PHYTOREMEDIATION OF EXPLOSIVES

USING *POPLUS DELTOIDES*

by

GREGORY ALLEN SEALOCK, JR

(Under the direction of Lawrence A. Morris)

ABSTRACT

Both the commissioning and disposal of weapons at ammunition plants have led to soil, ground, and surface water contamination by the explosives 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX). In this study, eastern cottonwood (*Poplus deltoides*) was grown both hydroponically and in explosives-contaminated soil. The hydroponic nutrient solution was spiked with explosives and sampled daily for eleven days to determine the rate of uptake. TNT was rapidly removed, resulting in a mean pseudo-first-order rate constant of 0.13 hr^{-1} followed by RDX at 0.01 hr^{-1}. HMX was not significantly removed. Tissue analysis suggested plant-catalyzed transformation. Cuttings were also grown in columns containing munitions contaminated soil and harvested weekly for up to eight weeks. Soil concentrations did not change significantly, and tissue analysis resulted in the identification of RDX only. In both studies, up to 60% of the identified RDX was found in leaf tissues.

INDEX WORDS: Phytoremediation, Explosives, Munitions, TNT, RDX, HMX, *Poplus deltoides*

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DEDICATION

This is dedicated to Robin and Jake. Without your help, love, and tolerance this work would not have been possible.

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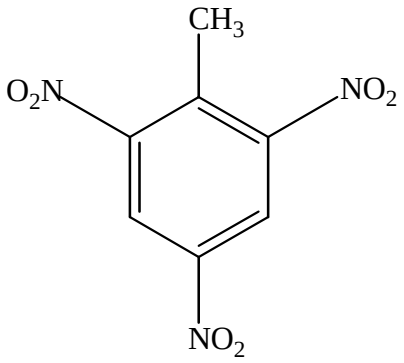
CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

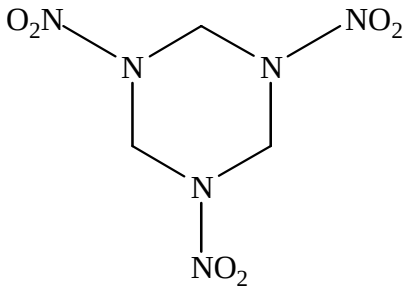
2,4,6-Trinitrotoluene (TNT), hexahydro-1,3,5,trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) are the most widely used compounds to manufacture explosive devices. Varying mixtures of these compounds form the base of most common military explosives including amatol, pentolite, tetrytol, torpex, tritonal, picratol, ednatol, cyclotol, composition A, composition B, composition C, HBX, and H-6. Before World War II, little thought was given to the hazardous effects that would result from manufacturing, storage, and disposal of these munitions. However, during World War II, when batch production and manufacture of munitions drastically increased, there was much concern due to the presence of TNT and its metabolites in waste effluents after large-scale production. Due to discoloration of the waste effluent, it was often termed “red” or “pink” water (Ruchoft 1945). The presence of these munitions eventually led to an abundance of research on the toxicity of some explosives, and methods that could be used to ameliorate the contamination of soil and water surrounding production facilities.

TNT, RDX, and HMX are classified as high explosives, as opposed to primary explosives. Primary explosives are readily ignited by contact with a flame or spark. While primary explosives are extremely sensitive to shock, friction, and heat; high explosives, are relatively insensitive to these conditions. High explosives detonation proceeds quickly, resulting in molecular rearrangement. This combustion reaction releases large amounts of energy, which forms highly stable gases which, such as CO,



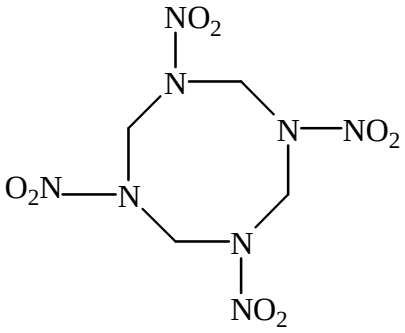
2,4,6 trinitrotoluene

(**TNT**)



hexahydro-1,3,5-trinitro-1,3,5-triazine

(**RDX**)



octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine

(**HMX**)

Figure 1.1: Structures of selected explosives that are common pollutants of soil and water found at munitions facilities.

CO_2, and N_2 (Yinon and Zitrin 1981). Their chemical structures indicate that TNT is a nitroaromatic munition, whereas RDX and HMX are heterocyclic nitroamines (Figure 1.1).

Also referred to as Triton, Trotyl, Trilite, Trinol, and Tritolo, TNT, is one of the most stable high explosives. It is relatively insensitive to blows or friction, however, it quickly reacts with alkaline compounds forming unstable mixtures that are highly sensitive to heat and impact. In 1863, Wilbrand became the first person to synthesize TNT by the nitration of toluene in a mixture of nitric and sulfuric acid (Zitting *et al.* 1982). RDX, also termed Cyclonite, Hexogen, cyclotrimethylenetrinitramine, and T_4; was initially formulated by the German scientist Hans Henning in 1899 from nitric acid and hexamethylenetetramine (Bachman and Sheehan 1949), and the British military adapted the acronym, RDX, which stands for Research Department Explosive. RDX is considered the most powerful of the military high explosives. HMX, high melting explosive, is also referred to as Octogen or cyclotetramethylenetetramine. RDX often contains low levels of HMX, because HMX is a by-product of RDX synthesis (Cataldo *et al.* 1987). It is seldomly used alone for detonation purposes, but functions effectively when mixed with one of the other high explosives.

TOXICITY

As a result of numerous health-problems reported by munitions plant workers, a substantial amount of work has been performed, investigating the toxicity of explosives to various species of mammals and aquatic organisms. In the U.S., TNT toxicity accounted for approximately 17,000 poisonings and 475 deaths during World War I (Yinon 1990). Studies have shown that TNT is mutagenic, and carcinogenic (Won *et al.*

1976; Honeycutt *et al.* 1996; Berthe-Corti *et al.* 1998). Human ingestion of TNT can cause a range of adverse health conditions including headaches and fatigue to liver damage, aplastic anemia, hepatitis, and death (Lewis 1992; Tchounwou *et al.* 2001). Additionally, TNT is toxic to aquatic organisms such as marine copepods (*Tigriopus californicus*), oysters (*Crassostrea gigas*), and freshwater unicelluar green algae (*Selenastrum capricornutum*) (Won *et al.* 1976). The studies conducted with these organisms, revealed that TNT concentrations as low as 2.5 to10 mg/L could inhibit physiological functions. Based on this toxicity data, the recommended maximum drinking water concentration is 140 μg/L (Stahl and Aust 1995).

Although it is not mutagenic, ingestion of RDX adversely affects the central nervous system, gastro-intestinal tract and kidneys. The toxicity of RDX to many organisms is well documented. Its acute toxicity towards rodents has led to its use as a rat poison (Levine *et al.* 1990a). Hypotriglyceridemia, behavioral changes, and mortality have been identified as signs of RDX intoxication in F344 rats (Levine *et al.* 1990b). Other studies have shown effects on reproduction, such as low birth weights and still births in rats administered RDX (Cholakis *et al.* 1980). Hexahydro-1,3,5-trinitroso-1,3,5-triazine, a degradation product of RDX has been used as an experimental tumorigen (Lewis 1992). In humans, common symptoms of RDX intoxication include nausea, vomiting, unconsciousness, and epileptic seizures (Kaplan *et al.* 1965; Etnier 1989). For this reason, it has been estimated that intake above 0.21 mg/d for a 70 kg human and a water concentration above 105 μg/L is unacceptable (Etnier, 1989).

Much less is known about HMX toxicity in humans. However, some aquatic organisms have shown signs of HMX toxicity, including fathead minnows (*Pimephales*

promelas) and the freshwater microcrustacean *Daphnia magna* (Talmage *et al.* 1999). It has also been shown that HMX is toxic to rats and mice (Talmage *et al.* 1999). Most recently, the toxicity of HMX was determined using the earthworm (*Eisenia andrei*) reproduction test (Robidoux *et al.* 1999; Robidoux *et al.* 2001). Based on various reproduction parameters, earthworm fecundity was reduced when exposed to 280 mg/kg and 2500 mg/kg respectively.

EXTENT OF CONTAMINATION

In the past, before environmental concern was realized, munitions wastewater was held in lagoons to allow the solid wastes to settle before releasing the water to nearby streams (Harvey *et al.* 1991). When this practice was discontinued, the lagoons evaporated, leaving these areas highly contaminated with munitions and their transformation products (Klausmeier *et al.* 1973; Traxler 1974). Due to manufacturing and disposal practices, these explosives and their transformation products are major pollutants in soils, groundwater, and surface water throughout the world. The chemical and physical properties of these compounds suggest their persistence in the environment (Table 1.1). Proof of these compounds' environmental recalcitrance lies in the fact that much of the contamination is a result of practices that occurred in some cases over 50 years ago. In the U.S., these sites are primarily on land owned by the Department of Defense (DOD). These facilities include commercial production operations, former munitions manufacturing plants, munitions assembly facilities, demilitarization operations, and burn and disposal sites. According to the U.S. Army Environmental Center (USAEC) there are at least 50 military installations that have explosives contamination within the U.S. (Figure 1.2)(Jerger and Woodhull 2000). In Europe,

Table 1.1: Chemical and physical properties of TNT, RDX, and HMX

	TNT	RDX	HMX
Molecular Formula	$C_7H_5N_3O_6$	$C_3H_6N_6O_6$	$C_4H_8N_8O_8$
CAS #	118967	121824	2691410
Molecular Weight (g/mol)[1]	227.113	222.117	296.156
Density (g/cm3)[1]	1.56	1.63	1.71
Melting Point (C)[2]	80.75	200	273
Boiling Point (C)[1]	380.5	581.4	771.1
Vapor Pressure (Torr at 25 C)[1]	2.13×10^{-6}	3.78×10^{-11}	2.71×10^{-16}
Log K_{ow}[3]	1.86	0.87	0.42
Aqueous Solubility (mg/L at 25 C)[1]	100.4	42	2.3

1 = SPARC, 2002; 2 = Yinon, 1990; 3 = Haderlein, 1996

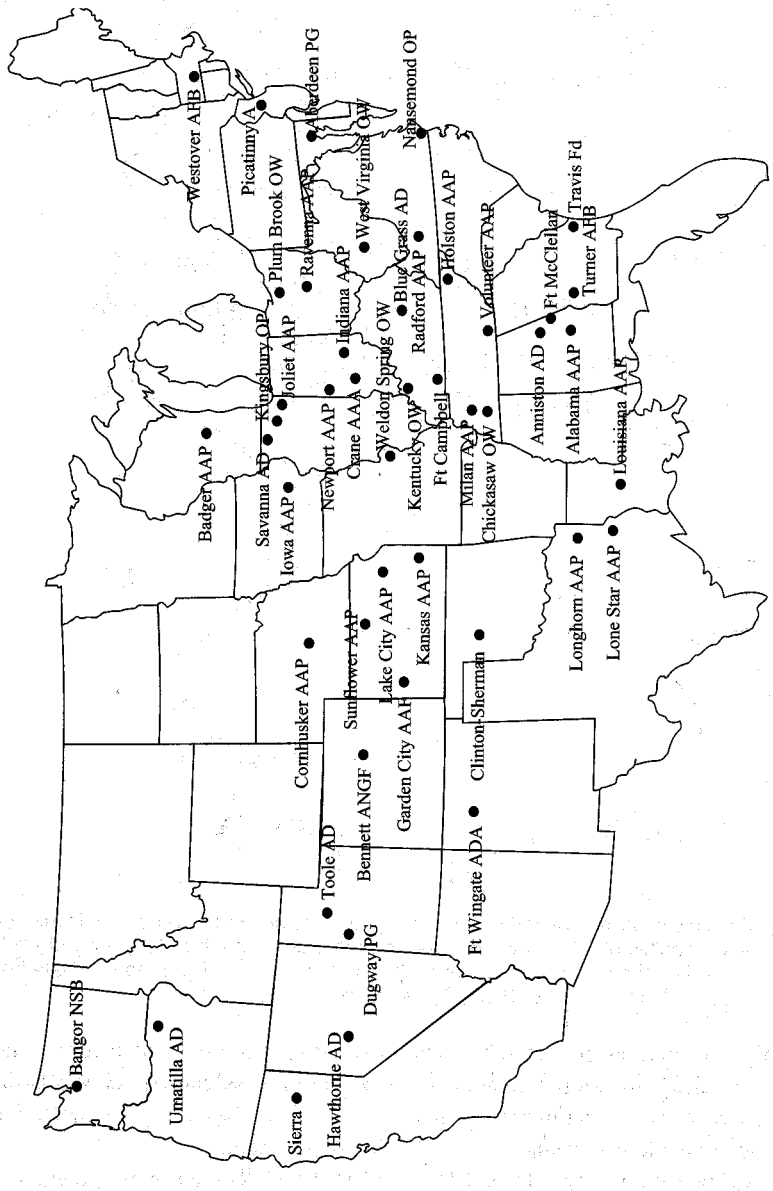


Figure 1.2: Explosives contaminated facilities in the United States (Spain 2000).

specifically Germany, most manufacturing facilities were demolished at the conclusion of World War II. Due to new construction on these sites, little characterization has been completed and even fewer remediation methodologies have been implemented. Other countries with munitions contamination include the United Kingdom, Canada, and Australia, yet, little has been done to address the problem (Spain 2000). In the remainder of the world, the extent of contamination is either unknown or undisclosed. Within the U.S., the cleanup of DOD sites is regulated by state and local environmental statutes as well as the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), as amended by the Superfund Amedments and Reauthorization Act (SARA), and the Resource Conservation and Recovery Act (RCRA) (Jerger and Woodhull 2000).

In the U.S., the primary method for remediating contaminated soil is excavation followed by composting or incineration. These methods are laborious, costly and there is some question about the toxicity and mutagenicty of the transformation products generated (Tan *et al.* 1992; Jarvis *et al.* 1998). Incineration produces unusable ash, and due to concerns over air quality has poor public acceptance (Hundal *et al.* 1997). Estimated cost of implementing this technology range between $200 to $1000 per cubic yard (Vanderford 1996). Munitions contaminated wastewater is typically treated using pump and treat methods, and carbon adsorption columns to remove the contaminants. Then, the treated water is discharged into streams (Harvey *et al.* 1991; Selim *et al.* 1995). It is estimated that up to 2 million gallons of wastewater with concentrations up to 12 mg/L can be generated in a single day at munitions facilities (McCormick *et al.* 1981; Jenkins *et al.* 1986). These current treatment methods have been widely accepted, but considered expensive. Based on the human health effects and chemical characteristics of

these compounds, there is an overwhelming demand for cost effective and environmentally “friendly” methods of remediation.

PHYTOREMEDIATION

In the mid-1990s phytoremediation emerged as a potential low cost alternative for ameliorating explosives contaminated soil and water (Cunningham and Ow 1996). The term “phytoremediation” combines the Greek word “phyton” (plant) with the Latin word “remediare” (to remedy) to describe the process by which aquatic or terrestrial plants, and the microbial communities associated with their rhizospheres, degrade, extract, contain, or immobilize contaminants from both soil and water. The term was first introduced in 1991 to describe the use of plants to accumulate metals from soil and water, but in 1995, the definition was expanded to include the breakdown of organic chemicals (Schnoor *et al.* 1995).

Many plant biochemical processes do not distinguish between contaminants and nutrient sources, leading to the direct translocation or transformation of pollutants. However, attenuation mechanisms involved in phytoremediation are not limited to the direct metabolism of contaminants (Burken *et al.* 2000). Indirect attenuation mechanisms include the modification of the physical and chemical properties of the soil, increases in organic soil carbon by the release of root exudates, increased soil aeration and porosity, and reversal of the hydraulic gradient by extraction of available water; thereby decreasing vertical and lateral migration of pollutants to ground water (Chang and Corapcioglu 1998).

There are several processes involved in plant-assisted transformation of contaminants. Phytoaccumulation, also termed phytoextraction or hyperaccumulation,

utilizes cation pumps and sorption to withdraw metals, salts, and organic compounds from soil via uptake of plant available water (Schnoor *et al.* 1995). In the process of phytostabilization, plants temporarily control soil properties such as gas exchange, and redoximorphic conditions. Phytostabilization may be able to control the movement of heavy metals, phenols, and chlorinated solvents (Cunningham *et al.* 1995). Volatile metals such as mercury (Hg) and selenium (Se), as well as chlorinated solvents, can be taken up and transpired by a process termed phytovolatilization (Cunningham *et al.* 1996). Polyaromatic hydrocarbons, polychlorinated biphenyls, and BTEX compounds can be transformed or degraded by rhizosphere bioremediation. This type of bio-remediation utilizes enzymatic activity of mycorrhizal fungi and other microorganisms to degrade the contaminant (Cunningham *et al.* 1995). Phytotransformation, as the name implies, involves the uptake, and metabolism of organic compounds to secondary and tertiary transformation products, which may be harmless in some cases. This process is effective in the transformation of some munitions, chlorinated solvents, and phosphorus and chlorine based pesticides (Schnoor *et al.* 1995). The metabolic processes involved in phytotransformation resemble human metabolism of xenobiotics (Burken *et al.* 2000). For this reason, a "green liver" model is often used to describe the mechanisms involved with phytotransformation (Sanderman 1994).

In contrast to microorganisms, plants use photosynthesis as an energy supply and do not need to metabolize organic compounds for an energy source. Similarly, plants detoxify foreign contaminants, much like humans metabolize xenobiotics. During this detoxification process, xenobiotics are transformed, conjugated, and sequestered. Initially, transformation occurs as a result of enzymes, which catalyze oxidation,

reduction, and hydrolysis reactions. Then, the secondary product undergoes conjugation with an organic molecule within the plant. This conjugation process generally leads to a reduction in toxicity to the plant (Coleman *et al.* 1997; Bhadra *et al.* 1999b). Several sequestration processes can follow conjugation. These can include storage in cell vacuoles, or covalent bonding, which results in conjugates being incorporated into lignin (Coleman *et al.* 1997). The latter case is characterized by unextractable, or bound residues (Burken *et al.* 2000).

Phytoremediation has many characteristics that make it a desirable form of contamination removal. Many methods of site remediation such as excavation and pump and treat involve expensive, laborious procedures which only transfer the pollutant from one medium to another (Mitsch 1993; Fox 1997). Phytoremediation is a more affordable alternative to conventional clean-up methods. Cleaning the top 15 centimeters of petroleum contaminated soil with phytoremediation costs between $2,500 and $15,000 per hectare, compared to $7,500 to $20,000 per hectare for on-site microbial remediation (Sustainable Strategies, 1997). In addition, the use of plants for detoxification also leads to a more aesthetically pleasing appearance. It has been suggested that phytoremediation is suitable for use at large scale field sites, sites with low concentrations of contaminants, and in conjunction with other methodologies where vegetation is used as a final cap and closure of the site (Schnoor *et al.* 1995).

There are, however, several limitations to this new technology. It is only effective in treating shallow soils, ground water, and surface water. Plants can only effectively remediate contaminates near their root zone (Schnoor *et al.* 1995). Phytotoxicity is also a limitation of this approach. Many plants are slow growing, difficult to establish, or

cannot survive in areas with high concentrations of contaminants. This limits some applications to areas with low concentrations surrounding the primary site of contamination (McCutcheon 1998). Finally, the secondary and tertiary transformation products of many compounds are also toxic and need to be disposed of properly. In some instances, the characteristics of by-products are not known, therefore, remediation to this point may not always be acceptable.

PLANT METABOLISM OF EXPLOSIVES

An abundance of work has been conducted to test the ability of aquatic and terrestrial plants to remove TNT from aqueous solutions. Yellow nutsedge (*Cyperus esculentus*) was the first species used to trace the fate of TNT within a plant (Palazzo and Leggett 1986). TNT and its aminated transformation products 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT), were found in all plant tissues, including leaves, roots, rhizomes, and tubers. Since only TNT was added to the solution and the method ensured that no metabolites were in the applied nutrient solution, it was suggested that these two products were formed within the plant; however, the study was not designed to differentiate between plant metabolism and microbial degradation.

The intrinsic ability of plants to metabolize TNT was shown using axenic Parrot feather (*Myriophyllum aquaticum*), native Eurasian water milfoil (*Myriophyllum spicatium*), and hairy root cultures (*Catharanthus roseus*) (Hughes *et al.* 1997; Vanderford *et al.* 1997). Each plant was exposed to uniformly labeled ^{14}C-TNT and evaluated. In all cases, TNT was completely transformed, but there was a lack of mineralization, which is consistent with the “green liver” concept. Most recently, hybrid

poplar trees (*Poplus deltoides* X *nigra*, DN34) were studied for their ability to remove TNT from contaminated hydroponic media (Thompson *et al.* 1998a; Thompson *et al.* 1998b). Those results concluded that most transformation products localized in the root tissue with very little accumulating within the aerial leaves. As in previous studies, both 2-ADNT and 4-ADNT were identified within the plant. Less than 10 % of the applied ^{14}C-TNT was extractable.

The primary transformation pathway of TNT within a plant is depicted in Figure 1.3. TNT lacks functional groups that would make conjugation possible. Thus, transformation must occur in order for further metabolism to proceed. Although oxidation has been suggested as a possible transformation mechanism (Bhadra *et al.* 1999a), nitroreduction is generally accepted as the primary pathway by which TNT transforms. Reduction proceeds with a series of electron transfers resulting first in the formation of the hydroxylamino isomers 2-hydroxylamino-4,6-dinitrotoluene (2HA 4,6 DNT) and 4- hydroxylamino-2,6-dinitrotoluene (4HA 2,6 DNT). Progressive reduction then leads to the sequential formation of the 2-ADNT and 4-ADNT isomers. Some researchers have postulated that diamino-derivatives are also formed within the plant, but conclusive research is needed (Thompson *et al.* 1998b).

Only a few published studies focus on the fate of RDX in plants. The first examination of RDX fate in plant systems, used bush beans (*Phaseolus vulgaris*), grown from seed (Harvey *et al.* 1991). The plants were initially grown in hydroponic nutrient solutions for 21 to 26 days. After the seedlings were established, the plants were transferred to nutrient solutions amended with 10 mg/L containing 5.6 μCi/500 mL uniformly labeled RDX. Plants were exposed to the RDX solution for either

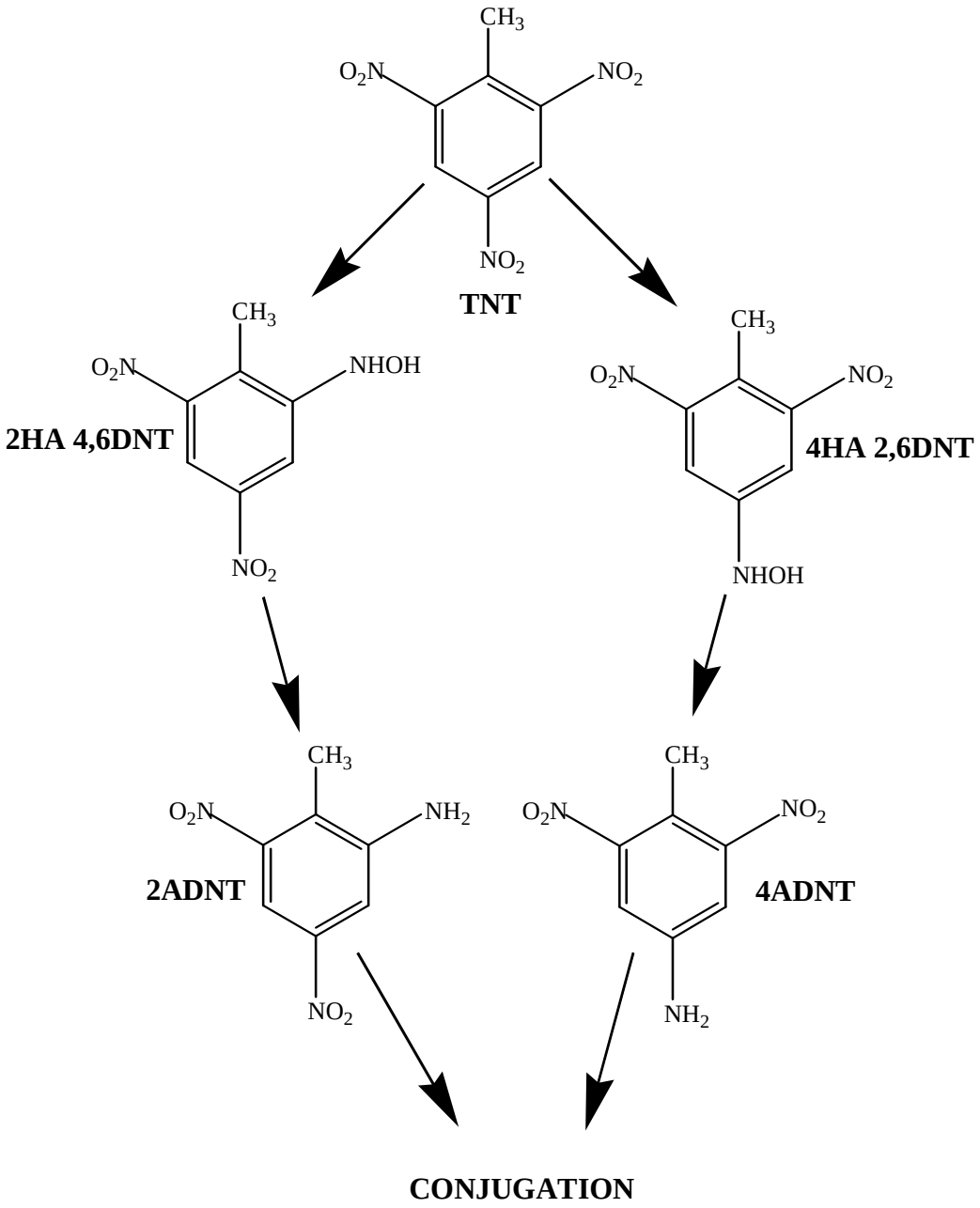


Figure 1.3: Hypothetical pathway of 2,4,6-trinitrotoluene (TNT) metabolism in plants to 2-hydroxylamino-4,6-dinitrotoluene (2HA 4,6DNT), 4-hydroxylamino-2,6-dinitrotoluene (4HA 2,6DNT), 2-amino-4,6-dinitrotoluene (2 ADNT), and 4-amino-2,6-dinitrotoluene (4 ADNT).

one or seven days. At each harvest, plants were separated into roots, stems, and leaves, then prepared for chemical analysis. Sampling the hydroponic solutions at harvest time established plant uptake of RDX. The initial mass of RDX, 5.14 ± 0.02 mg, decreased to 4.53 ± 0.04 mg after one day and 1.80 ± 0.73 mg after seven days. Transformation products of RDX were not found in the hydroponic solutions, suggesting a lack of microbial and rhizosphere degradation. After one day of exposure, RDX concentrations in leaf, stem, and root tissues were 19 mg/kg, 11 mg/kg, and 9 mg/kg, respectively. Based on the extraction process, and recovery of radiolabel, it was determined that RDX was not metabolized. Evidence of bioaccumulation was seen in the seven-day exposures. Leaf, stem, and root tissues contained 97 mg/kg, 11 mg/kg, and 6 mg/kg RDX, respectively. Limited metabolism of RDX was suggested based on the nonextractable fractions of the radiolabel. Emission of $^{14}CO_2$ and volatile organics did not reach detectable levels.

Hybrid poplar trees (*Poplus deltoides* X *nigra*, DN34) have also been evaluated for their ability to transform RDX (Thompson *et al.* 1999). Using hydroponic solutions containing radiolabeled RDX, uptake was monitored by sampling the hydroponic solution for approximately two days. As in previous studies, the mass of RDX decreased with time but no metabolites were detected. Plants remained in the system for up to seven days. Plant extractions resulted in mass balances that averaged 79.7 $\pm$ 7.1% recovery. Approximately 60% of the absorbed RDX was translocated to the leaves allowing 15 to 20% to remain in root and stem tissue. The author suggested that the bound fraction, which was approximately 15% of the radiolabel, may be an RDX

transformation product. As in previous studies plant respiration of $^{14}CO_2$ and volatile organics was negligible.

To date, there is a lack of research focusing on the fate of HMX in plant systems, and uptake of explosives from aged-contaminated soils. A review of relevant literature published through 2001 indicates no refereed journals which published research on this subject.

In contrast to TNT, there has been little documentation of RDX transformation within plants. However, a vegetation survey conducted at the Iowa Army Ammunition Plant (IAAP) produced results that suggest RDX transformation may occur within plants (Schneider 1995). RDX accumulated in the leaf tissues of black locust (*Robinia pseudoacacia*), red cedar (*Juniperus virginiana*), bromegrass (*Bromus inermis*), pigweed (*Amaranthus spp*.), reed canary grass (*Phalaris arundinacea*), Canadian goldenrod (*Solidago canadensis*), and ragweed (*Ambrosia artemislilfolia*). The highest RDX concentration found in the leaf tissues was 38.6 mg/kg although soil RDX concentrations were as high as 114 mg/kg. These tissue concentrations are low when compared to previous laboratory studies. Laboratory studies that implemented significantly lower RDX concentrations in hydroponic media resulted in high leaf tissue concentrations. The low RDX concentrations found in leaf tissues collected in the field suggest that RDX metabolism may occur over a longer period of time than what has been previously tested in the laboratory.

Based on the research reviewed, it is obvious that many important aspects of RDX uptake and transformation by plants are not well characterized. Previous studies were conducted for only short time periods. Also, previous work studied plants subjected to

only one explosive at a time, whereas most contaminated sites contain more than one explosive compound within the same media. The rates of uptake and transformation may differ when plants are grown in an environment containing TNT, RDX, and HMX. Further work needs to focus on the long-term fate of explosives in plants, specifically terrestrial species; identification of transformation products in plant tissues, and explosives uptake from aged-contaminated soil.

CHAPTER II

UPTAKE OF THE EXPLOSIVES TNT, RDX, AND HMX BY HYDROPONICALLY GROWN *POPLUS DELTOIDES*

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ABSTRACT

The explosives 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) have been used extensively by the United States military to manufacture munitions. Since World War II, both the commissioning and disposal of weapons at ammunition plants have led to soil, ground, and surface water contamination by these and other recalcitrant pollutants. It has been reported that certain plants have the ability to absorb and transform some explosives from contaminated water. In this study, eastern cottonwood (*Poplus deltoides*) was grown hydroponically. Cottonwood cuttings were cultivated in 2L Erlenmeyer flasks containing a hydroponic nutrient solution spiked with TNT, RDX, and HMX. The nutrient solution was sampled daily for eleven days to determine the rate of uptake. Both TNT and RDX concentrations in solution declined over the study period resulting in pseudo-first-order rate constants of 0.13 hr^{-1} and 0.01 hr^{-1} respecitvely. HMX was not significantly removed. Plant tissue analysis resulted in the identification of TNT transformation products. The mean RDX recovery was approximately 30% of initial application. Approximately 60% of the RDX identified in plant tissues translocated to leaves.

INTRODUCTION

The nitrogen containing compounds 2,4,6-trinitroltoluene (TNT), hexahydro-1,3,5,-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) are commonly used military explosives and regulated toxic substances. It has been estimated that there are at least 50 explosives-contaminated sites in the US (Spain 2000). Significant risk is posed by their presence in the environment, and many studies

have been conducted to examine the effects of mammalian exposure (Won *et al.* 1976; Etnier 1989; Talmage *et al.* 1999). Current methods utilized to treat explosives-contaminated soil include excavation followed by composting or incineration. Traditional pump and treatment with carbon adsorption is the most common methodology used to ameliorate explosives-contaminated wastewater. These methods have been considered laborious and expensive, and have received little public acceptance due to their damaging effects on the environment.

Since the early 1990s, phytoremediation has emerged as a new, cost-effective technology with the capabilities of treating media contaminated with organic compounds. Phytoremediation is defined as the use of aquatic and terrestrial plants, in conjuction with root associated microbial communities; to treat soil and water polluted with inorganic and organic compounds. Recent studies have examined the potential of using this technology to remediate explosives-contaminated media. In order for this technology to be accepted, the fate of these compounds and their transformation products within plant tissues must be identified.

The transformation of foreign compounds by plants differs greatly from microbial metabolism. Plant metabolism of xenobiotics strongly resembles the functions of the human liver. For this reason, the "green liver" paradigm is often used to describe the fate of organic compounds and their transformation products within plants (Sanderman 1994). The detoxification that occurs via the "green liver" model involves transformation, conjugation, and sequestration (Figure 2.1). Initially, transformation occurs as a result of enzymes, which catalyze oxidation, reduction, and hydrolysis reactions. Then, the secondary product undergoes conjugation with an organic molecule within the plant.

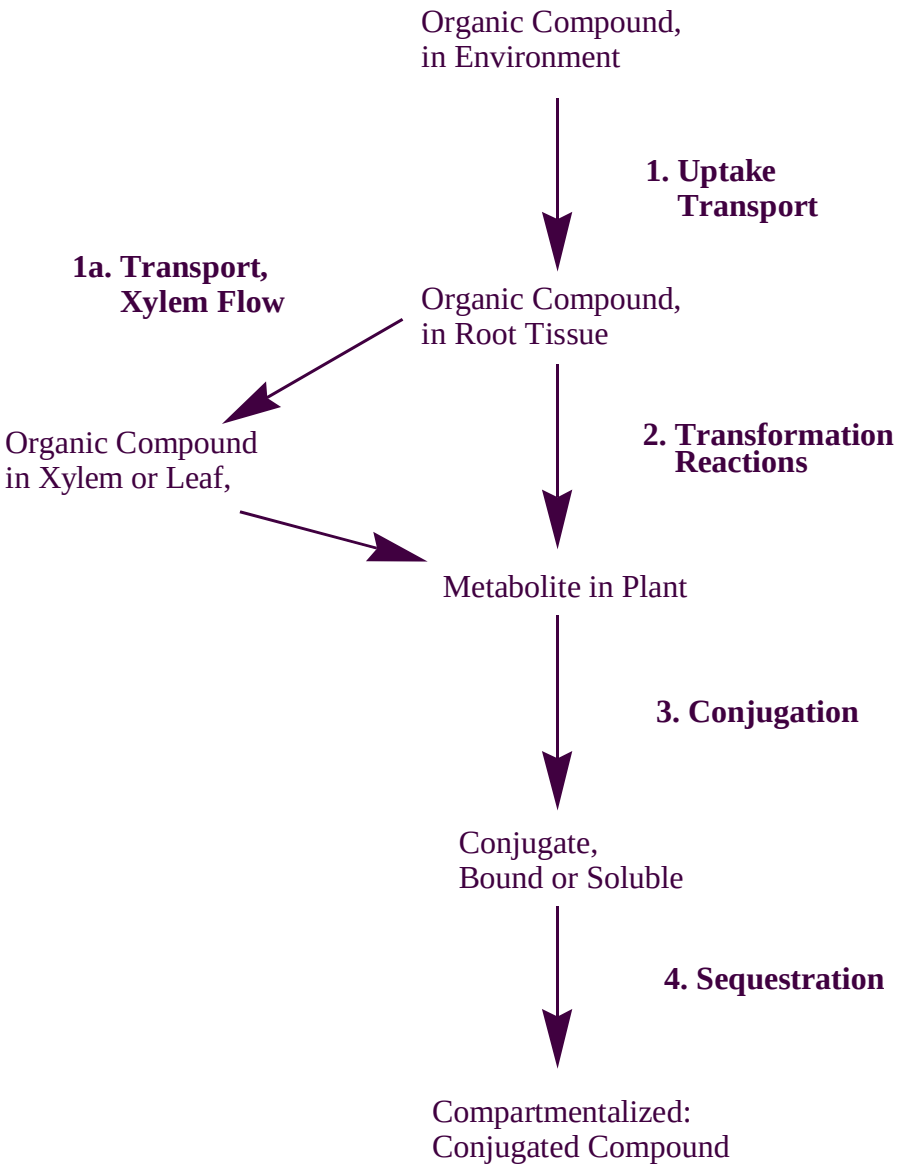

Figure 2.1: Schematic of the green liver model for metabolism of xenobiotics in plants (Burken *et al*. 2000).

This conjugation process generally leads to a reduction in toxicity to the plant (Coleman *et al.* 1997; Bhadra *et al.* 1999b). Several sequestration processes can follow conjugation and can include storage in cell vacuoles or covalent bonding, which results in conjugates being incorporated into lignin or other bio-polymers in the cell wall (Coleman *et al.* 1997). The latter case is characterized by unextractable, or bound residues (Burken *et al.* 2000).

TNT has been the most studied explosive in plant systems. Palazzo and Leggett (1986) initially examined the fate of ^{14}C using *Cyperus esculentus* grown hydroponically. TNT and the aminated isomers 2-amino-4,6-dinitrotoluene (2-ADNT), and 4-amino-2,6-dinitrotoluene (4-ADNT) were identified within plant tissue. Plant metabolism was suggested as the catalyst for product formation; however, the study was not designed to differentiate between plant and microbial degradation. Using axenic tissue cultures of *Myriophyllum aquaticum*, Hughes *et al*. (1997) showed the cability of a plant to metabolize TNT in the absence of culturable microbes.

Little work has been done to address the fate of RDX and HMX in plant systems, specifically HMX. Harvey *et al*. (1991) determined *Phaseolus vulgaris* readily removed RDX from hydroponic media. However, the observed removal kinetics differed significantly than those identified using hybrid poplar (Thompson *et al.* 1999). The re-occurring theme in both studies suggested that RDX was recalcitrant to transformation and accumulated in aerial tissues. Unlike TNT, the metabolic transformation products of RDX are unknown. Most recently, HMX was studied using *Myriophyllum aquaticum* and hairy root cultures of *Catharanthus roseus* (Bhadra *et al.* 2001). The results of the study indicated minimal biological activity. The above mentioned studies implemented

short exposure times, such as one to seven days. Considering the significant differences in the rate of plant uptake of such compounds, it is reasonable to predict the transformation kinetics will also differ. Moreover, the previous studies only exposed the plant to one explosive. A better representation of field conditions would incorporate multiple explosives within the same media.

Due to the lack of knowledge concerning the species specific uptake and transformation of explosives, and in order to expand on previous studies, a hydroponic study was conducted using Eastern cottonwood (*Poplus deltoides*). This species was chosen because most members of the *Salicaceae* family are considered ideal candidates for usage in phytoremediation technologies due to their rapid growth, high water usage, and ability to grow in most regions of the U.S. The specific objectives of this research were to (1) examine the rates of translocation and transformation of TNT, RDX, and HMX when contained in the same hydroponic media, and (2) identify transformation products and determine where they are localized within plant tissues.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade acetonitrile (CH_3CN) was purchased from Sigma-Aldrich, (St. Louis, MO). Calcium chloride ($CaCl_2$) was purchased from J.T. Baker (Phillipsburg, NJ). DD-6 alumina (Al_2O_3) was obtained from Alcoa Port Allen Works (Port Allen, LA). The magnesium silicate adsorbent Florisil was purchased from Fisher Scientific (Pittsburgh, PA). High Pressure Liquid Chromatography (HPLC) external standards for TNT, RDX, HMX were purchased from Accustandard (New Haven, CT). The TNT, RDX, and HMX standards were 1.0 mL volumes containing 1.0 mg of the compound in

an acetonitrile-methanol (1:1 v/v) mixture. Since separation of 2-ADNT and 4-ADNT could not be resolved using HPLC, a standard mixture was used. The 2-ADNT and 4-ADNT standard mixture was a 500.0 mL volume containing 500.0 mg/L of each compound. Solid TNT, RDX, and HMX were obtained from the U.S. Army Center for Environmental Health Research (Fort Detrick, MD).

Experimental Design

Eight-inch Eastern Cottonwood (*Poplus deltoides*) cuttings were purchased from the Greenwood Nursery (McMinnville, TN). Each cutting was placed in a hydroponic reactor, which consisted of a two liter Erlenmeyer flask covered with aluminum foil and fixture to support the plant stem. The growth medium was a half strenghth Hoagland's solution. The solutions were constantly aerated using aquarium pumps. The cuttings were cultivated using controlled greenhouse conditions for two months. After the two-month period, the cuttings were suplimented with fresh nutrient media spiked with TNT at 0, 5, and 25 mg/L, RDX at 0, 3, and 15 mg/L, and HMX at 0, 0.5, and 2 mg/L. The maximum concentrations used were limited by the solubility of the compounds in the growth media. The experimental design consisted of three replications of each treatment arranged in a completely randomized design. Unplanted reactors that contained TNT at five and 25 mg/L, RDX at three and 15 mg/L, and HMX at 0.5 and two mg/L were monitored in duplicate as controls. To compensate for water loss due to transpiration, fresh water was added daily to keep the total volume constant. The nutrient media from each reactor was sampled daily, for 11 days, by removing two milliliters for HPLC analysis. After the 11-day study period, the plants were harvested and stored at -40°C until analysis.

Plant Tissue Extractions

After incubation, plants were harvested in order to identify and quantitate the explosives and transformation products within the tissues. The extraction procedure utilized methods established by Larson *et al*. (1999). After plant harvest, the leaves, roots, and stems were separated and weighed. Then, each tissue sample was washed using deionized water and blotted dry with Chem Wipes. The samples were subsequently lyophilized for 24 hours using a Labconco Freeze Dryer 4.5 (Kansas City, MO). After drying, the plant material was weighed and ground to a 30-mesh particle size using a Thomas Scientific Wiley mill (Philadelphia, PA). Duplicate 0.5 g samples of each tissue were dried in a forced air oven at 110°C for 24 hours. Using scintillation vials, duplicate 0.25 g samples of the lyophilized plant material were combined with 10 mL acetonitrile, vortex swirled for one minute, and shaken on a temperature controlled incubator for 24 hours at 25°C. Each replicate was centrifuged for five minutes at 5000 rpm to pellet the insoluble plant residue. Then, five milliliters of the supernatant were removed and filtered. Filters were prepared by placing a small amount of glass wool in a serological pipette. The pipette was packed with glass wool and covered with 0.5 g florisil, which was then covered with 0.5 g DD-6 alumina. To equilibrate the column, five milliliters acetonitrile was passed through the filter and discarded. Five milliliters of the supernatant was subsequently passed through the filter and collected in a scintillation vial. Then, five milliliters of acetonitrile was passed through the filter and collected in the same vial. The vials were vortexed for one minute and the resulting solution was removed and placed in a disposable syringe and filtered through a 0.20 μm Acrodisc® syringe filter. The first milliliter was discarded, retaining the rest for HPLC analysis.

HPLC Conditions

Identification and quantification of TNT, RDX, HMX, 2-ADNT, and 4-ADNT was determined by reverse phase HPLC using a Hewlett Packard series 1100 quaternary pump, a diode-array UV detector set to 230 nm and 254 nm, and an autosampler with an injection volume of 15.0 μL. Separation was accomplished using a Hamilton PRP-1 column (10-μm particle size, 250mm × 4.1mm), a 12 minute isocratic run, with a mobile phase consisting of an acetonitrile-water (60:40 v/v) mixture at a flow rate of 1.0 mL/min. All water was ultrapure and at least 18 MΩ.

The TNT, RDX, and HMX external standards were removed from their vials and combined with one milliliter of the 2-ADNT and 4-ADNT mixture and 36.0 mL acetonitrile in a 40 mL volumetric flask, producing a stock solution which contained 25.0 mg/L of each compound. Calibration was achieved by diluting the stock solution with acetonitrile, producing standards of 20.0, 10.0, 5.0, 2.5, 1.0, and 0.5 mg/L. In order to retain analytical accuracy, all samples were injected twice, and a known standard, or deionized water was analyzed every 10 samples.

Results and Discussion

In order to test the accuracy of the extraction method, a standard that contained 5.0 mg/L of TNT, RDX, HMX, and ADNT respectively, was prepared in triplicate. Each replicate was handled and analyzed following the method described above. Compound recovery was determined to be 100.9 ± 3.3 %, 110.6 ± 0.8 %, 92.4 ± 0.9 %, and 100.6 ± 2.0 % for the respective explosives.

The uptake of explosives by *P. deltoides* was studied by temporal sampling of the nutrient media in each incubation reactor for approximately 11 days (Figure 2.2 to 2.4).

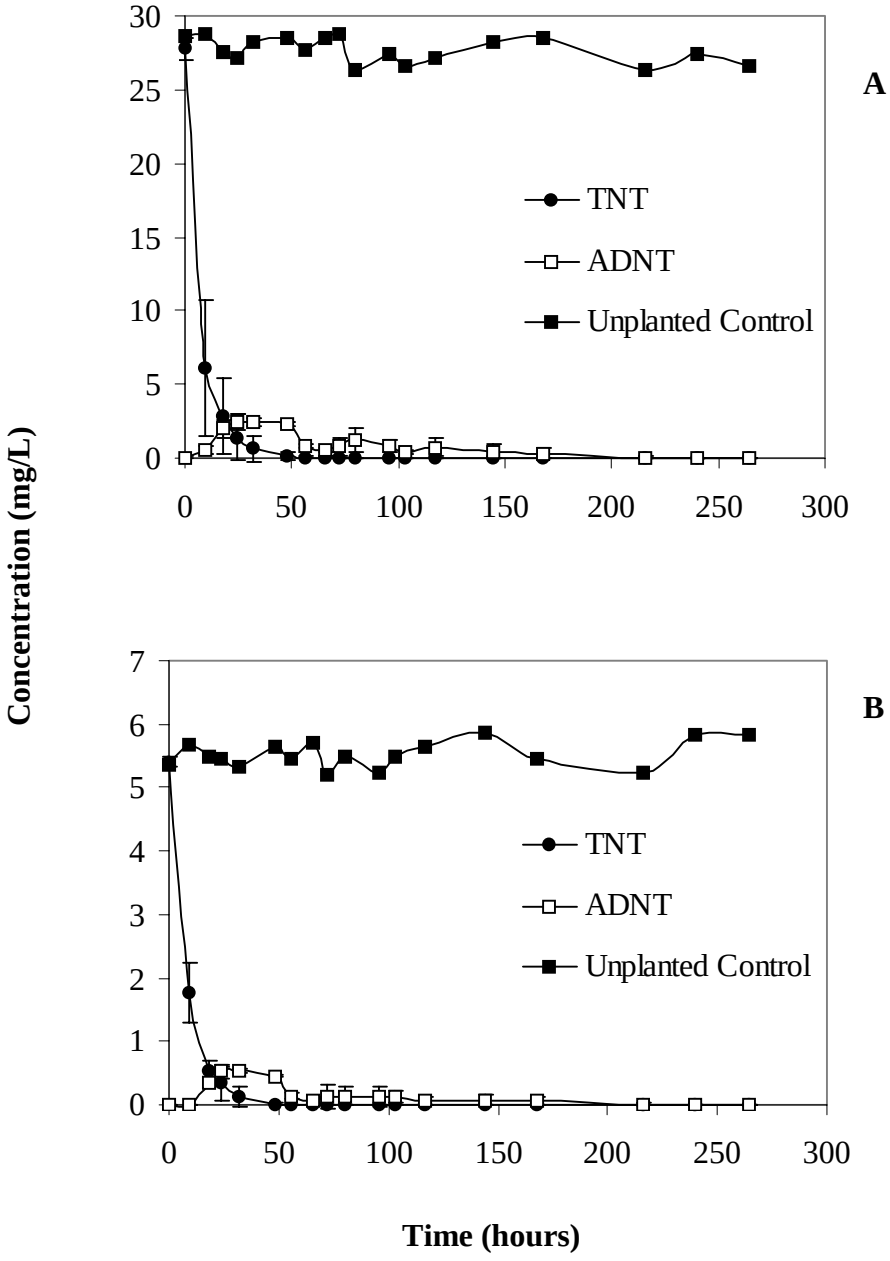

Figure 2.2: Disappearance of 2,4,6-trinitrotoluene (TNT), 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene (ADNT) from a hydroponic solution planted with *Poplus deltoides* with initial TNT concentrations A) 30 mg/L and B) 6 mg/L where *n* = 3 and error bars represent ± 1 standard deviation.

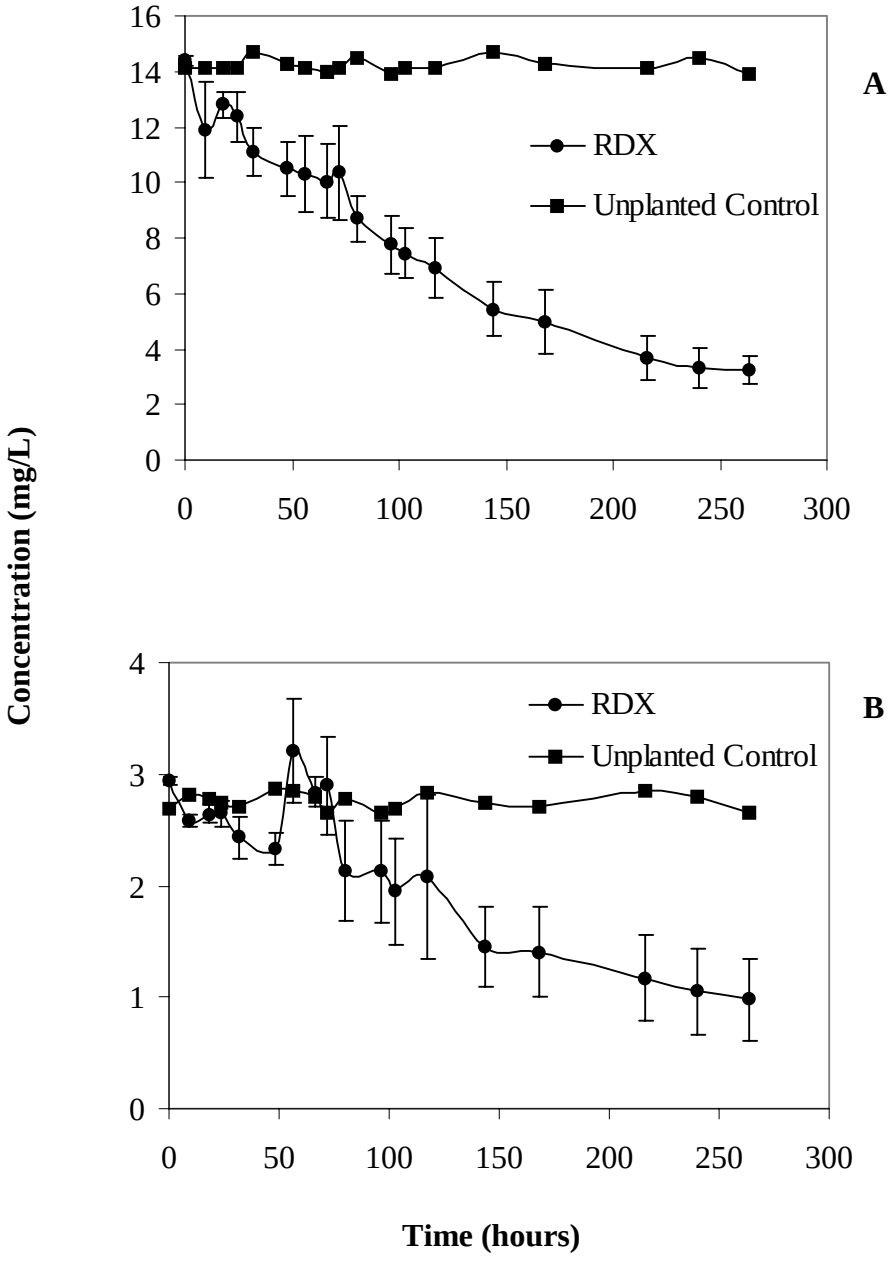

Figure 2.3: Disappearance of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from a hydroponic solution planted with *Poplus deltoides* with initial RDX concentrations A) 15 mg/L and B) 3 mg/L where *n* = 3 and error bars represent ± 1 standard deviation.

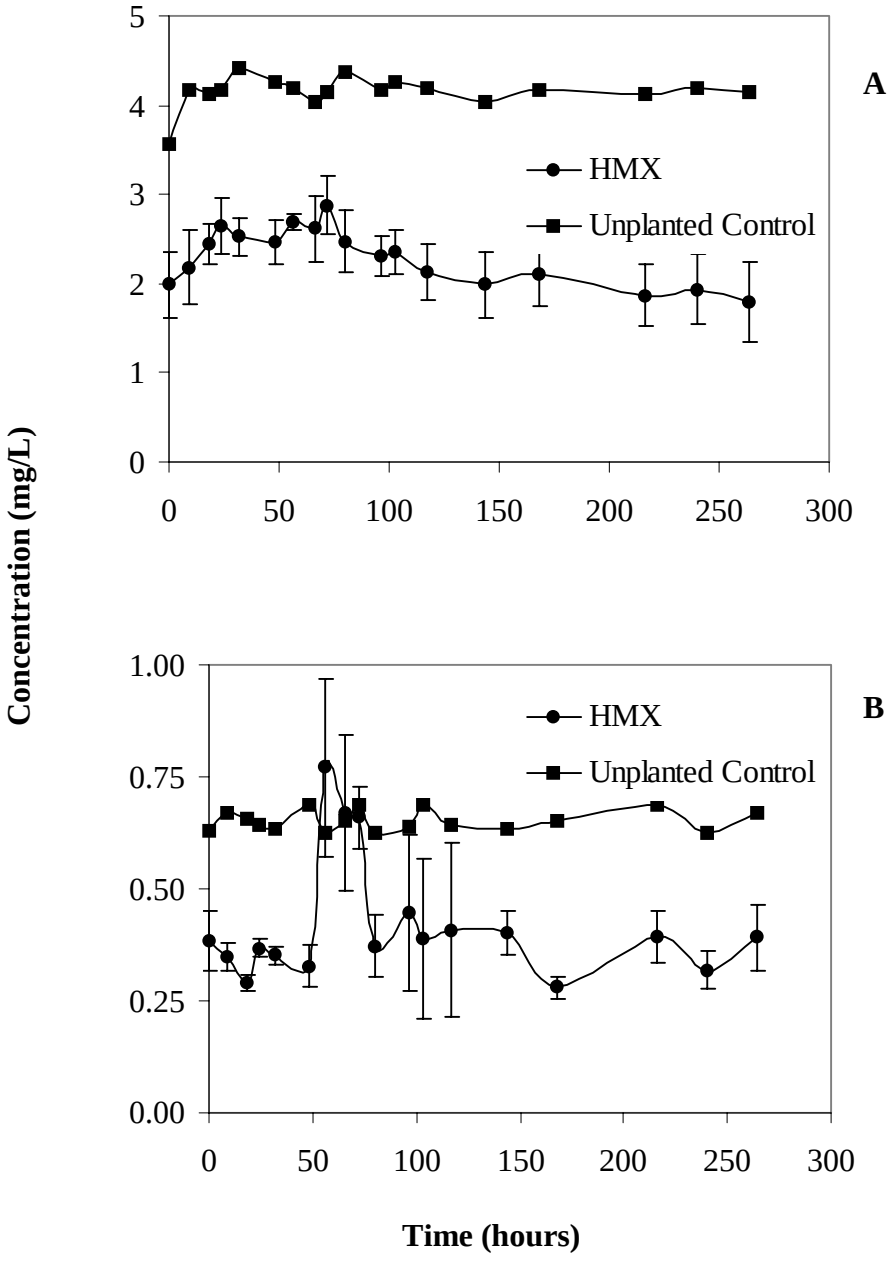

Figure 2.4: Disappearance of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) from a hydroponic solution planted with *Poplus deltoides* with initial HMX concentrations A) 2 mg/L and B) 0.5 mg/L where *n* = 3 and error bars represent ± 1 standard deviation.

Each reactor contained a combination of TNT, RDX, and HMX. This data was compared with reactors that contained the same compounds in the absence of vegetation. These unplanted controls were used to assess the occurrence of abiotic reactions with each compound. Planted reactors that did not contain explosives were also monitored to detect plant exudates that coelute with the respective explosives.

Figure 2.2 depicts the uptake of TNT in both the high and low concentration, treatment. TNT was rapidly removed from the solution with non-detectable levels at approximately 48 hours. As TNT disappeared, formation of 2-ADNT and 4-ADNT isomers was identified in the medium. The identification of the aminated transformation products in solution may explain that plant metabolism occurs at the root surface. However, it is also possible that these products can diffuse between the root tissue and growth media. Although these explanations are most likely, microbial transformation cannot be excluded because axenically grown plants were not used. TNT losses due to sorption to glassware or abiotic reactions in solution were negligible. This can be seen by the steady concentration of TNT in the unplanted controls.

The uptake of TNT by *P. deltoides* was significantly faster than that of RDX (Figure 2.3). Unlike TNT, only a portion of the initial RDX was removed from solution. However, 65 to 75% of the initial application was removed during the incubation time, which concluded at 264 hours. Again, sorption and abiotic phenomena were absent due to the lack of RDX removal in the unplanted controls. Transformation of RDX in solution was not considered as a mechanism to explain a decrease in RDX concentration. Although the transformation products of RDX are unknown, no unidentifiable peaks were detected during analysis. The increase in RDX concentration at 66 and 72 hours in the

low concentration treatment may have been a result of sampling error. As stated in the experimental design, daily transpirational losses were replenished by the addition of fresh water. An attempt was made to insure the total volume of growth medium remained at two liters. It is possible that the growth medium was not completely filled to the appropriate volume, which would result in an increase in concentration.

In contrast to the plant-catalyzed disappearance of TNT and RDX, HMX was not significantly removed in either treatment when compared to unplanted controls (Figure 2.4). Some loss of HMX from the solution may be attributed to sorption to root tissue. However, the difference between the initial HMX concentration in controls and planted reactors resulted from dilution error. Similar to RDX, an increase in HMX concentration was observed at 66 and 72 hours, apparently due to variability in replenishing the reactor solutions to the same volume.

Previous work with organic compounds has established relationships between plant uptake and the compound's physical and chemical properties (Briggs *et al.* 1982; Burken and Schnoor 1998). These studies suggest that uptake is directly related to the compound's hydrophobicity and the logarithm of the compound's octanol-water partition coefficient, log K_{ow}. This factor is considered important because the compound must pass the symplast of the endodermis in order to be translocated from the roots (Trapp *et al.* 1994). Organic compounds with log K_{ow} values ranging from 0.5-3.0 are considered thermodynamically favorable to enter root tissues (Schnoor *et al.* 1995). However, a log K_{ow} of 1.8 is considered to be the optimum (Briggs *et al.* 1982). Chemicals with log K_{ow} > 1.8 can enter root tissues but cannot enter the xylem and be translocated (Burken and Schnoor 1998). The findings of this study support these predictive relationships (Figure

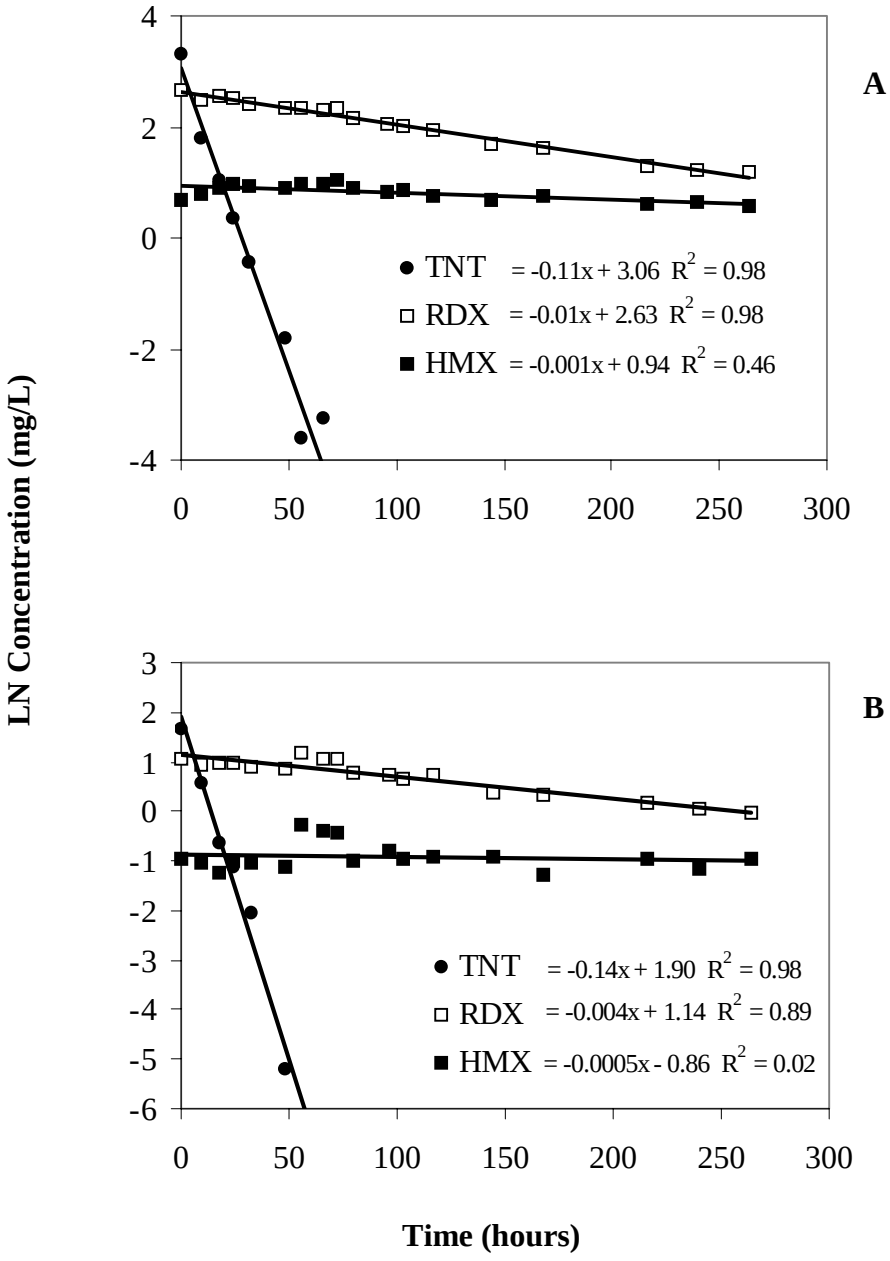

Figure 2.5: Kinetics of 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) removal from hydroponic solution by *Poplus deltoides* where *n* = 3 and initial concentrations were A) TNT = 30 mg/L, RDX = 15 mg/L, HMX = 2 mg/L and B) TNT = 6 mg/L, RDX = 3 mg/L, HMX = 0.5 mg/L.

2.5). TNT, which has the highest log K_{ow}, 1.86, was rapidly removed from the growth media, followed by RDX and HMX, with log K_{ow} values of 0.87 and 0.42 respectively TNT was found to have a pseudo-first-order rate constant of 0.10 hr^{-1}. RDX was removed at a rate of 0.01 hr^{-1} while HMX concentration remained constant with a 0.001 hr^{-1} removal rate. This relationship can also explain the presence of ADNT within the growth medium. Once TNT is taken into the root tissue, plant metabolism produces the 2-ADNT and 4-ADNT isomers. The production of these transformation products results in an increase in log K_{ow}. This increase in membrane permeability may result in diffusion back into the growth medium in order to obtain equilibrium.

At the end of incubation, the plants were harvested and processed for extraction to determine if the compounds had been metabolized within the plant. The fate of RDX and HMX exposed to hydroponically grown *P. deltoides* is shown in Table 2.1. A majority of the initially applied HMX remained in the nutrient solution. The intracellular fraction represents what was recovered during analysis of plant tissue extractions. The relatively small amount of HMX that was found in plant tissues was exclusively localized to root

Table 2.1: The fate of RDX and HMX after exposure to hydroponically grown *Poplus deltoides* where *n* = 3 and initial concentrations were A) RDX = 15 mg/L, HMX = 2 mg/L and B) RDX = 3 mg/L, HMX = 0.5 mg/L.

Fractions	Fraction of Initial RDX		Fraction of Initial HMX	
	A	B	A	B
Nutrient Media	0.21	0.33	0.96	1.02
Intracellular unbound	0.04	0.06	0.01	0.00
Intracellular bound	na.	na.	na.	na.
Total Recovered	0.25	0.39	0.97	1.02

tissue. This data suggests that HMX was adsorbed on the surface of the root tissues rather than being actively taken up from solution. In contrast, the average RDX recovery for the two treatments ranged from 25 % to 39 %. Approximately 21 % and 33 % of the initially applied RDX remained in the growth media of both high and low concentration treatments. However, only four and six percent of the initially applied RDX was recovered within the plant. This data suggests that approximately 60 % to 75 % of the initially applied RDX was unaccounted for. Figure 2.6 depicts the localization of RDX within various plant tissues. Bioaccumulation is suggested to have occurred because up to 62 % of the recovered compound was detected in leaf tissue, as opposed to 30 % and 8 % in stem and root tissues. Two unidentifiable peaks appearing more polar than RDX were detected in leaf, root, and stem tissues. These peaks could represent RDX transformation products. The portion of RDX unaccounted for could also represent intracellular, bound residues consisting of conjugated, unextractable transformation products. This explanation would be consistent with the "green liver" model of plant metabolism. These possible explanations may suggest that RDX was transformed within the plant. This result is significantly different than what has been observed in previous research (Harvey *et al.* 1991; Thompson *et al.* 1999). These findings could be the result of longer incubation times, or faster enzymatic transformation using this species.

At the conclusion of this study, there was no identifiable TNT or ADNT remaining in the growth media. Tissue analysis did not result in further TNT recovery in either the leaf, root, or stem. However, approximately 0.07 mg of ADNT was recovered from the root tissue. This finding was consistent with the "green liver" model and the

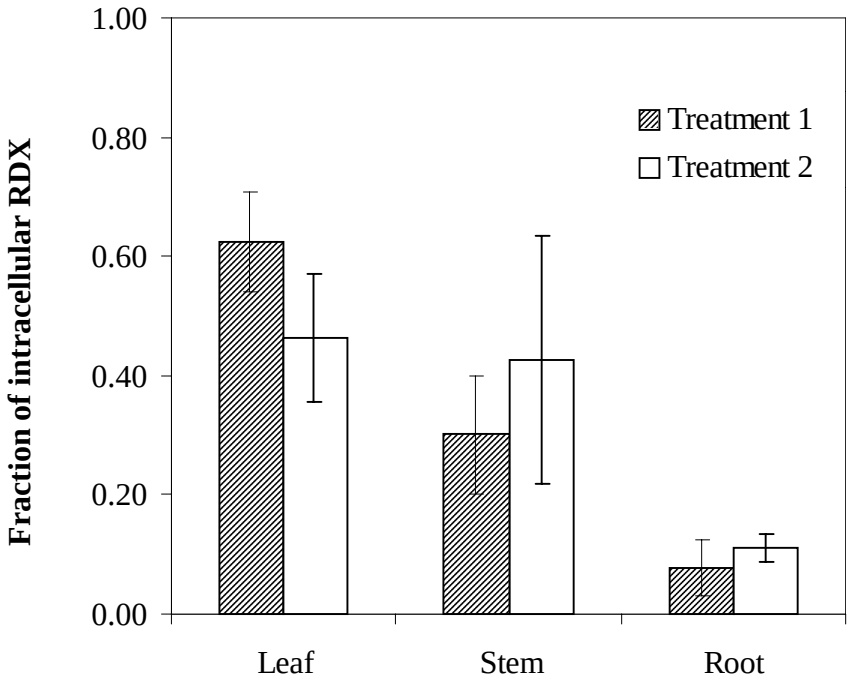

Figure 2.6: Fraction of intracellular hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) that localized in the root, stem, and leaf tissue of hydroponically grown *Poplus deltoides* where *n* = 3 and error bars represent ± 1 standard deviation. Treatment 1 and 2 represent initial RDX concentrations of 15 mg/L and 3 mg/L.

work of other researchers (Palazzo and Leggett 1986; Hughes *et al.* 1997; Thompson *et al.* 1998b). These studies suggest that TNT was transformed by plants enzymatically, resulting in the formation of 2-ADNT and 4-ADNT. Over time, further transformation produced bound residues which were irreversible (Bhadra *et al.* 1999b). The fact that little ADNT was recovered in this study supports the theory that conjugation and sequestration processes may result in unextractable products.

CONCLUSIONS

The uptake of the explosives TNT, RDX, and HMX was investigated by growing *Poplus deltoides* in hydroponic solutions containing three levels of explosives amendment ranging from 0 to 30 mg/L. Plants were harvested after approximately 11 days growth in the contaminated media and separated in to leaf, root, and stem. No visual symptoms of stress or growth inhibition were identified for the respective treatments. TNT was rapidly removed from the nutrient media in approximately 48 hours. The aminated isomers 2-ADNT and 4-ADNT were detected in solution as TNT was removed. The disappearance of RDX was significantly slower than TNT, with approximately 20 to 30% of the initial application remaining in solution after 11 days. In contrast to the plant uptake of TNT and RDX, HMX was not removed from the contaminated nutrient media. The uptake of these compounds appeared to be correlated to their hydrophobicity, measured by log K_{ow}. Tissue extractions did not result in the recovery of TNT within the plant. Approximately 0.07 mg ADNT was recovered in root tissues. Approximately 4 to 6% of the initially applied RDX were found in plant tissues. The majority of the RDX, 60%, localized in the leaf tissues. The total recovery of RDX averaged 25 to 40%. The fraction of TNT and RDX that was unaccounted for, coupled

with the detection of unidentified peaks during HPLC analysis suggest plant catalyzed transformation.

If phytoremediation is to be used as a mechanism to remove explosives from contaminated sites, future work needs to examine the toxicity of transformation products including bound residues. Radiolabeled studies are also needed to trace the fate of conjugated metabolites. And finally, to truly understand explosives transformation, studies are needed that determine the enzymes involved.

CHAPTER III

UPTAKE OF THE EXPLOSIVES TNT, RDX, AND HMX FROM CONTAMINATED SOIL BY *POPLUS DELTOIDES*

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To be submitted to *Int. J. Phytoremed*

ABSTRACT

The production, storage, and dismantling of conventional military munitions has led to 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) contamination in soil at many US facilities. In this study, batch equilibrium experiments were used to calculate adsorption model parameters for soil obtained from the Naval Surface Warfare Center (Crane, IN). In most cases, the data was best fit using the Freundlich equation. *Poplus deltoides* seedlings were also grown in columns containing explosives contaminated soil. Trees were harvested for up to eight weeks. Results suggest that TNT, and HMX were unavailable for plant uptake. RDX in leaf tissues increased linearly with concentrations reaching 120 g/kg.

INTRODUCTION

Many facilities operated by the U.S. Department of Defense (DOD) are contaminated with explosives due to the manufacture, storage, and disposal of conventional munitions. The most common compounds found at these sites are 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and their transformation products. Typically these contaminants are present in soil, groundwater, and in some cases surface water. The U.S. ceased production of explosives at many sites in the 1980s, however, contamination still exists due to the environmental recalcitrance of these compounds. The presence of these substances in the environment is a problem because they are carcinogenic and mutagenic (Won *et al.* 1976; Etnier 1989). In many cases, the transformation products may be more toxic than the parent compound (Hawari *et al.* 1999). Human ingestion of explosives can

result in nausea, vomiting, unconsciousness, epileptic seizures, and death (Ruchoft 1945; Kaplan *et al.* 1965). In the past, during ordnance production, munitions wastewater was discharged and held in lagoons to allow the solid wastes to settle before releasing the water to nearby streams (Harvey *et al.* 1991). When this practice was discontinued, the lagoons evaporated, leaving these areas highly contaminated with munitions and their transformation products (Klausmeier *et al.* 1973; Traxler 1974). In many locations, precipitated or solid-phase explosives are still present in surface soils (Singh *et al.* 1998). Typically, explosives contaminated soil is excavated and composted or incinerated for remediation purposes. However, these methods are expensive, produce unusable ash, and due to concerns over air quality and the toxicity of transformation products, have poor public acceptance (Tan *et al.* 1992; Hundal *et al.* 1997; Jarvis *et al.* 1998). Phytoremediation is a promising, new technology, which utilizes plants to remove and transform organic contaminants. Previous studies have shown that some plant species have the ability to remove explosives from hydroponic media (Palazzo and Leggett 1986; Harvey *et al.* 1991), and it has also been speculated that enzymatic activity within plants, catalyze explosives transformation (Schnoor *et al.* 1995). Although the results are promising, hydroponic studies do not provide information on the ability of plants to remove explosives from soil systems where soil particle interactions control solutions**.**

Adsorption isotherms are typically determined by equilibrating subsamples of a soil at constant temperature with a number of aliquots of solutions containing different concentrations of the adsorbate of interest, then determining the amount remaining in solution after adsorption. The time to equilibrium must be determined for accurate results, and this will vary. The plot of amount adsorbed against the equilibrium

concentration is termed the adsorption isotherm. Although most adsorption isotherms are nonlinear, the adsorption process may often be assumed linear for low solute concentrations or narrow concentration ranges (Equation 3.1). Additional models used to describe nonlinear adsorption are the Freundlich (Equation 3.2) and Langmuir (Equation3.3).

$$S = K_d C \quad [3.1]$$

$$S = K_d C^{1/n} \quad [3.2]$$

$$S = \frac{kKC}{1 + KC} \quad [3.3]$$

In these equations, S represents the amount adsorbed to the soil, expressed in mass of adsorbate per mass of soil, and C is the equilibrium solution concentration. K_d is the partition coefficient, expressed in volume of solvent per mass of soil, and n is an empirical constant. K is the adsorption coefficient related to enthalpy, and k is the solid phase concentration corresponding to all available sites being filled. Parameters for the nonlinear, Freundlich and Langmuir isotherms were determined by fitting the data to their linearized forms using Equation 3.4 and Equation 3.5 respectively.

$$\log S = \log K_d + 1/n \, (\log C) \quad [3.4]$$

$$\frac{1}{S} = \frac{1}{k} + \frac{1}{kKC} \quad [3.5]$$

Adsorption of nonpolar organic compounds has been shown to be highly correlated with the organic matter content of the soil (Hassett *et al.* 1980). The partition coefficient between organic matter and water, K_{OC}, is often used to describe this correlation. The K_{OC} of a specific compound can be predicted using Equation 3.6.

$$\log K_{OC} = 0.72 \log K_{OW} + 0.49 \quad [3.6]$$

Using the predicted value for K_{OC} in Equation 3.6, experimentally determined values of K_d, can be compared to calculated values, where OM represents the fraction of organic matter in the soil (Equation 3.7)(Schwarzenbach and Westall 1981).

$$K_d = OM \times K_{OC} \quad [3.7]$$

The specific objectives of this study were to 1) develop adsorption coefficients for TNT, RDX, and HMX on selected soil, and determine the model which best fit data for these compounds and 2) evaluate uptake of these compounds from a field-contaminated soil.

MATERIALS AND METHODS

Site Description

The Naval Surface Warfare Center Crane (NSWCC), Indiana occupies 25,278 hectares in southwest Indiana, primarily Martin County. The average annual precipitation for this area of the state is 113.67 centimeters. The average monthly temperature for the months of October thru March is 4°C and April thru September is 22°C. Current munitions disposal occurs at an area known as the Ammunition Burning Ground (ABG), that consists of approximately 20 hectares in the eastern part of the NSWCC and is located in the northwest corner of Section 28 and the southwest corner of Section 21, Township 5N, Range 3W.

The NSWCC has disposed of ammunition and explosives-contaminated waste materials since the early 1940's by open burning at the ABG. The largest quantities were destroyed between 1956 and 1960, when 15,000 pounds per day of smokeless powder and 48,000 pounds per day of high explosives were burned. From 1970 to 1981, over 10,000 major weapons were destroyed. The Environmental Protection Division,

NSWCC has monitored groundwater quality quarterly at the ABG by the sampling of 72 wells and seven natural springs since September 1987. Contaminants detected at these locations were the explosives TNT and RDX, the volatile organic TCE, and the heavy metal barium (Murphy 1994).

Soil Collection

Explosives contaminated soil was obtained from the NSWCC in April 2001. At the time of sampling, an environmental consulting firm had been contracted by the NSWCC to implement a composting procedure to decontaminate the soil. The soil that was retrieved was a composite sample that was excavated from the top meter of earth in the Ap and Bt horizons. It was determined to be a fine, silty, mixed, mesic, ultic, hapludalf. An uncontaminated soil sample was also obtained. Sample collection occurred in an area adjacent to the ABG along side a small stream. The soil was best described as a coarse, silty, mixed, non-acidic, mesic, aeric, fluvaquent. Samples were obtained from the upper 50 cm in the Ap and C horizons. The soil was sieved on site with a 1.27 cm wire mesh to remove any large rocks or debris. After returning to the laboratory, the soil was homogenized in a cement mixer. A portion of the explosives-contaminated soil was mixed (1:1 m/m) with washed sand. After mixing, the samples were extracted to determine the initial explosives concentration. The chemical and physical characteristics of the two soils are shown in Table 3.1. Nutrient concentrations were determined by double acid extraction (Mehlich 1953). Percent organic matter was determined by the "loss on ignition" method for three hours at 360°C (Ben-Dor and Banin 1989). Percent sand, silt, and clay was determined using the hydrometer method

Table 3.1: Properties of soils obtained from NSWCC where A represents an explosives contaminated sample and B represents an uncontaminated sample.

Sample		A	B
TNT	**(mg/kg)**	8.36	0
ADNT	**(mg/kg)**	4.02	0
RDX	**(mg/kg)**	1417.78	0
HMX	**(mg/kg)**	222.12	0
Ca	**(kg/ha)**	6171.3	4188.6
K	**(kg/ha)**	163.6	97.0
Mg	**(kg/ha)**	735.6	474.0
Mn	**(kg/ha)**	77.4	101.6
Na	**(kg/ha)**	118.5	104.9
P	**(kg/ha)**	106.9	12.0
Zn	**(kg/ha)**	7.9	30.6
OM	**%**	2.49	3.28
Sand	**%**	34	42
Silt	**%**	60	44
Clay	**%**	6	14
Soil Type		Silt Loam	Loam
CEC		18.52	13.42
pHb		7.8	7.75
pHw		7.6	7.4

(Bouyoucos 1951). Cation Exchange Capacity (CEC) was calculated based on the sodium and lime index.

Chemicals and reagents

Analytical grade acetonitrile (CH_3CN) was purchased from Sigma-Aldrich, (St. Louis, MO). Calcium chloride ($CaCl_2$) was purchased from J.T. Baker (Phillipsburg, NJ). DD-6 alumina (Al_2O_3) was obtained from Alcoa Port Allen Works (Port Allen, LA). The magnesium silicate adsorbent Florisil was purchased from Fisher Scientific (Pittsburgh, PA). High Pressure Liquid Chromatography (HPLC) external standards for TNT, RDX, HMX were purchased from Accustandard (New Haven, CT). The TNT, RDX, and HMX standards were 1.0 mL volumes containing 1.0 mg of the compound in an acetonitrile-methanol (1:1 v/v) mixture. Since separation of 2-ADNT and 4-ADNT could not be achieved using HPLC a standard mixture was used. The 2-ADNT and 4-ADNT standard mixture was a 500.0 mL volume containing 500.0 mg/L of each compound. Solid TNT, RDX, and HMX were obtained from the U.S. Army Center for Environmental Health Research (Fort Detrick, MD).

Experimental Design

To determine required equilibrium times, soil samples were air-dried to a constant weight and ground to pass through a 30-mesh sieve. Samples were prepared in duplicate using a 1:3 soil to solution ratio and initial concentrations of 5 mg/L TNT, RDX, ADNT, and 1 mg/L HMX. Three grams of soil and 10 mL of solution were equilibrated in scintillation vials. Controls without soil were also prepared. The vials were shaken in the dark on a wrist action shaker table at 25ºC for 24, 48, 72, or 96 hours. After incubation, the samples were centrifuged at 5000 rpm for five minutes. The supernatant

was removed, placed in a disposable syringe, and filtered through a 0.20 μm Arcodisc® syringe filter for analysis.

Based on the results of the initial sorption kinetics, the samples were incubated for 24 hours. Adsorption isotherms were produced by batch equilibrium. Samples were prepared in triplicate using a 1:3 soil to solution ratio and initial concentrations ranging from 0 to 50 mg/L of the respective explosives. The concentration of explosives adsorbed to soil was determined as the difference between initial and final solution concentrations.

Planted Soil Column Study

Eight-inch Eastern Cottonwood (*Poplus deltoides*) cuttings were purchased from Greenwood Nursery (McMinnville, TN). To evaluate uptake from contaminated soil, twenty soil columns were constructed using 10.2 cm diameter polyvinyl chloride (PVC) pipe cut to a length of 75 cm. Female adapters were attached to the bottom of each column using PVC primer and cement. PVC screw caps had holes drilled in them and fitted with T-shaped tubing connectors using silicone caulk. In order to prevent soil from exiting the column, a filtration system, consisting of washed gravel and polyester fiber, was placed in the bottom of each column. Half of the columns were packed with the contaminated soil while the remaining half were packed with the soil that had been mixed with sand.

The study consisted of four randomized treatments, maintained under controlled greenhouse conditions. Sixteen columns were planted with single cottonwood cuttings that were established in potting soil for two months. Two columns containing the soil-sand mixture and two columns containing contaminated soil were used as unplanted controls. Four cuttings were grown in potting soil to observe the health of the trees in the

absence of explosives. Plants were harvested every seven days, separated into leaf, root, and stem, and stored until analysis at -40°C. After harvesting the trees, soil columns were excavated and soil samples were recovered from 0 to 10 cm, 25 to 35 cm, and 45 to 55 cm. The soil samples were air dried to a constant weight and extracted for explosives.

Plant Tissue and Soil Extractions

Extraction of explosives from harvested plants utilized methods established by Larson *et al*. (1999). After plant harvest, leaves, roots, and stems were separated and weighed. Then, each set of tissue samples was washed using deionized water and blotted dry with paper towels. The samples were subsequently lyophilized for 24 hours using a Labconco Freeze Dryer 4.5 (Kansas City, MO). After the drying process, the plant material was weighed and ground to a 30-mesh particle size using a Thomas Scientific (Philadelphia, PA) Wiley mill. Duplicate 0.5 g sub-samples from each set of tissues were dried in a forced air oven at 110°C for 24 hours. In a scintillation vial, duplicate 0.25 g samples of the dried plant material was covered with 10 mL acetonitrile, vortex swirled for one minute, and shaken on a wrist action table for 24 hours at 25°C. Each replicate was centrifuged for five minutes at 5000 rpm to pellet the insoluble plant residue. Then, five milliliters of the supernatant was removed and filtered. Filters were prepared by placing a small amount of glass wool in a serological pipette. The glass wool was covered with 0.5 g florisil, which was then covered with 0.5 g alumina. Five milliliters acetonitrile was passed through the filter, then discarded. Five milliliters of the supernatant was passed through the filter and collected in a clean scintillation vial. Then, 5 mL of acetonitrile was passed through the filter and collected in the same vial. The vials were vortex swirled for one minute and the resulting solution was removed and

placed in a disposable syringe and filtered through a 0.20 μm Acrodisc® syringe filter. The first milliliter was discarded, retaining the rest for HPLC analysis.

In order to test the accuracy of the extraction method, a standard that contained 5.0 mg/L of TNT, RDX, HMX, and ADNT respectively, was prepared in triplicate. Each replicate was handled and analyzed following the method described above. Compound recovery was determined to be 100.9 ± 3.3 %, 110.6 ± 0.8 %, 92.4 ± 0.9 %, and 100.6 ± 2.0 % for the respective explosives.

Soil extractions were conducted following EPA Method 8330 (EPA 1998). Each sample was ground with a mortar and pestle and passed through a 20-mesh sieve. Two grams of soil was placed in 20 mL scintillation vials and covered with 10 mL acetonitrile. Samples were vortex swirled for one minute, sonicated for one minute using a Braun-Sonic U (Allentown, PA), and shaken on a wrist action table for 24 hours at 25°C. Afterwards, the samples were centrifuged at 5000 rpm for five minutes. Five milliliters of supernatant was removed and combined with 5.0 mL of 5.0 g/L calcium chloride in a scintillation vial. Each vial was vortex swirled for one minute, then placed in a disposable syringe, and filtered through a 0.20 μm Arcodisc® syringe filter. The first three milliliters was discarded, retaining the remainder for HPLC analysis.

HPLC Conditions

Identification and quantification of TNT, RDX, HMX, 2-ADNT, and 4-ADNT was determined by reverse phase HPLC using a Hewlett Packard series 1100 quaternary pump, a diode-array UV detector set to 230 nm and 254 nm, and an autosampler with an injection volume of 15.0 μL. Separation was accomplished using a Hamilton PRP-1 column (10-μm particle size, 250mm × 4.1mm), a 12 minute isocratic run, with a mobile

phase consisting of an acetonitrile-water (60:40 v/v) mixture at a flow rate of 1.0 mL/min. All water was ultrapure and at least 18 MΩ.

The TNT, RDX, and HMX external standards were removed from their respective, break-seal vials and combined with 1.0 mL of the 2-ADNT and 4-ADNT mixture and 36.0 mL acetonitrile in a 40 mL volumetric flask, producing a stock solution which contained 25.0 mg/L of each compound. Calibration was achieved by diluting the stock solution with acetonitrile, producing standards of 20.0, 10.0, 5.0, 2.5, 1.0, and 0.5 mg/L. In order to retain analytical accuracy, all samples were injected twice, and a random standard, or deionized water was analyzed every 10 samples.

Results and Discussion

To determine when adsorption equilibrium occurred, each compound was incubated with the soil obtained from NSWCC for up to 96 hours. Figure 3.1 shows that TNT obtained equilibrium in approximately 24 hours. This result is inconsistent with previous studies that suggested TNT may require months to obtain equilibrium (Pennington and Patrick 1990). However, an incubation period of 24 hours is typically used to evaluate TNT adsorption (Pennington and Patrick 1990; Xue *et al.* 1995). Similar results were identified for ADNT, RDX, and HMX equilibrium (Figure 3.2 to 3.4).

Using an incubation period of 24 hours, batch equilibrium data was used to calculate adsorption model parameters (Figure 3.5 to 3.8). The adsorption of TNT by NSWCC soil is shown in Figure 3.5. The data was best fit using the Freundlich model when compared to the simple linear and Langmuir models. ADNT and HMX were also best fit using the Freundlich model in Figure 3.6 and Figure 3.7 respectively. Although RDX adsorption visually appears to follow a linear trend, the Langmuir model produced

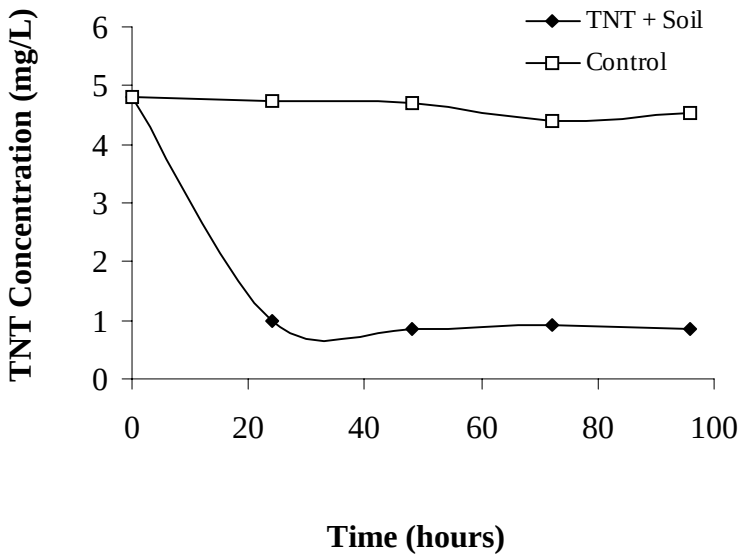

Figure 3.1: Equilibrium concentration of 2,4,6-trinitrotoluene (TNT) with NSWCC soil at 25°C

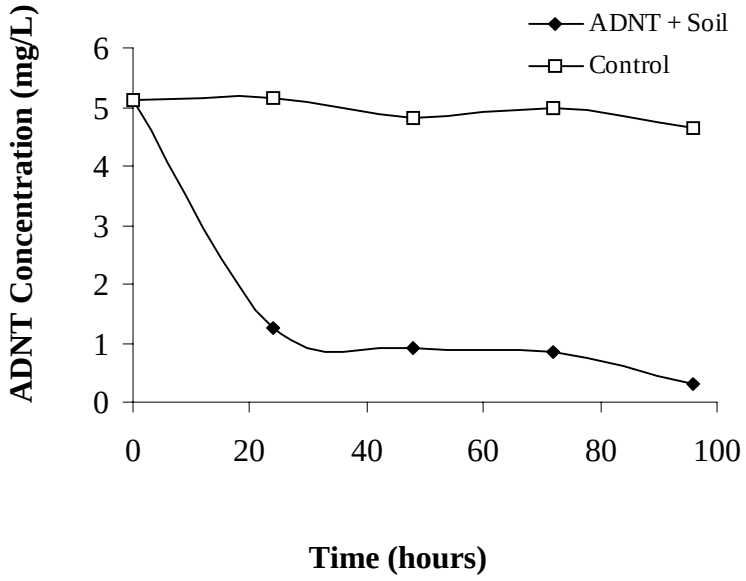

Figure 3.2: Equilibrium concentration of a mixture of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene (ADNT) with NSWCC soil at 25ºC

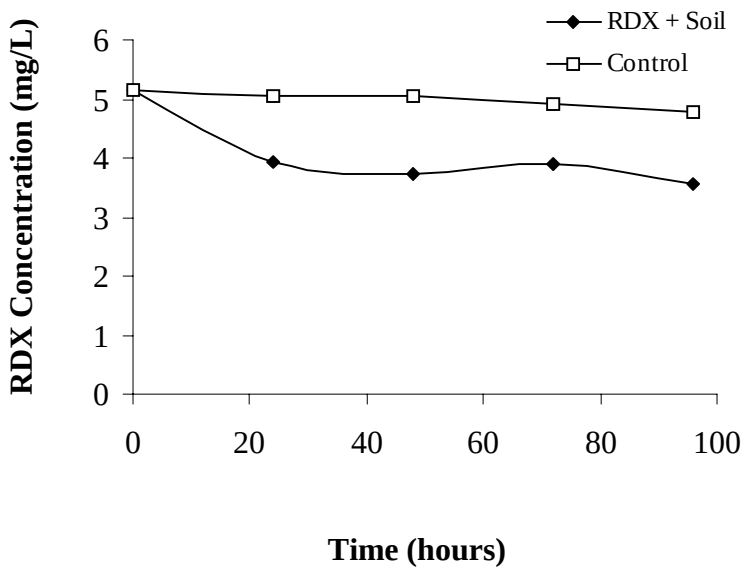

Figure 3.3: Equilibrium concentration of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with NSWCC soil at 25°C

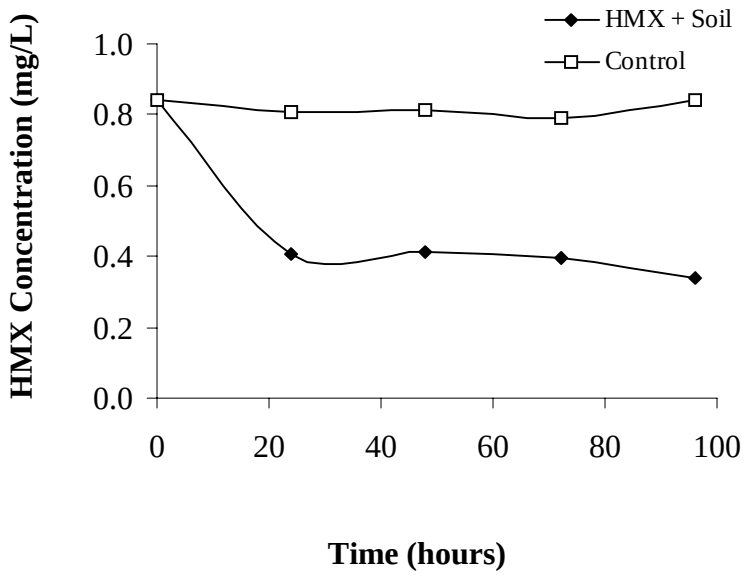

Figure 3.4: Equilibrium concentration of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) with NSWCC soil at 25ºC

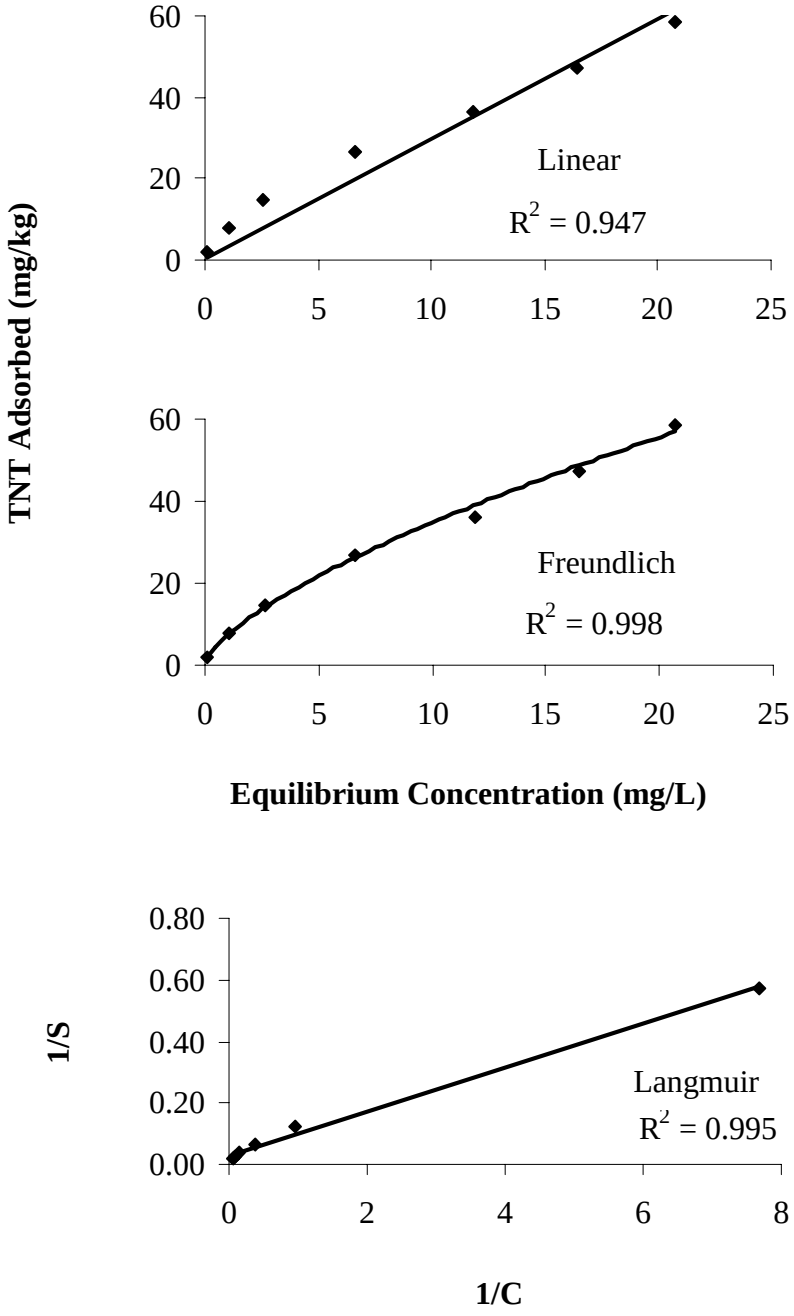

Figure 3.5: 2,4,6-Trinitrotoluene (TNT) adsorption isotherms using NSWCC soil at 25ºC where C represents equilibrium concentration (mg/L) and S represents amount adsorbed (mg/kg).

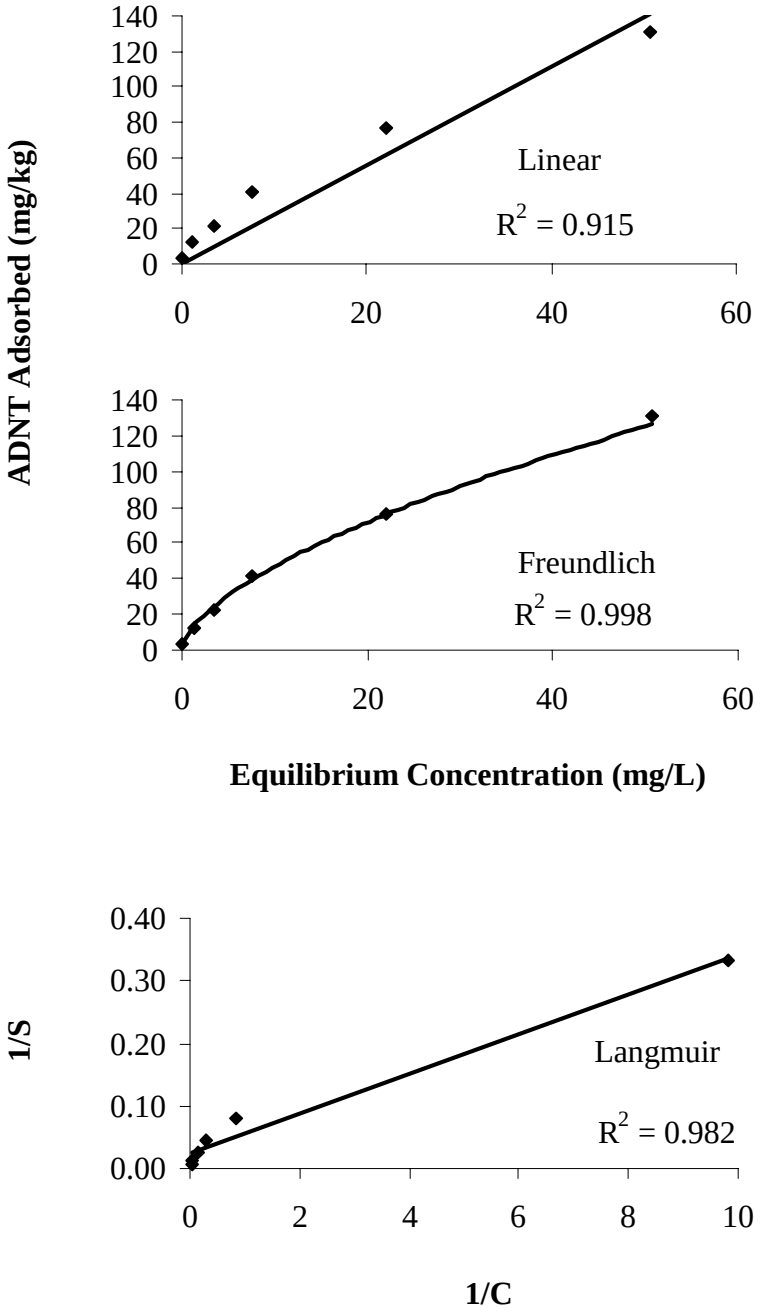

Figure 3.6: 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene (ADNT) adsorption isotherms using NSWCC soil at 25°C where C represents equilibrium concentration (mg/L) and S represents amount adsorbed (mg/kg).

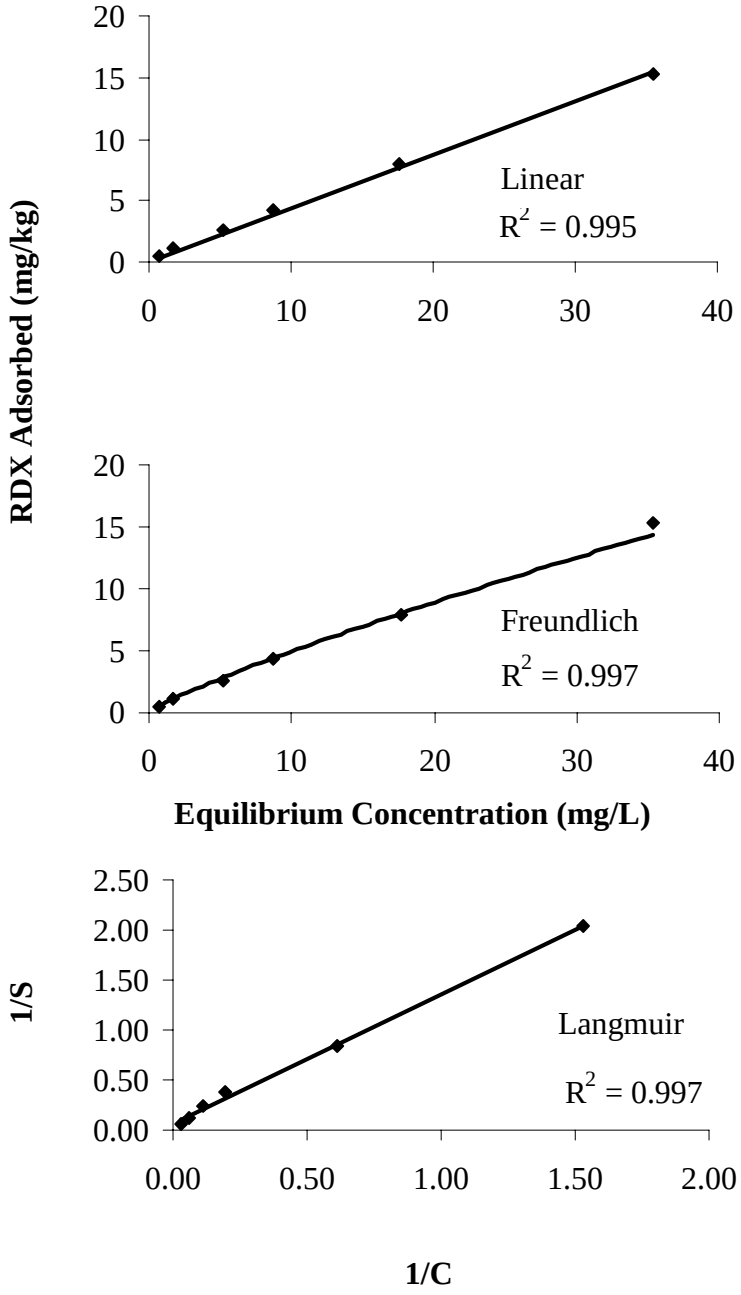

Figure 3.7: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) adsorption isotherms using NSWCC soil at 25ºC where C represents equilibrium concentration (mg/L) and S represents amount adsorbed (mg/kg).

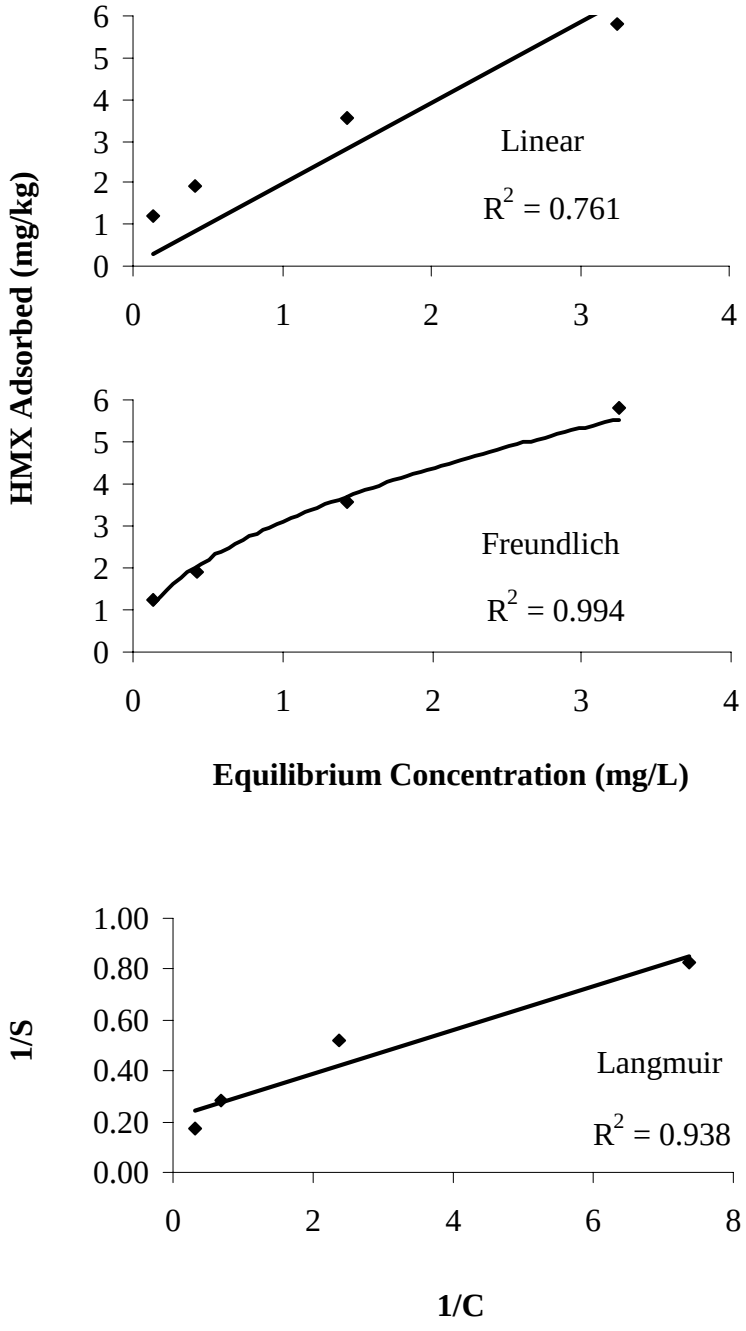

Figure 3.8: Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) adsorption isotherms using NSWCC soil at 25°C where C represents equilibrium concentration (mg/L) and S represents amount adsorbed (mg/kg).

Table 3.2: Calculated and experimentally determined adsorption model parameters

	Calculated K_d	Linear K_d	Linear R^2	Freundlich K_d	Freundlich R^2	Langmuir k	Langmuir R^2
TNT	2.21	2.96	0.947	7.31	0.998	105.26	0.995
ADNT	6.59	2.78	0.915	11.54	0.998	166.67	0.982
HMX	0.20	1.95	0.761	3.11	0.994	6.97	0.938
RDX	0.43	0.44	0.995	0.71	0.997	77.52	0.997

the best fit. For the equilibrium models given by Equations 3.1 to 3.3, parameter estimates are given in Table 3.2. These experimentally derived values are compared to the calculated values obtained from Equation 3.7. The calculated K_d values for TNT and RDX are very similar to the values obtained from the linear isotherms for those compounds. However, both compounds had better correlation using the Freundlich equation. The calculated K_d, for ADNT represents an average of the 2-ADNT and 4-ADNT isomers. The calculated K_d for ADNT and HMX are significantly different than those obtained experimentally. It appears that for these compounds, experimentally derived values are needed to describe adsorption. Using the data obtained from the adsorption isotherms, the general relationship of compound affinity to NSWCC soil is ADNT > TNT > HMX > RDX. This provides evidence that RDX will be most available for plant uptake, followed by HMX, TNT, and ADNT.

To test the availability of these chemicals for plant uptake, *P. deltoides* cuttings were grown in columns containing explosives contaminated soil. The treatments consisted of an unamended soil and a soil that was mixed (1:1 m/m) with washed sand to dilute the explosives concentration. Unplanted controls were also monitored. In both treatments, all replicate cuttings showed signs of toxicity after 10 days. The leaves showed signs of chlorosis and gradual necrosis of older leaves. However, throughout the

study all plants continued to produce new growth. Although the nutrient content of tissue samples was not analyzed, these symptoms resemble those of nutrient deficiencies. A possible explanation for growth inhibition is that the presence of explosives in the soil impacted the uptake of essential macro and micronutrients. Considering the nitrogen containing ring structure of RDX is similar to herbicides such as Atrazine, the presence of RDX in tissues may have caused the negative growth responses.

Tissue analysis resulted in the identification of RDX in leaf, root, and stem tissue of all replicates. The other explosives found in the soil were not identified within the plant. These findings support the results of the sorption study. The results of that study suggested that RDX exhibited the lowest affinity for the equilibrated soil. Bio-accumulation of RDX occurred in leaf tissues. RDX concentrations in leaf tissues increased linearly over the study period (Figure 3.9). Concentrations were highest in the cuttings grown in the contaminated soil amended with sand. The lower explosives concentrations in the amended treatment may explain the increased uptake capacity. RDX transformation may have occurred within the plant but cannot be verified since the transformation products of RDX are unknown. Although the cuttings were able to remove RDX in both treatments, the RDX concentration in soil did not significantly change over the study period (Table 3.3).

CONCLUSIONS

The affinity of TNT, ADNT, RDX, and HMX to soil obtained from NSWCC was determined by batch equilibrium. It was determined that ADNT adsorbed the most followed by TNT, HMX, and RDX. These results suggested that RDX would be the

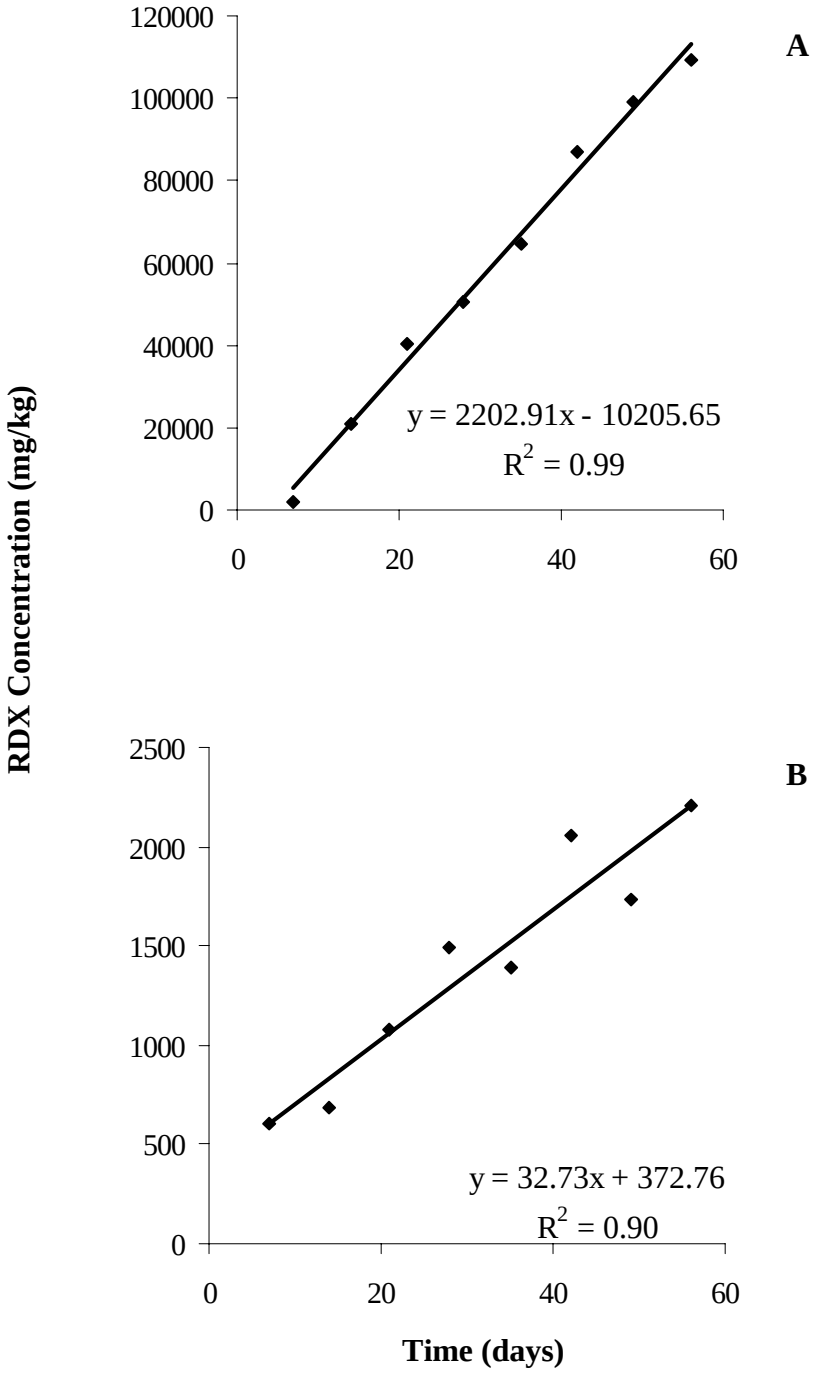

Figure 3.9: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) dry weight concentration in leaf tissues of *Poplus deltoides* grown in explosives contaminated soil where A represents soil amended with sand and B represents unamended soil.

Table 3.3: Explosives concentration in soil columns planted with *Poplus deltoides*, where A is unamended soil and B is soil amended with washed sand.

		A	B
Time (days)	**Chemical**	**(mg/kg)**	
1	**RDX**	1417.78 ± 39.70	529.28 ± 43.94
	HMX	222.12 ± 10.33	72.27 ± 1.77
	TNT	8.36 ± 0.03	4.31 ± 0.28
	ADNT	4.02 ± 0.08	1.89 ± 0.02
56	**RDX**	1249.91 ± 25.41	397.41 ± 0.39
	HMX	197.27 ± 0.37	70.93 ± 0.01
	TNT	7.35 ± 0.67	6.69 ± 0.10
	ADNT	1.46 ± 0.14	1.45 ± 0.08

most available for plant uptake. This finding was supported by the results of the column experiment. *P. deltoides* cuttings that were grown in explosives contaminated soil readily removed RDX. No other explosives were identified within the plant tissues. RDX was bioaccumulated in the leaves, with concentrations reaching 120 g/kg. Although *P. deltoides* removed RDX from contaminated soils, the cuttings showed signs of chlorosis and necrosis.

The biaccumulation of RDX in leaf tissues could pose a threat to wildlife. If phytoremediation of explosives contaminated soil is to be implemented, more research needs to focus on identifying RDX transformation products, determining the enzymes involved in metabolism, and investigate the irreversible binding of these chemicals to soil and plant tissues.

CHAPTER IV

CONCLUSIONS

The uptake of the explosives TNT, RDX, and HMX was investigated by growing *Poplus deltoides* in hydroponic solutions containing three levels of explosives amendment ranging from 0 to 30 mg/L. Plants were harvested after approximately 11 days growth in the contaminated media and separated in to leaf, root, and stem. No visual symptoms of stress or growth inhibition were identified for the respective treatments. TNT was rapidly removed from the nutrient media in approximately 48 hours. The aminated isomers 2-ADNT and 4-ADNT were detected in solution as TNT was removed. The disappearance of RDX was significantly slower than TNT, with approximately 20 to 30% of the initial application remaining in solution after 11 days. In contrast to the plant uptake of TNT and RDX, HMX was not removed from the contaminated nutrient media. The uptake of these compounds appeared to be correlated to their hydrophobicity, measured by log K_{ow}. Tissue extractions did not result in the recovery of TNT within the plant. Approximately 0.07 mg ADNT was recovered in root tissues. Approximately 4 to 6% of the initially applied RDX were found in plant tissues. The majority of the RDX, 60%, localized in the leaf tissues. The total recovery of RDX averaged 25 to 40%. The fraction of TNT and RDX that was unaccounted for, coupled with the detection of unidentified peaks during HPLC analysis suggest plant catalyzed transformation.

The affinity of TNT, ADNT, RDX, and HMX to soil obtained from NSWCC was determined by batch equilibrium. It was determined that ADNT adsorbed the most

followed by TNT, HMX, and RDX. These results suggested that RDX would be the most available for plant uptake. This finding was supported by the results of the column experiment. *P. deltoides* cuttings that were grown in explosives contaminated soil readily removed RDX. No other explosives were identified within the plant tissues. RDX was bioaccumulated in the leaves, with concentrations reaching 120 g/kg. Although *P. deltoides* removed RDX from contaminated soils, the cuttings showed signs of chlorosis and necrosis.

If phytoremediation is to be used as a mechanism to remove explosives from contaminated sites, future work needs to examine the toxicity of transformation products including bound residues. Radiolabeled studies are also needed to trace the fate of conjugated metabolites. And finally, to truly understand explosives transformation, studies are needed that determine the enzymes involved.

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