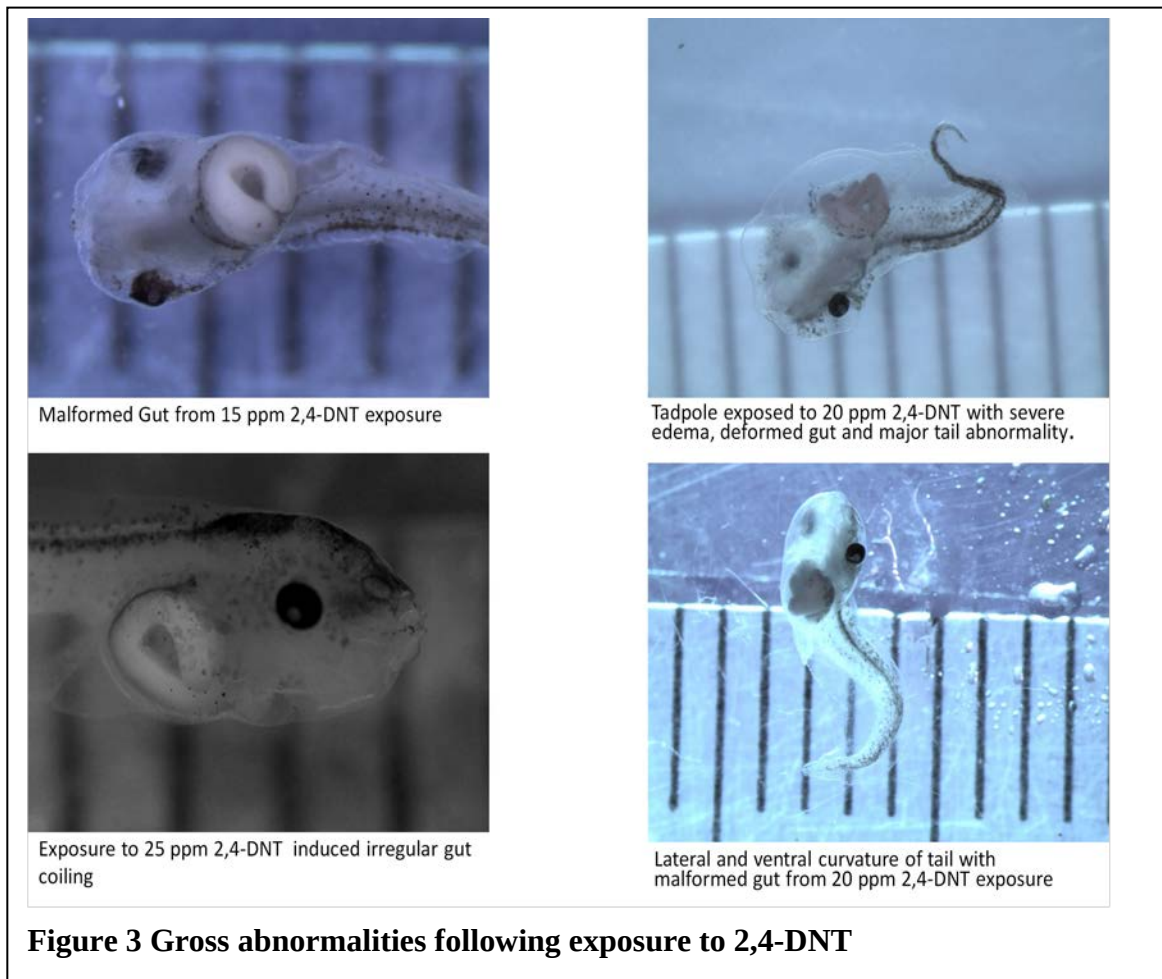
affect percent of tadpoles that hatched, there was a clear indication of the effects of 2,4-DNT on survivorship and the percentages of induced abnormalities. These are summarized in Figure 1. Deformities observed were scoliosis and varying degrees of edema in the abdominal and optic regions. A mortality rate of 100% was observed in larvae exposed to a concentration greater than 25 mg/L 2,4-DNT.

Characterization of 2,6-DNT revealed that developmental retardation was associated with *Xenopus* exposed to varying concentration of this toxicant. The controls and the 40 mg/L 2,6-DNT treatment groups were determined to be at stage 46, while those exposed to all other concentrations of 2,6-DNT were at stage 45. Larval mean body length of the 2,6-

There were no differences between the male and female calculated values. An estimated LD₅₀ of 350 mg/Kg was calculated for 2,6-DNT for female *Xenopus laevis*. Based on an assumed sigma of 0.5 the testing range is 85.78-2080 mg/Kg with an approximate 95% confidence interval. In contrast the Up-down method revealed male *Xenopus laevis* were less sensitive to 2,6-DNT with an estimated LD₅₀ of 1320 mg/Kg. Based on an assumed sigma of 0.5 the testing range is 1100-2000 mg/Kg with an approximate 95% confidence interval. The LD₅₀ for 2,4-DNT and 2,6-DNT was 1,098 mg/kg BW in adult bullfrogs. Both compounds elicited similar symptoms of toxicity including changes of skin color, body weight, development of seizures, liver and kidney necrosis, and lung cyanosis. Relative organ weights did not show significant change (Paden et al. 2008).

Retarded development was observed in tadpoles exposed to all concentrations of 2,4-DNT. The controls were determined to be at stage 47, while those exposed to 2,4-DNT were at stage 46. Larval mean body length of the 2,4-DNT treated tadpoles were shorter than the matched controls. The treated tadpoles ranged from 9.4-9.8 mm compared to 10.4 mm for the controls. While 2,4-DNT did not

DNT treated tadpoles were approximately the same length of matched controls. The treated tadpoles ranged from 8.6-8.9 mm compared to 9.0 mm for the controls. While

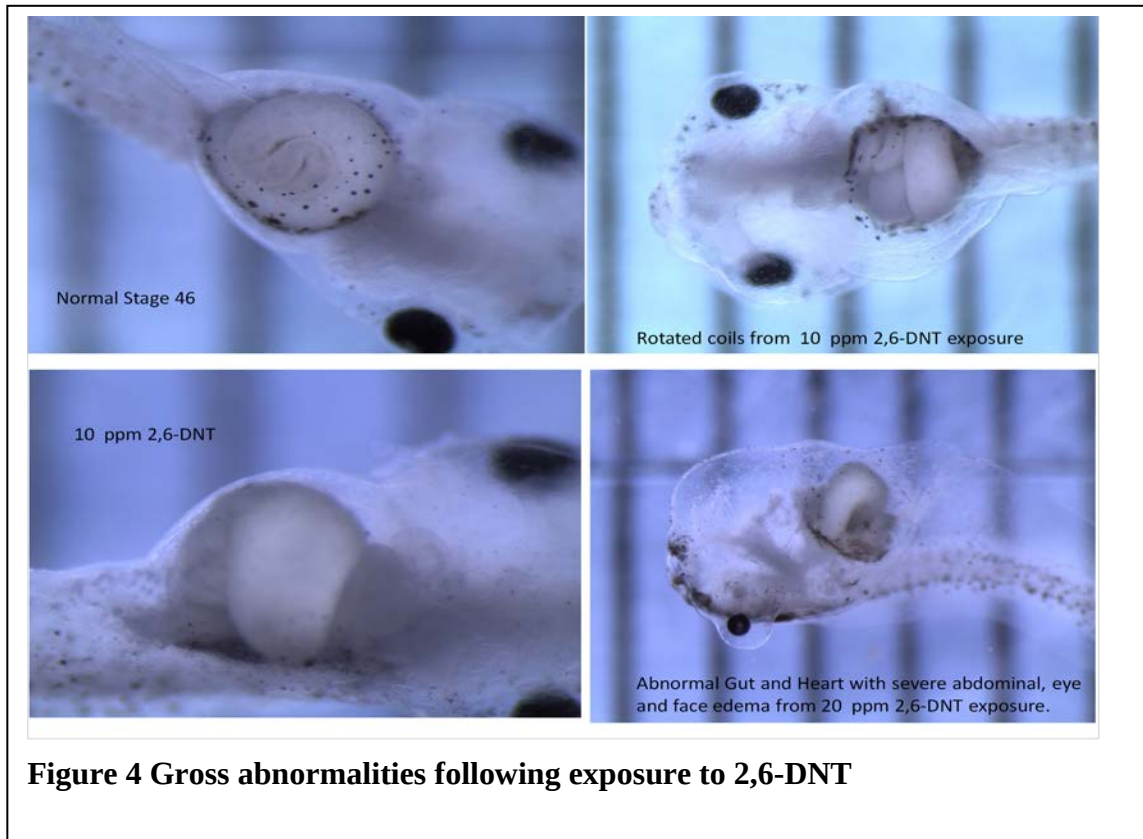


2,6-DNT did not affect percent of tadpoles that hatched, there was a clear indication of the effects of 2,6-DNT on survivorship and the number and types of induced abnormalities. Selected representative specimens are presented in Figure 2. Deformities observed were scoliosis and varying degrees of edema in the abdominal and optic regions as well as heart development and displacement. A mortality rate of 100% was observed in larvae exposed to a concentration greater than 40 mg/L 2,6-DNT. Representative abnormalities of 2,4-DNT and 2,6-DNT are presented in Figures 3 and 4.

Chronic exposure to 2,4-DNT and 2,6-DNT: 2,4-Dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are the most common isoforms of dinitrotoluene. The goal of this study was to determine the chronic toxic effects of 2,4-DNT, and 2,6-DNT to bullfrogs.

Exposure to 2,4 DNT had a significant effect on body weight (Nested ANOVA, $F_{(4,64)}=13.3$, $p=0.002$) and SVL (Nested ANOVA, $F_{(4,64)}=4.3$, $p=0.046$) of tadpoles. In contrast there were no significant effects on stage of development (Nested ANOVA, F

(4,64)=3.9, $p=0.0542$). However, 2,6 DNT had significant effects on the three parameters analyzed: weight (Nested ANOVA, $F_{(3,62)}=17.6$, $p=0.0012$), SVL (Nested ANOVA,



$F_{(3,62)}=7.1$, $p=0.016$), and stage of development ($F_{(3,62)}=15.9$, $p=0.0017$).

Abnormal swimming and gross developmental abnormalities were observed in tadpoles exposed in this study. Throughout the study period 2,6-DNT was associated with the greatest frequency of abnormal swimming. Linear regression analysis revealed dose-dependent values for incidence of abnormalities. An R^2 value of 0.8 was determined for 2,6-DNT. The most common swimming abnormality was swimming in circles. The incidence of abnormalities was higher for tadpoles exposed to 2,6-DNT. Dorsal flexure, tail flexure, ocular edema and abdominal edema, were the most observed abnormalities.

Gross histology analysis of gonad tissues did not reveal any apparent changes in the morphology and size of the gonad. One way ANOVA results for width ($F_{(1,16)}=0.1$, $p=0.76$) and length ($F_{(1,16)}=2.1$, $p=0.17$) indicated no significant difference compared to the controls.

16.0 DISCUSSION:

This study was designed to determine the acute effects of 2,4-DNT and 2,6-DNT individually on developing *Xenopus laevis* and bullfrogs. Exposure resulted in a concentration-dependent increase in frequency and severity of abnormalities. The main abnormalities that were observed in treated larvae were blisters, severe optic and thoracic

edema, dorsal and lateral flexure of the tail, enlarged intestine, and incomplete coiling and lateral displacement of the intestine and heart. Based on these observations, it is inconclusive whether these chemicals are teratogenic in this species. The incomplete coiling and distended gastrointestinal tract observed are likely due to edema or differential disruption of scheduled developmental events. These abnormalities were primarily evident after the second day of exposure.

Neither hatch rate nor hatch date was affected by exposure to any of the chemicals at the concentrations used in this study. These results suggest that 2,4-DNT and 2,6-DNT at the concentrations used in this study appear to induce developmental toxicity to *Xenopus laevis* and bullfrogs during the early stages of development. These toxic effects seen in response to exposure to these chemicals appear to perturb biochemical homeostasis that might be related to critical regulatory pathways in the early developmental stage.

Anuran development is divided into three distinct periods, premetamorphosis, prometamorphosis, and metamorphic climax (Dodd and Dodd, 1976; Etkin, 1964). During premetamorphosis, which is the period of embryonic and early larval stage, organogenesis and some advanced morphological changes such as hind limb bud development occurs without the influence of thyroid hormone. The prometamorphosis is marked with more specific morphogenesis, such as differentiation of the toes and elongation of the hind limbs. The final period which is metamorphic climax, is associated with metamorphosis, including tail resorption and tail forelimb development. Thus, to determine the advanced stage of developmental toxicity of these chemicals it would be necessary to investigate exposure over these periods to stage NF-66. The results from this study suggest that these chemicals are potentially hazardous to *Xenopus laevis* and *Rana catesbeiana*. The effects of these contaminants on native amphibian species that would be found at DoD contaminated sites, however, requires a full life cycle characterization and evaluation in such species (Theodorakis, 2004).

In the chronic and acute studies the results demonstrated that 2,4 DNT and 2,6 DNT had significant effects on the survival. These concentrations are within the range of concentrations detected in environment for 2,4 and 2,6 DNT respectively (Spanggord and Suta 1982; Spanggord et al. 1982). This indicates that effluents containing waste by-products in the water may have a negative effect on the survival of earlier stages of bullfrog development.

Overall tadpoles exposed to 2,4 and 2,6 DNT showed a trend of greater body weight, SVL, and stage of development at the end of the study compared to the controls. There are two possible explanations for this pattern. It has been reported that density can affect growth and development of amphibian tadpoles. In this study decreasing density of tadpoles was associated with increasing concentration of each treatment group with increased body weight, SVL and developmental stage (Newman, 1986; Dever, 1997). It is possible that DNT can stimulate metamorphosis similar to that demonstrated with stress hormones (Boone & Bridges 2003). *Rana clamitans* tadpoles exposed to carbaryl showed the same trend of our findings: greater body weight, SVL, and development stage compared to the controls after multiple exposures (Boone & Bridges 2003) indicating a

density dependent effect following exposure. Recent studies have demonstrated that some amphibian tadpoles chronically exposed to contaminants showed accelerated metamorphosis and body mass (Jung et al. 1996; Christensen et al. 2004; Johansson et al. 2006).

Bullfrog tadpoles chronically exposed to 2,4 DNT and 2,6 DNT showed a relationship between increasing dose and percentage of abnormal swimming. Thus, it is likely that at relevant environmental concentrations explosive residues will have direct effects on swimming behavior and secondary effects on survival due to increased vulnerability to attacks from predators. The ability of aquatic organisms to maintain their posture requires the integration of visual and vestibular sensory information that is processed by the central nervous system (Pronych et al., 1996). Our results demonstrated that at the early stages of bullfrog development (Gosner stages 25-40) 2,4 DNT, and 2,6 DNT had a 2.5 % and 3.5 % incidence of abnormalities, respectively. These values are small compared to those observed in *Rana blairi* tadpoles exposed to acute and sublethal levels (Bridges, 1997). However, further research is needed to analyze the mechanism of action of explosive residues in developing tadpoles.

In the chronic study, as early as 43 days of exposure, tadpoles exposed to 2,4 DNT and 2,6-DNT showed signs of abnormalities. A previous report of *Xenopus* tadpoles exposed to TNT (Saka, 2004), identified abnormalities that are similar to the abnormalities that were observed in our study. These included edema and irregular gut coiling, curved tail, deformed head and dorsal flexure. Environmental contamination with explosive residues such as DNT is believed to be one of the causes for the presence of frog deformities (Saka, 2004). Our data supports Saka's findings in terms of a strong relationship between incidence of deformities and increased dose for bullfrog tadpoles exposed to 2,6-DNT.

This study only examined survival, growth, incidence of abnormal swimming, morphological and gonad histological changes. The information provided in the present study combined with field studies of bullfrogs exposed to 2,4-DNT and 2,6-DNT will be beneficial to risk assessment of native amphibian populations exposed to explosive residues. Further research is needed to validate our findings and analyze the same endpoints at different stages of development.

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.

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TITLE: Development of Polycyclic Aromatic Hydrocarbon (PAH)
Toxicity Benchmarks for Avian Species

STUDY NUMBER: PAH-07-01

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Development Program
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RESEARCH INITIATION: September 2006

RESEARCH COMPLETION: August 2008

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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:

Phil N. Smith
Principal Investigator

Date

1.0 DESCRIPTIVE STUDY TITLE:

Development of Polycyclic Aromatic Hydrocarbon (PAH) Toxicity Benchmarks for Avian Species

2.0 STUDY NUMBER:

PAH-07-01

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:

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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 09/2006
Termination: 08/2008

6.0 KEY PERSONNEL:

Philip N. Smith	Principal Investigator
George P. Cobb	Co-Principal Investigator
Scott T. McMurry	Co-Principal Investigator
Blake Beall	Study Director
Dr. Ronald Kendall	Testing Facility Manager

7.0 STUDY OBJECTIVES / PURPOSE:

The purpose of this project was to conduct acute, sub-acute, and sub-chronic toxicity tests on Northern bobwhite quail (*Colinus virginianus*) with three polycyclic aromatic hydrocarbons (PAHs) and to approximate LD₅₀ values for all three compounds and NOAEL, and LOAEL values for the compound determined to be most toxic among the three.

8.0 STUDY SUMMARY:

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants of aquatic and terrestrial ecosystems. PAHs are known to induce biochemical alterations in exposed animals. The cytochrome P450 enzyme system is known to be a significant Phase I metabolic pathway for the breakdown of PAHs that enter animal systems. Little is known about the effects of PAHs in Northern bobwhite quail (*Colinus virginianus*) and other terrestrial avifauna. The objectives of this study were to 1) determine if benz[a]anthracene, pyrene, and naphthalene exposure are acutely toxic in quail, and 2) determine if sub-acute exposure to benz[a]anthracene in quail would render mortality and

produce alterations in enzyme activity, and 3) determine if sub-chronic exposure to benz[a]anthracene in quail would produce alterations in enzyme activity. Quail, acutely dosed with benz[a]anthracene, pyrene, and naphthalene experienced no mortality at the limit dose of 2000 mg/kg bodyweight. Additionally, there were no alterations in animal behavior. Sub-acute exposure of quail to benz[a]anthracene did not produce mortality. Quail were exposed to benz[a]anthracene at concentrations of 0, 0.1, 1, 10, 100, and 1000 mg/kg feed for 5 days. Alterations in cytochrome P450 1A and P450 2B were observed. An increasing trend in mean ethoxyresorufin-O-deethylase (EROD) activity in the liver was observed as exposure level increased. Pentoxyresorufin-O-deethylase (PROD) activity in the liver in exposed animals was significantly different when compared to the control. EROD activity in the kidney in the sub-acute study was observed to be significant when the interaction between exposure group and time was considered. Sub-chronic exposure of quail to benz[a]anthracene produced alterations in cytochrome P450 1A and P450 2B. Quail were exposed to benz[a]anthracene at concentrations of 0, 0.1, 1, and 10 mg/kg feed for 1, 3, 9, 30, and 60 days. EROD activity in the liver was observed to be significant when the interaction between exposure group and time was considered. An increasing trend in mean EROD and PROD activity in the liver and kidney was observed as exposure concentration increased. This study indicates that benz[a]anthracene, pyrene, and naphthalene are not acutely toxic in exposed quail, and benz[a]anthracene exposure affects enzyme activity in quail exposed sub-acutely and sub-chronically. Overall, this study provides evidence that metabolic alterations are experienced by Northern bobwhite quail exposed to PAHs.

9.0 TEST MATERIALS:

Naphthalene, Pyrene, and Benz[a]anthracene were acquired from Fisher. Subsequent orders of pyrene and benz[a]anthracene were from Sigma.

10.0 JUSTIFICATION OF TEST SYSTEM:

While the toxicity of many polycyclic aromatic hydrocarbons (PAH) has been described for aquatic and some terrestrial fauna, there is little data regarding the toxicological effects of singular PAH on avian species. When evaluating the Army Risk Assessment Modeling System (ARAMS) Terrestrial Toxicity Database for PAH toxicity data, very little information is available for avifauna. The data that are available consider only embryo toxicity in the evaluation of seven PAHs. In general most PAH induce biological responses in invertebrates, fishes, and mammals because they are carcinogenic, mutagenic, and potent immunosuppressants, which affect antibody responses to a variety of T cell-dependent and T cell-independent antigens (EPA, 1980; Klaassen, 2001). Many PAH considered to be carcinogenic are not acutely toxic to mammals when administered in small amounts (ATSDR, 1993). However, PAH exert carcinogenic and mutagenic effects after being metabolized by P450 enzymes (e.g., CYP1A) to more toxic metabolites (Klaassen, 2001). Laboratory studies have shown that PAHs may stimulate the induction of hepatic monooxygenase activity in birds, although PAHs are rapidly metabolized, and the bulk load is excreted from the body (Custer, 2001; Naf, 1992). When avifauna are considered for the evaluation of PAH toxicity, primary exposure most

likely occurs via oral intake. However, topical exposure is also important in some circumstances such as when incubating birds become oiled and subsequently transfer oils from feathers to the egg shell (Douben, 2003; Naf et al., 1992). Oiled birds are also orally exposed to PAH because they ingest oil when preening (Douben, 2003). Therefore, the ingestion of PAH and their molecular breakdown by the P450 enzyme to more toxic metabolites may significantly affect the physiological health of avifauna directly and through the formation of adducts. Reduced fitness may lead to alteration of thermoregulation, foraging, and breeding. These alterations may also lead to increased pathogen susceptibility, predation due to mobility impairment, and population declines.

Acute toxicity values have been reported for four PAH compounds administered to red-winged blackbirds via oral gavage. The LD₅₀ values for acenaphthene, fluorine, anthracene, and phenanthrene were 101, 101, 111, and 113 mg/kg bw, respectively (Schafer et al., 1983). The toxicity of crude oil PAH fractions to birds has also been determined, and the effects linked to the chemical composition of the PAH fraction. Herring gull nestlings administered a single (12 ml) dose orally had reduced growth, and increased adrenal and nasal gland weights within 8 days of exposure (Peakall et al., 1982). The fraction that produced the greatest effects was the methylated series of chrysenes, benzanthracenes, phenylanthracenes, binaphthyls, and traces of benzopyrenes (Douben, 2003). Dose-response studies with crude oil have demonstrated that transfer of minute amounts of crude oil to the eggshell can result in toxic effects (Douben, 2003). An LD₅₀ of 1.3 and 2.2 µl/egg has been reported for Prudhoe Bay and Hibernia crude oil, respectively, following application to the shell on day 8 of incubation (Lee et al., 1986). Gross pathological effects, including liver necrosis, renal lesions, extensive edema, growth retardation and teratogenicity have also been reported in chicken and mallard embryos following the application of PAH compounds to the eggshell (Hoffman et al., 1981; Matsumoto et al., 1986, 1988; Couillard, 1989, 1990). In reference to, and in continuation of the latter studies, it is important to assess the impact of single PAH compounds upon acute/chronic toxicity of avifauna.

11.0 TEST ANIMALS:

All animals used in this study were cared for according to Texas Tech University (Lubbock, TX, USA) Animal Care and Use Committee protocol 06033-09. Quail were maintained in a temperature controlled room at 25-26°C and 30-40% relative humidity on a light/dark cycle of 12 hour light: 12 hours dark (12L:12D). Quail were housed in stacked quail cages and acclimated for at least 7 days prior to dosing. Quail were provided food and water *ad libitum* during acclimation periods.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Quail were maintained in cages labeled with individual animal identification, study protocol number, age, sex, and the name of the persons responsible for their care. Likewise all samples derived from these animals were labeled accordingly.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Experimental designs for all portions of this project are described in detail in section 14.0 Methods below.

14.0 METHODS:

PAH Acute Study: Animal Husbandry

All animals used in this study were cared for according to Texas Tech University (Lubbock, TX, USA) Animal Care and Use Committee protocol 06033-09. In October 2006, 66 12-week old quail were purchased from Rush Creek Quail Farm in Fort Worth, TX, USA. Quail were maintained in a temperature controlled room at 25-26°C and 30-40% relative humidity on a light/dark cycle of 12 hour light: 12 hours dark (12L:12D). Quail were housed in stacked quail cages and acclimated for 7 days prior to dosing. Quail were provided food and water *ad libitum* during the acclimation period.

PAH Acute Study: Dosing Method

Birds in this study were subjected to 2000 mg/kg limit dose as recommended by the Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemical #425 for Acute Oral Toxicity – Up-and-Down Method (OECD, 2001). Birds were dosed via an oral gavage (stainless steel intubation cannula). Polyethylene glycol served as a carrier for BAA, and corn oil served as a carrier for pyrene and naphthalene. Dosing solutions were warmed to $30 \pm 3^\circ\text{C}$ on a hot plate/stirrer (Corning Inc., USA) to enhance homogenization and solubility. Dose volume was adjusted based on USEPA Ecological Effects Test Guideline 712-C-96-139 which suggests the use of 5 ml/kg bodyweight (USEPA, 1996a). Quail were restrained and the dose administered. Birds were then placed back in their cages and monitored at 0.5, 1, 4, and 8 hours, and three times per day thereafter for signs of toxicity. Acute mortality of birds at the limit dose would result in a reduction/progression of dose. If an animal dies at the 2000 mg/kg limit dose the next animal receives a dose a step below the level of the best estimate of the LD50 (OECD, 2001). If the animal survives, the dose for the next animal is increased by a factor of 3.2 times the original dose; if it dies, the dose for the next animal is decreased by a similar dose progression (OECD, 2001). When observational criteria are satisfied, dosing is stopped at which time an estimate of the LD50 and a confidence interval are calculated for the test substance based on the status of all the animals at termination (OECD, 2001). Observational criteria is 1) 3 consecutive animals survive at the upper bound, 2) 5 reversals occur in any 6 consecutive animals tested, and 3) at least 4 animals have followed the first reversal (OECD, 2001). Monitoring of birds continued for 48 hours after dosing, and birds were then euthanized via carbon dioxide narcosis/asphyxiation. Necropsy immediately followed euthanization to identify any potential pathological anomalies.

PAH Sub-acute Study: Animal Husbandry

All animals used in this study were cared for according to Texas Tech University

(Lubbock, TX, USA) Animal Care and Use Committee protocol 06033-09. In January 2007, 90 seven-day old Northern bobwhite quail were purchased from WW Quail Ranch in Wardville, OK, USA. Quail were acclimated for 7 days prior to experimental exposure. Quail were housed in a stacked, Georgia Quail Farm (GQF) deck game bird/poultry battery brooder for the duration of the study. Brooder temperature was maintained at $\sim 37\pm 3^\circ\text{C}$ with a light/dark cycle of 12L:12D. Food and water were provided *ad libitum*.

PAH Sub-acute Study: Exposure Method

Sub-acute toxicity for BAA was determined using the USEPA Ecological Effects Test Guideline 712-C-96-140 (USEPA, 1996b). Prior to initiation of the study all birds were weighed and then randomly placed into one of six groups to be exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, and 1000 mg/kg. The control group contained 30 quail and all treatment groups contained 13 quail. Birds were not identified by sex. BAA was dissolved in acetone and then mixed with Purina Game Bird Startena at the concentrations listed above. Control animals were provided with feed treated with acetone only. Acetone was allowed to volatilize from feed for 3 days prior to storage in opaque plastic containers at $\sim 4^\circ\text{C}$. Refrigerators used for storage did not allow light to interact with treated feed.

A five day dosing trial was initiated in which quail were exposed to BAA via diet. Mortality and signs of intoxication were monitored for the first 0.5, 1, 4, and 8 hours, and then at least twice daily thereafter. On the fifth day, 3 control animals along with 3 animals from each exposure group were euthanized and necropsied. On day 8, remaining study animals were euthanized and necropsied. The liver and kidney of each bird was excised, weighed, and immediately flash frozen in liquid nitrogen. Frozen tissues were wrapped in clean aluminum foil and stored at -80°C until time of analysis.

PAH Sub-chronic Study: Animal Husbandry

All animals used in this study were cared for according to Texas Tech University (Lubbock, TX, USA) Animal Care and Use Committee protocol 06033-09. In February 2007, 90 seven day-old Northern bobwhite quail were purchased from WW Quail Ranch in Wardville, OK, USA. Quail were acclimated for 7 days prior to experimental exposure. Quail were housed in a stacked, GQF deck game bird/poultry battery brooder for the first 30 days of the study. Brooder temperature was maintained at $\sim 37\pm 3^\circ\text{C}$ with a light/dark cycle of 12L:12D. Food and water were provided *ad libitum*. On day 30, quail were moved to stacked quail cages for the remainder of the study.

PAH Sub-chronic Study: Exposure Method

Sub-chronic toxicity of BAA in quail was determined using USEPA Health Effects Test Guideline 712-C-98-199 (USEPA, 1998). Prior to initiation of the study all birds were weighed and then randomly placed into one of four treatment groups exposed to BAA at nominal concentrations of 0, 0.1, 1, 10 mg/kg. The 0, 0.1, 1, 10 mg/kg treatment groups contained 27, 20, 21, and 20 animals, respectively. Birds were not identified by sex. BAA was dissolved in acetone and then mixed with Purina Game Bird Startena at

concentrations listed above. Control animals were provided feed treated with acetone only. Acetone was allowed to volatilize from feed for 3 days prior to storage in opaque plastic containers at ~ 4° C. Refrigerators used for storage did not allow light to interact with treated feed.

A 60 day dosing trial followed in which the quail were exposed to BAA via diet. Mortality and signs of intoxication were monitored for the first 0.5, 1, 4, and 8 hours, and then at least twice daily thereafter. On the first, third, ninth, and thirtieth day, 3 control animals along with 3 animals from each exposure group were euthanized. On day 60, the remaining study animals were euthanized and necropsy ensued. The liver and kidney of each specimen was excised, weighed, and then immediately flash frozen in liquid nitrogen. Frozen tissues were wrapped in aluminum foil and stored in liquid nitrogen until time of analysis.

Sub-acute/-chronic Microsomal Preparation and Cytochrome P450 Isozyme Assay

Induction of hepatic and renal metabolic enzymes was quantified by measuring ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-deethylase (PROD) activity in quail liver and kidney microsomal preparations, using modifications of previously described methods (Prough et al., 1978; Smith, 2000). Each sample was kept on ice at all times during the microsomal preparation procedure. Microsomes of both liver and kidney samples were prepared by homogenizing tissues in a 20mM Tris (pH=7.4), 250 mM Sucrose buffer. Each tissue was weighed and then combined with Tris buffer at three times the volume of the tissue weight in pre-chilled 10 ml homogenization tubes. Tissues were then homogenized using a Wheaton Overhead Stirrer (Wheaton Corporation, Millville, NJ, USA), fitted with a teflon pestle, until the slurry was uniform. Homogenized samples were transferred to pre-chilled centrifuge tubes and then placed in a high speed centrifuge for 10 minutes at 10,000 x g (4°C) to remove large chunks. The supernatant was then removed and centrifuged for 20 minutes at 15,000 x g (4°C), and the process was repeated for an additional 70 minutes at 105,000 x g rpm (4°C). Following the final centrifugation, the supernatant was removed and the remaining pellet was washed with an 80 mM Tris (pH=7.4) / 250 mM Sucrose buffer. The washed pellet was then combined with an 80 mM Tris (pH=7.4) / 250 mM Sucrose / 25 mM KCl resuspension buffer equal to ½ the mass of the original tissue sample. The pellet was then homogenized with an overhead stirrer fitted with the teflon pestle. The sample was transferred to pre-chilled cryo-storage vials and placed in -80° C (sub-acute samples) or liquid nitrogen (sub-chronic samples) storage for future enzymatic analysis.

Characterization and optimization of all enzymatic assays for Northern bobwhite quail tissues was conducted prior to EROD and PROD bioassays. A kinetic assay performed on a fluorometer with a 96-well plate reader was used to detect and quantify resorufin formation after microsomal delacylation of two resorufin ethers (Smith, 2000). Dilutions of enzyme (microsomes) and substrate (EROD and PROD) were varied to optimize conditions for quail liver and kidney samples. The optimization procedure ensured conditions that would prevent the premature enzymatic depletion of substrate and substrate inhibition (Smith, 2000). Nicotinamide adenine dinucleotide phosphate (NADPH, reduced form) concentration was kept constant at 10⁻³ M (in 0.1 M Tris buffer,

pH=7.8) in both studies (Smith, 2000). Quantification of EROD and PROD activity in both liver and kidney samples of each experimental group was undertaken.

EROD activity was analyzed kinetically using an fmax Fluorescence Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA). Samples were run in triplicate in a 96-well plate with a final assay volume of 180 μ L per well. The EROD assay was conducted at 26° C, in a 0.1 M Tris-HCl Buffer (pH=7.8) + 1.6 mg/mL Bovine Serum Albumin (BSA). Enzymes (microsomes) were diluted in a 0.1 M Tris-HCl Buffer (pH=7.8) + 0.5 mg/mL BSA (Hofius, 1992). Ethoxyresorufin and pentoxyresorufin were prepared in methanol at stock concentrations of 88.9 μ M. EROD or PROD was combined with 0.1 M Tris-HCl buffer (pH=7.8) to final assay concentrations. Optimal substrate concentrations were determined for EROD (25×10^{-8} M) and PROD (1.6×10^{-6} M). Microsomal (enzyme) preparations were diluted by a factor of 4 for measurement of EROD and PROD. Fresh dilutions of EROD or PROD were prepared every two hours during the isozyme assay to prevent use of degraded substrates. NADPH, at a final assay concentration of 50 μ M, was prepared in 0.1 M Tris-HCl buffer, aliquoted, stored in dark conditions at -20° C, and thawed daily as needed (Hofius, 1992).

Protein content of all microsomal preparations was quantified using a bicinchoninic acid (BCA) protein assay kit produced by Pierce Biotechnology (Rockford, IL). Microsomal samples were diluted in a 0.05 M Tris buffer (pH = 7.4). Dilutions were 10-40X, microsome to buffer. Standards for protein quantification were prepared using bovine serum albumin (BSA) combined with 0.05 M Tris buffer (pH = 7.4) at concentrations ranging from 0 – 2 mg/ml. A nine point standard curve was generated to determine actual protein concentrations of microsomal dilutions. Twenty-five μ L of each standard or enzyme dilution, and 200 μ L of BCA solution containing sodium carbonate, sodium bicarbonate, BCA, sodium tartate in 0.1 M sodium hydroxide, and 4% cupric sulfate were added to a clear flat bottom 96-well plate. Each plate was incubated at 37°C for 30 minutes on a slide warmer before analysis. Plates were cooled to 27°C and absorbance of 562 nm light was measured in each well using a on a SpectraMax Plus spectrophotometer (Molecular Devices). Each standard or microsomal dilution was run in triplicate. Mean plate blank readings were subtracted from each sample reading. Protein concentration was determined using Softmax Pro software which generated a regression line from the standard curve.

Activity was quantified by combining tris buffer, substrate (EROD or PROD), and diluted enzymes (1000 mg protein/mL) in incubated 96-well plates for two minutes at 25° C. Following incubation, 10 μ L of NADPH was added to each well, mixed, and kinetic analysis immediately performed on the fluorometer. Assay solutions were analyzed at 544 nm (excitation) and 590 nm (emissions). Readings were integrated over 30 seconds for 5 minutes for a total of 10 time points. Kinetic data was evaluated by plotting Δ fluorescence over time for each sample replicate. The mean Δ fluorescence of the sample was then divided by the slope of the corresponding standard curve to express substrate activity in pmol/min/1000 mg_{protein}.

Sub-acute/-chronic Hepatosomatic Index

The Hepatosomatic Index (HSI) was evaluated as liver mass as a percentage of whole body mass (Mora et al., 2006). HSI was used to determine somatic proliferation within the liver and explain its relationship with experimental exposure to BAA. Each animal's liver and body mass were recorded. Liver mass was then divided by body mass and multiplied by 100 to achieve an HSI value for each individual bird.

Analytical Measurement of Actual Concentrations in Treated Feed

Determination of actual concentrations of BAA in treated feed was conducted using a modified method of PAH analysis in solids (Zaugg et al., 2006). Samples consisting of 5 g of feed from each treatment group were combined with 3.5 g NaSO₄. Samples were then ground into a powder using a mortar and pestle, and loaded into Accelerated Solvent Extraction (ASE) cells. Samples were then loaded on an ASE instrument and extracted using a hexane/acetone mixture (50:50 volume-to-volume ratio). Each sample was extracted at 1400 psi and 120°C for one 10 minute cycle on the ASE. Extracts were then concentrated in a Buchi Rotavapor R-124 (Buchi, Switzerland) rotating evaporator. Compounds were then isolated using florisil solid phase extraction (SPE) cartridges activated with 2 ml of hexane. Sorbed compounds were eluted from the SPE cartridges using a dichloromethane/diethyl ether mixture (80:20 volume-to-volume ratio). Cleaned extracts were then solvent exchanged into ethyl acetate and dried with nitrogen gas in an N-EVAP 111 (Organomation Assoc. Inc. Berlin, MA, USA) nitrogen evaporator to a final volume of 2 ml. Extracts were then transferred to 2 ml gas chromatography vials and placed in -20°C storage until analysis.

BAA extracted from feed was analyzed on a Hewlett Packard HP 6890 Series GC (gas chromatograph) system equipped with a 5973 Mass Selective Detector. A 30 m X 0.25 mm HP-5MS with 0.25 µm film thickness column was used with helium gas as a carrier (65.9 ml/min flow rate). Standards for BAA quantification were prepared using ethyl acetate combined with BAA at concentrations ranging from 0.1 – 100 mg/L. A four point standard curve was generated to determine actual BAA concentrations in extracted samples. Mean recoveries of BAA were 98.9% – 101% with an initial method detection limit (MDL) of 13.4 µg/kg.

Statistical Methods

Measures of central tendency are expressed as mean ± standard error. All data were checked for normality using a Shapiro-Wilk test. Assumptions regarding homogeneity of variances were checked using Bartlett's test. Food consumption and body mass were tested using a one-way analysis of variance (ANOVA) in both the sub-acute and sub-chronic studies. In the sub-acute study, the effect of treatment was compared among groups using an ANOVA. Birds euthanized on day five of the sub-acute study were compared separately from birds euthanized after the three day recovery period. Significant differences among treatment groups were then identified using Dunnett's post hoc comparison. In the sub-chronic study the effect of treatment and time on EROD and PROD activities were tested using a two-way ANOVA. Any differences among treatment group means were further analyzed using a post hoc Tukey test. Hepatic and renal, EROD and PROD activities were compared using multiple analysis of variance (MANOVA). HSI values were analyzed using a one-way ANOVA followed by a

Dunnett's test to distinguish which means of treated birds differed from the control in both the sub-acute and sub-chronic studies. All data analyses were conducted with the statistical program R version 2.5.1 (R Development Core Team, Boston, MA, USA). Statistical tests were considered significant when $p < 0.05$.

15.0 RESULTS:

PAH Acute Study: Mortality and Pathology

BAA, pyrene, and naphthalene were not acutely toxic to northern bobwhite quail. No quail died at the limit dose nominal concentration of 2000 mg/kg bodyweight for any of the PAHs studied. Actual mean concentrations of BAA, pyrene, and naphthalene were 1990 ± 46.7 , 2050 ± 34.4 , and 2070 ± 44.7 , respectively. No signs of intoxication were observed at any point within the acute exposure studies. Necropsy revealed no gross physiological anomalies. Mean weights of quail were 200 ± 39.1 grams and body mass did not change ($p = 0.817$) during acute studies. Food consumption among quail dosed at 2000 mg/kg body weight ranged from 10.2 – 15.6 g/day, and was not significantly different during acute exposure ($p = 0.582$).

PAH Sub-acute Study: Mortality and Pathology

There was no mortality in the controls or any of the treatment groups exposed to BAA. Actual concentrations of BAA in the sub-acute study were 0 ($< \text{MDL}$), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed. No signs of toxicity were observed at any point in the sub-acute study. Necropsy revealed no gross physiological anomalies. Quail consumed equivalent amounts of feed during the course of the study through day 5 ($p = 0.281$) and during the 3 day recovery period (day 8) ($p = 0.986$) (Table 1) with no differences among treatment groups. There was no difference in body mass among the treatment groups after sub-acute treatment ($p = 0.739$). Mean daily exposure/bird to BAA was 0 ($< \text{MDL}$), 1.43 ± 0.55 , 5.89 ± 0.13 , 67.5 ± 0.51 , 526 ± 0.67 , and 6210 ± 2.25 mg/kg/day (Table 2).

PAH Sub-acute Study: Cytochrome P450 Analysis, Hepatic EROD and PROD

Mean hepatic EROD activities of quail euthanized on day 5 were not significantly different ($p = 0.287$) among treatment groups. Although day 5 EROD activities were not significantly higher than controls, they tended to be elevated when compared to controls in regard to all treatment groups except that of the 1 mg/kg group (Table 3). Mean EROD activities from quail euthanized after the 3 day recovery period (day 8) were significantly different among treatments ($p = 0.001$). Mean EROD activity of quail from the 100 mg/kg exposure group was significantly higher than controls ($p = 0.001$) (Figure 1).

Mean hepatic PROD activities after the fifth day of exposure were not significantly different ($p = 0.105$) among treatment groups, but activities were elevated when compared to controls. However, after the 3 day (day 8) recovery period, mean PROD activities were significantly different ($p = 0.008$) among treatment groups. Mean PROD activity in the 10 mg/kg treatment group was significantly higher than that of controls ($p < 0.001$) (Figure 2). However, no significant difference was observed for all other treatments when

compared to controls. Hepatic PROD activities for quail euthanized after the 3 day recovery period are reported in Table 4.

PAH Sub-acute Study: Cytochrome P450 Analysis, Renal EROD and PROD

There was a significant difference ($p = 0.012$) in mean renal EROD activities among treatment groups. Mean EROD activity of quail exposed to 1000 mg/kg BAA was significantly higher than that of controls ($p = 0.016$) (Figure 3) and was elevated when compared to all other treatment groups. After the 3 day recovery period (day 8), mean EROD activities were not significantly different among treatments ($p = 0.619$). Mean renal EROD activities after the 3 day recovery period were either equivalent to or lower than controls (Table 5).

Mean renal PROD activities of quail euthanized on day 5 ($p = 0.352$) or after the 3 day recovery period (day 8) ($p = 0.958$) were not significantly different among treatment groups. Mean PROD activities of quail from all treatment groups were similar when compared to controls (Table 5 and 6).

Sub-chronic Study: Mortality and Pathology

There was no mortality in quail exposed to control feed, or BAA at 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. No signs of toxicity were observed at any point in the sub-chronic study. Necropsy revealed no gross physiological anomalies. Quail in each treatment group consumed equivalent amounts of feed during the course of the study with no differences among groups ($p = 0.56$) (Table 7). There was no significant difference in body mass among treatment groups ($p = 0.846$). Mean food consumption was not significantly different among treatment groups ($p = 0.067$) which allowed exposure to BAA (mg/kg/day) to be congruent with treatment group (Table 8).

Sub-chronic Study: Cytochrome P450 Analysis, Hepatic EROD and PROD

Regarding the two-way ANOVA model, mean hepatic EROD activity of quail orally exposed to BAA changed in response to an interaction between time and treatment ($p = 0.011$). As time progressed and BAA concentration increased, EROD activity increased (Figure 4). However, mean EROD activity from quail exposed to 10 mg/kg BAA increased until day 30 and then decreased by day 60 (Figure 4).

For mean hepatic PROD activity, there was no significant ($p = 0.559$) interaction between treatment group and time of exposure. However, mean PROD activities were significantly different among treatments ($p < 0.001$), but time had no effect ($p = 0.998$). Mean PROD activities of quail exposed to 0.1 ($p < 0.001$), 1 ($p < 0.001$), and 10 mg/kg BAA ($p < 0.001$) were significantly higher than controls in the post-hoc comparison (Figure 5). Mean EROD and PROD activities from days 1, 3, 9, 30, and 60 are reported in Tables 9–13.

Sub-chronic Study: Cytochrome P450 Analysis, Renal EROD and PROD

There was no significant interaction ($p = 0.124$) between treatment group and time with regards to mean renal EROD activities. A significant difference in mean EROD activities ($p < 0.001$) among treatments was observed. Mean EROD activities from quail exposed

to 0.1 ($p = 0.043$), 1 ($p = 0.002$), or 10 mg/kg BAA ($p = 0.001$), were significantly different among treatments in the post-hoc comparison (Figure 6).

For mean renal PROD activities, there was no interaction between treatment group and time of exposure ($p = 0.431$). However, mean PROD activity was significantly different ($p = 0.029$) among treatment groups (Tables 14 –18), but time was not a significant factor in the model ($p = 0.734$). Mean PROD activity from quail exposed to 10 mg/kg BAA in feed ($p = 0.020$) was the only significantly elevated treatment when compared to all other treatments in the post-hoc comparison. All other treatments renal PROD activities fluctuated over time. Mean EROD and PROD activities for each individual day are reported in Tables 14 –18.

Sub-acute/-chronic Hepatic and Renal Enzyme Activity Comparison

There were no significant differences between EROD and PROD activities among treatment groups ($p = 0.353$), ($p = 0.595$), respectively, when hepatic and renal tissues were compared in the sub-acute study. However, after the fifth day of exposure hepatic EROD activities were approximately 3 times higher in controls and over 4 times higher in each treatment group as compared to renal EROD activities. Also, hepatic EROD activities were more than 3 times higher than renal EROD activities following the 3 day recovery period. Hepatic PROD activities ranged from 5 - 38 times higher than renal PROD activities with regard to the 5 day treatment period, and hepatic PROD activities were 5 -10 times higher than renal PROD activities following the 3 day recovery period.

Mean hepatic and renal EROD activities were significantly different when treatment groups were compared in the sub-chronic study ($p < 0.001$). Also, there was a significant difference among treatment groups with regard to mean hepatic and renal PROD activities ($p = 0.001$). Hepatic EROD and PROD activities were higher than renal EROD and PROD activities on each day sampled in the sub-chronic study.

Sub-acute/Sub-chronic Hepatosomatic Index

Mean HSI values of quail exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg feed in the sub-acute study were compared to controls. Mean HSI of quail exposed to 1000 mg/kg on day 5 were significantly different ($p < 0.001$) than controls (Figure 7). Mean HSI from quail euthanized after the 3 day (day 8) recovery period were significantly different from controls ($p < 0.001$) (Figure 8). Mean HSI of the 1000 mg/kg exposure group was significantly different than controls ($p < 0.001$). All mean HSI values are reported in Table 19.

HSI was also calculated for quail following the sub-chronic study. Mean HSI values from quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg feed on day 1, 3, 9, 30, or 60 were compared to controls. Days 1, 3, 30, and 60 presented HSI values that were significantly different from controls in several treatment groups. The 10 mg/kg treatment group was significantly higher than controls on each day sampled except day 9, and the 0.1 and 1 mg/kg treatment groups were significantly higher than controls on an inconsistent basis throughout the time course. All treatments HSI values on day 9 were not significantly different from controls ($p = 0.212$) (Figures 9 – 12). Mean HSI

values are reported in Table 20.

16.0 DISCUSSION

This study characterized the acute toxicity of three PAHs in northern bobwhite quail. Acute results indicated that exposure to BAA, pyrene, or naphthalene was not toxic to adult northern bobwhite quail at doses below 2000mg/kg. Initially, we expected mortality or some non-lethal toxicity associated with acute exposure to BAA because of its structural similarity to BAP, acenaphthene, fluorine, anthracene, and phenanthrene. We hypothesized that the upper limit dose (2000 mg/kg) would produce mortality, however, in this study that was not observed. In past studies, PAHs were observed to cause mortality in small mammals, fish, and some birds which were acutely exposed (Douben, 2003; Morris et al., 1989). Druckrey et al. (1967) observed BAP to be acutely toxic to rats at 50 mg/kg. Schafer et al. (1983) observed acenaphthene, fluorine, anthracene, and phenanthrene to be acutely toxic in red-winged blackbirds at concentrations of 101, 111, and 113 mg/kg, respectively, and anthracene to be acutely toxic in house sparrows at 244 mg/kg (Douben, 2003; Schafer et al., 1983). Although, no toxicological studies examining BAA in pre-natal avian life forms were located we expected toxicity among bobwhite quail to be similar to that of red-winged blackbirds and sparrows exposed to other PAHs. Although, quantitative and qualitative differences have been observed in response to toxic substances among species, and phylogenetically similar species may exhibit large variation in toxic response to xenobiotics (Klaassen, 2001). Therefore, the lack of toxicity associated with acute BAA exposure in adult quail was not entirely unexpected, but acute exposure to developing northern bobwhite quail embryos would likely result in toxicity based on data from Brunstrom et al. (1991) who found BAA to be acutely toxic in chicken embryos at concentrations of 79 mg/kg. Adult animals are more readily able to detoxify xenobiotics that enter the body when compared to developing individuals (Klaassen, 2001). However, factors such as animal strain, age, type of feed and water, caging, pretrial fast time, method of administration, volume and type of suspension medium, and duration of observations may have influenced the lack of toxic response observed in adult quail used in the acute study (Klaassen, 2001). The delivery vehicle (suspension medium) may also have prevented efficient absorption of the PAHs across the gastro-intestinal tract, although the two vehicles employed in this study are widely used in acute toxicity studies (OECD, 2001). Ultimately, when a chemical does not produce an acutely toxic response in test subjects, USEPA and OECD guidelines suggest terminating the further tests; however based upon the known long term toxic effects associated with PAH exposure the sub-acute/-chronic toxicity of BAA was evaluated. Acute, sub-acute, and sub-chronic exposure to BAA did not cause mortality in quail in the experiment, but sub-lethal biochemical responses were observed.

The sub-acute and sub-chronic studies characterized the effects of BAA by evaluating mortality, EROD activity, PROD activity, and HSI as endpoints. PAHs are known inducers of CYP1A within tissues of animals (Davis, 1997). CYP1A activity is primarily identified via EROD induction within hepatic and renal tissues of mammals (Nims and Lubet, 1995), however, PROD induction generally recognized as CYP2B activity, has also been observed to be catalyzed by CYP1A to a certain degree (Liu et al., 2003).

Furthermore, hepatic tissues contain low levels of CYP2B even when untreated (Klaassen, 2001). This may explain some of the observed PROD activity in treated and untreated quail in the preceding study. Prior studies have used PROD as an endpoint to describe the effects of PAH exposure. Dickerson et al. (1994) measured both EROD and PROD activity in hepatic and renal tissues of deer mice (*Peromyscus maniculatus*) exposed to PAHs in the field to describe biochemical effects associated with exposure to benz[a]anthracene and other selected PAHs. Although the effects upon PROD were less pronounced than the effects upon EROD in the preceding study described, the observations support PROD induction via PAH exposure.

The sub-acute study indicated that a 5 day, repetitive, oral exposure with BAA may not cause mortality in Northern bobwhite quail. We hypothesized that P450 metabolic enzymes would be induced by BAA exposure and that enzyme activities would be related to the concentration of BAA in feed following a 5 day exposure regimen. Numerous studies have documented increases in hepatic enzyme activity in response to PAH exposure in both laboratory and field settings (Custer et al., 2000; Custer et al., 2001; Trust et al., 1994; Trust et al., 2000; Boersma et al., 1986; Cortright and Craigmill, 2006; Walters et al., 1987; Peakall et al., 1989; Jellinck and Smith, 1973), but these studies considered enzyme activity in the first 1-48 hours in lab settings and singular time points in field settings. To our knowledge this is the first study that has characterized enzyme activity following a five day exposure regimen of BAA and then characterized enzyme activity associated with a three day recovery period.

Custer et al. (2001) found hepatic EROD activities to be significantly higher in tree swallows (*Tachycineta bicolor*) nesting near PAH contaminated sites when compared to swallows nesting at reference sites. We observed hepatic EROD activity to be approximately three times higher, and PROD activity approximately fourteen times higher in the lowest treatment group compared to controls (sub-acute study; Table 3). Renal EROD and PROD activities were affected to a lesser degree following BAA exposure. Renal EROD activity was approximately four times higher, but renal PROD activity was not affected in the highest treatment group compared to controls (Table 6). Numerous studies employing the use of both EROD and PROD, when examining samples of the same microsomal dilution, have documented that total enzyme activity is much greater in the liver than the kidney (Liukkonen-Anttila et al., 2003; Russell et al., 2004; Dickerson et al., 1994). EROD and PROD activities of hepatic tissues following the 3 day recovery period (day 8) were generally higher than activity of hepatic tissues taken on day 5 of the sub-acute study, however renal EROD and PROD activities were effected to a much lesser degree than hepatic tissues (Tables 3 – 6). A longer recovery period may have been required to see a decrease in hepatic and renal enzyme activity effected by BAA exposure. However, day 5 enzyme activities were generated with smaller sample sizes than day 8 activities. Perhaps the increased sample size on day 8 allowed a more definitive evaluation of enzyme activity. Although, day 5 and day 8 activities were not statistically compared because of differences in experimental treatment, a general comparison detects the length of time needed for enzyme activity to return to typical levels.

Typical time trials characterizing P450 activity associated with BAA exposure observe initial induction within the first six hours of exposure, and induction peaks within the 24-48 hours following exposure (Muto et al., 2003). This supports our observations of significant elevations in EROD and PROD activities following day 1 of exposure in the sub-chronic study. However, EROD activity continued to increase in the 10 mg/kg treatment group to day thirty and then declined to day sixty (Figure 4). The sub-chronic study indicated that BAA exposure over a 60 day period did not induce mortality in juvenile or sub-adult northern bobwhite quail. We hypothesized that sub-chronic exposure to BAA would induce P450 enzymes in quail and that induction would be related to increased concentrations of BAA and exposure time. The biochemical enzyme induction observed among sub-chronically exposed quail was congruent with increasing treatment concentration and time. Hepatic EROD activity increased over treatment concentration and time (Figure 4) except for the highest treatment group. Prolonged exposure to xenobiotic can result in hepatic injury which may account for the decrease in EROD activity (Gomes et al., 1999). However, in vitro studies utilizing cultured cells from rats exposed to dimethylbenz[a]anthracene at varying concentrations have demonstrated that initial increases in enzyme activity occur within the first 48-hours of exposure and are maintained at constant levels or decrease over time (Muto et al., 2003). Although, enzyme activity has been thoroughly studied within the first 24-48 hrs of induction, we chose to observe activities over a 60 day exposure period. To our knowledge this is the first study that has measured changes in enzyme activity induced by BAA for a prolonged exposure period.

In most assessments of renal and hepatic enzyme function in birds, hepatic tissues generally have greater enzymatic activity (Liukkonen-Anttila et al., 2003; Russell et al., 2004; Dickerson et al., 1994). Although, the liver is the major source of phase I enzyme induction, the kidney contains a significant source of P450 enzymes in avian systems (Pan and Fouts, 1979; Rennick, 1976). Renal EROD activity in the sub-chronic study was significantly different among treatment groups when days 1, 3, 9, 30, and 60 were compared, and renal EROD activity was elevated when compared to controls. This provides further support that the avian renal system can transform xenobiotics that directly enter the kidney via the renal portal system (Pan, 1978, 1979). Increased renal activity was observed in both the sub-acute and sub-chronic study.

According to the United States Environmental Protection Agency, the lowest level of a stressor that causes statistically and biologically significant differences in test samples as compared to other samples subjected to no stressor forms the basis for establishing a lowest observed adverse effect level (LOAEL) (USEPA, 1997). While exposure to PAHs may or may not cause a detriment in animals that experience enzyme induction, an effect is observed. Therefore, the data may not support the calculation of an LOAEL, but a lowest observable effect level (LOEL) is justified. Hence, hepatic EROD activity of the 0.1 mg/kg exposure group was observed to be significantly different from the control. This suggests a LOEL of 0.11 mg/kg/day BAA exposure in Northern bobwhite quail be established because of its effects upon hepatic EROD activity. Such metabolic alterations induced by BAA in quail may or may not have detrimental effects upon quail. Therefore, a more comprehensive evaluation of PAH effects upon metabolic disposition is needed to

elucidate impacts at individual or population levels.

Conclusion

Due to the limited numbers of studies on the effects of individual PAHs in terrestrial avifauna, this study provides valuable information for wildlife management and ecological risk assessment. It is clear that PAHs have biochemical effects in northern bobwhite quail, and therefore may have effects in other terrestrial birds. While PAHs did not produce mortality associated with acute, sub-acute, and sub-chronic exposure, they did induce hepatic and renal enzymatic activity in quail. Further studies on other avian species are warranted since they may have varying capacities for dealing with toxicants in the environment (Pan, 1978, 1979). A more complete assessment of PAH effects within all terrestrial avifauna would allow for improvements in future ecological risk assessments and decisions in regard to management.

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FIGURES

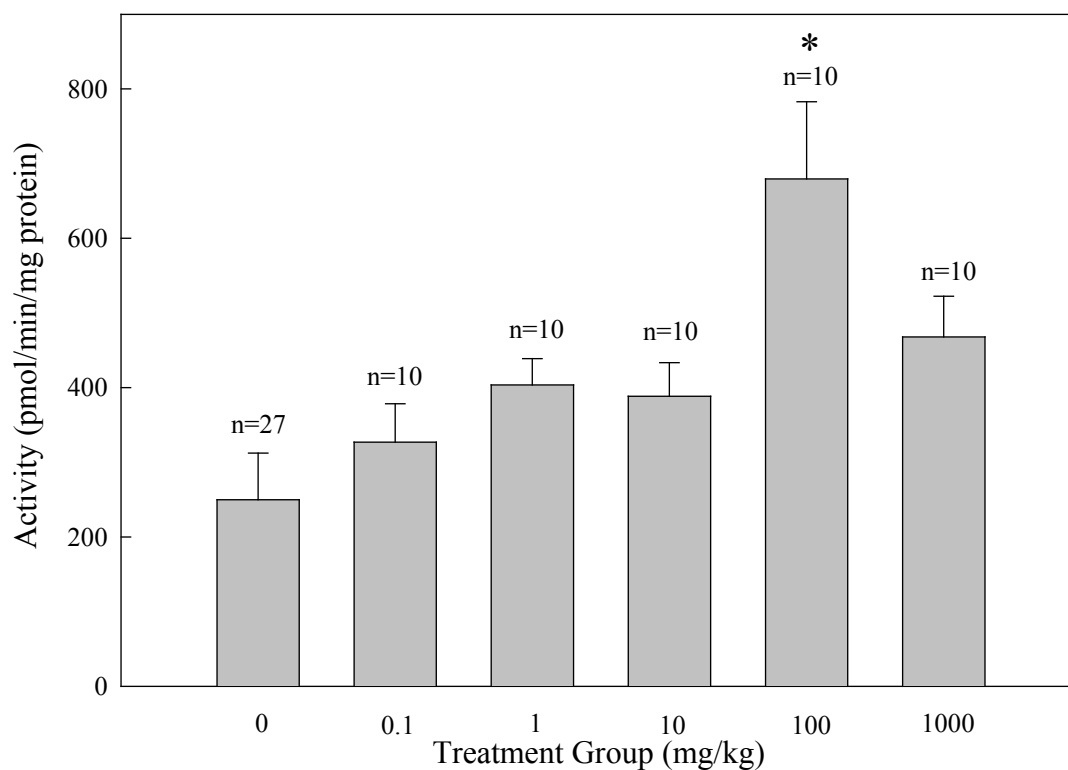


Figure 1. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase activities of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed. Quail euthanized on day 8 of the sub-acute study are shown. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

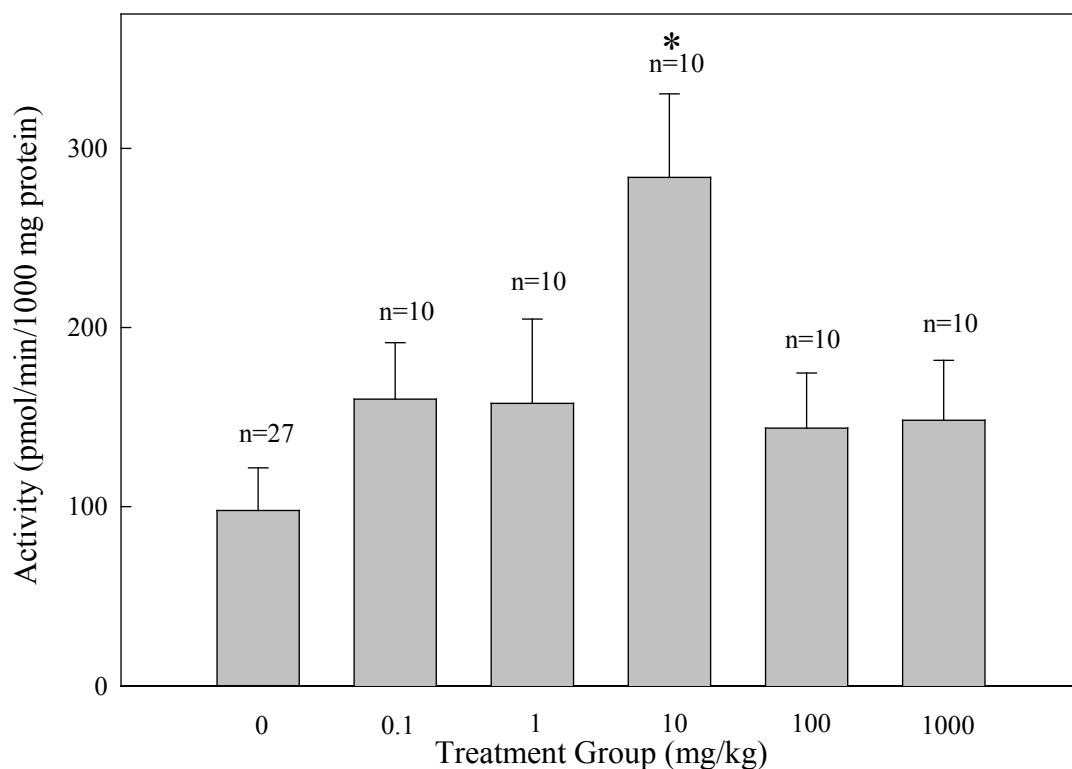


Figure 2. Mean $\pm$ (SE) hepatic pentoxoresorufin-O-deethylase activities of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed. Quail euthanized on day 8 of the sub-acute study are shown. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

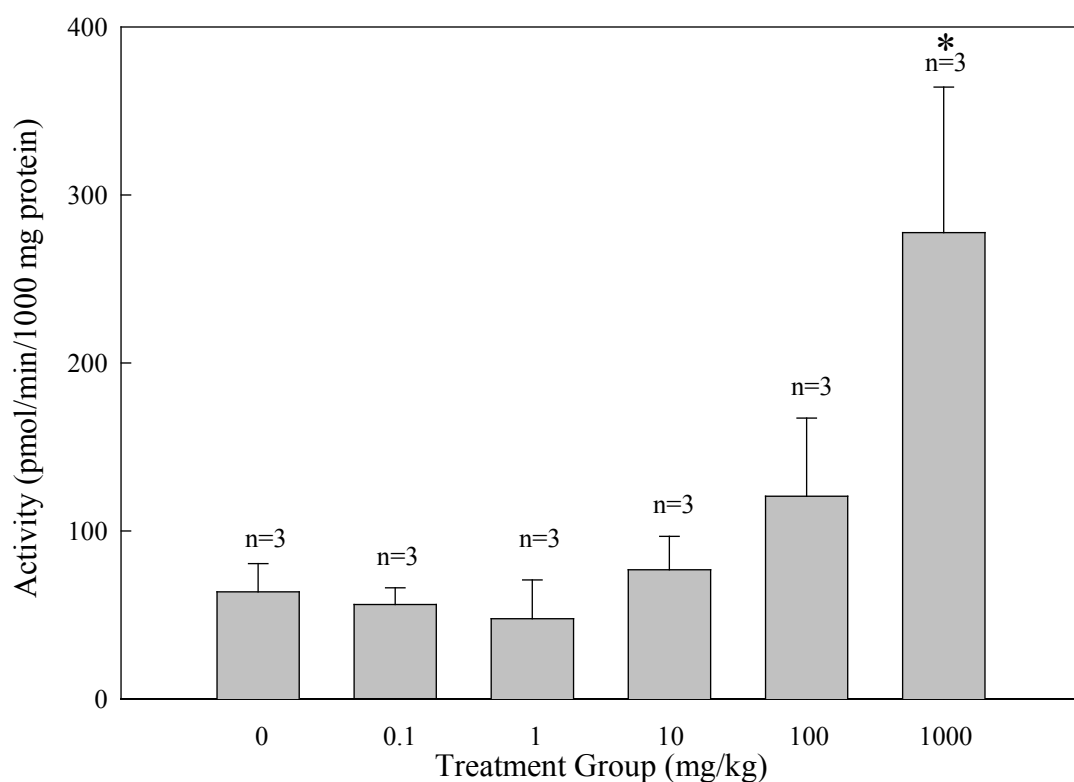


Figure 3. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase activities of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed. Quail euthanized on day 5 of the sub-acute study are shown. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

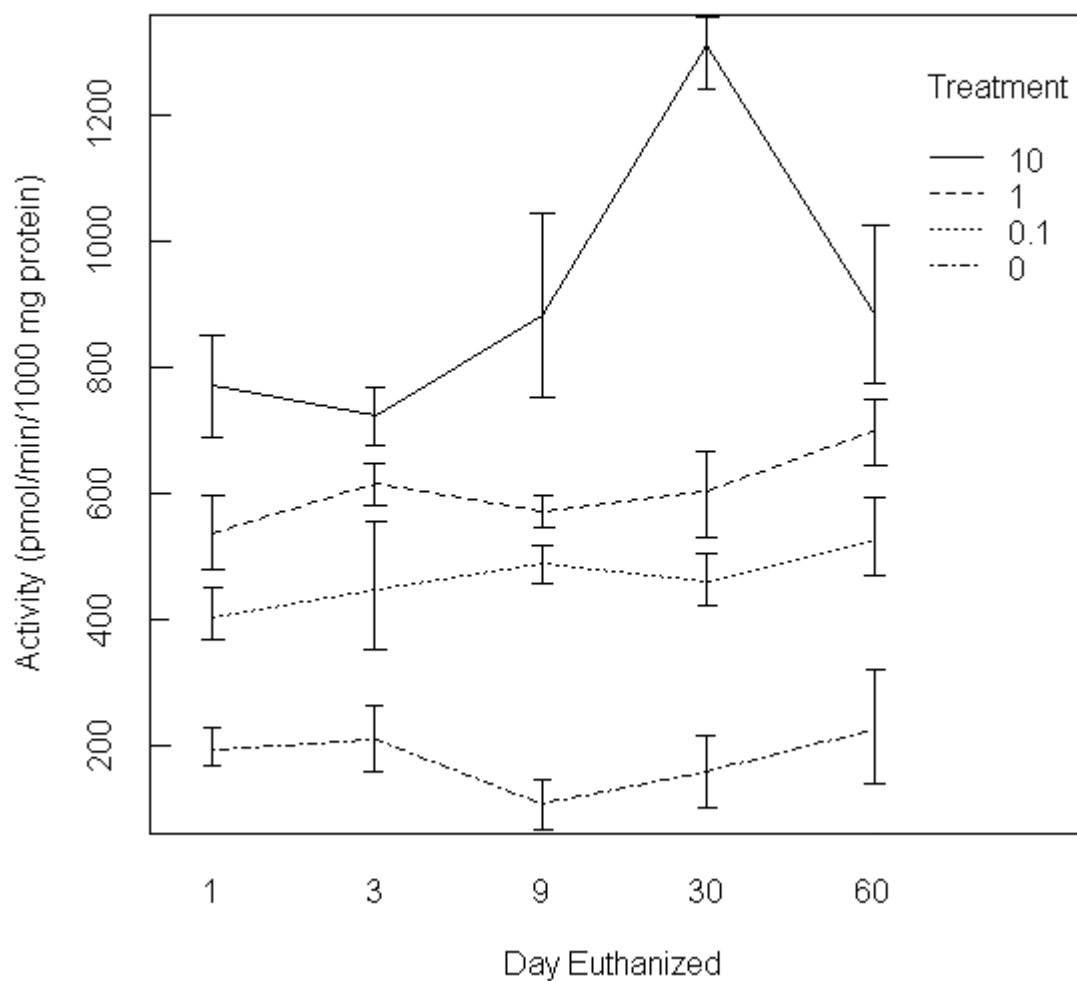


Figure 4. Mean hepatic EROD activities of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed with respect to time and treatment in the sub-chronic study. Northern bobwhite quail were exposed to benz[a]anthracene for 1, 3, 9, 30, or 60 days.

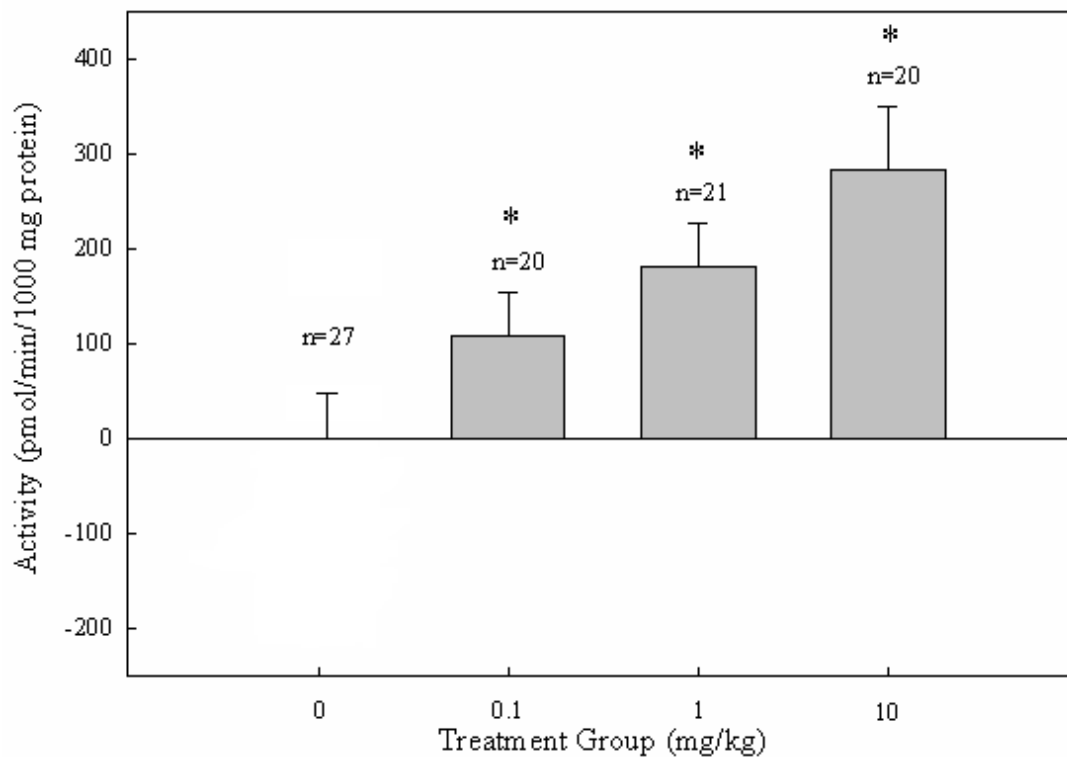


Figure 5. Mean $\pm$ (SE) hepatic pentoxoresorufin-O-deethylase activities of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. All samples from quail euthanized on days 1, 3, 9, 30, and 60 of the sub-chronic study are compared and evaluated via a two-way analysis of variance. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from all other treatments ($P < 0.05$).

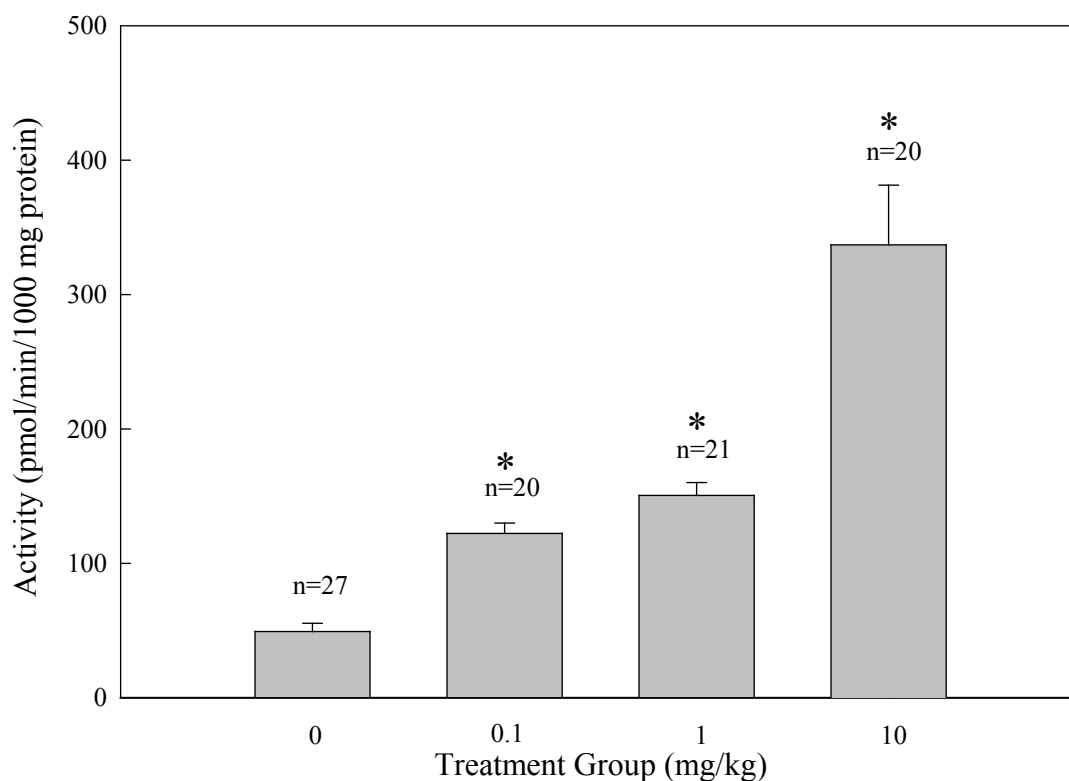


Figure 6. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase activities of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. All samples from quail euthanized on days 1, 3, 9, 30, and 60 of the sub-chronic study are compared and evaluated via a two-way analysis of variance. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from all other treatments ($P < 0.05$).

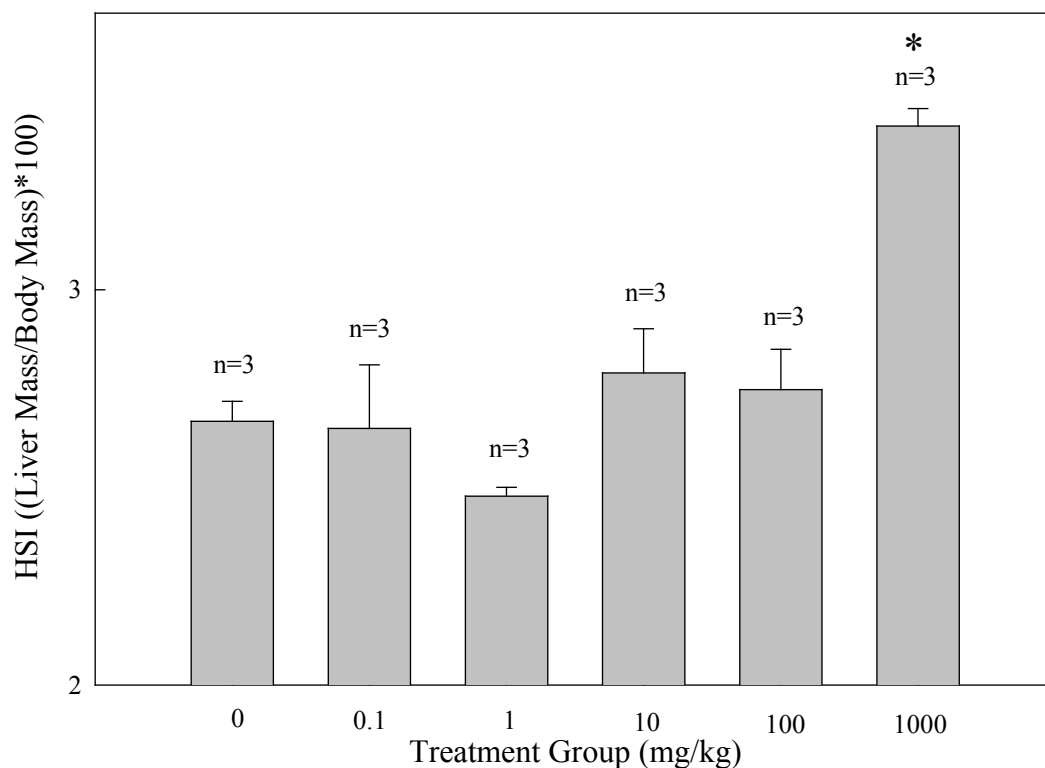


Figure 7. Mean $\pm$ (SE) Hepatosomatic index (HSI) values of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg. Samples from quail euthanized on day 5 of the sub-acute study are shown. HSI is expressed as liver mass as a percentage of bodyweight. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

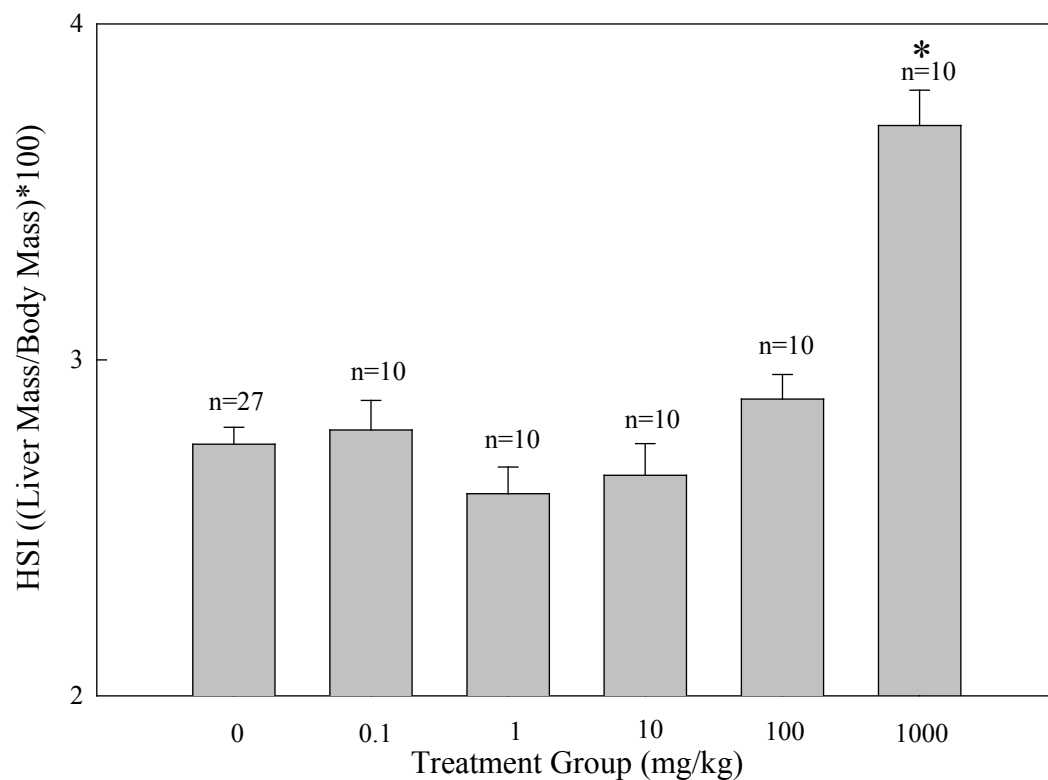


Figure 8. Mean $\pm$ (SE) Hepatosomatic index (HSI) values of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg. Samples from quail euthanized after the 3 day recovery period (day 8) of the sub-acute study are shown. HSI is expressed as liver mass as a percentage of bodyweight. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P<0.05$).

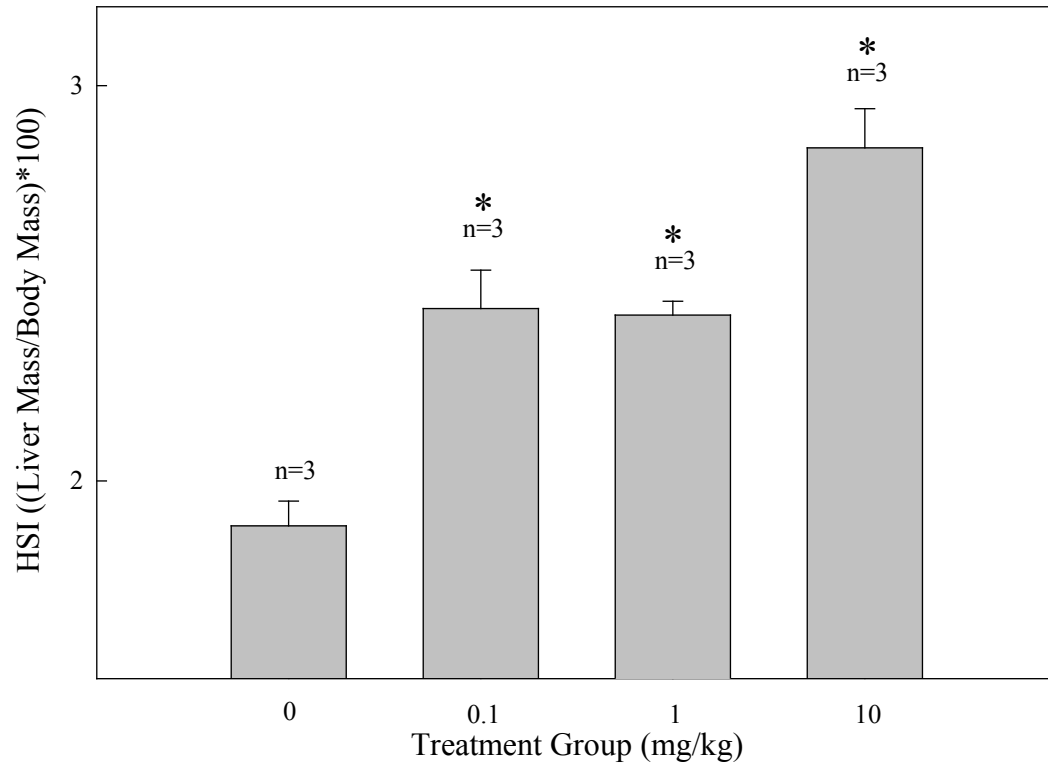


Figure 9. Mean $\pm$ (SE) Hepatosomatic index (HSI) values of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Samples from quail euthanized on day 1 of the sub-chronic study are shown. HSI is expressed as liver mass as a percentage of bodyweight. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

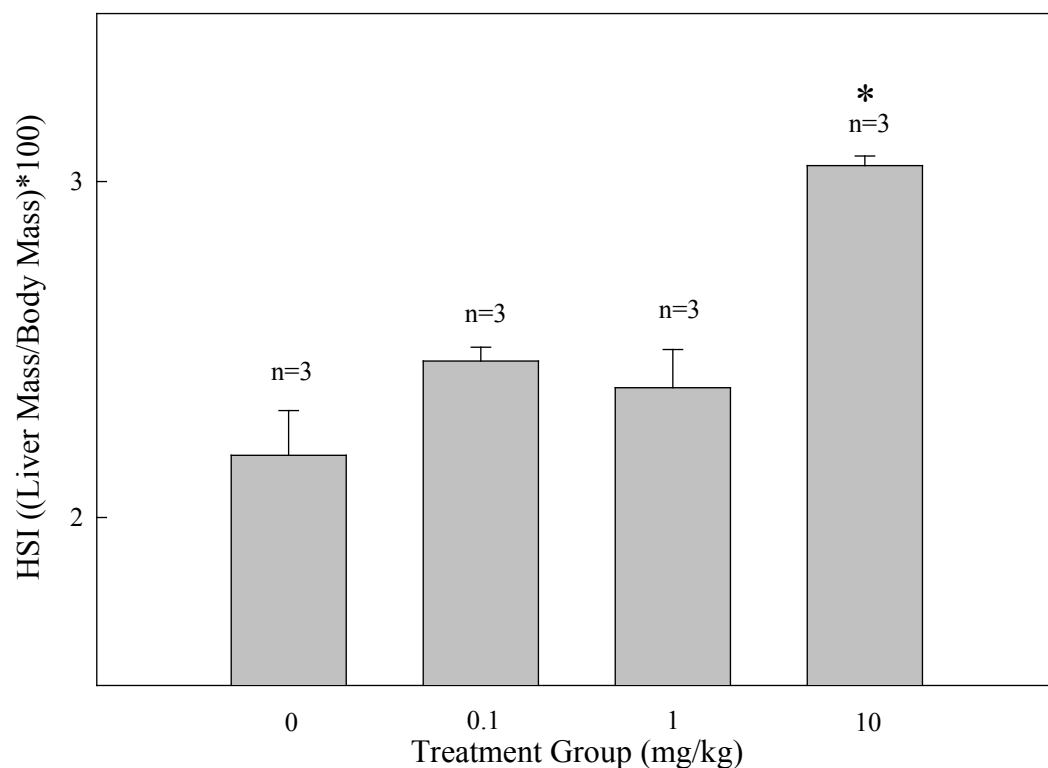


Figure 10. Mean $\pm$ (SE) Hepatosomatic index (HSI) values of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Samples from quail euthanized on day 3 of the sub-chronic study are shown. HSI is expressed as liver mass as a percentage of bodyweight. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

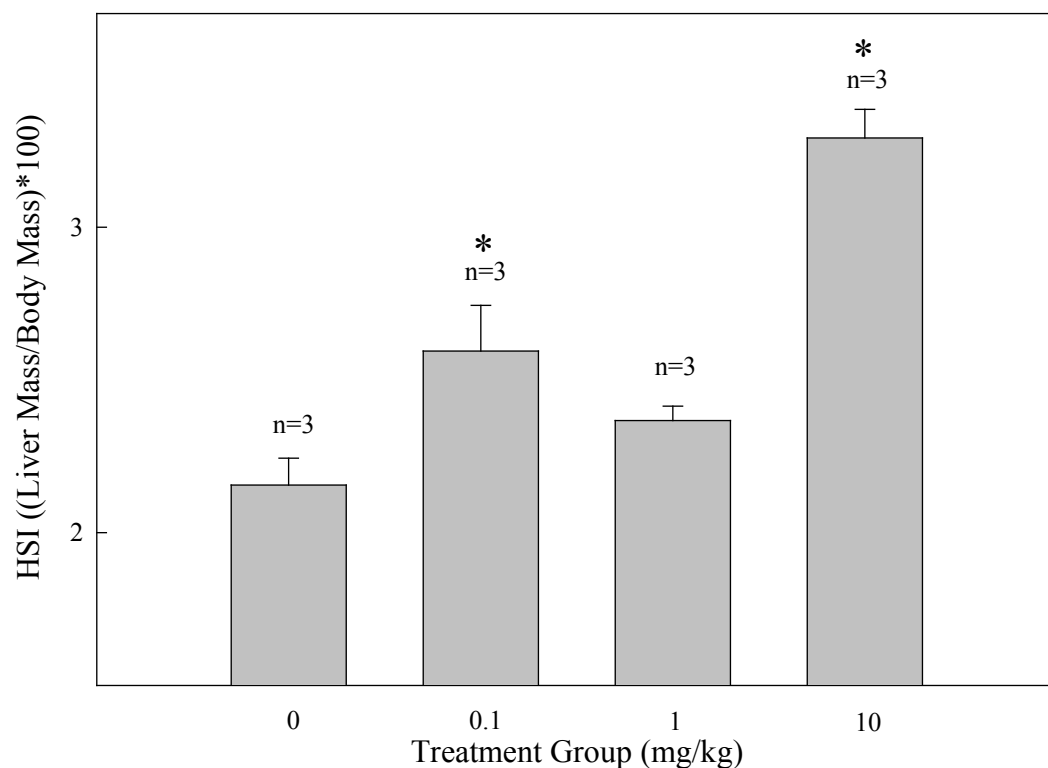


Figure 11. Mean $\pm$ (SE) Hepatosomatic index (HSI) values of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Samples from quail euthanized on day 30 of the sub-chronic study are shown. HSI is expressed as liver mass as a percentage of bodyweight. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

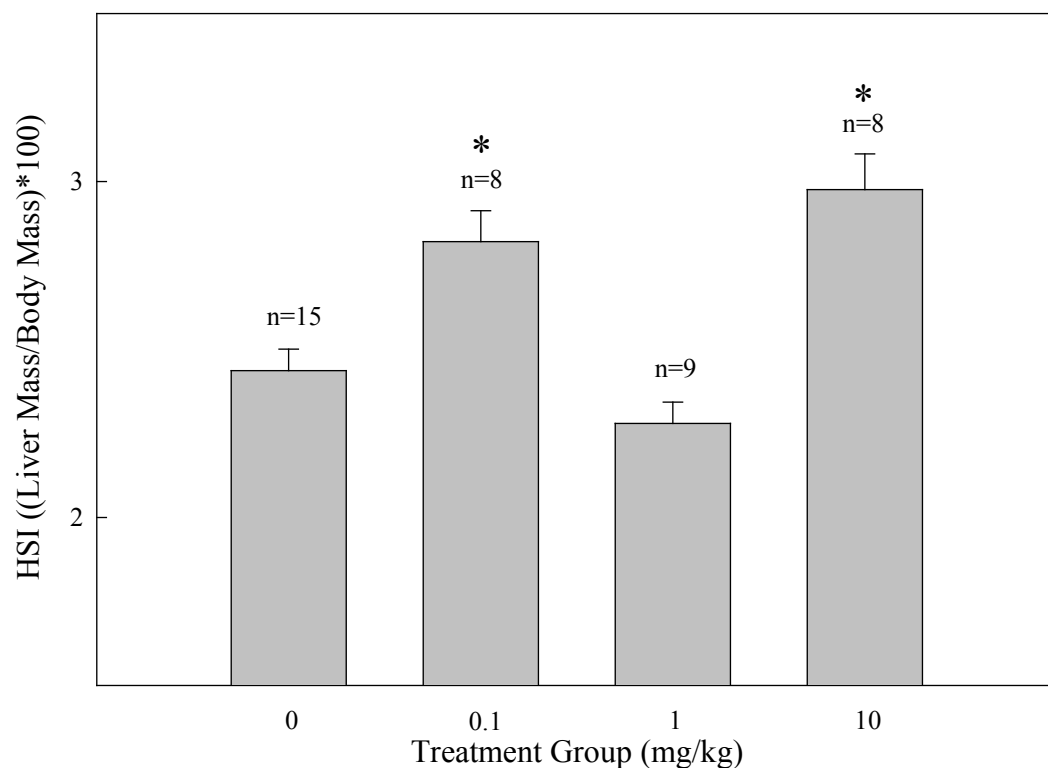


Figure 12. Mean $\pm$ (SE) Hepatosomatic index (HSI) values of Northern bobwhite quail exposed to benz[a]anthracene via contaminated feed at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Samples from quail euthanized on day 60 of the sub-chronic study are shown. HSI is expressed as liver mass as a percentage of bodyweight. Values above bars denote sample size (n). The symbol (*) above bars denotes significantly different means from controls ($P < 0.05$).

TABLES

Table 1. Mean daily food consumption. Northern bobwhite quail were exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed for 5 days. A 3 day recovery period followed the 5 day exposure period.

Day	<u>Mean Daily Food Consumption</u>					
	<u>Treatment Groups</u>					
	Control	0.1 (mg/kg)	1 (mg/kg)	10 (mg/kg)	100 (mg/kg)	1000 (mg/kg)
1	5.69	6.18	4.30	4.36	4.26	4.10
2	5.89	6.77	5.47	4.90	4.73	5.70
3	6.47	7.46	6.32	5.31	5.63	6.43
4	7.10	6.53	6.16	6.13	5.95	4.60
5	8.00	7.33	6.52	8.82	6.24	7.50
6	7.70	9.65	7.10	9.96	9.43	9.95
7	7.90	6.46	8.95	8.13	8.31	12.2
8	8.65	8.73	12.7	13.3	10.8	11.1
Grand Mean	7.18 (0.38)	7.39 (0.43)	7.19 (0.91)	7.61 (1.07)	6.91 (0.82)	7.70 (1.07)

Table 2. Nominal concentrations (mg/kg feed), actual concentrations (mg/kg feed) $\pm$ (SE), mean (g) $\pm$ (SE) food consumption per bird per day, and mean dose benz[a]anthracene (mg) $\pm$ (SE) per bird in the sub-acute exposure study. Northern bobwhite quail were exposed to benz[a]anthracene at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed for 5 days. Control (0 mg/kg), actual concentration was less than method detection limit (<MDL). Not available (NA).

Nominal Concentration (mg/kg)	Actual Concentration (mg/kg)	Food Consumption/Bird/Day (g)	Dose/Bird/Day (mg)
0	0 (<MDL)	6.12 (1.12)	NA
0.1	0.21 (0.03)	6.85 (0.54)	1.43 (0.55)
1	1.02 (0.04)	5.75 (0.40)	5.89 (0.13)
10	11.4 (0.38)	5.90 (0.78)	67.5 (0.51)
100	98.1 (3.93)	5.36 (0.37)	530 (0.67)
1000	1100 (51.7)	5.67 (0.61)	6200 (2.25)

Table 3. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed which were euthanized on day 5 of the sub-acute study.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	168 (38.0)	16.1 (6.00)
0.1	3	463 (129)	216 (52.0)
1	3	198 (54.0)	112 (76.0)
10	3	310 (58.0)	231 (44.0)
100	3	411 (156)	432 (67.0)
1000	3	345 (90.0)	262 (190)

Table 4. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed which were euthanized on after the 3 day recovery period (day 8) of the sub-acute study.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	27	250 (62.0)	97.9 (23.0)
0.1	10	327 (51.0)	160 (31.0)
1	10	403 (35.0)	158 (47.0)
10	10	389 (45.0)	284 (47.0)
100	10	680 (103)	144 (31.0)
1000	10	468 (55.0)	148 (33.0)

Table 5. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.7 mg/kg feed which were euthanized after the 3 day recovery period (day 8) of the sub-acute study.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	27	102 (27.0)	31.1 (22.0)
0.1	10	103 (13.0)	33.8 (9.00)
1	10	85.2 (13.0)	28.7 (6.00)
10	10	106 (10.0)	47.3 (8.00)
100	10	84.0 (11.0)	29.7 (9.00)
1000	10	46.2 (6.00)	14.9 (6.00)

Table 6. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.21 ± 0.03 , 1.02 ± 0.04 , 11.4 ± 0.38 , 98.1 ± 3.93 , and 1100 ± 51.67 mg/kg feed which were euthanized on day 5 of the sub-acute study.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	63.7 (17.0)	11.1 (6.00)
0.1	3	56.1 (10.0)	44.6 (9.00)
1	3	47.7 (23.0)	11.5 (23.0)
10	3	76.9 (20.0)	6.07 (12.0)
100	3	121 (47.0)	34.1 (13.0)
1000	3	278 (87.0)	13.0 (15.0)

Table 7. Mean food consumption for days 1-30. Grand mean ($\pm$ SE) food consumption. Northern bobwhite quail were exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 ($<$ Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.4 ± 0.54 mg/kg feed for 1, 3, 9, 30, or 60 days.

Day	Food Consumption/Treatment Group			
	Control	0.1 (mg/kg)	1 (mg/kg)	10 (mg/kg)
1	6.18	6.34	5.76	5.98
2	6.77	6.73	6.89	6.76
3	7.46	7.23	7.39	7.37
4	6.53	6.21	6.43	6.67
5	7.33	7.45	7.27	7.29
6	9.65	9.71	9.89	9.41
7	6.46	6.25	7.49	7.51
8	8.73	8.97	8.46	8.42
9	8.56	6.43	8.49	8.19
10	8.69	9.01	8.76	8.56
11	9.12	9.17	9.26	9.03
12	8.95	9.34	8.65	9.37
13	9.16	9.51	9.82	9.27
14	8.75	8.72	8.64	9.01
15	9.43	9.67	9.71	9.41
16	9.13	9.52	9.29	9.65
17	9.58	9.51	9.27	9.11
18	9.56	9.42	9.91	10.1
19	9.25	9.85	8.91	8.67
20	9.76	9.56	9.43	9.76
21	9.84	10.0	9.54	9.22
22	9.68	10.0	9.42	9.23
23	9.95	9.93	9.67	9.95
24	10.0	10.3	8.99	10.2
25	9.65	9.72	10.2	9.47
26	9.98	10.1	9.56	9.95
27	10.6	10.5	11.0	10.8
28	10.4	10.3	10.1	10.4
29	11.7	11.3	11.9	11.5
30	10.7	10.9	11.3	11.1
Grand	9.05	9.05	9.05	9.05
Mean	(0.24)	(0.27)	(0.25)	(0.24)

Table 8. Total benz[a]anthracene consumed (g) per bird per exposure group on days 1, 3, 9, and 30 of the sub-chronic study. Nominal concentrations (mg/kg feed) of benz[a]anthracene in feed and actual concentrations (mg/kg feed) $\pm$ (SE) of benz[a]anthracene in feed are shown. Northern bobwhite quail were exposed to benz[a]anthracene at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed for 1, 3, 9, 30, or 60 days.

Nominal	<u>Treatment Groups</u>			
	0	0.1	1	10
Actual	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
	0 (<MDL)	0.11 (0.01)	1.1 (0.04)	11.5 (0.54)
Day 1	NA	6.34e-7	5.76e-6	5.98e-5
Day 3	NA	2.06e-6	2.01e-5	2.01e-4
Day 9	NA	6.59e-6	6.83e-5	6.70e-4
Day 30	NA	2.69e-5	2.71e-4	3.00e-3

Table 9. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 1 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	193 (33.0)	< MDL
0.1	3	401 (17.0)	< MDL
1	3	535 (26.0)	175 (196)
10	3	769 (31.0)	212 (19.0)

Table 10. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 3 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	209 (71.0)	< MDL
0.1	3	448 (21.0)	64.3 (68.0)
1	3	616 (55.0)	< MDL
10	3	723 (12.0)	216 (104)

Table 11. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 9 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	108 (52.0)	< MDL
0.1	3	488 (39.0)	32.3 (74.0)
1	3	571 (11.0)	100 (6.00)
10	3	975 (113)	164 (44.0)

Table 12. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 30 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	158 (9.00)	< MDL
0.1	3	458 (20.0)	282 (200)
1	3	604 (40.0)	162 (123)
10	3	1290 (104)	67.8 (42.0)

Table 13. Mean $\pm$ (SE) hepatic ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 60 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	15	220 (41.0)	< MDL
0.1	8	530 (8.00)	77.6 (29.0)
1	8	707 (19.0)	287 (76.0)
10	8	870 (61.0)	489 (155)

Table 14. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 1 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	5.32 (2.00)	3.49 (0.60)
0.1	3	96.9 (13.0)	262 (244)
1	3	124 (9.00)	27.2 (12.0)
10	3	220 (9.00)	46.4 (7.00)

Table 15. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 3 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	16.7 (2.00)	12.7 (5.00)
0.1	3	114 (3.00)	55.3 (34.0)
1	3	138 (15.0)	26.6 (10.0)
10	3	306 (77.0)	96.4 (25.0)

Table 16. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 9 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	17.3 (8.00)	18.3 (9.00)
0.1	3	132 (11.0)	24.0 (7.00)
1	3	127 (19.0)	10.3 (9.00)
10	3	257 (35.0)	57.2 (15.0)

Table 17. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 30 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	3	39.2 (7.00)	10.8 (8.00)
0.1	3	160 (19.0)	11.7 (4.00)
1	3	158 (34.0)	90.0 (67.0)
10	3	316 (69.0)	97.3 (36.0)

Table 18. Mean $\pm$ (SE) renal ethoxyresorufin-O-deethylase and pentoxyresorufin-O-deethylase activity (pmol/min/1000 mg protein) from Northern bobwhite quail exposed to BAA at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed. Activities of quail euthanized on day 60 are shown.

Treatment Group (mg/kg)	n	EROD Activity (pmol/min/1000 mg protein)	PROD Activity (pmol/min/1000 mg protein)
0	15	72.9 (5.00)	9.89 (6.00)
0.1	8	117 (15.0)	86.3 (84.0)
1	8	169 (17.0)	103 (68.0)
10	8	430 (99.0)	235 (109)

Table 19. Mean $\pm$ (SE) Hepatosomatic Index (HSI) values from Northern bobwhite quail exposed to benz[a]anthracene at concentrations of 0, 0.1, 1, 10, 100, or 1000 mg/kg feed. All samples, day 5 samples, and samples taken after the 3 day recovery period (day 8) of the sub-acute study are shown.

Treatment Group	n	HSI (all)	n	HSI (day 5)	n	HSI (day 8)
0 (mg/kg)	30	2.74 (0.04)	3	2.70 (0.06)	27	2.75 (0.05)
0.1 (mg/kg)	13	2.76 (0.08)	3	2.65 (0.16)	10	2.79 (0.09)
1 (mg/kg)	13	2.57 (0.06)	3	2.48 (0.02)	10	2.60 (0.08)
10 (mg/kg)	13	2.69 (0.08)	3	2.79 (0.11)	10	2.66 (0.09)
100 (mg/kg)	13	2.85 (0.06)	3	2.75 (0.1)	10	2.88 (0.07)
1000 (mg/kg)	13	3.63 (0.09)	3	3.41 (0.04)	10	3.70 (0.11)

Table 20. Mean $\pm$ (SE) Hepatosomatic Index (HSI) values from Northern bobwhite quail exposed to benz[a]anthracene at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.46 ± 0.54 mg/kg feed. All samples as well as days 1, 3, 9, 30 and 60 samples of the sub-chronic study are shown.

Treatment Group	0 (mg/kg)	0.1 (mg/kg)	1 (mg/kg)	10 (mg/kg)
n	27	20	21	20
HSI All	2.28 (0.05)	2.61 (0.06)	2.34 (0.04)	3.03 (0.06)
n	3	3	3	3
HSI Day 1	1.89 (0.06)	2.44 (0.09)	2.42 (0.04)	2.84 (0.09)
n	3	3	3	3
HSI Day 3	2.18 (0.13)	2.47 (0.04)	2.39 (0.11)	3.05 (0.03)
n	3	3	3	3
HSI Day 9	2.13 (0.05)	2.36 (0.09)	2.36 (0.22)	2.67 (0.26)
n	3	3	3	3
HSI Day 30	2.16 (0.09)	2.60 (0.15)	2.37 (0.05)	3.29 (0.09)
n	15	8	9	8
HSI Day 60	2.44 (0.06)	2.82 (0.09)	2.28 (0.06)	2.98 (0.11)

Table 21. Actual P values of the interaction (Treatment * Time), treatment, and time evaluating the variables EROD and PROD activity in the sub-chronic study are shown. Northern bobwhite quail were exposed to benz[a]anthracene at nominal concentrations of 0, 0.1, 1, or 10 mg/kg, and actual concentrations of 0 (< Method Detection Limit), 0.11 ± 0.01 , 1.10 ± 0.04 , and 11.5 ± 0.54 mg/kg feed for 1, 3, 9, 30, or 60 days.

	<u>Liver</u>		<u>Kidney</u>	
	EROD	PROD	EROD	PROD
Interaction	1.08e-2	0.56	0.12	0.43
Treatment	< 2.00e-16	7.50e-4	1.77e-14	0.03
Time	5.95e-4	0.99	0.18	0.73

TITLE : Effects of RDX on microbial communities in high bioavailability and low bioavailability soils

STUDY NUMBER: RDX-07-01

SPONSOR: Strategic Environmental and Research
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RESEARCH INITIATION: September 2006

RESEARCH COMPLETION: August 2008

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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:

Stephen Cox
Principal Investigator

Date

1.0 DESCRIPTIVE STUDY TITLE:

Effects of RDX on microbial communities in high bioavailability and low bioavailability soils.

2.0 STUDY NUMBER:

RDX-07-01

3.0 SPONSOR:

Strategic Environmental Research and Development Program
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4.0 TESTING FACILITY NAME AND ADDRESS:

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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 09/2006
Termination: 08/2008

6.0 KEY PERSONNEL:

Stephen B. Cox	Principal Investigator
John Zak	Research Assistant
Jennifer Humphries	Research Assistant
Dr. Ronald Kendall	Testing Facility Manager

7.0 STUDY OBJECTIVES / PURPOSE:

Many military sites, historically involved in the manufacture, packaging or disposal of explosive compounds, remain highly contaminated with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Few studies have examined the potential long-term effects of high concentrations of RDX on microbial communities in soil.

8.0 STUDY SUMMARY:

In this study, a sandy loam soil and a silt loam soil (high and low bioavailability, respectively) were artificially-contaminated with RDX (0, 50, 500, 1500, 5000, 10000, and 15000 mg/kg soil). Microbial communities from each treatment were monitored over 63 days to characterize the effects of RDX exposure on microbial activity, biomass, functional diversity (Biolog microtiter plates), and structural diversity (denaturant gradient gel electrophoresis (DGGE) of 16S rDNA). Microbial communities native to the high bioavailability soil were inherently different than microbial communities native to the silt loam soil, not only in terms of microbial activity and biomass, but also in terms of microbial community functional and structural diversity. Soil RDX contamination was correlated with decreased microbial biomass in the silt loam soil treatments and with

decreased microbial activity in the sandy loam soil treatments on day 7. RDX contamination did not cause a significant shift in the functional diversity of the microbial communities native to the silt loam soil, but was correlated with a shift in identities of substrates utilized by microbial communities native to the sandy loam soil on Day 7. Microbial community structure was insensitive to the gradient of RDX concentrations at the beginning of the incubation. However, the identities of carbon substrates utilized by microbial communities in both soil types were affected by long-term incubation with RDX.

9.0 TEST MATERIALS:

RDX was obtained from the Explosives Analytical Core.

10.0 JUSTIFICATION OF TEST SYSTEM:

Microbial communities are critically important for maintaining ecological function within soil ecosystems. Although the potential for microbial communities to biodegrade explosive compounds, especially within anaerobic marine sludges, has received considerable attention, the potential toxicity of explosive compounds on soil microbial communities has received relatively little attention. Understanding the potential changes in microbial communities, which may arise as a consequence of exposure to explosive compounds, is critical for achieving an ecologically relevant measure of the potential risk of explosive compounds to natural environments.

11.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Soil from two sampling locations were selected for use in this study (see methods section below).

12.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Hexahydro-1,3,5-trinitroso-1,3,5-triazine (purity, >99%) (SRI International, Menlo Park, CA, USA) was added in granular form to both the Harlan County, NE (N1-N7) and Terry County, TX (T1-T7) soils to create a total of seven treatments (0, 50, 500, 1500, 5000, 10000, and 15000 mg/kg soil). For each treatment, granular RDX and 50 ml ultrapure H₂O (>18 MΩ) were evenly incorporated into 3 kg soil by mixing for 30 min with a hand-held electric food mixer. The unamended control treatment for each soil type (N1 and T1, respectively) received 0 mg/kg RDX and 50 ml ultrapure H₂O, and was mixed for 30 min with the electric mixer.

Following spiking of soils with RDX, 140 g of soil was added to plastic “conetainers” (Stuewe & Son, Corvallis, OR, USA), creating 20 identical sacrificial replicates for each treatment. Conetainers were sealed with perforated polyfilm to both prevent contamination and excessive drying of the soils through evaporation. Conetainers were stored in racks at room temperature. On day 0, 10 ml ultrapure H₂O was added to each conetainer, and soils were allowed to drain freely. Soil moisture was monitored and was adjusted weekly by addition of ultrapure H₂O, to maintain between 12% - 20% moisture (based on dry weight).

13.0 METHODS:

Site Description and Sampling Procedure:

Soil from two sampling locations [Terry County, TX, USA (sandy loam) and Harlan County, NE, USA (silt loam)] have been characterized previously [14] and were selected for use in this study based on soil type and differences in bioavailability (high and low bioavailability soils, respectively). In April of 2007, soils from each site were sampled to a depth of 15 cm, and soil was sieved to remove the > 2 mm fraction. Approximately 24 kg of soil from each site was homogenized for 2 h in a cement mixer rotating at slow speed. Soils were stored at 4 °C for three weeks prior to initiation of the experiment.

Sampling and Analytical Analysis of Soils:

On day 0, 5 g of soil from each RDX-amended treatment was extracted with 50:50 acetonitrile:water using Accelerated Solvent Extraction (Dionex ASE 200, Sunnyvale, CA, USA) [15]. Briefly, each cycle included a 4 min preheat, 5 min heat and 5 min static extraction at constant temperature (100 °C) and pressure (1500 psi). Extracts (approximately 26 -30 ml) were collected in glass vials, were filtered through 0.45 µM nylon syringe filters (PALL Gellman, Fisher Scientific, Pittsburg, PA, USA), and were analyzed using a Hewlett Packard 1100 Liquid Chromatograph with UV detection. Isocratic separation utilized a reverse phase Discovery C18 (25 cm x 4.6 mm with 5 µm i.d., Supelco, Bellefonte, PA, USA) analytical column with a flow rate of 1 ml/min using 50:50 ultrapure water:acetonitrile. The injection volume was 50 µl and the total run time was 8 min. RDX was quantified based on calibration standards ranging from 0, 20, 50, 100, 150, and 200 µg/ml (Supelco, Bellefonte, PA, USA). HPLC-grade acetonitrile (Fisher Scientific, Pittsburg, PA, USA) and ultrapure water was used for all analytical methods.

Additionally, on Day 0, soil nutrient [nitrate (NO₃), ammonium (NH₄), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca)], and physicochemical [pH, % organic matter (OM), cation exchange capacity (CEC), particle size distribution] profiles were analyzed by Waters Agricultural Laboratories, Owensboro, KY, USA.

On days 7, 21, and 63, five replicate conetainers from each treatment were sacrificed. A sub-sample of soil from each sacrificial conetainer was placed in sterile tubes and was stored at 4 °C for 2 weeks prior to microbial analysis. Remaining soil was used to quantify RDX concentration, as well as to monitor shifts in soil physicochemical and nutrient profiles over time.

Soil Microbial Analysis:

Biolog GN microtitre plates (BIOLOG Inc., Hayward, CA, USA) were used to characterize microbial activity and community functional diversity, by monitoring carbon substrate utilization over time. Soil (10 g dry weight) from each sacrificial conetainer (n=5 per treatment, per timepoint), was emulsified in 0.2% water agar using an electric food processor and was serially diluted using sterile water [16-18]. Biolog 96-well plates were inoculated with 150 µl of the 10⁻⁴ dilution and were incubated at 25 °C for 72 h. Carbon substrate utilization was analyzed every 12 h at a wavelength of 590 nm.

Biomass:

The effects of RDX contamination on microbial biomass was measured over the duration of the study (n=5 per timepoint, per treatment) using a modified chloroform-fumigation-extraction method [19].

Denaturing Gradient Gel Electrophoresis (DGGE):

DGGE was used to characterize the effects of soil RDX contamination on microbial community structural diversity. Genomic DNA was extracted from 1 g of soil (n=5 per timepoint, per treatment) using UltraClean Soil DNA Extraction kits, and following the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA, USA). Extracted DNA was PCR-amplified using the bacterial primer 341f with a GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and the universal primer 519r (5'-ATT ACC GCG GCT GCT GG-3') (Integrated DNA Technologies, Coralville, IA, USA) as follows: each 25 ul reaction contained: 20 pmol of each primer, 0.4 mM of each dNTP, 1X Buffer (containing 2 mM MgCl₂), 1.5U Takara Ex Taq (Fisher Scientific, Pittsburg, PA, USA) and sterile water. Following PCR amplification (Thermocycler conditions: initial denaturation at 94 °C for 2 min; followed by 35 cycles of denaturation, annealing, and extension (at 98 °C for 10 s, 54 °C for 40 s, 72 °C for 1 min, respectively); followed by a final extension at 72 °C for 10 min), PCR products were stored at -20 °C.

Previously established DGGE protocols [20, 21] were modified and optimized to separate PCR-amplified 16S rDNA on a BioRad DCode DGGE system. Each 8% w/v polyacrylamide gel contained a denaturant gradient ranging from 35-36% (where 100% denaturant contains 40% formamide (Sigma-Aldrich, St. Louis, MO, USA) and 7M Urea (BioRad Laboratories, Richmond, CA, USA). After allowing 2-3 h for gel polymerization, PCR products were loaded, pulsed through the wells at 90 V for 15 min, and run at a constant current and temperature (60 V, 60 °C) for 16 h. A standard marker containing a mixture of *Pseudomonas aeruginosa*, *Shewanella putefaciens*, *Sphingomonas sp.*, *Ralstonia sp.*, *Desulfovibrio sp.*, run in the beginning, middle and end lanes of each gel, was used for normalization. After being stained in ethidium bromide, polyacrylamide gels were digitally photographed under UV light (Kodak Molecular Imaging Systems, New Haven, CT, USA). GelComparII software was used to analyze gel images, as described below.

Data Analysis:

Soil RDX concentrations in each treatment were analyzed on days 0 and 63, and measured values were utilized when characterizing treatment effects. Shifts in soil physicochemical and nutrient profiles were also monitored over the course of the study. Because there were no observed shifts in the soil nutrient and physicochemical profiles in each treatment over time (data not shown), soil data analyzed on days 0, 7, 21, and 63 were pooled and ANOVA was used to compare the means within treatment groups. Tukey's multiple comparison test was used to 1) compare each treatment to its respective site-specific unamended control, and 2) detect differences between the two unamended soil types (i.e., comparing soil profiles between treatments N1 and T1).

Biolog plates were analyzed as follows. The number of substrates utilized (substrate richness) and the amounts of substrates utilized (microbial activity) were calculated based on raw difference data (i.e., the absorbance registered in the control well subtracted from the absorbance registered in each of the 95 substrate-containing wells [16]). Boxplots, representing the median, upper and lower quartiles of the distribution and the extreme outliers (o) in each treatment, were used to visualize treatment effects. Significant differences between treatments were analyzed by ANOVA, and Tukey's multiple comparison test was used to a) compare each treatment to its respective site-specific unamended controls and b) detect differences between the two unamended soil types (N1 and T1). Additionally, spearman rank correlation was used to correlate microbial biomass, activity, and substrate richness with the soil physicochemical profiles and RDX concentrations in each soil and across the different timepoints.

Non-metric multidimensional scaling analysis (NMDS) and analysis of similarity (ANOSIM) was used to explain the differences in the substrate utilization profiles (SUPs) among treatments (in terms of the identity of substrates utilized). Ellipses, representing the standard error of the multivariate means of each treatment, depict the differences in SUPs among treatments. Vectors were used to illustrate significant correlations between soil physicochemical properties or RDX concentration and the NMDS ordination axes.

PCR-DGGE gels were analyzed by Gel CompareII professional software (Applied Maths, Austin, TX, USA) by first normalizing each gel using the standard markers. Gel lanes were assigned, visible bands within each gel lane were manually identified, and bands were quantified using a best-fit Gaussian curve. Gel images were compiled and band lanes across all gels were assigned (optimization was adjusted to 0.50 and position tolerance was adjusted to 1.00) (Heather Christensen, Applied Maths, personal communication, 2007). Binary band tables, representing the presence/absence and intensity of bands in each lane, were generated and the differences in community DGGE banding profiles was characterized by NMDS, as described above. All statistical tests were conducted using R [22].

14.0 RESULTS:

The high bioavailability soil collected from Terry County, TX was classified as sandy loam soil (71.4% sand, 2.6% silt, and 26.0% clay) and supported 1.1% organic matter (OM). The low bioavailability soil from Harlan County, NE was classified as silt loam soil (33.8 % sand, 10.2% silt, and 56.0% clay) and supported 2.5% OM. Soil characteristics were monitored over the course of the study to assess the effects of incubation and RDX contamination on physicochemical properties. Soil physicochemical profiles were not observed to shift over the course of the experiment as a result of incubation (data not shown). However, as was seen in both soil types, some soil properties were significantly affected by RDX amendment (Tables 1 and 2). For instance, soil NO₃ levels were significantly elevated in the most highly contaminated treatments (N4-N7, and L4-L7), relative to the unamended control treatments, following RDX contamination ($p < 0.001$). Likewise, soil OM was significantly elevated in both soil types in the most highly contaminated treatments (N6-N7, and L7, $P < 0.001$).

Extraction efficiency of RDX was highly variable in both soil types, and total extractable RDX was considerably lower than nominal concentrations in all treatments (Table 1a and 1b). Despite the poor extraction efficiency of RDX in this study, both soil types received similar concentrations of RDX. Extractable RDX concentrations in each treatment were not significantly different between soil types ($P>0.05$). RDX concentrations were not observed to vary over the course of the experiment, and no significant degradation was observed (data not shown).

Despite the fact that the two soil types had significantly different soil physicochemical profiles, the microbial communities native to the unamended N1 and T1 treatments supported similar levels of substrate richness (i.e., the numbers of substrates utilized) and microbial biomass at the beginning of the incubation (Day 7) (Figures 1, 3). Microbial activity (i.e., the amounts of substrates utilized) was lower in the T1 soil (Figure 2). The numbers of substrates utilized by microbial communities native to the two different soil types remained similar throughout the duration of the experiment and were not significantly affected by amendment with RDX in a dose-dependent manner (Figure 1). Microbial activity was observed to decrease in both soil types and in all treatments over the course of the experiment, likely as a result of incubation stress (Figure 2). This inhibition was most pronounced in the high bioavailability sandy loam soil, relative to the low bioavailability silt loam soil. By Day 63, the unamended T1 treatment supported significantly lower levels of microbial activity, relative to N1 (Figure 2, $p<0.001$). Microbial activity was significantly correlated with RDX concentration in the sandy Texas soil on Day 7; however, it was not correlated with RDX concentration at later time points or in the low bioavailability Nebraska soil (Tables 3, 4). Microbial biomass, on the other hand, was observed to decrease in the low bioavailability silt loam soil, relative to the sandy loam soil over the course of the experiment (Figure 3). Microbial biomass was significantly correlated with RDX concentration in the low bioavailability Nebraska soil on Day 7, however was not correlated with RDX concentration at later time points or in the high bioavailability Texas soil (Tables 3, 4).

As seen by their proximity in 2-dimensional space, the microbial communities native to the Nebraska soil treatments were significantly different than the microbial communities native to the Texas soil, in terms of the identities of substrates utilized (Figure 4, ANOSIM $R=0.2428$, $p<0.001$). This observed trend was consistent over the course of the experiment and was highly correlated with differences in the OM content, cation exchange capacity (CEC), pH, Ca, P, K, and NH_4 levels between the two soil types. The overlapping substrate utilization profiles (SUPs) of all seven Nebraska soil treatments (N1-N7) indicates that the microbial communities in this soil remained similar, in terms of the identities of substrates utilized, regardless of the large gradient in RDX concentrations within these treatments (Figure 4). ANOSIM of the Nebraska soil treatments shows that there were significant treatment-associated differences in the identities of substrates utilized ($R=0.1003$, $p<0.05$), however RDX concentration was not correlated with this shift ($p>0.05$, data not shown). On the other hand, RDX concentration weakly correlated with the shift in the identities of substrates utilized within the Texas soil treatments on Days 7 and 21 ($0.1<p<0.05$), indicating that the Texas

soil communities were slightly more sensitive to RDX contamination than Nebraska soil communities, at the beginning of the experiment.

Similar trends in SUPs between the two soil types were observed on both day 21 (ANOSIM $R=0.2026$, $p<0.001$) and day 63 (ANOSIM $R = 0.3061$, $p<0.001$). There were no treatment-associated differences in the Nebraska soil communities on day 21 (ANOSIM $R=-0.05023$, $p>0.05$) or on day 63 (ANOSIM $R = 0.02444$, $p>0.05$). RDX concentration was correlated with the shift in carbon substrate utilization observed within the Texas soil communities on day 21 ($p<0.05$), however, was not able to explain the shifts in the shift in the identities of substrates utilized in Texas soil treatments on Day 63.

Similar to the trends observed in microbial community functional diversity, the microbial communities native to the two different soil types were unique in terms of their structural diversity at the onset of the experiment (Figure 5). Over the course of the experiment, the differences in microbial community structural diversity between the two soil types were correlated with the same set of physicochemical properties that were previously observed to affect microbial functional diversity in soils (i.e., OM content, CEC, pH, Ca, P, K, and NH_4 , $p<0.05$). On day 7, there were no treatment-associated differences in the structural diversity of microbial communities native to the Nebraska soil (ANOSIM $R= -0.04364$, $p>0.05$) or the Texas soil (ANOSIM $R=0.01556$, $p>0.05$) (data not shown). On day 21, no significant treatment-associated differences were observed in the structural diversity of microbial communities native to the Nebraska soil (ANOSIM $R=0.04705$, $p>0.05$). Treatment-associated differences were observed in the structural diversity of microbial communities native to the Texas soil (ANOSIM $R=0.0192$, $p<0.05$), which was significantly correlated with soil NO_3 and P concentrations ($p<0.05$). Finally, treatment-associated differences in the structural diversity of microbial communities native to the Nebraska soil (ANOSIM $R=0.1444$, $p<0.05$) and Texas soil (ANOSIM $R=0.1635$, $p<0.05$) existed on Day 63. RDX concentrations were weakly correlated with the shift observed in microbial community structure in Nebraska soil treatments ($0.1<p<0.05$) and was significantly correlated with the shift observed in Texas soil communities ($p<0.05$).

15.0 DISCUSSION

RDX is a suspected human carcinogen and is listed as a priority pollutant by the Environmental Protection Agency (EPA) [1]. As a result, studies designed to assess the effects of RDX contamination on humans and wildlife have increased in recent years. Among other things, RDX exposure has been linked to decreased growth and egg production in earthworms [23], decreased serum triglyceride levels, food intake and increased mortality in rats [24], as well as seizures, delirium and neurotoxicity in humans [25]. With the exception of studies optimizing bioremediation, metabolism and degradation of explosive, few studies have characterized the effects of explosive compounds on microbial communities in soils. To our knowledge, only one other study has attempted to characterize the effects of RDX contamination on microbial community structure [26]. Limited numbers of studies have characterized the effects of RDX on microbial community activity and function, however those studies are largely focused on remediation and degradation of RDX by microbial populations [1, 5, 7, 27, 28]).

Understanding the potential changes in microbial communities, which may arise as a consequence of exposure to explosive compounds, is critical for achieving an ecologically relevant measure of the potential risk of explosive components to natural environments. Therefore, the purpose of this study was to assess the effects of a range of RDX concentrations on soil microbial community structural and functional diversity in high bioavailability and low bioavailability soils.

The soils selected for use in this study have been well-characterized, not only in terms of soil type, but also in terms of the bioavailability of energetic compounds. Previously in these soils, explosive compounds (TNX and MNX) were observed to be more toxic to earthworms housed in the sandy loam soil (Terry County, TX) compared to earthworms housed in silt loam soil (Harlan County, NE) [14]. Similarly, TNX and MNX were more readily transferred from the sandy loam soil to C18 semi-permeable membrane devices, relative to silt loam soil [29]. These results indicate higher bioavailability of explosives in the sandy soil, relative to the silt loam soil. This may be a function of elevated organic matter in the silt loam Nebraska soil, which has previously been noted to effectively bind RDX in soils [29].

Both soil types were amended with a wide range of RDX concentrations; however, the highest concentrations used in this study would only be likely to occur in the most extreme environmental scenarios. In 2000, Gong et al. amended their soils with a similarly high range of concentrations, to characterize the effects of RDX on microbial activity (nitrogen fixation, nitrification, dehydrogenase, and respiration) [27]. That study documented a lowest observable adverse effect concentration (LOAEC) of 1,235mg/kg RDX, and reported significant inhibition of select microbial activities at concentrations exceeding the LOAEC. Since few studies have been conducted to gauge the effects of RDX on microbial communities in soils, several treatments exceeding the previously established LOAEC were included in this study, in an effort to document an inclusive range of effect concentrations for these microbial structural and functional endpoints.

As was seen in a previous study, RDX recovery was highly variable and was significantly lower than nominal levels [27]. Poor recovery was consistent across soil type and treatment, and did not appear to be related to bioavailability in soils. Actual RDX concentrations in treatments were used for comparisons and correlations. In both soil types, RDX amendment was correlated with significantly increased soil NO₃ levels. The elevated NO₃ levels in the most highly contaminated treatments likely were a function of the high nitrogen content of RDX [1]. RDX amendment was also correlated with significantly increased organic matter in both soil types. Underlying mechanisms for observed increases in soil organic matter remain to be determined; however increased organic carbon content in RDX contaminated soils was observed previously in field-contaminated soils [1].

As would be expected based on location and soil type, and the spatial heterogeneity of microbes in soils, the microbial communities native to the two different soils were unique to each soil and were inherently different in terms of both their structural and functional diversity [30]. Following amendment with RDX, soil microbial communities from both

soil types remained functionally unique over the course of the experiment. RDX contamination was not observed to cause a shift in the functional diversity of the microbial communities in either soil type, regardless of the large gradient of RDX concentrations within the RDX-amended treatments.

Unlike the results of Gong et al., who reported significant decreases in microbial activity in soils where RDX concentrations exceeded 1235 mg/kg, no significant decreases in microbial activity were detected in soils contaminated with up to 15000 mg/kg RDX, which far exceeds the previously reported LOAEC for RDX [27]. Likewise, reduced soil quality and decreased soil microbial biomass carbon was previously reported in RDX, TNT, and HMX contaminated field soil [1], however, with the exception of the Day 7 Nebraska soil treatments, RDX was not correlated with decreased microbial biomass in this study. While the high bioavailability Texas soil appeared to be slightly more sensitive to RDX contamination in terms of its overall functional diversity on Day 7, this trend was not consistent over the course of the experiment and soil bioavailability did not seem to significantly influence RDX toxicity to microbial communities in this study.

The sole endpoint that appeared to be affected by RDX contamination in this study was microbial community structure; however, shifts in microbial community structure (i.e., the number and abundance of species) were slow to develop and only became apparent after 63 days of incubation. Effects of RDX contamination on microbial community structure have been reported previously in microbial communities native to the highly contaminated Iowa Army Ammunition Plant, which supported decreased proportions of gram-positive organisms, relative to uncontaminated soils at the same site [31]. Conversely, Juck et al., failed to find shifts in microbial community structure (measured by DGGE) in soil columns amended with 1000 mg/kg RDX [26]. One reason for the discrepancy between Juck et al. (2002) and the present study may be due to the difference in RDX concentrations assessed, where the present study used 15-fold higher concentrations. Nevertheless, further molecular-based studies are needed to better understand the potential effects of long-term low level RDX contamination on microbial community structure and function in soils.

Shifts in microbial community structure may or may not correspond with a shift in microbial community function, depending on the degree of functional overlap within microbial functional guilds [32]. Evidence from this study demonstrates that the microbial communities native to these two soil types support enough functional overlap to allow them to perform similar ecological roles within the soils (relative to carbon substrate utilization), despite RDX-induced shifts in community structure. From an ecological perspective, effects of RDX contamination on microbial community activity and function are more relevant when trying to assess environmental implications, because loss of critical functions in soils could be correlated with larger ecosystem-level effects (i.e., nutrient cycling, plant growth, soil fertility and health). So, in this regard, it is encouraging to note that the high concentrations of RDX used in this study did not result in significant shifts in microbial community function, at least in terms of carbon substrate utilization. Future work using more ecologically relevant functional endpoints would

help to assess how more critical soil functions in could be affected by long-term RDX contamination.

16.0 REFERENCES:

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Table 1: Soil physicochemical properties (mean (Standard error)) within Harlan County, NE soil treatments. Significant differences of RDX amended treatments (N2-7) relative to experimental control (N1) are denoted ** (p<0.001) and * (p<0.05). Significant differences between soil properties within Harlan County, NE control soil (N1) and Terry County, TX control soil (L1, Table 1b) are denoted ^{aa} (p<0.001) and ^a (p<0.05).

Property	Treatments													
	N1		N2		N3		N4		N5		N6		N7	
NO ₃ (mg/kg)	37.1	(21.3)	42.6	(11.1)	127.1	(13.8)	194.2	(21.7)**	207.9	(24.6)**	215.1	(25.4)**	211.9	(24.0)**
NH ₄ (mg/kg)	1.9	(0.5)	2.0	(0.2)	1.4	(0.1)	1.5	(0.2)	1.5	(0.5)	1.8	(0.2)	1.5	(0.0)
pH	7.6	(0.1) ^{aa}	7.7	(0.0)	7.7	(0.0)	7.7	(0.1)	7.7	(0.1)	7.7	(0.0)	7.7	(0.0)
P (mg/kg)	271.0	(1.8) ^{aa}	284.7	(6.2)	282.0	(4.4)	281.2	(1.5)	760.2	(15.8)	290.5	(1.3)	273.0	(3.7)
K (mg/kg)	776.7	(43.2) ^{aa}	809.3	(16.8)	786.7	(7.8)	781.8	(19.8)	363.7	(1.9)	792.7	(11.3)	734.7	(17.9)
Mg (mg/kg)	388.0	(24.8)	389.3	(9.4)	378.5	(5.6)	374.7	(3.2)	3241.3	(97.1)	381.2	(8.2)	357.2	(7.1)
Ca (mg/kg)	3588.7	(399.5) ^a	3448.0	(165.9)	3363.2	(114.1)	3351.8	(86.6)	6482.7	(194.2)	3390.7	(143.0)	3180.5	(112.7)
CEC (meq/100g)	24.8	(2.3) ^a	24.7	(0.6)	23.8	(0.5)	23.8	(0.1)	22.9	(0.3)	23.9	(0.7)	22.5	(0.5)
OM (%)	2.7	(0.2) ^{aa}	2.7	(0.1)	2.6	(0.1)	2.7	0.1	3.2	0.1	3.7	(0.2)**	4.2	(0.2)**
RDX (mg/kg)	ND	ND	ND	ND	296.3	(115.5)**	672.3	(173.1)**	3731.3	(449)**	5298.7	(867.2)**	7170.0	(1271.3)**

Table 2: Soil physicochemical properties (mean (SE)) within Terry County, TX soil treatments. Significant differences of RDX amended treatments (L2-7) relative to experimental control (L1) are denoted ** (p<0.001) and * (p<0.05).

Property	Treatments													
	L1		L2		L3		L4		L5		L6		L7	
NO ₃ (mg/kg)	49.2	(4.5)	34.2	(4.7)	126.5	(15.4)	183.4	(22.3)**	208.9	(28.6)**	209.7	(26.9)**	209.0	(26.9)**
NH ₄ (mg/kg)	1.8	(0.5)	1.1	(0.2)	0.9	(0.1)	0.9	(0.2)	1.9	(0.8)	0.8	(0.2)	0.6	(0.3)
pH	8.2	(0.0)	8.3	(0.0)	8.2	(0.0)	8.2	(0.0)	8.2	(0.0)	8.2	(0.0)	8.2	(0.0)
P (mg/kg)	41.7	(0.7)	37.0	(0.3)	37.5	(0.8)	35.3	(0.6)	35.5	(0.05)	35.5	(0.0)	36.2	(0.8)
K (mg/kg)	559.8	(11.2)	567.0	(4.1)	563.7	(10.9)	546.7	(17.5)	557.0	(6.3)	530.2	(10.5)	537.0	(19.8)
Mg (mg/kg)	386.2	(5.9)	392.2	(8.5)	391.5	(12.4)	379.2	(10.8)	389.3	(10.1)	374.7	(8.3)	357.2	(7.1)
Ca (mg/kg)	5975.7	(387.8)	6130.7	(488.3)	6122.5	(571.5)	5987.5	(488.2)	36.2	(2.6)	5939.0	(528.7)	6016.5	(599.3)
CEC (meq/100g)	35.7	(1.9)	3.6	(2.5)	36.5	(3.0)	35.7	(2.5)	6058.7	(505.4)	35.4	(2.7)	35.8	(3.2)
OM (%)	1.2	(0.1)	1.2	(0.1)	1.2	(0.1)	1.3	(0.1)	1.5	(0.1)	1.9	(0.1)	2.4	(0.2)**
RDX (mg/kg)	ND	ND	ND	ND	327.0	(21.1)**	500.3	(24.4)**	3314.7	(266.3)**	4987.0	(502.9)**	6670.0	1025.4**

Table 3: Results of Spearman Rank Correlation. Tabled values represent correlation coefficients (r_s) for soil RDX concentrations or physicochemical attributes which were significantly related to substrate richness, microbial biomass and microbial activity and species richness in Nebraska soil treatments, where ** ($p < 0.001$), * ($p < 0.05$) and @ ($0.1 < p < 0.05$).

	Biomass			Activity			Richness		
	Day 7	Day 21	Day 63	Day 7	Day 21	Day 63	Day 7	Day 21	Day 63
NO3	-0.40*	-0.36*							
NH4				-0.35*				-0.35*	
pH	-0.39*					0.34*			-0.49*
P									
K									
Mg									
Ca	0.37*								
CEC						0.34*			
OM	-0.47*		-0.35*						
RDX	-0.48*								

Table 4: Results of Spearman Rank Correlation. Tabled values represent correlation coefficients (r_s) for soil RDX concentrations or physicochemical attributes which were significantly related to substrate richness, microbial biomass and microbial activity and species richness in Texas soil treatments, where ** ($p < 0.001$), * ($p < 0.05$) and @ ($0.1 < p < 0.05$).

	Biomass			Activity			Richness		
	Day 7	Day 21	Day 63	Day 7	Day 21	Day 63	Day 7	Day 21	Day 63
NO3		-0.36*		0.36*					
NH4									
pH				-0.20*				0.34*	0.44*
P									0.37*
K		0.34*							
Mg		0.38*							0.38*
Ca								0.34*	
CEC		0.38*							
OM				0.46*					
RDX				0.43*					

FIGURE LEGENDS

Figure 1: Total substrate richness supported by of bacterial communities within the RDX-amended Nebraska (N1-N7) and Texas (T1-T7) soil treatments on days 7, 21, and 63. Boxplots represent the median, upper and lower quartiles of the distribution and the extreme outliers (o) in each treatment. Whiskers include data points within 1.5 times the interquartile range. Significant differences between RDX-amended treatments and their respective unamended controls are denoted (** $p < 0.001$, * $p < 0.05$). Significant differences between soil types (N1 vs T1) are denoted (^{aa} $p < 0.001$, ^a $p < 0.05$), based on Tukey's multiple comparison test.

Figure 2: Total microbial activity supported by of bacterial communities within the RDX-amended Nebraska (N1-N7) and Texas (T1-T7) soil treatments on days 7, 21, and 63. Boxplots represent the median, upper and lower quartiles of the distribution and the extreme outliers (o) in each treatment. Whiskers include data points within 1.5 times the interquartile range. Significant differences between RDX-amended treatments and their respective unamended controls are denoted (** $p < 0.001$, * $p < 0.05$). Significant differences between soil types (N1 vs T1) are denoted (^{aa} $p < 0.001$, ^a $p < 0.05$), based on Tukey's multiple comparison test.

Figure 3: Total microbial biomass supported by of bacterial communities within the RDX-amended Nebraska (N1-N7) and Texas (T1-T7) soil treatments on days 7, 21, and 63. Boxplots represent the median, upper and lower quartiles of the distribution and the extreme outliers (o) in each treatment. Whiskers include data points within 1.5 times the interquartile range. Significant differences between RDX-amended treatments and their respective unamended controls are denoted (** $p < 0.001$, * $p < 0.05$). Significant differences between soil types (N1 vs T1) are denoted (^{aa} $p < 0.001$, ^a $p < 0.05$), based on Tukey's multiple comparison test.

Figure 4: Indirect gradient analysis of microbial carbon substrate utilization. Variation in the identities of substrates utilized by bacterial communities within the Nebraska (outlined polygon) and Texas soil treatments (shaded polygon) are projected onto two axes via non-metric multidimensional scaling (based on Jaccard's index of similarity). Ellipses represent 95% confidence ellipses of the multivariate means of each treatment. Arrows represent significant correlations of soil variables with ordination axes, where ** $p < 0.001$, and * $p < 0.05$.

Figure 6: Indirect gradient analysis of DGGE banding patterns. Tables, representing the presence and intensity of bands were generated using GelCompareII Software. Variation in these banding fingerprints is projected onto two axes via non-metric multidimensional scaling (based on Jaccard's index of similarity). Ellipses represent 95% confidence ellipses of the multivariate means of the bacterial communities within the Nebraska (outlined polygon) and Texas soil treatments (shaded polygon). Arrows represent significant correlations of soil variables with ordination axes, where ** $p < 0.001$, and * $p < 0.05$.

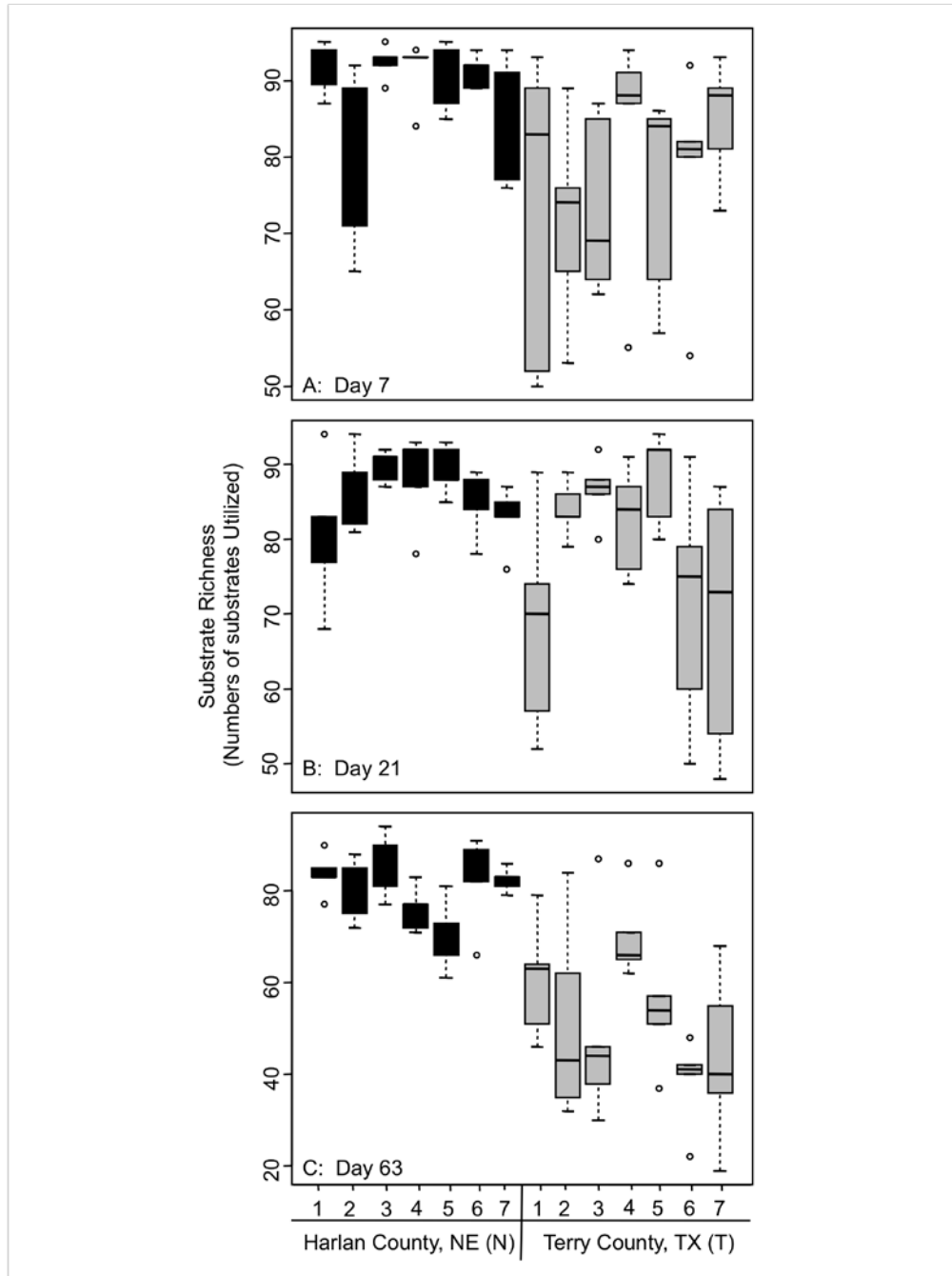


Figure 1.

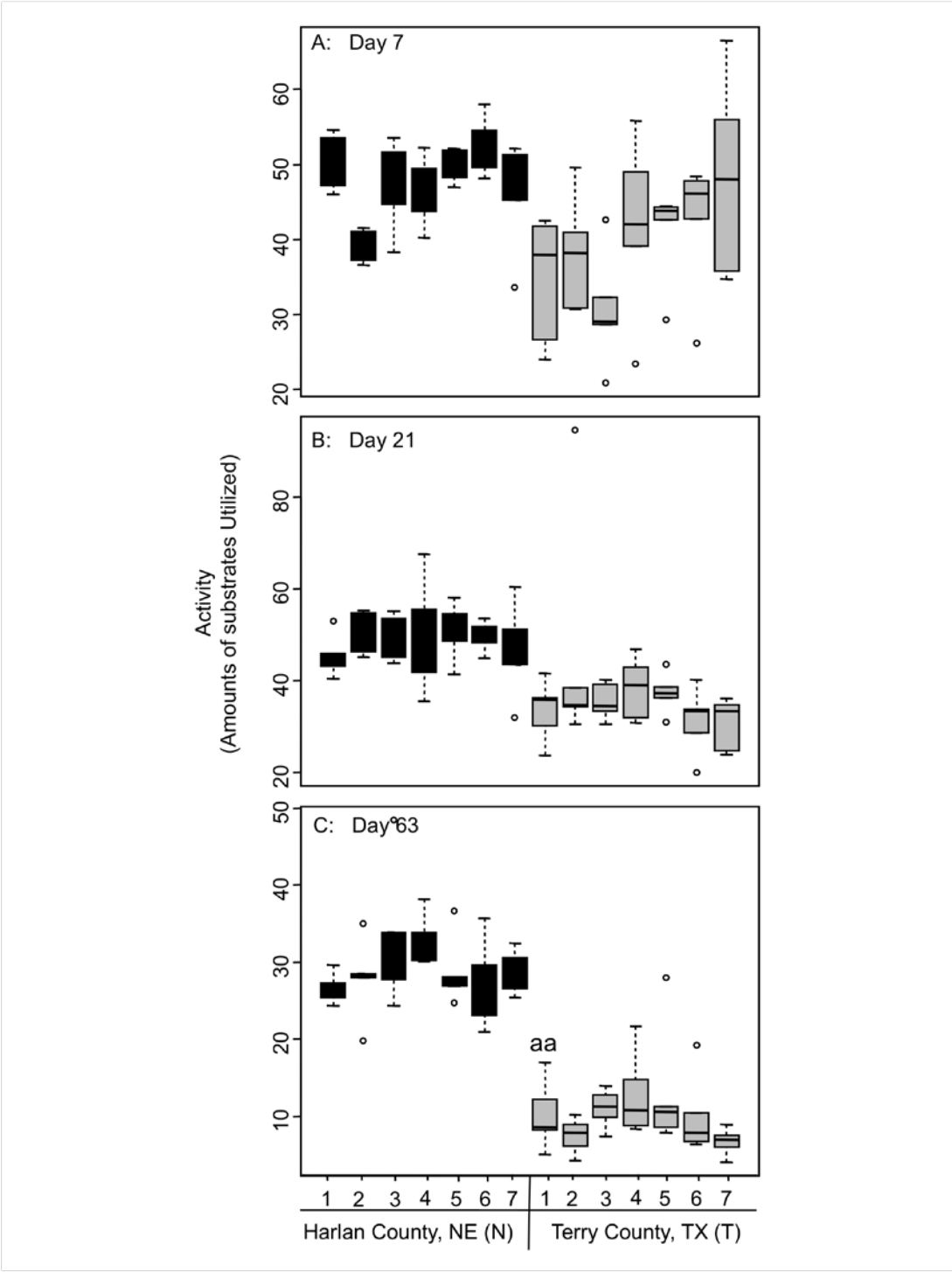


Figure 2.

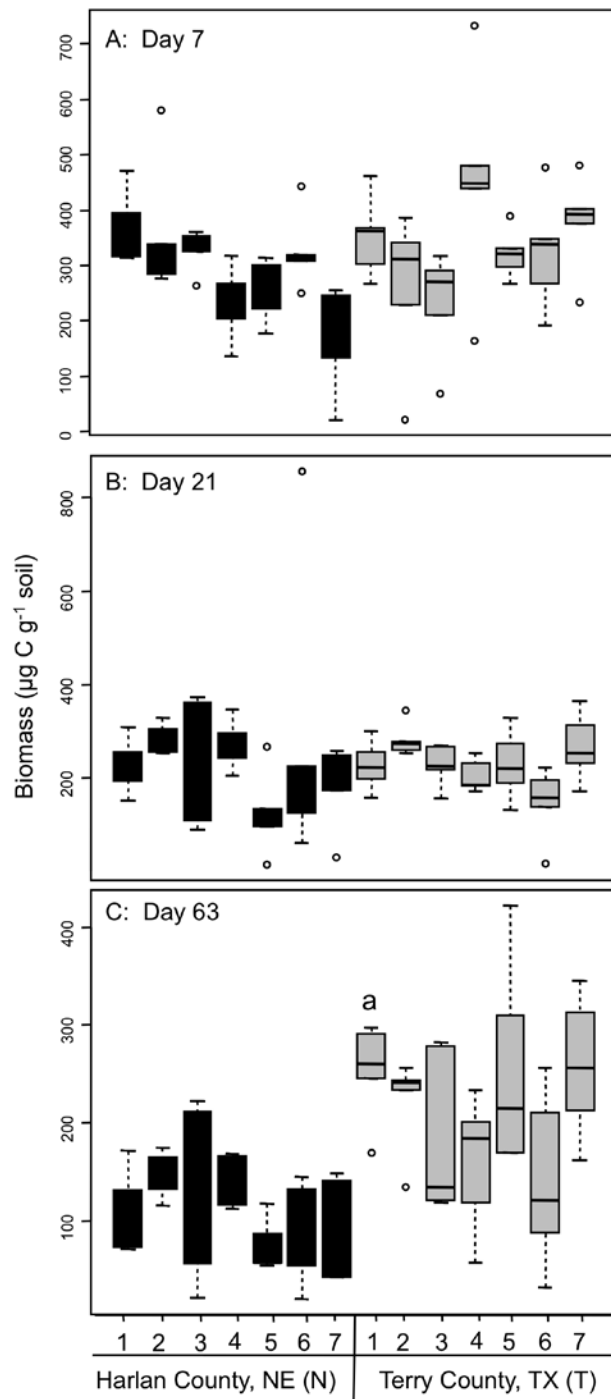


Figure 3.

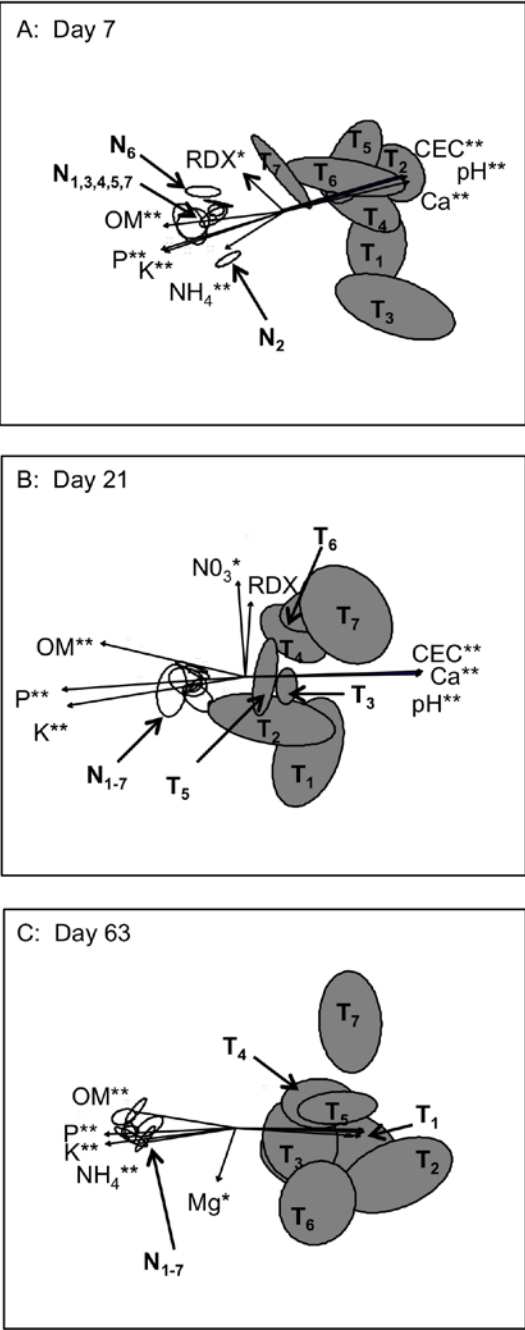


Figure 4.

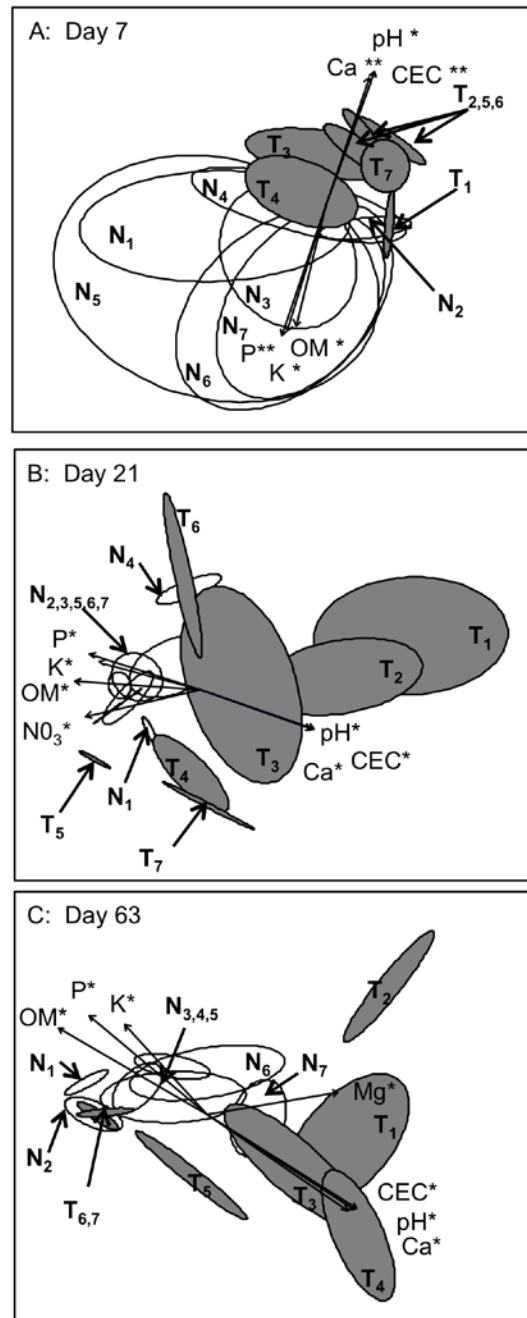


Figure 5.