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Environmental Contamination and Toxicology

Effects of Energetic Compounds on the Northern Bobwhite Quail and Biotransformation Applications of the Intestinal Flora

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Energetic compounds, such as Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and 2,4,6 Trinitrotoluene (TNT), have been detected in more than one-million cu yards of soil at 28 military bases and production facilities in the United States (Preslan *et al*, 1993). These sites are often valued habitats for wildlife; therefore effects from exposure to these compounds need to be evaluated. In recent years, methods to evaluate the toxicity of these compounds in mammals, and to remove them from the environment have been conducted (Dilley *et a.*, 1982). Acute RDX exposure has been associated with neurological effects including tremors, convulsions, and decreased motor activity at single oral exposures as low as 12.5 mg/kg (Cholakis *et al.* 1980, MacPhail *et al.*, 1985). Relatively higher acute oral HMX exposure was required for death in mice and rats, where adverse neurological symptoms were also reported (Cuthbert *et al.*, 1985). Acute exposures to TNT have been reported to cause a hypochromic anemia, splenomegaly and hepatomegaly, decreased body weight, and hypercholesterolemia in dogs (Dilley *et al*, 1982).

Bioremediation of these compounds may provide an environmentally safe alternative method of disposal. Boopathy *et al* (1998) were able to develop an aerobic/anoxic soil slurry reactor that completely degraded TNT. Williams *et al* (1992), used a composting method that reduced TNT levels from over 11,000 mg/kg to 3 mg/kg, reduced HMX levels from over 700 mg/kg to 26 mg/kg and reduced RDX levels from over 5000 mg/kg to 45 mg/kg in 150 d. Our study evaluated the effects of an acute oral exposure to a subtoxic dose of RDX, HMX and TNT on the Northern Bobwhite and the potential of the intestinal flora to break down these compounds *in vitro*.

MATERIALS AND METHODS

Northern Bobwhite quail (*Colinus virginianus*), approximately 1 yr of age, were hatched and grown at the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA. All birds were housed under Virginia Tech's Animal Care and Use Committee's guidelines. The study consisted of 3 phases; phase I to set up protocol for media composition and HPLC analysis; phase II was the oral dosing, phase III was to test the ability of the bacterial isolates to break down the compounds in vivo.

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For the first phase, M-9 glucose deficient minimal media was prepared (Sambrook et al, 1989) supplemented with a 10% casamino acids solution and Bacto agar (Difco Sparks, MD). Each of the 3 energetic compounds was dissolved in acetone then supplemented into media to achieve media concentrations of 12.5 mg/kg, 125 mg/kg, and 1250 mg/kg of the individual energetic compound. Media was poured into petri dishes under a Class II sterile hood, and left with the lids tilted for a minimum of 9 hr to allow for acetone evaporation, then incubated for 24 hr at 37 °C.

Six birds (3 male, 3 female) were euthanized via electrocution. The ceca and duodenum were isolated, their contents aseptically removed, and diluted 1:10 with sterile physiologically buffered saline (Media Tech Manassas, VA). During phase I, control cecal-saline and duodenal-saline suspensions were streaked on the following plates: control (ie: no energetic compound supplemented), RDX, HMX or TNT supplemented at one the following concentrations 12.5 mg/kg, 125 mg/kg, 1250 mg/kg. Once inoculated plates. were incubated aerobically at 37 °C for 2 wk. Individual colonies selected based on morphology were streaked onto 2° plates supplemented with the same compound at the same concentration as the 1° plate, then incubated aerobically at 37 °C for 2 wk.

Three d before the start of the second phase, thirty quail were moved into side by side individual cages in an alternating male:female pattern. The control group consisted of 6 birds, while each of the 3 treatment groups (RDX, HMX, TNT) consisted of 8 birds with a 1:1 male:female ratio. The quail were fasted for 24 hr prior to oral gavage, and obtaining baseline weight. An aqueous 125 mg/kg suspension of the solid form of each explosive compound was prepared and administered at a dosage of 125 mg/kg of body weight. Control birds were administered water only. After dosing, the quail were given feed trays filled with Southern States Sporting Bird Feed to a total wt of 600g. Birds and feed trays were weighed again on d 3 and 7. On d 3 and 7, birds (n=15/d) were euthanized and cecal samples were obtained. Cecal-saline suspensions (1:10 dilutions in PBS) from treatment and control groups were used to streak a control plate and 2 plates with the prospective compound at a concentration of 125 mg/kg. Minimal media for the second phase was prepared in the same manner as above; except the energetic compounds were only supplemented at a level of 125 mg/kg of media and no casamino acids were supplemented.

On d 1,3 and 7, blood was collected via right jugular venipuncture, and blood smears were made manually as described by Gogal *et al* (2001). A 5-point differential was performed; which enumerated lymphocytes, monocytes, heterophils, basophils, and eosinophils (100 leukocytes/slide). In preparation for HPLC analysis, blood samples were centrifuged (500 x g, 15 min, 23°C) through a YM-30 micron centrifugal filter device by Millipore (Bedford, MA). Filtrate was analyzed on a Beckman Gold HPLC system with a variable wavelength detector model number 165 set at 254 nm, a solvent delivery module pump model 114M, and an analog interface module model 406. The mobile phase consisted of

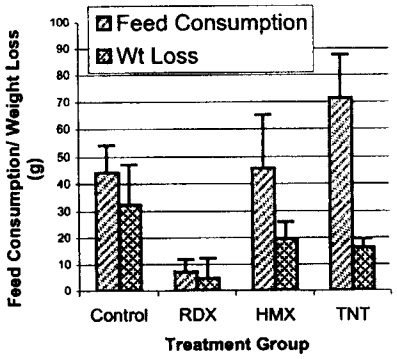

Figure 1 Feed Consumption and Wt Loss (d 3).

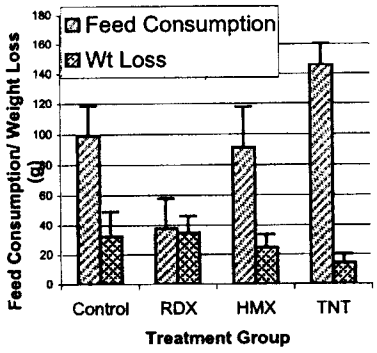

Figure 2 Feed Consumption and Wt Loss (d 7).

a 50/50 methanol/water mixture that ran at 1.5 mL/minute. A 100 µL aliquot of each sample was injected into a 4.6 x 250 mm column, C18 5 micron with a standard guard. On d 1, 3 and 7; fresh feces were collected and mixed in deionized water until an even consistency was obtained. These samples were then dried in an oven at 65°C. Once dried, the samples were crushed and mixed into a powder-like consistency. Two grams of powdered feces was resuspended in 2 ml acetonitrile via vortexing. The suspension was centrifuged at 1800 x g, 15 min, at 23°C, and then 100 µL of supernatant was subjected to HPLC analysis as previously stated.

For phase III glucose deficient M-9 minimal media broth was prepared as described by Sambrook *et al.* (1989) then supplemented with the appropriate amount of an RDX, HMX, or TNT acetone solution to make a 125 mg/L final compound concentration in the media. Media was left under a class II sterile hood overnight to allow for acetone evaporation. Pure cultures from treatment groups, that grew on 2° plates were inoculated into 50 mL of broth media; then incubated at 37°C in a reciprocal shaking water bath for 4 wk. Bacterial growth was estimated by measuring turbidity on a Klett-Summerson Photoelectric Colorimeter model 800-3 (Klett Manufacturing INC, New York) weekly at the same time that 1.0 mL samples were taken for HPLC analysis. One ml media samples were prepared for HPLC analysis by oven drying them at 65°C. Dried samples were resuspended in 200 µL of acetonitrile, and centrifuged through a YM-30 micron filter by Millipore (Bedford, MA) at 500 x g for 15 min at 23°C. Next, 100 µL of supernatant was analyzed on a Beckman Gold HPLC System with the previous stated specification based on methods described by Lyter (1983).

RESULTS AND DISCUSSION

During the first phase, growth was observed on the 12.5, 125, and 1250 mg/kg plates supplemented with casamino acids for all compounds and the controls.

However, the 125 mg/kg plates with casamino acids for all compounds had the highest yield of individual colonies/plate. Growth occurred on RDX plates at all 3 concentrations inoculated with cecal contents in an average of 2 d, while plates inoculated with duodenal samples grew in an average of 3 d. Primary control plates without casamino acids had limited growth. However, 2° plates of the controls prepared without casamino acids had no growth. Plates prepared with RDX, HMX or TNT at a concentration of 125 mg/kg without casamino acids grew. During the second phase, there was no difference in colony numbers/plate or morphological colony types/plate when comparing treatment group plates to controls. However, in the RDX treatment group there was a direct correlation between increases in the number of morphological types of colonies/plate and feed consumption. This may have been due to variations in avian GI tract.

Blood smears from all 3 treatment groups contained large numbers of vacuolated monocytes and heterophils which suggests compound related stress from exposure. Average heterophil/lymphocyte ratios were greater than 1 on d 1, 3 and 7; except for the TNT treatment group, which normalized by d 7. Further, all treatment groups exhibited a basophilia on d 1,3 and 7.

Average RDX and HMX blood concentrations were inversely related to feed consumption; as concentrations increased, consumption decreased. TNT treatment groups consumed more feed than controls on d 3 and 7; however, feed consumption in all treatment groups was highest on d 7 when levels of compounds in the blood were low. On d 3 and 7, RDX and HMX treatment groups lost more wt than controls **(Figs.1-2)**. TNT treatment group lost less wt than control birds on d 3 and 7. Results of HPLC analysis of blood and fecal samples are shown in **Table 1**.

Table 1. Mean concentration of energetic compounds in the blood and feces.

Blood	d 1	d 3	d 7	Feces	d 1	d 3	d 7
RDX	35.0 ± 4.0	50.0 ± 9.6	11.8[B]	RDX	84 ± 82	0	17.5[c]
HMX	5.5 ± 1.6	2.6 ± 1.4	0	HMX	2079 ± 565	294 ± 132	0
TNT	165.± 140	41.0 ± 9.7	4.9 [B]	TNT	0	0	0

[A] Concentrations of the energetic compounds were measured in µg/ml. [B] RDX and TNT were cleared from the blood of all birds by d 7 except in one male.
[C] RDX was detected in the feces of only one bird

RDX, HMX and TNT have a variety of effects on mammals (Dilley *et al*, 1982, MacPhail *et al*,1985, Cuthbert *et al.*, 1985). The 1° effect of acute oral exposure in mammals is on the neuromuscular system leading to ataxia, convulsions and sometimes death (Dilley *et al.* 1982). Few data regarding the toxicity of energetic compounds on birds are available (Gogal *et al.* 2001, 2003). Gogal *et al.* (2001, 2003) found that higher exposures were required to produce similar acute effects in rodents. Evaluation of the compounds effects on feed consumption and weight loss revealed some measurable trends. RDX and HMX blood levels seemed to have an inverse relationship with feed consumption.

Accordingly, both treatment groups lost wt when compared to controls. In our study the TNT treatment group exhibited increased feed consumption and decreased wt loss compared to controls; suggesting it acted as an appetite stimulant; however, recent unpublished data using common doves suggests that TNT can suppress feed consumption (Johnson in prep.) Increased feed consumption on day 7, while TNT blood levels are lowest suggests a TNT metabolite is responsible for the increase. Mammalian studies have shown similar results. Dilley *et al* (1982) found that Sprague-Dawley rats administered 0.05% and 0.25% TNT diets showed increased feed consumption and wt gain after TNT treatment was discontinued. Control birds also exhibited a significant wt loss that can be attributed to increased activity levels due to handling stress.

Analysis of blood compound levels revealed all compounds were absorbed to some degree into the blood via the gut. Concentrations of RDX were relatively high and were cleared from the blood of 75% of the birds by d 7. Similar to mammalian studies, HMX was absorbed into the blood at relatively low concentrations in quail. Wilson (1985) found only a small portion of the orally administered HMX dose was absorbed from the gut in rats and mice. TNT was absorbed at the highest levels of the three compounds and metabolized the quickest. Fecal analysis yielded results similar to mammalian studies. Concentrations of RDX found in the feces were relatively small when the dosage is considered. Similarly, Schneider *et al* (1977) found that after an RDX oral gavage at a dosage of 100 mg/kg to Pittman-Moore Miniature swine and Sprauge-Dawley rats less than 3% was recovered in the feces. Conversely, a vast majority of HMX administered was found in the feces on d 1, a much lower concentration on d 3 and levels were below detectable limits by d 7. Mammalian studies have reported similar findings. Wilson (1985) found that 85% of the orally administered HMX had been excreted in the feces 4 d after the initial dosing. TNT was not detected in the feces, suggesting it is readily absorbed and metabolized. TNT treatment group had maroon colored white caps on their feces, which is similar to reports in beagles and Sprague-Dawley rats (Dilley *et al*, 1982).

Phase III, broth analysis revealed some decreases in compound concentration. In the first phase, several methods were tested for the broth media analysis; however, the drying down method was most accurate. During the second phase, variations in compound concentrations in controls can most likely be accounted for by the compounds water insolubility leading to uneven distribution throughout the media. *Bacillus Thermoglucosidasius* consistently showed a peak with a retention time of 2.5 min, and had RDX levels below the detection limits by the 4th wk of culturing. *Sanguibacter Inulinus* and *Sphingomonas Paucimobilis* also showed decreases in TNT concentrations of 3312 µg/ml and 3140 µg/ml prospectively, and may be metabolizing the compounds. However, further analysis is needed to confirm if these three isolates are metabolizing the compounds.

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