DEVELOPMENT AND APPLICATION OF NON-TRADITIONAL VERTEBRATE MODELS TO INVESTIGATE TERRESTRIAL ECOLOGICAL RISK TO 2,4,6-TRINITROTOLUENE EXPOSURE

By

Mark Steven Johnson

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Veterinary Medical Sciences

APPROVED BY:

_____

Steven D. Holladay, Committee Chairman

_____	_____	_____
Stephen A. Smith	John L. Robertson	Katherine S. Squibb

_____

Wilfred C. McCain

8 December 1998

Blacksburg, Virginia

Keywords: Amphibians, *Ambystoma*, TNT, Exposure, non-specific immunity, Dermal, skin, *Peromyscus*

DEVELOPMENT AND APPLICATION OF NON-TRADITIONAL MODELS TO INVESTIGATE TERRESTRIAL ECOLOGICAL RISK TO 2,4,6-TRINITROTOLUENE EXPOSURE

Mark S. Johnson

Abstract

Assessing ecological risk to wildlife exposed to anthropogenic contamination in soil has traditionally been problematic. Attempts to standardize an approach to evaluate risk for various community types in North America have been challenging, given the variation in terrestrial communities and the values in which policy makers are bound to protect. This has resulted in vague, yet flexible guidance from the U.S. Environmental Protection Agency and other interested parties (e.g., the U.S. Army Corps of Engineers, and the Tri-Service Ecological Risk Assessment Working Group). Interpretation of these and other guidance has been variable, often resulting in conflicting opinions on how best to address the question of ecological risk to receptors that are exposed to xenobiotics in a soil matrix.

This work reports the results of research designed to address the question of ecological risk to terrestrial vertebrates. Objective, ecologically-relevant criteria were used in the selection and development of models in this research. Several lines of logic were considered: 1) substance sensitivity, 2) ecological sensitivity (i.e., the species importance to the system; e.g., keystone species); and, 3) probability and extent of exposure.

A primary soil contaminant at many U.S. Army installations is 2,4,6-trinitrotoluene (TNT). This was a result of the mass manufacturing, storing, and assembly of weapons from the early 1900's until the 1950s. The Army has reported soil concentrations of TNT ranging from 0.12 to 38,600 *u*g/g (Walsh and Jenkins 1992) and 0.08 to 64,000 *u*g/g (Hovatter et al. 1997). The chemical-physical properties of TNT result in a relatively unique compound, not easily amenable to

current modeling techniques to estimate exposure to terrestrial wildlife. Moreover, there are few data describing the effects of exposure to TNT in other than mammals, fish, and specific invertebrates.

In this research, the pathways of exposure and selected potential toxic effects from TNT exposure were investigated in a terrestrial salamander: *Ambystoma tigrinum* (tiger salamanders). *A. tigrinum* was chosen since they are exclusively carnivorous, relatively long-lived, have a thin integument, and are large enough to investigate individual effects. These investigations were designed to mimic natural conditions as closely as possible, though maintain a degree of homogeneity in a laboratory environment. All studies exposed salamanders to soil and food (earthworms) in identical preparations. As such, these exposures were considered complete, eliminating assumptions made regarding daily food consumption, systemic dermal dose, etc.

The first study examined the relative contribution of dermal or oral exposures to the whole-body burdens of TNT and primary metabolites. A poly-chlorinated biphenyl (PCB) mixture (Aroclor® 1260) was used with TNT to simultaneously to assist in the evaluation of each pathway, since the fate and transport of PCBs are well characterized. Tiger salamanders were exposed 28 days *in situ.* The dermal route of exposure contributed the most to the final burdens of TNT in salamanders, with the primary reduction products, 2-amino-4,6-dinitrotoluene and 4-amino, 2,6-dinitrotoulene reaching higher concentrations than of parent compound. Other TNT metabolites were found in insignificant quantities. The concentrations of PCBs were higher in the oral treatment, as expected. These results were corroborated in a subsequent study using *Ambystoma maculatum* (spotted salamanders).

The second series of investigations evaluated the potential toxic effects from TNT exposure. Two treatments consisting of TNT and a control were used to evaluate these effects to *A. tigrinum*. The salamanders were exposed *in situ* for 14 days to TNT in soil and food (earthworms of which were exposed to TNT in the soil in similar preparations). Non-specific immune effects were evaluated through the characterization of splenic phagocytes in their ability

to: 1) phagocytize foreign particles, and 2) digest (through oxygen radicals) phagocytized material. This was conducted using fluorescent microspheres and a fluorescent chemical probe specific to hydrogen peroxide, measured per each cell using flow cytometry. Other data collected included histological examination (e.g., liver, kidney, and other miscellaneous organs), blood differentials, weight changes over time, organ/ body weight comparisons, and an analysis of organ-specific metabolism. No significant effects were noted in salamanders exposed to these conditions.

Coordinated with the preceding study included a search for biomarkers of exposure and an investigation of the metabolites of TNT *in situ*. Biotransformation products of TNT were found including primary (e.g., 2-amino-4,6-dinitrotoluene) and secondary (e.g., 2,4-diamino-6-nitrotoluene) in relative concentrations in skin, liver, and kidney. Biomarkers of exposure included an analysis of cytochrome p450, b5, and the glutathione antioxidant enzymes in liver, kidney, skin, lung, and serum, respectively. Traces of parent compound were found in the skin and liver only. Levels of 2,4-diamino-6-nitrotoluene were found only in the liver and kidney, suggesting that TNT is reduced primarily in or on the skin. Levels of p450 were higher in TNT exposed salamanders than controls. Glutathione and related enzyme levels are reported. This work suggests that salamanders have levels of detoxification enzymes capable of the biotransformation of anthropogenic substances in soil rivaling that of mammals.

Another investigation evaluated these same immunological parameters in white-footed mice (*Peromyscus leucopus*). This species was chosen based on the relative importance of small mammals to the community structure in many North American ecosystems. Mice were exposed to TNT in the feed at 0.264, 0.066, 0.033, and 0.017%, where actual daily dose estimates for males were 604, 275, 109, and 65; and for females was 544, 282, 143, and 70 mg/kg/d. An investigation to evaluate the specificity of commercially-available monoclonal antibodies specific to cell surface markers for thymocytes and splenocytes in inbred mice was unsuccessful. These results suggest the recognition epitopes of monoclonal antibodies prepared against Old-World mice are not conserved into *Peromyscus*, a New-World species. However, high dose

males and females had larger spleens consistent with the hemolytic effects previously reported for mammals exposed to TNT. Further, males exposed at all levels had reduced phagocytic activity of splenocytes, and reduced hydrogen peroxide production associated with the two highest doses relative to controls. Females showed no response relative to treatment.

This research has shown the feasibility for these types of investigations, and provides toxicity information valuable for modeling estimates of ecological risk. Further, the *in situ* exposures have provided media concentrations that are or are not toxic for species of concern. This type of information reduces the uncertainty associated with ingestion modeling estimates, dermal exposure estimates, and other factors not traditionally considered in toxicity studies.

DEDICATION

I dedicate this effort to my wife, Denise, and to our sons Christopher and Ryan. To my wife, I thank her for all her support, patience, and time I spent absent working away from home. Throughout my "later-life" educational endeavors her support was consistent and unwavering. I am thrilled to be able to fulfill a life-long dream and I am sure she is glad I have completed a "terminal" degree.

To my sons I leave this as an example of what you can do if you try. Thanks for understanding when dad had to work.

ACKNOWLEDGEMENTS

I wish to thank all whom have helped support and contribute to this effort. Firstly, I thank Dr.s Robertson, Smith, and Squibb for their input and encouragement as Committee Members. I thank Dr. Steven Holladay for his patience, technical support, encouragement, and particularly for his confidence in my remote research plans. I appreciate his help in every way especially his quick and complete e-mail responses and for being a good friend. I thank each of my instructors at Tech and UM for making complex ideas simple and memorable in my courses (specifically Dr.s Ehrich, Squibb, Kimmel and Caceci). Thanks to Jennifer Ferguson for allowing me to participate in her study. Thanks to Dr.s Glenn Leach and David Young for their encouragement throughout this process and allowing me to mix these pursuits with those confluent with work at the U.S. Army Center for Health Promotion and Preventive Medicine.

I thank all those who have assisted me in these projects: Patricia Beall (for coordinating lab efforts at necropsy), Laurie Franke (for day-to-day husbandry support and her compatible sense of humor), Kelly Lippenholz (for general technical and husbandry support), and Ross Smith. Most of all I thank Dr. Will McCain for his encouragement and for the “brain seed” he planted that started me on this track; thanks, Will!

I also wish to thank Dr. John Robertson for his individual attention and instruction helping me to appreciate and understand salamander pathology (and pathology in general). Also thanks go to Ms. Linda Price for all her help in getting me through the logistical hurdles of the graduate school process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Present legislation (e.g., CERCLA, SARA, RCRA, etc., e.g., CFR 29) requires an ecological risk assessment be conducted for all contaminated sites on the National Priorities List (NPL), including many on military installations. Stemming from a lack of specific procedural guidelines, the adaptation of the human-health approach for ecological decision making, and from the literature written on the subject, specific exposure modeling techniques are often used in risk assessment (USEPA 1993a, b). Most modeling attempts begin with media-specific chemical concentrations that are used to estimate dietary intake to species (receptors) of concern. This includes biomagnification estimates for many hazardous substances as they accumulate through the food web (Calabrese and Baldwin 1993, Suter 1993). However, for terrestrial vertebrates, these approaches only consider the ingestion pathway, and all other exposures are assumed insignificant (Urban and Cook 1986, Calabrese and Baldwin 1993, Opresko et al. 1994; see Moriarty 1988 for a review). Moreover, the current accepted methodologies used are subject to criticisms in that they:

i) Are based upon limited toxicological data, where species specific data are often not available;

ii) Are based upon inappropriate laboratory LD50 results, which are often unrealistic contaminant concentrations that are higher than what would affect factors influencing population stability (i.e., reproductive atrophy, teratogenic effects, mate recognition behavior, reproductive success, etc.);

iii) Often ignore indirect contamination effects (e.g., biomagnification of substances that affect changes in predator densities, changes in the balance of interspecific competition, etc.), that have the potential to be more important than direct effects (Orians 1986);

iv) Are often confounded by human altered landscapes;

v) Ignore the combined effects of multiple contaminants, i.e., possible synergistic responses;

vi) Inaccurately assume exposure for many secondary and tertiary consumers, i.e., predators with large home ranges where estimates of exposure are often conjecture; and,

vii) Ignore the selective processes of R-selected species in habitats where contamination has occurred 40 + years in the past (i.e., adaptation).

Assessing reproductive performance is vital in predicting the continued viability of populations and consequently predicting changes in community structure. However, cycles of reproductive success are often variable, habitat- and species-specific, and currently are not adequately characterized for many populations. Confounding variables such as changes in food abundance, predator densities/activity, pathogen exposure, and phytotoxic effects that could alter plant community physiognomy have the potential to affect current or future reproductive performance for many terrestrial vertebrates. However, recognizing and identifying sensitive indicators of stress that could ultimately affect reproductive output (i.e., mortality or stress that reduces the reproductive output of mature adults as well as survival of offspring) is important when making inferences based on chemical exposures to assess ecological risk.

The immune system has been suggested to be a conservative indicator of environmental stress (Weeks et al. 1992). Evidence has linked a depressed immune system from the effects of malnourishment and/or chemical insult to affect reproduction and/or offspring health (Porter et al. 1984, Fine et al. 1990 and Holladay et al. 1991). Moreover, the list of many immunologic depressive anthropogenic 'substances of concern' has been growing with our knowledge of these compounds and how organisms respond to exposure. Immunological biomarkers of effect are feasible indicators of organism health, in that they are sensitive, relevant, informative, and have the potential to be measured by non-invasive means (Wester et al. 1994). Further, it is logical to infer that certain, specific indirect effects that induce stress (through corticosteroid production that induces immunosuppression) may adversely affect population density through disease, whereby individuals in non-stressed populations may be more resistant. In this scenario, pathogen exposure may have greater populational implications that may either be directly or indirectly attributable to xenobiotic exposure (Carey 1995).

There are relatively few *in vivo* toxicity data for many terrestrial organisms, particularly secondary consumer (predator) level organisms. Tiger salamanders (*Ambystoma tigrinum*) are carnivorous, predominantly terrestrial amphibians that occupy the surface soil levels of strata. They have permeable skin, live in sub-surface soil environments and small ephemeral pools at the larval stage and briefly when breeding (Bishop 1943). They, and members of their genus, are fairly ubiquitous in the Nearctic and are found in the forests and old field habitats of eastern, central, and southwestern North America. *A. tigrinum* can be found from Long Island to north Florida; Ohio to Minnesota and south to the Gulf of Mexico, though they are absent from much of the Appalachians (Conant 1986). In addition, salamanders have been reported to have relatively small home ranges (Merchant 1972, Semlitsch 1981, Kleeberger and Werner 1982, Duellman and Trueb 1986, Martin et al. 1986) therefore, making them excellent indicators of exposure in small, contaminated areas (e.g., hot spots).

Recently, there have been many reports citing alarming evidence that amphibian densities are declining (Phillips 1990, Wake 1991, Blaustein et al. 1994). Amphibians are considered as "sentinel species" of environmental stresses by many investigators (Cooke 1981, Burkhardt and Gardner 1997, Schmidt 1997). Amphibians have been chosen to assess the toxicity of many substances (Slooff and Baerselman 1980, Freda et al. 1990, Herkovits et al. 1977), and their usefulness in the assessment of environmental mixtures has been reported (Cooke 1981, Birge et al. 1985). Metamorphic life stages, relatively thin integument, and diverse life histories may contribute to their selection as a model. Moreover, amphibians (specifically salamanders) can constitute a significant portion of the biomass in many specific habitats. Burton and Likens (1975) found approximately 1,770 g/ ha wet wt. of salamanders in a deciduous forest in New Hampshire. This was twice that of breeding birds during peak breeding season and was reported to be about equal to that of small mammals. It is for these reasons (e.g., propensity for exposure, sensitive life histories, and ecologically relevant) that salamanders were chosen to investigate the effects of contaminant exposure through soil in this terrestrial vertebrate model.

This research addresses several questions pertinent to investigations involving ecological risk to terrestrial receptors. First, the relative contribution of two exposure routes (i.e., ingestion and dermal) were investigated in *A. tigrinum* to 2, 4, 6-trinitrotoluene (TNT). Limited data are available for actual bioaccumulation of nitroaromatic compounds in the food web, particularly for soil-dwelling organisms. Virtually no data are available for dermal *in vivo* absorption of these compounds for endemic species, and there are few species-specific data available quantifying the bioaccumulation of explosives as they may affect secondary consumers (predators). Further, potential toxic effects were investigated using a holistic exposure regime, described as a method for the evaluation of toxicity in a non-traditional laboratory species. Endpoints of toxicity investigated included relevant non-specific immune characterization, pathology of main organs, and hematological parameters. Potential biomarkers of exposure were investigated, including biochemical indicators of metabolism (e.g., cytochrome p450, EROD, PROD), specific antioxidant concentrations (e.g., glutathione), and the relative concentrations of metabolites to gain insight on the intraorganismic fate and transport of TNT. Lastly, these same non-specific immunological parameters were investigated in an important prey species (the white-footed mouse: *Peromyscus leucopus*) in a feeding study using TNT. Hopefully, these data and future work may be applicable in current and future investigations involving terrestrial ecological risk and may be used as general approaches for further investigations for other species.

CHAPTER 2: LITERATURE REVIEW

2.1.1: Ambystomid Physiology:

General: The class Amphibia, within the phylum Chordata, are considered to be phylogenetically advanced of fish (branched of the Subclass: Crossopterygii), and primitive of reptilies (Class: Reptilia). Consequently, the physiological changes found within this class are quite diverse, ranging from the exclusively aquatic Sirens (Family: Sirenidae, Order: Urodela), to the terrestrial spadefoot toads (Family: Pelobatidae, Order: Anura). Adaptive radiation within this class is diverse, and thus includes a wide range of life histories. Salamanders are of the Order Urodela (formerly Caudata), and are primitive among amphibians (second to Caecilians, Order: Gymnophiona).

Mole salamanders (Family: Ambystomidae) are the most primitive among the terrestrial salamanders. Metamorphosis includes a reduction and subsequent assimilation of gill filaments, and the functional use of a lung (although not highly vascularized, supported through a bucco-pharyngeal pump). Most gas exchange occurs cutaneously (Turner 1988). In salamanders, 80% of the CO_2 release and 65 – 74% of the O_2 uptake is by the skin (Whitford and Hutchison 1966). Larvae resemble adults at birth (except having gill filaments), and in high density situations can develop into cannabalistic morphs (e.g., *A. tigrimun;* Collins and Cheek 1983). Reproduction in *A. tigrinum* occurs from November to June (depending upon latitude and population) in ponds or wetland areas for approximately two weeks (Bishop 1943). Males arrive and subsequently deposit a spermatophore. The males then begin to coax females into accepting it through tactile cues (Bishop 1943). Eggs are subsequently laid in masses (range 23 – 110 eggs/mass, $\bar{x}$ = 52; Bishop 1943) and develop into larvae from 14 to 40 days depending on latitude and climate; hatching can occur from January to May (Hassinger et al. 1970). Larvae (and adults) are mostly nocturnal; larvae metamorphose in about 2 – 4 months (Gordon 1968, Hassinger et al. 1970). Metamorphosis consists of gill degeneration and specific atrial/ heart changes (e.g., atrial division) to facilitate cutaneous gas exchange and systemic circulation (Turner 1988).

Salamanders have reknown limb regeneration capabilities (Nebeker et al. 1994) and are a source of subject matter in developmental investigations (e.g., axolotyl). Their integument contains numerous mucous-producing glands that protect against dessication and potentially through non-specific efflux against pathogens (Elkan 1976). The skin is specialized for ion regulation (in Anurans) and can be adversely affected by exposures to cyclic aromatics (Blankemeyer and Bowerman 1993). No information was found regarding the metabolic potential of the skin.

2.1.2: Hematopoetic Centers: Tissues associated with hematopoiesis in amphibians are diverse, given the significant radiation within this class. Specific structure and function relationships are less morphologically distinct as they are with higher organisms (such as mammals).

In salamanders, (e.g., *Notophthalmus* and *Amphiuma*), granulocyte production is mixed: eosinophils, neutrophils, and thrombocytes are produced in the liver; basophils are produced in the spleen (Cowden et al. 1964, Cowden 1965, and Hightower and Haar 1975). All red cell production (and destruction) occurs in the spleen in adult Ambystomids (Turner 1988) which is also an active site for mature cells of all types. The kidney may be an important source of granulocyte and lymphocyte production in larval Ambystomids, and gut-associated lymphoid tissue (GALT) has been reported for some species (Turner 1988). Bone marrow has been reported to be an active site for hemopoiesis, though is restricted to a single family of lungless salamanders (Plethodontidae) and is responsible for granulocyte stem cell production only (Turner 1988). This adaptative shift to bone marrow hematopoeisis is seen as a response to the natural radiation hazards of a terrestrial existance (Cooper et al. 1980).

The cortex of the liver consists of a 4 – 8 cell thick layer of hematopoietic tissue (i.e., granulopoetic). The spleen contains these areas also, though more diffuse throughout the tissue with no specific structural organization (i.e., not associated with arterial structure; no definable white pulp areas). However, the spleen is reported to be the primary site for the production of mature blood cells in Ambystomids (Turner 1988). The intertubular tissue of the kidney has

been reported to produce granulocytes in some species, though some lymphoid tissue does occur in these regions also in diffuse organization (Turner 1988).

In newts (*Triturus cristatus carnifex*), the spleen has been reported to be responsive to changing oxygen demands (either through changes in activity or through environmentally-induced hypoxia) where erythrocytes are shunted back and forth into and from circulation (Frangioni and Borgioli 1991). The liver is reported to possess this function in frogs (Frangioni and Borgioli 1993). However, investigations using *A. tigrinum* in hypoxic conditions for 8 days resulted in decreased organic phosphate concentrations (ATP and DPG) that enhanced oxygen affinity, while there was no change in hematopoeisis (Wood et al. 1982).

Significant variation (interspecific and intraspecific seasonal) also exists in the morphology of blood cell types among species of amphibians. Erythrocytes are nucleated, oblong and can range from 20 to 70 μ in length (Mitruka and Rawnsley 1981). Granular lysosomes have been identified in erythrocytes in salamanders (through acid phosphatase cytochemistry) and are capable of digestion (Turner 1988). However, these may be immature erythrocytes since maturation occurs in open circulation. Lymphocytes are relatively smaller, spherical, with minimal cytoplasm (similar to those of other vertebrates). Thrombocytes are similar to lymphocytes, though are mostly oblong in shape (as are the nuclei) due to two spindle-shaped extensions of the cytoplasm (Turner 1988). Monocytes are similar in appearance to lymphocytes with greater amounts of cytoplasm and usually a single, kidney-shaped nucleus. However, positive identification requires evidence of phagocytic activity or of peroxidase activity (the latter of which is lacking in lymphocytes; Turner 1988). Basophils are variable in shape and abundance in amphibians. They usually have a large, unsegmented nucleus and large metachromatic granules (Mitsui 1965, Turner 1988). Neutrophils (heterophils), distinguished by a highly segmented nucleus, are abundant and conspicuous in many amphibians (Turner 1988). Eosinophils have distinct cytoplasmic granules and are less common then neutrophils. Enzyme characterization of eosinophils is less diagnostic in amphibians (Turner 1988).

Non-specific esterases can be demonstrated in lymphocytes, through peroxidases cannot (only reported for neutrphils and monocytes in *Ambystoma*; Mitsui 1965, Fey 1966, 1967 a,b). β-Glucoronidase and aryl sulphatase activity has not been demonstrated in amphibian lymphocytes (Cannon and Cannon 1979).

The number of circulating erythrocytes in amphibians can vary greatly (Roofe 1961). Frog erythrocyte counts can range from 0.5 to 0.7 million / mm^3. In *Ambystoma tigrinum*, they have been reported to range from 0.130 – 2.0 million / mm^3 (Myers and Alexander 1945, Roofe 1961). The number of frog leukocytes can range from 14,000 – 1,000 / mm^3 (Mitruka and Rawnsley 1981). Leukocyte counts in *A. tigrinum* have been reported to range from 3200 to 7300 cells / mm^3 (Roofe 1961). The differential ratio has been reported: lymphocytes, 92%; neutrophils, 4%; monocytes, 3%; and eosinophils, 2% (Roofe 1961). Specific hematological data are presented in Table 2.1.

2.1.3: Liver: In addition to hematopoesis, the liver is important in the transition from ammonotelism to ureotelism during metamorphosis, specifically through specific enzyme production necessary in this process (Duellman and Trueb 1986). The liver is a single and undivided organ in urodeles, and is important in bile production (Reeder 1964). Bile is stored in an adjoining gall bladder. Melanin pigments have been reported in the liver of amphibians, as well as the observations that report the "brown pigments" contain ionic iron (Brown 1964). These pigments have been reported to be correlated to seasonality and fasting conditions (in *Bufo arenarum*), and that the parenchymal cells are involved in the metabolism of these pigments (Brown 1964).

Table 2.1. Characteristics of the blood for some species of amphibians (from Duellman and Trueb 1986).

Species	RBC volume [ml/(100 ml blood)$^{-1}$]	RBC Volume (u^3)	Hemoglobin [g/(100 ml blood)$^{-1}$]	Concentration [g/(100 ml (RBC)$^{-1}$] *(sic)*	RBC Hb content (pg)	O_2 capasity [ml O_2/100 ml blood)$^{-1}$]
Caecilians						
Boulengerula taitanus	40.0	588	10.3	25.7	151	14.00
Salamanders						
Cryptobranchus alleganiensis	49.0	4,425	13.3	27.1	2,010	-
Necturus maculosus						
Amphiuma means	40.0	13,857	9.4	23.5	3,287	7.26
Taricha granulosa	36.7	3,336	9.5	13.2	837	9.70
Dicamptodon ensatus	24.2	4,938	4.4	15.6	880	5.60
Anurans						
Tematobius culeus	27.9	394	8.1	28.1	281	8.02
Rana catesbeiana	29.3	670	7.8	26.9	179	10.43
Rana esculenta	27.3	659	7.8	28.9	187	-
Rana pipens	24.6	768	6.7	27.2	208	11.70

2.1.4: Kidney: The kidney is developed to excrete urea as the primary nitrogenous waste in adults (Eckert and Randall 1978). Since Ambystomids are sensitive to dessication, they produce dilute urine, and therefore the nephrons have a reduced medullarly penetration (i.e., limited counter current ion exchange; Eckert and Randall 1978). However, the excretory system of terrestrial amphibians does include a urinary bladder (first appearance among vertebrates) that can absorb both water and solutes from the urine (Wilson 1972). During relatively dry periods, amphibians may not excrete urine, rather conserve water and solutes by increasing bladder absorption. In amphibians, nephrons are relatively large and few compared to other vertebrates (Prosser 1973). The glomeruli are within a layer close to the mesial border of the kidney. Briefly, beyond each glomerulus is a ciliated neck, a thick-walled proximal tubule, a narrow intermediate tubule, and ending with the distal tubule, that empties into a collecting duct. Supporting circulation to the glomeruli and the tubules are separate (Prosser 1973). The distal tubule is relatively water impermeable thus the urine is hypotonic to the plasma (Prosser 1973). The mesonephric tubules form a strand-like network which is shared with the reproductive system. The gonad intercalates with the sexual portion of the kidney (anterior end) and is linked through a longitudinal marginal canal (Francis 1934, Duellman and Treub 1986).

2.1.4: Immune System: There have been few investigations regarding the immune system in salamanders, fewer still relevant to Ambystomids. The bulk of amphibian investigations involve graft transplant assays and evaluations of autoimmune function. Of these, most of the immunological work has focussed on anurans (e.g., *Xenopus laevis*); few studies have been published regarding salamanders (Table 2.2). Of those investigating graft rejection, it can be briefly noted that Urodeles and legless apodans exhibit chronic or slow rejection, while anurans exhibit faster or acute rejection (Cooper 1976).

The bulk of the amphibian lymphoid system consists primarily of the thymus, spleen and areas involved in granulocyte production (e.g., kidney and liver, Cooper 1976). Studies involving anuran lymphocytes have identified them as the antibody forming cells, though no other clear distinction has been made (Turner 1988).

Table 2.2. Lymphocyte functions within the Amphibia (from Turner 1988).

Function		Anura	Urodela
Allograft rejection		acute	chronic
Mixed lymphocyte reactivity		strong	weak
Graft-vs-host reactivity		strong	weak
Cytotoxic T-cells		present	?
Lymphokine production (migration inhibition factor)		present	present
Antibodies:	IgM	present	present
	IgRAA	present	present?
	IgA	absent	absent
	IgE	absent	absent
Regulatory cells:	helpers	present	present?
	suppressors	present	present

Precise structural details on salamander immunoglobulins are lacking. Only one class of antibody has been identified (IgM) in amphibians. Antibody purified from Axolotls (*Ambystoma mexicanum*) exposed to bacterial antigens has been found to form quickly sedimenting IgM subunits with a *u*-type heavy chain (Manning and Turner 1978). Further, these investigations have found that bacterial antigens provoked good, but delayed antibody response, while they failed to respond to soluble antigens (e.g., pig serum and ferritin).

Evidence of cellular cooperation in antigen recognition has been found in the red-spotted newt (*Notophthalmus viridescens*) (Ruben and Edwards 1980). Briefly, a hapten-carrier system comprized of trinitrophenol (TNP) coupled to erythrocytes was measured using immunocytoadherence tests (Manning and Turner 1978). Preimmunization with the carrier erythrocytes, followed four days later by injection of carrier coupled with hapten, produced cells capable of forming rosettes with anti-hapten (TNP) specificity. These rosette-forming cells were associated with lymphoid populations of the liver, spleen, and kidney. These results were confirmed with axolotls where adult thymectomy dramatically reduced the number of anti-TNP rosette-forming cells (B-cells) (Tahan and Jurd 1981). The specificity of these reactions are most likely associated with T-cell (helper) function, however, sub-populations as such have not been specifically established for amphibians (Manning and Turner 1978).

The thymus is relatively large in tiger salamander larvae and is organized as nodules associated with each pharygeal pouch (5 paired thymic buds). As they mature, 2 disappear leaving those from pouches 3, 4, and 5 to develop and form the thymus (Francis 1934). In adult Ambystomids this three-lobed organ resides in the connective tissue behind the mandible and is difficult to excise (pers. obs). The development of the thymus and spleen is a slow process and subsequently the development of cell-mediated immunity is also relatively slow. Axolotls reared at 21°C did not respond effectively to skin allografts until they were about three months old (Turner 1988).

Other lymphoid tissues in salamanders show little or no functional advance over that of the fishes (Turner 1988). There are no lymph nodes in urodeles, no well organized GALT, and the bone marrow is not active (except for *Plethodontidae*).

Glucocorticoids are important in water and electrolyte regulation and carbohydrate metabolism in amphibians (Novales et al. 1973 in Prosser 1973). They are also important in facilitating ovulation in amphibians (Chang and Witschi 1957). Elevated levels occur in animals subjected to stressful conditions and have been reported to result in immunosuppression (Fletcher 1986, Fowles et al. 1993). Therefore, a reduction in immunocompetence may be an indirect result of xenobiotic exposure that subsequently creates a stressful condition.

2.2: Amphibian Toxicity Testing: Toxicity testing using amphibians is not new. While acute, as well as chronic tests have focussed on the sensitive embryo-larval stage (Hall and Swineford 1980, Huey and Beitinger 1980, Cooke 1981, Geen et al. 1984, Birge et al. 1985, Freda et al. 1990); few experiments have been conducted on adults (but see Beatty et al. 1976 and Safe et al. 1976). Field studies have concentrated on tissue accumulation of environmental contaminants (Korfmacher et al. 1986a & b, Russell et al. 1995) and physical attributes of the environment (Portnoy 1990, Horne and Dunson 1994).

Two amphibian models, in particular, have received much attention. The African clawed frog (*Xenopus laveis*) is an aquatic frog that can be reared relatively easily in laboratory environments. *Xenopus* have been used in many assays, the most common is the FETAX assay (Frog Embryo Tetratogenicity Assay Xenopus). The FETAX assay was originally developed as a non-mammalian alternative to evaluate developmental toxicity (Dawson et al. 1989, Bantle et al. 1994, Bantle 1995), though subsequently refined to also address environmentally-relevant amphibian exposures also (Birge et al. 1985, Dumpert 1986).

Axolotls (*Ambystoma mexicanum*) have been used extensively in laboratory investigations (Armstrong and Malacinski 1989). Axolotls used today are the descendants of six individuals

shipped to Europe in the early 19th century (Smith 1989) and are therefore considered to be sufficiently inbred. They are neotenic (i.e., never metamorphose and retain aquatic characteristics) and as such are easily maintained. They have been primarily used in developmental, endocrine, and physiological (specifically eye innervation) investigations.

No information was found regarding the use of terrestrial salamanders in toxicity tests in the literature.

2.3: 2,4,6-Trinitrotoluene

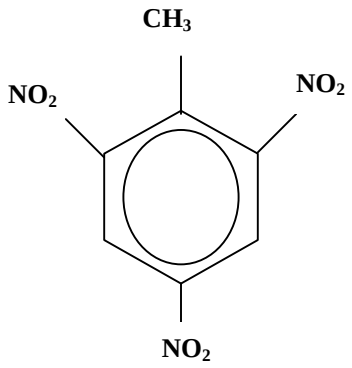
2.3.1: General:

The nitroaromatic, 2,4,6-trinitrotoluene (TNT) is a yellow-white crystalline solid used as a high explosive in military armaments and as a chemical intermediate in the manufacture of dyes and photographic materials (Sax and Lewis 1987). Other synonyms include trotyl, tolit, triton, tritol, and 1-methyl-2,4,6-trinitrobenzene (Army 1984). There are six other possible isomers, however, only the alpha isomer (i.e, methyl at the 1st position) is of military interest and used in military production (Army 1984). Production and load/pack operations using this isomer were widespread in the military, thus TNT continues to be a major problem in water and soil contamination at many Army installations (Walsh 1990, Walsh and Jenkins 1992). This contamination has occurred as a result of activities related to production, load/pack operations, and open burning and detonation of armaments, some of which are outdated, suboptimal, or recently discovered (Walsh 1990). TNT has been identified in at least 20 of the 1,397 hazardous waste sites on the EPA National Priorities List (NPL) (ATSDR 1995).

TNT has been classified as a high explosive, though it is relatively insensitive to shock, has good chemical and thermal stability, is compatible with other explosives, and has a low melting point that is favorable for casting (Army 1984, ATSDR 1995). It has been used either as a pure substance or mixed in combination with RDX (i.e, cyclotols), HMX (i.e., octols), ammonium nitrate (i.e., amatols), or with aluminum (i.e., tritonals; Gibbs and Poplato 1980). In addition to military applications, TNT is also used commerically in industrial explosive applications (HSDB 1998).

2.3.2: Physical / Chemical Properties: Structurally, TNT consists of three nitro groups at the 2, 4, and 6th position of a benzene ring, with a methyl group at the 1st position (see Figure 1., Sax and Lewis 1987).

Figure 2.1. Chemical structure of 2,4,6-trinitrotoluene.



2, 4, 6-Trinitrotoluene

TNT has a molecular weight of 227.13 and a melting point of 80 - 81°C (Army 1984). The freezing point is more reproduceable than the melting point, yet is very sensitive to impurities (Army 1984). TNT boils at 345°C, and can be without explosion for short durations (Army 1984). Unlike many other explosives, TNT does not undergo partial decomposition when melted. At ordinary room temperatures, TNT is essentially non-volatile (Army 1984). Other physical / chemical properties are presented in Tables 2.3-2.4.

Table 2.3. Physical and chemical properties of 2,4,6-trinitrotoluene (from ATSDR 1995).

Property	Information	Reference
Molecular weight	227.13	Budvari et al. 1989
Color	Yellow-white	Budvari et al. 1989
Physical state	Monoclinic needles	Budvari et al. 1989
Melting point	80.1°C	Budvari et al. 1989
Boiling point	240°C	HSDB 1990
Specific gravity	1.654	Budvari et al. 1989
Odor	Odorless	NIOSH 1990
Solubility:		
Water at 20°C	130 mg/L	HSDB 1990
Organic solvents	Soluble in acetone, and benzene; soluble in either alcohol and ether	Budvari et al. 1989
Partition coefficients:		
Log K_{ow}	1.60; 2.2 (measured), 2.7 (estimated)	HSDB 1990; Spanggord et al. 1985
K_{oc}	300 (estimated), 1,100 (measured)	Spanggord et al. 1985
Vapor pressure at 20°C	1.99E-04 mm Hg	HSDB 1990
Henry's law constant:		
at 20°C	4.57E-07 atm m^3/mole	HSDB 1990
Flammability and reactivity	4.4	NIOSH 1994
Conversion factors	1 ppm = 9.28 mg/m^3 1 mg/m^3 = 0.108 ppm	NIOSH 1973
Explosive temperature	464°F	HSDB 1994

Table 2.4. Solubility of 2,4,6-trinitrotoluene. Solubility in grams per 100 grams of solvent at °C. (from Army 1984).

Solvent	0	20	25	30	40	50	60	75
Ethanol (95%)	0.65	1.23	1.48	1.80	-	4.61	-	19.5
Ether	1.73	3.29	3.80	4.56	-	-	-	-
Acetone	57	109	132	156	-	346	-	-
Carbon tetrachloride	0.20	0.65	0.82	1.01	-	3.23	-	24.35
Chloroform	6	19	25	32.5	-	150	-	-
Ethylene chloride	-	18.7	22	29	-	97	-	-
Benzene	13	67	88	113	-	284	-	-
Toluene	28	55	67	84	-	208	-	-
Carbon disulfide	0.14	0.48	0.63	0.85	-	-	-	-
Methyl acetate	-	72.1	80	99	-	260	-	-
Triacetin	-	-	37.7	-	-	-	-	-
Butyl carbitol acetate	-	24	-	-	-	-	-	-
Sulfuric acid	-	4	-	-	-	-	-	-
N, N-dimethylformaide	90	119 @ 15°C	142	-	-	-	-	-
Dimethyl sulfoxide	-	-	128	-	-	-	-	-
1-Methyl-2-pyrrolidinone	-	-	118	-	-	-	-	-
Pyridine	-	137	-	-	-	-	-	-

2.3.3: Environmental Fate and Transport: The distribution of TNT at many U.S. military sites is substantial. At least 17 Army installations have reported soil concentrations ranging from 0.08 to 64,000 *u*g/g (Hovatter et al. 1997). Of those that had detectable concentrations, 5 installations had samples in which surface soils exceeded 10,000 *u*g TNT/g soil dry weight (Walsh and Jenkins 1992).

An important route for the contamination of surface water, ground water and surface soils with TNT has historically been due to large aqueous effluents of rinse water ("pink water", Walsh and Jenkins 1992, ATSDR 1995). Some sources have reported wastewater emissions ranging from 61 – 210 pounds/day (Rosenblatt et al. 1973). Due to the its relatively low vapor pressure, and relatively high water solubility, TNT does not actively partition from surface waters to the atmosphere (ATSDR 1995). Photolysis studies, comparing river waters and distilled water, have shown that the rate of TNT photolysis is directly related to increases in pH and organic matter content (Spanggord et al. 1981). Generally, TNT is not expected to hydrolyze or bioconcentrate in aquatic systems under normal environmental conditions. (HSDB 1997)

Soil contamination of TNT can result from spills, disposal of solid waste, open incineration and detonation of explosives, or leaching from poorly engineered impoundments (Burrows et al. 1989). Retrieval and subsequent destruction of Unexploded Ordinance (UXO) can result in soil contamination as well (includes Open Burning / Open Detonation, OB/OD areas). Based primarily upon the physical and chemical properties of TNT (i.e., log K_{ow} and water solubility) TNT is not expected to bioaccumulate or biomagnify in terrestrial systems (HSDB 1997).

Based on the measured and estimated soil organic carbon adsorption coefficient (K_{oc}) of 300 – 1,100, TNT is not expected to significantly partition to sediment (from surface waters) or sorb to soil particles (HSDB 1997, ATSDR 1995). However, the biotransformation of TNT in soil can be significant, and can be readily reduced under anaerobic conditions. These anaerobic reactions occur through microbial reduction, primarily through successive reduction of the nitro groups (Burrows et al. 1989). Several bacteria have been identified in these reactions. They include

species of *Pseudomonas, Escherichia, Bacillus, Citrobacter, Enterobacter, Klebseilla, Veillonella,* and *Clostridium* (Burrows et al. 1989). Fungii are also capable of reducing TNT (Burrows et al. 1989, ATSDR 1995). Microbial transformation of TNT leads to a variety of reduction products, including 2-amino and 4-amino dinitrotoluene and azoxydimers (Burrows et al. 1989, HSDB 1997), though some oxidation products have been identified (Won et al. 1974). Biological transformation by bacterial and fungal species occurs slowly in the environment, with slightly higher rates in the presence of other carbon sources. However, biological degradation may not extend to cleavage of the TNT ring (the successive reductions of each nitro groups may be reduced to amino groups followed by oxidative deamination to a phenol that releases an ammonia or nitrite (HSDB 1997). Accurate mass balance without the use of radio-labeled compound is difficult with TNT based on its crystal forming tendencies, low organic solubility, and relatively low water solubility (M. Major, pers.comm).

Another process that can affect the fate and transport of TNT in the environment is photolysis. Photolysis has been reported to produce "pink water" from TNT contaminated surface water (ATSDR 1995). Numerous transformation products have been identified in pink water, the predominant ones including 1,3,5-trinitrobenzene, 4,6-dinitroanthranil, 2,4,6-trinitrobezaldehyde, 2,4,6-trinitrobenzonitrile, in addition to several azo and azoxy derivatives formed by the coupling of nitroso and hydroxyamine products (Jerger et al. 1976, Spanggord et al. 1980) TNT is not expected to hydrolyze in soils or groundwater under normal environmental conditions, though photolysis is probable in aerobic conditions (HSDB 1997).

2.3.4: Aquatic Toxicity: The Army has sponsored many toxicity tests that involve aquatic receptors, including fish, invertebrates, and some algae. These original internal reports (some of which are only in abstract form) are difficult to obtain. However, Dacre and Rosenblatt (1974) and Burrows et al. (1989) have completed extensive reviews that are specific and informative. The following are presented in this section.

The tests that were completed were not all uniform. Differences in test conditions (flow-through vs static water renewal), determination of the concentrations of TNT in the media, and the endpoints measured are varied. Burrows et al. (1989) has sorted these tests based on these criteria, results of which are summarized here (see Table 2.5). Most of these tests are acute exposures where lethality was the endpoint. Few full-life cycle chronic tests were conducted (Burrows et al. 1989). In addition, several tests were conducted on pink water effluent (i.e., mixtures), the results of which are not reported here.

Generally, flow-through conditions were more toxic than static ones. Pederson (1971) found that in two, 96-hr tests involving bluegills (*Lepomis macrochirus*) (one test in which that water was renewed daily, the other was not), TNT concentrations fell to 10% of the original (this may have been due to biotransformation or photoylsis). Water was deionized reconstituted with to 60 or 180 ppm hardness with $CaCO_3$. The lowest lethal dose was determined to be 2.3 ppm TNT at either hardness at 10°C. These results are roughly consistent with those presented in a thesis by Gring (1971) where lowest lethal dose was determined to be 2.6 and 2.0 ppm for 24 and 96-hr exposures, respectively (Dacre and Rosenblatt 1974).

Chronic toxicity tests of TNT were conducted with fathead minnows (*Pimephales promelas*) and water fleas (*Daphnia magna*). Early life stage tests (30, 60, and 90 day hatchlings) were conducted with fathead minnows, rainbow trout (*Salmo gairgneri*), and channel catfish (*Ictalurus punctatus*) (Burrows et al. 1989). The chronic studies produced adverse effects at the lowest concentrations tested (0.04 mg/L). The effects included reduced growth, egg hatching, and fry survival (Bailey et al. 1984, 1985). Multiple generation investigations using fathead minnows found that adverse effects were more pronounced in later generations (Burrows et al. 1989). Higher concentrations were needed to produce adverse effects in fry. In addition, TNT exposure has been found to adversely affect earthworm mass (i.e., growth) in concentrations > 110 mg/kg in artificial soil (Phillips et al. 1993)

Table 2.5. Acute toxicity (LC50) of TNT to selected species of fish and invertebrates under flow-through conditions in mg/l (from Burrows et al. 1989).

Species	48-hr	96-hr	Incipient[b]	Duration (hr)
Fathead Minnow	5.9	3.7	1.5 (0.9 – 2.5)	384
Channel Catfish	5.6	3.3	1.6 (0.9 – 3.0)	288
Rainbow Trout	2.0	2.0	1.9 (1.3 – 3.3)	240
Bluegill	2.6	2.5	1.4 (0.8 – 2.5)	312
Water Flea	>4.4	1.2	0.2 (0.1 – 1.0)	192

Data from Liu et al. 1984. [b] Represent concentrations that above which 50% of the organisms would not survive "indefinitely". Data in parentheses represent 95% confidence limits.

2.3.5: Mammalian Toxicity: Acute: Oral LD_{50} values of 660 mg/kg in male and female mice and 1320 and 795 mg/kg in male and female rats, respectively have been reported (Dilley et al. 1982a). These animals developed seizures (grand mal), followed by mild convulsions 1 – 2 hours after exposure. All deaths occurred within 24 hours after exposure; red urine and lethargy were other signs of exposure (Dilley et al. 1982a). Animals that survived the convulsions were still alive 14 days following the exposure (Dilley et al. 1982b). Variation in response for dogs was considered significant (Voegtlin et al. 1921). Cyanosis was evident 12 hours following administration of 100 mg/kg TNT. Severe incoordination and tremors followed. However, the authors note that some dogs receiving 100% of the 100 mg/kg dose did not exhibit the same symptoms as those receiving 50% or less (Voegtlin et al. 1921). Most species showed signs of ataxia after dosing (Voegtlin et al. 1921, Dilley et al. 1982b).

Cats injected intraperitoneally with 0.10 to 0.15 g/kg TNT died within 5.5 hours (Bredow and Jung 1942). Injections of 0.04 g/kg caused convulsions, paralysis of the hindlimbs, decrease in body temperature, and enhanced saliva secretion. Methemoglobin was also present in the blood. Cats given daily subcutaneous injections of 50 mg/kg TNT died within 4 to 9 days (Lillie 1943). Their spleens were congested. Livers had fat accumulation (steatosis) and Kupffer cell hemosiderosis.

2.3.6: Mammalian Toxicity: *Sub-chronic*: Sub-chronic exposures to rats, mice, and dogs have produced consistent hematologic effects (von Oettingen et al. 1944, Dilley et al. 1982b, Levine et al. 1990a, b). Exposures of 13 weeks were sufficient to produce anemia (consisting of reduced number of red blood cells, reduced hemoglobin and hematocrit) in all of these species. Increases in immature red blood cells (reticulocytes), reduction in blood volume, and in hematocrit and corpuscle volume was evident after only 15 days in dogs administered TNT in gelatin capsules of dosages ranging 5 – 33 mg (Voegtlin et al. 1921). TNT exposure is reported to result in direct hemolysis within circulating blood, leading to an increase in spleen weight. Dilley et al. (1982a, b) reported similar findings including pathological assessment of the spleen that suggested hemolytic anemia in beagles. Other important effects included increased liver weight (including

hepatocytomegaly), intestinal inflammation (and mucoid stools), enlarged kidneys, and splenic congestion in mice, rats, and dogs (Dilley et al. 1982b, Levine et al. 1990a, b). Most animals in the highest dose group of all species displayed some degree of hemosiderosis of the spleen (Dilley et al. 1982b). Rats and dogs had dose-related increased serum cholesterol and lower iron and serum glutamic-pyruvic transaminase (SGPT) levels following the 13 week exposure period; mice seemed to be more resistant to treatment (Dilley et al. 1982b). Increased serum cholesterol was consistent with dose in rats and dogs (Levine et al. 1984, Dilley et al. 1982b). Other endpoints consistent with anemia were depression of erythrocyte numbers, hemoglobin and hematocrit values, and occasionally bone marrow hyperplasia.

Testicular atrophy was most pronounced in rats (Dilley et al. 1982b), and consisted of dose-related degeneration of the germinal epithelium lining the seminiferous tubules and hyperplasia of interstitial Leydig cells (in high dose group, 300 mg/kg/d; Levine et al. 1984). The No Observable Effect Levels (NOELs) for these three species were: dogs, 0.20; rats, 1.42; and mice, 7.76 mg/kg/d, suggesting that dogs were the most sensitive (Dilley et al. 1982b). Dilley et al. (1982b) also mention that the effects appear to be totally reversible (up to a 4-week exposure) following a 4-week recovery period.

A single study investigating the functional response of splenic phagocytes to TNT in NMRI mice was conducted (through chemiluminescent analysis) from exposure TNT metabolites (2,4-diamino-dinitrotoluene, 2,4,6 triaminotoluene, 2-amino-6-nitrotoluene, 4-amino,-3,5-dinitrotoluene, and 2-amino-4,6-dinitrotoluene) *in vitro* (Thierfelder and Masihi 1995). This assay quantifies intracellular activated oxygen species. Relatively high doses of metabolites were associated with reduced response relative to controls. Specifically, > 1 mg/l of 2,4-diaminotrinitrotoluene, >50 mg/l for 4-amino-3,5-dinitrotoluene, and > 100 mg/l for 2-amino-4,6-dinitrotoluene caused a plateau of 57 – 65% inhibition (Thierfelder and Masihi 1995).

2.3.7: Mammalian Toxicity: Chronic: Effects from chronic exposures were consistent with those of sub-chronic exposures. Two studies using Fisher 344 rats (Furedi et al. 1984) and beagle dogs

(Levine et al. 1990a) reported dose-dependent indicators suggesting hemolytic anemia (e.g., reduced hemoglobin, hematocrit, and erythrocyte counts, increased quantities of reticulocytes). These effects were different from controls at doses ≥ 8.0 (i.e., and 32 mg/kg/d for dogs; Levine et al. 1990a) and for all TNT treatments for rats (i.e., 0.4, 2.0, 10.0, and 50.0 mg/kg/d; Furedi et al. 1984). Exposures for the rat study lasted 106 weeks and 26 weeks for dogs. Compensatory responses to anemia were minimal in rats (e.g., erythrocytic macrocytosis and reticulocytosis; Furedi et al. 1984). Methemoglobinemia was apparent in both studies in animals of the higher dose groups. Reduction in body weight was apparent in rats exposed to 10 mg/kg/d or greater, and at 8 mg/kg/d or greater for dogs (Furedi et al. 1984, Levine et al. 1990a). Dose-related hepatomegaly (and increased kidney weights) was evident in rats receiving > 2.0 mg/kg/d; this was only evident in the high dose group for dogs. Splenomegaly was evident in rats and dogs in the higher dose groups. Hemosiderosis in Kupfer's cells was seen in various dogs at most dose levels (Levine et al. 1990a). Renal injury was supported by gross and tissue morphological examinations (in high dose groups; Furedi et al. 1984). Increased pigment deposition occurred in the kidneys (as did evidence of bone marrow fibrosis) of rats exposed to 2.0 mg/kg/d or greater (Furedi et al. 1984). Enteritis of the small intestine was reported to be related to TNT treatment in dogs (Levine et al. 1990a). Urinary bladder carcinomas were evident in some rats (2 males and 4 females of 1794 and 1754 rats, respectively) exposed for 106 weeks (Furedi et al. 1984). Given the rate occurance for these types of neoplasias, this finding was considered significant. A No Effect Level was determined to be 0.4 mg/kg/d for rats (Furedi et al. 1984); none was found for dogs (Levine et al. 1990a). TNT was found to be mutagenic (without S9 activation) in *Salmonella typhimurium;* the reduced metabolites were less potent mutagens (Tan et al. 1992).

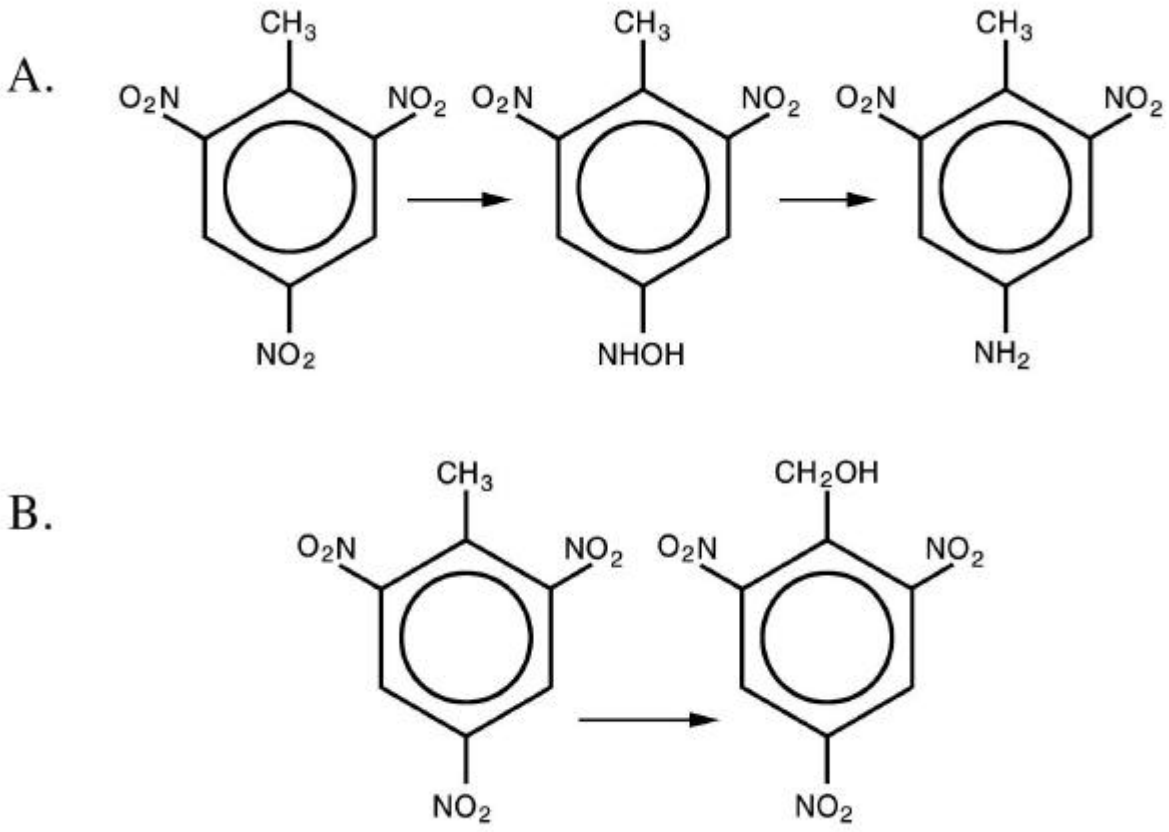
2.3.8: Metabolism: In mammals, the principal metabolites of TNT formed and excreted are 2-amino-4, 6-dinitrotoluene (2ADNT) and 4-amino-2,6,-dinitrotoluene (4ADNT), although smaller amounts of 2, 4-diamino-6-dinitrotoluene and 2, 6-diamino-4-nitrotoluene are formed (Dacre and Rosenblatt 1974, Rickert 1987, Burrows et al. 1989, Yinon 1990). It has been suggested (and supported with empirical evidence) that these reduction reactions can reach saturation at relatively low doses (Yinon 1990).

There is evidence that TNT is conjugated to a glucuronide, roughly equivalent to half the dose (Channon et al. 1944). Channon et al. (1944) found that rabbits given powdered TNT via intragastric administration produced urine containing 47% glucuronides and 30% as aromatic amino derivitives. They concluded that since glucuronic acid was found naturally combined only with compounds having an aliphatic or aromatic hydroxyl group, that glucuronides of TNT must have originated from an oxidation product such as trinitrobenzyl (TNB) alcohol (see Figure 2). They also attribute the red coloration in the urine of these animals to TNB alcohol production. These compounds (in addition to 2ADNT, 4ADNT, and diaminonitro-compounds) have also been found in the urine of munition workers; the hydroxy-amino compound has not been found in rats but has been found in mice and dogs (El-hawari et al. 1981, Yinon 1990). These products of TNT oxidation have also been identified as products of metabolism by pseudomonas-like organisms *in vitro* (Won et al. 1974).

Experiments conducted by El-hawari et al. (1981) *in vivo* using rats, mice and dogs found a greater majority of TNT metabolites were eliminated through glucuronide conjugation. This was based on an increase in radioactivity after hydrolysis with β-glucuronidase. Large amounts of congugates were found in the bile; mice had the least amounts of conjugates (El-hawari et al. 1981). Since the greatest amounts of TNT reduction products were recovered through billiary cannulation, the authors suggested that the liver was the primary site for TNT reduction (El-hawari et al. 1981).

The predominant route of excretion is through the urine in mammals. The disposition of TNT was studied using radiolabelled TNT administered via an intragastric tube in rats by Lee et al. (1975). Expired CO_2, feces, and urine were collected. At 24 hours post dosing, aortic blood, kidneys, liver, brain, lungs, and thigh muscle were collected and analyzed. These authors reported that 26% of the radioactivity was found in the GI tract and feces; 53% of the administered dose was recovered in the urine. Small but significant amounts were found in the kidneys and liver; traces were found in expired air.

Figure 2.2. Two-step reduction of TNT to 4-amino-2, 6-dinitrotoluene (A); Oxidation of TNT to trinitrobenzyl alcohol (B; from Yinon 1990).



2.4: References

Agency of Toxic Substances and Disease Registry (ATSDR). 1995. Toxicological Profile for 2,4,6-Trinitrotoluene. U.S. Department of Health & Human Services, Public Health Service.

Army. 1984. Military Explosives. Technical Manual No. TM 9-1300-214, Headquarters, Department of the Army, September 1984.

Armstrong, J. B., and G. M. Malacinski (Eds). 1989 *Developmental Biology of the Axolotl*. Oxford University Press, New York, NY.

Bailey, H. C., R. J. Spanggord, H. S. Javitz, and, D.. H. W. Liu. 1984. Toxicity of TNT wastewaters to aquatic organisms. Vol. IV. Chronic Toxicity of 2, 4-Dinitrotoluene and Condensate Water. Final report No. AD-A153536 prepared for the U.S. Army Medical Research and Development Command by SRI International, Menlo Park, CA.

Bailey, H. C., R. J. Spanggord, H. S. Javitz, and, D. H. W. Liu. 1985. Toxicity of TNT wastewaters to aquatic organisms. Vol. III. Chronic Toxicity of LAP Wastewater and 2, 4, 6-Trinitrotoluene. Final report No. AD-A1642820 prepared for the U.S. Army Medical Research and Development Command by SRI International, Menlo Park, CA.

Bantle, J. A., D. T. Burton, D. A. Dawson, J. N. Dumont, R. A. Finch, D. J. Fort, G. Linder, J. R. Rayburn, D. Buchwalter M. A. Maurice, and S. D. Turley. 1994. Initial interlaboratory validation study of FETAX: Phase I testing. J. Appl. Toxicol. 14:213-223.

Bantle, J. A. 1995. FETAX – A developmental toxicity assay using frog embryos. Pp. 207-230 *in* Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment. (G. M. Rand, Ed.) 2nd Ed., Taylor and Francis, Washington D.C.

Beatty, P. W., M. A. Holscher, and R. A. Neal. 1976. Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in larval and adult forms of *Rana catesbeiana*. Bull. Environ. Contam. Toxicol. 16:578-581.

Birge, W. J, J. A. Black, and A. G. Westerman. 1985. Short-term fish and amphibian embryo-larval tests for determining the effects of toxicant stress on early life stages and estimating chronic values for single compounds and complex effluents. Environ. Toxicol. Chem. 4:807-821.

Bishop, S. C. 1943. *Handbook of Salamanders*. Cornell University Press, Ithaca, NY. USA.

Blankemeyer, J. T., and M. C. Bowerman 1993. Effect of cyclic aromatics on sodium active transport in frog skin. Bull. Environ. Contam. Toxicol. 50:132-137.

Blaustein, A. R., D. B. Wake, and W. P. Sousa. 1994. Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. Conservation Biol. 8:60-71.

Bredow, M., and F. Jung. 1942. Studies on methemoglobin formation. Comparative toxicity of some aromatic nitro compounds, *Naun. Schmiedelbergs* Arch. Exp. Pathol. Pharmakol. 200:335 *in* Yinon 1990 .

Brown, G. W. Jr. 1964. The metabolism of amphibia. Pp. 18 *in* Physiology of the Amphibia (J. A. Moore, Ed.). Vol. I, Academic Press, New York, NY.

Burton, T. M., and G. E. Likens. 1975. Salamander populations and biomass in the Hubbard Brook Experimental Forest, New Hampshire. Copeia 3:541-546.

Burkhart, J. G., and Gardner, H. S. 1997. Non-mammalian and environmental sentinels in human health: back to the future? Human and Ecol Risk Assess. 3:309-328.

Burrows, E. P, D. H. Rosenblatt, W. R. Mitchell, and D. L. Parmer. 1989. Organic explosives and related compounds: environmental and health considerations. Technical Report No. AD – 8901. U. S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, MD.

Calabrese, E. J., and L. A. Baldwin. 1993. Performing ecological risk assessments. Lewis Publishers, Boca Raton.

Cannon, M. S., and A. M. Cannon. 1979. The blood leucocytes of *Bufo alavarius*: a light, phase contrast and histochemical study. Can. J. Zool. 57:314-322.

Carey, C. 1995. Potential role of immunosuppression and disease in the decline of amphibians in the mountains of Colorado. (Abstract). Modulators of Immune Resonses: Phylogeny of Regulators of Immune Responses and Immunotoxicants. July 8-13, Breckenridge, CO.

Channon, H. J., Mills, G. T., and R. T. Williams. 1944. The metabolism of 2, 4, 6-trinitrotoluene (TNT). Biochem. J. 38:70.

Chang, C. Y., and E. Witschi. 1957. Facilitation of ovulation by cortisone. Endocrinology 61:514-519.

Collins, J.P. and J.E. Cheek. 1983. Effect of food and density on development of typical and cannibalistic salamander larvae in *Ambystoma tigrinum nebulosum*. Amer. Zool. 23:77- 84.

Conant, R. 1986. Reptiles and amphibians. 2nd Edition. Houghton Mifflin, Boston.

Cooke, A. S. 1981. Tadpoles as indicators of harmful levels of pollution in the field. Environ. Pollut. 25:123-133.

Cooper, E. L. 1976. Immunity mechanisms. Pp. 163-271 *in* Physiology of the Amphibia (B. Lofts, Ed.) Vol III. Academic Press, New York, NY.

Cooper, E. L., A. E. Klempau, J. A. Ramirez, and A. G. Zapata. 1980. Sources of stem cells in evolution. Pp. 3-14 *in* Development and Differentiation of Vertebrate Lymphocytes (J. D. Horton, Ed.). Elsevier Press, Amsterdam.

Cowden, R. R., A. Narain, and G. C. Beveridge. 1964. Basophil leukocytes and tissue mast cells in the newt, *Diemyctylus viridescens*. Acta Haematol. 32:250-255 *in* Turner 1988.

Cowden, R. R. 1965. Quantitative and qualitative cytochemical studies on the Amphiuma basophil leukocyte. Z. Zellforsuch., 67:219-233 *in* Turner 1988.

Dacre, J. C., and D. H. Rosenblatt. 1974. Mammalian toxicology and toxicity to aquatic organisms of four important types of waterborne munitions pollutants – an extensive literature evaluation. Technical Report No. AD 778725-7403. U.S. Army Medical Bioengineering Research and Development Laboratory. Aberdeen Proving Ground, MD.

Dawson, D. A., D. J. Fort, D. L. Newell, and J. A. Bantle. 1989. Developmental toxicity testing with FETAX: evaluation of five compouds. Drug Chem. Toxicol. 12:67-75.

deRobertis, E. 1939. Origin and localization of pigment in amphibian liver. Rev. Soc. Argent. Biol. 15:87-93 *in* Brown 1964.

Dilley, J. V., Tyson, C. A., R. J. Spanggord, et al. 1982a. Short-term oral toxicity of 2,4,6-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine mixture in mice, rats, and dogs. J. Toxicol. Environ. Health 9:587-610.

Dilley, J. V., Tyson, C. A., R. J. Spanggord, et al. 1982b. Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats, and dogs. J. Toxicol. Environ. Health 9:565-585.

Duellman, W. E., and L. Treub. 1986. Biology of Amphibians. McGraw-Hill, New York.

Dumpert, K. 1986. Tests with the Souh African clawed toad (*Xenopus laevis*) for detecting chemical causes of the decrease of amphibians. Chemosphere 15:807-811.

Eckert, R, and D. Randall. 1978. Animal Physiology. W. H. Freeman and Co., San Francisco.

El-hawari, A. M., J. R. Hodgson, M. D. Winston, M. D. Sawyer, M. Hainje, and C. C. Lee. 1981. Species differences in the disposition and metabolism of 2,4,6-trinitrotoluene as a function of route administration. Final Report No. ADA114025. Midwest Research Institute. Project No. 4274-B, Kansas City, MO.

Elkan, E. 1976. Ground substance: an anuran defense against desiccation. Pp. 101-110 *in* Physiology of the Amphibia. Vol. 3 (B. Lofts, Ed.). Academic Press, New York, NY.

Fey, F. 1966. Vergleichende Hamozytologie neiderer Vertebraten. II Thrombozyten. Folia Haematol. 86:205-217 *in* Turner 1988.

Fey, F. 1967a. Vergleichende Hamozytologie neiderer Vertebraten. III Granulozyten. Folia Haematol. 86:1-20 *in* Turner 1988.

Fey, F. 1967b. Vergleichende Hamozytologie neiderer Vertebraten. IV Monozyten, Plasmozyten, Lymphozyten. Folia Haematol. 86:133-147 *in* Turner 1988.

Fine, J. S., A. E. Silverstone, and T. A. Gasiewicz. 1990. Impairment of prothymocyte activity by 2,3,7,8-tetrachlorodibenzene-p-dioxin. J. Immunol. 144: 1169-1176.

Fletcher, T. C. 1986. Modulation of nonspecific host defenses in fish. Vet. Immun. Immunopath. 12:59-67.

Fowles, J. R., A. Fairbrother, M. Fix, S. Schiller, and N. I. Kerkvliet. 1993. Glucocorticoid effects on natural and humoral immunity on mallards. Dev. Comp. Immuno. 17:1-13.

Francis, E. T. B. 1934. Anatomy of the Salamander. Clarendon Press, Oxford, UK.

Frangioni, G., and G. Borgioli. 1991. Effect of spleen congestion and decongestion on newt blood. J. Zool. Lond. 223:15-25.

Frangioni, G., and G. Borgioli. 1993. The role of the liver in the cutaneous respiratory compensation of the frog: (*Rana esculenta*). J. Zool. Lond. 230:483-493.

Freda, J., V. Cavdek, and D. G. McDonald. 1990. Role of organic complexation in the toxicity of aluminum to *Rana pipiens* embryos and *Bufo americanus* tadpoles. Fish. Aquat. Sci. 47:217-224.

Furedi, E. M., B. S. Levine, D. E. Gordon, V. S. Rac, and P. M. Lish. 1984. Determination of the chronic mammalian toxicological effects of TNT (Twenty-four month chronic toxicity /carcinogenicity study of trinitrotoluene (TNT) in the Fischer 344 rat. ADA168637 Final report Phase II Vol. I, U.S. Army Medical Research and Development Command, Ft. Detrick, Frederick, MD.

Geen, G. H., B. A. McKeown, T. A. Watson, and D. B. Parker. 1984. Effects of acephate (orthene) on development and survival of the salamander, *Ambystoma gracile*. J. Environ. Sci. Health. 19:157-170.

Gibbs, T. R., and A. Poplato (Eds). 1980. LASL explosive property data. Berkeley, CA. University of California Press, pp. 163-171 *in* ATSDR 1995.

Gordon, R. E. 1968. Terrestrial activity of the spotted salamander, *Ambystoma maculatum.* Copeia 4:879-880.

Gring, D. M. 1971. Biological effects of trinitrotoluene (TNT), PhD Thesis, Department of Zoology, Indiana University *in* Dacre and Rosenblatt (1974).

Hall, R. J., and D. Swineford. 1980. Toxic effects of endrin and toxaphene on the southern leopard frog: *Rana sphenocephala.* Environ. Pollut. 23:53-65.

Hassinger, D. D., J. D. Anderson, and G. H. Dalrymple. 1970. The early life history of *Ambystoma tigrinum* and *Ambystoma opacum* in New Jersey. Am. Mid. Nat. 84:474-495.

Hazardous Substance Data Base (HSDB). 1997. CDROM Tomes/ Micromedix Inc. National Library of Medicine, Bethesda, MD., April 1997.

Herkovits, J., P. Cardellini, C. Pavanati, and C. S. Perez-Cole. 1977. Susceptibility of early life stages of *Xenopus laevis* to cadmium. Environ. Toxicol. Chem. 16:312-315.

Hightower, J. A., and J. L. Haar. 1975. A light and electron microscopic study of myelopoietic cells in the perihepatic subscapular region of the liverin the adult aquatic newt, *Notophthalamus viridescens*. Cell Tissue Res. 159:63-71.

Holladay, S. D., P. Lindstrom, B. L. Blaylock, C. E. Comment, D. R. Germolec, J. J. Heindel, and M. I. Luster. 1991. Perinatal thymocyte antigen expression and postnatal immune development altered by gestational exposure to tetrachlorodibenzo-p-dioxin (TCDD). Teratology 44:385-393.

Holladay, S. D., B. L. Blaylock, C. E. Comment, J. J. Heindel, and M. I. Luster. 1993. Fetal thymic atrophy after exposure to T-2 toxin: selectivity for lymphoid progenitor cells. Toxicol. Appl. Pharmacol. 121:8-14.

Horne, M. T., and W. A. Dunson. 1995. Exclusion of the jefferson salamander, *Ambystoma jeffersonianium*, from some potential breeding ponds in Pennsylvania; effects of pH, temperature, and metals on embryonic development. Arch. Environ. Contam. Toxicol. 27:323-330.

Hovatter, P. S., Talmage, S. S., Opresko, D. M., and R. H. Ross. 1997. Ecotoxicity of nitroaromatics to aquatic and terrestrial species at army superfund sites. Pp. 117-129 *in* Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment. Sixth Vol. (T. R. Doane and M. L. Hinman, Eds.) American Society for Teating and Materials.

Huey, D. W., and T. L. Beitinger. 1980. Toxicity of nitrite to larvae of the salamander *Ambystoma texanum*. Bull. Environm. Contam. Toxicol. 25:909-912.

Jerger, D. E., P. B. Simon, R. L. Weitzel, and J. E. Schenk. 1976. Aquatic field surveys at Iowa, Radford, and Joliet Army Ammunition Plants. Volume III. Microbial Investigations, Iowa and Joliet Army Ammunition Plants. AD AO36778, U.S. Army Medical Research and Development Command, Wash. D.C.

Kleeberger, S. R., and J. K. Werner. 1982. Home range and homing behavior of *Plethodon cinereus* in northern Michigan. Copeia 2:409-415.

Korfmacher, W. A., E. B. Hansen, Jr., and K. L. Rowland. 1986a. Use of bullfrogs (*Rana catesbeiana*) as biological markers for 2,3,7,8-tetrachlorodibenzo-p-dioxin contamination in the environment. Sci. Total Environ. 57:257-262.

Korfmacher, W. A., E. B. Hansen, Jr., and K. L. Rowland. 1986b. Tissue distribution of 2,3,7,8-TCDD in bullfrogs (*Rana catesbeiana*) obtained from a 2,3,7,8-TCDD contaminated area. Chemosphere 15:121-126.

Lee, C. C., J. V. Dilley, J. R. Hodgson, D. O. Helton, W. J. Wiegand, D. N. Roberts, B. S. Andersen, B. N. Halfpap, L. D. Kurtz, and N. West. 1975. Mammalian toxicity of munition compounds. Phase I. Acute oral toxicity, primary skin and eye irritation, dermal sensitization, and disposition and metabolism. Project No. 3900-B (ADB011150), Midwest Research Institute, Kansas City, MO. *in* Yinon 1990.

Levine, B. S., E. M. Furedi, D. E. Gordon, P. M. Lish, and J. J. Barkley. 1984. Subchronic toxicity of trinitrotoluene in Fischer 344 rats. Toxicology 32: 253-265.

Levine, B. S., J. S. Rust, J. J. Barkley, E. M. Furedi, and P. M. Lish. 1990a. Six-month oral toxicity study of trinitrotoluene in beagle dogs. Toxicology 63:233-244.

Levine, B. S., E. M. Furedi, D. E. Gordon, J. J. Barkley, and P. M. Lish. 1990b. Toxic interactions of munition compounds TNT and RDX in F344 rats. Fund. Appl. Toxicol. 15:373-380.

Lillie, R. D. 1943. Notes on the pathology of experimental trinitrotoluene poisoning. Public Health Report No. 58, U.S. Public Health Service *in* Yinon 1990.

Liu, D. H. W, R. J. Spanggord, H. C. Bailey, H. S Javitz, and D. C. L. Jones. 1984. Toxicity of TNT wastewaters to aquatic organisms, Vol. I. Acute Toxicity of LAP Wastewater and 2,4,6-

Trinitrotoluene. Final report No. AD-A138408 prepared for U.S. Army Medical Research and Development Command by SRI International, Menlo Park, CA *in* Burrows et al. (1989).

Manning, M.J. and R. J. Turner. 1976. Comparative Immunobiology. John Wiley and Sons, New York, NY.

Martin, D. L., R. G. Jaeger, and C. P. Labat. 1986. Territoriality in an *Ambystoma* salamander? Supprort for the null hypothesis. Copeia 3:725-730.

Merchant, H. 1972. Estimated population size and home range of the salamanders *Plethodon cinereus*. J. Wash. Acad. Sci. 62:248-257.

Mitruka, B. M., and H. M. Rawnsley. 1981. Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals and Normal Humans. 2nd Ed. Masson Publishing, New York.

Mitsui, T. 1965. Light microscope and electron microscope study of the peroxidase reaction of the eosinophil leukocytes in cold-blooded animals. Okajimas Folia Anat. Japan. 40:893-909 *in* Turner 1988.

Moriarty, F. 1988. Ecotoxicology: the study of pollutants in ecosystems. 2nd Ed. Academic Press, London.

Myers, R. B., and G. Alexander. 1945. Erythrocyte counts in Colorado *Ambystoma*. Copiea, 1945:46.

Nebeker, A. V., G. S. Schuytema, and S. L. Ott. 1994. Effects of cadmium on limb regeneration in the northwestern salamander *Ambystoma gracile.* Arch. Environ. Contam. Toxicol. 27:318-322.

Opresko, D. M., B. E. Sample, and G. W. Suter II. 1994. Toxicological benchmarks for wildlife: 1994 revision. ES/ER/TM-86/R1, USDOE, Wash. D. C.

Orians, G. D. 1986. Ecological knowledge and problem solving. National Academy Press, Washington D.C.

Pedersen, G. L. 1971. Evaluation of toxicity of selected TNT wastes on fish. Phase I – Acute toxicity of alpha-TNT to bluegills, 1 January 1970 – 31 October 1970. Sanitary Engineering Special Study No. 24-007-70/71. U.S. Army Environmental Hygiene Agency, Edgewood Arsenal, MD. *in* Dacre and Rosenblatt (1974).

Phillips, K. 1990. Where have all the frogs and toads gone? BioScience 40:422-424.

Phillips, C. T., R. T. Checkai, and R. S. Wentsel. 1993. Toxicity of selected munitions and munition-contaminated soil on the earthworm: (*Eisenia foetida*). ERDEC-TR-037, U. S. Army Chemical and Biological Defense Agency, Edgewood Research, Development and Engineering Center, APG, MD, USA.

Porter, W. P., R. Hinsdill, A. Fairbrother, L. J. Olson, J. Jaeger, T. Yuill, S. Bisgaard, W. G. Hunter, and K. Nolan. 1984. Toxicant-disease-environment interactions associated with suppression of immune system, growth, and reproduction. Science. 224:1014-1017.

Portnoy, J. W. 1990. Breeding biology of the spotted salamander *Ambystoma maculatum* in acidic temporary ponds at Cape Cod, USA. Biol. Conserv. 53:61-75.

Prosser, C. L. 1973. Comparative Animal Physiology. (3rd Ed.) W. B. Saunders Co., Philadelphia, PA.

Reeder, W. G. 1964. The digestive system. Pp. 132-135 *in* Physiology of the Amphibia (J. A. Moore, Ed.). Vol. I, Academic Press, New York, NY.

Rickert, D. E. 1987. Metabolism of nitroaromatic compounds. Drug Metabolism Rev. 18(1):23-53.

Roofe, P. G. 1961. Blood constituents of *Amblystoma tigrinum* (*sic*). Anat. Rec. 140:337-340.

Rosenblatt, D. H., M. J. Small, and J. J. Barkley. 1973. Munitions production products of potential concern as waterborne pollutants – Phase I. Report No. 73-07. Edgewood Arsenal, MD: U. S. Army Medical Environmental Engineering Research Unit.

Ruben, L. N., and B. F. Edwards. 1980. Phylogeny of the emergence of T-B collaboration in humoral immunity. Contemp. Topics in Immunobiol. 9:55-90.

Russell, R. W., S. J. Hecnar, and G. D. Haffner. 1995. Organochlorine pesticide residue in southern Ontario spring peepers. Environ. Toxicol. Chem. 14:815-817.

Safe, S., D. Jones, J. Kohli, L. O. Ruzo, O. Hutzinger, and G. Sundstrom. 1976. The metabolism of chlorinated aromatic pollutants by the frog. Can. J. Zool. 54:1818-1823.

Sax, N. I, and R. J. Lewis. 1987. Hawley's condensed chemical dictionary. 11th Ed., New York, NY, Van Nostrand Reinhold Co.

Schmidt, C. W. 1997. Amphibian deformities continue to puzzle researchers. Environ. Sci. Technol. News 31:324-326.

Semlitsch, R. D. 1981. Terrestrial activity and summer home range of the mole salamander (*Ambystoma talpoideum*). Can. J. Zool. 59: 315-322.

Sloof, W., and R. Baerselman. 1980. Comparison of the usefulness of the Mexican axolotl (*Ambystoma mexicanum*) and the clawed frog (*Xenopus laevis*) in toxicological bioassays. Bull Environm Contam Toxicol 24:439-443.

Smith, H. M. 1989 Discovery of the axolotl and its early history in biological research. Pp. 3-11 *in* Developmental Biology of the Axolotl (J. B. Armstrong and G. M. Malacinski, Eds.). Oxford University Press, New York, NY.

Spanggord, R. J., T. Mill, and T. W. Chou. 1980. Environmental fate studies on certain munition wastewater constituents. Final Report, Phase II – Laboratory Studies. SRI international, Menlo Park: AD A099256, U.S. Army Medical Research and Development Command, Ft. Detrick, Frederick, MD.

Suter, G. W. II. 1993. Ecological Risk Assessment. Lewis Publishers, Boca Raton.

Tan, E. L., C. H. Ho, W. H. Griest, and R. L. Tyndall. 1992. Mutagenicity of trinitrotoluene and its metabolites formed during composting. J. Toxicol. Environ. Health 36:165-175.

Tahan, A. M., and R. D. Jurd. 1981. Thymus dependancy in antitrinitrophenyl binding responses in the spleen of *Ambystoma mexicanum:* effects of thymectomy and anti-thymocyte serum treatments. Dev. Comp. Immunol. 5:85-94.

Thierfelder, W., and K. N. Masihi. 1995. Effects of trinitrotoluene (TNT) metabolites on chemiluminescence response of phagocytic cells. Int. J. Immunopharmac. 17:453-456.

Turner, R. J. 1988. Amphibians. Pp. 129-209 *in* Vertebrate Blood Cells. (A. F. Rowley and N. A. Ratcliff, Eds.). Cambridge University Press, Cambridge, MA.

Urban, D. J., and N. J. Cook. 1986. Hazard Evaluation, Standard Evaluation Procedure, Ecological Risk Assessment. EPA-540/9-85-001. USEPA, Wash. D.C.

USEPA. 1992. Report on the ecological risk assessment guidelines strategic planning workshop. EPA/630/R-92, U.S. Environmental Protection Agency, Feb.

USEPA. 1993a. Wildlife exposure factors handbook, Vol. 1., Office of Research and Development, EPA/600/R-93/187a, U.S. Environmental Protection Agency, Dec.

USEPA. 1993b. Wildlife exposure factors handbook, Vol. 2., Office of Research and Development, EPA/600/R-93/187b, U.S. Environmental Protection Agency, Dec.

Voegtlin, C., C. W. Hooper, and D. M. Johnson. 1921. Trinitrotoluene poisoning – its nature, diagnosis, and prevention. J. Ind. Hygiene. 3:239-254 *in* Dacre and Rosenblatt (1974).

Von Oettingen, W. F., D. D. Donahue, R. K. Synder, B. L. Horecker, A. R. Monoco, A. H. Lawton, T. R. Sweeney, and P. A. Neal. 1944. Experimental studies on the toxicity and potential dangers of trinitrotoluene (TNT). Public Health Bulletin No. 285. U.S. Public Health Service, Washington D.C.

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

Walsh, M. E. 1990. Environmental transformation products of nitroaromatics and nitroamines: literature review and recommendations for analytical method development. Special Report 90-2, U.S. Army Corps of Engineers, Cold Regions Research & Engineering Laboratory.

Walsh, M. E., and T. F. Jenkins. 1992. Identification of TNT transformation products in soil. Special Report 92-16, U.S. Army Corps of Engineers, Cold Regions Research & Engineering Laboratory.

Weeks, B. A., D. P. Anderson, A. P. DuFour, A. Fairbrother, A. J. Goven, G. P. Lahvis, and G. Peters. 1992. Immunological biomarkers to assess environmental stress., Pp. 211 - 234 *in* Biomarkers, biochemical, physiological, and histological markers of anthropogenic stress. (Huggett et al. Eds.) Lewis Publishers, Boca Raton.

Wester, P. W., A. D. Vethaak, and W. B. Van Muiswinkel. 1994. Fish as biomarkers in immunotoxicology. Toxicol. 86:213-232.

Whitford, W. G., and V. H. Hutchison. 1966. Pulmonary and cutaneous gas exhange in salamanders. Copeia 573-577.

Wilson, J. A. 1972. Principles of Animal Physiology. The Macmillan Co., New York, NY.

Won, W. D., R. J. Heckly, D. J. Glover, and J. C. Hoffsommer. 1974. Metabolic disposition of 2,4,6-trinitrotoluene. Appl. Microbiol. 27:513-516.

Wood, S. C., R. W. Hoyt, and W. W. Burggren. 1982. Control of hemoglobin function in the salamander, *Ambystoma tigrinum*. Mol. Physiol. 2:263-272.

Yinon, J. 1990. Toxicity and Metabolism of Explosives. CRC Press. Boca Raton, LA.

CHAPTER 3. Bioaccumulation of 2,4,6-Trinitrotoluene (TNT) and PCBs Through Two Routes of Exposure in a Terrestrial Amphibian: Is the Dermal Route Significant?

(Accepted: Environmental Toxicology and Chemistry)

Mark S. Johnson[1†], Laura S. Franke[1], Robyn B, Lee[2], and Steven D. Holladay[3] [1]Oak Ridge Institute of Science and Education, [2] DAKKRO Corp., [3]Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech.

[†] Current address: U.S. Army Center for Health Promotion and Preventive Medicine, 5158 Blackhawk Rd., Attn: MCHB-TS-THE, Toxicology Directorate, Aberdeen Proving Ground, MD 21010-5422

Corresponding author: Mark S. Johnson, U.S. Army Center for Health Promotion and Preventive Medicine, 5158 Blackhawk Rd., Attn: MCHB-TS-THE, Toxicology Directorate, Aberdeen Proving Ground, MD 21010-5422
P: 410-671-3980
e-mail: Mark_S._Johnson@chppm-ccmail.apgea.army.mil

3.1: Abstract -- Tiger salamanders (*Ambystoma tigrinum*) were exposed via soil and/or food (earthworms) to 2,4,6-trinitrotoluene (TNT) and a PCB mixture (Aroclor 1260) at environmentally relevant concentrations. Four exposures were considered: (1) uncontaminated food + uncontaminated soil (Control group); (2) contaminated soil + uncontaminated food (Dermal group); (3) contaminated food + uncontaminated soil (Oral group); and, (4) contaminated soil + contaminated food (Dual-Exposure group). The chemical exposure was estimated for each group by analysis of both soil and earthworms. Body-burdens of TNT and its primary metabolites were highest in groups dermally exposed while PCB burdens were highest in the Oral group. Concentrations of the primary TNT metabolites evaluated, 2-amino-DNT and 4-amino-DNT, exceeded that of unmetabolized TNT. These results provide evidence that dermal exposures to nitroaromatics in terrestrial salamanders may make an important contribution to total body burden, and thus may be important when considering the health consequences of such exposures. Further, the demonstration of the accumulation of TNT and TNT metabolites in a primitive vertebrate may have food web modeling implications.

Keywords: Dermal, PCB, TNT, Bioaccumulation, *Ambystoma tigrinum*

3.2: INTRODUCTION

Present legislation requires an assessment of risk to the environment from exposures to anthropogenic contamination. Currently, there are several guidance documents that are useful [1,2]. Although these agree that all potential exposure pathways should be considered, there are few suggestions concerning dermal exposures to wildlife.

Given the variety of organisms comprising any particular terrestrial community, and the lack of species-specific data available and applicable to dermal exposure pathways, it is clear why there are few ecological risk assessments that quantify and/or characterize risk from dermal exposures. However, the potential for dermal exposures to some compounds may in fact be considerable in organisms with less impermeable integument. This is logical in fauna that actively respire or osmoregulate through their skin such as amphibians [3].

In addition, the relatively recent declines in amphibian populations and increases in the rate of developmental abnormalities have provided incentives for investigating these issues [4-6]. The use of amphibians as "sentinel species" in toxicity evaluations has been suggested by various investigators [7-9]. Furthermore, the contribution of amphibians to the biomass of communities has been shown to be considerable, in some cases exceeding that of nesting birds and equaling that for small mammals [10]. These attributes, coupled with the higher trophic level of Urodeles and the terrestrial life history of mole salamanders (Family: Ambystomidae) provided the basis for using tiger salamanders (*Ambystoma tigrinum*) as a model to investigate questions concerning exposures to xenobiotics in a soil matrix. *A. tigrinum* are fairly long-lived (>10 years; [11]). Furthermore, they (and members of their genus) exist predominantly below the soil surface [12,13] and presumably have relatively small home ranges [14].

We chose to investigate the relative contribution of the dermal in relation to the ingestion pathway of exposure to a nitroaromatic, 2,4,6-trinitrotoluene (TNT), in a realistic scenario (in situ) for *A. tigrinum*. The relatively unique chemical/physical properties of TNT compared to those of other organic compounds and the number of military sites where TNT is a soil

contaminant of concern provided the initiative for this project [15]. In addition, Aroclor 1260 was also used in combination with TNT since the fate and transport of PCBs in the food chain have been well characterized [16,17].

3.3: METHODS

3.3.1: Treatment Preparation – Soil was collected (from a location where species of *Ambystomidae* were known to be present), dried, pulverized, and sifted through two screens (Nalgene, 1 mm^2, and 0.5 mm^2 mesh). A polychlorinated biphenyl (PCB) mixture (Aroclor 1260; Alltech Assoc., Deerfield, IL USA) was transferred to an acetone diluent, added and mixed into the dry, processed soil and allowed to evaporate. A portion of this stock mixture was then mechanically mixed for 20 minutes using a Hobart feed mixer (Model A120T, Hobart Corp., Troy, OH USA) in a 12 qt stainless steel bucket to achieve an Aroclor 1260/soil concentration of 67 *u*g/g dry weight. A re-distilled stock of crystalline 2,4,6-trinitrotoulene (TNT) was obtained from U.S. Army Research Laboratory (W. Hilstrom, Aberdeen Proving Ground, MD USA) and determined to be at least 99.4% pure through flame ionization detection gas chromatography. TNT crystals were weighed and added to the previously-mixed soil containing PCBs to achieve a concentration of 1000 *u*g/g dry weight. These test concentrations were determined through a 10-day preliminary range-finding study exposing *A. tigrinum* individuals to 1/10 LC50 concentrations, in two, 2/10 log intervals above and below (i.e., 5 concentrations [18,19]). The concentrations selected for the 28-day study were found not to produce overt toxicity in the preliminary assay (unpub. data). Manual mixing (versus using a total acetone diluent for PCBs) of the final soil mixture was necessary to preserve the natural microflora of the soil to best emulate natural conditions. Soils used for the control treatments were treated identically to the methods above except without the addition of PCBs or TNT. Approximately 125 g of control soil or chemically-treated soil were added to each 2 gallon terrarium (Carolina Biological, Burlington, NC USA) and subsequently hydrated with approximately 20 ml deionized water.

Salamanders were divided into one of four exposure groups: 1) untreated soil and untreated food (Control); 2) treated soil and untreated food (Dermal); 3) untreated soil and treated food (Oral);

and 4) treated soil and treated food (Dual-Exposure group). Earthworms (*Lumbricus terrestris*) and redworms (*Lumbricus rubellus*; Carolina Biological) were exposed to either of two soil treatments (i.e., untreated, (for Control and Dermal treatments); or treated, (PCBs+TNT for Oral and Dual-Exposure treatments)) from the identical soil stocks previously discussed. Treated earthworms were fed ad libitum to salamanders in the appropriate treatments. Worms were maintained in the treated soil for a minimum of 10 days prior to feeding. Earthworms served as the only food for the salamanders during the course of the treatments.

3.3.2: Animal Husbandry – Adult tiger salamanders (N = 48; field collected and obtained from NASCO; Ft. Atkisson, WI USA) were evenly distributed by weight into 4 treatments to minimize any age-specific differences (ANOVA, df = 3, $p > 0.96$, overall $\bar{x} = 34.6$ g $\pm$ 1.52). Sex could not be determined reliably in this group of animals, and thus was unknown at the initiation of treatment. Given the sporadic availability of these animals and the established experimental chronology, animals were placed into treatment upon arrival. All animals were housed individually. Husbandry techniques generally followed Jaeger [20] and included proper temperature monitoring, maintaining proper humidity, and ensuring at least one worm was added, per animal, every two days. More food was added as necessary. Presence or absence of food and/or observation of feeding determined consumption. Animals exhibiting profound overt stress (e.g., excess mucus production, cutaneous/sub-cutaneous septicemia, i.e., red-leg or moribund) were treated and removed from the study. Animals were exposed for 28 days, euthanized by decapitation and necropsied, with the bulk of the tissues retained for the chemical analysis of body burdens.

3.3.3: Toxicity – To investigate potential mechanisms and other potential responses to treatment, basic indices of toxicity were measured. These included weekly body weight measures (bw) and organ/bw measures of spleen, liver, and kidney at necropsy. Slices of these excised organs were taken for subsequent histological examination.

3.3.4: Chemical Analyses – Soil and food samples were pooled for each treatment and gathered at the beginning, midpoint, and end of the study. Entire carcasses of salamanders with the exception of small slices taken for histological examination were immediately frozen at –30°C until analyzed. TNT concentrations were determined by the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Directorate of Laboratory Sciences. Samples for PCB analysis were prepared and analyzed by the Pesticide and Organic Chemistry Program in accordance with a modified USACHPPM - POCP SOP 37.2, solvent extraction with gel permeation clean-up, electron-capture, gas chromatography. Samples for explosive analysis were prepared and analyzed by the Military Unique and Special Chemistry Program in accordance with USACHPPM - MUSCP SOP 51.4. Estimated concentrations were used (i.e., J-values) when available if estimable below the Method Detection Limit (MDL). Duplicates were averaged. Concentrations not detected were assumed to be zero. This research was conducted in accordance to Good Laboratory Practices where appropriate.

3.3.5: Data Analysis – This study was a factorial design, with the two factors of exposure consisting of the dermal and ingestion pathways. Salamanders were exposed to TNT/PCBs either in their food, soil, both food and soil, or neither food nor soil. The data were tested for normality using a Shapiro-Wilk, W-test at the $p < 0.05$ level. If the data failed to fit either a normal or log-transformed distribution, they were ranked and analyzed using the ranks. To test the hypotheses of dermal and ingestion effects and their interaction, a two-way ANOVA was used. To test the equal effects of the four treatments, a one-way ANOVA was used. Statistical significance was defined at the $p < 0.05$ level; adequate power was defined as 80% or greater. The mean and standard error of the mean were reported for the data having a normal distribution. The geometric mean and the geometric standard error of the mean were reported for the data having a lognormal distribution. The median and the 5^{th} and 95^{th} percentiles were reported for data that had neither distribution.

A similar study using *A. maculatum* (obtained from Glade Herp., Ft. Meyers, FL) was conducted to investigate the possibility of TNT/PCB facilitated uptake. Sufficient numbers of *A. tigrinum*

were not available for this investigation, thus, the syngeneric *A. maculatum* were used. These methods were identical to those described above with the following exceptions: 1) four treatment groups were composed of a control, TNT exposed, PCB exposed, and TNT/PCB exposed. All contaminated treatments consisted of exposure to contaminated soil and food.

3.4: RESULTS

3.4.1: Soil Concentrations – Initial soil concentration of TNT at the time of mixing, prior to hydration and 10 days prior to treatment was 1101 *u*g/g (Table 3.1). PCBs were considered relatively stable and not analyzed until the beginning of treatment. However, natural attenuation of TNT in soil reduced the soil concentration dramatically (about one order of magnitude) in 10 days and reached an approximate steady state at about 15 days after initial hydration (i.e., 5 days after the start of treatment). This was duplicated in the *A. maculatum* study. PCB concentrations were variable which may be due to an uneven distribution of compound and/or may be an artifact of pooled sampling. However, average concentrations across each sample interval approximated the intended mixed concentration. All control soils had undetectable explosive residues. Control soils had background concentrations of Aroclor 1260 < 1.3 *u*g/g.

3.4.2: Food concentrations -- Concentrations of TNT in the worms were highest at the beginning of treatment (i.e., after 10 days of initial exposure). Concentrations of primary metabolites (2 and 4-amino-DNT) were also detected at this time. The latter were found to be higher than parent compound concentrations in both experiments. Mean concentrations of PCBs in worms were stable for both studies (though variable), in the range of one order of magnitude less than the soil concentrations.

3.4.3: Body burdens – Since logistical problems prevented a sustained quarantine, infrequent incidences of septicemia (i.e., red-leg) occurred, irrespective of treatment. Moreover, appetite was varied within and among treatments. Therefore, only animals exposed ≥ 20 days and those that had eaten ≥ 2 times during the experiment were included in the analyses. Of these the average salamander ate 6.11 ± 0.55 times.

Power was adequate, greater than 98% in the dermal comparisons that were made for TNT, 2-amino-4, 6-dinitrotoluene (2-amino-DNT) and for 4-amino-2, 6-dinitrotoluene (4-amino-DNT) (Table 3.2). The data for the PCBs did not confirm a normal or a lognormal distribution, therefore the power was not determined. Analyses of these data were performed on the ranks. No other biotransformation product of TNT approached the concentrations of these primary reduction products.

The results of the two-way ANOVA revealed a dermal contribution for TNT uptake, but no ingestion contribution or interaction. This implies that dermal exposures to TNT/PCBs made the greatest contribution to the bioaccumulation of TNT. The results of the one-way ANOVA showed an overall group effect. The Control and the Oral groups had lower TNT concentrations than the Dermal and Dual-exposure groups (Table 3.3).

The two-way ANOVA showed both a dermal and an ingestion effect contributing to 2-amino-DNT concentrations, but no significant interaction. This implies that both dermal and ingestion exposures to PCBs/TNT contributed to the bioaccumulation of 2-amino-DNT. The results of the one-way ANOVA revealed an overall group effect. The Control and Oral groups had lower 2-amino-DNT concentrations than the Dual-Exposure group.

The results of the two-way ANOVA demonstrated a dermal effect and an ingestion effect compared to controls, but no interaction for 4-amino-DNT. This implies that both dermal and ingestion exposure to PCBs/TNT were related to the bioaccumulation of 4-amino-DNT. The one-way ANOVA resulted in an overall group effect. The Control and the Oral groups had lower concentrations of 4-amino-DNT than the Dermal and Dual-Exposure groups.

The results of the two-way ANOVA revealed an ingestion/dermal interaction contributing to PCB burdens. The one-way ANOVA showed an overall group effect. The Control group had lower PCB concentrations than the Oral and Dual-Exposure groups only.

Since availability of animals was limited for the *A. maculatum* study, each treatment included 6 individuals. At the completion of the study, 4/6 controls, 2/6 TNT, 5/6 PCB, and 2/6 PCB/TNT treated animals survived until necropsy; too few to do meaningful statistical comparisons. However, mean concentrations of the compounds of interest were calculated for comparison purposes. These were comparable to those of the previous study in *A. tigrinum*

3.4.4: Treatment-Related Effects – No treatment-related changes in relative organ weight were found (Fig. 3.1). There were no significant changes in body mass over the course of the exposures (ANOVA; $p \geq 0.67$ for all treatments).

Tissues were processed using routine histological techniques, stained with hematoxylin and eosin, and examined by light microscopy. Since these animals were field collected, and thus previous histories were not known, these results must be considered preliminary. Histopathological examinations of the spleen, liver, and kidney revealed an increased incidence of alterations were associated with contaminant exposure (i.e., Oral, Dermal and Dual-Exposures) relative to control animals (data not shown). Briefly, kidneys of all control animals were essentially normal, with no obvious patterns of kidney changes among treatments. However, three salamanders in the Dual-Exposure Group exhibited generalized renal nephrosis and/or necrosis. Livers and spleens were adversely affected in all but one animal in the Dual-Exposure group. In spleens, the predominant effect included focal to multifocal necrosis with varying degrees of severity (i.e., size of the necrotic area). Three animals in the Dermal group displayed essentially normal tissue in all three organs, while others had incidences of liver and spleen necrosis and focal to diffuse splenic lymphoid loss. All animals in the Oral group displayed varying degrees of liver necrosis; one spleen was characterized with mild, diffuse lymphoid loss. A few cases of parasitic infestations were noted.

Some individuals in both studies, predominantly the *A. maculatum* study, were affected by septicemia and skin ulcerations. Cultured samples from infections in animals in the *A.*

maculatum study indicated the presence of *Staphylococcus epidermidis* and *S. hominis*, two ubiquitous bacteria. An investigation of septicemia in one *A. tigrinum* individual detected *Vibrio sp*. Histological examinations of these ulcerated skin lesions (predominanlty associated with the caudal region) were equivocal. Since cases were roughly equivalent across treatments, the exact causes are unknown. However, the development of these incidences is consistent with shipping-related stresses. Fewer cases were observed as the studies progressed.

3.5: DISCUSSION

Risks from exposure to xenobiotics are rarely assessed in amphibians, although there are many indications that these animals may represent sensitive sentinel organisms with potential efficacy in toxicological studies [7-9]. Permeable integument, complex life histories, and physiological metamorphosis add to the difficulties in assessing risks of chemical exposure to amphibians, particularly in a modeled effort. However, given these constraints, we have attempted to evaluate the relative importance of two routes of exposure (dermal and oral) in a terrestrial amphibian genus, and have identified a significant gap in the estimation of risk to these organisms when the dermal route is not considered. A similar relative result was reported for American toads (*Bufo americanus*) where dermal exposures to methoxychlor in water contributed an order of magnitude greater to the body burdens than the oral exposures [21].

The amphibian integument is a complex, vital organ. The majority of respiration occurs through the gas exchange capabilities of the skin, as much as 70% in mole salamanders (Family Ambystomidae) to 95% in species of lungless salamanders (Family Plethodontidae) [3]. Many species of these families are predominantly terrestrial [12-14], including the *Ambystoma spp*. considered in these present studies. Given these physiological differences between salamander skin and the skin of terrestrial non-amphibian vertebrate species, it is reasonable to assume that amphibian integument may differ in its permeability to certain pollutants within the soil matrix, as compared to the skin of many other terrestrial vertebrate species. Therefore, as the results of this study indicate, any comprehensive ecological risk evaluation neglecting the dermal route of

exposure in amphibians would likely underestimate the total systemic exposure and result in inaccurate characterization of potential effects.

In mammals, TNT has been shown to absorb well across the skin. Using carbon-14 labeled TNT in a soil matrix, 3-5% of the label was found to traverse excised pig skin in an ex vivo situation [22]. These data were consistent with in vivo assays conducted by the same author, leading to the conclusion that dermal absorption was a predominant route of exposure for TNT uptake from a contaminated soil matrix applied to the skin. Considering the relatively thin, moist, physiologically active integument of many amphibians, it seemed reasonable to hypothesize that dermal absorption may similarly constitute a significant route of TNT uptake in species such as *A. tigrinum* and *A. maculatum*. Although there were differences (i.e. in species, media and compound) our results were consistent with the findings of Hall and Swineford [21].

The relative contribution of contaminated food consumption to overall body burden of chemical pollutants has been the focus of several studies in aquatic organisms [23,24], less so for terrestrial vertebrate systems [25,26]. Measures of fat solubility (often evaluated through N-octanol-water partition coefficient, K_{ow}) have been used to determine the probability and magnitude of uptake for organic compounds through ingestion [27,28] and are heavily relied upon in risk assessments. However, field verification and replication of food chain exposures are often problematic [29-31].

Salamanders are opportunistic foragers. A study investigating the metabolic demands and food availability of *Plethodon jordani* revealed that many individuals operated on a periodic daily negative energy budget [32]. Rainfall events and ambient temperature were criteria cited as most important in determining capture rate, and as such suggests that some species are “pulse feeders”, eating when opportunities are maximized. If this is true of *Ambystoma*, then oral exposures may vary and be most important during (or after) pulse feeding events. We found appetite to be varied, often most voracious once weekly, though food was offered every other day. Moreover, capture rate of earthworms was less than accurate, capturing prey in about 1:3

attempts when hand fed (pers. obs). In these species, estimation of food intake would require a greater understanding of food capture rates, as well as an understanding of differential fat accumulation and metabolism that may liberate lipophilic compounds and thus periodically enhance systemic contaminant concentrations.

Ignoring these criteria may cause daily estimates on exposure to be inaccurate, and as such suggest the need for species-specific, holistic evaluations in the determination of chemical bioaccumulation in potential target/sentinel species such as terrestrial salamanders. These studies were therefore designed to evaluate the relative contributions of oral and/or dermal exposures to chemical uptake in salamanders and the potential differences between the exposures of two dissimilar compounds in a realistic setting.

These studies proved difficult for several reasons, including obtaining a sufficient number of similar-sized research animals and maintaining sufficient numbers healthy for the duration of the studies (particularly the *A. maculatum* study). This is common in studies that depend on field-collected individuals. The loss of study animals to septicemia seemed to be related to shipping stress, since incidences of such were not associated with treatment and decreased with time. This is consistent with other studies we have conducted where extended periods of quarantine were necessary until all incidences of septicemia had ceased. Further, other confounding variables associated with field-collected amphibians must also be considered (e.g., age differences, parasite load, previous environmental stresses, bacterial exposures, etc.). Logistical constraints did not allow for an extended quarantine in these assays, but these and other confounding criteria must be considered in the proper evaluation of these data. However, we consider it unlikely that these influences would have any significant effect in the alteration of contaminant load in the total burden of these salamanders. A subsequent study conducted with only healthy *A. tigrinum* has corroborated these exposure data (unpub. data).

The most interesting observation in these studies is the importance of the dermal route of exposure to the accumulation of TNT and particularly the primary metabolic reduction products

of TNT, which may be of toxicological importance to higher trophic level species. Specifically, though both a dermal and ingestion route were shown to be important to the total body accumulations, the dermal route was found to be more important than the oral route for the bioaccumulation of both the parent compound and its metabolites in salamanders. In contrast, PCB burdens were found to be highest in salamanders exposed by the oral route. The latter observation is reasonable, considering the lipophilic nature of PCBs and the moist skin of the study salamanders. An understanding of the specific physio-chemical properties of chemicals that are most important in amphibian dermal uptake is greatly needed for any future model development that accurately characterizes exposure.

3.6: ACKNOWLEDGEMENT

We thank Warren Hilstrom for providing the TNT, Lawrence Clark and John Suprock of CHPPM for providing the chemical analysis expertise, LTC David Young for the pathology, Jenni Jenkins of the National Aquarium at Baltimore for the investigative hematology, and everyone who helped out at necropsy (especially Patricia Beall). This work was funded by U. S. Army Environmental Center, Installation Restoration Program.

3.7: REFERENCES

1. U.S. Environmental Protection Agency. 1997. Ecological Risk Assessment Guidance for Superfund: Process for Designing and Conducting Ecological Risk Assessments. EPA 540/R-97/006. Washington, D.C. USA.

2. Wentsel RS, LaPoint TW, Simini M, Checkai RT, Ludwig D, Brewer LW. 1996. Tri-Service Procedural Guidelines for Ecological Risk Assessments. 56015/408/05/0000. Washington, DC, USA.

3. Duellman WE, Trueb L. 1986. *Biology of Amphibians.* McGraw-Hill, New York, NY, USA.

4. Phillips K. 1990. Where have all the frogs and toads gone? *BioScience* 40: 422-424.

5. Wake DB. 1991. Declining amphibian populations. *Science* 253: 860.

6. Schmidt CW. 1997. Amphibian deformities continue to puzzle researchers. *Environmental Science and Technology News* 31: 324-326

7. Burkhart JG, Gardner HS. 1997. Non-mammalian and environmental sentinels in human health: back to the future? *Human and Ecol Risk Assess* 3: 309-328.

8. Cooke AS. 1981. Tadpoles as indicators of harmful levels of pollution in the field. *Environ Pollut* 25: 123-133.

9. Sloof W, Baerselman R. 1980. Comparison of the usefulness of the Mexican axolotl (*Ambystoma mexicanum*) and the clawed frog (*Xenopus laevis*) in toxicological bioassays. *Bull Environm Contam Toxicol* 24: 439-443.

10. Burton TM, Likens GE. 1975. Salamander populations and biomass in the Hubbard Brook Experimental Forest, New Hampshire. *Copeia* 3: 541-546.

11. Spector WS. 1956. *Handbook of Biological Data*. W. B. Saunders, Philadelphia, PA, USA.

12. Bishop SC. 1943. *Handbook of Salamanders*. Cornell University Press, Ithaca, NY. USA.

13. Gordon RE. 1968. Terrestrial activity of the spotted salamander, *Ambystoma maculatum*. *Copeia* 4: 879-880.

14. Semlitsch RD. 1981. Terrestrial activity and summer home range of the mole salamander (Ambystoma talpoideum). Can J Zool 59: 315-322.

15. Walsh ME, Jenkins TF. 1992. Identification of TNT Transformation Products in Soil. 92-16. Special Report. U.S. Army Corps of Engineers, Cold Regions Research & Engineering Laboratory. Hanover, NH, USA.

16. National Research Council (NRC). 1979. Polychlorinated Biphenyls. National Academy of Sciences, Washington DC, USA.

17. Rice CP, O'Keefe P. 1995. Sources, pathways, and effects of PCBs, dioxins, and dibenzofurans. In Hoffman DJ, Rattner BA, Burton GA Jr, and Cairns J Jr, eds, *Handbook of Ecotoxicology*. Lewis Publishers, Boca Raton, LA, USA, pp 424-468.

18. Dilley VJ, Tyson CA, Spanggord RJ, Sasmore DP, Newell GW, Dacre JC. 1982. Short-term oral toxicity of 2,3,4-trinitrotoluene in mice, rats, and dogs. *J Toxicol Environ Health* 9: 565-585.

19. DiPinto LM, Coull BC, Chandler GT. 1993. Lethal and sublethal effects of sediment-

associated PCB Aroclor 1254 on a meiobenthic copepod. *Environ Toxicol Chem* 12: 1909-1918.

20. Jaeger RG. 1992. Housing, handling, and nutrition of salamanders. In Shaeffer et al., eds, *The Care and Use of Amphibians, Reptiles and Fish in Research*. Scientist Ctr. For Animal Welfare, Bethesda, MD, USA, pp. 25-29.

21. Hall RJ, Swineford D. 1979. Uptake of methoxychlor from food and water by the American Toad (*Bufo americanus*). *Bull Environm Contam Toxicol* 23: 335-337.

22. Reifenrath WG. 1994. Assessment of skin penetration of environmental contaminants in air and bioremediated soil utilizing the pig skin model. DAMD17-93-C-3167. Final Report. U.S. Army Medical Research, Development, Aquisition and Logistics Command (Provisonal), Ft. Detrick, Frederick, MD, USA.

23. Biddinger GR, Gloss SP. 1984. The importance of trophic transfer in the bioaccumulation of chemical contamination in aquatic ecosystems. *Residue Rev* 91: 103-145.

24. Ogata M, Fujisawa K, Ogino Y, and Emiko M. 1984. Partition coefficients as a measure of bioconcentration potential of crude oil compounds in fish and shellfish. *Bull Environ Contam Toxicol* 33: 561-567.

25. Kenaga EE. 1980. Correlation of bioconcentration factors of chemicals in aquatic and terrestrial organisms with their physical chemical properties. *Environ Sci Technol* 14: 553-556.

26. Garten CT, Trabalka JR. 1983. Evaluation of models for predicting terrestrial food chain behavior of xenobiotics. *Environ Sci Technol* 17: 590-595.

27. Travis CC, Arms AD. 1988. Relationship between dietary intake of organic chemicals and their concentrations in human adipose tissue and breast milk. *Arch Environ Contam Toxicol* 17: 473-478.

28. Suter GW Jr. 1993. *Ecological Risk Assessment*. Lewis Publishers, Boca Raton, LA, USA.

29. Connell DW, Markwell RD. 1990. Bioaccumulation in the soil to earthworm system. *Chemosphere* 20: 91-100

30. Esser HO. 1986. A review of the correlation between physicochemical properties and bioaccumulation. *Pestic Sci* 17: 265-276.

31. Hansen LG, Lambert RJ. 1987. Transfer of toxic substances by way of food animals: selected examples. *J Environ Qual* 16: 200-205.

32. Jaeger RG. 1980. Fluctuations in prey availability and food limitation for a terrestrial salamander. *Oecologia* 44: 335-341.

Table 3.1. Soil and worm concentrations of 2,4,6-trinitrotoluene, 2-amino-4, 6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and Aroclor 1260 in *u*g/g dry weight

	Soil *u*g/g				Worms *u*g/g			
	TNT	2-A-DNT	4-A-DNT	PCB	TNT	2-A-DNT	4-A-DNT	PCB
Sample dry at mixing	1101	<1.3	<1.3	ND				
A. tigrinum assay								
Control – Initial sample	<0.23	<0.23	<0.23	0.12	<0.02	<0.01	<0.01	<0.040
Control – Midpoint	<0.23	<0.23	<0.23	0.40	<0.02	<0.01	<0.01	<0.040
Control – Final	<0.23	<0.23	<0.23	1.27	<0.02	<0.01	<0.01	<0.040
PCB+TNT – Initial sample	79.0	38.0*	38.0*	56.6	0.133	17.3	12.1	6.34
PCB+TNT – Midpoint	76.0	15.5*	15.5*	34.0	0.122	1.82	1.62	0.33
PCB+TNT – Final	60.0	12.0*	12.0*	45.0	0.02	0.141	0.152	2.33
A. maculatum assay								
Control – Initial sample	<1.8	<1.8	<1.8	<1.0	<0.05	<0.05	<0.05	<0.05
Control – Midpoint	<2.0	<2.0	<2.0	<1.0	<0.05	<0.05	<0.05	<0.05
Control – Final	<1.7	<1.7	<1.7	<1.0	<0.05	<0.05	<0.05	<0.05
TNT – Initial sample	738	<1.7	<1.7	<1.0	1.8	5.6	6.4	<0.05
TNT – Midpoint	164	60	90	<1.0	0.45	3.4	3.5	<0.05
TNT – Final	184	56	82	<1.0	0.33	2.10	2.0	<0.05

	Soil *u*g/g				Worms *u*g/g			
	TNT	2-A-DNT	4-A-DNT	PCB	TNT	2-A-DNT	4-A-DNT	PCB
Sample dry at mixing	1101	<1.3	<1.3	ND				
PCB – Initial sample	<1.7	<1.7	<1.7	55.6	<0.05	<0.05	<0.05	2.13
PCB – Midpoint	<1.9	<1.9	<1.9	56.5	<0.05	<0.05	<0.05	5.07
PCB – Final	<1.8	<1.8	<1.8	60.8	<0.05	<0.05	<0.05	1.09
TNT+PCB – Initial sample	692	<1.6	<1.6	62.3	0.86	3.9	4.1	2.58
TNT+PCB – Midpoint	123	60	91	71.3	0.38	2.75	2.85	3.65
TNT+PCB – Final	214	61	91	55.5	0.40	2.25	1.95	3.30

ND – No data

*Method used failed to separate analytes; totals evenly divided between analytes 2- and 4-amino-DNT

MDL for PCBs in soil was <0.10 *u*g/g

Table 3.2. Distribution, power and statistical test results of compounds detected in *A. tigrinum* body burdens.

Compound	Distribution	Power: 2-way ANOVA	2-way ANOVA	Power 1-way ANOVA	1-way ANOVA
TNT	lognormal	Dermal - 99% Ingestion – 22% Interaction – 5%	Dermal $p < 0.001$	99%	Overall group effect $p < 0.001$
2-A-DNT	lognormal	Dermal – 99% Ingestion – 97% Interaction – 12%	Dermal $p < 0.001$ Ingestion $p = 0.001$	99%	Overall group effect $p < 0.001$
4-A-DNT	lognormal	Dermal – 99% Ingestion – 75% Interaction – 36%	Dermal $p < 0.001$ Ingestion $p = 0.01$	99%	Overall group effect $p < 0.001$
PCBs	neither	NA	Interaction $p = 0.023$	NA	Overall group effect $p = 0.004$

Table 3.3. Concentrations of 2,4,6-trinitrotoluene; 2-amino-4,6-dinitrotoluene; 4-amino-2,6-dinitrotoluene; and Aroclor 1260 in *A. tigrinum* and *A. maculatum* in ug/kg dry weight. All presented with geometric means, SEM; except medians and 5%-95% for PCB data. Treatment values with different superscripts are different at $p < 0.05$. Sample sizes in parentheses.

Treatments	TNT	2-A-DNT	4-A-DNT	PCBs
A. tigrinum				
Control	0.00 ± 0.00^{a} (6)	0.00 ± 0.00^{a} (6)	0.00 ± 0.00^{a} (6)	$9.0\ (5.0 – 110)^{a}$ (5)
Oral	2.39 ± 0.19^{a} (6)	16.46 ± 0.58^{b} (6)	22.10 ± 0.82^{b} (6)	$1960.0\ (1010 – 2870)^{b}$ (6)
Dermal	16.78 ± 0.47^{b} (6)	67.03 ± 0.35^{c} (6)	532.79 ± 0.49^{c} (6)	$550.0\ (500 – 570)^{a}$ (4)
Dual-exposure	29.97 ± 1.34^{b} (6)	291.95 ± 2.19^{c} (6)	971.63 ± 2.46^{c} (6)	$1965\ (1210 – 3170)^{b}$ (6)
A. maculatum				
Control	0.00 ± 0.00 (2)	0.00 ± 0.00 (2)	0.00 ± 0.00 (2)	150.0 ± 5.00 (2)
TNT	170.0 ± 0.00 (2)	940.0 ± 46.0 (2)	755.0 ± 85.0 (2)	ND (2)
PCB	0.00 ± 0.00 (3)	0.00 ± 0.00 (2)	0.00 ± 0.00 (2)	2136.7 ± 960 (3)
PCB/TNT	< 49.0 (1)	1450.0 ± 0.00 (1)	840.0 ± 0.00 (1)	4770.0 ± 0.00 (1)

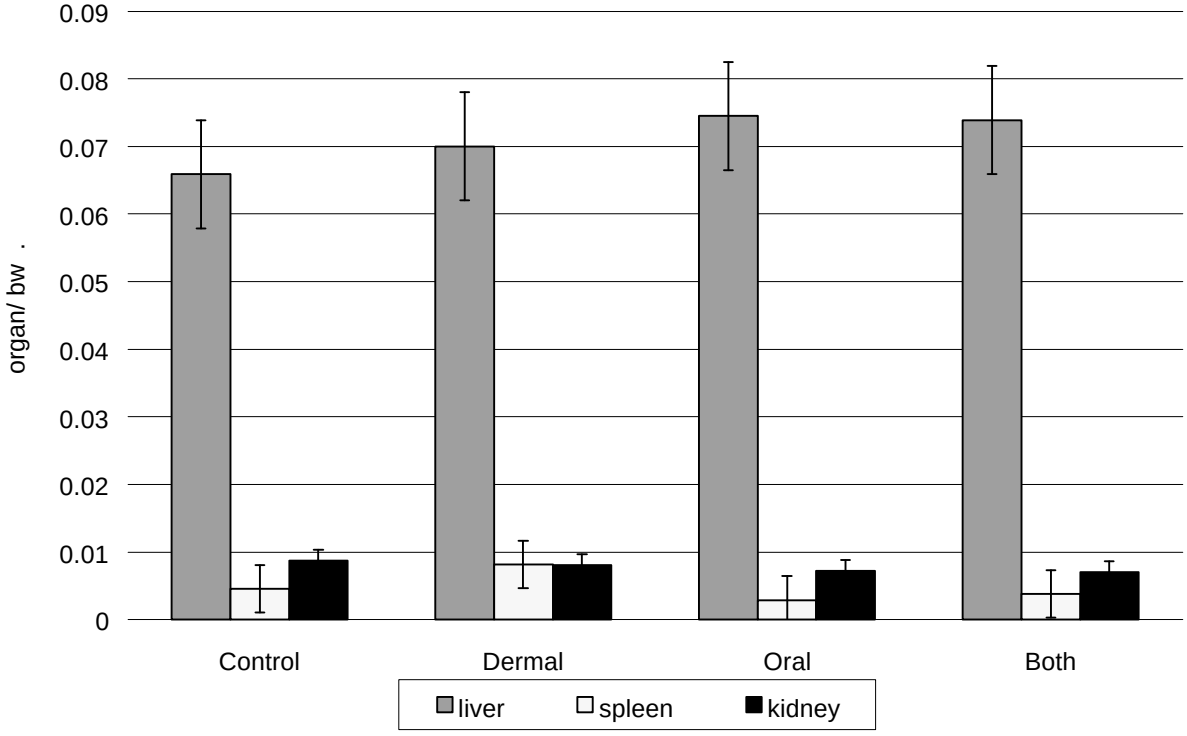


Fig. 3.1: Relative organ/body weight ratios of *A. tigrinum* according to treatment.

CHAPTER 4. Effects of 2,4,6-Trinitrotoluene in a Holistic Environmental Exposure Regime to a Terrestrial Salamander: *Ambystoma tigrinum.*

Mark S. Johnson[†*], Steven D. Holladay[‡], Kelly S. Lippenholz[§], Jennifer L. Jenkins[¶], and Wilfred C. McCain[†], [†]U.S. Army Center for Health Promotion and Preventive Medicine, [‡] Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, [§]Oak Ridge Institute for Science and Education, [¶]National Aquarium at Baltimore.

Corresponding author: Mark S. Johnson, U.S. Army Center for Health Promotion and Preventive Medicine, 5158 Blackhawk Rd., Attn: MCHB-TS-THE, Toxicology Directorate, Aberdeen Proving Ground, MD 21010-5422

P: 410-671-3980

e-mail: Mark_S._Johnson@chppm-ccmail.apgea.army.mil

4.1: Abstract – 2,4,6-trinitrotoluene (TNT) is a defense-related environmental contaminant present at high concentrations in soil at some military installations. Tiger salamanders (*Ambystoma tigrinum*, Family: Ambystomidae) were exposed to TNT in a soil matrix and fed earthworms which had also been exposed to TNT via contaminated soil. Such exposure was previously shown to result in significant accumulation of both TNT and TNT metabolites by salamanders. Following 14 days of combined oral and dermal exposures, salamanders were evaluated for signs of toxicity. Control and TNT-exposed salamanders gained weight ($p < 0.025$). In addition, organ to body weight ratios (kidney, liver and spleen) were not affected by treatment. The function of splenic phagocytic cells was evaluated, since these cells have been found to be sensitive to certain environmental chemical exposures. Neither the chemoluminescence response (H_2O_2 production) nor the phagocytic capacity of such cells were different between controls and treatment. In like manner, no change was seen in the peripheral hematological parameters investigated. Histopathological evaluations were inconclusive, yet the liver revealed a high concentration of heavily pigmented, iron-rich phagocytes (melanomacrophages). Too few treatment representatives were available for an accurate characterization of these cells via image density to be made. This investigation presents a realistic approach and preliminary data for investigating the effects from xenobiotic exposure in a soil matrix for a terrestrial vertebrate.

Keywords: salamanders, *Ambystoma tigrinum*, melanomacrophages, TNT

4.2: INTRODUCTION

Increasingly, toxicological information for amphibians exposed to environmental xenobiotics is demanded. In the past it has been presumed that data evaluated for birds, fish, and mammals would be protective of amphibians [1]. Moreover, the reported declines in abundance [2,3] and recent increases in developmental abnormalities [4] have suggested that amphibians may be more susceptible to these exposures and thus may act as sentinel organisms for environmental exposures to some compounds [5].

Worse case vertebrate exposures from contaminants in a soil matrix may indeed be experienced by terrestrial salamanders. Members of the genus *Ambystoma* exist primarily in soil [6], are carnivorous, fairly long-lived [7], and have a relatively thin integument compared to that of birds and mammals.

In addition, several important nitroaromatic pollutants found in soil have been described as a result of manufacture and loading of military explosives [8]. The presence of these compounds in the sediments and soils from munitions facilities has been shown at concentrations high enough to adversely effect some algal, vascular plant, invertebrate, and fish species [9,10]. Toxic effects of TNT exposure to mammals have been investigated [11-13] however information specific to amphibian exposures is lacking. Moreover, TNT and metabolites in a soil matrix have been shown to bioaccumulate in terrestrial salamanders [14]. These demonstrations provided the incentive to investigate potential adverse effects from exposures to 2,4,6-trinitrotoluene (TNT) in a soil matrix for Tiger salamanders (*Ambystoma tigrinum*) *in situ*, and characterize several general physiological criteria (based on earlier work on other species) as indicators of exposure. These were: inhibition of splenic phagocyte activity, histopathological changes in the liver and kidney, and hematologic alterations in serum cell populations. We chose an *in situ* exposure regime to best emulate natural conditions in habitats where species of *Ambystomidae* are present.

Further, we present specific physiological information for *A. tigrinum* to add to baseline data needed for these species and to potentially assist in the development of salamanders for use as bioindicators of effect in the evaluation of toxicity at contaminated sites.

4.3: METHODS

4.3.1: Treatment Preparation – Soil was collected (from a location in central Maryland where species of *Ambystomidae* were known to be present), dried, pulverized, and sifted through two screens (Nalgene, 1 mm^2, and 0.5 mm^2 mesh). A re-distilled stock of crystalline 2,4,6-trinitrotoulene (TNT) was obtained from U.S. Army Research Laboratory (W. Hilstrom, Aberdeen Proving Ground, MD) and determined to be at least 95% pure through flame ionization detection, GC. TNT crystals were weighed and added and then mechanically mixed for 20 minutes using a Hobart feed mixer (Model A120T, Hobart Corp., Troy, OH) in a 12 qt stainless steel bucket to achieve a TNT/soil concentration of 1000 *u*g/g dry weight. This test concentration was determined through a preliminary range finding study using *A. tigrinum* and was found not to produce overt toxicity after hydration (unpub. data). Soils used for the control treatments were treated identically to the methods above except without the addition of TNT. Approximately 400 g of control soil or chemically treated soil were added to each terrarium and subsequently hydrated with 20 ml deionized water 6 days prior to exposure. A small piece of sheet moss was added and hydrated at day-1 with deionized water to reduce stress and emulate natural conditions.

Salamander treatments included two exposure groups: 1) untreated soil and untreated food (Control); 2) treated soil and treated food (TNT). Earthworms (*Lumbricus terrestris*; Manchester Worm Farm, Manchester, CT) were exposed to either of two soil treatments: untreated (Control), or treated (containing TNT) from the identical soil stocks previously discussed with the addition of worm food (Carolina Biological, Burlington, NC) and sphagnum moss. Treated earthworms were fed ad libitum to salamanders in the appropriate treatments. Worms were maintained in the treated soil for a minimum of 10 days prior to feeding. Earthworms served as the only food for the salamanders during the course of the treatments. Worms were sampled and pooled for chemical analysis at the beginning, midpoint, and end of the study to determine levels of TNT and metabolites in oral exposures. Soil from each terrarium (1 g) was removed and pooled according to treatment at the beginning and end of study determine the concentrations of TNT and the primary metabolites in soil during the study for the salamanders.

4.3.2: Animal Husbandry – Tiger salamanders (N = 36; field collected and obtained from Glade Herp., Ft. Meyers, FL) were quarantined in separate 2.5 gal polystyrene aquariums (Carolina Biological) for approximately one month. During this period, any salamanders exhibiting signs of overt stress (e.g., septicemia, red-leg) were treated by daily oral gavage with doxycycline

monohydrate (Pfizer Laboratories, NY) at 5 mg / 30 g bw suspended in sterile isotonic saline until they improved or became moribund. No salamander received any treatment 16 days prior to the beginning of the study. Of the remaining salamanders, 18 were selected and evenly distributed by weight into the two treatments. This was done to minimize any age-specific differences (overall $\bar{x}$ = 40.1 g ± 1.7). Selection was based on health (i.e., appetite, appearance and general growth). Sex could not be determined reliably in this group of animals, and thus was unknown at the initiation of treatment; however, qualitative classification of cloaca enlargement was made and tested for sex post necropsy. The treatment cages were commercially available (Rubbermaid, 29 x 10 x 18 cm polypropylene boxes) with eight 0.5 cm holes symmetrically placed along the sides, and six 0.25 cm holes placed longitudinally in the lid. Husbandry techniques generally followed Jaeger [15] and included temperature monitoring (maintained between 16 - 20°C), maintaining proper humidity, and offering at least one worm, per animal, every two days. An additional worm was offered with a total consumption never exceeding two worms/salamander/day. Most often salamanders were hand-fed.

4.3.3: Toxicity - During the study, salamanders were weighed at the beginning, midpoint and end. Chemical exposure lasted 14 days, after which the salamanders were euthanized by decapitation and necropsied. All spleens were retained for immunological characterization. The initial study design included metabolic analyses in liver and kidney (in prep) as well as livers and kidneys for histopathological examination. All organs mentioned were weighed to the nearest 0.001 g. All organs identified for histological examination were fixed in formalin, processed into paraffin, sectioned, and stained with hematoxylin and eosin. Iron staining was done using a Gomori stain. Tissues were examined through light microscopy using an Olympus BH2 equipped with a Spot sp900 (Optic Elements Corp., Sterling, VA) digital camera.

Blood was removed via caudal venipuncture prior to euthanasia. Samples were collected in heparin-prepared vaccutainers to prevent clotting. Hematocrit, Mean Corpuscular Volume, and enumeration of a five part differential were conducted using light microscopy. Smears for cell enumeration were stained with Wright–Geimsa.

4.3.4: ROI Production – Salamander spleens were collected by dissection and placed in a culture dish containing 1 ml of culture medium (RPMI containing glutamine). Cells from each spleen were released by mechanical disruption using gentle abrasion of the tissue against a stainless screen in

media. The resulting cell suspension was washed twice in culture medium and resuspended in 1 ml culture medium. Cells were counted by using a Coulter ZM (Hialeah, FL) and adjusted to $5x10^5$ cells/ml.

Intracellular ROI production (specifically H_2O_2) was measured through individual cell analyses by using flow cytometry [16]. Oxidation of the probe 2',7'-dichlorofluoroescein diacetate (DCF-DA) produces a highly fluorescent product that fluoresces in the Fluorescein Isothiocyanate (FITC) range (500 - 600 nm). From each adjusted cell sample, 200 *u*l were taken and dispensed into 7 x 35 mm polypropylene round bottom tubes. Next, 5 *u*l of DCF-DA obtained from Molecular Probes (5 mM; Eugene, OR) were dispensed into each tube and incubated at room temperature for 15 minutes. Following this incubation, 10 *u*l of a 5 nM solution of phorbal myristate acetate (PMA; Sigma Chemicals, St Louis, MO) were added and incubated at room temperature for 30 minutes. After this latter incubation, samples were placed on ice and immediately analyzed using flow cytometry. Cell viability was determined through a fluorescent cell exclusion dye (propidium iodide, PI; ICN Pharmaceuticals, Costa Mesa, CA). Only cells that did not exclude PI were analyzed through a back-gating technique using the flow cytometer.

4.3.5: Phagocytosis of fluorescent microspheres - Characterization of phagocytic activity of the splenic macrophages was determined by a modification of the method of Dunn and Tylor [17]. Briefly, cells were aliquoted as above in polypropylene round-bottom tubes (Fisher Scientific, Pittsburgh, PA) at $5x10^5$ cells/ml in 0.5 ml in culture media. Fluoresbrite microspheres (1.16 mm; Polysciences, Inc., Warrington, PA) were washed twice at 1140 G for 15 minutes in standard buffer, and then sonicated (Branson Ultrasonic Cleaner, Shelton, CN) for 1 minute to disrupt aggregated microspheres. A 100 *u*l volume of microspheres was added to 280 *u*l media, and dispensed in 10 *u*l aliquots to give an initial ratio of 50 beads/cell. Fresh culture media was then added to each tube to give a final volume of 4.0 ml/tube. This latter step was designed to prevent the development of acidic media conditions resulting from metabolic activity of the cells during incubation. Each tube also received 50 *u*l of penicillin/streptomycin to discourage bacterial growth. A positive control (to inhibit phagocytosis) [18] was 10 *u*l of a 0.5 mg/l solution of gadolinium (III) chloride (Aldrich Chemical, Milwaukee, WI) added to control samples that had sufficient cell numbers to permit additional analyses (i.e., surplus cells). Cells were then incubated overnight (18 hr) at 20 °C. Following incubation, cells were washed twice with media to remove non-phagocytized

microspheres, after which cells were resuspended in 0.5 ml standard buffer and immediately analyzed by flow cytometry (Coulter EPICS XL, Hialeah, FL) in the 500 - 600 nm range. Background fluorescence was addressed through proper gate adjustment after analysis of cells without beads. Further, a solution of only beads was analyzed to determine the light scatter position of a population of undigested beads. Thus, peaks on the fluorescence histogram resulting from cells containing 1 or >1 microspheres could be identified. For each sample, 5,000 events were collected. This technique was standardized using additional animals not in the study. Cell viability was assessed through PI-exclusion as mentioned above.

4.3.6: Statistical Analyses - Statistical significance was determined at the $\alpha < 0.05$ level with a Student's *t*-test when comparing the data from two sets when the data were normally distributed (Jandel Scientific, San Rafael, CA). A Mann-Whitney Rank Sum Test was used in cases where the data failed a normality test. Repeated measures such as body weight and digital analyses of melanomacrophage densities were analyzed with a one-way Repeated Measures ANOVA. Differences between groups were then compared using the Bonferroni method.

4.4: RESULTS

4.4.1: Exposure regime – Maintaining stable soil concentrations of TNT in septic conditions was difficult. Our immediate, post-mixing sample (i.e., before hydration) was analyzed and determined to be at 1200 *u*g TNT/g soil dry weight. Six days after hydration (i.e., start of exposure), soil concentrations of parent compound were reduced almost one order of magnitude and mono-amino reduction products were present (Table 1). TNT concentrations were lower at the end of study, while the primary reduction products were relatively unchanged.

One composite sample of worm soil was also collected at the beginning of the study for analysis. Concentrations of TNT and the primary reduction metabolites were lower compared to salamander treatment soils, however worm soils were hydrated 3 days prior to the salamander treatment enclosures.

Earthworms contained consistently higher levels of the primary reduction products than of parent compound. In addition, trace concentrations of 2,4-diamino-6-nitrotoluene were detected. No

pattern of attenuation was conspicuous in the earthworms, i.e., TNT metabolites were relatively consistent throughout the exposure period.

4.4.2: Weight changes – Control and TNT exposed salamanders gained weight over the 14-day period (Control, $t = 7.63$, N = 9, $p < 0.05$; TNT, $t = 3.62$, N = 9, $p < 0.05$; Fig. 1). There were no differences between treatments for the kidney, liver, or spleen weights when evaluated in relation to individual body weights (Table 2).

4.4.3: Flow Cytometry – Well defined (i.e., clearly separate) splenic leucocyte populations were not observed on the Forward Angle Light Scatter (FALS) and Side Angle Light Scatter (SALS), histograms (Fig. 2a). Therefore, the larger, relatively granular cells were analyzed as reported under similar circumstances for other non-mammalian species (e.g., fish) [19]. Sufficient cell densities were obtained from each spleen to permit individual analyses. A positive control for phagocytosis (i.e., Gadolinium treatment) was used in cases where excess cells were available. Additional centrifugation necessary to concentrate cells for enumeration of splenocyte composition through light microscopy was not conducted due to cell fragility.

4.4.4: ROI production – There were no differences in intracellular H_2O_2-mediated oxidation of DCFH in the splenic phagocytes of the TNT-exposed salamanders relative to the controls (ANOVA, $p > 0.21$; Fig. 2b-d). However, the power of this test was well below a desired level (i.e., 0.11 vs 0.80), and as such these negative findings should be considered cautiously.

4.4.5: Phagocytosis – No differences in median fluorescence were found between the phagocytic splenocytes of the control and TNT-exposed salamanders (Kruskal-Wallis ANOVA on Ranks; Fig. 3). However, gadolinium-treated positive controls did display suppressed phagocytosis as expected relative to the other treatments ($p < 0.05$).

4.4.6: Hematology – No differences were found between the two treatments (two-tailed Student's *t*-test, $p < 0.05$; Table 2). Problems with sample quality made some evaluations difficult. Significant variation between samples was consistently present in these analyses. However, trends in hematocrit, mean corpuscular volume, and neutrophil composition were consistent with a possible TNT-related effect.

4.4.7: Pathology – No treatment-related effects were found in the liver, kidney, or gut in the pathological examinations relative to controls. However, due to other study constraints for those tissues, sample sizes were small (sections evaluated from 5 control, and 2 treated salamanders).

Initial H&E staining of liver sections revealed the presence of dark-staining interstitial cells (Fig. 4). These cells were observed to be in close association with eosinophils, neutrophils, and lymphocytes. They were present in livers from salamanders in both treatments. Digital areal extent of the melanomacrophages consisting of 6 random regions of each liver section from each individual were analyzed according to treatment and were not diagnostic, though sample sizes were again limited due to other constraints on organ availability (i.e., livers from some individuals were designated before hand for metabolic studies). Subsequent staining for iron (Gomori stain) revealed a high iron content. Discrete heavily stained granules were evident in these cells (Fig. 4). Moreover, these cells appeared phagocytic, consistent with macrophage function (Kupffer Cells), and were associated with localized inflammation.

4.4.8: Other Observations – Detailed observations were recorded at each feeding. All were offered earthworms by hand, and a few salamanders refused food throughout the exposure period (one TNT exposed salamander refused food twice, another, once only). No obvious patterns of food consumption consistent with treatment were observed. Appetite appeared consistent throughout the exposure period. None appeared lethargic or displayed any obvious signs of stress (e.g., increased mucus production, red discoloration, hiding in soil, etc.) during the experiment, nor were there any indications of ataxia or incoordination.

4.5: DISCUSSION

The predominant effect of TNT exposure to mammals has been anemia [11-13]. We therefore looked for indicators of anemia in salamanders exposed to 1200 mg/kg of TNT prepared in soil. This effect was not observed, possibly the result of the dynamics of the substrate (capable of significantly reducing the parent compound), duration of the experiment, and the variability among the field-caught animals. Indicators of anemia such as increases in immature red blood cells and spleen weight, reduction in blood volume, hematocrit, and mean corpuscle volume were not found. In agreement with these endpoints were the data used for the characterization of non-specific immune response, where there were no differences in phagocytosis or ROI production between treatments. This, however, is consistent with mammalian data where only high doses of the mono-amino metabolites of TNT were found to adversely affect ROI production [20]. Moreover appetite appeared consistent for all salamanders across treatments, control and treated animals gained weight during the study.

Dilley et al. [11] reported a treatment-related pathological assessment of the spleen that suggested hemolytic anemia in beagles. Histopathology of the spleen was not part of the present study because the spleens were used to provide cells for the phagocyte immunoassays. However, an earlier investigation involving exposures to polychlorinated biphenyls and TNT (the latter at similar concentrations) where the pathology of the spleen was examined did not reveal any obvious treatment-related effects [14].

Of histological interest is the presence of dark staining, iron-rich cells found in the livers of *A. tigrinum.* The structures of these cells and their position between the parenchymal tissue are consistent with a phagocytic function. These are similar to the melanomacrophages (MM0s) found in many species of fish [21-23], yet none were found in the kidney or were common in the spleen (data not shown), nor were they associated in aggregations in the liver (i.e., melanomacrophage “aggregates” or “centers” [21]). The density of the Gomori stain was extensive in these cells (i.e., high concentrations of iron; hemosiderin or ferritin). Future investigation may show them to be low in melanin content if consistent with the MM0s of some species of fish [22].

The capsular appearance of these iron particles is similar to that reported in the hepatocytes and Kupffer cells in rats [24]. The latter authors noted an age effect, yet no evidence of toxicity and thus concluded that the containment of iron may be a mechanism of sequestration to isolate free iron.

This scavenging function has been attributed to MM0s in fish, where density and size of MM0s have increased proportionally with anemic state, and subsequently decreased coincident with recovery of the erythroid population [25]. In future investigations we hope to determine if the density of MM0s may serve as a subtle indicator of TNT-induced erythrolysis. These cells were present in control animals also, and the present analyses were based on few samples, thus a possible relationship between TNT exposure and MM0 density was not detected.

We realize that any discussion of the function of these structures in *A. tigrinum* is merely speculation. We know of no other reported incidence of these structures in salamanders, with the possible exception of vaguely described "dark staining cells" in deRobertis [26]. Furthermore, many potential function(s) of these structures have been described in fish, yet are based primarily on circumstantial evidence [21,27]. Our observations suggest that these structures in *A. tigrinum* may be associated with inflammatory responses, including phagocytosis. However, given these limited observational data, their role may be more of a scavenging one, potentially becoming an iron sink for erythropoesis in hypoxic conditions or functioning as a protective mechanism against free iron toxicity [24]. This scavenging role is consistent with the function suggested by Meseguer et al. [22] in fish, yet they found no association with MM0's and an inflammatory response.

Other factors such as age, sex, and seasonality have been reported to be highly correlated to MM0 density in toads and turtles [26,27]. These patterns have yet to be established for Ambystomidae, as were any speculative function(s). Therefore, we recommend that the presence of these cells not be used as biomarkers until better characterized and calibrated with toxicant-induced exposures.

This study was designed to address soil exposures of TNT to a terrestrial vertebrate in a holistic scenario. By exposing the animals in this way, many assumptions necessary in determining exposure in a risk assessment context need not be made. However, other factors must be considered in the extrapolation of these and any other laboratory tests for ecotoxicological uses. Firstly, these exposures ignored the effect of natural environmental stress (e.g., intraspecific and interspecific competition, predator avoidance and food abundance, etc.). These salamanders gained weight appreciably during quarantine and the exposure period simply because food was offered ad libitum. Moreover, it was observed that food put directly in front of the salamanders often resulted in several tries before it was captured. It may be, for instance, that capture rate in the environment is lower.

Therefore, animals exposed to similar concentrations of TNT in the soil in the environment may illicit effects where animals in this study did not.

Many site-specific short-term evaluations of amphibian health have not been diagnostic [27,28] however, substances which induce stress at relatively low concentrations may act to immunologically suppress an organism. This may make survival against ubiquitous bacterial infections difficult. Observations from earlier investigations have suggested that bacterial infections of organisms that are common in the habitats where salamanders are found can become lethal in stressed individuals (pers obs). Xenobiotics acting in these ways may be more insidious in terms of being diagnostic of exposure. We found no significant differences between treatments in any of the histological or functional parameters that we investigated. However, some trends were consistent with exposure (e.g., slower rate of growth during treatment), possibly indicating that longer exposures or more animals may have provided definitive results.

4.6: ACKNOWLEDGEMENTS

We thank Patricia Beall (for necropsy support), LTC David Young and John Roberston (for pathological support), and Maj. Terry Besch for veterinary assistance. This study was funded by U. S. Army Environmental Center, Installation Restoration Program.

4.7: REFERENCES

1. Hoffman DJ. 1997. Wildlife toxicity testing. In Hoffman DJ, Rattner BA, Burton GA Jr, Cairns J Jr, eds, *Handbook of Ecotoxicology*. Lewis Publishers, Boca Raton, LA, USA, pp 47-69.

2. Phillips K. 1990. Where have all the frogs and toads gone? *BioScience* 40: 22-424.

3. Wake DB. 1991. Declining amphibian populations. *Science* 253: 860.

4. Schmidt CW. 1997. Amphibian deformities continue to puzzle researchers. *Environ Sci Technol* 31: 324-326

5. Burkhart JG, Gardner HS. 1997. Non-mammalian and environmental sentinels in human health: back to the future? *Human and Ecol Risk Assess* 3:309-328.

6. Bishop SC. 1943. *Handbook of Salamanders*. Cornell University Press, Ithaca, NY. USA.

7. Spector WS. 1956. *Handbook of Biological Data*. W. B. Saunders Co., Philadelphia, PA, USA.

8. Walsh ME, Jenkins TF. 1992. Identification of TNT transformation products in soil. Special Report No. 92-16, U.S. Army Corps of Engineers, Cold Regions Research and Engineering Laboratory.

9. Burrows EP, Rosenblatt DH, Mitchell WR, Parmer DL. 1989.Organic explosives and related compounds: environmental and health considerations. TR-8901. Technical Report. U.S. Army Biomedical Research and Development Laboratory, Frederick, MD USA.

10. Talmage SS, Opresko DM. 1995. Ecological criteria document for 2,4,6-trinitrotoluene. Ecological Monitoring Research Division, Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH, USA.

11. Dilley JV, Tyson CA, Spanggord RJ, Sasmore DP, Newell GW, Dacre JC. 1982. Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats, and dogs. *J Toxicol Environ Health* 9: 565 -585.

12. Levine BS, Rust JS, Barkley JJ, Furedi EM, Lish PM. 1990a. Six-month oral toxicity study of trinitrotoluene in beagle dogs. *Toxicology* 63: 233-244.

13. Levine BS, Furedi EM, Gordon DE, Barkley JJ, Lish PM. 1990b. Toxic interactions of munition compounds TNT and RDX in F344 rats. *Fund Appl Toxicol* 15 :373-380.

14. Johnson MS, Franke LS, Lee RB, Holladay SD. 1998. Bioaccumulation of 2,4,6-trinitrotoluene (TNT) and PCBs through Two Routes of Exposure in a Terrestrial Amphibian: Is the Dermal Route Significant? *Environ Toxicol Chem* (in press).

15. Jaeger RG. 1992. Housing, handling, and nutrition of salamanders. In Shaeffer et al., eds, *The Care and Use of Amphibians, Reptiles and Fish in Research*. Scientist Ctr. For Animal Welfare, Bethesda, MD, USA, pp. 25-29.

16. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J immuno* 130: 1910-1917.

17. Dunn PA, Tyrer HW. 1981, Quantitation of neutrophil phagocytosis, using fluorescent latex beads. *J Lab Clin Med* 98: 374-381.

18. Husztik E, Lazar G, Parducz A. 1980. Electron microscopic study of Kupffer cell phagocytosis blockade induced by gadolinium chloride. *Br J Exp Pathol* 61: 624-630.

19. Holladay SD, Smith SA, El-Habback H, Caceci T. 1996. The influence of chlorpyrifos, an organophosphate insecticide, on the immune system of tilapia (*Oreochromis niloticus*). *Aquatic Toxicol* 41: 17-19.

20. Thierfelder W, Masihi KN. 1995. Effects of trinitrotoluene (TNT) metabolites on chemiluminescence response of phagocytic cells. *Int J Immunopharmac* 17: 453-456.

21. Wolke RE. 1992. Piscine macrophage aggregates: a review. *Annual Rev of Fish Diseases* pp. 91-108.

22. Meseguer J, Lopez-Ruiz A, Esteban MA. 1994. Melano-macrophages of the seawater teleosts, sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*): morphology, formation and possible function. *Cell Tissue Res* 277: 1-10.

23. Tsioros KK, Youson JH. 1997. Intracellular distribution of iron (and associated elements) in various cell types of larvae and juveniles of the sea lamprey (*Petromyzon marinus*). *Tissue & Cell* 29: 137-162.

24. Masson R, Roome NO. 1997. Spontaneous iron overload in Sprague-Dawley rats. *Toxicol Pathol* 25: 308-316.

25. Herraez MP, Zapata AG. 1986. Structure and function of the melano-macrophage centres of the goldfish (*Carassius auratus*). *Vet Immuno Immunopathol* 12: 117-126.

26. deRobertis, E. 1939. Origin and localization of pigment in amphibian liver. Rev. Soc. Argent. Biol. 15:87-93 *in* Brown GW Jr. 1964. The metabolism of amphibia. *In* Moore JA, ed, *Physiology of the Amphibia* Vol. I. Academic Press, New York, NY. pp. 18.

27. Agius C. 1985. The melano-macrophage centers of fish: a review. In. Manning MJ, Tatner MF eds, *Fish Immunology*. Academic Press, London, UK pp. 85-105.

28. Christensen J L, Grzybowski JM, Kodama RM. 1996. Melanomacrophage aggregations and their age relationships in the yellow mud turtle, *Kinosternon flavescens* (Kinosternidae). *Pigment Cell Res* 9: 185-190.

29. Blaustein, AR, Wake DB, Sousa WP. 1994. Amphibian declines: judging stability, persistence, and susceptibility of populations to local and global extinctions. *Conservation Biology* 8: 60-71.

30. Harris ML, Bishop CA, Struger J, van den Huevel MR, Van Der Kraak GJ, Dixon GD, Ripley, B, Bogart JP. 1998. The functional integrity of northern leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*) populations in orchard wetlands. I. Genetics, physiology, and biochemistry of breeding adults and young-of-the-year. *Environ Toxicol Chem* 17:1338-1350.

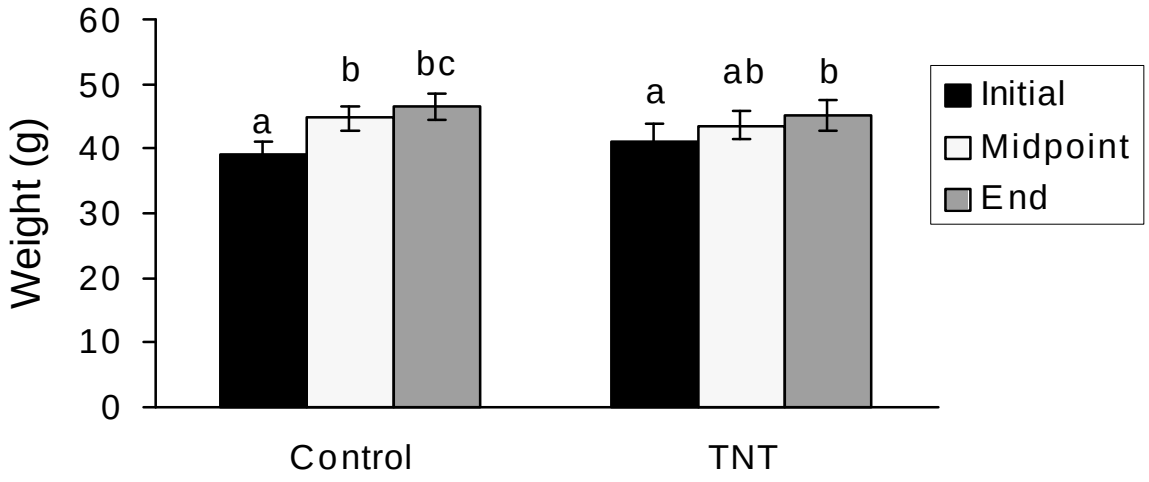


Figure 4.1. Mean body weight of *A. tigrinum* for each treatment during the exposure period. Means with different superscripts are different at $p < 0.05$. Bars represent standard errors (N = 9).

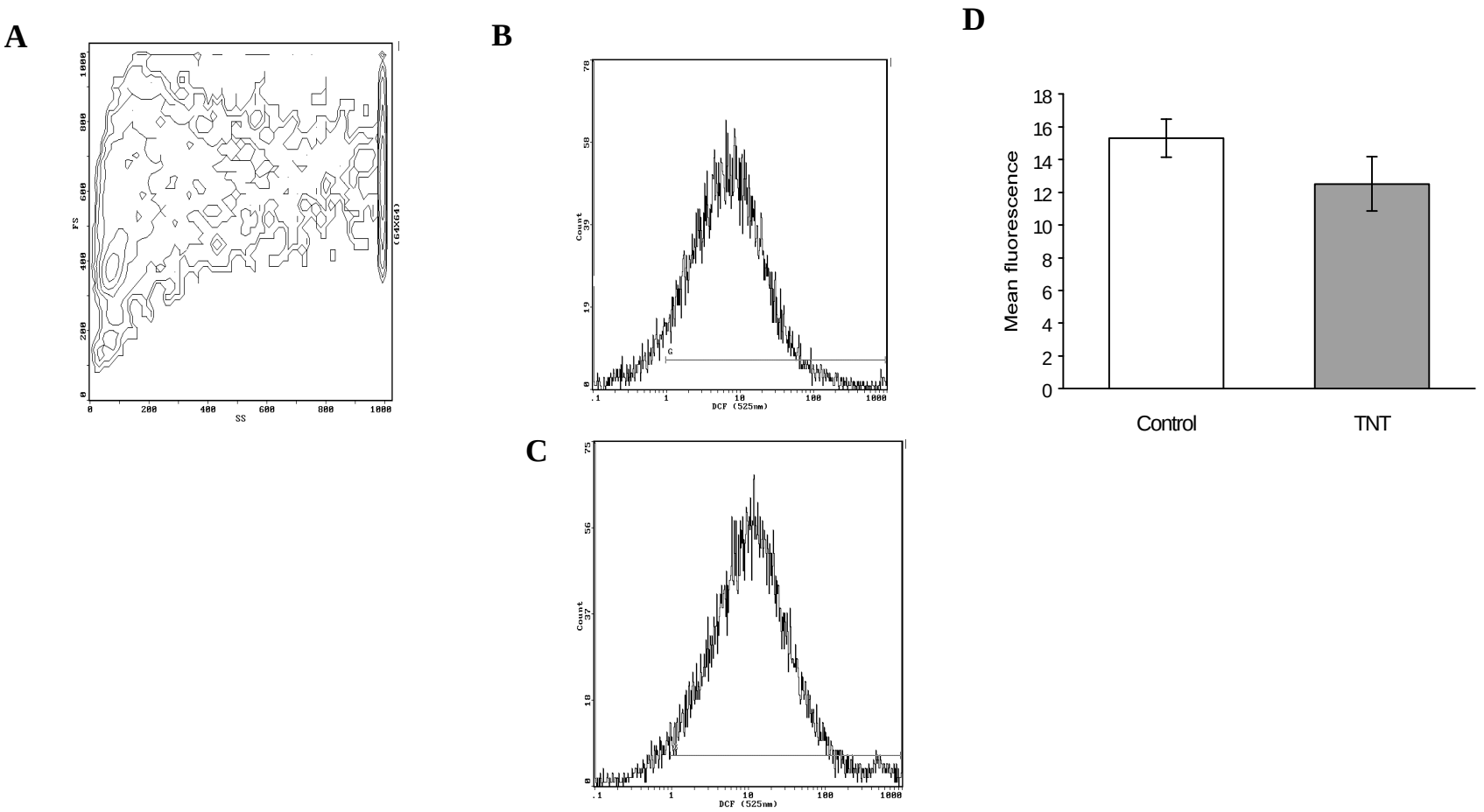
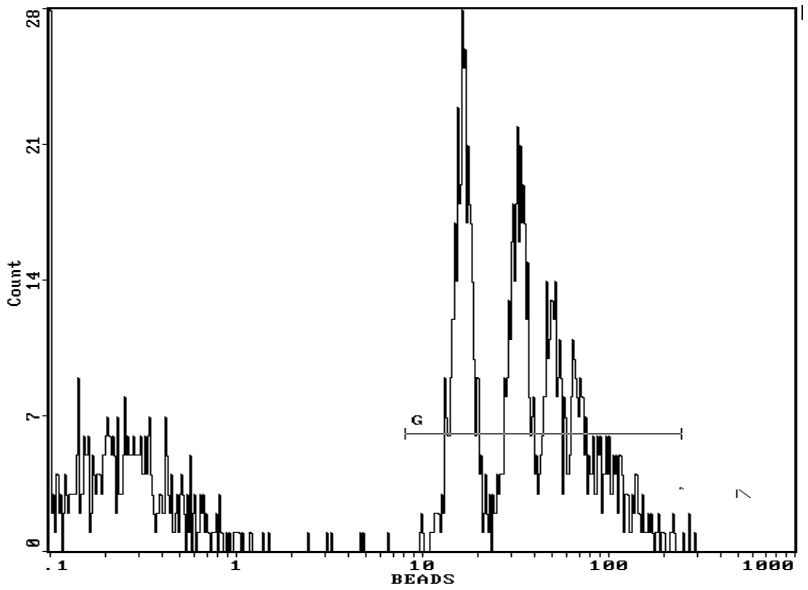


Figure 4.2. Effects of TNT-exposed splenic phagocytes in *A. tigrinum.* Flow cytomeric analysis of splenic phagocytes using Forward Angle Light Scatter (FALS) and Side Angle Light Scatter (SALS) (2a). An example of a FITC-filtered DCF peak measured an analysis of ROI production for controls (2b) and TNT-exposed splenic phagocytes (2c). Mean ROI production of controls and TNT-exposed splenic phagocytes. Bars represent standard errors.

A



B

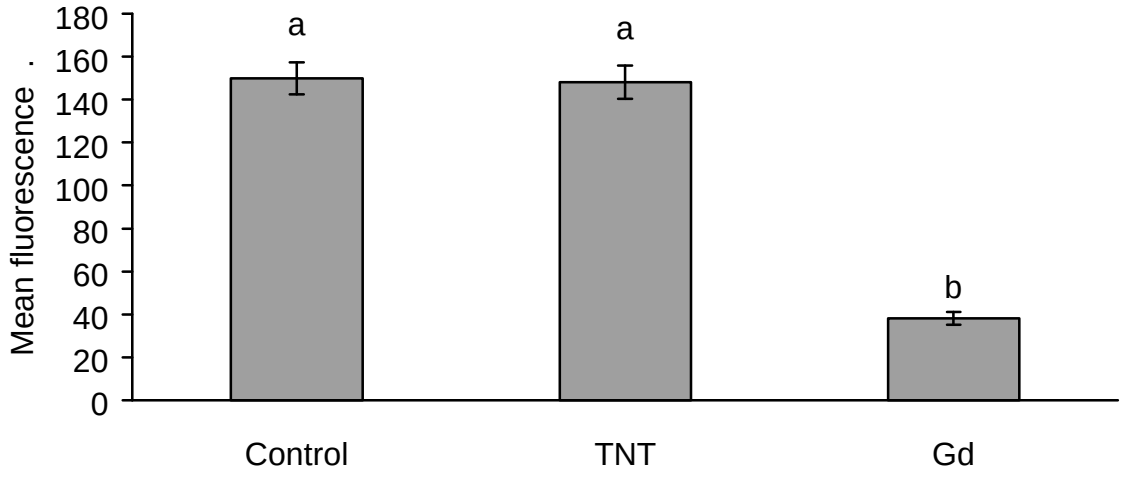
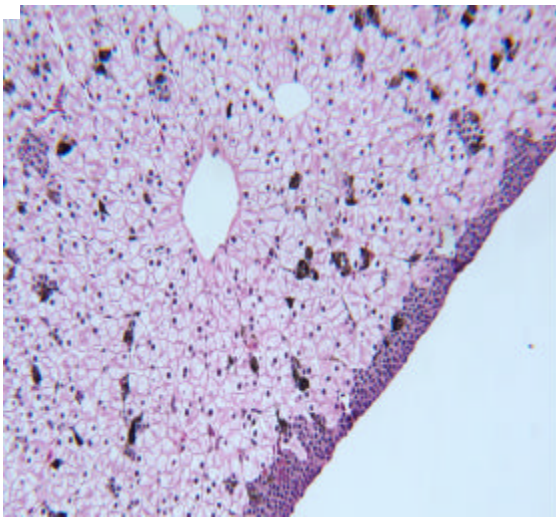
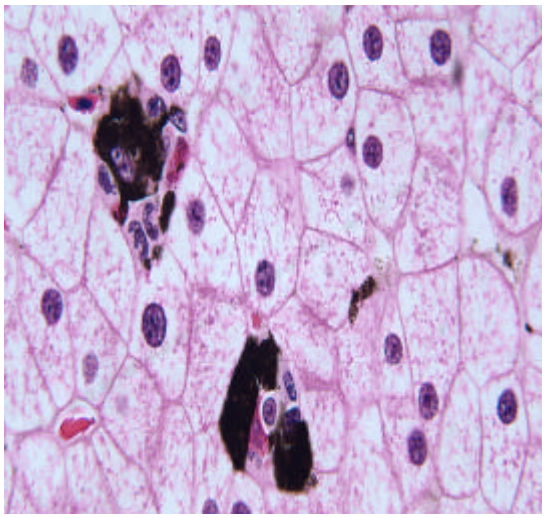


Figure 4.3. Analysis of phagocytosis of fluorescent microspheres in the splenocytes of *A. tigrinum* exposed to TNT and of Controls measured by flow cytometry. An example is presented for *A. tigrinum* where the first three peaks from the left correspond to cells phagocytosizing 1, 2, and 3 microspheres, respectively. B represents the mean fluorsecence of intracellular microspheres; bars represent SEMs. *Indicates different from controls at $p < 0.05$; Gd = gadolimium chloride administered *in vitro* (see text).

A



B



C

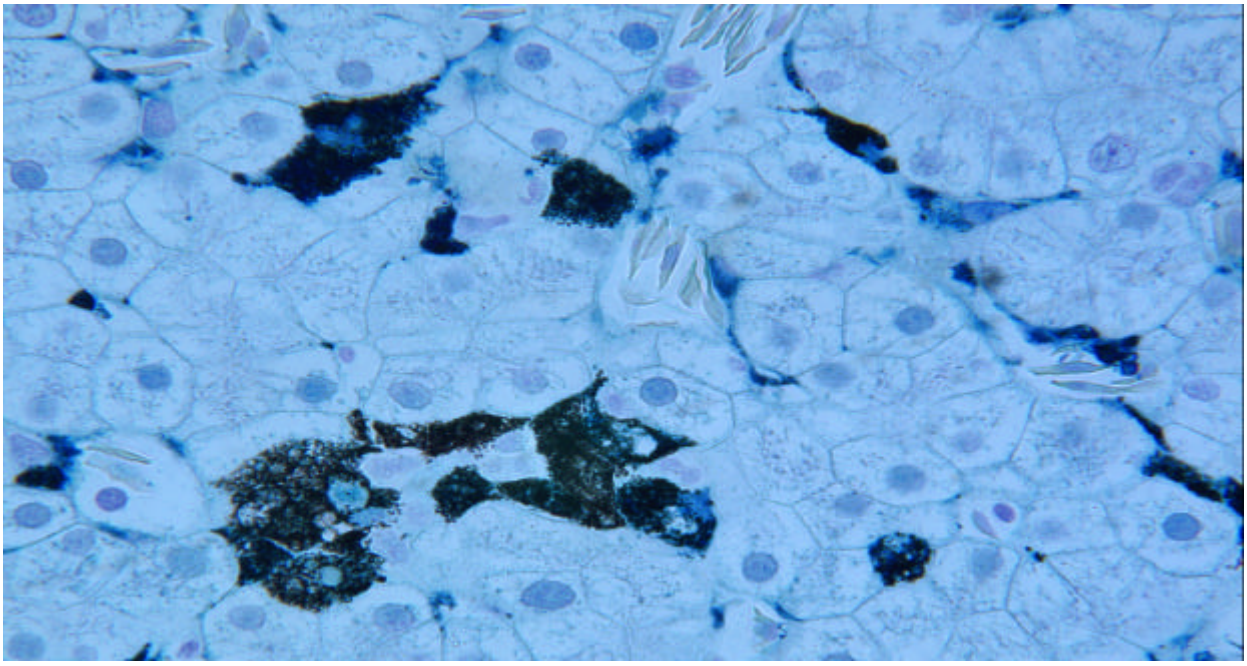


Figure 4.4. Images of liver sections of *A. tigrinum* prepared in H&E (A, B) and Gomori stain (C). Low power (60x) view of liver showing distribution of melanomacrophages and outer granulopoetic tissue (A). High power (200x) view of melanomacrophages in proximity of eosinophils, neutrophils, and lymphocytes (B). High power view of iron-stained granules (C).

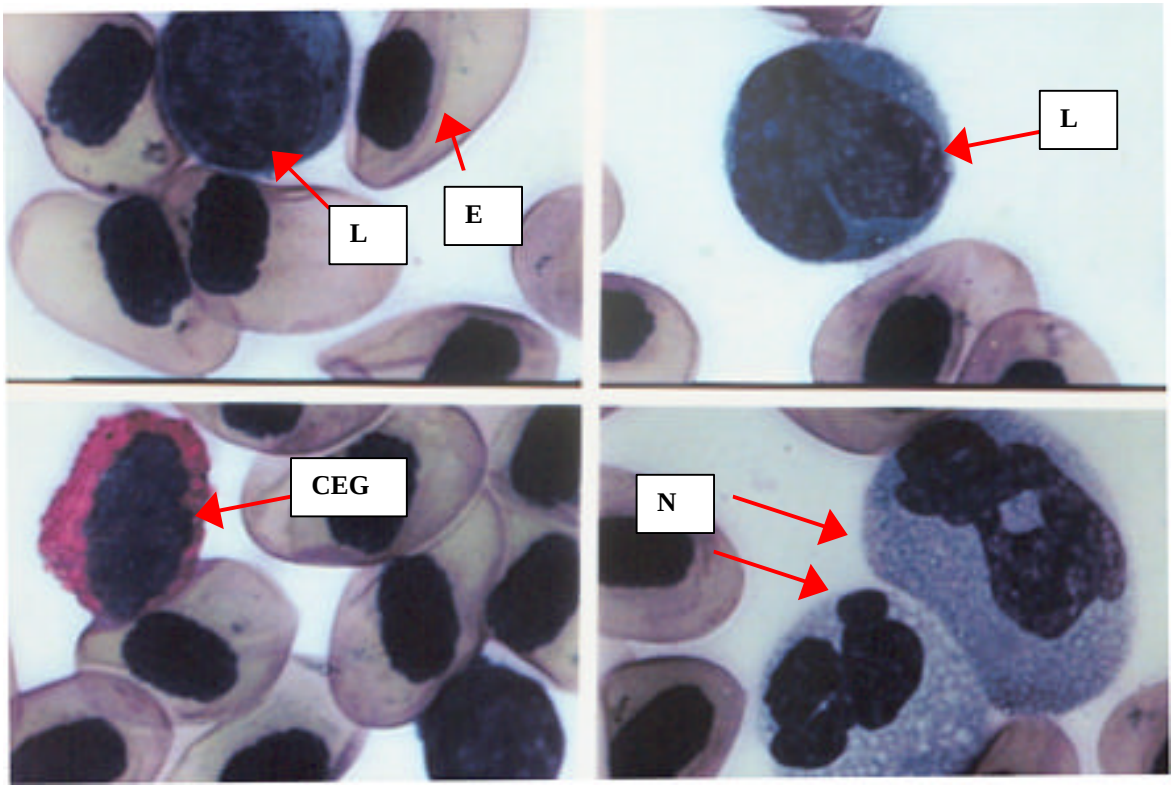


Figure 4.5. Blood cell types stained with Wright-Giemsa (180 x). Course eosinophilic granulocytes (CEG), neutrophils (N), lymphocytes (L), and erythrocytes (E) identified by arrows.

Table 4.1. Concentrations of TNT and primary reduction products (2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and 2,4-diamino-6-nitrotoluene) in soil and food taken at the beginning and the end of exposure (expressed in *u*g/g). Control soils and worms were all below detection levels (>0.50 *u*g/g and >0.0005 *u*g/g, respectively). † = Worm soil sample taken at the beginning of treatment. ND = No Data.

	Soils				Worms		
	Post-mixing (dry)	Initial	Final	Worm soil†	Initial	Midpoint	Final
2,4,6-TNT	1200	280	59	81	0.25	0.79	0.62
2-A-DNT	<0.51	39	58	29	2.10	2.60	2.30
4-A-DNT	<0.51	62	78	43	2.50	2.10	2.10
2,4-DA-6-N	ND	ND	ND	ND	0.43	0.18	0.26

Table 4.2. Mean and standard errors of the organ/body weight ratios for the primary blood conditioning organs of *A. tigrinum* for controls and TNT-exposed individuals.

Organ	TNT-exposed	Control	*t*	p
Spleen (organ/bw $x10^{-3}$)	1.88 ± 0.180	1.85 ± 0.144	-0.131	0.90
Liver (organ/bw $x10^{-2}$)	5.63 ± 0.270	5.13 ± 0.208	-1.46	0.16
Kidney (organ/bw $x10^{-3}$)	5.15 ± 0.652	5.26 ± 0.447	-0.134	0.89

Table 4.3. Effects on the hematology of *A. tigrinum* from soil exposures to 2,4,6-trinitrotoluene. Means are presented with standard errors; sample sizes in parentheses.

Parameter	Control	TNT-exposed	p >
RBC (M/ul)	0.183 ± 0.02 (8)	0.198 ± 0.02 (8)	0.60
WBC (K/ul)	3.26 ± 0.61 (8)	3.47 ± 0.38 (8)	0.77
Hematocrit (%)	32.1 ± 1.94 (9)	37.0 ± 3.13 (9)	0.20
MCV (fl)	1842.0 ± 201.4 (8)	1973.1 ± 251.4 (8)	0.69
Eosinophils (%)	45.6 ± 7.01 (8)	37.6 ± 4.97 (8)	0.36
Neutrophils (%)	9.38 ± 2.91 (8)	15.71 ± 3.65 (9)	0.19
Basophils (%)	3.50 ± 1.66 (4)	2.14 ± 0.55 (7)	0.36
Total protein (g/dl)	2.39 ± 0.12 (9)	2.69 ± 0.18 (9)	0.18

CHAPTER 5. Evaluation of Fate and the Biochemical Effects of 2,4,6-Trinitrotoluene (TNT) Exposure to Tiger Salamanders: (*Ambystoma tigrinum*).

Mark S. Johnson[1], Steven D. Holladay[2], Jayant Vodela[3], and Gunda Reddy[1]U.S. Army Center for Health Promotion and Preventive Medicine, [2] Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, [3]Dynamac Corp.

Corresponding author: Mark S. Johnson, U.S. Army Center for Health Promotion and Preventive Medicine, 5158 Blackhawk Rd., Attn: MCHB-TS-THE, Toxicology Directorate, Aberdeen Proving Ground, MD 21010-5422

P: 410-671-3980

e-mail: Mark_S._Johnson@chppm-ccmail.apgea.army.mil

5.1: Abstract - Biochemical changes in animals exposed to environmental contaminants are of potential use in the exposure assessment of a toxicant. We examined biotransformation, metabolic enzyme profiles and the glutathione antioxidant system in tiger salamanders *(Ambystoma tigrinum*) from a 14-day exposure to 2,4,6-trinitrotoluene (TNT) *in situ*. The indicators evaluated included cytochrome p450 (p450) and cytochrome b5 (b5) content and their dependent isozyme activities, ethoxy resorufin O-dealkylation (EROD), pentoxy resorufin O-dealkylation (PROD), and the glutathione antioxidant system in the skin, liver, lung, kidneys, and blood. Concentrations of parent compound and metabolites were also measured. Considerable differences were found in relative concentrations of TNT and its metabolites in the tissues studied. Trace amounts of TNT were detected only in the skin and liver of exposed animals while one of the secondary reduction metabolites, 2,6-diamino-nitrotoluene (2,6-DANT), was found only in liver and kidney. Differences in metabolite concentrations between systemic organs (liver, kidneys) and the skin suggest that the skin may be important in the primary reduction of TNT. In addition, measurable levels of these basal enzyme indicators were found, yet of those evaluated only hepatic p450 content was found to be affected by TNT exposure. These results indicate that qualitative and quantitative differences in TNT and its metabolites in tissues suggest the fate and metabolism of the TNT in salamanders. Furthermore, our results indicate that these salamanders possess considerable levels of xenobiotic metabolizing and antioxidant enzymes in these tissues. These indicators were not sensitive to TNT exposure.

Keywords: p450, TNT, EROD, PROD, amphibian, salamander

5.2: INTRODUCTION

Anthropogenic substances have been distributed throughout the environment yet their potential impact on many species is not fully understood. The measurement of levels of detoxifying enzymes in exposed animals as potential indicators of environmental contamination has a conceptual and practical basis. These biotransformation enzymes are primarily measured in hepatic tissue, a major site of bioaccumulation for lipophilic chemicals (Lubet et al., 1990). Such biotransformation enzymes are highly induced by a variety of environmental chemicals and are generally involved in the metabolism of a wide variety of environmental pollutants. The induction of these enzymes can significantly alter susceptibility following repeated or chronic exposures to many xenobiotics (Batt et al. 1992).

It has been suggested that amphibians are an ideal model in which to study the metabolism of chlorinated aromatics (Safe et al. 1976). These authors considered the amphibian metabolic pathway similar to that of mammals, and reported a higher detoxification capacity for frogs compared to fish. Earlier investigations in our laboratory have shown that dermal exposures were more important than ingestion for nitroaromatics in soil to a terrestrial salamander (e.g., TNT, Johnson et al. *in press*). Further, reference ranges and the relative distribution of activity for glutathione and related antioxidant enzymes in other various tissues (liver, skin, kidney, hemolysate) have not been reported for many amphibians. We therefore investigated the metabolic potential of these tissues through the relative proportion of biotransformation products and of the parent compound (i.e., 2, 4, 6-trinitrotoluene; TNT) in the skin, liver, and kidney. We further chose to investigate the relative concentrations of cytochrome b5, cytochrome p450 and its isozymes (e.g., PROD and EROD) for these tissues in Tiger salamanders (*Ambystoma tigrinum*).

5.3: METHODS

5.3.1: Aminal Husbandry - Tiger salamanders (N = 36; obtained from Glade Herp., Ft. Meyers, FL) were quarantined in separate 2.5 gal polystyrene aquariums (Carolina Biological) for approximately one month. During this period, any salamanders exhibiting signs of overt stress (e.g., septicemia, red-leg) were treated by daily oral gavage with doxycycline monohydrate (Pfizer Laboratories, NY) at 5 mg / 30 g bw suspended in sterile isotonic saline until they

improved or became moribund. No salamander received any treatment 16 days prior to the beginning of the study. For this study, 18 salamanders were selected and evenly distributed by weight into the two treatments.

5.3.2: Treatment Preparation – Soil was prepared and mixed with a re-distilled stock of crystalline 2,4,6-trinitrotoulene (TNT) determined to be at least 95% pure (through flame ionization detection, GC) to a concentration of about 1200 mg/kg dry weight. Approximately 400 g (± 10 g) of treated soil was added to each terrarium and subsequently hydrated with 20 ml deionized distilled water six days prior to exposure. A small piece of sheet moss was added and hydrated on day-1 to reduce stress and emulate natural conditions.

Food consisted exclusively of earthworms (*Lumbricus terrestris*; Manchester Worm Farm, Manchester, CT) exposed to identical soil stocks previously discussed. Food was offered every other day *ad libitum* yet not to exceed two worms/salamander/day. Salamanders were maintained at 12 hour light/dark cycles at 17 – 19 °C. Humidity was maintained through the hydration of the soil and sheet moss, when necessary, for each terrarium. Approximately 1 g of soil from each terrarium was removed and pooled according to treatment at the beginning and end of study determine the concentrations of TNT and the primary metabolites in soil throughout the study.

5.3.3: Collection Procedures – Blood was collected from hand-restrained individuals, prior to euthanasia, through the caudal vein and then the salamanders were euthanized by decapitation. Samples of liver, lung, kidney, and skin were collected and were removed for chemical and histopathological analysis; and remaining tissues were stored at -140°C (control, N = 4; TNT, N = 3). Tissues collected for chemical analyses were stored at -32°C in borrosilicate glass containers with teflon lids in the dark until analyzed. No attempt was made to separate by sex since this variable could not be determined reliably at the time of treatment.

5.3.4: Isolation of Red Blood Cells (RBC) - The blood samples were collected in heparinized tubes and stored at room temperature for one hour and centrifuged at 2000 G for 15 minutes at room temperature to separate plasma after Vodela and Dalvi (1997). After separation of plasma

an equal amount of chilled water was added to erythrocytes and samples were stored at -20 C for an hour to lyse red blood cells. These samples were centrifuged at 2000 G for 10 minutes and the supernatant was collected. This procedure was repeated for 3 times for each sample and the mean calculated.

5.3.5: Enzyme Assay Procedures - Skin samples were washed in phosphate buffered saline and dissected free of fat before using for microsomal preparation via differential centrifugation (cell fractioning) (Singhal et al 1993). Prior to analysis each sample was aliquoted into cryovials and stored at -140°C. Tissues samples from individual salamanders were pooled according to treatment for skin, kidneys, and lungs (control, N = 4; TNT, N = 3) and prepared for microsomal and cytosolic enzyme activity according to Reddy et al. (1997). Three replicates were then conducted for each pooled sample and the mean reported. Livers were of sufficient size to permit individual analyses.

Cytochrome p450 (p450) and cytochrome b5 (b5) contents were determined by the method of Omura and Sato (1964). The ethoxyresorufin O-dealkylation (EROD) and pentoxyresoruf O-dealkylation (PROD) were determined based on the fluorometric method of Lubet et al. (1990) using a Spex Fluoromax Spectrofluorometer at an excitation wavelength of 540 and an emission wavelength of 585 nm. Glutathion S-transferase (GST) activities with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate were determined as described by Habig et al. (1974). Reduced (GSH) and oxidized (GSSH) glutathione levels were analyzed using fluorometric method of Hissin and Hilf (1976). Glutathione reductase (GSH-GR) (Carlberg and Mannervick 1985) and glutathione peroxidase (GSH-PX) (Flohe and Gunzler 1985) were determined as described. The methods of Stich (1990) were used to estimate protein concentrations in hemolysate, cytosol, and microsomes using Pierce Bicinchoninic acid protein assay reagent (Pierce Chemical, Rockport, IL).

5.3.6: Chemical Analysis - Chemical analyses followed U.S. Army Center for Health Promotion and Preventive Medicine, Directorate of Laboratory Sciences, Analytical Method Program Standing Operating Procedure No. 51.6. Briefly, tissues were frozen via liquid nitrogen infusion and then ground up manually with mortar and pestle. Tissue was dried using anhydrous $NaSO_4$.

TNT and metabolites were extracted using acetalnitrile and analyzed using gas chromatography, mass spectrometry. Six point curves were created using deuterated TNT and TNT metabolites in spiked matrixed dupicates. If the detected concentration exceed this range, they were diluted by a factor of 10 and reanalyzed. J-values were used when estimable; non-detects were assumed at ½ the detection limit.

5.3.7: Statistical Analyses - Tissue-specific concentrations of TNT metabolites were tested with a Two Way Repeated Measures Analysis of Variance on Two Factors Balances Design. Since the data were not normally distributed, nor were the variances equal, they were analyzed using the Student-Newman-Kuels Method. Statistical significance was determined at the $p < 0.05$ level for all tests. Glutathione, related enzymes, and liver p450 and related markers were tested with a Student *t*-test. Samples otherwise indicated were pooled and as such could not be statistically compared.

5.4: RESULTS

5.4.1: Exposure regime – Natural attenuation of TNT in soil was significant. Soil concentrations of TNT were 1200 *u*g/g in dry soil sampled at the time of preparation. However, 6 days after hydration (day 1 of exposure) soil concentrations of TNT had dropped an order of magnitude and significant amounts of the mono-amino reduction products were present (TNT = 280 *u*g/g, 2-amino-dinitrotoluene (2A-DNT) = 39 *u*g/g, 4-amino-dinitrotoluene (4A-DNT) = 62 *u*g/g). TNT concentrations were lower at the end of study (59 *u*g/g), though the primary reduction products were relatively unchanged (58 and 78 *u*g/g, 2A-DNT and 4A-DNT, respectively). Earthworms had higher levels of the primary reduction products than of parent compound ($\bar{x}$ = 2.33 and 2.33 *u*g/g 2A-DNT and 4A-DNT, respectively) yet far below that of soil. Detectable traces of 2,4-diamino-6-nitrotoluene were also detected in earthworms (unpublished data).

5.4.2: Tissue Residue – Concentrations of metabolites in the liver and kidney were different from those of the skin (Fig. 5.1.). There were no differences between the kidney and liver samples. There were no differences between the overall concentrations of the two secondary reduction products, 2,4-diamino-nitrotoluene (2,4-DANT) and 2,6-diamino-nitrotoluene (2,6-DANT), yet there were differences between those and one of the primary reduction products, 4A-DNT. The

4A-DNT concentrations were also higher than 2A-DNT for all tissues. Only a single sample from the liver and one from the skin had detectable concentrations of parent compound (TNT). Concentrations of 2A-DNT, 4A-DNT, and 2,4-DANT were comparable to that in earthworms (see Chapter #3).

5.4.3: Biochemical Indicators - Detectable levels of p450, b5, and its isozymes are presented in Table 5.1. The p450 content was highest in the liver followed by skin, kidneys, and lung of control salamanders while the b5 content was high in liver but low in skin. The microsomal ERODs were detectable in liver and skin but low (i.e., not detectable) in lung and skin at the protein levels used. However, PROD activities were detected in liver, skin, lung and kidneys. There were no obvious treatment differences in these enzyme systems except in the increase in cytochrome p450 content in the livers of TNT exposed animals.

The results of the glutathione antioxidant system are presented in Table 5.2. High basal levels of GSH were found in liver, skin, lung, kidney, and blood of salamanders when compared to GSSH. Considerable GST, GSH-PX, and GSH-R activities were also detected in varying degree for liver, skin, lung, kidney, and erythrocytes. No TNT treatment-related effects were noticed in any of these glutathione antioxidant components.

5.5: DISCUSSION

The fate and biochemical effects of TNT in *A. tigrinum* were investigated to identify sensitive biochemical indicators of exposure and to provide baseline information regarding tissue-specific enzymatic activities for a terrestrial salamander. Our results show that salamanders exposed to TNT in the soil at these concentrations do contain TNT reduction metabolites in the tissues investigated. The qualitative and quantitative differences of these metabolites in the skin, liver, and kidney may be due to differences in the disposition or metabolic capacity of the organs examined as a result of a 14 day exposure. During this period, no overt toxicity was observed (see Chapter 3).

We present the first information regarding the relative, tissue specific concentrations of certain biochemical indicators in an Ambystomid salamander. Relatively low levels of p450 and

isozymes activity have been reported for African clawed frog (*Xenopus laevis*) (Saito et al. 1997) and the newt (*Pleurodeles waltl*) (Marty et al. 1992). However, our results for basal p450 levels are lower than those found for three species of freshwater turtles (Yawetz et al. 1997).

These data suggest that the liver is of primary importance in TNT biotransformation in *A. tigrinum*. However, the systemic concentrations of TNT metabolites combined with the relative concentrations of parent compound in the soil suggest that the primary reduction processes may occur at (or on) the skin. This may be accomplished through surface microbes or through processes within the skin. Secondary reduction metabolites (2,4 and 2,6-DANT) were found in the liver and kidney, yet only traces of 2,4-DANT and no detectable amounts of 2,6-DANT were found in the skin. Further, the only detectable amounts of TNT were found in the skin and liver. However, since these organs were not perfused, there exists the possibility that these concentrations may represent serum levels.

In our study, salamanders had high levels of GSH-GR and GSH PX activity in liver, skin, lung and kidney. Glutathione metabolizing enzymes play an important role in the protection of cells against oxidative and alkylating agents. Cartana et al (1992) similarly observed organ specific activity of GSH-GR in rats, which may relate to the different susceptibilities of different organs to a toxic compound. However there were no significant differences between the present control and TNT exposed salamanders in liver, kidney, skin and lungs. Zitting et al. (1982) found differences in reduced glutathione in the livers, brains, and kidneys of male Wistar rats as a result of intraperitoneal TNT exposure. Ultrastructural analysis of the brain, liver, and kidney of these rats suggested TNT-induced toxicity and led to the postulation that the cyclic reduction of nitroaromatics create superoxide anions that may be involved in lipid peroxidation. This may explain the increased hemolysis frequently observed in mammalian studies (Dilley et al. 1982, Levine et al. 1990, Hathaway 1977). Glutathione would be important as a potential peroxide scavenger. Our data may suggest this, however, sample sizes were too small to support such a hypothesis at this time.

TNT is a strong oxidizing agent that is most often reduced. Therefore perhaps it is axiomatic that the induction of oxidizing enzymes may not be a relevant bioindicator of TNT exposure.

Zitting et al. (1982) found no correlation of p450 content in rats exposed to TNT. Igbo et al. (1997) examined EROD activity as a potential indicator of 2,4-dinitrotoluene exposure in Japanese medaka (*Oryzias latipes*) and reported a slight induction in liver preparations, yet this was of a pooled sample. Our investigations support limited value of these indicators as biomarkers of exposures to TNT. However, some of these indicators do present an interesting basis into the evolutionary value of tissue-specific differences in mechanisms of metabolism and protection for terrestrial amphibians as a result of exposures to xenobiotics in soil.

5.6: ACKNOWDEGEMENTS

We thank Dr.s Howard Bausum and Glenn Leach for previous review and suggestions. This research was funded by the U.S. Army Environmental Center, Installation Restoration Program.

5.7: REFERENCES

Batt, AM, Siest G, Magdalou J, Galteau, MM. Enzyme induction by drugs and toxins. *Clinical Chemistry Acta* 209:109-121:1992.

Carlberg I, and Mannervick B. 1985. Glutathione reductase. *Methods Enzymol* 113:484-490.

Cartanta J, Romeu A, and Arola L. 1992. Effects of copper, cadmium and nickel on liver and kidney glutathione redox cycle of rats. *Comparative Biochemistry and Physiology* 101:209-213.

Dilley JV, Tyson CA, Spanggord RJ, Sasmore DP, Newell GW, Dacre JC. 1982. Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats, and dogs. *J Toxicol Environ Health* 9:565 -585.

Flohe L, and Gunzler WA. (1985). Assay of Glutathione Peroxidase. *Methods Enzymol* 105:115-121.

Habig WH, Pabst MJ, and Jakoby WB. 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chemistry* 35:7130-7139.

Hathaway JA. 1977. Trinitrotoluene: a review of reported dose-related effects providing documentation for a workplace standard. *J Occup Med* 19: 341-345.

Hissin PJ, and Hilf R. 1976. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem* 74:214-226.

Igbo I, Obih P, Huang T. 1997. Use of AChE and EROD as biomarkers to 2,4-dinitrotoluene (DNT) exposure in Japanese medaka. Poster, SETAC 18th Annual Meeting, San Francisco, CA.

Levine BS, Rust JS, Barkley JJ, Furedi EM, and Lish PM. 1990. Six-month oral toxicity study of trinitrotoluene in beagle dogs. *Toxicology* 63:233-244.

Lubet RA, Guengerich PF, and Nims RW. 1990. The induction of alkoxyresorufin metabolism: a potential indicator of environmental contamination. *Arch Environ Contam Toxicol* 19:157-163.

Marty J, Riviere JL, Guinaudy MJ, Kremers P, Lesca P. 1992. Induction and characterization of cytochrome p450IA and –IIB in the newt, *Pleurodeles waltl*. *Ecotoxicol Environ Safety* 24:144-154.

Omura T, Sato R. 1964. The carbon monoxide-binding pigment of the liver microsomes.I. Evidence for its hemoprotein nature. *J Biol Chem* 239:2370-2378.

Reddy G, Qualls CW, Hampton AEG, Yelamanchili, A, and Kim S. (1997). Acute pathological and biochemical effects of 2,4,6-trinitrophenyl-N-methylnitramine (Tetryl) in male rats. *Res Comm Pharmacol Toxicol* 2:1-11.

Safe S, John D, Kohli J, Ruzo LO, Hutzinger O, and Sundstrom G. 1976. The metabolism of chlorinated aromatic pollutants by the frog. *Can J Zool* 54:1818-1823.

Saito H, Ohi H, Sugata E, Murayama N, Yoshiaki F, Higuchi S. 1997. Purification and characterization of a cytochrome p450 from liver microsomes of *Xenopus laevis. Arch Biochem Biophys* 345:56-64.

Singhal SS, Saxena M, Awasthi S, Mukhtar H, Zaidi SI, Ahmad H, and Awasthi YC. 1993. Glutathione S-transferase of human skin: qualitative and quantitative differences in men and women. *Biochem Biophys Acta* 1163:266-272.

Stich T. 1990. Determination of protein covalently bound to agarose supports using bicinchoninic acid. *Anal Biochem* 191:343-346.

Vodela JK, Dalvi RR. 1997. Erythrocyte glutathione S-transferase activity in animal species. *Vet Human Toxicol* 39:9-10.

Yawetz A, Benedek-Segal M, Woodin B. 1997. Cytochrome p4501a immunoassay in freshwater turtles and exposure to pcbs and environmental pollutants. *Environ Toxicol Chem* 16:1802-1806.

Zitting A, Szumanska G, Nickels J, and Savolainen H. 1982. Acute toxic effects of trinitrotoluene on rat brain, liver, and kidney: the role of radical production. *Arch Toxicol* 51:53-64.

Table 5.1. Cytochrome p450, b5 and its isozymes in *A. tigrinum* exposed to TNT *in situ* for 14 days.*

Parameter	Control	Treatment	P value
Cytochrome p450[1]			
Liver	0.47 ± 0.05 (4)	0.71 ± 0.01 (3)	0.01
Skin	0.37	0.44	
Lung	0.26	0.34	
Kidney	0.37	0.26	
Cytochrome b5[2]			
Liver	0.46 ± 0.14 (4)	0.46 ± 0.07 (3)	0.97
Skin	0.26	0.33	
Lung	0.31	0.42	
Kidney	0.36	0.29	
EROD[3]			
Liver	29.3 ± 5.7 (4)	28.5 ± 8.6 (3)	0.94
Skin	35.0	95.0	
Lung	ND	ND	
Kidney	ND	ND	
PROD[3]			
Liver	2.45 ± 0.36 (4)	2.46 ± 0.72 (3)	0.99
Skin	2.3	2.0	
Lung	6.6	8.8	
Kidney	2.9	4.1	

[1]*u*moles of cytochrome p450/mg protein.

[2]*u*moles of cytochrome b5/mg protein.

[3]picomoles of resorufin formed/min/mg protein.

[ND]Not detected at 11.56 and 7.69 mg protein in lung and kidney, respectively.

Sample sizes in parentheses. Means presented with standard errors. Skin, lung and kidney samples were pooled, yet represent average values of 3-5 replicates.

Table 5.2. Glutathione antioxidant enzymes in *A. tigrinum* exposed to TNT *in situ* for 14 days*

Parameter	Control	Treatment	P value
Reduced Glutathione[b]			
Erythrocytes	0.028 ± 0.006 (4)	0.024 ± 0.007 (4)	0.33
Liver	29.9 ± 3.7 (4)	34.7 ± 0.5 (3)	0.7
Skin	21.5	23.3	
Lung	11.2	10.7	
Kidney	11.2	10.5	
Oxidized Glutathione[a]			
Erythrocytes	0.016 ± 0.003 (4)	0.015 ± 0.003 (4)	0.84
Liver	3.53 ± 0.36 (4)	3.08 ± 0.18 (3)	0.36
Skin	0.36	0.40	
Lung	0.18	0.22	
Kidney	0.16	0.23	
Glutathione S-transferase[c]			
Erythrocytes	0.050 ± 0.0004 (4)	0.040 ± 0.005 (4)	0.16
Liver	0.74 ± 0.06 (4)	0.99± 0.13 (3)	0.11
Skin	0.004	0.010	
Lung	0.002	0.010	
Kidney	0.050	0.040	
GSH-Peroxidase[d]			
Liver	1.68 ± 0.15 (4)	1.72 ± 0.19 (3)	0.86
Skin	1.03	1.18	
Lung	1.10	1.55	
Kidney	0.69	0.76	
GSH-Reductase[e]	5.08 ± 0.34 (4)	5.18 ± 0.08 (3)	
Liver	0.18	0.001	0.83
Skin	0.05	0.05	
Lung	0.02	0.07	
Kidney			

[a,b]nmoles/*u*g protein.

[c]nmoles/*u*g protein/minute.

[d]Units/*u*g protein/minute.

[e]nmole activity/min/mg protein.

*Liver and erythrocytes represent analyses of individual samples; all others pooled. Sample sizes in parentheses. Means presented with standard errors.

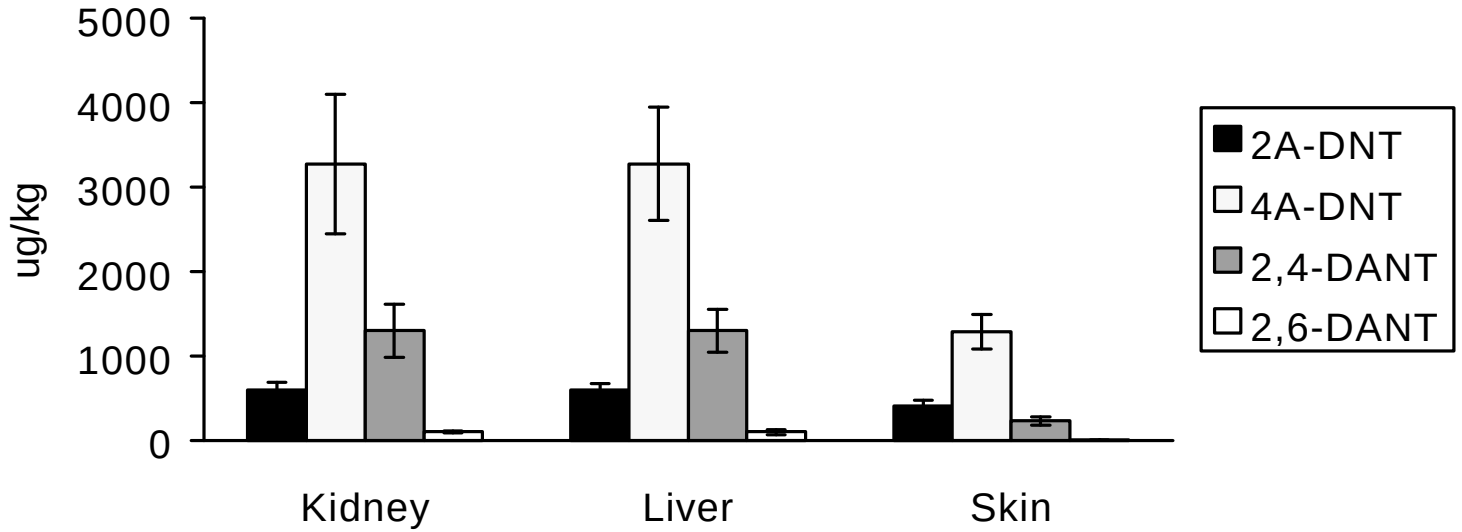


Figure 5.1. Mean metabolite concentrations relative to organ of tiger salamanders (*A. tigrinum*) exposed *in situ* to 2,4,6-trinitrotoluene (TNT) in a soil matrix (dry weight). Metabolites include: 2-amino-dinitrotoluene (2A-DNT), 4-amino-dinitrotoluene (4A-DNT), 2,4-diamino-nitrotoluene (2,4-DANT), and 2,6-diamino-nitrotoluene (2,6-DANT). Bars are standard errors. Concentrations of metabolites in skin were different from liver and kidney (for a complete description of relationships, see text).

CHAPTER 6. An Evaluation of Immune Effects of Oral 2,4,6-Trinitrotoluene (TNT) Exposure to the White-Footed Mouse (*Peromyscus leucopus*).

Mark S. Johnson*[†], Steven D. Holladay[‡], Jennifer W. Ferguson[§], and Wilfred C. McCain[†], [†]U.S. Army Center for Health Promotion and Preventive Medicine, [‡]Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, [§]Oak Ridge Institute of Science and Education.

*Corresponding author: Mark S. Johnson, U.S. Army Center for Health Promotion and Preventive Medicine, 5158 Blackhawk Rd., Attn: MCHB-TS-THE, Toxicology Directorate, Aberdeen Proving Ground, MD 21010-5422

P: 410-671-3980

e-mail: Mark_S._Johnson@chppm-ccmail.apgea.army.mil

6.1: Abstract – We examined indicators of immune toxicity associated with exposure to 2,4,6-trinitrotoluene (TNT) in a 14-day feeding study in white-footed mice (*Peromuscus leucopus*). Equal numbers of each sex (N = 10) were exposed to 0.017, 0.033, 0.066, and 0.264% TNT in feed for 14 days. Based upon average consumption and body weight, these diets resulted in approximate daily doses of 65, 109, 275, and 604 mg TNT /kg bw for males and 70, 143, 282, and 544 mg/kg/d for females. At the end of the exposure period the mice were euthanized and several indicators of non-specific immunity were examined, using the National Toxicology Program (NTP) Tiered approach as a guide. These indicators included primary (thymus) and secondary (spleen) lymphoid organ/body weight ratio, and characterization of non-specific immune responses (phagocytosis and radical oxygen intermediate (ROI) production). No deaths occurred even though the high dose group was equivalent to the reported LD_{50} in Swiss-Webster mice. Spleen weight increased in the high dose group (0.264% TNT) for both sexes, where males, but not females, displayed inhibited splenic macrophage phagocytosis and ROI production. These results illustrate significant species specific differences between laboratory (genus: *Mus*) and Nearctic (genus: *Peromyscus*) mice. In addition, this investigation reports that these immunological indicators appear more sensitive than other toxicological endpoints that have been reported as most descriptive of TNT-related effects in mammals.

Keywords: TNT, immune, *Peromyscus*, phagocytosis, spleen, thymus, hydrogen peroxide

6.2: INTRODUCTION

Since World War I, 2,4,6-trinitrotoluene (TNT) has been the most commonly used high explosive (Hathaway 1977). In the course of TNT production, handling, loading, and ultimate use, there have been many environmental releases (Burrows et al. 1989). Many U.S. Army installations have detectable residues in soil, with reported concentrations of TNT in soil ranging from 0.12 to 38,600 (Walsh and Jenkins 1992) and 0.08 to 64,000 *u*g/g (Hovatter et al. 1997). Consequently, questions regarding the effects from TNT exposure in wildlife have developed.

Effects of oral TNT exposure in laboratory rodents have been well characterized (Dilley et al. 1982, Levine et al. 1984), yet to date, no investigations have characterized these effects in an indigenous wildlife species, nor has a characterization of potential immune effects been conducted in any laboratory animal. Here we report the results of a preliminary evaluation of immune effects, utilizing tests outlined in the NTP protocol (Luster et al. 1988).

6.3: METHODS

6.3.1: Treatments – Each treatment consisted of 10 male and 10 female white-footed mice (Peromyscus Stock Center, University of South Carolina, Columbia, SC). All animals were quarantined for two weeks prior to treatment. Mice were housed in individual stainless wire mesh cages and maintained at 12 hr photoperiod cycles at 17°C and 75-85% relative humidity. Feed and water were provided *ad libitum*.

Treatments were based on the LD_{50} for Swiss-Webster mice (660 mg/kg body weight, Dilley et al. 1982) and results from previous investigations using TNT with *P. leucopus* (McCain et al.

unpub data.). Dosing in the feed began at the reported LD_{50} and included three sequential 50% reductions in concentration to arrive at 4 treatments (i.e., 660, 330, 165, 83 mg TNT/kg bw/d). These doses were viewed as approximate, in that they were based upon intake of *Mus musculus* and previous investigations using TNT in *P. leucopus* (McCain et al. *unpub data*) at 4 gm/d and using an average weight of 20 g/mouse. This resulted in 0.017, 0.033, 0.066, and 0.264% TNT in feed. Feed was weighed on days 0, 1, 3, 7, and 14 to estimate actual dose.

Feed was ground dry mix commerically available (Zeiglar Bros., Gardner, PA) and mixed for 20 minutes using a 12 qt feed mixer (Model A120T, Hobart Corp., Troy, OH) in a 12 qt stainless steel bucket. The TNT was purchased from a re-distilled stock from the U.S. Army Research Laboratory (W. Hillstrom, Aberdeen Proving Ground, MD) and determined to be at least 95% pure through flame ionization detection, gas chromatography (GC). Concentrations in the feed were verified by the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Directorate of Laboratory Sciences and determined through acetylnitrile extraction, flame ionization GC. A 14-day stability study was conducted in feed and found not to significantly change in concentration (Ferguson unpub data). Fresh feed was mixed every week and stored in the dark at room temperature.

At the conclusion of the treatment period, mice were euthanized with CO_2 and necropsied. Initiation of treatment according to sex was staggered for males and females, therefore daily necropsy resulted in random processing (by treatment, but not by sex) of mice on different days. Spleen, thymus, and whole body were weighed to the nearest 0.0001, 0.0001, and 0.01g, respectively. Since weight varied between mice, organ weight and total leucocyte cell counts

(cellularity) were both normalized as a ratio of total body weight. Spleens from half of each treatment (i.e., 5 males and 5 females) were used for the flow cytometric assays. Others were used for histopathological examination.

6.3.2: Immunocharacterization – Tier I bioindicators consisted of pathological examinations of the thymus and spleen, thymus/bw and spleen/bw comparisons, and cellularity enumerations (Luster et al. 1988). Tier II indicators consisted of assays that characterize non-specific immunological function (i.e., phagocytosis and hydrogen peroxide production (H_2O_2)). Other investigations involved the interspecies characterization (staining) with monoclonal antibodies developed in *Mus* species to identify several cell surface markers of interest (e.g., CD-4, CD-8, CD-45R). However, the results of these studies indicated that the commercial monoclonal antibodies developed against Old World *Mus spp*. immune cell surface antigens do not all cross react with *Peromyscus spp*., a New World mouse (data not shown). These same conclusions were reached in a subsequent investigation by Holladay that yielded identical results (unpub. data).

Characterization of splenic phagocyte function was conducted using assays that evaluated phagocytosis and radical oxygen intermediate (ROI) production. Both assays were conducted with splenocytes isolated through mechanical disruption of the spleen on a stainless steel mesh screen into isotonic medium (RPMI, Sigma Chemical, St. Louis, MO). Following two washings at 300G, cells were diluted and counted with a Coulter Z-1 Particle Counter (Coulter Corp., Hialeah, FL).

6.3.3: ROI Production – ROI production was quantified for each cell using an intracellular probe that fluoresces in the FITC range when oxidized by H_2O_2 (Bass et al. 1983). Specifically, the dichlorofluoroscein diacetate (DCF-DA) probe penetrates cell membranes, then becomes trapped within the cell after becoming relatively polar following deacetylation by non-specific esterases (Bass et al. 1983). The deacetylated probe is not fluorescent until oxidized. Briefly, 300 ul of cells standardized to concentrations of 5.0 x 10^5 cells/ml media were added to 7 x 35 mm polypropylene round bottom tubes for each sample. Next, 5 *u*l of 5 mM DCF-DA (Molecular Probes; Eugene, OR) were dispensed into each tube and incubated at room temperature for 15 minutes. Following incubation, 10 *u*l of a 5 nM solution of phorbal myristate acetate (PMA; Sigma Chemicals) were added and the tubes were incubated at room temperature for 30 minutes. After this latter incubation, samples were placed on ice and immediately analyzed using flow cytometry (using the criteria mentioned below). Cell viability was determined through a fluorescent cell exclusion dye (propidium iodide, PI; ICN Pharmaceuticals, Costa Mesa, CA). Only viable cells (i.e., cells that excluded PI) were included in the analysis.

6.3.4: Phagocytosis – This assay was conducted using a method described in Dunn and Tyrer (1981). Phagocytosis was quantified through the fluorescence of yellow-green fluorescent microspheres (Fluoresbrite microspheres, 1.16 mm, Polysciences, Inc. Warrington, PA) ingested by each phagocyte using flow cytometry. Microspheres were washed twice at 1420 G for 15 minutes in RPMI, and then sonicated (Branson Ultrasonic Cleaner, Shelton, CN) for 1 minute to disrupt aggregated microspheres. Cells were washed twice at 300G for 15 minutes, resuspended and standardized to a concentration of 5x10^5 cells/ml. A volume of 300 ul was taken from each cell sample and transferred to polypropylene round bottom tubes. Microspheres were then

added to each sample to achieve a concentration of about 50 beads/cell. An additional 3 mls of media were then added to each tube to prevent acidic media, after which cells were incubated at 37°C and at 5% CO_2 for 60 minutes. Following incubation, cells were washed and analyzed with a Coulter Epics XL Flow Cytometer (Coulter Corp., Hialeah, FL) excited with a 388 nm argon laser and quantified between 500 - 600 nm at the Fluorescein Isothiocyanate (FITC) range. Background fluorescence was determined by an analysis of cells without beads. Further, a solution of only beads was analyzed to determine the light scatter position of undigested beads. Thus, the peak on the fluorescence histogram resulting from cells containing 1 or >1 microspheres could be identified. For each sample, 10,000 events were collected. This technique was standardized using additional animals not in the study. Cell viability was assessed through PI-exclusion as mentioned above.

6.3.5: Histopathology – Spleens and thymuses not used for immunological analyses described herein were trimmed, fixed in formalin, and embedded in paraffin. These tissues were then sectioned at 6 microns, stained with hematoxylin and eosin, and examined via light microscopy. Treatment was not known to the pathologist until after the histopathologic examination was completed.

6.3.6: Statistical Analyses - The data were tested for normality using a Shapiro-Wilk, W-test at the $p < 0.05$ level. If the data failed to fit a normal distribution, they were ranked and analyzed using an ANOVA using the ranks. Normally distributed data were analyzed using an ANOVA then using the Bonferroni's method for a comparison to controls. Similar comparisons for non-parametric data were further analyzed using the Dunnett's method. Statistical significance was

defined at the $p < 0.05$ level. An outlier (determined to be > 3 standard deviations from mean) was removed from the analyses (for phagocytosis). Since half of the spleens were retained for histopathological examination, sample sizes within sex were small. Therefore, to be consistent, means and standard errors of the mean are presented for all data.

6.4: RESULTS

6.4.1: Dose estimate – P. leucopus consumed less food, on average, than 4 g/d. Average consumption was 3.11 ± 0.21g food/d for males and 2.99 ± 0.12 g food/d for females making actual oral dose estimates less than those projected. Mean weight was about 20g (males - $\bar{x}$ = 20.1 ± 0.25g; females - $\bar{x}$ = 19.13 ± 0.27g). Therefore, actual daily dose estimates for males were 604, 275, 109, and 65; and for females was 544, 282, 143, and 70 mg/kg/d for 0.264, 0.066, 0.033, and 0.017%, respectively. No deaths occurred in any treatment of either sex.

6.4.2: Organ/body weights – Spleens of the highest dose were different from controls for both sexes (males, Dunnett's q' = 3.11; females, Bonferroni t = 4.21) suggesting hemolysis and congestion. No response was evident in relative thymus weights in either sex (Fig. 6.1, a & b), suggesting no thymocyte response as a result of TNT exposure.

6.4.3: Cellularity – There were no differences in total cellularity for either spleen or thymus for either sex (Fig. 6.2, a & b; spleens, males $p = 0.33$, females $p = 0.15$; thymus, males $p = 0.18$; females $p = 0.35$) suggesting no obvious TNT-related effects, yet a dose-dependent trend in reduced thymus cellularity was suggested in males.

6.4.4: ROI production – The pattern in DCF oxidation was similar with that for phagocytosis, with a response in males between both high dose groups and controls (Kruskal-Wallis ANOVA on Ranks, Dunnett's $q > 2.5$). No difference was found within females (Fig. 6.3a). All female responses were lower than male controls (Bonferroni's $t > 3.65$ for all comparisons).

6.4.5: Phagocytosis – Since phagocytosis of fluorescent microspheres was primarily unimodal after a 60 minute incubation, peak height was used for statistical comparisons. All treatments were different from controls for males (Bonferroni's $t > 3.13$), but not for females ($p = 0.11$; Fig. 6.3b). Male controls were different from female controls (two-way ANOVA, Bonferroni's $t = 5.72$) as were all other comparisions except the 0.066% group (Bonferroni's $t > 4.11$ for all comparisons; male controls vs female 0.066%, $t = 3.44$).

6.4.6: Histopathology – Mice in the 0.066% and 0.264% TNT groups exhibited an increased accumulation of red blood cells which was noted as congestion (Fig. 6.4). Often associated with congestion was an increased level of extramedullary hematopoiesis (EMH) in the spleen and in the adrenal cortex of a small number of mice (Fig. 6.5). This finding is not uncommon in *Peromyscus ssp.* (D. Young, pers. comm.). No other histological findings were found attributable to exposure to test material.

6.5: DISCUSSION

The immune system has the potential to be a highly sensitive target organ of toxicity from exposure to a variety of xenobiotics. Others have suggested that immunological assays that investigate functional differences in response (such as those presented in this paper) provide

accurate indicators of exposure and may provide populationally-relevant indicators of effect as a result of environmental exposures to contaminants (Weeks et al. 1992, Wester et al. 1994). Weeks and Warinner (1984) found phagocytic activity was inhibited in two species of fish collected from a known PAH-contaminated estuary compared to fish collected of another nearby estuary of reduced industrialization. Weeks et al. (1986) found reduced chemotactic response of macrophages harvested from fish captured from the relatively polluted estuary. This response appeared to recover when these fish were held in the reference area for three weeks.

Laboratory analysis of phagocyte function appears to be sensitive to some environmental contaminants. An organophosphate insecticide (chlorpyrifos) altered macrophage activity at exposure levels not associated with other signs of toxicity in fish (1 ppb in water; Holladay et al. 1996). In addition to inhibiting phagocyte activity, chlorpyrifos reduced total leucocyte counts in the fish pronephros. Two polycyclic aromatic hydrocarbon (PAH) agents (7,12-dimethylbenzanthracene and benzo[a] pyrene) inhibited phagocyte function, but only at levels associated with signs of systemic toxicity in fish (Hart et al. 1998). Roszell and Anderson (1994) found that phagocytosis and superoxide generation were inhibited from exposure to 5 mg/l and 10 mg/l, respectively, in phagocytes isolated from the pronephos of *Fundulus heteroclitus* exposed to pentachlorophenol *in vitro*.

Effects reported previously from oral TNT exposures suggest that the blood is the primary target organ (Dilley et al. 1982, Levine et al. 1990, Hathaway 1977). The gross indicators of anemia in our report were consistent with that of others (e.g., splenomegaly) (Dilley et al. 1982, Levine et al. 1990). Histological examination of the spleen also indicated TNT-related effects. This was

found in both sexes. However, a dose-related decrease in H_2O_2 production was observed in male mice only; phagocytosis appeared to be affected at all treatments in males.

Any conclusions made from the sexual differences in non-specific immune response must be considered preliminary. Firstly, sexes were analyzed on separate days and therefore the possibility exists that differences may be due to slight variances in assay preparation. Also, sample sizes were small (N = 5) for each treatment, decreasing the power of the statistical comparisons. Others have measured sexual differences in response (e.g., spleen weight) in mice, rats, and dogs exposed orally to TNT (Dilley et al. 1982, Levine et al. 1990). Moreover, sexual differences in immune response have been recorded in many species (Saad and Shoukrey 1988, Konstadoulakis et al. 1995, Fox 1995, Spitzer and Zhang 1996) and have been attributed to differences in endocrine function possibly as a result of sexual selective pressures (Zuk and McKean 1996).

Overall, *P. leucopus* appeared more tolerant to oral TNT exposures than were other species of rodents tested previously (Dilley et al. 1982, Levine et al. 1990). In particular, the present high dose was roughly equivalent to the LD_{50} in Swiss-Webster mice (Dilley et al. 1982) but had limited effect in *P. leucopus*.

This investigation presents evidence that the immune system may be adversely affected from TNT exposure, and as such may provide an indicator of effect from exposure, either through direct or indirect mechanisms. Clearly future work including specific hematopoetic, hematolytic, and hepatotoxic indicators should be compared to these data for a more accurate characterization.

Our data are also consistent with evaluations of non-specific immunological characterization of in vitro splenic phagocytes as a result of exposure to TNT metabolites (Thierfelder and Masihi 1995) and in other non-mammalian assays (Johnson unpub. data). Pidemsky et al. (1968) reported that rats given 30 mg TNT/kg body weight daily for 6 days exhibited progressively decreased phagocytosis, while an 1mg/kg niacin subcutaneous injection resulted in an 50% increase in phagocytic activity relative to controls. However, these reports, among others, provide a profile that illustrates the considerable variation in response of organisms to TNT exposure (Dacre and Rosenblatt 1974). This report adds to the knowledge of significant species-specific as well as individual variation of TNT toxicity in mammals.

6.6: ACKNOWLEDGEMENTS

We thank Warren Hillstrom from the U.S. Army Research Laboratory for the TNT, Patricia Beall for technical support, George Parker for the histopathology, and T. V. Reddy and B. Daniels for encouragement. This work was funded by the U.S. Environmental Protection Agency.

6.7: REFERENCES

Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J immuno* 130: 1910-1917.

Burrows EP, Rosenblatt DH, Mitchell WR, Parmer DL. 1989. Organic explosives and related compounds: environmental and health consideration. TR-8901 U.S. Army Biomedical Research & Development Laboratory, Ft. Detrick, Frederick, MD.

Dacre JC, Rosenblatt DH. 1974. Mammalian toxicology and toxicity to aquatic organisms of four important types of waterborne munitions pollutants – an extensive literature evaluation. AD 778725. Technical Report 7403. U.S. Army Medical Bioengineering Research and Development Laboratory, Aberdeen Proving Ground, MD, USA.

Dilley JV, Tyson CA, Spanggord RJ, Sasmore DP, Newell GW, Dacre JC. 1982. Short-term oral toxicity of 2,4,6-trinitrotoluene in mice, rats, and dogs. *J Toxicol Environ Health* 9: 565-585.

Dunn PA, Tyrer HW. 1981, Quantitation of neutrophil phagocytosis, using fluorescent latex beads. *J Lab Clin Med* 98: 374

Fox HS. 1995. Sex steriods and the immune system. *Ciba Found Symp* 191: 203-211.

Hart LJ, Smith SA, Robertson JL, Smith BJ, Besteman E, Holladay SD. 1998. Subacute immunotoxic effects of 7,12-dimethylbenzanthracene on leukocyte number and macrophage activity in spleen and pronephros of tilapia (*Oreochromis niloticus*). *Aquatic Toxicol* 41: 17-19.

Hathaway JA. 1977. Trinitrotoluene: a review of reported dose-related effects providing documentation for a workplace standard. *J Occup Med* 19: 341-345.

Holladay SD, Smith SA, El-Habback H, Caceci T. 1996. The influence of chlorpyrifos, an organophosphate insecticide, on the immune system of tilapia (*Oreochromis niloticus*). *Aquatic Toxicol* 41: 17-19.

Hovatter PS, Talmage SS, Opresko DM, Ross RH. 1997. Ecotoxicity of nitroaromatics to aquatic and terrestrial species at army superfund sites. Pp. 117-129 *in* Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment. Sixth Vol. (T. R. Doane and M. L. Hinman, Eds.) American Society for Teating and Materials.

Konstadoulakis MM, Syrigos KN, Baxevanis CN, Syrigou EI, Papamichail M, Peveretos P, Anapliotou M, Golematis BC. 1995. Effect of testosterone administeration, pre- and postnatally, on the immune system of rats. *Horm Metab Res* 27: 275-278.

Levine BS, Furedi EM, Gordon DE, Lish PM, Barkley JJ. 1984. Toxic interactions of munition compounds TNT and RDX in F344 rats. *Fund Appl Toxicol* 15: 373-380.

Levine BS, Rust JS, Barkley JJ, Furedi EM, Lish PM. 1990. Six-month oral toxicity study of trinitrotoluene in beagle dogs. *Toxicology* 63: 233-244.

Luster MI, Munson AE, Thomas PT, Holsapple MP, Fenters JD, White KL Jr, Lauer LD, Germolec DR, Rosenthal GJ, Dean JH. Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Programs's guidelines for immunotoxicity in mice. *Fund Appl Toxicol* 10: 2-19.

Pidemsky EI, Chukicher EM, Mul'menko. 1968. Niacin action of phagocytosis in chronic trinitrotoluene poisoning. *Farmakol Toksikol* (Moscow) 31: 365-366 *in* Dacre and Rosenblatt 1974.

Roszell LE, Anderson RS. 1994. Inhibition of phagocytosis and superoxide production by pentachlorophenol in two leukocyte subpopulations from *Fundulus heteroclitus. Marine Environ Res* 38: 195-206.

Saad AH, Shoukrey N. 1988. Sexual dimorphism on the immune responses of the snake, *Psammophis sibilans*. *Immunobiology* 177: 404-419.

Spitzer JA, Zhang P. 1996. Gender differences in neutrophil function and cytokine-induced neutrophil chemoattractant generation in endotoxic rats. *Inflammation* 20: 485-498.

Thierfelder W, Masihi KN. 1995. Effects of trinitrotoluene (TNT) metabolites on chemiluminescence response of phagocytic cells. *Int J Immunopharmac* 17: 453-456.

Walsh ME, Jenkins TF. 1992. Identification of TNT transformation products in soil. Special Report No. 92-16, U.S. Army Corps of Engineers, Cold Regions Research and Engineering Laboratory.

Weeks, BA, Anderson DP, DuFour AP, Fairbrother A, Goven AJ, Lahvis GP, Peters G. 1992. Immunological biomarkers to assess environmental stress. In Huggett RJ, Kimerle RA, Mehrle PM Jr, Bergman HL, eds, Biomarkers Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis Publishers, Boca Raton, LA, USA, pp 211-336.

Weeks BA, Warinner JE. 1984. Effects of toxic chemicals on macrophage phagocytosis in two estuarine fishes. *Marine Environ Res* 14: 327-335.

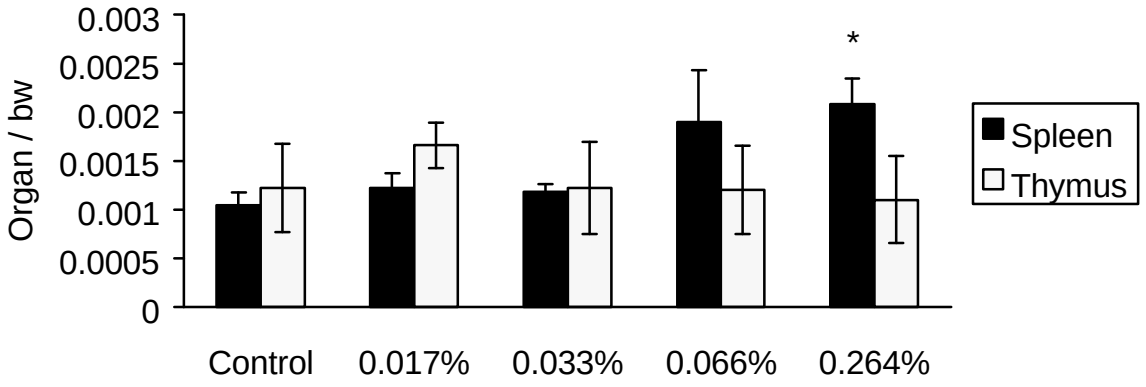
Weeks BA, Warinner JE, Mason PL, McGinnis DS. 1986. Influence of toxic chemicals on the chemotactic response of fish macrophages. *J Fish Biol* 28:653-658.

Wester PW, Vethaak AD, van Muiswinkel WB. 1994. Fish as biomarkers in immunotoxicology. *Toxicol* 86:213-232.

Zitting A, Szumanska G, Nickels J, and Savolainen H. 1982. Acute toxic effects of trinitrotoluene on rat brain, liver, and kidney: the role of radical production. *Arch Toxicol* 51:53-64.

Zuk M, McKean KA. 1996. Sex differences in parasite infections: patterns and processes. *Int J Parasitol* 26: 1009-1023.

A



B

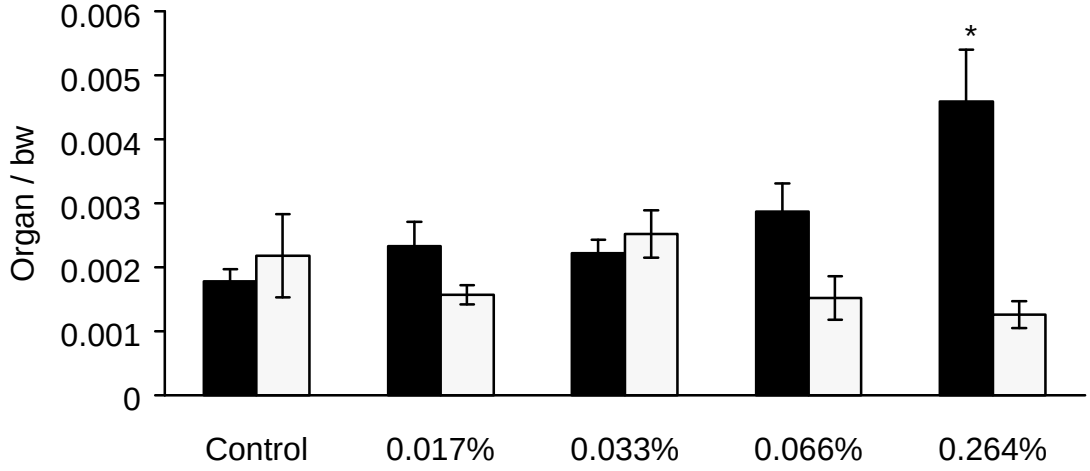
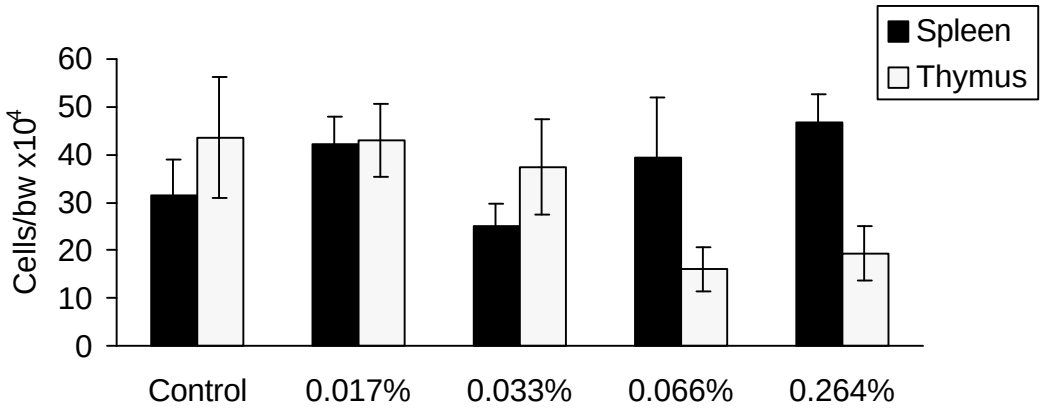


Figure 6.1. Thymus/body weight and spleen/body weight comparisons for male (A) and female (B) *P. leucopus* exposed to 0.017, 0.033, 0.066, and 0.264% TNT. Means are different at $p < 0.05$ from controls noted by asterisk; bars are SEMs.

A



B

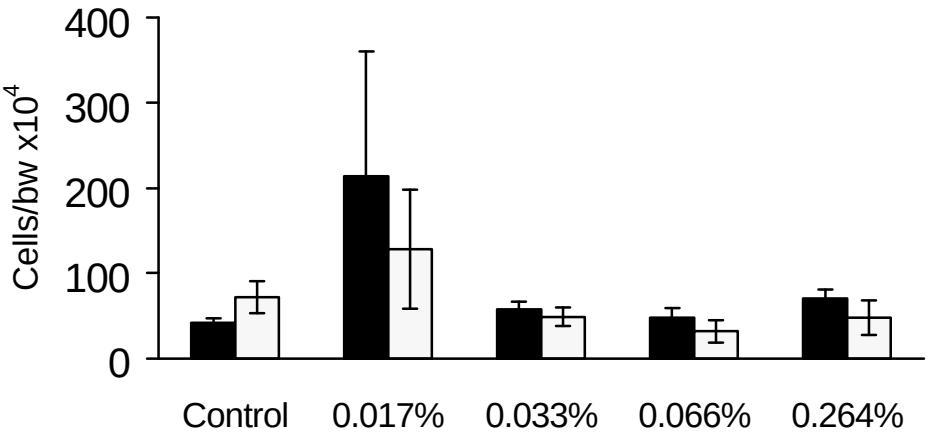
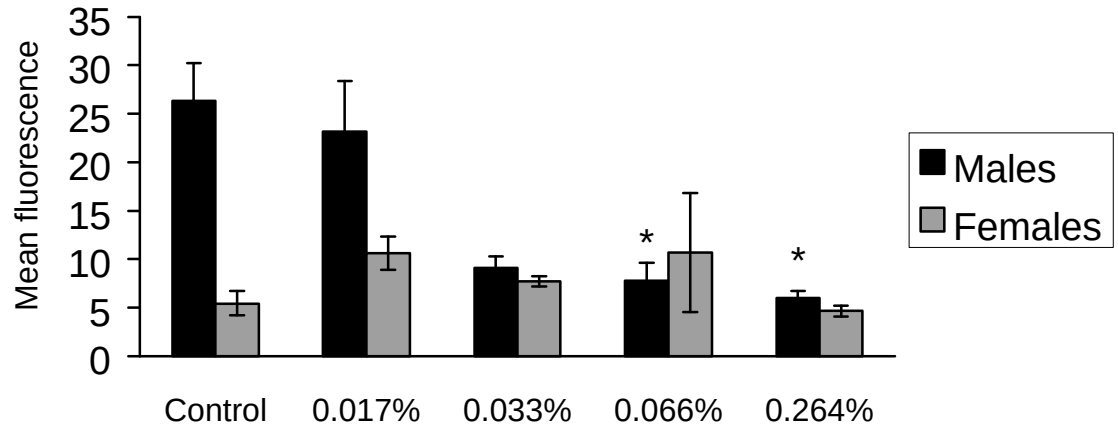


Figure 6.2. Ratio of cellularity of thymus and spleen to body weight in male (A) and female (B) *P. leucopus* exposed to 0.017, 0.033, 0.066, and 0.264% TNT. Total cells divided by body weight. Means presented; bars are SEMs.

A



B

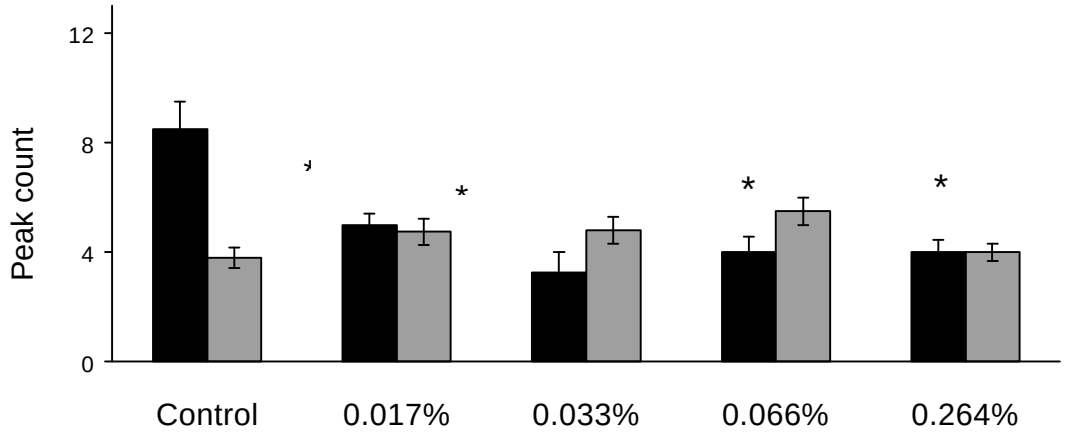
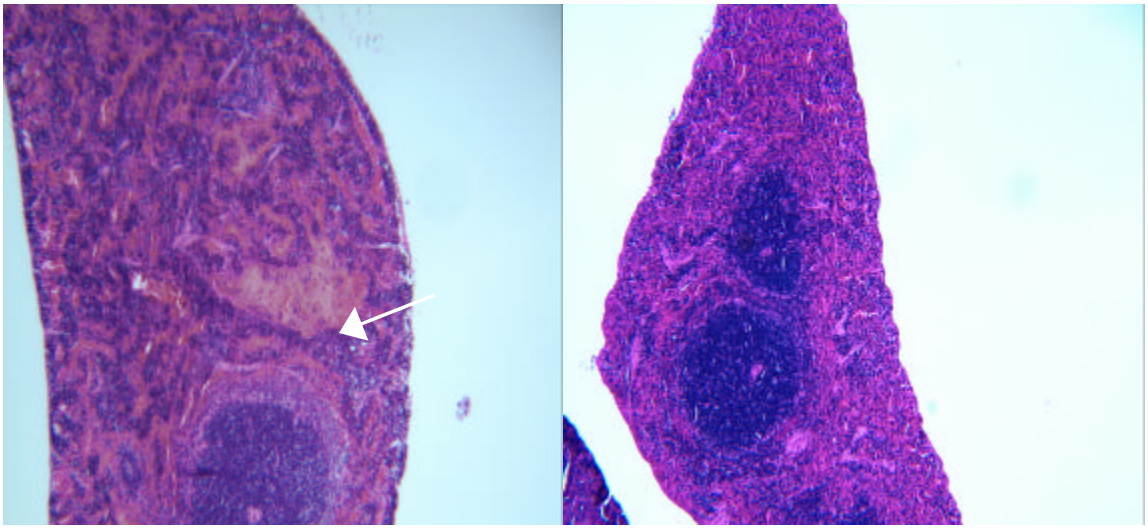


Figure 6.3. Analysis of non-specific immune response through: A) intracellular DCF oxidation within *P. leucopus* splenic phagocytes quantified through flow cytometry; and, B) phagocytosis of fluorescent microspheres by *P. leucopus* splenic phagocytes after an 1-hour incubation relative to treatment. Means presented; bars are SEMs. Means with different superscripts are different at $p < 0.05$. Only within sex comparisons shown.

A B



C

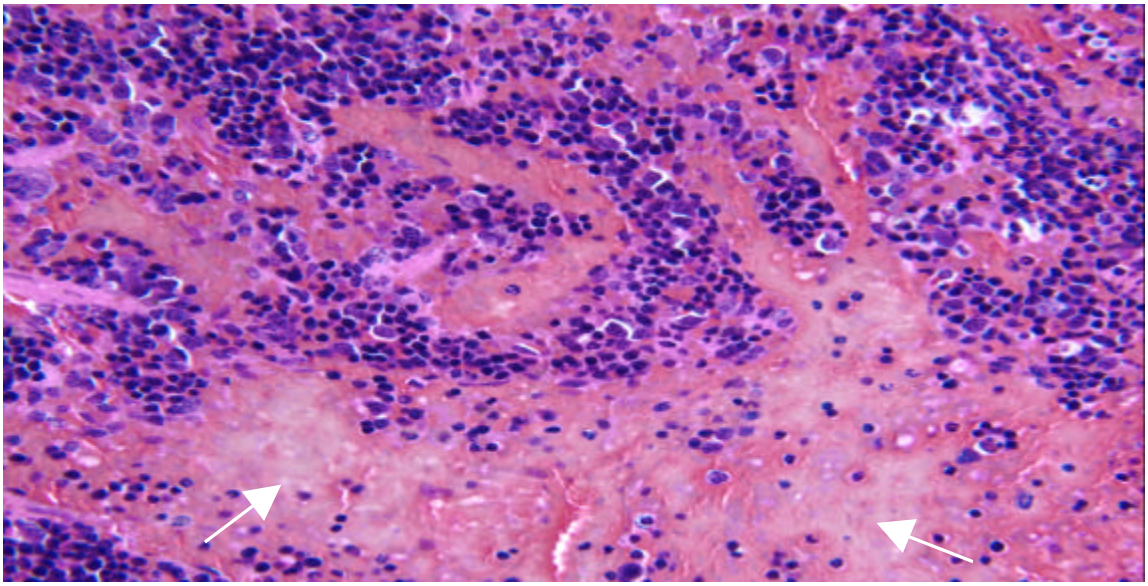


Figure 6.4. Spleens of *P. leucopus* exposed 14 days to 0.264% TNT (A) in feed and of control (B) mice. Areas of increased blood volume noted as congestion indicated by arrows. High power view of congested areas (C) 240x; H&E stained.

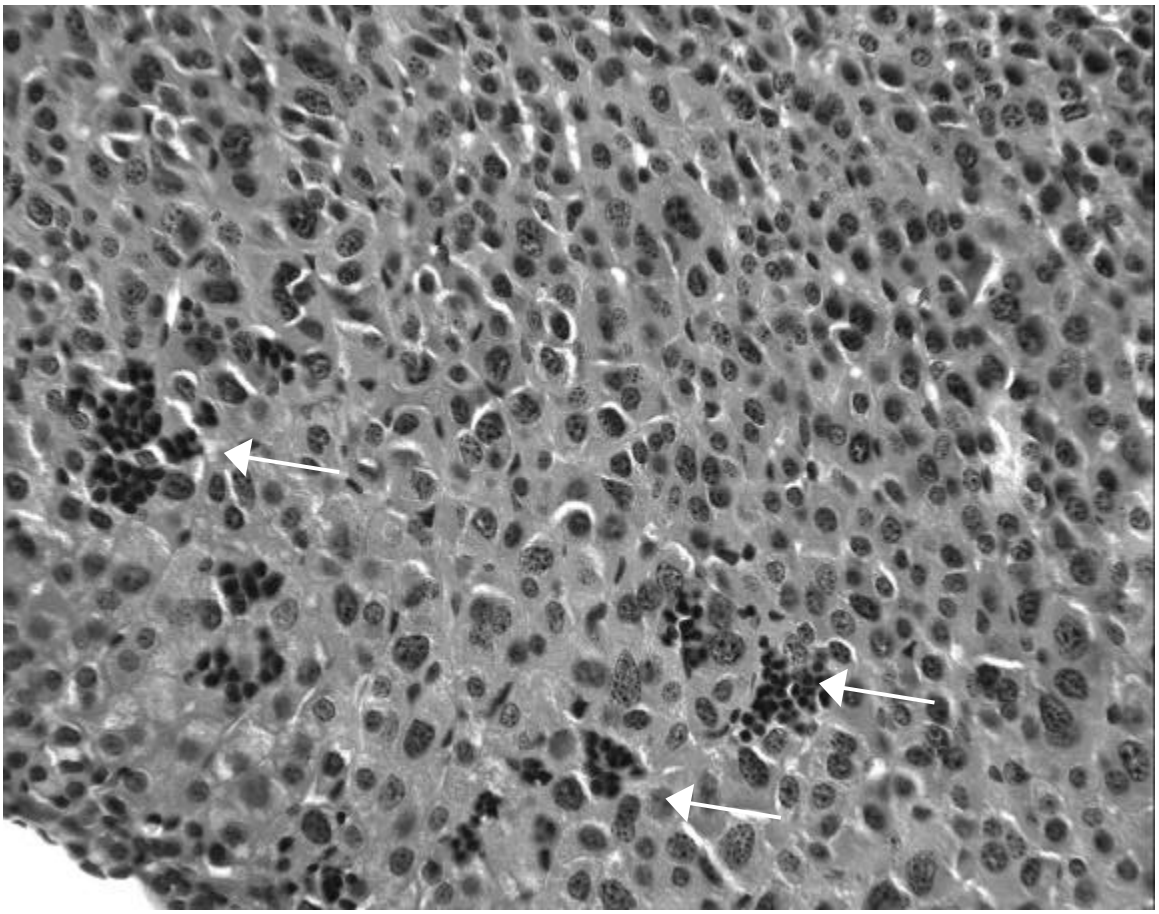


Figure 6.5. Extramedullary hematopoesis (EMH) in the adrenals of *Peromuscus leucopus* exposed to 0.264% TNT in feed for 14 days. Stained in H&E; 180x.

CHAPTER 7 CONCLUSIONS

Exploring the impacts of anthropogenuc compounds in the environment to wildlife is a relatively recent science. Increased public opinion arising from issues raised by Rachel Carson, among others, have contributed to the increased awareness concerning xenobiotics in the environment. The issues of the food web transport of DDE and the resulting toxicity to higher trophic level raptors have been well published and the evidence is convincing. As the Peregrine Falcon was an indicator species of DDE exposure, many are concerned over the relatively recent perceptions of amphibian population declines.

Few ennvironmental impact studies have focused on terrestrial amphibians. Fewer still have used salamanders in toxicological studies. This work has provided important baseline information for the further development of salamanders in toxicological evaluations of xenobiotics in soil.

Dermal exposures were found to be significant in Ambystomid salamanders from TNT in soil. Moreover, concentrations of the primary reduction metabolites of TNT were found to bioaccumulate, consistent with the saturation of these reductive processes. However, exposure to TNT at the dose level and the duration tested did not produce any apparent toxicity. The information gained through the identification and enumeration of the hematological parameters and histological examination of the liver provided important information not previously published. Further, the investigations of enzyme activity and content (i.e., p450, b5, and glutathione antioxidant system) provided needed baseline information in which to develop further research. The tissue-specific analysis of the biotransformation of TNT suggests that the skin may be an important first contact organ that assists in the metabolism (reduction) of TNT. In addition, the activity of the isozymes investigated suggest that the selective effects of life history characteristics (i.e., fossorial) may be more important than endothermia in determining metabolic capasity.

The studies of immune effects in *P. leucopus* provided needed data for sensitive indicators of stress from exposure to TNT as a potential biomarker of effect. In terms of lethality, *P. leucopus* appeared to be more resistant from oral exposures to TNT than the laboratory rodent species

previously tested. However, for males, effects were seen in immune activity at levels below the highest doses of TNT used. No functional immune effects were observed in females, yet the spleens of both sexes were larger at the highest dose (0.264% TNT in feed). This may have been the result splenic congestion caused by lysed erythrocytes. Histopathological examination of the spleen supported that conclusion. A review of the literature indicated that this is the first report of potential immune effects of TNT exposure in a mammal. These specific functional assays (i.e., relative phagocytosis activity and ROI production) may therefore show promise as a sensitive bioindicator of effect in *P. leucopus* males from exposure to TNT. Further development will be necessary to determine the applicability of these endpoints in a field situation.

Many important issues are and will most likely remain unresolved in ecological risk assessment. As is often the case, the present work has demonstrated that many more questions are created when we attempt to initially address even a limited, well-defined set of endpoints. The demostration that Ambystomid salamanders accumulate a significant amount of TNT through the skin raises questions about other contaminants in soil that have similar structure and physical properties. Specifically, dermal exposures are not addressed in ecological risk assessments, nor are inhalation exposures that have the potential to be significant, particularly to burrowing fauna (in functionally confined spaces).

This work investigated a holistic exposure regime in which a terrestrial amphibian was exposed to a xenobiotic in a soil matrix. An attempt was made to address not only bioaccumulation, but also functional and biochemical alterations in salamanders caused by exposure to similar concentrations. Endpoints were identified which, with further development and modification, may have potential for extension to a field application to assess ecological risk. Indeed, based on these studies a propsal has been submitted to extend the present observations into a smaller, more available salamander species *Plethodon cinereus* (Red-backed Salamander) (Gunnison et al. *submitted*). Additional proposals include an analysis of the fate and effect of TNT in the environment utilizing radioactive-labeled TNT in two soil types and investigating the presence of labeled compound in exposed salamanders.

Testing of the type we have done and propose to continue may be of direct use in the U.S. EPA Ecological Soil Screening Level (ESSL) Initiative. This specific project is working toward the development of soil screening values that are protective of wildlife for Superfund sites. One of the top 20 substances to be investigated is TNT; thus it is hoped that the present work may be useful in that effort particularly in the exposure modeling for amphibians.

Traditional toxicity testing (i.e., feeding study) also yielded a potential for a new biomarker of effect in an important mammalian prey species, *P. leucopus*. The differences observed between the sexes in non-specific immune activity may provide a base from which to further develop research directed toward biomarkers of effect. Further calibration of such biomarkers may provide indicators specific to important ecological parameters resulting from TNT exposure in the soil, including effects important to the population and the community at large. Currently, extrapolations of individual-based toxicity information are rarely conducted in relation to populational or community-level effects. This is due to a lack of data permitting such extrapolations, yet potential effects are often inferred. It is hoped that the present work may provide the seed that encourages toxicity testing with ecologically-relevant criteria mind.

Many difficulties were associated with the development of a relatively new animal model to investigate ecotoxicological endpoints. This proved trying, particularly since funding was distributed on an annual basis. Animal availability varied due to field conditions and a host of abiotic and biotic factors. This was coupled with the *a priori* constraints of doing non-traditional animal research at our laboratory designed for rodent and lagomorph species. All things considered, this effort was worthwhile in that it enhanced the knowledge base of a species where few specifics of this kind were initially available.

Hindsight related to this project will become valuable in developing future efforts. Larger sample sizes and long-term exposures would be two primary considerations in such development. In addition, the use of a substance that was less reactive with the soil (to allow for a dose-response assay to be conducted) would seem to be axiomatic. However, funding constraints and animal availability dictated such criteria in the past. Investigations concerning mechanisms and toxicokinetics of TNT in salamanders need to be addressed, yet it may be that

only collaborative efforts involving laboratories with appropriate expertise can accomplish such initiatives.

The present research suggests that additional ecologically-relevant endpoints (e.g., immune function) need to be considered in toxicity studies that investigate toxic effects associated with environmental chemical exposure for use in ecological risk assessments. This is an example of a prototype that considers such endpoints and a comprehensive exposure regime. However, other ecologically-relevant interactions are required to make these physiological data useful. Food advoidance, behavioral (e.g., mate recognition, predator avoidance, etc.), reproductive, and developmental endpoints should be investigated before any risk evaluation is complete. This work has provided some of the baseline experience needed to develop a program to investigate the relevance of a vertebrate model to address toxicity of xenobiotics in a soil matrix.

MARK STEVEN JOHNSON

WORK: United States Army Center for Health Promotion and Preventive Medicine
Health Effects Research Program
Aberdeen Proving Gnd. MD 21010-5422
E-MAIL: Mark.Johnson@amedd.army.mil

HOME: 3204 Bryson Ct.
Baldwin MD 21013
E-MAIL: piranga@bellatlantic.net

QUALIFICATIONS SUMMARY

Current Position: Research Biologist, Army Center for Health Promotion and Preventive Medicine, Health Effects Research Program. Sitting advisor on the Army's Biological Technical Assistance Group (BTAG) and Tri-ServiceEcological Risk Assessment Working Group (TSERAWG); member of the Task I Group (Toxicity Profile and Toxicity Reference Value Development) of the U.S. EPA Ecological Soil Screening Level Initiative. Current position includes conducting research on improving eco-risk methodology including site-specific evaluations, Installation Restoration Program review / providing guidance and comments on Risk Assessments, consulatory toxicological services involving human health and wildlife. Current projects include derivation of 1-hour acute inhalation values for soldiers and the general population. Position involves presenting research at national meetings and publishing results. Supervisor Glenn Leach, PhD DABT.

Research Experience - Recent research concentrates on the improvement of the current methodologies used in Ecological Risk Assessments. Projects currently underway involve the development of a conservative, secondary consumer model for subsequent use in ERAs. This research involves assessing the proportional contribution to bioaccumulation of two pathways (dermal and ingestion) of a predatory salamander: *Ambystoma tigrinum*. This effort also includes analysis of immunological response to exposure and proposed subsequent field validation of response. Previous work involved assessing reproductive performance, estimation of the bioaccumulation through modeling and confirmation of model and development of variation estimates, and integrated density estimation techniques for avian and amphibian representatives at J-Field, APGEA. Other work involves modeling indirect exposures of chemical agent incineration for RCRA compliance, and developing food web models in Risk Assessment, including developing Toxicity Reference Values for use in ecological and human risk assessments. Further, recent research concentrates on developing a battery of non-animal tests (e.g., cell culture, stress gene induction, electron transport, etc.) for complex environmental mixtures in assessing relative toxicity, and the development of alternative methods for the evaluation of immunotoxicity using flow cytometry.

Field Techniques – Experienced in aural and visual amphibian surveys. Mist-netting and banding birds, censusing birds (both visually and aurally), locating and monitoring nests, spot mapping and evaluating reproductive potential, and conducting vegetation/habitat surveys. Certified in field identification of breeding avifauna in compliance with the Maryland Dept. of Natural Resources, Critical Areas Commission to conduct and assess avian censusing in Maryland. Master permitted bird bander with the NBS/USF&WS. Experienced in conducting amphibian sensusing techniques including aural indentification techniques.

Computer Skills - Experienced in word processing, graphics, and statistical analysis using a variety of Microsoft products (Word 7.0, Excel, Access, Powerpoint), Word Perfect 6.1, Lotus 123 ver. 5.0, Cricket Graph- Macintosh, and Statistical Analysis System (SAS). Experience with PICO, EMACS, X and K-edit main frame editors, CMS and UNIX operating systems. Experienced in moderate level Basic programming skills and in internet communications including hypertext environments (World Wide Web). Most familiar in Windows/IBM environments.

Pertinent Courses - Reproductive and Developmental Toxicology, Biochemistry, Advanced Toxicology, Principles of Toxicology, Pharmacology and Toxicology Testing, Veterinary Histology, Ornithology, General Ecology, Population Ecology, Insect Taxonomy, Mammalogy, Topics in Wildlife Conservation, Insect Ecology, Insect Physiology, Herpetology, Research Methods, Ecology Seminar, Comparative Animal Physiology, Botany, Flow Cytometry, Risk Assessment; and HAZWOPER (40 hr) trained.

EDUCATION

Virginia Polytechnic Institute and State University, Virginia-Maryland College of Veterinary Medicine, PhD candidate. Expected graduation: Dec 98.
Advisor: Dr. Steven D. Holladay

University of Delaware, Department of Entomology and Applied Ecology.
Master of Science in Entomology. Grade Point Average = 3.81. December 1993.
Thesis Title: The Effects of Age and Habitat on the Reproductive Performance of Wood Thrushes (*Hylocichla mustelina*). Research Assistant: 1993-1994, Teaching Assistant for Avian Taxonomy and Insect Taxonomy., 1992-1993.
Advisor: Dr. Roland R. Roth

University of Delaware, Masters of Science in Entomology and Applied Ecology awarded in May 1994.

Towson State University, B. S. in Biology (Zoology concentration). (GPA = 3.79), 1991.

Harford Comm. College, Associate of Arts, 101 credits (GPA = 3.8) 1984.

CONTINUING EDUCATION

Currently PhD student (candidate) of the Virginia Polytechnic Institute and State University, Maryland-Virginia Regional College of Veterinary Medicine. Research involves those mentioned previously. Coursework involved the following:
Amphibian Pathology (Independant Study):Virginia Tech; J. Robertson, Dec 1997.
Veterinary Histology: Virginia Tech; T. Caceci, Dec. 1997.
Laboratory Animal Management: Virginia Tech; D. Moore, May 1997.
Biochemistry I,II: Virginia Tech; W. McCain, Dec. 1996, May 1997.
Pharmacology and Toxicology Testing: Virginia Tech; M. Ehrich.
Priniples of Toxicology: University of Maryland, Baltimore; K. Squibb, May 1996.
Advanced Toxicology: University of Maryland, Baltimore; K. Squibb, December 1995.
Immunology: Towson State University, B. Masters, December 1995.

Reproductive and Developmental Toxicology: University of Maryland, Baltimore; C. Kimmel and G. Kimmel, May 1995.

Risk Assessment Training: Navy Environmental Health Center, Environmental Programs Directorate, TRC Environmental Corp., March 1994.

40-Hour Basic Health and Safety Training (HAZWOPER): University of Pittsburg Applied Research Center, Center for Hazardous Materials Research, August 1994.\

Flow Cytometry: Coulter Epics XL conducted September 23 - 27, 1996. Miami, Fla.

Risk Communication, CHPPM, Edgewood, MD, Nov 4-6, 1998.

RESEARCH EXPERIENCE

Ecologist/Toxicologist Internship - Reviewed draft documents (e.g., work plans, Remedial Investigations, Feasibility Studies) for DOD. Completed food chain model for indirect exposures related to agent incineration. Assisted in toxicological profiles and advisories for the army relating to human and ecological risk assessment.

These profiles have involved benchmark formulation (Toxicity Reference Values) for screening purposes. I serve as an advisory member of the BTAG group (Biological and Technical Assistance Group) for the Army and part of a Tri-Services Ecological Working Group. Conducted peliminary qualitative ecological risk assessments and provided assistance in remediation concerns. Currently developing techniques to identify bioindicators of soil exposure and effects of military unique compounds to amphibians and other vertebrates. Conducted terrestrial ecological risk assessment of J-Field, Aberdeen Proving Ground, Maryland, where a holistic, integrated approach was used. A similar approach is being used to address military-related effects to amphibians at Twin Cities Army Ammunition Plant. Also conducted qualitative ecological risk assessments for the Presidio, San Francisco, California. Served as an Army consultant in regards to ERAs at Dugway and Tooele Army Depot, Louisania Army Ammunition Plant, and Lone Star Army Installation..

Research Assistant - Tested the relative effects of age and habitat on the reproductive success of Wood Thrush. Evaluated the effect of reproductive success on age structure within habitat types. Tested reproductive success and age of owners with degree of vegetational nest concealment and nest defense to an avian predator. Monitored nests, color banded owners and nestlings at the University of Delaware Woodlot at the Agricultural Farm in Newark, DE. 1991-1993.

Neotropical Research: Central America - Conducted mist-netting and banding activities, identified, aged, and measured mass and wingcord of Neotropic migrant and resident bird species, and assisted in vegetational surveys and species counts for U.S.F.& W.S. Supervisors: Chandler Robbins and Barbara Dowell. Belize, January 1990, 1991; Guatemala, February 1992, 1993.

Winter Bird Research - Patuxtent Wildlife Research Center: Collected data, banded and measured passerines captured at P.W.R.C. for ongoing research involving population trends, return rates, and survivorship of winter birds. Supervisor: Kathleen Klimkiewicz. November - April, 1991-1993.

Further Banding Activities - Irvine Natural Science Center: Conducted banding activities during migration and during the breeding season and as part of the M.A.P.s project (Monitoring Avian Productivity). Supervisor: Barbara Ross, 1990 - Present. Granted master permit through NBS.

Ecological Consultant - Biohabitats Inc.: Conducted forest interior breeding bird surveys in compliance with the Critical Areas Commission of the Chesapeake Bay. Conducted Bald Eagle breeding assessments in the Perryman area. Completed three publications associated with these surveys, available for public record through Biohabitats Inc. 1991-1992.

PUBLICATIONS

Johnson, M. S., and R. R. Roth. (*submitted July 98)* The Effects of Age and Habitat on the Reproductive Performance of Wood Thrush (*Hylocichla mustelina*). Auk.

Johnson, M. S., L. S. Franke, R. B., Lee, and S. D. Holladay. 1998. Bioaccumulation of 2,4,6-trinitrotoluene (TNT) and PCBs through Two Routes of Exposure in a Terrestrial Amphibian: Is the Dermal Route Significant? Environmental Toxicology and Chemistry (*accepted May 1998)*.

Johnson, M. S. 1997. The effect of age on nest concealment and its complimentary effect on production of Wood Thrush. Wilson Bull. 109(1): 66-73.

McCain, W. C., R. Lee, M. S. Johnson, J. E. Whaley, J. W. Ferguson, P. Beall, and G. Leach. 1997. Acute oral toxicity of pyridostigmine bromide, permethrin, and DEET in the laboratory rat. J. Toxicol. Environ. Health, 50: 113-124.

Johnson, M. S., and G. J. Leach. 1997. Selection and Rationale for the Determination of Acute Reference Values Protective of the General Population from Inhalation Exposures to Products of Incomplete Combustion. Toxicol. Study No. 87-1402-97. USACHPPM, Feb-July.

R. R. Roth, M. S. Johnson and T. J. Underwood. 1996. Wood Thrush (*Hylocichla mustelina*). In The Birds of North America, No. 256 (A. Poole and F. Gill, Eds.) The Academy of Natural Sciences, Philadelphia, PA, and The American Ornithologists' Union, Washington D.C.

Johnson, M. S. 1995. An early ovenbird nest for Maryland. Maryland Birdlife, 51: 3-4.

Johnson, M. S., L. S. Franke, W. C. McCain, and J. E. Whaley. 1995. Ecological effects of anthropogenic contamination to amphibians and birds at J-field, Aberdeen Proving Ground, Maryland. U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Health Effects Research Study No. 87-23-3250-96, October 1995.

Johnson, M. S., and G. L. Leach. 1994. Determination of acute inhalation risk values concerning products of incomplete chemical warfare incineration. USACHPPM, Health Effects Research Program No. 87-51-1399-95, Dec. 1994.

Johnson, M. S. 1994. Assessing the risk of volatile organic compounds and metal uptake in game animals. Final Report, Toxicology Study No. 75-51-Y176-94, USAEHA, May 1994.

Chesapeake Bay Critical Area Forest Interior Dwelling Bird Survey for the Sharon Alignment/Perryman Pipeline Project. August 1992. B.G.&E. Publication prepared for by Biohabitats Inc.

Impact Assessment of the Proposed Power Plant at Perryman as it Relates to the Status of the Bald Eagle *(Haliaeetus leucocephalus)*. March 1992. B.G&E. Publication prepared for Biohabitats Inc.

AWARDS AND HONORS

Received the following awards:

Compton Crook Memorial Award : (Outstanding Junior in Biology) from Towson State University as an undergraduate, 1990.

James Mondiodis Scholarship: awarded from TSU as an undergraduate, 1991.

Deans Merit Scholarship: awarded from TSU as an undergraduate, 1991.

Lois O'Dell Award: (Outstanding Senior in Biology) from Towson State University, 1992.

BLOC Tuition Scholarship: awarded as a incoming graduate student at University of Delaware, 1992.

Teaching Assistantship: Department of Entomology and Applied Ecology, University of Delaware, 1992.

Research Assistantship: Department of Entomology and Applied Ecology, University of Delaware, 1993.

American College of Toxicology Travel Award: 1998 Annual Meeting, 9 November, 1998, Orlando, Fl.

ORGANIZATIONS AND AFFILIATIONS

Society of Environmental Toxicology and Chemistry: Student member 1995-present.

Maryland Ornithological Society, Harford Chapter: State Director, Chapter Sanctuary Representative, and member of Field Trip Committee, 1991-1997; President Harford Co. Chapter 1995-1997.

Partners in Flight, Maryland Dept. Natural Resources: member Research Committee; involved in prioritizing research activities relative to the concerns of recent declines in Neotropical migrants. Duties include peer review of research proposals, providing consultation to land managers, and defining research needs in conservation.

Eastern Bird Banding Association: Councilor, 1992-1995, member 1990 to 1995.

American Ornithologists' Union: member 1991 to present.

Association of Field Ornithologists: member 1991-1993.

Wilson Ornithological Society: member 1991 to present.

Beta Beta Beta Biological Honor Society: member 1991 - present.

Alpha Sigma Lambda Honor Society: member 1991 - present.

SYMPOSIA AND POSTERS PRESENTED

Symposia on Environmental Contaminants and Terrestrial Vertebrates: Effects on Populations, Communities, and Ecosystems. (SETAC). An In Situ Method to Investigate Exposures to a Terrestrial Vertebrate: A Holistic Exposure Regime using *Ambystoma tigrinum*. Oct 19-21, 1998. University of Maryland, MD.

Society of Environmental Toxicology and Chemistry (SETAC) 18th Annual Meeting, Nov. 1997. Bioaccumulation of TNT and PCBs through Two Routes of Exposure: Is the Dermal Route Significant? M. S. Johnson, L. S. Franke, R. B. Lee, and S. D. Holladay, San Francisco, CA.

Workshop on Ecological Risk Assessment and Military Related Compounds: Current Research and Future Needs. Addressing Data Gaps in Ecological Risk Assessment. Mark S. Johnson and Laura S. Franke, July 96, Denver, Colorado.

Conference on Advances in Toxicology and Applications to Risk Assessment. April 1996. Wright-Patterson Air Force Base, Dayton, Ohio.Application of Ecological Risk Assessments and Case Studies in the Army, (Symposia).

Conference on Advances in Toxicology and Applications to Risk Assessment. April 1996. Wright-Patterson Air Force Base, Dayton, Ohio. Estimating Ecological Risk to Terrestrial Receptors at J-Field, Aberdeen Proving Ground, Maryland (poster).

Society of Environmental Toxicology and Chemistry (SETAC) 2nd International Conference, November 1995, Vancouver, BC; Estimating Ecological Risk to Amphibians Through Reproductive Performance at J-Field, Aberdeen Proving Ground, Edgewood Area, Symposia.

Eastern Bird Banding Association, April 1995, Cape May, NJ; The Effects of Age and Habitat on the Reproductive Performance of Wood Thrush, Symposia.

USACHPPM / Directorate of Laboratory Services Seminar. April 1995, How Contaminaton Affects the Ecology of Terrestrial Systems.

Ecological Society of America, August 1994. The Effects of Age and Habitat on the Reproductive Performance of Wood Thrush, (poster).

Occupational and Environmental Medicine Residency Program. Sept. 1994, Fate and Transport and Ecological Risk Assessment.

AOU/COS/WOS Joint Meeting, June 1994, Missoula, MT; The Effects of Age and Habitat on the Reproductive Performance of Wood Thrush, Symposia.

SCIENTIFIC REVIEWS

Peer Review, Ecological Risk Assessment Guidance for Superfund: Process for Designing and Conducting Ecological Risk Assessments. EPA 540-R-97-006 June 1997.

Peer Review, Ecological Risk Assessment of Savanna Army Depot. Nov. 1997.

Peer Review, D.E. Burhans and Frank R. Thompson III, The effect of time on nest concealment and its importance in songbird nesting studies. Condor. March 1998

Peer Review, Friesen , L. E., V. E. Wyatt, and M. D. Cadman. Nest re-use by wood thrushes and rose-breasted grosbeaks. Wilson Bull. July 1998

INVITED SEMINARS

Co-Chair, Addressing Data Gaps and Uncertainty in Ecological Risk Assessments. Summary of a Workshop on Ecological Risk Assessment and Military-Related Compounds: Current Research Needs. Denver, CO. Aug. 1996.

Participant in Task I, Derivation of Toxicity Reference Values (TRVs) in the Ecological Soil Screening Level Initiative.

Presentation of USACHPPM methods in immune characterization using flow cytometry, Russian Federation, Science Research Institute of Pathology and Human Ecology, St. Petersburg, RF; Sept. 98.

INTERESTS

Hiking and Camping

Birdwatching, Cross-country skiing

Raku pottery; Volleyball (USVBA mens B division)

REFERENCES

Glenn J. Leach, PhD DABT
Program Manager, Health Effects Research
USACHPPM
APGEA, MD 21010-5422

Roland Roth, PhD
Dept. of Entomology and Applied Ecology
University of Delaware
Newark, DE 19717-1303

Douglas Tallamy, PhD
Dept. of Entomology and Applied Ecology
University of Delaware
Newark, DE 19717-1303

Mrs. Barbara M. Ross (President, Eastern Bird Banding Assoc.)
308 Thornhill Rd.
Baltimore, MD. 21212

Dr. Chandler Robbins or Ms. Barbara Dowell
U.S. Fish and Wildlife Service
Department of Migratory Bird Management
Laurel, MD 20708

Tel. # 301-498-0383

Ms. M. Kathleen Klimkiewicz
U.S. Fish and Wildlife Service
Patuxtent Wildlife Research Center
Laurel, MD. 20708