NITROAROMATICS CONTAMINATION AND DEGRADING BACTERIA

By

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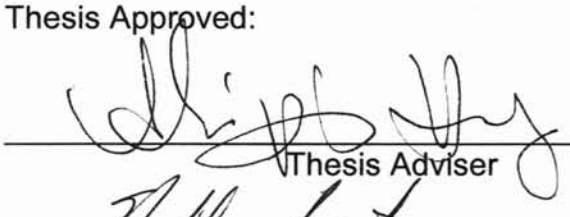

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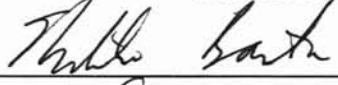

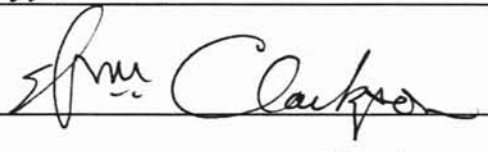

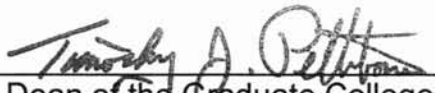

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FORMAT OF THESIS

This thesis is presented in a combination of the Archives of Environmental Contamination and Toxicology, Microbiology, Biodegradation and formats outlined by the Oklahoma Sate University graduate college style manual. This format allows the independent chapters to be suitable for submission to scientific journals. A complete chapter contains an abstract, introduction, materials and methods, results, discussion, and reference section.

Chapter I

INTRODUCTION

Nitramine and nitroaromatic compounds are a class of contaminants that are of environmental concern, with at least 20 nitroaromatics-contaminated sites on the National Priority List (Pennington, 1998). Most of these sites resulted from past or current activity in the manufacture and the load-assemble-package (LAP) processes of explosives (ATSDR, 1995). The principal contaminants polluting these areas include 2,4,6-trinitrotoluene (TNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). The latter is often used in combination with octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Pennington, 1998).

These contaminants inhibit the growth and survival of bacteria, fungi, and actinomycetes (Fuller and Manning, 1997; Klausmeier et al., 1973), soil fauna (Parmelee et al., 1993), and higher plants (Palazzo and Leggett, 1986; Peterson et al., 1996). For example, TNT is cytotoxic and genotoxic to bacterial and mammalian cells (Honeycutt et al., 1996; Lachance et al., 1998; Berthe-Corti et al., 1998). Soil microbial activities decreased with increasing TNT contamination (Gong et al., 1999). In fact, high levels of TNT contamination may even lead to inhibition of vegetative growth (Gong et al., 1999).

In addition to direct toxicity effects from the nitroaromatic compounds, these contaminants can also lead to nitrate toxicity, resulting from the

accumulation of removed nitro groups from ring structures during biodegradation (umbbd.ahc.umn.edu/tnt/tnt_map.html; umbbd.ahc.umn.edu /tnt2/tnt2_map.html). Elevated nitrate levels in drinking water may cause chronic toxic effects, such as methemoglobinemia in babies, and even cancer if bacteria in the stomach can form N-nitroso compounds (Hill 1999). Therefore, it is important to remove the contaminants from the environment.

Many remediation strategies have been evaluated for soils contaminated with nitroaromatic compounds. These include composting (Garg et al., 1991; Goodfellow et al., 1984; Pasti-Grigsby et al., 1996), bioslurry reactors (Shen et al., 2001; Funk et al., 1993; Hawthorne et al., 2000), natural attenuation (Khan and Husain, 2001; Filz et al. 2001), phytoremediation (Harvey et al., 1991; Bhadra et al., 2001), and incineration (www.epa.gov/region07/programs/spfd/nplfacts/Nebraska_army_ordinance. pdf; www.frtr.gov/matrix2/section414_26.html). For example, RDX uptake occurs readily in plants (Harvey et al., 1991; Bhadra et al., 2001) allowing the removal of this compound from soil by phytoremediation. Incineration can be costly and additional treatment of gases and combusted materials are required before disposal or release can occur. Natural attenuation is the most economical procedure, but it is slow, thus, poses a potential risk by allowing contaminants to leach into groundwater. Bioremediation by composting and slurry bioreactors are more effective, although more costly.

For example, windrow composting was used at Umatilla Army Depot site in Hermiston, Oregon, a National Priority Listed site. Original levels of contamination in the south lagoon were reported as high as 88,000 mg TNT kg^{-1} soil, 731 mg RDX kg^{-1} soil, and 127 mg HMX kg^{-1} soil, as well as lower levels of the metabolites 1,3,5-trinitrobenzene (TNB), 1,3-dinitrobenzene (DNB), 2,4-dinitrotoluene (DNT), and nitrobenzene (NB). Reduction to 30 mg kg^{-1} of TNT and RDX, the remedial goal, was accomplished by using composting (www.denix.osd.mil).

As a promising remediation strategy, bioremediation has gained considerable attention. Numerous studies have been conducted to reveal the biodegradation processes of nitroaromatic compounds. TNT may be broken down through an aerobic or anaerobic pathway (umbbd.ahc.umn.edu), leading to production of various metabolites that can enter a common biological process (umbbd.ahc.umn.edu), or humification to become part of the organic matrix of the soil (Bruns-Nagel et al. 2000). Although less is known about biodegradation of RDX and HMX, it has been shown that the biotransformation of cyclic nitramines occurs under both anaerobic (McCormick et al., 1981), and aerobic conditions (Binks et al., 1995). Once the cyclic structure is broken, these contaminants can be easily degraded to small nitrogen/carbon containing compounds (Melius, 1990), eventually leading to complete degradation.

There are two major limiting factors in bioremediation of soil contaminants: microbial activity and availability of the contaminants to the

microbes. TNT contamination could decrease microbial activity to undetectable levels and lead to eventually no vegetation (Gong, 1999). This implies that natural attenuation may not be a viable option. At low levels of contamination where microbial activity persists, additives, such as surfactants, can increase the effectiveness of bioremediation (Zappi et al., 1994; Boopathy and Manning, 1999), by enhancing the solubility of many contaminants, especially those that are hydrophobic (Mulligan et al., 2001). It has been shown that up to 70% of the TNT metabolites may irreversibly bind to the soil matrix (Shen et al., 1998). Surfactant molecules adsorbed on the surface of the contaminant cause repulsion between the head group of the surfactant and the soil particles (Deshpande et al., 1999), which may reduce adsorption to the soil matrix. Unfortunately, many surfactants can affect growth of microorganisms, animals and plants (Mulligan et al., 2001) by interfering with extracellular transport, inhibiting cell growth, or changing the membrane permeability (Volkering et al., 1998). Bioremediation is also limited by availability of microbial strains that can effectively degrade nitroaromatic compounds under a range of soil and environmental conditions. Therefore, the objectives of this study were (1) to determine the effects of nitroaromatic and nitramine contamination on soil chemical and microbiological properties; (2) to isolate microorganisms involved in the biodegradation of nitroaromatics in soils; and (3) to assess the impact of surfactants on the growth of nitroaromatic-degrading microorganisms.

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Chapter II

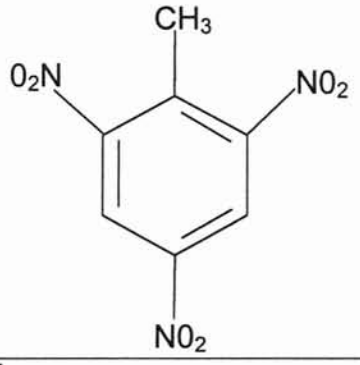
LITERATURE REVIEW

Explosive contamination is of concern because microorganisms, soil fauna and plant life are affected, but also potentially humans as well. To effectively remove these contaminants, a basic understanding of characteristics of each contaminant is required. This review includes the sources of nitroaromatic contaminants, pathways of degradation, microorganisms involved in degradation, biotic and abiotic treatment technologies for remediation, and factors impeding treatment efficiency.

Structure and Properties of TNT, RDX, and HMX

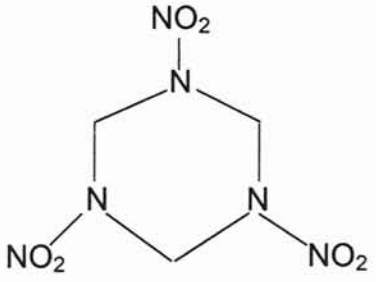
TNT, RDX, and HMX are xenobiotic chemicals developed for use as munitions. The DuPont Company first began producing TNT in 1880 (World Book Online Americas Edition, 2002), and during World War I it was the most used high explosive (Encarta Online, 2001). Extremely efficient new high explosives, such as RDX and HMX, were developed before and during World War II (Encarta Online, 2001). These munitions are similar in function but differ in chemical and physical properties (Tables 2.1, 2.2, and 2.3). One common characteristic of munitions is their relatively high nitrogen content, ranging from 18.5% to 37.8%.

Table 2.1. Chemical and physical properties of TNT.

Chemical	2,4,6-trinitrotoluene
Synonyms	TNT; sym-trinitrotoluene; trinitrotoluene; 2-methyl-1,3,5-trinitrobenzene;entsufon; 1-methyl-2,4,6-trinitrobenzene; methyltrinitrobenzene; tolite; trilit; s-trinitrotoluene; s-trinitrotoluol; trotyl; sym-trinitrotoluol; alpha-trinitrotoluol; trinitrotoluene
Molecular Formula	$C_7H_5N_3O_6$
CAS Number	118-96-7
Chemical Structure	
Density (g/cm^3)	1.65
Vapor Pressure (mm Hg)	5.51×10^{-6}
Molecular Weight	227.133 g/mol
Physical State	Pale yellow crystalline solid
Water Solubility (mg/L)	150
Partition Coefficient (K_{ow})	2.00

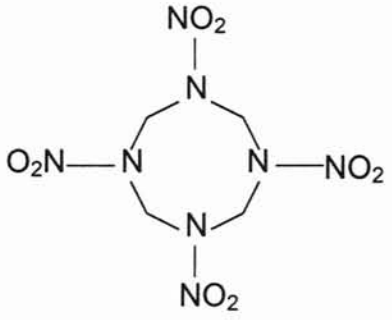
Modified from denix.osd.mil/denix/Public/Library/Remedy/Umatilla/umati02.html. Defense Environmental Network and Information Exchange

Table 2.2. Chemical and physical properties of RDX.

Chemical Name	Hexahydro-1,3,5-trinitro-1,3,5-triazine
Synonyms	RDX; cyclonite; cyclotrimethylenetrinitramine; hexogen; trimethylenetrinitramine; hexolite; 1,3,5-trinitrohexahydro-p-triazine;1,3,5-trinitrocyclohexane; trinitrohexahydrotriazine; hexahydro-1,3,5-trinitro-s-triazine; 1,3,5-trinitro-1,3,5-triazacyclohexane
Molecular Formula	$C_3H_6N_6O_6$
CAS Number	121-82-4
Chemical Structure	
Density (g/cm^3)	1.83
Vapor Pressure (mm Hg)	4.03×10^{-9}
Molecular Weight	222.117 g/mol
Physical State	White crystalline solid
Water Solubility (mg/L)	60
Partition Coefficient (K_{ow})	0.87

Modified from denix.osd.mil/denix/Public/Library/Remedy/Umatilla/umati02.html. Defense Environmental Network and Information Exchange

Table 2.3. Chemical and physical properties of HMX.

Chemical Name	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
Synonyms	HMX; octagen; HW4; LX 14-0; cyclotetramethylenetetranitramine; 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane; cyclotetramethylene tetranitramine
Molecular Formula	$C_4H_8N_8O_8$
CAS Number	2691-41-0
Chemical Structure	
Density (g/cm^3)	1.90
Vapor Pressure (mm Hg)	3.33×10^{-14}
Molecular Weight	296.156 g/mol
Physical State	White crystalline solid
Water Solubility (mg/L)	5
Partition Coefficient (K_{ow})	0.26

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Toxicology

For humans, TNT exposure through inhalation or dermal exposure can cause headaches, skin irritation, weakness, cartaracts, anemia, and liver injury (Merck's Index, 1983; McConnell and Flinn, 1946; Hathaway, 1985; Morton et al., 1976). Indirectly, metabolites of TNT, including 2,4-dinitrotoluene (DNT) and 2,6-DNT, have shown toxicity towards *Poecilia reticulzata*, with 14-hour LC_{50} values of 12.5 mg 2,4-DNT L^{-1} and 18 mg 2,6-DNT L^{-1} (Deneer et al., 1998). These metabolites formed in the uniary tract are mutagenic, and cause hemolysis, hepatotoxicity, and changes in hepatic enzyme levels (Dilley et al., 1982; Levine et al., 1990). It has been demonstrated that many munitions-related chemicals have been subjected to the Ames test for mutagenicity (George et al., 2001). Studies indicated that TNT is a direct acting mutagen, while the monoamino- and diamino-metabolites were less mutagenic, with the exception of 2-amino-4,6-DNT and 2,6-diamino-4-nitrotoluene, which demonstrated similar levels of mutagenicity to TNT (George et al., 2001). The U.S. Army (1980) has reported that TNT may leach to groundwater from soil. The U.S. Environmental Protection Agency (1990) has designated TNT to be a hazardous waste.

In contrast to TNT, which has been produced for over 120 years, widespread use of RDX and HMX began during World War II. Limited information is available on their toxicity to a biological system. Exposure to RDX can be through inhalation and dermal adsorption (Kaplan et al., 1965). RDX has been found in human cerebrospinal fluid, plasma, urine, and feces

(Woody et al., 1992). RDX is classified as a class C carcinogen, and has been known to cause unconsciousness and epileptiform seizures (Harvey et al., 1991). Solubilized RDX can leach into the groundwater as well as bind strongly with soil (U.S. Army, 1986). The U.S. EPA identified it as a regulated hazardous compound (Bhadra et al., 2001) and a minimal risk level (MRL) of 0.03 mg kg^{-1} day^{-1} has been set (Mclellan et al., 1998). The MRL is an estimation of a dose that is not likely to cause adverse systemic effects. The water solubility of RDX is 60 mg L^{-1} (Table 2.2). Assuming a person weighs 75 kg and drinks 2 liters of RDX-saturated water a day, this person would be taking 1.6 mg kg^{-1} day^{-1}, which is 53-fold of MRL.

No adverse effects were reported in workers exposed to unknown concentrations of HMX. However, animal studies indicate that it may be harmful to the liver and central nervous system if swallowed, inhaled, or contacts the skin (ATSDR, 1995). The solubility of HMX is 5 mg L^{-1} in water (Table 2.3), which is only one-twelfth of that of RDX and one-thirtieth of TNT. Nevertheless, the U.S. Army (1979) reports that HMX is likely to leach into the groundwater, especially in sandy soils. Therefore, the U.S. EPA (1990) regulates waste containing HMX as hazardous, and has set restrictions on landfill disposal (1991). Later, the U.S. EPA Office of Drinking Water set a limit of 0.4 mg L^{-1} for drinking water (1994).

K_{ow}, an indicator of toxicity, is the partition coefficient for a compound between n-octanol and water (Newman, 1998). It is used to reflect the lipophilicity of a compound and to imply relative partitioning of a xenobiotic

between aqueous phases of the environment and lipids in the organism. In a simple K_{ow} approach, the organism is envisioned as a membrane-enveloped pool of emulsified lipids, and uptake as well as elimination of hydrophobic organic compounds is controlled by permeation through the aqueous phase (Newman, 1998; Connell, 1990). The lower the K_{ow} value, the higher partitioning in the aqueous phase, and the more bioavailable the contaminant is to living organisms (Donnelly et al, 1994). This has been observed widely in benthic species in sediment (Landrum and Robbins, 1990). On the other hand, uptake increases and excretion decreases with increasing K_{ow} values in the log K_{ow} values of 3 to 6 (Newman, 1998). The increased uptake and reduced excretion imply increasing in biomagnification, another measure of toxicity. Biomagnification is the increase in the concentration of a contaminant as it progresses to higher trophic levels of the food chain (Newman, 1998). Predators tend to live longer than prey and therefore, have more time to accumulate a higher concentration of contaminants (Moriarity, 1983). An example of this transfer through trophic webs has been seen with the pesticide DDT. It has been suggested that Log K_{ow} values must fall between 4 (Connolly and Pedersen, 1988) and 6 (Gobas et al., 1993) to cause biomagnification. Below 4, decreased uptake and increased elimination (excretion) rates limits the capacity for biomagnification. Biomagnification is inhibited by low assimilation efficiencies above 6 (Thomann, 1989). The K_{ow} of TNT, RDX, and HMX are 2.00, 0.87, and 0.26, respectively

(www.denix.osd.mil). The K_{ow} values of TNT, RDX and HMX do not fall into this range, thus, it is unlikely that biomagnification will occur.

Sources of TNT, RDX, HMX, and Other Nitroaromatics

Nitroaromatics, such as TNT, RDX and HMX, are common military explosives that are found in soils at sites such as destruction ranges, explosive dumping grounds, manufacturing processes, firing ranges and ammunition factories (Shen et al., 2001). Production grade RDX contains impurities such as significant amounts of HMX and trace amounts of 1-acetylhexahydro-3,5-dinitro-1,3,5-triazine (Rosenblatt et al., 1991). HMX becomes the major product and RDX the impurity by modification of the RDX manufacturing process (Binks et al., 1995).

Although site contamination is due to current or past activity in the manufacture and the load-assemble-package (LAP) processes of explosives (ATSDR, 1995), large quantities of nitroaromatic compounds are also released to the environment from manufacture and use of pesticides, dyes, plastics, and pharmaceuticals (Davis et al., 1997). RDX is also used as a rodenticide (Wildman and Alvarez, 2001). Other nitroaromatic pesticides include dinoseb, dinitrocresol, parathion, and methylparathion, which are intentionally released into the environment during agricultural use (Spain, 2000). Nitrophenols and nitrotoluenes are used extensively as feedstocks in industry and are often released into surface water from waste streams (Spain, 2000). In contrast to

In the aerobic reduction pathway TNT is reduced to two different dinitrotoluenes (DNT) to an aminodinitrotoluene (ADNT) (Figure 2.1). In the anaerobic pathway, TNT is reduced to hydroxyaminodinitrotoluene (HADNT), ADNT, diaminonitrotoluene (DANT) and triaminotoluene (TAT). Once TAT is formed, it can be further degraded to an intermediate in the toluene pathway or organic acids (Figure 2.2). Thus, this represents a more complete degradation of TNT. Moreover, humification of the metabolites from aerobic/anaerobic systems can lead to formation of humic compounds and become part of the organic matrix of the soil (Bruns-Nagel et al. 2000) (Figure 2.3).

The cyclic structure of RDX and HMX can be broken down under both aerobic and anaerobic conditions (Binks et al., 1995; McCormick et al., 1981), then degraded to small nitrogen/carbon containing compounds and eventually to CO_2 (Melius, 1990). In an anaerobic degradation:

RDX ➡ MNX ➡ DNX ➡ TNX

In this pathway, RDX is reduced to hexahydro-1-nitroso-3,5,-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5,-triazine (TNX). The nitroso compounds undergo further degradation to unstable hydroxyl derivatives (Hawari, 2000). The only detailed biotransformation pathway published for cyclic nitramines

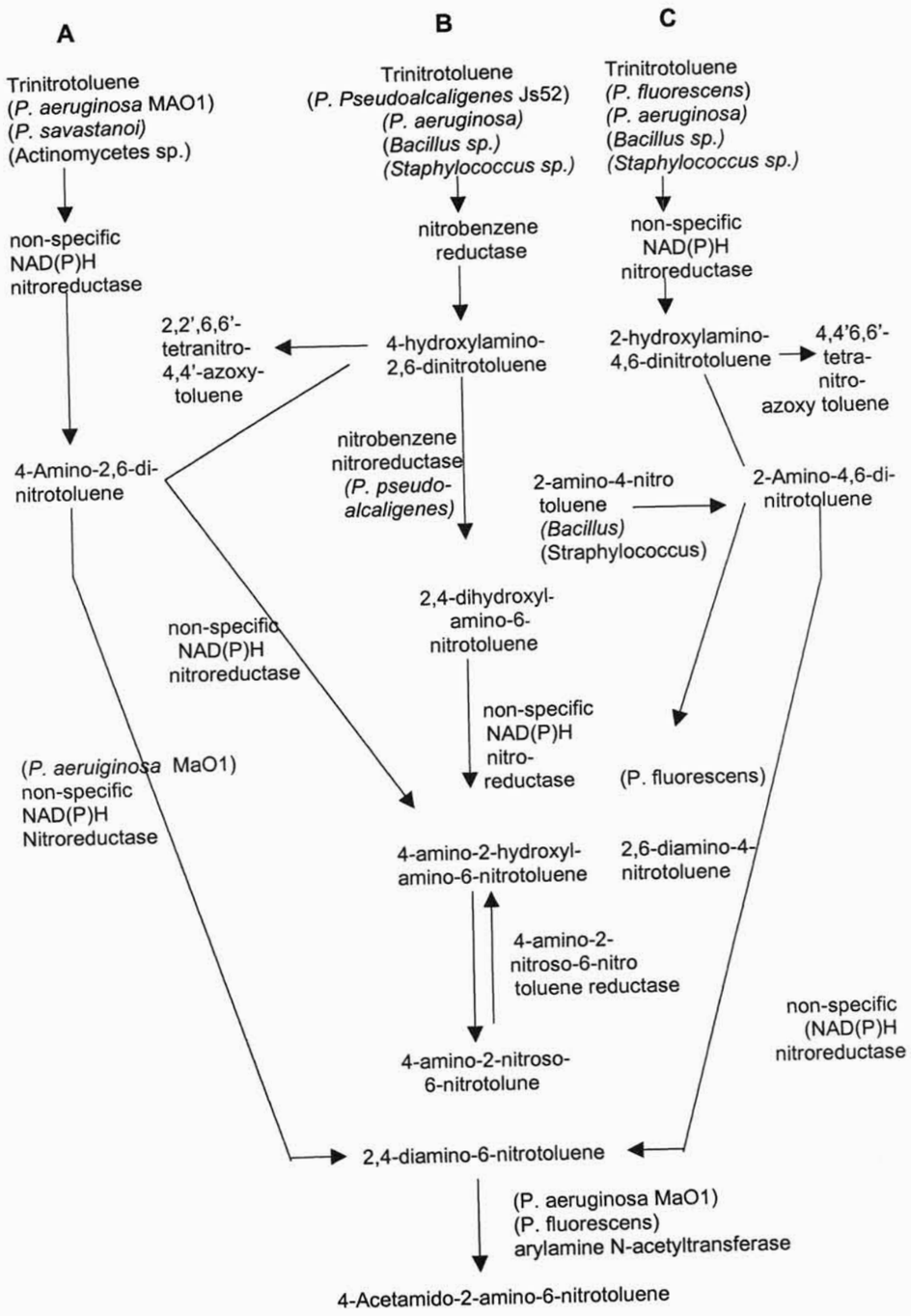
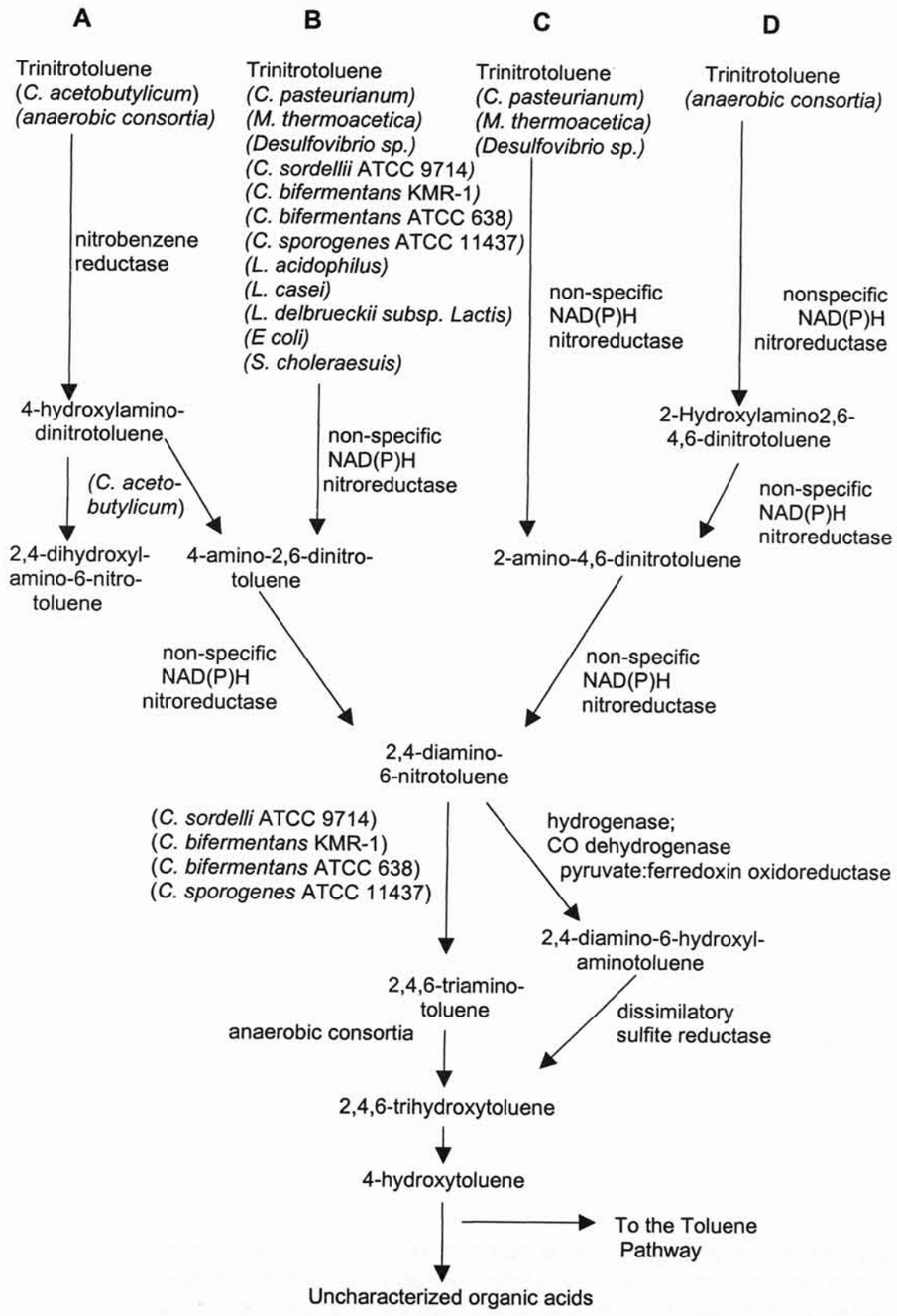


Figure 2.1. Three possible aerobic degradation pathways of 2,4,6-trinitritoluene. Pathway A involves two bacteria or actinomycetes using nonspecific NAD(P)H nitroreductases during the entire process with a final product 4-acetamido-2-amino-6-nitrotoluene. Pathway B involves only bacterial species, but uses some specialized enzymes such as nitrobenzene reductase and 4-amino-2-nitroso-6-nitrotoluene reductase. Degradation is less complete, with the final product 4-amino-2-nitroso-6-toluene. Pathway C involves bacteria as well, but uses nonspecific NAD(P)H nitroreductases like pathway A. Pathway C differs in the metabolites produced during degradation, ending with the same final metabolite as pathway A. (Adapted from the University of Minnesota Biocatalysis / Biodegradation Database. umbbd.ahc.umn.edu/tnt/tnt_map.html, 2002)

Figure 2.2. Four possible anaerobic degradation pathways of 2,4,6-trinitrotoluene. Pathway A involves a bacterial species or consortia that uses specific enzymes such as nitrobenzene reductase during the reduction of TNT. The metabolites produced include the dead end intermediate 2,4-dihydroxyl-amino-6-nitrotoluene and 4-amino-2,6-dinitrotoluene which joins into the main pathway. Pathway B has many possible bacterial species which also produces the metabolite 4-amino-2,6-dinitrotoluene, but with the a nonspecific NAD(P)H nitroreductase that merges into the center pathway. Pathway C involves three possible bacterial species using a nonspecific NAD(P)H reductase producing 2-amino-4,6-dinitrotoluene which merges with the main pathway. Pathway D uses an anaerobic consortia and a nonspecific NAD(P)H nitroreducases producing the intermediate 2-amino-4,6-dinitrotoluene which merges into the center pathway. After the four pathways merge the final products of degradation include 4-hydroxytoluene, which can be used in the toluene pathway or uncharacterized organic acids. (Adapted from the University of Minnesota Biocatalysis / BiodegradationDatabase. umbdd.ahc.umn.edu/tnt2/tnt2_map.html).



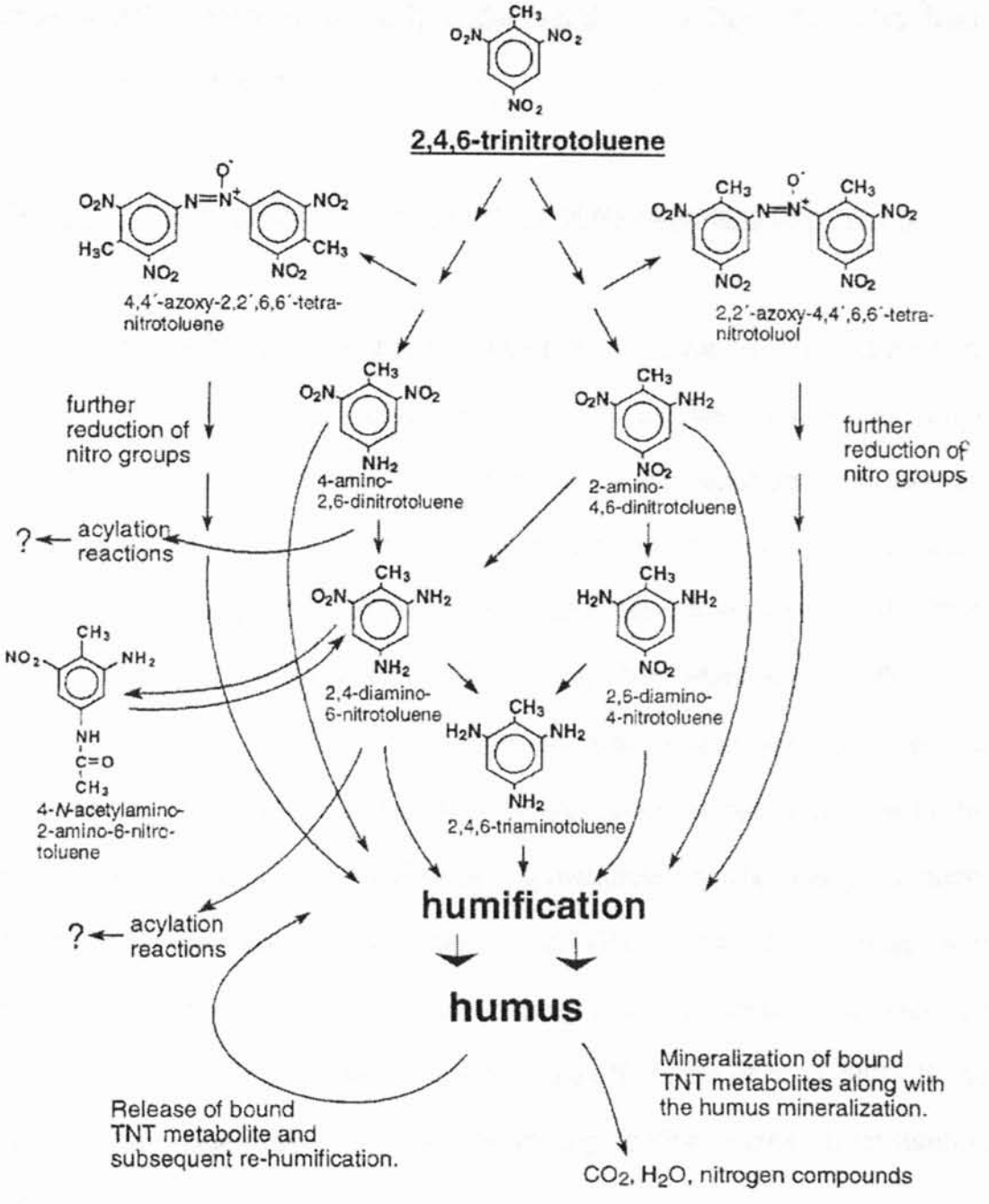


Figure 2.3. Humification of 2,4,6-trinitrotoluene occurring under both aerobic and anaerobic composting conditions. Humification can occur with many metabolites during the reduction of TNT (Bruns-nagel, 2000).

was in 1981 by McCormick et al. (Figure 2.4). More recently, a partial biodegradation pathway of RDX under aerobic conditions has also been reported (Binks et al., 1995).

Microorganisms Involved in the Degradation of Nitroaromatic Compounds

Degradation of nitroaromatic compounds involves the activities of aerobic, facultative, and anaerobic bacteria as well as some fungi. Transformation of TNT by microorganisms has been reported under aerobic and anaerobic conditions with complete reduction of one or more nitro groups observed (Crawford, 1995; French et al., 1998; Marvin-Sikkema et al., 1994; Michels et al., 1995; Preuss et al., 1995; Spain, 1995; Stahl et al., 1995).

An example of the aerobic pathway was researched with DNT, a precursor of TNT and once the most widely manufactured explosive in the world (Nishino et al, 2000). DNT is also a metabolite of TNT biodegradation. *Burkholderia* sp. reduces nitro groups of DNT using dioxygenase and monooxygenase enzymes. Ligninolytic fungi produce extracellular enzymes that increase the mineralization of TNT as well (Fritsche et al., 2000). Since all fungi are obligate aerobes, this type of degradation occurs under aerobic conditions.

An example of the anaerobic pathway is the use of *Clostridia,* obligate anaerobes, which have enzymes that are capable of rapid reduction of nitro groups (Ahmad and Hughes, 2000). Studies have demonstrated transforming

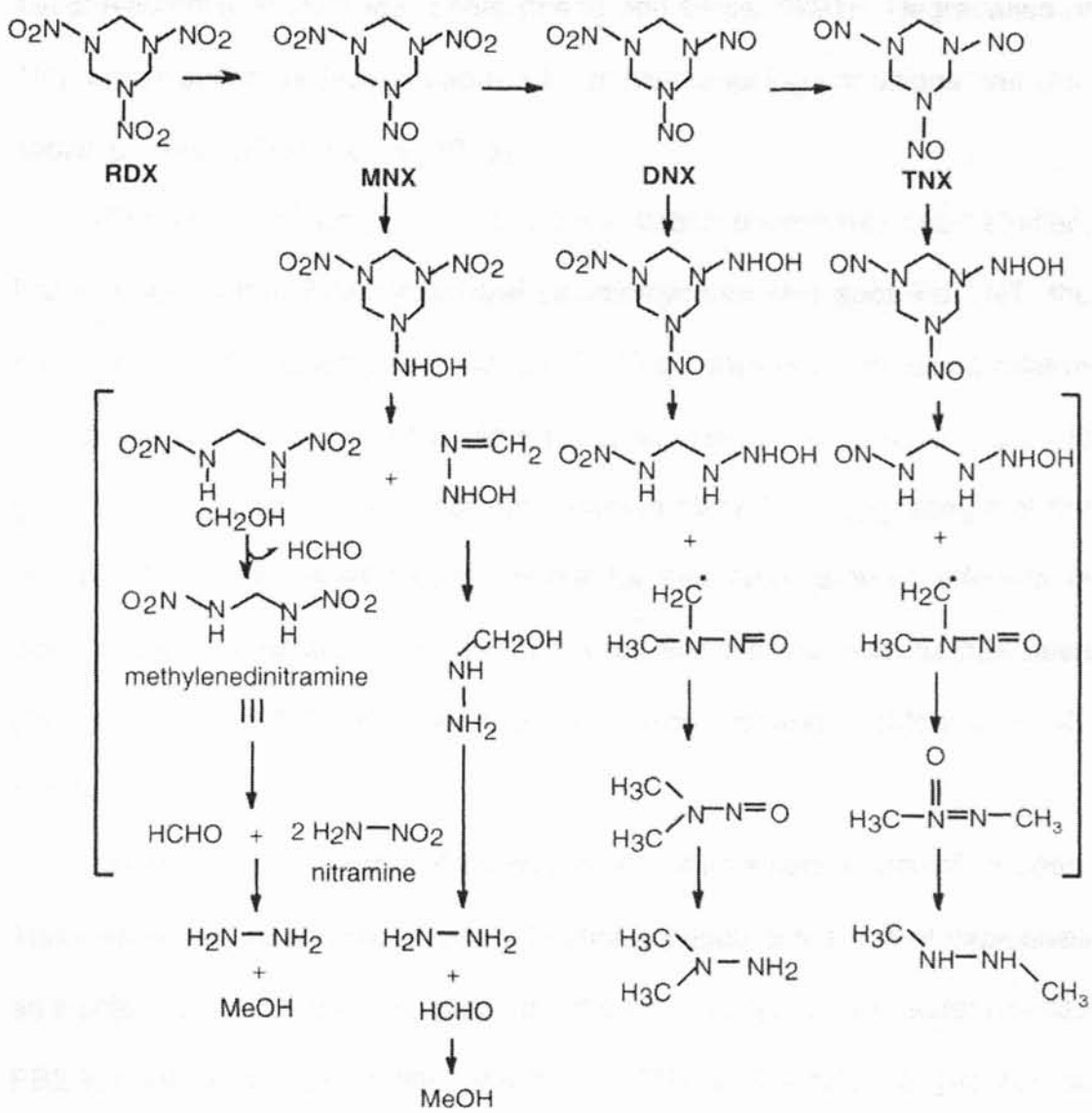


Figure 2.4. The only comprehensive degradation pathway known for RDX. Some known degradation metabolites include MNX, DNX, and TNX. The products in brackets were not observed during the experiment (McCormick et al., 1981).

TNT to an intermediate TAT under anaerobic conditions (McCormick et al., 1976; Naumova et al., 1989; Schackmann and Müller 1991). Degradation of TNT under sulfate reducing followed by nitrate reducing conditions has also shown promise (Preuss et al., 1993).

The use of nitroaromatics as a sole carbon source has been studied. For example, when *Ectomycorrhizal basidiomycetes* was supplied TNT, the rate of biotransformation under nitrogen limiting conditions decreased relative to nitrogen sufficient conditions (Meharg et al., 1997). Also, no decrease in growth was observed under short-term carbon starvation suggesting that the use of TNT as the sole carbon source for this fungi is most effective in biodegradation (Meharg et al., 1997). Also, *Serratia marcescens* has been shown to degrade TNT as a sole source of carbon and energy (Montpas et al., 1997).

Some microorganisms also use explosives as a sole source of nitrogen. This option may be the most favorable choice because the use of explosives as a sole source of carbon may lead to nitrogen toxicity. *Enterobacter cloacae* PB2 is capable of slow aerobic growth with TNT as the sole nitrogen source (French et al. 1998). *Stenotrophomonas maltophilia* PB1 was isolated under aerobic, nitrogen limiting conditions with RDX as a sole source of nitrogen for growth from soil (Binks et al., 1995). In fact, the disappearance of RDX in crude cell extracts from *S. maltpphilia* PB1 provides evidence of a specific enzyme for RDX degradation (Binks et al., 1995).

Nitroaromatic-degrading microbes have also been found in anaerobic sewage sludge, which were able to degrade the explosives in bioslurry reactors in a relatively short period of time (Shen et al., 2001). *Actinomycetes* sp. isolated from uncontaminated environments could also cause biotransformation of TNT (Pasti-Grigsby, 1996) and be used as an inoculant.

An additional carbon source may also be beneficial if indigenous bacteria or inoculants cometabolize the contaminants. *Phanerochaete chrysosporium* needed another carbon source to efficiently reduce TNT concentrations (Rho et al., 2001). Co-substrates, including glycerol, glucose (Boothpathy et al., 1994; Daun et al., 1998; Stahl & Aust, 1994; Sublette et al., 1992), toluene (Tharakan & Gordan, 1999), and acetate (Zappi et al., 1995), have been used to simulate the transformation of explosives. Although many microbes have shown promise for use in bioremedation of nitroaromatic-contaminated soil, many more remain unidentified.

Biotic Treatment of Explosives Contamination

Current biotic practices include composting (Garg et al., 1991; Goodfellow et al., 1984; Pasti-Grigsby et al., 1996;), bioslurry reactors (Shen et al., 2001; Funk et al., 1993; Hawthorne et al., 2000), phytoremediation (Harvey et al., 1991; Bhadra et al., 2001) and natural attenuation (Khan and Husain, 2001; Filz et al. 2001).

Composting

Many of the microorganisms discussed could be used in composting. *Actinomycetes* are important decomposers in composts (Goodfellow et al., 1984), and have been reported to transform TNT into recalcitrant intermediates, regardless of any previous TNT exposure (Pasti-Grigsby et al., 1996). However, none of the actinomycetes grew when TNT was supplied as the sole source of nitrogen or carbon in any medium, which indicated a nutrient-rich environment can help overcome the inhibition of growth resulting from the presence of TNT (Patsi-Grigsby et al., 1996).

Composting explosives is a proven technology. Windrow composting was used at the Umatilla Army Depot. During treatment of this superfund site many lessons were learned about composting explosives. Amendment composition affects biodegradation rates, and a soil-loading rate of 30% soil and 70% amendments produced the best results. Moisture content should remain at 60% water holding capacity, and appropriate temperatures allowed thermophilic organisms to enhance biodegradation. Mixing the windrow for aeration allows more rapid degradation, higher operating temperatures, and reduced odor. However non-aerated windrows exhibited equal or better removal of contaminants (www.denix.osd.mil/denix/Public/Library/Remedy/Umatilla/umati02.html).

Bioslurry Reactors

The degradation rate by microorganisms can be increased by the use of mechanically mixed, bioslurry reactors. The reactor "enhances the mass transfer between the solid and aqueous phases and the contact between the microorganisms and the hazardous compounds" (Shen et al., 2001). In addition, nutrients and microbial activities can easily be manipulated and are therefore not site specific (Shen et al., 2001). A simple static vessel was used to treat soils contaminated with explosives with subcritical (hot) water. The treatment was successful with complete degradation at 275°C, although TNT degradation started at somewhat lower temperatures (Hawthorne et al., 2000). In contrast, RDX persistence has been observed, indicating that indigenous microorganisms were unable to degrade RDX in soil slurry reactors containing multiple explosives (Funk et al., 1993). Supporting evidence has shown that RDX mineralization in a reactor was inhibited by the presence of TNT (Shen et al., 1998).

Phytoremediation

Phytoremediation, also known as plant-driven bioremediation, could provide a cost-effective approach to remediation of explosives, and has generated increasing interest recently (Medina et al., 1998). RDX uptake occurs readily in hydroponic bush bean plants, and RDX accumulates in aerial parts like the leaves (Harvey et al., 1991). *M. aquaticum* plants were able to significantly remove RDX, but were unable to remove HMX, while *C. roseus*

plants had the intrinsic capability to remove both RDX and HMX (Bhadra et al., 2001).

Natural Attenuation

The term "monitored natural attenuation" is used when cleanup of a site is reliant on natural attenuation (Khan and Husain, 2001). Policymakers have shifted to a risk-based approach to become more cost effective, which in many cases includes the use of natural attenuation (Khan and Husain, 2001). It is recognized that there are significant risks associated with soil and groundwater contamination in application of natural attenuation (Khan and Husain 2001). Thus, a new method, combining natural attenuation with barrier controls, has been developed for cleaning up groundwater. This method is called barrier-controlled monitored natural attenuation (BCMNA). This strategy uses a low-permeability, nonreactive barrier to release contaminants at a rate that optimizes natural attenuation of contaminated groundwater (Filz et al., 2001). It has also been determined that this technology reduced the contaminant concentrations to safe levels (Filz et al., 2001). A similar approach, using a barrier, such as a liner, to stop leacheate from reaching the groundwater could be used with soil remediation. However, a notable uncertainty on fate of the explosives in the environment exists, and it is difficult to monitor the type and extent of on-site contamination (Hawari, 2000). This strategy needs to be further strengthened.

Bioaugmentation

Another option to consider when degrading TNT, RDX, HMX, and their metabolites, is addition of exogenous microorganisms. Bioaugmentation with special microorganisms may be useful if the indigenous community lacks the catabolic potential of certain pollutants (Blumenroth and Wagner-Döbler, 1998). Under natural environmental conditions, however, inoculated microorganisms must compete with indigenous microbes for survival. Research has proven that adding amendments, such as preferred nutrients and C source, to the environment provides a temporary advantage for the introduced strain, which would potentially ensure survival of the inoculant. Otherwise, the inoculant must be able to exploit a specific niche that is not occupied by the naturally occurring community (Blumenroth and Wagner-Döbler, 1998). For example, an additional carbon source proved to be beneficial when establishing an inoculant (Blumenroth and Wagner-Döbler, 1998).

Abiotic Treatment of Explosives Contamination

Abiotic practices currently used to treat explosives-contaminated soils include iron reduction (Brannon et al., 1998; Shen et al., 2001; Wildman and Alavarez 2001; Blowes et al., 1995; Kaplan et al., 1996), alkaline hydrolysis/oxidation (Emmrich, 2001) electrolysis (Rodgers et al., 2001) and incineration (http://www.epa.gov/region07/programs/spfd/nplfacts/Nebraska_

army_ordnance.pdf; http://www.frtr.gov/matrix2/section414_26.html).

Iron Reduction

Research has shown the presence of 4-amino-2,6-dinitrotoluene in sterilized soil slurry, suggesting a portion of TNT was transformed due to chemical degradation explained by the presence of reductive components such as sulfide (Shen et al., 2001). Abiotic transformation of TNT is a function of pH, and the possibility of a Fe^{2+} reduction pathway in reduced soils has been confirmed (Brannon et al., 1998).

Zero-valence iron, Fe(0), can be used as a reactive material to remove redox-sensitive contaminants (Wildman and Alavarez 2001). Fe(0) can be buried as a broad continuous curtain (Blowes et al., 1995), or injected as colloids (Kaplan et al., 1996). Usually Fe(0) is used to treat chlorinated solvents and hexavalent chromium plumes (Vidic and Pohland, 1996), although Singh et al. (1998) reported that Fe(0) is capable of RDX reduction in contaminated soil. Permeable reactive Fe(0) barriers can intercept and degrade RDX plumes (Wildman and Alvarez, 2001). The rate and extent of RDX biotransformation can be enhanced by anaerobic microorganisms that feed on cathodic hydrogen (Wildman and Alvarez, 2001). Nitro groups attached to the RDX ring attract electrons released during iron corrosion (Wildman and Alvarez, 2001). This electron transfer might remove the nitro groups from the ring structure, releasing NO_2^-, which can be used as a nitrogen source for bacteria (Singh et al., 1998).

Alkaline Hydrolysis

Alkaline hydrolysis is another possible abiotic treatment method. TNT content dropped to almost zero, and the content of the intermediates, aminodinitrotoluenes (2A-4,6-DNT, 4A-2,6-DNT) and 2,4-dinitrotoluene (2,4-DNT), decreased by 75% using this technology (Emmrich, 2001). However, dinitrotoluenes may undergo only partial hydrolysis through this pathway. The reaction rate of TNT hydrolysis increased with increasing TNT concentration in the solid and aqueous phases. This method is sufficient at pH 12, which shortens treatment time, and is recommended when TNT is the only nitroaromatic contaminant in the soil (Emmrich 2001).

Electrolysis

The use of electrical energy to drive unfavorable reactions, electrolysis, is emerging as a possible remediation technology (Rodgers et al., 2001). Electrochemical techniques have benefits such as the use of electrons as reagents instead of harmful chemicals (www.icpt.nrc/projects/electr_e.html). It offers higher energy efficiency versus other techniques such as photolysis (treatment with light) or thermal treatment (Rodgers et al., 2001). Although drawbacks can occur, such as parasitic reactions including water electrolysis, which lowers the energy efficiency by competing with the electrolysis of the contaminants, TNT and its metabolites decreased approximately 80% by using electrolysis (Rodgers et al., 2001).

Incineration

The U.S. Army Corps of Engineers implemented the on-site incineration of 16,500 cubic yards of contaminated soils at the Nebraska Ordnance Plant by supplying oxygen and using temperatures between 870-1200°C. Combustion has been known to remove 99.99% of the contamination, although the gases and combustion residue generally require further treatment before disposal (www.epa.gov/region07/programs/spfd/nplfacts/nebraska_army_ordnance.pdf; www.frtr.gov/matrix2/section414_26.html). Even though incineration is effective, treatment of highly contaminated soils by direct incineration is expensive and dangerous (Emmrich, 2001). Among the technologies listed above, the most commonly used treatments are incineration and composting (Tables 2.4 and 2.5).

Factors Impeding Treatment

Research has demonstrated that nitroaromatic compounds may adsorb specifically and reversibly to clay minerals, but nitramine compounds like RDX do not (Haderlein et al., 1996). TNT has the highest affinity to clay minerals (Haderlein, 1996), and eventually will become humus in soil (Bruns-Nagel, 2000). This may lead to less TNT leaching into the groundwater. Soil properties such as soil pH, soil type, cation exchange capacity (CEC), particle size, soil permeability and the type of contaminants can promote or discourage

Table 2.4. Examples of remediation technologies used at installations where soil remediation is complete.

Installation	NPL	Remediation Technology	Amount of Soil	Year Completed
Alabama AAP	X	Incineration	32,000 yd^3	1994
Cornhusker AAP	X	Incineration	46,000 tons	1998
Camp Mead		Incineration	16,000 yd^3	1998
Weldon Spring		Incineration	58,000 yd^3	1998
Louisiana AAP	X	Incineration	119,000 tons	1989-90
Savanna AD	X	Incineration	42,000 tons	1992
Camp Navaho (NG)		Composting	4,000 yd^3	1999
Umatilla AD	X	Composting	15,000 yd^3	1999

AAP=Army Ammunition Plant
AD=Arm Depot
NG=National Guard
NPL=National Priority List
Modified from Broder MF and Westmoreland RF (1998).

Table 2.5. Examples of remediation technologies used at installations where cleanup activity is in progress.

Installation	NPL	Remediation Technology	Amount of Soil
NWS Crane		Composting	100,000 yd^3
Hawthorne AD		Composting	64,000 yd^3
Iowa AAP	X	Low temp thermal stripping	10,000 yd^3
Joliet AAP	X	Composting	200,000 yd^3
Milan AAP	X	Composting	58,000 yd^3
Newport AAP		Composting pilot test complete	9,000 yd^3
Pueblo AD		Composting	21,000 yd^3
Sierra AD		Composting	2,000 yd^3
Tooele AD	X	Composting pilot test complete	15,000 yd^3

AAP=Army Ammunition Plant
AD=Army Depot
NPL = National Priority List
Modified from Broder MF and Westmoreland RF (1998).

the ability of microorganisms to degrade pollutants (Mulligan et al., 2001). Bioavailability of contaminants, for instance, can be reduced by high percentage of clay and high organic matter content in soil.

Surfactants

One possible solution to overcoming treatment inefficiency is the addition of surfactants. The availability of contaminants is limited by solubility (Deshpande et al., 1999), and the use of surfactants enhances the bioavailability of the contaminants to the microbes and therefore increases the rate of biodegradation (Boopathy and Manning, 1999). Surfactant molecules adsorbed on the surface of contaminants cause repulsion between the head group of the surfactant and the soil particles (Deshpande et al., 1999). Research has shown that up to 70% of TNT metabolites may irreversibly bind to the soil matrix (Shen et al., 1998). Surfactants could help solublize many contaminants, especially those that are hydrophobic (Mulligan et al., 2001). Previous research demonstrated the need to enhance RDX bioavailability in soil-slurry reactors for soils from ammunition plants at Parsons, Kansas and Amarillo, Texas for a feasible bioremediation approach under aerobic, nitrate-reducing, sulfate-reducing, and mathanogenic conditions (Light, 1997).

Desirable surfactant characteristics include biodegradability, low toxicity, and solubility at ambient temperatures if directly applied to the soil

surface (Mulligan, 2001). Many surfactants are of low toxicity to humans, but can affect animals and plants (Mulligan et al., 2001).

An alternative could be used in situations where microorganisms are sensitive to commercially produced surfactants. Biosurfactants, naturally produced by certain bacteria and yeast, are produced from various substrates including sugars, oils, alkanes and wastes (Lin, 1996). Biosurfactants may be more biodegradable, more tolerant to pH, salt and temperature variation, and in some cases less expensive (West and Harwell, 1992). It has been shown that rhamnolipid surfactants from *P. aeruginosa* UG2 enhanced the solubilization of four-ring polyaromatic hydrocarbons (PAHs) more significantly than three-ring PAHs and were five times more effective than sodium dodecyl sulfate (SDS) (Deschênes et al., 1994). PAHs are recalcitrant, much like TNT, RDX and HMX are in the soil. When considering the use of biosurfactants, emulsification is a cell density dependent phenomenon, and as a result, the higher the concentration of cells, the more biosurfactant is produced (Ron and Rosenberg, 2001). The cell concentration in an open system, like a polluted environment, never reaches a high enough density to emulsify organics effectively (Ron and Rosenberg, 2001). Therefore, biosurfactants are possible additives for bioremediation in a closed system where cell density can be controlled.

With the combined efforts of microbiologists, engineers, and regulatory agencies, bioremediation can become the most efficient and most selected remediation strategy.

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Chapter III

ACETONITRILE EXTRACTABLE AND WATER LEACHABLE 2,4,6-TRINITROTOLUENE (TNT), HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX), AND OCTAHYDROL-1,3,5,7-TETRANITRO-1,3,5,7-TETRAZOCINE (HMX) IN SOILS AND THEIR IMPACT ON MICROBIAL COMMUNITY

Abstract. Impact of 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) contaminations on soil chemical and microbiological properties were evaluated. Four soils were obtained from munitions manufacture and loading sites that have been operating since World War II. Tests indicated that acetonitrile extracts of these soils contained up to 6435 mg TNT kg^{-1} soil, 2933 mg RDX kg^{-1} soil, and 2135 mg HMX kg^{-1} soil, in addition to other detectable levels of degradation intermediates. Nitrate-N levels in these soils reached as high as 315 mg N kg^{-1} soil and ammonium-N measured as high as 150 mg N kg^{-1}. The long-term contamination resulted in undetectable fungal populations in all four soils tested. The recoverable bacterial population was as low as 5 bacterial colony forming units g^{-1} soil. Activity of dehydrogenase, an intracellular enzyme that is active only in viable cells, was also not detected in the highly contaminated soil. Microbial biomass carbon was as low as 3 mg C kg^{-1} soil. Thus, natural attenuation in these soils may not be a feasible remediation technology. Interestingly, reduction in the total recoverable microbial population was not observed when an agricultural soil was spiked

with up to 5000 mg TNT kg^{-1} soil. Short-term amendment of TNT, however, did result in a shift in microbial community structure as indicated by distribution of r/K microbial growth strategists. TNT also affected germination of alfalfa and cotton. Less than 10% alfalfa seeds germinated when 100 mg TNT L^{-1} was supplemented. Results obtained from this study may be used to guide development of bioremediation strategies that maximize the use of natural resources while minimize environmental risk.

Introduction

Microorganisms are bioindicators of soil health because they maintain soil fertility, are in close contact with the soil environment, and may rapidly respond to contamination (Torstensson, 1997; Turco et al., 1994; van Beelen and Doelman, 1997).

Nitroaromatic compounds, such as 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), are common military explosives that are found in soils at destruction ranges, explosive dumping grounds, industry production sites, firing ranges and ammunition factories (Shen et al., 2001). These compounds may inhibit the growth and survival of bacteria, fungi, and actinomycetes (Fuller and Manning, 1997; Klausmeier et al., 1973), soil fauna (Parmelee et al., 1993), and higher plants (Palazzo and Leggett, 1986; Peterson et al., 1996). TNT is cytotoxic and genotoxic to bacterial and

mammalian cells (Berthe-Corti et al., 1998; Honeycutt et al., 1996; Lachance et al., 1998). Soil microbial activities decreased as TNT contamination increased (Gong et al., 1999). Contamination of these compounds could result in extremely low microbial activities and inhibit growth of plants (Gong et al., 1999). Because of the decreased indigenous population, natural attenuation may not be a viable strategy for remediation of nitramine and nitroaromatics-contaminated soil environments. Moreover, nitroaromatic compounds and their degradation metabolites can leach through soil into the groundwater (U.S. Army, 1980), which results in degraded water quality. One well-known example is nitrate toxicity. The nitro groups on the nitroaromatic compounds are removed from the ring structures during degradation (Nishino et al., 2000), which leads to production of nitrate. Nitrate is mobile in soil and may leach to ground water if it is not assimilated timely by microorganisms and/or plants (Hill, 1991), which results in nitrate contamination of groundwater and surface water (Yadav, 1997). Nitrate is known to cause chronic toxic effects (Hill, 1999). Ingestion of nitrate by babies can cause a disease, methemoglobinemia (Hill, 1999). This is because nitrate can be reduced to nitrite, which reacts with hemoglobin to form methemoglobin in the blood (Hill, 1999). Methemoglobin has reduced oxygen binding affinity, causing the infant turning blue. More over, nitrate and nitrite in a digestive system of humans can be converted to N-nitroso compounds by bacteria, which cause cancer (Hill, 1999). In addition, nitrite is known to be a potent antimicrobial agent (Hill, 1999). The decreased activity of *Nitrosomonas europaea* was attributed to

inhibition of nitrite to the enzyme ammonia monooxygenase (Stein and Arp, 1998).

Toxicity of nitroaromatic compounds and their degradation products has been studied extensively. TNT exposure through inhalation or dermal exposure can cause headaches, skin irritation, weakness, cataracts, anemia, and liver injury (Hathaway, 1985; McConnell and Flinn, 1946; Merck's Index, 1983; Morton et al., 1976). Indirectly, metabolites of TNT, including 2,4-dinitrotoluene (DNT) and 2,6-DNT, have shown toxicity towards *Poecilia reticulzata*, with 14-hour lethal concentration (LC_{50}) values of 12.5 mg 2,4-DNT L^{-1} and 18 mg 2,6-DNT L^{-1} (Deneer et al., 2001). Studies indicated that TNT is a direct acting mutagen, while the monoamino- and diamino-metabolites were less mutagenic. However, 2-amino-4,6-DNT and 2,6-diamino-4-nitrotoluene have demonstrated similar levels of mutagenicity to TNT (George et al., 2001). Exposure to RDX can be through inhalation and dermal absorption (Kaplan et al., 1965), and has been found in human cerebrospinal fluid, plasma, urine, and feces (Woody et al., 1992). RDX is classified as a class C carcinogen, and has been known to cause unconsciousness and epileptiform seizures (Harvey et al., 1991). Animal studies indicate that HMX may be harmful to the liver and central nervous system if swallowed, inhaled, or contacted (ATSDR, 1995). Therefore, the U.S. EPA (1990) regulates these munitions as hazardous, and has set restrictions on landfill disposal (1991).

Although numerous studies have been directed to evaluate the toxicity of nitroaromatics, most of these studies have focused on the inhibition of

growth of certain microorganisms (Fuller and Manning, 1997; Klausmeier et al., 1973) or the ability to biodegrade nitroaromatic compounds (e.g. Binks et al., 1995; French et al. 1998; Marvin-Sikkema et al. 1994; McCormick et al., 1976; Meharg et al., 1997; Montpas et al., 1997; Naumova et al., 1989; Preuss et al., 1993; Schackmann and Müller 1991). Little work has been conducted to investigate the effect of munitions on soil microbiological community, with even less work on microbial community structure.

This study was directed to evaluate TNT, RDX, and HMX contaminations in soil and impacts of their long-term contamination on levels of NO_3-N, NH_4-N, and TNT degradation intermediates, on microbial biomass C content, dehydrogenase activity, and culturable microbial communities. In addition, studies were also conducted to assess impact of spiked TNT on soil microbial activity and community, and toxicity of pure TNT on germination of cottonseed and alfalfa.

Materials and Methods

Soil samples

Three soil samples were taken from the Pantex Facility (Amarillo, Texas), referred to as PF1, 2 and 3. PF1 was taken from the edge of the facility as a control soil. All three soils are clay loam. PF1 has a mean particle size distribution of 20% sand and 40% clay. PF2 and PF3 have a mean particle

size distribution of 24% sand, 28% clay. The Pantex facility was originally called Pantex Ordnance Plant during World War II. This facility processed millions of pounds of high explosives before 1942. For example, bomb loading line number one, one of the 11 zones, loaded 8,248 five hundred pound bombs on a 7 day week basis, requiring about two and one half million pounds of TNT per day (www.pantex.com). The Pantex facility was deactivated after World War II ended and was recaptured by the Atomic Energy Commission in 1951 as a nuclear weapons facility, the only one in the United States (www.pantex.com). The explosives contamination has been present for over 50 years.

One soil sample was taken from the Kansas Army Ammunition Plant (Parsons, Kansas), referred to as KP. The KP soil is a sandy clay loam with a mean particle size distribution of 50% sand and 25% clay. This facility was built in 1941, and was called Kansas Ordnance Plant during World War II (www.parsonsks.com/history.htm). The facility still loads, assembles, and packs ammunition products for the military and contractors today (www.dayzim.com).

A soil sample was also taken from a century-long continuous winter wheat (*Triticum aestivum* L.) experiment located in central Oklahoma, U.S.A., referred to as OK soil. The OK soil is a silt loam with a mean particle-size distribution of 37.5% sand and 22.5% clay. Cattle manure from a feedlot was applied to the soil every four years at 269 kg N ha^{-1} since 1899. This soil was

used as a healthy soil for comparisons and for evaluating impact of spiked TNT on microbial activity and community structure.

Soil chemical properties

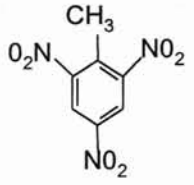
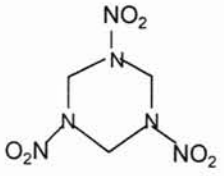
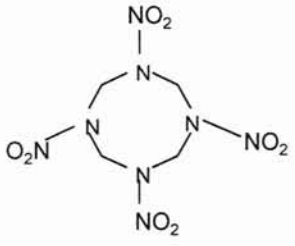
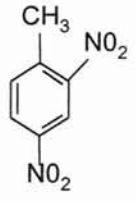
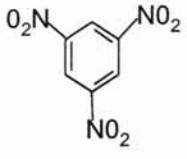
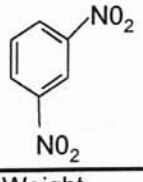
Soil pH values were determined using a combination glass electrode (soil:0.01 *M* $CaCl_2$ ratio = 1:2.5), and soil organic C (C_{org}) and total N by dry combustion using a Carlo-Erba NA 1500 Nitrogen/Carbon/Sulphur Analyzer (Schepers et al., 1989). Ammonium and nitrate in soil were extracted by shaking with 2 *M* KCl (1:5 soil:2 *M* KCl) for 30 minutes, and filtering through a Whatman number 42 filter paper (Bremner, 1965). NH_4^+ and NO_3^- in the extracts were analyzed by QuickChem® lachet using methods 12-107-06-2-A for ammonium and 12-107-04-1-B for nitrate.

Soil organic C and total N were determined using soil samples with particle size less than 180 μm. Soil pH was determined with air-dried samples that passed a 2-mm sieve.

Nitroaromatic compounds in soil

Structures and properties of TNT, RDX, HMX and their degradation intermediates are shown in Table 3.1. Their concentrations in soil were determined using the U.S. Environmental Protection Agency Method 8330 (1997). Briefly, soil and acetonitrile (1:5 ratio) suspension was placed in an

Table 3.1. Structure and chemical properties of contaminants and degradation intermediates.

Compound	Structure	M.W.	Solubility	N Content	C Content
			(mg L^{-1})	-------%	------
TNT		227.133	150[a]	19	37
RDX		222.117	60[a]	39	16
HMX		296.156	5[a]	39	16
DNT		182.135	270[b]	15	46
TNB		213.106	350[c]	20	34
DNB		168.109	469[b]	17	43

M.W. = Molecular Weight

[a] www.denix.osd.mil/denix/Public/Library/Remedy/Umatilla/umati02.html

[b] chemfinder.cambridgesoft.com

[c] Merck's Index (1993)

ultrasonic bath for 18 hrs at 4°C. After settling for 30 min, a portion of the supernatant was removed, diluted with 0.05 *M* $CaCl_2$ (1:1 ratio), and then filtered through a 0.45-μm filter before analysis using a C-18 reverse phase HPLC column (representative separation is shown in Figure 1, Appendix). The concentration of nitroaromatics in the extracts was calculated using calibration curves developed with standard grade (> 98%) TNT, RDX, HMX, and their degradation intermediates 1,3,5-trinitrobenzene (TNB), 2,6-dinitrotoluene (DNT), and m-dinitrobenzene (DNB) standards (ChemService, West Chester, PA, USA). In addition, water leachates were collected by passing 10-ml of deionized water through a 5-g soil sample that was held on a plastic funnel with a Whatman No 2 folded filter paper. Concentrations of nitroaromatic compounds and their degradation intermediates in the leachates were determined using HPLC as described above.

Soil Biochemical and Microbiological Properties, and Microbial Community Structure

Dehydrogenase activity was assayed using the method described by Casida et al. (1964). The microbial biomass C was determined by the chloroform-fumigation-incubation method (Jenkinson and Ladd, 1981), using a k_c factor of 0.45 with subtraction of the control.

Bacteria were cultured on 0.1-strength Tryptone soya agar (TSA) plates at 25°C (Katoh and Itoh, 1983; Lawley et al., 1983). All bacterial colonies

appearing within 24 h were designated as r-strategists, and the remaining as K-strategists (De Leij et al., 1993). Unless specified, colonies were enumerated on a daily basis for 5 consecutive days and on day 10. Plates were examined at low magnification (1.5X), and each day colonies that were visible were marked and enumerated. Thus, 6 counts (classes) were generated per plate. Plates with 20 to 200 colonies were selected for enumeration. When plates became too crowded, the next dilutions were used for enumeration. Distribution of bacteria in each class as a percentage of the total counts gave insight into the distribution of r- and K-strategists in each sample. Bacteria counts obtained were expressed in colony forming units (CFU) per gram soil.

Fungi were cultured on 0.1-strength malt extract agar (MEA) plates at 23°C. Fungal colonies were observed after 72 hours of incubation with countable plates of the highest dilution being enumerated.

An agriculture soil was spiked with 0, 250, 500, 1000, 2500 and 5000 mg TNT kg^{-1} soil, respectively, and incubated for 10 days at 23°C and 60% field-moisture content. Then bacterial and fungal populations in these soils were determined as described above.

All the experiments were conducted in triplicate incubations. Plate counts were conducted with 5 replicated-plates for each dilution.

Seed Germination

Water agar plates were spiked with 0, 1.0, 3.2, 10, 32, and 100 mg TNT L^{-1}, respectively. Since TNT obtained was dissolved in acetonitrile, equivalent amount of solvent was added to the control and all the treatments. The seeds were sterilized by soaking in 1.5% hypochloride for 5 minutes, followed by 95% ethanol for 30 seconds and rinsed with water 6 times. Fifty alfalfa seeds were placed on each plate. Five replicates were performed. The percent germination was recorded daily for 5 days.

All results are expressed on a moisture-free basis. Moisture was determined after drying at 105°C for 48 h. Significant differences among treatments were determined using one-way analysis of variance (ANOVA). Comparison of treatment means was performed using the least significant difference (LSD) test. Percentage data were transformed using arc sine transformation before analysis (Gomez and Gomez, 1984). All results reported are averages of replicated assays and analyses.

Results

Chemical properties and nitrogenous compounds

The pH values of the contaminated soils were similar, ranging from 7.4 to 7.9 (Table 3.2). The total nitrogen in the contaminated soils ranged from 1.9 to

Table 3.2. Properties of the soils studied.

Soil	pH[a]	Organic C[b]	Total N[b]	Sand	Clay
		---g kg^{-1}soil---		-----%-----	
PF1	7.7	15.1	1.9	20.0	40.0
PF2	7.9	18.4	6.9	25.0	27.5
PF3	7.6	28.8	22.0	22.5	27.5
KP	7.4	35.3	10.6	50.0	25.0
OK	5.6	10.2	0.9	37.5	22.5

[a] pH was determined by using soil : 0.1 M $CaCl_2$ (1 : 2.5).

[b] Organic carbon (C) and total nitrogen (N) was determined by total combustion using a Carlo-Erba NA 1500 Nitrogen/ Carbon/Sulfur Analyzer.

[c] Particle size distribution was determined using pipette analysis (Kilmer and Alexander, 1949).

22.0 g N kg^{-1} soil, while total organic carbon ranged from 15 to 35 g C kg^{-1} soil (Table 3.2). Levels of NH_4^+-N in the contaminated soils were from 45.2 to 150.3 mg NH_4^+-N kg^{-1} soil, and NO_3^--N from 5.8 to 487.6 mg N kg^{-1} soil (Table 3.2). NH_4^+-N level in PF3 soil was 18.6-fold of that in the OK soil and over 3-fold of those in the PF1 and PF2. NO_3^--N in PF3 was 143-fold of that in the OK soil and 84-fold of that in the PF1 and over 4-fold of that in PF2. The highest levels of HMX, RDX, and TNB were found in PF3 soil, and were 2138, 2933, and 441 mg kg^{-1} soil, respectively (Table 3.3). The highest TNT concentration was found in the KP soil, which was about 6438 mg kg^{-1}soil. TNT was the only contaminant that was detectable in the PF1 soil, but was less than 1 mg kg^{-1} soil. Levels of TNB and DNB were low in all the soils tested, but detectable in the PF2 and PF3 soil. Total N in the forms of contaminant tested reached as high as 2512 mg N kg^{-1} soil, which represented about 11.4% of the total N detected in the PF3 soil.

Moreover, nitroaromatic contaminants are quite mobile in soil and can be leached out with water (Figure 3.1). Water leachates of the contaminated soils showed concentrations of contaminants up to 52 mg HMX, 76 mg RDX, 106 mg TNB, and 134 mg TNT kg^{-1} soil (Table 3.4).

Microbial Activity, Population, and Community Structure

Activity of dehydrogenase, an enzyme that is active only in viable cells, was not detectable in the PF3 soil and less than 10 mg triphenyl formazan (TPF)

Table 3.3. Nitroaromatic compounds and inorganic N detected in the soils studied.

Soil	HMX [a]	RDX [a]	TNT [a]	TNB [a]	DNT [a]	DNB [a]	NH_4^+-N [b]	NO_3^--N [b]	N detected	As % of total N
	---------- mg N kg^{-1} soil ----------									%
PK1	ND (ND)	ND (ND)	< 1.0 (< 5.0)	ND (ND)	ND (ND)	ND (ND)	51.1	5.8	57.9	< 4.0
PK2	546 ±161 (1445 ± 302)	806 ± 285 (2133 ± 533)	102 ± 7 (551 ± 28)	23± 12 (117 ± 43)	0.4 ± 0.5 (2.7± 2.6)	11 ± 0 (64± 0)	45.2	113.4	1648.6	23.9
PK3	808 ± 110 (2138 ± 290)	1109 ± 153 (2933 ± 403)	504 ± 24 (2726 ± 131)	87 ± 8 (441 ± 41)	ND (ND)	3 ± 2 (82 ± 29)	150.3	487.6	3150.0	14.3
KP	ND (ND)	42 ± 24 (112 ± 65)	1191 ± 101 (6438 ± 547)	58 ± 12 (296 ± 59)	ND (ND)	ND (ND)	73.5	314.7	1679.2	15.9

[a] Nitroaromatic compounds were extracted with acetonitrile and analyzed by HPLC using a C-18 column for separation (EPA method 8330). Concentations reported are means ± standard error. Results in parentheses reported in mg contaminant kg^{-1} soil. HMX = Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine, TNB = Trinitrobenzene, TNT= 2,4,6-trinitrotoluene, DNT= Dinitrotoluene, DNB = Dinitrobenzene, and ND=Not Detected.

[b] Ammonium and nitrate were determined by extracting with 5:1 2 M KCL and analyzed by Lachet Quick Chem® methods 10-107-04-1-A and 10-107-06-2-D.

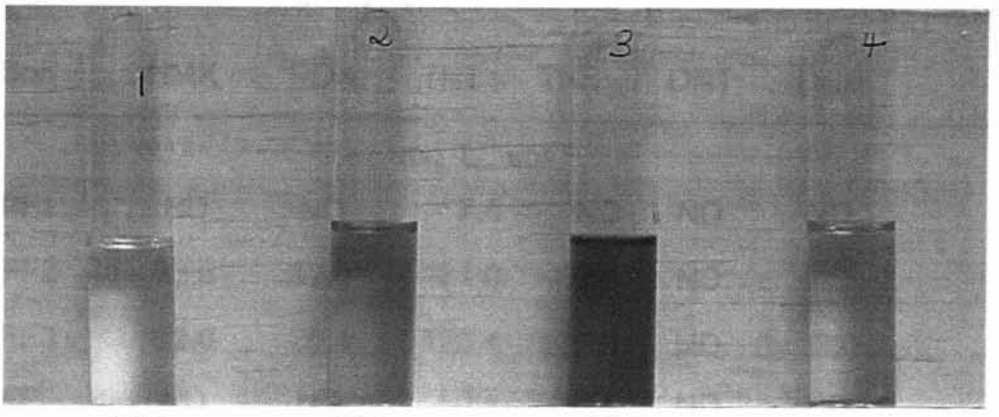


Figure 3.1. Water leachates of nitroaromatic-contaminated soils tested in this study. Colors of the leacheates are likely associated with the contaminants TNT, RDX, and HMX or the intermediates of their biodegradation. The major contaminants in PF2 and PF3 soils are RDX followed by HMX and TNT. The major contaminant of KP soil is TNT. A detectable level of TNT was found in PF1.

Table 3.4. Nitroaromatic compounds detected in water leachates. [a]

Soil	HMX	RDX	TNT	TNB	DNT	DNB
	---------- mg L^{-1} soil ----------					
PF1	ND	ND	7.6	ND	ND	ND
PF2	23.6	62.8	134.0	57.4	ND	ND
PF3	52.0	76.4	122.4	106.0	ND	13.0
KP	ND	ND	50.4	36.4	ND	ND

[a] Nitroaromatic compounds were extracted with water and analyzed by HPLC using a C-18 column for separation (EPA method 8330).
HMX = Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine,
RDX = Hexahydro-1,3,5-trinitro-1,3,5-triazine, TNB = Trinitrobenzene,
TNT = 2,4,6-trinitrotoluene, DNT = Dinitrotoluene,
DNB = Dinitrobenzene,and ND = Not Detected

kg^{-1} soil in the PF2 and KP soil, while activity of this enzyme was over 40 and about 60 mg TPF kg^{-1} soil in the PF1 and OK soils, respectively (Figure 3.2). Interestingly, microbial biomass C was not only detectable in the PF3 soil, but also the detected value was about 5-fold of that in the KP soil, which are 15 and 3.4 mg C kg^{-1} soil, respectively (Figure 3.2). In general, microbial biomass C contents were less than 20 mg C kg^{-1} soil in the contaminated soils with the exception of PF1, which had 66 mg C kg^{-1} soil (Figure 3.2).

Culturable bacteria population was the highest in PF1 soil, 6.3×10^4 CFU g^{-1} soil, and the lowest in KP soil, only 5 CFU g^{-1} soil. The PF2 and PF3 soils had culturable bacterial populations under 158 CFU g^{-1} soil and 20 CFU g^{-1} soil, respectively (Figure 3.3). Fungi were not even detectable in all the soils tested.

In the bacterial community detected, percentage of r-strategists increased with increasing contamination levels in the PF soils, ranging from 8 to 56% (Figure 3.3). Interestingly, r-Strategists bacteria were not detected in the KP soil.

When an agricultural soil was spiked with various concentrations of TNT, the total recoverable bacterial population was not affected by addition of TNT up to 2500 mg TNT kg^{-1} soil. Following addition of 5000 mg TNT kg^{-1} soil, significantly higher bacterial population was found (Figure 3.4). However, bacterial community structure within the community did change as indicated by distribution on percentages of r-strategists (Figure 3.4). In general, percentage

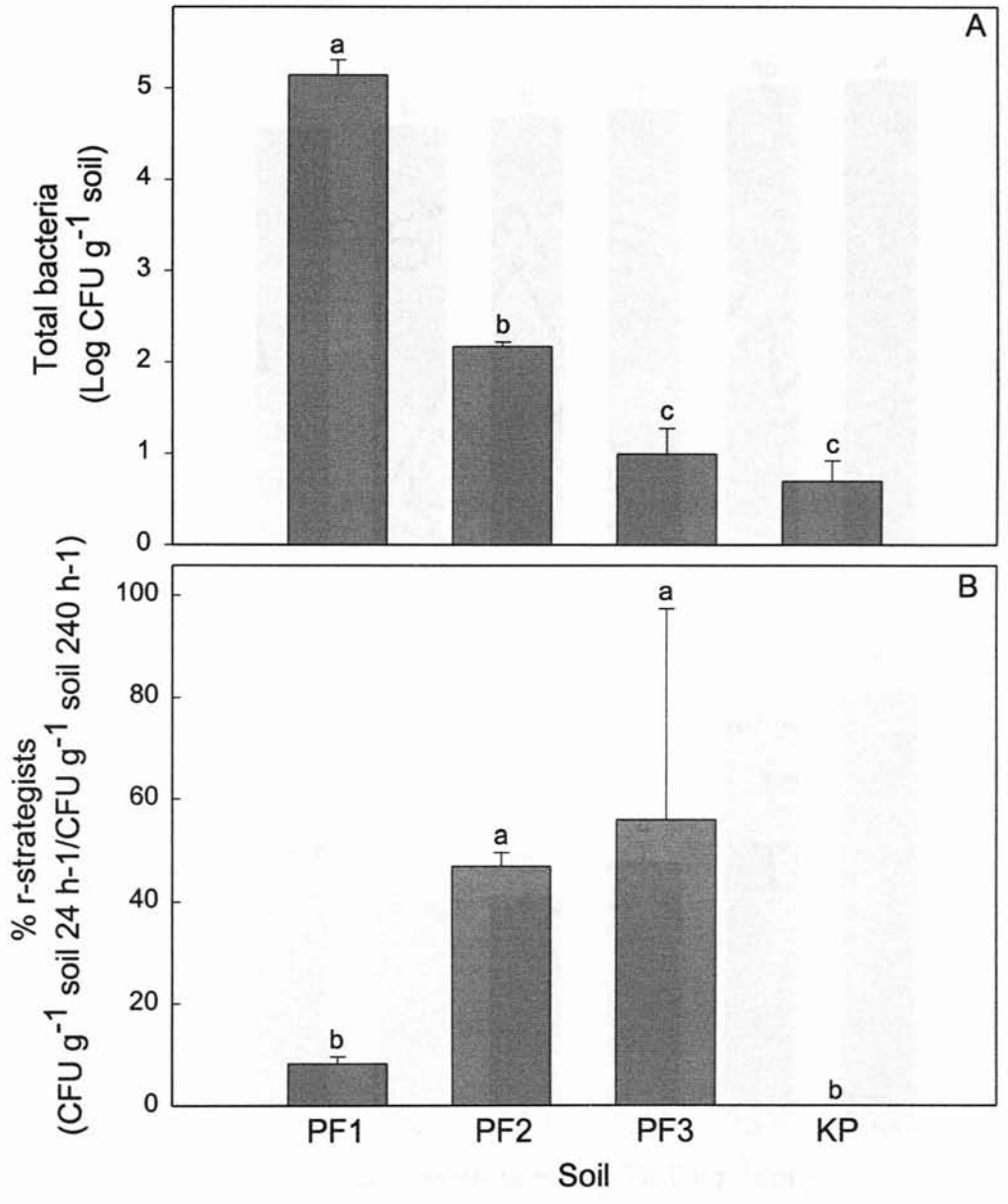


Figure 3.3. Total recoverable bacteria (A) and the distribution of bacterial growth strategists (B) in the contaminated soils studied. All bacterial colony forming units (CFU) that appeared within 24 hours were designated as r-strategists, and the remaining K-strategists. Bars indicate standard error. Different letters indicate significantly different means at $P < 0.05$ according to the least significant difference test.

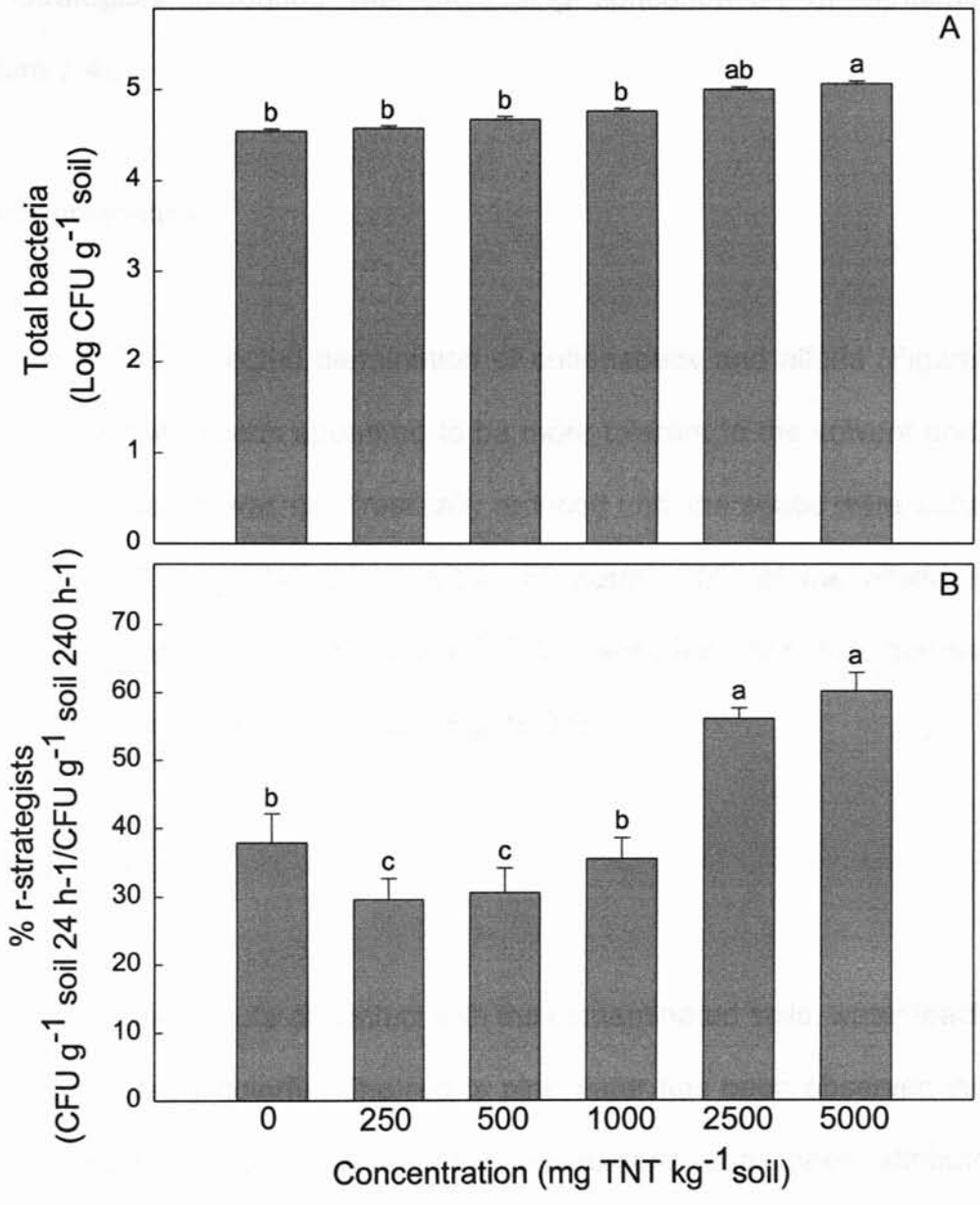


Figure 3.4. The impact of 10 days of TNT contamination in a spiked agricultural soil (OK) on total recoverable bacterial population (A) and the distribution of bacterial growth strategists (B). All colony forming units (CFU) that appeared within 24 hours were designated as r-strategists, and the remaining K-strategists. Bars indicate standard error. Different letters indicate significantly different means at $P < 0.05$ according to the least significant difference test.

of r-strategists increased with increasing concentration of contaminants (Figure 3.4).

Seed germination

Addition of TNT affected germination of cottonseeds and alfalfa (Figures 3.5 and 3.6). Alfalfa seeds appeared to be more tolerant to the solvent and TNT Alfalfa germination was not drastically reduced until the seeds were subjected to 32 and 100 mg TNT L^{-1}. Within 60 hours, 60% of the alfalfa seeds germinated when exposed to 32 mg TNT L^{-1}, while less than 10% germination upon exposure to 100 mg TNT L^{-1} (Figure 3.6).

Discussion

In less than one minute of contact with the contaminated soils, water leachates were undoubtedly colorful. The red or pink water has been observed in most munition-contaminated sites, and this phenomenon has been attributed to color change when exposed to sunlight (Howard et al. 1991). Although solubility of TNT and RDX were considered low (Merck Index, 1993), the color of water leachates from the contaminated soils strongly indicates the presence of these contaminants. In fact, TNT, RDX, and HMX have been reported to leach into groundwater (U.S. Army 1979; 1980). Results from this study

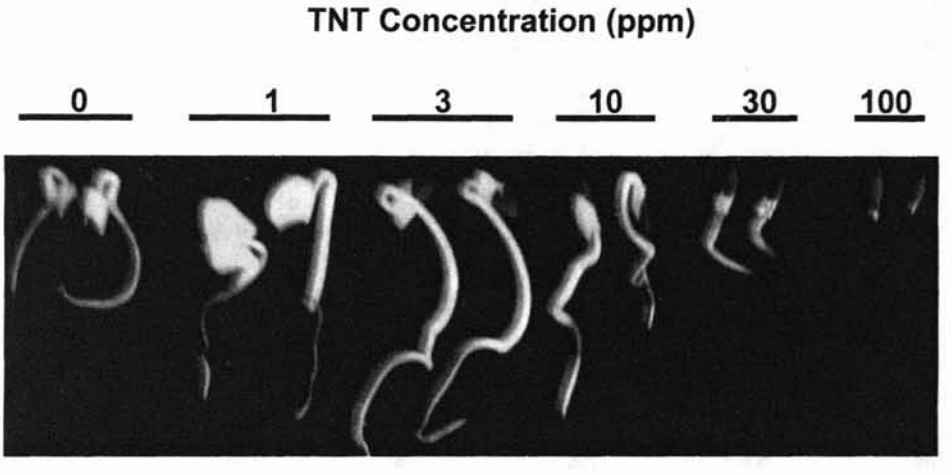


Figure 3.5. Germination of cotton at various levels of TNT. Seeds were germinated on water agar plates. Standard TNT solution (1000 ppm) obtained was dissolved in acetonitrile. Thus, seeds in the control were geminated on plates supplemented with acetonitrile concentration that was equivalent to 100 ppm TNT standard solution, which is the highest acetonitrile concentration among the plates tested.

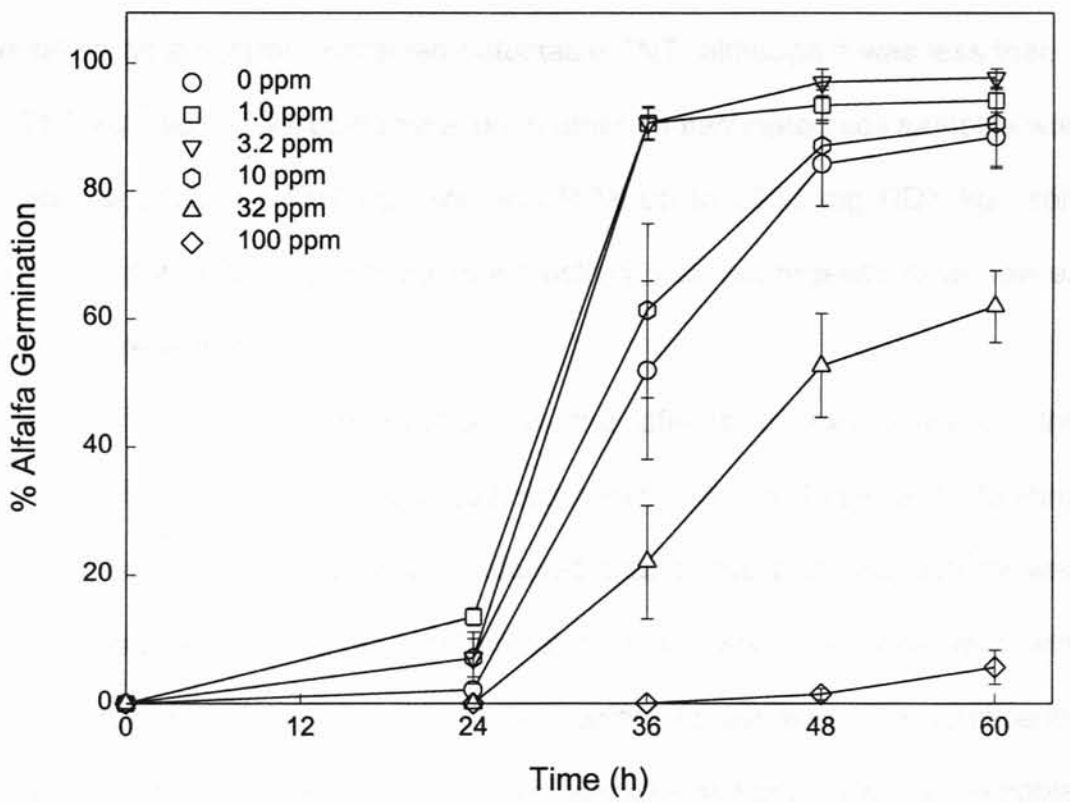


Figure 3.6. Germination of alfalfa seeds on various levels of TNT. Seeds were germinated on water agar plates. Standard TNT solution (1000 ppm) obtained was dissolved in acetonitrile. Thus, seeds in the control were geminated on plates supplemented with acetonitrile concentration that was equivalent to those in the 100 ppm TNT plates, which is the highest acetonitrile concentration among the plates tested. Bars indicate standard error.

suggest that these contaminants might have been leaching from the sites over 50 years.

Contamination levels varied among the soils tested. PF1 soil, which was taken as a control, contained detectable TNT, although it was less than 1 mg TNT kg^{-1} soil. TNT contamination in other contaminated soil samples was as high as 6435 mg TNT kg^{-1} soil and RDX up to 2933 mg RDX kg^{-1} soil. Thus, it is not surprising that microbial activity was suppressed to as low as undetectable levels.

There is limited information on the effects of explosives on the indigenous community in a historically contaminated site (Fuller and Manning 1998). Results from this study indicated that dehydrogenase activity was reduced dramatically in the contaminated soils, which is consistent with previous findings (Gong et al. 1999). Microbial biomass was also significantly reduced due to contamination. However, biomass and activity of soil microbial communities are not the same in principle. Early effects on microbial processes may be detected by measurements of activity rather than by determination of microbial biomass content (Landi et al. 2000). This explains the different trend in dehydrogenase activity and microbial biomass for the KP soil, suggesting that there were dormant microorganisms present in the KP soil.

Although plate counts are limited to revealing a small fraction (approximately 1-10%) of soil microbial community (Zuber 1994), long-term contamination of nitroaromatics undoubtedly has left these soils nearly

sterilized. Fungal population was undetectable and bacterial populations were as low as 5 CFU g^{-1} soil.

Among the microorganisms that survived, percentages of r-strategists increased. This is unexpected because r-strategist organisms are fast growers, which are not efficient at metabolizing recalcitrant substrates or dealing with stress conditions (Luckinbill 1978; De Leij et al. 1993). K-strategists, on the other hand, were expected to do well in a contaminated environment because they have been shown to be less sensitive to toxins (Luckinbill 1978; De Leij et al. 1993). Results obtained from the KP soil supported these concepts. However, results obtained from the PF soils and the spiked soils are contradictory with the concepts. One possible explanation is that bacteria proliferating in nitroaromatic-contaminated environments happened to be dominated by r-strategists in these soils.

However, total recoverable bacterial populations were not reduced upon addition of TNT to an agriculture soil, and a significant increase was observed at the highest TNT concentration treated. This differs from a prior report that dehydrogenase activities were reduced in TNT-spiked soils (Gong et al. 1999).

Reduction in microbial populations in contaminated soils could be due to toxicity of the contaminants and/or their degradation intermediates or products. The levels of NH_4^+ and NO_3^- detected in the highly contaminated soils raise the question of NH_4^+ and NO_3^- toxicity. It is known that certain microorganisms, such as methanotrophs (Paul and Clark 1996), can be inhibited by high levels

of NH_4^+. Nitrate has been shown to inhibit the growth of bacteria, such as *Nitrosomonas europaea* (Stein and Arp 1998).

Contamination of nitroaromatic compounds inhibited not only the microbial community, but also higher plants as shown by the germination of cotton and alfalfa. Inhibition of nitroaromatics on higher plants has also been reported by Gong et al. (1999) and Peterson et al. (1996). In fact, vegetative and microbial growth are both indicative of contamination. As shown by Gong et al. (1999), soil samples taken from areas where grasses were present had much higher dehydrogenase activity. However, microbial activities are generally considered to be more sensitive to changes in the environmental conditions.

Apparently, some of the bacteria survived in the contaminated soils and possess the ability to use contaminants for nutrient and/or energy sources, implying potential release of nitrate to the environment. Even though nitrate toxicity no longer represents a threat to children once the gastric acid barrier reduces colonization in the upper gut, which minimizes nitrite formation, a risk of cancer in adults still remains (Hill 1999). Nitroaromatics contamination still poses a threat to the health of living organisms.

In summary, soil microbial population is sensitive to RDX, HMX and TNT contamination. Microbes existing in a contaminated soil ecosystem provide an excellent measure of the impact of toxicants on the soil environment. Indigenous microorganisms may be repressed by the toxicity of the contaminants, which could decrease the efficiency of natural attenuation.

Reduced microbial activity would lead to reduced degradation rates of the contaminants, which would potentially lead to nitrate toxicity and nitroaromatic contamination in the ground and surface water.

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Chapter IV

ISOLATION, CHARACTERIAZATION AND IDENTIFICATION OF NITROAROMATIC DEGRADING BACTERIA

Abstract — Nitramine and nitroaromatic compounds, including the explosives 2,4,6-trinitrotoluene (TNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), are a class of environmental contaminants that have been shown to affect not only microbial life, but also humans. Isolation, identification, and characterization of microorganisms that are capable of degrading these contaminants would facilitate bioremediation by optimizing conditions for growth of these microorganisms. Eight bacteria capable of using either TNT or RDX as a sole source of nitrogen were isolated from soil. Among the 8 bacteria, two were gram-positive, two cocci, 5 rods and one fibrous. All isolates were capable of fermenting glucose, lactose, and mannitol, but only isolates 5, 6, and 8 produced CO_2 with glucose. Isolate 5 was the only bacterium that was capable of producing alcohol and various acidic end products. All eight isolates produced catalase. Only isolates 7 and 8 were capable of utilizing starch, while isolates 4, 5, and 8 were capable of casein hydrolysis. All isolates, with the exception of isolate 5, produced lipase for fat hydrolysis. Tryptophanase was not produced by any of the bacterial isolates. Isolates 1, 2, and 3 produced urease and were capable of the hydrolysis of urea. Results from gram stain, morphology, biooxidation tests, and hydrolysis tests along

with 16S rDNA sequences suggest that isolate 2 is a *Sinorhizobium*, isolate 3 belongs to *Streptomyces*, both isolates 5 and 6 are from the same genus, *Bacillus*, isolate 7 is a *Pseudomonas*, and isolate 8 appears to be an *actinomyces*. Isolates 1 and 4 remain unidentified.

Key words: TNT, RDX, biodegradation, bacterial isolates

Contamination of nitramine and nitroaromatic compounds in the environment has been widespread and at least 20 of the National Priority Listed sites resulted from their contamination [1]. Commonly found contaminants include 2,4,6-trinitrotoluene (TNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). The latter is often used in combination with octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) [1]. TNT, RDX and HMX are common military explosives that are found in soils at sites such as destruction ranges, explosive dumping grounds, manufacturing processes, firing ranges and ammunition factories [2].

TNT is made up of 18.5% nitrogen, all of which is constituent nitro groups. Current use and past disposal practices have resulted in extensive TNT contamination of soils, sediments, surface waters, and groundwater [3, 4, 5], and it has also been shown to be toxic to various biological groups, including bacteria, fungi and soil fauna [6, 7, 8].

RDX, royal demolition explosive, has three nitrogen atoms in the hexacyclic ring, three nitro groups, and is classified as a polynitramine. RDX

is also used as an explosive as well as a rodenticide [9]. RDX is a class C carcinogen, and has been known to cause unconsciousness and epileptiform seizures [10]. Production grade RDX contains impurities such as significant amounts of HMX (high melting explosive) and trace amounts of 1-acetylhexahydro-3,5-dinitro-1,3,5-triazine [11].

Modification to the RDX manufacturing process allows HMX to become the major product and RDX the impurity [12]. HMX is an octacyclic ring, with 37.8% of nitrogen. HMX is also a polynitramine and is a regulated toxic hazardous compound with significant environmental risk from the metabolites formed during degradation [13, 14].

Degradation of these compounds involves the activities of aerobic, facultative, and anaerobic bacteria and some fungi. TNT transformation by microorganisms has been reported under aerobic and anaerobic conditions with complete reduction of one or more nitro groups observed [15, 16, 17, 18]. *Enterobacter cloacae* PB2 [19], a bacterium, and *Phanerochaete chrysosporium,* a white rot fungus [20, 21], have been shown to reduce TNT. Studies have demonstrated the transformation of TNT to the intermediate triaminotoluene (TAT) [22, 23, 24]. TAT is an intermediate of a normal toluene metabolic pathway in bacteria [25]. Research has also shown promise in the degradation of TNT under sulfate reducing followed by nitrate reducing conditions [26].

Numerous bacteria have been identified to be able to break down RDX, including *Stenotrophomonas maltophilia* PB1 [12], *Providencia rettgeri*,

Morganella morganii, and *Citrobacter freundii* [27], and *Rhodococcus* sp. strain DN22 [28]. They were all isolated from soil under aerobic, nitrogen limiting conditions. The fungus *Phanaerocheate chrysosporium* has also been shown to degrade RDX [29]. In fact, municipal anaerobic sewage sludge also effectively mineralized RDX [30]. However, bioremediation effort of nitroaromatics-contaminated soils is still hampered by lack of strains that effectively degrade nitroaromatic compounds for an economically feasible bioremediation approach.

Eight bacteria capable of using either TNT or RDX as a sole source of nitrogen were isolated. The isolated bacteria were characterized using gram stain, morphology, biooxidation, and hydrolysis tests, along with 16S rDNA sequences for their tentative identification.

MATERIALS AND METHODS

Growth media, isolation and maintenance. The growth media consisted of 980 ml of basal medium [26] supplemented with 5 µg of RDX, 20 ml of trace element solution and 1 ml vitamin solution [31]. The basal medium contained (all g liter^{-1}) KH_2PO_4, 0.16 g; $K_2HPO_4.3H_2O$, 0.42g; $Na_2HPO_4.2H_2O$, 2.2 g; $NaH_2PO_4.H_2O$, 1.1 g; $MgSO_4.7H_2O$, 0.1 g. When used in anaerobic conditions, 100 ml $NaHCO_3$ (10% w/v), 50 ml Na_2S (1 g $Na_2S.9H_2O$ in 100 ml H_20) and 0.1 g resazurin [26] were also included in one liter of basal medium. The media was sterilized by autoclave for 30 minutes for use under aerobic

conditions or by filter sterilization through a 0.22-μm-pore size filter if used under anaerobic conditions. Glucose (0.5 g liter^{-1}) was added to the medium as a carbon and energy source for microbial growth.

Microorganisms were isolated from soils taken from ammunition facilities with over 50 years of contamination. Detailed descriptions of these soils are given in Chapter 3. Soil samples were pre-incubated at room temperature at 60% field-moist capacity for 10 days prior to isolation. The isolates were obtained by spreading soil suspensions on basal medium plates with RDX or TNT as the sole source of nitrogen. Microbial colonies isolated were further purified by streaking each isolate at least three times on fresh plates.

Routine growth was done with tryptone soya broth (TSB) for enhanced growth. RDX or TNT (5 μg L^{-1}) was added to the medium as a selective pressure. Stock cultures of the isolates were grown up in TSB, mixed with 40% glycerol (1:1 ratio), frozen in liquid nitrogen and stored at -80°C.

Microscopic characterization. All cells used for microscopic characterization were grown in TSB or on tryptone soytone agar (TSA) or basal plates. Observations were made from microbial slides following gram stain under a Bauch & Lomb brightfield microscope, and pictures were taken with a Kodak Microscopy Documentation System 290 using a Kodak DC 290 Zoom Digital Camera.

DNA extraction, purification, and analyses. Genomic DNA was extracted using either liquid or freeze-dried bacterial culture following procedures described by Sambrook et al. [32]. Unless specified, PCR amplification was performed on an automated thermal cycler (PTC-100, MJ Research Inc., Watertown, MA, USA) with an initial denaturation (94°C for 120 sec), followed by 30 cycles of denaturation (94°C for 45 sec), annealing (65°C for 30 sec) and extension (72°C for 120 sec), and a single final extension (72°C for 10 min). The annealing temperature used for Isolate 8 was 60°C. The primers used during amplification are listed in Table 4.1. PCR products were purified using the UltraClean PCR clean up kit (Mo Bio Laboratories, Inc., Solana Beach CA, USA) and sequenced using an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequence similarity searches were conducted using the BLAST network service [34], and sequence alignments used the GAP program [35].

Biophysiological tests. Durham tubes with phenol red glucose, lactose, and mannitol broth were used to test for production of acid and/or gas from fermentation of a particular sugar, indicated by color change of the media used or appearance of gas bubbles [36]. These tubes were inoculated and incubated at 37°C for 48 hours. The lactose fermentation was slow and those tubes were monitored for 5 days.

Mixed acid fermentation was determined using the methyl red test. The isolates were grown in MR-VP medium at 37°C for 48 hours. The formation of

Table 4.1 Nucleotide sequence of primers used for polymerase chain reactions (PCR) in this study. [a]

Target gene	Primer	Sequence
16S rDNA	1	5'-CCT ACG GGA GGC AGC AG-3'
16S rDNA	2	5'-CGG TGT GTA CAA GGC CCG GGA AAC G-3'
16S rDNA	3	5'-AGA GTT TGA TCC TGG CTC AG-3'
16S rDNA	4	5'-AAG GAG GTG ATC CAG CCG CA-3'

[a] Toytal et al. [33]

acids was indicated by a color change from yellow to red immediately following addition of methyl red [36].

The Voges-Proskauer test was used to determine if butanediol fermentation occurred. Bacterial isolates were grown in MR-VP medium at 37°C for 4 days. Production of 2,3-butanediol and alcohols was tested by a color change from yellow to pink or red following addition of Barritt's reagent. This protocol actually tests for the presence of acetoin, a precursor of 2,3-butanediol [36].

Catalase production was determined by smearing a loopful of culture on a slide with a few drops of 3% hydrogen peroxide on it. If catalase was present the hydrogen peroxide effervesced [36].

Oxidase production was determined by placing a few drops of oxidase test reagent (1% dimethyl-ρ-phenylenediamine hydrochloride) on a Whatman No. 2 filter paper and then smearing a loopful of cells onto the reagent. Color change from pink to black indicates oxidase production [36].

Presence of nitratase, reducing nitrate to nitrite, was detected by growing the bacterial isolate in nitrate broth at 37°C for 48 hours under an anaerobic environment. In the presence of nitrites, the culture would turn to red following addition of sulfanilic acid and dimethyl-α-naphthylamine dissolved in acetic acid [36].

Starch hydrolysis is indicative of amylase presence. Following streaking the bacterial isolates to starch agar plates and incubating for 48 hours, Gram's

iodine was added to the plate's surface. A clear zone surrounding the growth indicates absence of starch [36].

Casein is the predominant protein in milk. Caseinase hydrolyzes this protein to produce more soluble derivatives. Bacterial isolates were streaked on skim milk agar plates and incubated for 48 hours at 37°C. A clear zone next to the growth indicates production of caseinase [36].

Lipase, which hydrolyzes fat into one glycerol and three fatty acids, was tested by growing bacterial isolates on spirit blue agar plates for approximately 48 hours at 37°C. Appearance of blue precipitate or clear zone around the cells indicates fat hydrolysis [36].

Tryptophanase, which splits tryptophan into indole and pyruvic acid, was detected by production of indole following growing bacterial isolates in tryptone broth at 37°C for 48 hours. Tryptophan hydrolysis is indicated by a red layer at the top of the medium following addition of Kovacs' reagent [36].

Urease, which splits urea and produces ammonia, was tested by growing bacterial isolates in urea broth at 37°C for seven days. Upon release of ammonia, phenol red in the medium will turn to pink, indicating urea hydrolysis [36].

RESULTS

Eight bacteria were isolated from long-term nitroaromatics-contaminated soils. Four isolates were obtained using TNT as the sole source

of nitrogen, and another four from using RDX as the sole nitrogen source. With the exception of isolate 8, the isolates were obtained under aerobic environments. Isolate 8 was obtained under anaerobic conditions, but was later shown to be facultative. Among the 8 bacteria, two gram-positive, two cocci, 5 rods and one fibrous were isolated (Table 4.2, Figure 4.1). Isolates 1, 2, 4, and 6 are round, smooth, convex, and cream-colored. Isolate 2 produced a polysaccharide slime layer. Isolate 3 also has cream-colored colonies, but the bacterial colonies adhered to the medium. This isolate also produced a brown colored compound released onto the agar plate. Colonies of isolate 7 on TSB plates are light orange with an L-form, irregular shape. Isolate 8 exhibits crumbly, textured growth that is green and turns brown after approximately 2 days of growth on TSB plates.

All isolates were capable of fermenting glucose, lactose, and mannitol but only isolates 5, 6, and 8 produced gas with glucose (Table 4.3). None of the isolates produced gas during lactose and mannitol fermentation. Isolate 5 was the only bacterium that was capable of producing alcohol and various acidic end products (Table 4.3). However, all eight isolates produced catalase, which allows for the detoxification of hydrogen peroxide, but not oxidase (Table 4.3). This suggests that none of the isolates use oxidase in the metabolism of oxygen. It is expected that most of the isolates are not able to reduce nitrate. However, isolate 8, though able to grow in an anaerobic environment, was also not able to use nitrate as the final electron acceptor for anaerobic respiration (Table 4.3).

Table 4.2. Description of bacteria isolated from soil, capable of using the specified nitroaromatic compound as the sole N source.

Isolate Number	Nitrogen Source	Soil	Gram Stain	Morphological Characteristics
1	TNT	PF1	Negative	Rod
2	TNT	PF1	Negative	Rod
3	TNT	PF1	Positive	Cocci
4	TNT	PF1	Negative	Cocci
5	RDX	PF1	Positive	Rod
6	RDX	PF3	Negative	Rod
7	RDX	KP	Negative	Rod
8	RDX	KP	Negative	Actinomyces

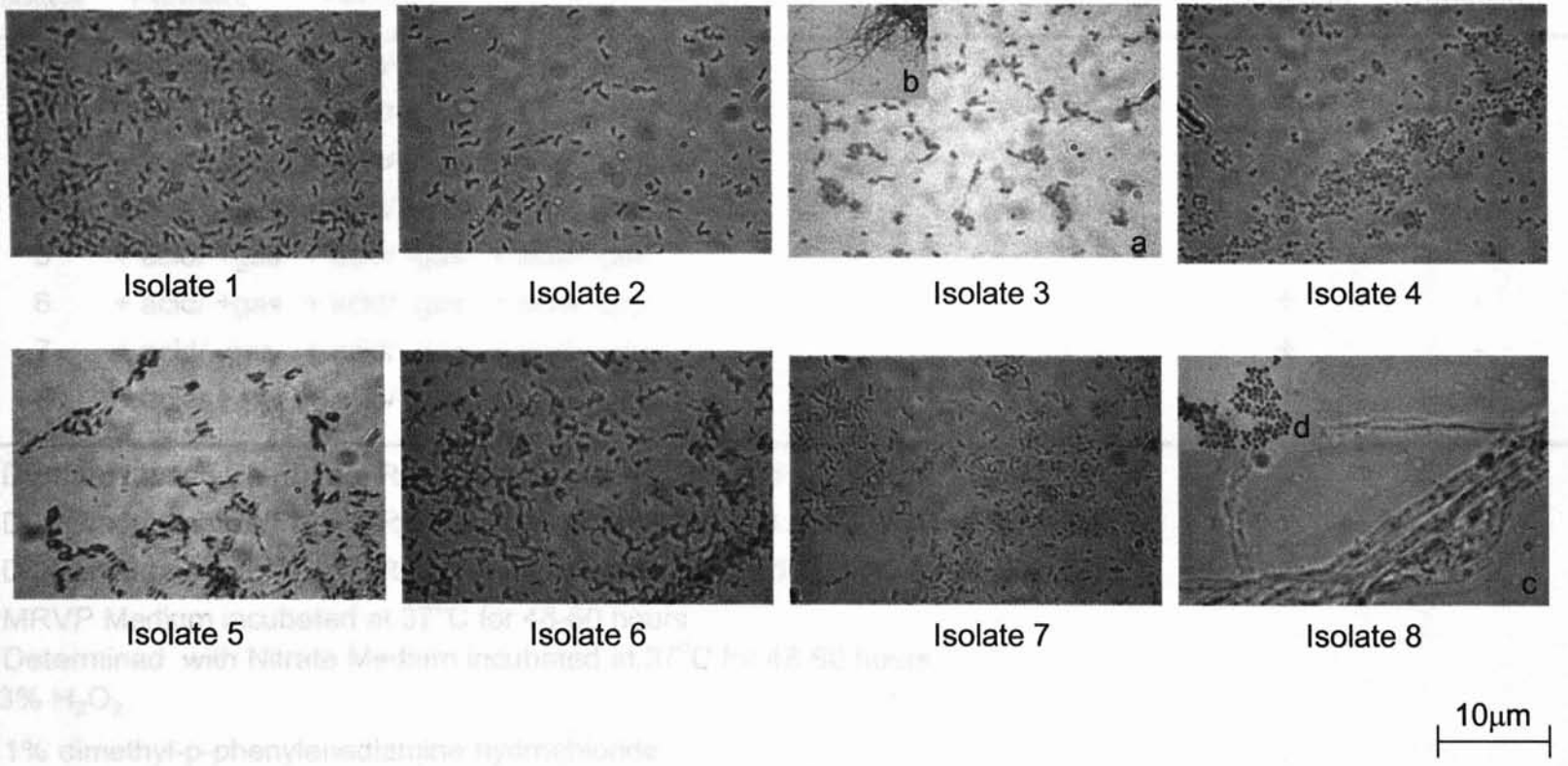

Figure 4.1. Bacterial isolates. Microorganisms were isolated from soils using basal medium plates supplemented with mineral and vitamin solution and 5 mg kg^{-1} TNT/RDX as the sole N source. Isolate 3 were fibrous (b) when initially isolated, but changed morphologically after consecutive culturing in the laboratory for over a year (a). Isolate 8 was isolated under anaerobic conditions, but later confirmed to be facultative. This bacterium was fibrous and produced spores (d).

Table 4.3. Biooxidation tests of the isolates.

Isolate	Glucose Ferment [a]	Lactose Ferment [b]	Mannitol Ferment [c]	Mixed Acid Ferment [d]	Alcohol Ferment [d]	Nitrate Reduction [e]	Catalase Production [f]	Oxidase Production [g]
1	+ acid/ -gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-
2	+ acid/ -gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-
3	+ acid/ -gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-
4	+ acid/ -gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-
5	+ acid/ +gas	+ acid/ -gas	+ acid/ -gas	+	+	-	+	-
6	+ acid/ +gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-
7	+ acid/ -gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-
8	+ acid/ +gas	+ acid/ -gas	+ acid/ -gas	-	-	-	+	-

[a] Durham tubes with Phenol Red Glucose Broth incubated at 37°C for 48 hours.

[b] Durham tubes with Phenol Red Lactose Broth incubated at 37°C for 48-60 hours.

[c] Durham tubes with Phenol Red Mannitol Broth incubated at 37°C for 48-60 hours.

[d] MRVP Medium incubated at 37°C for 48-60 hours.

[e] Determined with Nitrate Medium incubated at 37°C for 48-60 hours.

[f] 3% H_2O_2.

[g] 1% dimethyl-p-phenylenediamine hydrochloride.

Even though many bacteria are capable of hydrolyzing starch [38], results indicate that only isolates 7 and 8 are capable of utilizing starch (Table 4.4). Isolates 4, 5, and 8 were capable of casein hydrolysis and all isolates, with the exception of isolate 5, produced lipase fat hydrolysis (Table 4.4). Tryptophanase was not produced by any of the bacterial isolates (Table 4.4). Isolates 1, 2, and 3 produced urease and were capable of hydrolyzing urea (Table 4.4).

The results of the biooxidation and hydrolysis tests of bacterial isolates were compared with descriptions in the Bergey's Manual (1993) for possible genus identification of the isolates (Tables 4.5 - 4.12). The tentative classification of each isolate was further confirmed by partial sequences of bacterial 16S rDNA (Table 4.13). Isolate 2 is a *Sinorhizobium*, while isolate 3 belongs to *Streptomyces*. Isolates 5 and 6 are from the same genus, *Bacillus*, but isolate 7 is a *Pseudomonas*. Isolate 8 appears to be an *Actinomyces*. A tentative identification was determined using the 16S rDNA sequences and the results of the biophysiological tests (Table 4.14). Although isolate 1 and 4 remain unidentified, results from this study indicated that many different genera of bacteria could utilize TNT or RDX as a sole nitrogen source.

DISCUSSION

Oxidative microorganisms obtain their energy and carbon source from reduced organic substances and release end products such as carbon dioxide

Table 4.4. Hydrolysis of starch, casein, fat, tryptophan, and urea by the soil isolates at 37°C for 48 hours.

Isolate	Starch Hydrolysis [a]	Casein Hydrolysis [b]	Fat Hydrolysis [c]	Tryptophan Hydrolysis [d]	Urea Hydrolysis [e]
1	-	-	+	-	+
2	-	-	+	-	+
3	-	-	+	-	+
4	-	-	+	-	-
5	-	+	-	-	-
6	-	+	+	-	-
7	+	-	+	-	-
8	+	+	+	-	-

[a] Starch agar plates

[b] Skim milk agar plates

[c] Spirit blue agar plates

[d] Tryptone Broth

[e] Urea Broth

Table 4.5. Tentative identification of isolate 1 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Possible Species	Matching Characteristics		
Acinetobacter	ND	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose +
Pseudomonas	*syringaepathovars* *vesicularis* *viridiflava*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose +
Acetobacter	*diazotrophicus* *liquefaciens*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose + indole -
Xanthomonas	*albilineans* *acampestris*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose + NO_3^- reduction -
Arsenphonus	ND	gram (-) rod	catalase + oxidase -	acid from Glucose + MR - VP-
Bacillus	ND	gram variable polymorphic	catalase + oxidase -	spore formation +

ND = Not Determined

Table 4.6. Tentative identification of isolate 2 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Possible Species	Matching Characteristics		
Acinetobacter	ND	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose +
Pseudomonas	*syringaepathovars vesicularis viridiflava*	gram (-) rod	catalase + oxidase -	starch hydrolysis- acid from Glucose +
Acetobacter	*diazotrophicus liquefaciens*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose + indole -
Xanthomonas	*albilineans acampestris*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose + NO_3^- reduction -
Arsenphonus	ND	gram (-) rod	catalase + oxidase -	acid from Glucose + MR - VP-
Sinorhizobium	ND	gram (-) rod	catalase + oxidase -	extracellular slime
Beijerinckia	ND	gram (-) rod	cysts + giant colonies	extracellular slime highly viscous culture

ND = Not Determined

Table 4.8. Tentative identification of isolate 4 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Matching Characteristics	
Bacillus	gram variable polymorphic	catalase + oxidase - spore formation +
Sporosarcina	gram variable cocci	catalase + oxidase - spore formation +
Planococcus	gram (-) cocci NO_3^- reduction -	catalase + oxidase - starch hydrolysis -

Table 4.9. Tentative identification of isolate 5 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Possible Species	Matching Characteristics	
Bacillus	ND	gram variable polymorphic	catalase + oxidase - spore formation +
Erwina	*stewarti*	gram (+) rod NO_3^- reduction - urea hydrolysis -	catalase + oxidase - acid from Glucose + acid from Lactose + acid from Mannitol +
Curtobacterium	*luteum*	gram (+) rod starch hydrolysis -	catalase + oxidase - acid from Glucose + casein hydrolysis +

ND = Not Determined

Table 4.10. Tentative identification of isolate 6 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Possible Species	Matching Characteristics		
Acinetobacter	ND	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose +
Pseudomonas	*syringaepathovars* *vesicularis* *viridiflava*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose +
Acetobacter	*diazotrophicus* *liquefaciens*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose + indole -
Kingella	*kingae*	gram (-) rod	catalase + oxidase - **	acid from Glucose + urea hydrolysis - indole -
Arsenphonus	ND	gram (-) rod	catalase + oxidase -	acid from Glucose + MR - VP-
Bacillus	ND	gram variable polymorphic	catalase + oxidase -	spore formation +

** Actually oxidase +, but result is negative with the use of dimethyl-p-phenylenediamine hydrochloride

ND = Not Determined

Table 4.11. Tentative identification of isolate 7 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Possible Species	Matching Characteristics		
Pseudomonas	*syringaepathovars* *vesicularis* *viridiflava*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose +
Xanthomonas	*albilineans* *acampestris*	gram (-) rod	catalase + oxidase -	starch hydrolysis - acid from Glucose + NO_3^- reduction -
Legionella	ND	gram (-) rod	catalase + oxidase -	urea hydrolysis - NO_3^- reduction -
Sphingobacterium	ND	gram (-) rod	catalase + oxidase -	acid from Glucose + indole - endospores -
Arsenphonus	ND	gram (-) rod	catalase + oxidase -	acid from Glucose + MR - VP-

ND = Not Determined

Table 4.12. Tentative identification of isolate 8 based on biophysiological tests and the Bergey's Manual (1993).

Possible Genus	Matching Characteristics	
Actinomycetes	gram (+) rough crumbly texture optimum temp. 37°C acid from Glucose +	catalase + oxidase - indole- NO_3^- reduction - facultative anaerobe

Table 4.13. Tentative identification of bacterial isolates based on partial 16S rDNA sequence. The sequences are shown in Figures 2-6 in Appendix.

Isolate Number	Tentative Classification[a]	amplified 16S rDNA (bp)	BLAST Score	E Value	Number of Gaps	% Match
1	ND					
2	*Sinorhizobium*	538	1053	0	0	100
3	*Streptomyces*	597	1152	0	3	99
4	ND					
5	*Bacillus*	588	1136	0	1	99
6	*Bacillus*	1470	2857	0	4	99
7	*Pseudomonas*	746	1453	0	2	99
8	ND					

[a] highest BLAST score (www.ncbi.nlm.nih.gov/blast/). ND = Not determined.

Table 4.14. Tentative identification of bacterial isolates using 16S rDNA sequence, biophysiological test results, and descriptions in the Bergey's Manual (1992).

Isolate	Genus
1	Unidentified
2	*Sinorhizobium*
3	*Streptomyces*
4	Unidentified
5	*Bacillus*
6	*Bacillus*
7	*Pseudomonas*
8	*Actinomycete*

and water. Fermentative bacteria also use organic compounds for energy, but the end products are acids, aldehydes, and alcohol [36]. Some microorganisms produce mixed acids as end products, while others produce 2,3-butanediol and ethanol instead of acids. The Voges-Proskauer test accounts for the production of alcohol end products.

Some aerobic and facultative microorganisms utilize oxygen and produce hydrogen peroxide, which is a toxic byproduct [36]. The enzyme catalase is responsible for detoxification of hydrogen peroxide to water and oxygen [36]. All the isolates had catalase activity suggesting that they possess the ability to detoxify hydrogen peroxide.

Oxidase is an enzyme that is part of oxygen metabolism and allows converting O_2 to O_2^- using the coenzymes $FADH_2$ to FAD^+ [36]. All isolates were oxidase negative, suggesting that these microbes do not require antioxidant compounds or special enzymes for defense against toxic products (free radicals with an unpaired electron) formed in the process of electron transport in oxygen metabolism [37]. Free radicals often combine to form toxic compounds that damage lipids and proteins [37].

Hydrolysis of various substrates can also be useful in classification of unknown isolates. A general class of exoenzymes called hydrolases split organic compounds into smaller units in the presence of water [36]. A molecule of starch consists of amylose, a straight chain polymer of 200-300 glucose units, and amylopectin, a branched polymer with phosphate groups. Starch is the major nutrient polysaccharide of plants [37]. Starch hydrolysis is

completed with amylases, which yield molecules of maltose, glucose, and dextrins [36].

Protein hydrolysis, also known as proteolysis or peptonization, is done to produce soluble, transparent derivatives. The protein used in this study was casein, which is the predominant protein in milk. Caseinase hydrolyzes casein into smaller protein units [36].

The enzyme lipase hydrolyzes fat into one molecule of glycerol and three fatty acid molecules [36]. In many cases these products are used by bacteria to synthesize fats and other cell components, or are oxidized to yield energy [36]. Unlike the last two hydrolysis enzymes discussed, all isolates except number 5 tested positive for lipase.

With the information received from the biophysiological tests and the DNA sequences, the bacterial isolates can be tentatively identified and potentially be used in bioremediation. Determination of the identity of each bacterial isolate could enhance biodegradation efficiency by optimizing the growth conditions.

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Chapter V

EFFECTS OF SURFACTANTS ON THE GROWTH OF NITROAROMATIC DEGRADING BACTERIA

Key words: nitroaromatics, surfactants, growth density

Abstract

The nitroaromatic and nitramine compounds, TNT, RDX and HMX, are common military explosives that are persistent in the environment, which was attributed partially to their low solubility. The efficiency of removal from contaminated sites can be enhanced by the addition of surfactants. Although many surfactants are considered to have low toxicity to humans, they can affect microbial growth. Studies were conducted to assess the impact of surfactants on the microorganisms involved in the degradation of nitroaromatic compounds. Effects of SDS, Steol CA-230, Tween 20, Tween 60, and Tween 80 at 0.4, 4.0 and 25 times the critical micelle capacity (CMC) on growth of three bacteria isolated from soil were tested. These bacteria were capable of using TNT or RDX as a sole nitrogen source. SDS and Steol CA-230 equivalently reduced the growth of Isolate 3 with increasing surfactant concentration. Steol CA-230 greatly affected the growth of isolate 6, decreasing the absorbance from 1.5 to 0.1 with the addition of 19,125 mg L^{-1} (25 times the CMC). The growth of isolate 7 increased absorbance from 1.0 to

1.4 with 0.4 times the CMC (3 to 306 mg L^{-1}) and to 1.3 with 4 times the CMC (30.6 to 3060 mg L^{-1}) of SDS and Steol CA-230. In general, nonionic surfactants, including Tween 20, Tween 60, and Tween 80, had little effect, while anionic surfactants, SDS and Steol CA-230, generally inhibited growth of the bacteria tested. Results obtained from this study suggested that caution should be exercised in the use of surfactants to facilitate bioremediation because surfactants might inhibit, promote, or remain neutral to bacterial growth depending on the type and concentrations used.

Introduction

Nitroaromatics, such as 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydrol-1,3,5,7-tetrazocine (HMX) are common military explosives that are persistent in the environment (Spain 2000). One possible solution to help increase the biodegradation rate of nitroaromatic compounds is the use of additives, such as surfactants. The availability of contaminants has been limited by solubility (Deshpande et al. 1999), therefore utilization of surfactants can enhance the bioavailability of the contaminants to the microbes and therefore increase the rate of biodegradation (Boopathy and Manning 1999).

TNT, RDX and HMX are absorbed in humic materials (Pignatello and Xing 1996) or coat the surfaces of soil particles as non-aqueous liquids. It is also possible that contaminants fill voids between soil particles when the

concentration of the contaminant is 1% or higher (Karimi & Gray 2000). Surfactants solubilize the contaminants by adsorption on the surface of the contaminant, which causes repulsion between the head group of the surfactant and the soil particles (Deshpande et al. 1999). Desirable surfactant characteristics include biodegradability, low toxicity, and solubility at ambient temperatures if directly applied to the soil surface for biodegradation (Mulligan et al. 2001). Many surfactants are of low toxicity to humans, but can affect animals and plants (Mulligan et al. 2001). Therefore it is important to assess the impact of surfactants on the microorganisms involved in degradation of nitroaromatic compounds.

Materials and Methods

Bacterial strains used

Isolates 3, 6, and 7 (Chapter 4) were chosen for this study. Isolates 6 and 7 possess the ability to degrade RDX and were persistent in highly contaminated environments. Isolate 3 was able use TNT as the sole N source. All three bacteria were obtained from soils described in Chapter 3. The chemical properties of the soils are listed in Table 3.1 and Table 3.2. The characteristics of the three isolates are discussed in Chapter 4 and included in Tables 4.1, 4.2, 4.3 and 4.4.

Surfactants

Steol CA-230 (sodium laureth sulfate, Stephan Co., Northfield, IL), SDS (sodium dodecyl sulfate, L-4509 Sigma Chemicals), Tween 20 [POE (20) sorbitan monolaurate, P-1379 Sigma Chemicals], Tween 60 [POE (60) sorbitan monolaurate, P-1629 Sigma Chemicals], and Tween 80 [POE (80) sorbitan monolaurate, P-8074 Sigma Chemicals, St. Louis, MO], were used in this study. These surfactants were used because of their availability and varying characteristics. Only food-grade rated surfactants were used. The properties of the surfactants are listed in Table 5.1.

Growth and maintenance of isolates

Routine growth of isolates was done with tryptone soya broth (TSB). Isolates 6 and 7 were grown at 30°C, while isolate 3 was grown at 37°C. Stock cultures of the isolates were grown in TSB, frozen with 40% glycerol (1:1) using liquid nitrogen, and stored at -80°C.

Growth density curves

Nephelometry flasks with 100 ml of TSB were autoclaved for 30 minutes. After the medium cooled, 0.4, 4.0, and 25 times the CMC of a surfactant were added to each flask. The flasks were then inoculated with 200 µl of 12-hour-

Table 5.1. Properties of surfactants used.

Trade Name	Chemical Name	M.W.	Ionic State	CMC
		(g $mole^{-1}$)		(mg L^{-1})
Tween 20	POE (20) sorbitan monolaurate	1226	nonionic	0.05
Tween 60	POE (60) sorbitan monolaurate	1310	nonionic	0.03
Tween 80	POE (80) sorbitan monolaurate	1308	nonionic	0.01
SDS	Sodium dodecyl sulfate	288	anionic	8.40
Steol CA-230	Sodium laureth sulfate	375	anionic	765.00

M.W.= Molecular Weight
CMC= Critical Micelle Capacity

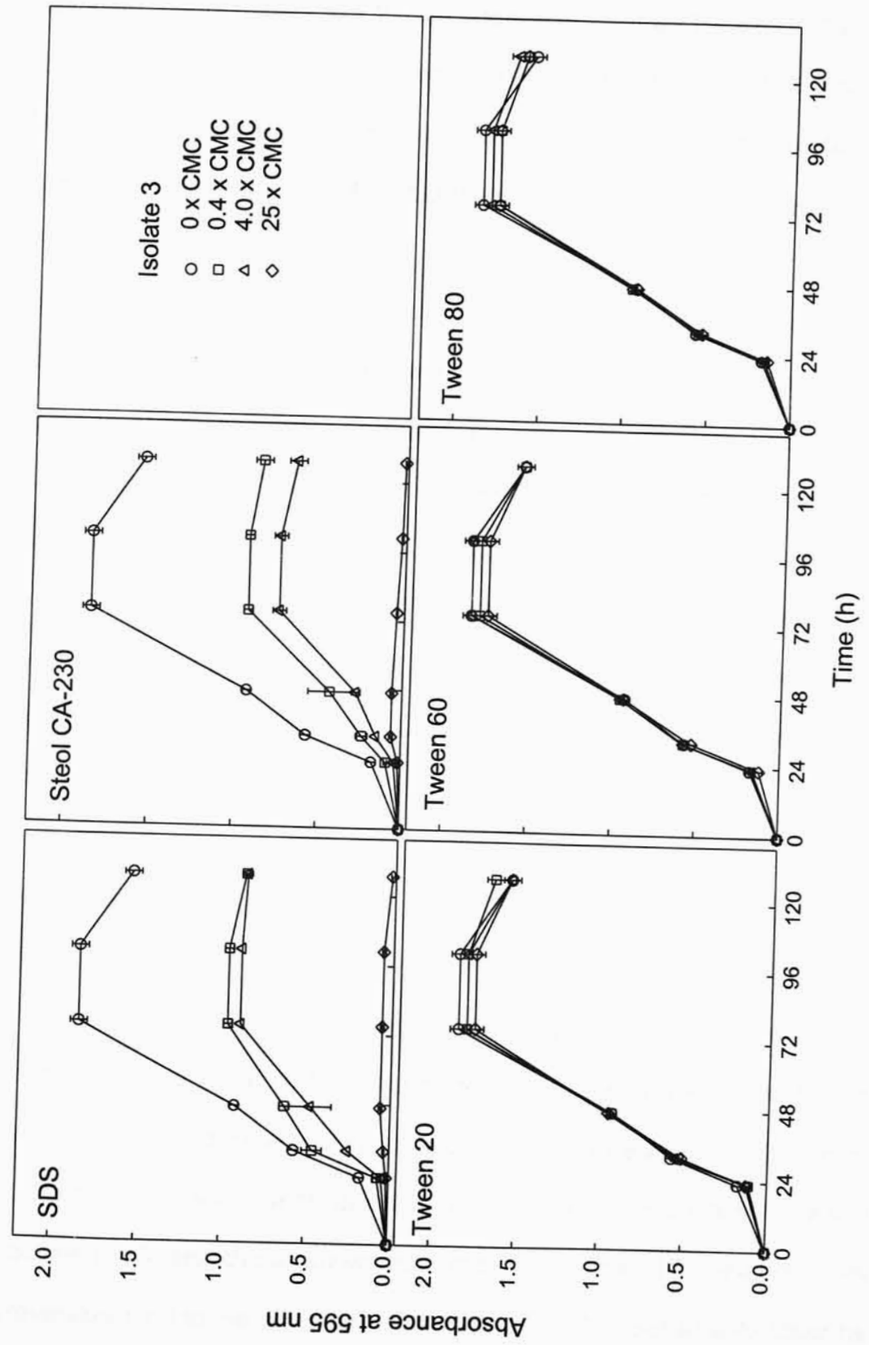
old culture of the isolate tested. After inoculation, the flasks were placed on a rotary shaker at 200 rpm at 30°C for isolates 6 and 7 and 37°C for isolate 3. Absorbance readings were taken periodically. Absorbance changes due to increase in culture density were recorded on a Bausch and Lomb Spectronic 20 at 595 nm. This procedure was repeated for all three isolates on all five surfactants. A blank with TSB was used for calibrating the Spectronic 20.

Results

Nonionic surfactants, Tween 20, Tween 60, and Tween 80, did not greatly affect the growth of isolate 3, with an average maximum absorbance of 1.9. Even at 25 times the CMC the absorbance was reduced only slightly (to 1.8). However, the anionic surfactants Steol CA-230 and SDS reduced bacterial growth considerably. At 25 times the CMC (210 and 19,125 mg L^{-1}) for both SDS and Steol CA-230, the growth density was reduced to an absorbance of less than 0.1 versus the absorbance of 1.9 in the control (Figure 5.1).

The growth of isolate 6 seemed unaffected by the addition 0.4 times the CMC of Tween 20, Tween 60, and Tween 80, all of which are nonionic surfactants. Increasing the amount of nonionic surfactant decreased the absorbance by approximately 0.1. At 25 times the CMC (1 mg L^{-1} of Tween 20 or Tween 60) a reduced absorbance of 1.0 was recorded compared to the absorbance of the control with no surfactant, which reached 1.5. SDS, an anionic surfactant, did affect growth of this isolate, but only reduced its

Figure 5.1. The effects of Steol CA-230, SDS, Tween 20, Tween 60, and Tween 80 on the growth of isolate 3 in tryptone soya broth (TSB) at 30°C. Growth was monitored by changes in culture turbidity at the wavelength 595 nm. Bars indicate standard error.



stationary culture density to 1.2 upon addition of 25 times the CMC. Steol CA-230, an anionic surfactant, affected its growth substantially. At 25 times the CMC the culture density never reached 0.1 absorbance, while the stationary phase culture density was 1.5 absorbance in the absence of Steol CA-230 (Figure 5.2).

In contrast to isolate 6, isolate 7 was not affected by the use of any addition of Tween 20, Tween 60, and Tween 80. No reduction of absorbance occurred at all. Also, this bacterium reacted very differently to the anionic surfactants. When Steol CA-230 and SDS were added, the growth actually increased compared to the control, indicating that isolate 7 may be using the surfactants as a nutrient source (Figure 5.3). Although, the addition of 3060 mg SDS L^{-1} or 19,125 mg Steol CA-230 L^{-1} (25 times the CMC) reduced the growth density absorbance to 1.0 verses the control, which reached an absorbance of 1.2 (Figure 5.3).

Discussion

Water solubility is a controlling removal mechanism of organic contaminants, and the efficiency of removing organic contaminants from soil can be enhanced by the addition of additives such as surfactants (Mulligan et al. 2001). Using surfactants may reduce adsorption of the contaminants to the soil matrix. Research has shown that up to 70% of the TNT metabolites may irreversibly bind to the soil matrix (Shen et al., 1998). Surfactants could help

Figure 5.2. The effects of Steol CA-230, SDS, Tween 20, Tween 60, and Tween 80 on isolate 6 in tryptone soya broth (TSB) at 30°C. Growth was monitored by changes in culture turbidity at the wavelength 595 nm. Bars indicate standard error.

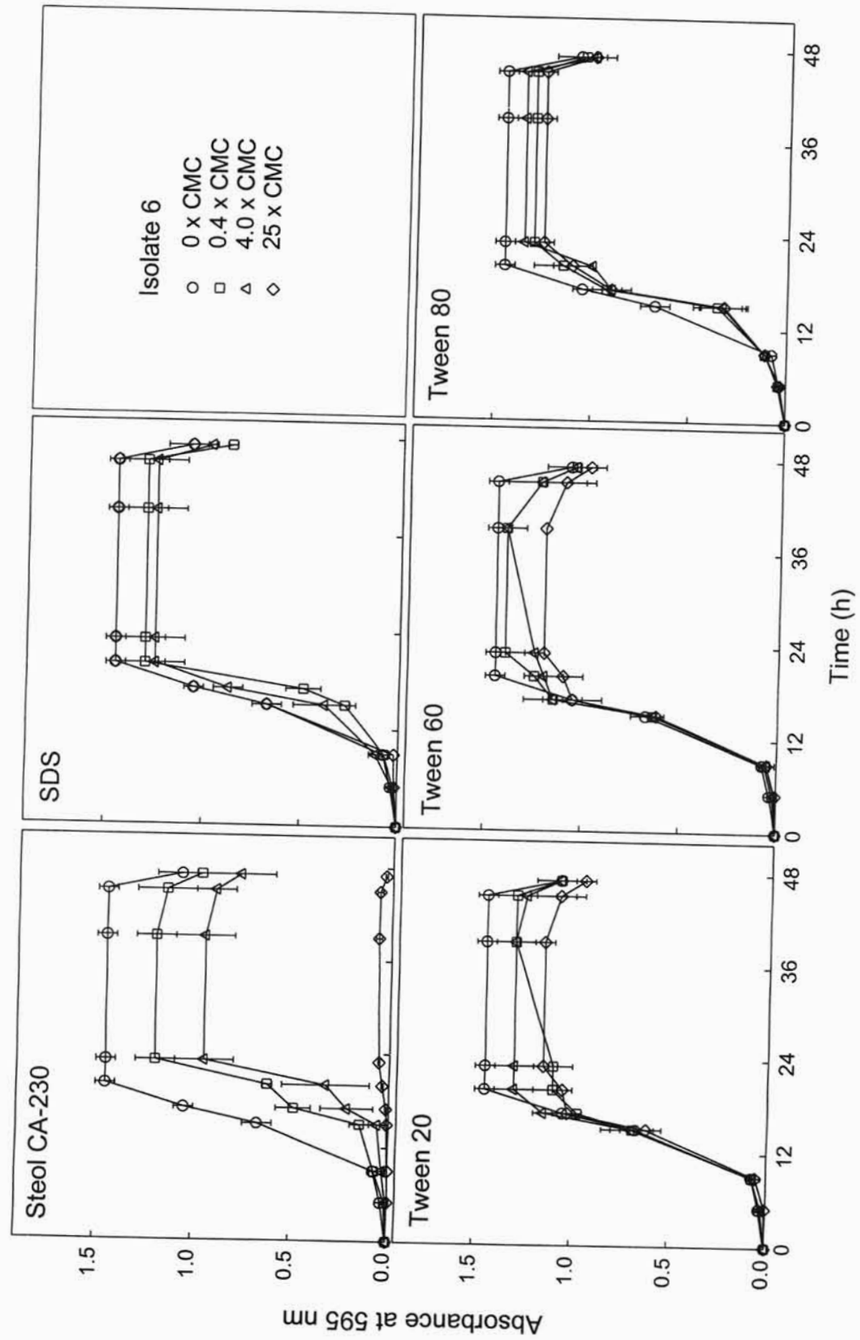
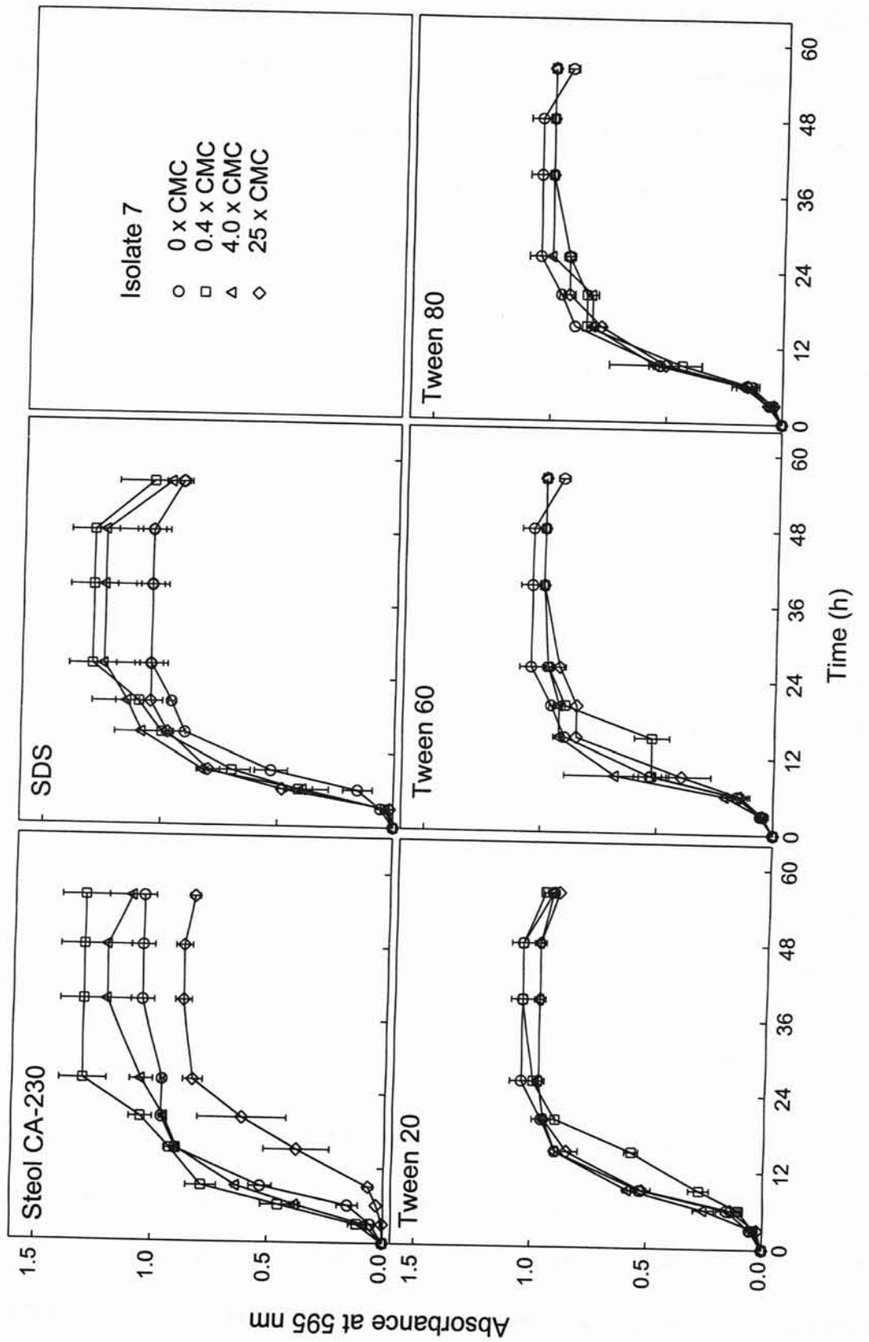


Figure 5.3. The effects of steol CA-230, SDS, Tween 20, Tween 60, and Tween 80 on isolate 7 in tryptone soya broth (TSB) at 30°C. Growth was monitored by changes in culture turbidity at the wavelength 595 nm. Bars indicate standard error.



solublize many contaminants, especially those that are hydrophobic (Mulligan et al. 2001). Surfactants have been referred to as non-aqueous phase liquids (NAPL). It has been reported that dispersing droplets of NAPL increases the interfacial area (Grimberg et al. 1994), solubilizes hydrophobic substrates (Grimberg et al. 1995), and enhances the dissolution of solid substrates (Volkering et al. 1998).

An important consideration when solubilizing contaminants is the critical micelle capacity (CMC) of the additive used. The CMC is the concentration at which micelles first begin to form (Deshpande et al. 1999). A micelle forms around a hydrophobic compound, with the hydrophilic part of the surfactant on the outside, allowing the contaminant to be brought into solution. It has been suggested that surfactants must be applied at concentrations above the CMC for a significant amount of the contaminants to be released into solution (Deshpande et al., 1991).

Prior research on the impact of surfactants on bacterial growth shows no significant difference occurred with growth of *Mycobacterium* species on glucose and the surfactant Triton X-100, suggesting that Triton X-100 is not toxic to that species of bacteria (Chen et al. 2000). Triton X-100 was not used as a sole source of carbon, did not inhibit bacterial growth on glucose, or did not act as a competitive substrate in the presence of anthracene, another organic contaminant (Chen et al. 2000). Their research findings are similar to the results obtained from this study using Tween 20, 60, and 80. Some surfactants can be utilized for co-metabolism or growth substrate (Volkering et

al. 1998). This may be the case with the use of Steol CA-230 and SDS, with additions of 3 to 3060 mg L^{-1}, which increased the growth of isolate 7 during this study.

The difference in growth density between the anionic surfactants could be partially explained by the CMC of each surfactant. The CMC of SDS is 8.4 mg $liter^{-1}$, while the CMC of Steol CA-230 is 765 mg $liter^{-1}$. Both are much greater when compared with Tweens 20, 60 and 80 (0.05, 0.03, and 0.012 mg L^{-1}, respectively). As a result, 25 times the CMC is 19,125 mg Steol CA-230 L^{-1}, or 210 mg SDS L^{-1}, comparing to 0.3 mg Tween 80 L^{-1}.

Surfactants can inhibit or increase degradation depending on the target contaminant, the bacterial species involved, and the surfactant used (Chen et al. 2000). Bacteria, that prefer to be attached to a surface for growth, would be restricted by the addition of surfactants that disperse them into the aqueous phase (Volkering et al. 1998). Surfactant can also interfere with extracellular transport, inhibit cell growth, or change the membrane permeability (Volkering et al. 1998). This is evidenced by addition of surfactant Dowfax 8390 that led to no growth of *Mycobacterium* at all concentrations tested (Chen et al. 2000). In this study, reduction in growth of isolates 3 and 6 was observed for Steol CA-230, and of isolate 3 for SDS. As a general rule, the sorption of surfactant occurs on the hydrophobic domains on the cells surfaces. For example, *Pseudomonas* cells are less hydrophobic than *Mycobacterium,* so the latter cells are more sensitive to surfactants (Chen et al. 2000).

The use of surfactants may also pose a threat to the environment because surfactant mobilizes pollutants, which promotes downward or horizontal movements of pollutants that may subsequently contaminate groundwater (Mulligan et al., 2001). Without confining walls or liners in place, an increase in nitrogen toxicity from any of the nitrogenous compounds could be observed. This is evidenced upon applying a 0.75% solution of the surfactant Witconol to a PCB-contaminated soil (Abdul et al., 1992). Therefore, caution should be exercised in applying surfactants to remediate nitroaromatic compounds contaminated soils.

Biosurfactants, an alternative to commercially produced surfactants, are naturally produced by certain bacteria or yeast from various substrates including sugars, oils, alkanes and wastes (Lin 1996). Biosurfactants may be more biodegradable, more tolerant to pH, salt and temperature variation, and in some cases less expensive (West & Harwell 1992). It has been shown that rhamnolipid surfactants from *P. aeruginosa* UG2 enhanced the solubilization of four-ring polyaromatic hydrocarbons (PAHs) more significantly than three-ring PAHs and that the biosurfactant was five times more effective than the surfactant SDS (Deschênes et al. 1994). PAHs are recalcitrant, much like TNT, RDX and HMX in soil. Emulsification is a cell density dependent phenomenon. The more cells that are in a location, the more biosurfactant are produced (Ron & Rosenberg 2001). The cell concentration in an open system, like a polluted environment, never reaches a high enough cell density to emulsify organics, like oil, effectively (Ron & Rosenberg 2001). However, if

used in ex-situ technologies, cell density could be maximized by optimizing growth conditions, which could make the use of biosurfactants feasible.

Results obtained from this study suggested that caution should be exercised in the use of surfactants to facilitate bioremediation because surfactants might inhibit, promote, or remain neutral to bacterial growth depending on the type and concentrations used. In general, nonionic surfactants, including Tween 20, Tween 60, and Tween 80, had little effect, while anionic surfactants, SDS and Steol CA-230, generally inhibited growth of the bacteria tested.

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Chapter VI

SUMMARY AND CONCLUSION

Nitroaromatic contamination is a threat to the welfare of people as well as smaller forms of life, including bacteria, fungi, and other soil fauna. Contaminations of TNT, RDX, and HMX have been in the environment since the production of high explosives during World War I. Biodegradation is a possible economical solution for removal of these pollutants. Understanding microorganisms involved in their biodegradation and their optimal growth conditions can lead to development of effective bioremediation strategies.

Results from this study indicate that nitroaromatic contamination can be so toxic that most of the microorganisms in the soil were killed from the exposure. Nonetheless, a few bacteria were persistent and survived in the contaminated environment. More importantly, some of these bacteria were capable of using RDX or TNT as a sole source of nitrogen. Thus, they may be potentially used for remediation of nitroaromatics-contaminated soils, providing that their growth conditions are well evaluated. Isolation and characterization of these isolates are essential steps of their potential application.

This study indicated that nonionic surfactants had little effect, while anionic surfactants generally inhibited growth of the bacteria tested. However, further evaluation of these isolates with respect to growth requirements would be beneficial prior to apply these bacteria for remediation of nitroaromatics-contaminated soils on a large scale.

APPENDIX

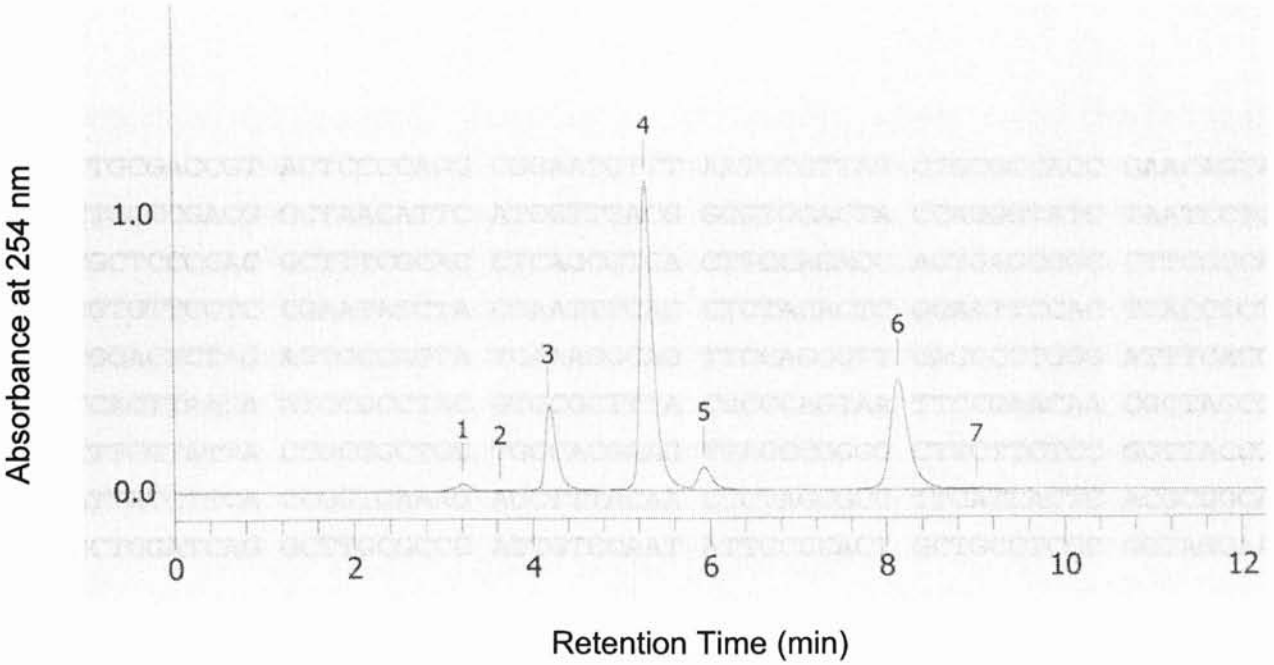

Figure 1. Representative separation of nitroaromatic compounds by the U.S. Environmental Protection Agency Method 8330 using a C-18 reverse phase HPLC column. Nitroaromatic compounds were extracted from soils using acetonitrile and a ultrasonicator as described in Chapter 3. Peaks 3, 4, 5 and 6 correspond to octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 1,3,5-trinitrobenzene (TNB) and 2,4,6-trinitrotoluene (TNT), respectively. Peak 1 is unidentified.

```
  1 TTGCGACCGT ACTCCCCAGG CGGAATGTTT AATGCGTTAG CTGCGCCACC GAACAGTAAA
 61 CTGCCCGACG GCTAACATTC ATCGTTTACG GCGTGGACTA CCAGGGTATC TAATCCTGTT
121 TGCTCCCCAC GCTTTCGCAC CTCAGCGTCA GTTCCAGACC AGTGAGCCGC CTTCGCCACT
181 GGTGTTCCTC CGAATATCTA CGAATTTCAC CTCTACACTC GGAATTCCAC TCACCTCTTC
241 TGGACTCTAG ATTGCCAGTA TGAAAGGCAG TTCCAGGGTT GAGCCCTGGG ATTTCACCCC
301 TCACTTAACA ATCCGCCTAC GTGCGCTTTA CGCCCAGTAA TTCCGAACAA CGCTAGCCCC
361 CTTCGTATTA CCGCGGCTGC TGGCACGAAG TTAGCCGGGG CTTCTTCTCC GGTTACCGTC
421 ATTATCTTCA CCGGTGAAAG AGCTTTACAA CCCTAGGGCC TTCATCACTC ACGCGGCATG
481 GCTGGATCAG GCTTGCGCCC ATTGTCCAAT ATTCCCCACT GCTGCCTCCC GGTAGGAA
```

Figure 2. Partial 6S rDNA sequence (538 bp) of isolate 2.

```
  1 ATGGGCCAAA GCCTGATGCA GCGACGCCGC GTGAGGGATG ACGGCCTTCG GGTTGTAAAC
 61 CTCTTTCAGC AGGGAAGAAG CGAAAGTGAC GGTACCTGCA GAAGAAGCGC CGGCTAACTA
121 CGTGCCAGCA GCCGCGGTAA TACGTAGGGC GCAAGCGTTG TCCGGAATTA TTGGGCGTAA
181 AGAGCTCGTA GGCAGGCTTG TCACGTCGGT TGTGAAAGCC CGGGGCTTAA CCCCGGGTCT
241 GCAGTCGATA CGGGCAGGCT AGAGTTCGGT AGGGGAGATC GGAATTCCTG GTGTAGCGGT
301 GAAATGCGCA GATATCAGGA GGAACACCGG TGGCGAAGGC GGATCTCTGG GCCGATACTG
361 ACGCTGAGGA GCGAAAGCGT GGGGAGCGAA CAGGATTAGA TACCCTGGTA GTCCACGCCG
421 TAAACGGTGG GCACTAGGTG TGGGCAACAT TCCACGTTGT CCGTGCCGCA GCTAACGCAT
481 TAAGTGCCCC GCCTGGGGAG TACGGCCGCA AGGCTAAAAC TCAAAGGAAT TGACGGGGGC
541 CCGCACAAGC GGCGGAGCAT GTGGCTTAAT TCGACGCAAC GCGAACAACC TTACCAA
```

Figure 3. Partial 16S rDNA sequence (597 bp) of isolate 3.

```
  1 TCCTACGGGA GGCAGCAGTA GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG
 61 CCGCGTGAGT GATGAAGGTT TTCGGATCGT AAAGCTCTGT TGTTAGGGAA GAATAAGTGC
121 AAGAGTAACT GCTTGCACCT GACGGTACCT AACCAGAAAG CCACGGCTAA CTACGTGCCA
181 GCAGCCGCGG TAATACGTAG GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC
241 GCAGGCGGTT TCTTAAGTCT GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA
301 AACTGGGAAA CTTGAGTGCA GAAGAGGAGA GTGGAATTCC ACGTGTAGCT GTGAAATGCG
361 TAGAGATGTG GAGGAACACC AGTGGCGAAG GCGACTCTCT GGTCTGTAAC TGACGCTGAG
421 GAGCGAAAGC GTGGGGAGCG AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT
481 GAGTGCTAAG TGTTAGGGGG TTTCCGCCCC TTAGTGCTGC AGCTAACGCA TTAAGCACTC
541 CGCCTGGGGA GTACGGTCGC AAGACTGAAA CTCAAAGGAA TTGACGGA
```

Figure 4. Partial 16S ribosomal DNA sequence (588 bp) of bacterial isolate 5.

```
   1 ACAGATGGGA GCTTGCTCCC TGATGTAGCG GCGGACGGGT GAGTAACACG TGGGTAACCT
  61 GCCTGTAAGA CTGGGATAAC TCCGGGAAAC CGGGGCTAAT ACCGGATGGT TGTTTGAACC
 121 GCATGGTTCA GACATAAAAG GTGGCTTCGG CTACCACTTA CAGATGGACC CGCGGCGCAT
 181 TAGCTAGTTG GTGAGGTAAC GGCTCACCAA GGCGACGATG CGTAGCCGAC CTGAGAGGGT
 241 GATCGGCCAC ACTGGGACTG AGACACGGCC CAGACTCCTA CGGGAGGCAG CAGTAGGGAA
 301 TCTTCCGCAA TGGACGAAAG TCTGACGGAG CAACGCCGCG TGAGTGATGA AGGTTTTCGG
 361 ATCGTAAAGC TCTGTTGTTA GGGAAGAACA AGTGCCGTTC AAATAGGGCG GCACCTTGAC
 421 GGTACCTAAC CAGAAAGCCA CGGCTAACTA CGTGCCAGCA GCCGCGGTAA TACGTAGGTG
 481 GCAAGCGTTG TCCGGAATTA TTGGGCGTAA AGGGCTCGCA GGCGGTTTCT TAAGTCTGAT
 541 GTGAAAGCCC CCGGCTCAAC CGGGGAGGGT CATTGGAAAC TGGGGAACTT GAGTGCAGAA
 601 GAGGAGAGTG GAATTCCACG TGTAGCGGTG AAATGCGTAG AGATGTGGAG GAACACCAGT
 661 GGCGAAGGCG ACTCTCTGGT CTGTAACTGA CGCTGAGGAG CGAAAGCGTG GGGAGCGAAC
 721 AGGATTAGAT ACCCTGGTAG TCCACGCCGT AAACGATGAG TGCTAAGTGT TAGGGGGTTT
 781 CCGCCCCTTA GTGCTGCAGC TAACGCATTA AGCACTCCGC CTGGGGAGTA CGGTCGCAAG
 841 ACTGAAACTC AAAGGAATTG ACGGGGGCCC GCACAAGCGG TGGAGCATGT GGTTTAATTC
 901 GAAGCACCGC GAAGAACCTT ACCAGGTCTT GACATCCTCT GACAATCCTA GATATAGGAC
 961 GTCCCCTTCG GGGGCAGAGT GACAGGTGGT GCATGGTTGT CGTCAGCTCG TGTCGTGAGA
1021 TGTTGGGTTA AGTCCCGCAA CGAGCGCAAC CCTTGATCTT AGTTGCCAGC ATTCAGTTGG
1081 GCACCTCTAA GGTGACTGCC GGTGACAAAC CGGAGGAAGG TGGGGATGAC GTCAAATCAT
1141 CATGCCCCTT ATGACCTGGG CTACACACGT GCTACAATGG ACAGAACAAA GGGCAGCGAA
1201 ACCGCGAGGT TAAGCCAATC CCACAAATCT GTTCTCAGTT CGGATCGCAG TCTGCAACTC
1261 GACTGCGTGA AGCTGGAATC GCTAGTAATC GCGGATCAGC ATGCCGCGGT GAATACGTTC
1321 CCGGGCCTTG TACACACCGC CCGTCACACC ACGAGAGTTT GTAACACCCG AAGTCGGTGA
1381 GGTAACCTTT ATGGAGCCAG CCGCCGAAGG TGGGACAGAT GATTGGGGTG AAGTCGTAAC
1441 AAGGTAGCCG TATCGGAAGG TGCGGCTGT
```

Figure 5. Partial 16S ribosomal DNA sequence (1469 bp) of bacterial isolate 6.

```
  1 TTGGACAATG GGCGAAAGCC TGATCCAGCC ATGCCGCGTG TGTGAAGAAG GTCTTCGGAT
 61 TGTAAAGCAC TTTAAGTTGG GAGGAAGGGC AGTAAGTTAA TACCTTGCTG TTTTGACGTT
121 ACCGACAGAA TAAGCACCGG CTAACTTCGT GCCAGCAGCC GCGGTAATAC GAAGGGTGCA
181 AGCGTTAATC GGAATTACCT GGGCGTAAAG CGCGCGTAGG TGGTTCGTTA AGTTGGATGT
241 GAAAGCCCCG GGCTCAACCT GGGAACTGCA TCCAAAACTG GCGAGCTAGA GTATGGCAGA
301 GGGTGGTGGA ATTTCCTGTG TAGCGGTGAA ATGCGTAGAT ATAGGAAGGA ACACCAGTGG
361 CGAAGGCGAC CACCTGGGCT AATACTGACA CTGAGGTGCG AAAGCGTGGG GAGCAAACAG
421 GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA CTAGCCGTTG GGATCCTTGA
481 GATCTTAGTG GCGCAGCTAA CGCATTAAGT CGACCGCCTG GGGAGTACGG CCGCAAGGTT
541 AAAACTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAA
601 GCAACGCGAA GAACCTTACC AGGCCTTGAC ATGCTGAGAA CCTGCCAGAG ATGGCGGGGT
661 GCCTTCGGGA ACTCAGACAC ACGTGCTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT
721 TGGGTTAAGT CCCGTAACGA GCGCAC
```

Figure 6. Partial16S rDNA sequence (746 bp) of isolate 7

VITA

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Personal Data: Born in Ponca City, Oklahoma, on September 13, 1976, the daughter of Max and Sandra Haines.

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