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Phytoremediation of energetic compounds at Eglin Air Force Base

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PHYTOREMEDIATION OF ENERGETIC COMPOUNDS AT EGLIN AIR FORCE BASE

by

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CHAPTER 1
INTRODUCTION AND OBJECTIVES

1.1 Background

The energetic compounds TNT (2,4,6-trinitrotoluene), RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) contaminate military testing ranges worldwide yet are known to be degraded by plants and microbes in the laboratory (Hawari, Beaudet, Halasz, Thiboutot, & Ampleman, 2000; Hawari et al., 2001; T. F. Jenkins et al., 2006; Van Aken & Agathos, 2001). However, these contaminants remain persistent in the environment and represent a health threat to both humans and ecosystems (Flokstra, Van Aken, & Schnoor, 2008; Thomas F. Jenkins, Bartolini, & Ranney, 2003). The use of traditional soil remediation technologies, such as landfilling or incineration, require large excavation costs and disrupt the ecology of the site . Phytoremediation, the use of green plants for the in situ treatment of contaminants, may be the most appropriate means of treating energetic residues present at military testing ranges (Hannink, Rosser, & Bruce, 2002).

Eglin Air Force Base (EAFB), located near Niceville, FL, is one of the largest military installations in the world and holds many plant and animal species which are threatened or endangered (Jacobson & Marynowski, 1997). The use of explosives during training exercises on firing ranges at EAFB has resulted in contamination of energetics on range soils. In an effort to increase range sustainability with respect to explosives contamination, EAFB has been established as the site where phytoremediation processes will be explored for this research.

1.2 The Use of Explosives on Military Training Ranges

Although the use of explosives on military ranges may result in widespread contamination of energetic materials, testing and training with conventional weapons is necessary for maintaining armed services combat readiness (Thiboutot, Ampleman, & Hewitt, 2002). Energetic residues in soil at training ranges are typically present at operational firing points, sites where munitions have undergone a low-order (partial) detonation, sites where demolition activities occur, and where unexploded ordinance (UXO) has been blown-in-place (Alan D. Hewitt et al., 2007). Explosives are classified as either "primary" or "secondary". Primary explosives are highly susceptible to ignition and are often used to ignite secondary explosives. Secondary explosives, which include TNT, RDX, and HMX, are much more prevalent on military ranges than primary explosives (Thiboutot, et al., 2002). The concentration, distribution, and type of energetic residue present on military ranges will vary depending on the type of training exercise and munitions used (T. F. Jenkins, et al., 2006).

1.3 Properties of RDX, HMX, and TNT

RDX is a cyclic nitramine and a major component of military explosives such as Composition B (Comp B) and Composition 4 (C4) (Alan D. Hewitt, et al., 2007). Originally prepared for medical use in 1899, the properties and preparation of RDX were fully developed during World War II (Akhavan, 2004). RDX is a white crystalline solid and has great explosive power compared with TNT. Contamination of RDX is of particular concern at military ranges due to its mobility in the environment and toxicity (Dontsova, Yost, Simunek, Pennington, & Williford, 2006; Flokstra, et al., 2008). RDX has migrated into the groundwater at sites such as the Massachusetts Military Reservation

and the Iowa Army Ammunition Plant causing regulatory action to take place (J. Clausen, Robb, Curry, & Korte, 2004; McCutcheon & Schnoor, 2003).

HMX is another cyclic nitramine used in military explosives, and is often found as an impurity of RDX (Alan D. Hewitt, et al., 2007). Military grade RDX contains approximately 10% HMX, therefore RDX and HMX are often found together in military range samples (T. F. Jenkins, et al., 2006). HMX is similar to RDX with respect to its chemical reactivity and is used as a high explosive in octol mixtures, which consist of 70% HMX and 30% TNT (Ampleman, Marois, & Thiboutot, 1999). Octol is typically used in rockets, causing extensive HMX contamination on anti-tank ranges (T. F. Jenkins et al., 1999).

TNT has been used extensively in explosives since 1902, resulting in soil and groundwater contamination from munitions manufacture and processing (Gorontzy et al., 1994). Low manufacturing cost, low sensitivity to impact and friction, and fairly high explosive power have made TNT popular for military use from WW I to the present. Although six different isomers of TNT exist, the symmetrical isomer 2,4,6-trinitrotoluene is used in the explosives industry (Akhavan, 2004). TNT is a nitroaromatic compound which biodegrades readily under both aerobic and anaerobic conditions (Hawari, et al., 2000). Although the nitro groups surrounding the aromatic ring of TNT are easily reducible, the aromatic ring is resistant to electrophilic attack, making mineralization of TNT rare (Spain, 1995). TNT is highly toxic and mutagenic to certain aquatic species and has caused toxic effects in workers at large-scale manufacturing operations (McCormick, Feeherry, & Levinson, 1976; Won, Disalvo, & Ng, 1976). The physical-chemical properties of TNT, RDX, and HMX are shown in Table 1-1.

1.4 Remediation Technologies for Explosives

Current remediation strategies for explosives contaminated soil include incineration, landfilling, composting, and bioaugmentation. Removing soil from contaminated sites such as military training ranges for incineration, landfilling, or composting is extremely expensive and disrupts the ecology of the site. Microbes which are introduced during bioaugmentation may compete poorly with native microbes, requiring amendments to the soil and increasing costs (Van Dillewijn et al., 2007). Phytoremediation is an ideal method for the *in situ* treatment of explosives due to cost effectiveness and minimal disruption of site ecology compared with traditional technologies (Hannink, et al., 2002). However, phytoremediation may require long periods of time to be effective.

1.5 Phytoremediation of Explosives

Phytoremediation is defined as the use of plants and their associated microbes for environmental cleanup. Phytoremediation is usually performed *in situ* and is viewed positively by the public as an alternative to chemical treatment systems and excavation equipment (Pilon-Smits, 2005). Plants can be used in a variety of ways to treat contaminants, including pollutant stabilization, extraction, degradation, and volatilization. Phytostabilization is used to reduce the mobility of contaminants, especially metals, by stabilizing them in the soil and reducing the interaction of contaminants with biota. Phytoextraction is the uptake of contaminants by plants, resulting in phytoaccumulation when the contaminant is not degraded. Plants can be harvested following phytoaccumulation of contaminants and disposed of in appropriate landfills. Phytodegradation (phytotransformation) occurs when plants tissue is capable of

degrading contaminants by plant enzymes or enzyme co-factors (Susarla, Medina, & McCutcheon, 2002). For example, nitroreductase and laccase enzymes have been shown to break down TNT and incorporate the broken ring structure into new plant material (Schnoor, Licht, McCutcheon, Wolfe, & Carreira, 1995). Phytovolatilization occurs through the uptake of a volatile compound, or conversion of a contaminant into a volatile form, resulting in the release of contaminants to the atmosphere (Pilon-Smits, 2005).

Another form of phytoremediation is rhizodegradation, the biological treatment of a contaminant by enhanced bacterial and fungal activity in the rhizosphere of plants (Susarla, et al., 2002). The rhizosphere is the volume of soil surrounding living roots which is influenced by root activity (Hinsinger, Bengough, Vetterlein, & Young, 2009). The symbiotic relationship between plants and microorganisms creates an area of increased microbial activity in the rhizosphere. Plant roots aerate the soil and produce root exudates which provide nutrients for bacteria. Bacteria in the rhizosphere produce beneficial exudates of their own and provide disease resistance by destroying pathogens. This beneficial relationship can result in both reduced toxicity and reduced nutrient deficiency in bacteria and plants (Wenzel, 2009).

The "green liver" model can be used to describe the processes by which xenobiotics such as explosives are metabolized by plants (Sandermann, 1994). The detoxification of xenobiotics in plants occurs in three stages: transformation, conjugation, and sequestration. Transformation of contaminants occurs through chemical reactions such as oxidation or reduction. Transformed compounds can then take place in conjugation reactions with plant compounds, resulting in less toxic products. The resulting compound can then undergo sequestration processes such as storage in plant

vacuoles (Singh & Jain, 2003). The fate of plant-transformed contaminants is an important consideration for phytoremediation applications due to the possibility of plant consumption leading to bioaccumulation of contaminants in the food chain (Hannink, et al., 2002).

1.5.1 Phytoremediation of RDX

Uptake of RDX occurs in a wide variety of plants with accumulation occurring mostly in leaves rather than in the roots (Laura B. Brentner, Mukherji, Walsh, & Schnoor, 2009; Hannink, et al., 2002). Multiple transformation processes have been observed following uptake and translocation of RDX to plant leaves. Transformation of RDX to polar metabolites in plant tissues has commonly been reported (Hannink, et al., 2002). The formation of bound residues containing RDX metabolites have also been observed and are likely the result of an initial transformation process followed by conjugation reactions (Just & Schnoor, 2004). Mineralization of RDX was observed in poplar tissue through phytophotolysis in a proposed three step pathway: (1) a light-independent reduction of RDX to MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine) and DNX (hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine) by plant cells, (2) a plant/light mediated breakdown of RDX, MNX, or DNX into metabolites (formaldehyde and methanol), (3) a light-independent mineralization of metabolites to CO_2 (Van Aken, Yoon, Just, & Schnoor, 2004). The reduction of RDX to MNX was also observed in whole plant hybrid poplar experiments with further loss to volatile products (Yoon, Van Aken, & Schnoor, 2006). Overall, RDX appears to be translocated to leaves relatively quickly followed by reductive transformation with possible mineralization to CO_2.

1.5.2 Phytoremediation of HMX

The uptake of HMX in plants appears to be hindered by its poor solubility (Hannink, et al., 2002). HMX does not translocate as readily as RDX, and is more recalcitrant than both RDX and TNT (Yoon, et al., 2006). HMX accumulates in leaves following translocation without any known transformation. Leaching of HMX from fallen poplar leaves has also been observed, causing further concern for phytoremediation applications (Yoon, Oh, Just, & Schnoor, 2002). Although uptake of HMX has been documented in aquatic, wetland, and terrestrial plants, no metabolites have yet been found (Best, Kvesitadze, Khatisashvili, & Sadunishvili, 2005). Further plant metabolism studies of HMX are warranted if phytoremediation is to be implemented for HMX treatment.

1.5.3 Phytoremediation of TNT

TNT typically degrades rapidly in the presence of both bacteria and plants but is poorly mineralized (Hannink, et al., 2002; Hawari, et al., 2000). Although TNT degradation has been observed in plant tissues, TNT translocation does not occur in many plant species (Schneider, Oltmanns, Radenberg, Schneider, & PaulyMundegar, 1996). The majority of TNT contamination typically remains in root tissues with little transport to stems and leaves (Laura B. Brentner, et al., 2009). The lack of TNT translocation in most plants is likely due to the high biochemical reactivity of the aromatic nitro group which forms oxidative coupling reactions on roots (Thompson, Ramer, & Schnoor, 1998).

The aerobic reduction of TNT is the most common transformation reaction performed by plants, resulting in the metabolites 2-ADNT (2-amino-4,6-dinitrotoluene)

and 4-ADNT (4-amino-2,6-dinitrotoluene) (Hannink, et al., 2002). These metabolites are also reactive and can undergo further binding or degradation reactions. The metabolites 2,4-DNT (2,4-diamino-6-nitrotoluene) and 2,6 DNT (2,6-diamino-4-nitrotoluene) have also been detected as transformation products, important as these compounds have been shown to be animal carcinogens (Schneider, et al., 1996). TNT transformation has been observed in many plant types including hybrid poplar trees, aquatic wetland plants, and various grass species (Hughes, Shanks, Vanderford, Lauritzen, & Bhadra, 1997; Scheidemann, Klunk, Sens, & Werner, 1998; Thompson, et al., 1998). Although reduction of TNT appears to be the dominant detoxification process in plants, oxidation processes have also been shown and may initiate a route to conjugation and sequestration (Best, et al., 2005). Gene expression analysis in poplar trees exposed to TNT have shown that glutathione S-transferases are the genes which may be responsible for detoxification of TNT in plants (L. B. Brentner et al., 2008). The evidence supporting plant metabolism of TNT is encouraging for future phytoremediation applications.

1.6 Phytoremediation of Explosives in Field Studies

Although there have been many laboratory and pilot studies published detailing the effectiveness of explosives phytoremediation, few full-scale phytoremediation field studies treating explosives have been performed to date (Hannink, et al., 2002; Singh & Jain, 2003). Phytoremediation was successfully implemented as a method for treating TNT and RDX using treatment wetlands at the Iowa Army Ammunition Plant (IAAP) in Middletown, Iowa (McCutcheon & Schnoor, 2003). Screening studies were performed using several species of native aquatic plants to determine the uptake kinetics of explosives from contaminated IAAP groundwater samples (Best et al., 1997). The

screening results showed significant TNT removal (94% to 100%) from plant incubations, although RDX results were not conclusive over the 10 day study. No particular plant species was shown to provide superior treatment in the pilot-screening studies, so aquatic species were chosen for the treatment wetlands based on ability to survive in the climate of the region. Following construction, the two treatment wetlands were monitored for 2 years during which TNT concentrations were undetectable in surface water and plant tissue. RDX concentrations in both wetlands remained below the EPA human health advisory level of 0.002 mg/L during the summer months when wetland water was discharged to adjacent surface waters.

1.7 Eglin Air Force Base

Eglin Air Force Base (EAFB) is located in the panhandle of Florida near the city of Niceville. EAFB is the largest forested military installation in the western hemisphere and is home to the critically endangered longleaf pine forest ecosystem. Much of EAFB is open to the public for recreational uses such as hunting, fishing, camping, hiking, and wildlife observation. Many of the plant and animal species inhabiting the base are classified as rare, of special concern, threatened, or endangered (Jacobson & Marynowski, 1997). The location of EAFB is shown in Figure 1-1.

The mission of EAFB as a major research, development, and test facility includes the evaluation of a wide range of non-nuclear munitions. EAFB also supports training activities involving ground troops, air operations, and special operations. To ensure continued access of land and airspace for military research and development, the natural resources of EAFB must be kept in a healthy condition (Corporation, 2007).

EAFB supports a wide variety of wildlife due to the variety of habitats found on the base. The types of habitats found on undeveloped portions of the base are generally in decline throughout off-base areas of Florida due to urban development. There are 11 federally listed threatened and endangered species which are being managed on EAFB. These species occur on the Eglin Reservation year round or seasonally and include: the red-cockaded woodpecker, bald eagle, piping plover, Okaloosa darter, Gulf sturgeon, flatwoods salamander, Eastern indigo snake, loggerhead sea turtle, green sea turtle, leatherback sea turtle, and Florida perforate lichen. These species depend on the natural conditions and habitat found throughout EAFB (Corporation, 2007).

EAFB is recognized as a globally significant area for biodiversity as many species have taken refuge on range land to escape rapid development throughout the surrounding region. Therefore, natural resources on the base must be managed while still providing as much flexibility to the military as possible for training missions (Corporation, 2007). Phytoremediation could play a significant role in natural resource management at EAFB by removing toxic contaminants from range environments while disrupting critical habitats as little as possible.

1.8 Objectives

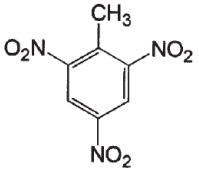
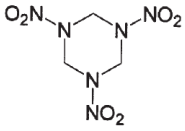
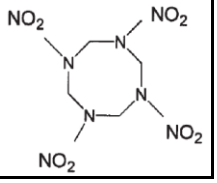
The main objective of this project is to determine whether phytoremediation can be used to improve long-term operation of EAFB and to prevent leaching of energetics, thus rendering the site to be more sustainable; and to understand the mechanisms by which energetic compounds (RDX, HMX, and TNT) are actually degraded in contaminated subsurface soils at EAFB. This research will be performed using three phytoremediation field plots at EAFB. Plot #1 is expected to contain high levels of

explosives, Plot #2 is expected to contain lower levels of explosives, and Plot #3 (control plot) is not expected to contain explosives and will be used to determine if explosives are toxic to vegetations through comparison to Plot #1 and Plot #2. This research is significant as there have been no field scale phytoremediation demonstrations on military ranges to date. The following specific objectives have been established for this research:

1. Determine whether plants significantly improve biodegradation of explosives at a contaminated site near the Open Burn/Open Detonation area at EAFB through comparison of the planted and unplanted portions of Plot #1
2. Determine whether plants can significantly uptake RDX and HMX at the site
3. Determine whether RDX, HMX, and TNT are biodegraded in unplanted soils from the contaminated area

To meet these objectives, the background degradation rates of RDX, HMX, and TNT in unplanted EAFB soils are investigated in Chapter 2 to provide comparison to field degradation rates. The results of microbial community characterization is presented in Chapter 3 to better understand the microbial structure of different EAFB soils and to draw conclusions on how microbial diversity may affect remediation strategies. The field work performed for this research is shown in Chapter 4, which details results from the ongoing phytoremediation field study at EAFB.

Table 1-1. Physical-chemical properties of TNT, RDX, and HMX.

	TNT	**RDX**	**HMX**
Molecular Formula (Akhavan, 2004)	$C_7H_5N_3O_6$	$C_3H_6N_6O_6$	$C_4H_8N_8O_8$
Molecular Weight (g) (Akhavan, 2004)	227.1	222.1	296.2
Solubility in Water (mg/L) (J. L. Clausen, Korte, Nic, Dodson, Robb, & Rieven, 2006)	130	42	5
Molecular Structure (Hannink, et al., 2002)			

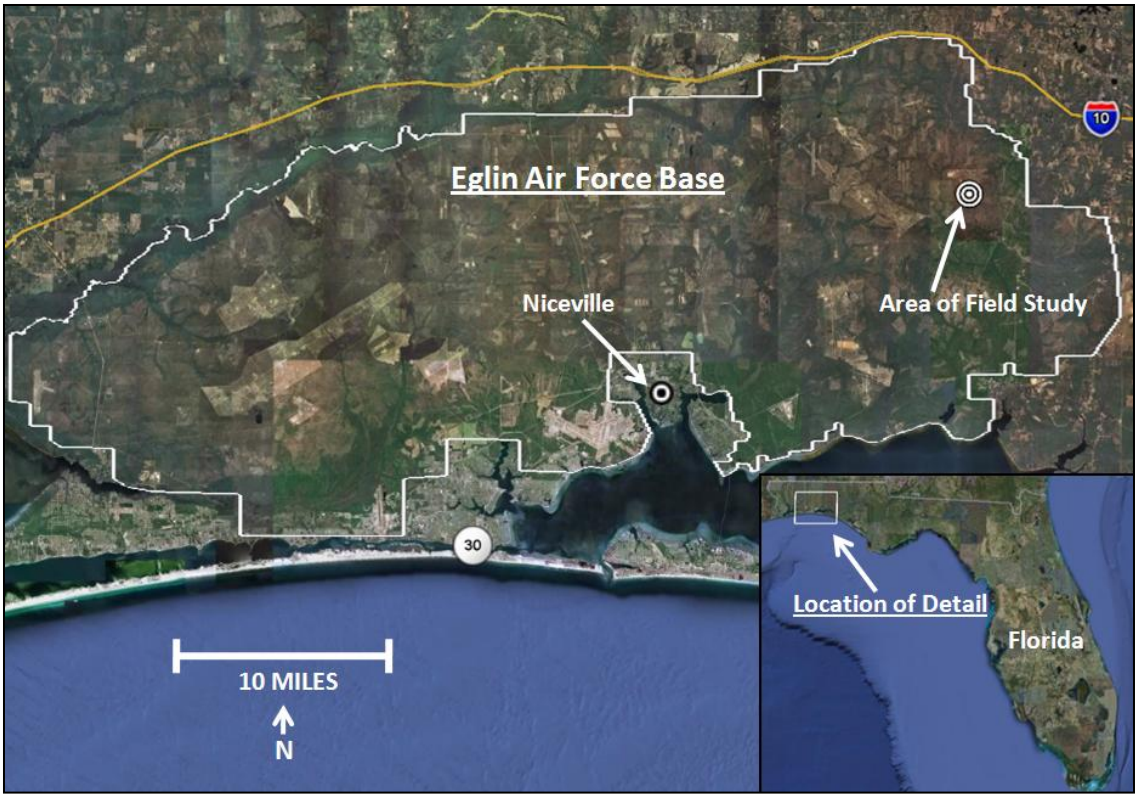


Figure 1-1. Location of Eglin Air Force Base and area of field study.

CHAPTER 2
BIODEGRADATION OF EXPLOSIVES IN UNPLANTED SOILS FROM EGLIN AIR FORCE BASE, FL

2.1 Introduction

To better understand and differentiate the removal mechanisms for the energetic compounds TNT, RDX, and HMX, a variety of microcosm studies have been performed using soils taken from Eglin Air Force Base (EAFB) in Okaloosa County, Florida. Examples in the literature show degradation and removal of TNT, RDX, and HMX from soil by both bacteria and plants (Hannink, et al., 2002; Hawari, et al., 2000). This indicates that both microbes and plants are likely to play important roles in any phytoremediation application. Microcosm studies using unplanted soil provide an estimate for background degradation rates for comparison to phytoremediation removal rates.

2.2 Materials and Methods

2.2.1 Soil Collection

The two types of soils investigated in these studies are Lakeland Soil and Dorovan Muck. Lakeland Soil is a sandy soil and is the predominant soil type at EAFB. Dorovan Muck, an organic rich clayey soil, is less common and is located in low-lying areas. Soil samples were collected during a site visit at EAFB on March 2, 2007 and shipped to The University of Iowa in 28-qt coolers at 4-8 °C. The soils were stored at 4 °C until used in experiments.

2.2.2 Soil Analyses

Samples of Lakeland Soil and Dorovan Muck were sent to A&L Analytical Laboratories (Memphis, TN) for nutrient, pH, bulk density, and texture analyses. The results of the laboratory analyses are provided in Table 2-1 and Table 2-2. The physical and chemical characteristics of the two soil types are very different as shown in Table 2-1 and Table 2-2. Dorovan Muck has a much higher clay content and organic content than Lakeland Soil, along with higher nutrient levels.

2.2.3 Experimental Setup

Soil used in the experiment was unplanted and either contaminated immediately prior to incubation or was contaminated and aged for 18 months while kept at 4 °C. The experimental set up for freshly contaminated soil consisted of soil which was: sterile and kept in the light, sterile and kept in the dark, non-sterile and kept in the light, or non-sterile and kept in the dark. The experimental setup for soil which was contaminated and aged for 18 months consisted of soil which was non-sterile and either kept in the light or dark. The experimental setup is provided in Table 2-3.

2.2.4 Experimental Conditions

Experiments were carried out in pint sized glass jars. The experiment was conducted at 30 °C under a 16:8 hour light:dark photoperiod (150 μmol s^{-1} m^{-2}). Jars kept in the dark were covered with aluminum foil, loosely enough to allow for passive gas exchange. The soil was kept moist with deionized, sterile water, but was not saturated to keep soil conditions aerobic. At each time step in the experiment, samples were taken from eight locations in each jar and composited to account for heterogeneity. All experiments were conducted in triplicate.

2.2.5 Chemicals and Materials

TNT and analytical standards were purchased through Chemservice (Westchester, PA). RDX was synthesized in house according to (Ampleman et al., 1995) and purified by recrystallization. Synthesized RDX was 99% pure or higher as indicated by High Performance Liquid Chromatography (HPLC) analysis. HMX was synthesized in house according to (Ampleman et al, 1999), and determined to be 99% pure or higher as indicated by HPLC and Neutron Magnetic Resonance (NMR) analysis.

2.2.6 Soil Sterilization

Soil was sterilized through gamma irradiation performed by Sterix Isometrics. Both soil types underwent gamma irradiation using ^{60}Cobalt at 30 – 40 kGy. Following sterilization, samples of each soil type were diluted in minimal salts media, spread onto full strength tryptic soy broth agar plates, and allowed to incubate for several weeks in the dark at 30 °C. No bacterial colonies formed during the incubation.

2.2.7 Soil Contamination

Soil to be aged was contaminated separately with TNT and RDX to a nominal concentration of 100 mg/kg and 50 mg/kg, respectively and then stored for 18 months at 4 °C prior to the start of the experiment. Freshly contaminated soil was contaminated with TNT, RDX, and HMX to a nominal concentration of 100 mg/kg. Explosives dissolved in acetonitrile were first mixed vigorously into 6% of soil mass, subsequently blended with the remaining soil for 20 seconds, mixed thoroughly, and then blended again for 20 seconds to obtain a homogeneous contamination. Soils were stored at 4°C until use in degradation experiments.

2.2.8 Explosives Extraction from Soil

Extraction of explosives from soil was performed using a modified version of EPA Method 8330B (Agency, 2006). Soil samples were dried at room temperature until a constant weight was reached. Each sample was then ground with mortar and pestle, placed in a 15 ml vial, and extracted with 10 ml acetonitrile to 1 g soil in an ultrasonic bath for 18 hours. The samples were then filtered under 0.20 µm Durapore membrane filters. Sample filtrate was then collected for analysis by HPLC.

2.2.9 Chemical Analyses

Analyses of TNT, TNT metabolites, RDX, and HMX were performed by HPLC (HP Series 1100; Hewlett-Packard, Palo Alto, CA) using a C_{18} Supelcosil® LC-18 column (Supelco, Bellefonte, PA). The mobile phase consisted of acetonitrile:deionized water 50:50 v/v at a flow rate of 1.0 ml/min. Compounds were detected by UV absorbance at 230 nm and 254 nm using a UV visible photodiode array detector (HP series 1100). Calibration standards and blanks were analysed before and and after sample runs to ensure quality control.

2.3 Results

2.3.1 RDX and HMX Degradation

As shown in Figure 2-1, Figure 2-2, and Figure 2-3, soil samples taken over the course of 56 days showed no statistically significant degradation of RDX or HMX in any of the soil microcosms. Minimal degradation of RDX and HMX could have occurred but may have been masked by the error associated with the variability in sampling each pot.

However, as the experiment took place over 56 days, any minimal degradation would still indicate very slow removal kinetics for RDX and HMX.

2.3.2 TNT Degradation

TNT degraded rapidly in microcosms which were freshly contaminated as shown in Figure 2-4. In freshly contaminated Dorovan Muck, greater than 50% removal was achieved within the first 7 days with the exception of the sterile soil which was kept in the light. Non-sterile Dorovan Muck kept in the light and dark achieved 90% and 91% removal, respectively, over 56 days. Less degradation occurred in sterile Dorovan Muck, which achieved 40% removal in the light and 89% removal in the dark over 56 days.

Lakeland Soil which was freshly contaminated with TNT showed similar results to Dorovan Muck. Rapid degradation occurred in non-sterile, freshly contaminated Lakeland Soil with 89% and 88% removal in the light and dark, respectively, over the first 28 days. Over 56 days, the non-sterile freshly contaminated Lakeland Soil achieved 97% removal in the light and 93% removal in the dark. Degradation in sterile, freshly contaminated Lakeland Soil occurred more slowly than in non-sterile Lakeland Soil. TNT removal of 94% in the light and 53% in the dark occurred in sterile, freshly contaminated Lakeland Soil.

Degradation of TNT in aged Dorovan Muck was much lower compared to aged Lakeland Soil over the 56 day experiment as shown in Figure 2-5, likely due to increased binding in the higher clay content of Dorovan Muck. TNT removal in aged Dorovan Muck was 19% in the light and 36% in the dark. Aged Lakeland Soil achieved 97% and 93% removal in the light and dark, respectively.

The target concentration of 100 mg/kg for TNT was not obtained in all of the experimental setups shown in Figure 2-4. This could be due to particles of undissolved TNT that may have been present in the stock solution used to contaminate the soils. Different starting concentrations, especially the higher TNT concentration present in the sterile Dorovan Muck in the light, could have made an impact on removal rates throughout the experiment.

The majority of metabolites detected during the TNT microcosm study were 2-ADNT (2-amino-4,6-dinitrotoluene) and 4-ADNT (4-amino-2,6-dinitrotoluene). Trace amounts of 2,4-DNT (2,4-diamino-6-nitrotoluene) and 2,6 DNT (2,6-diamino-4-nitrotoluene) were also detected at concentrations much lower than those of 2-ADNT and 4-ADNT. As shown in Figure 2-6 and Figure 2-7, the total ADNT concentrations are much higher in Dorovan Muck than in Lakeland Soil. ADNT concentrations increased rapidly (within 7 days) in freshly contaminated Dorovan Muck, with the exception of sterile soil kept in the light. Minimal ADNT concentrations were present in freshly contaminated Lakeland Soil over the 56 day experiment. As with freshly contaminated soil, aged Dorovan Muck contained higher levels of ADNTs than aged Lakeland Soil.

2.4 Discussion

The results of the degradation experiments provided in Figure 2-1, Figure 2-2, and Figure 2-3 show that HMX and RDX are recalcitrant under moist, aerobic conditions in the two soils which were investigated. These findings are consistent with other results in the literature showing the relative stability of RDX and HMX under aerobic conditions (Grant, Jenkins, Myers, & McCormick, 1995; Thomas F. Jenkins, et al., 2003; McCormick, Cornell, & Kaplan, 1981). The majority of explosives contamination at

military testing ranges is primarily present in the top 5 cm of the ground surface (generally aerobic), which may explain the recalcitrance of these compounds under actual field conditions (Alan D. Hewitt, et al., 2007).

In contrast to RDX and HMX, TNT disappeared relatively quickly under most experimental conditions. All microcosms with freshly contaminated soil showed significant removal of TNT, regardless of soil type or being sterile. As shown in Figure 2-4, TNT degraded most consistently in non-sterile soil, with approximately 90% removal for both soil types in the light and in the dark. More variable results occurred among sterile soils. TNT removal in sterile soil ranged from 40% (Dorovan Muck, light) to 94% (Lakeland Soil, light). Although degradation occurred more slowly in the sterile soils, significant removal did occur over the 56 day experiment. This may indicate irreversible binding to clay, an abiotic degradation mechanism for TNT, or bacterial contamination into the soil microcosms over the course of the experiment.

Results from the aged TNT microcosms showed significant differences between removal rates in the two soil types, as shown in Figure 2-5. Much lower removal was observed in aged Dorovan Muck compared to both aged Lakeland Soil and freshly contaminated soil of both types. Removal of only 19% (light) and 36% (dark) occurred in aged Dorovan Muck. The substantially greater TNT removal of 97% (light) and 93% (dark) in aged Lakeland Soil is likely due to the much higher clay content present in Dorovan Muck. TNT has been shown to adsorb to clay minerals, which may cause a decrease of TNT bioavailability to bacteria in the process (Haderlein, Weissmahr, & Schwarzenbach, 1996).

As expected, 2-ADNT and 4-ADNT were the dominant metabolites formed from TNT degradation throughout the experiment. As shown in Figure 2-6, higher concentrations of ADNTs remained in Dorovan Muck than in Lakeland Soil. This is likely due to decreased bioavailability in Dorovan Muck causing degradation to occur more slowly than in Lakeland Soil. Similar results were observed for ADNT concentrations in aged soils, as shown in Figure 2-7. As in freshly contaminated soil, higher concentrations of ADNTs were present in aged Dorovan Muck than in aged Lakeland Soil throughout the experiment.

In conclusion, this experiment has shown that RDX and HMX are recalcitrant under aerobic conditions in both soil types that are present at EAFB. These are expected results and are similar to the findings observed in the literature regarding the general recalcitrance of these compounds under aerobic conditions (Grant, et al., 1995; Thomas F. Jenkins, et al., 2003; McCormick, et al., 1981). In contrast, TNT was shown to disappear relatively quickly with some degradation to 2-ADNT and 4-ADNT, as well as trace amounts of 2,4-DNT and 2,6-DNT. This pathway for bacterial degradation of TNT under aerobic conditions has been well documented and is now confirmed to occur in EAFB soils (Esteve-Nunez, Caballero, & Ramos, 2001).

Table 2-1. Results of soil texture analysis.

	Dorovan Muck	**Lakeland Soil**
Sand (%)	11.1	70
Silt (%)	16.7	4
Clay (%)	72.2	26
Classification	Clay	Loamy sand

Table 2-2. Summary of physical-chemical soil characteristics.

	Dorovan Muck	**Lakeland Soil**
Soil pH	4.1	5.1
Buffer pH	6.14	6.93
Bulk density (g/cc)	0.94	1.45
Phosphorus (lb/acre)	22 (low)	16 (low)
Potassium (lb/acre)	40 (very low)	36 (very low)
Calcium (lb/acre)	1018 (high)	512 (med)
Magnesium (lb/acre)	182 (med)	38 (low)
Nitrate nitrogen (lb/acre)	16	14
Ammonical nitrogen (lb/acre)	20	0
CEC (meq/100g)	10.6	1.9
Organic matter content (%)	4.8	0.4

Table 2-3. Experimental setup for unplanted soils experiment using Lakeland Soil and Dorovan Muck.

	Freshly Contaminated	**Aged 18 Months**
Sterile, Light	TNT, RDX, HMX	-
Non-Sterile, Light	TNT, RDX, HMX	TNT, RDX
Sterile, Dark	TNT, RDX, HMX	-
Non-Sterile, Dark	TNT, RDX, HMX	TNT, RDX

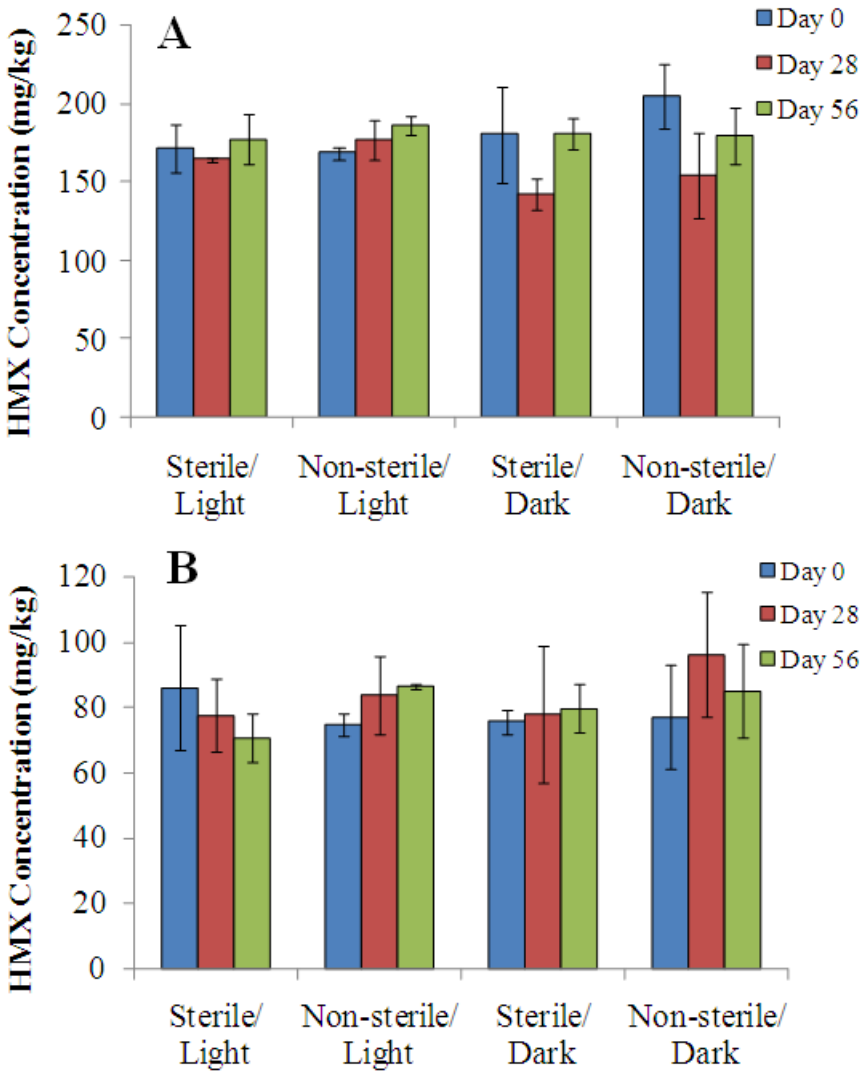


Figure 2-1. Degradation experiment of HMX incubated in (A) Dorovan Muck and (B) Lakeland Soil. Error bars represent one standard deviation.

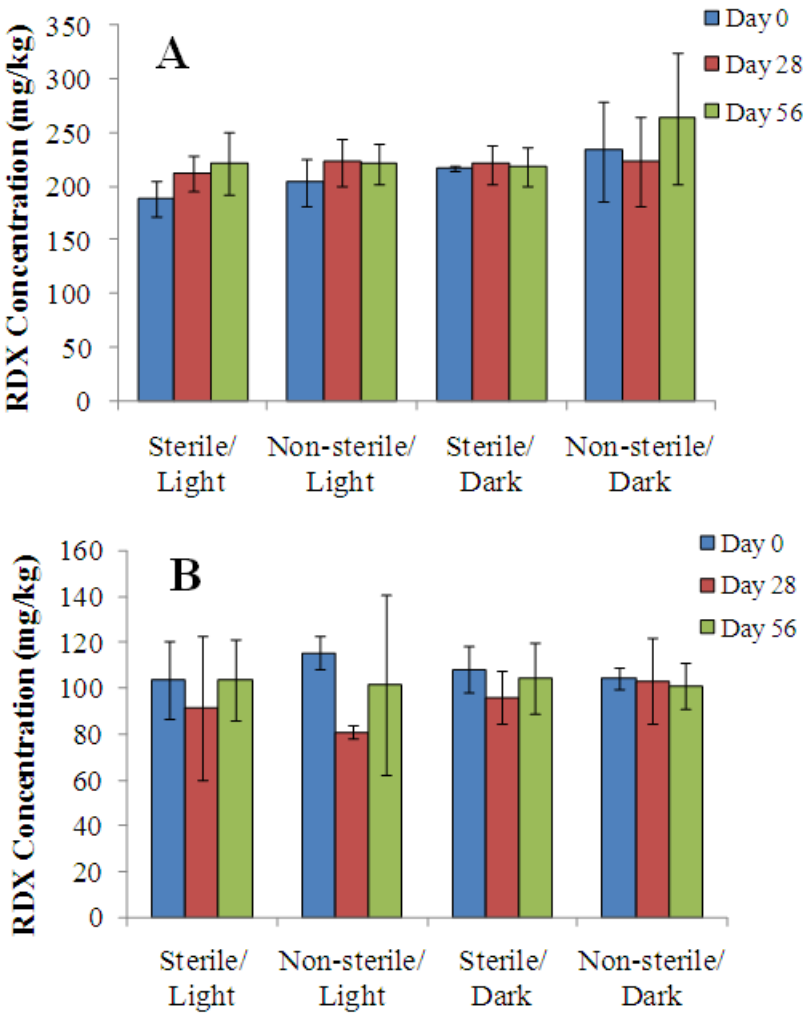


Figure 2-2. Degradation experiment of RDX incubated in (A) freshly contaminated Dorovan Muck, (B) freshly contaminated Lakeland Soil.

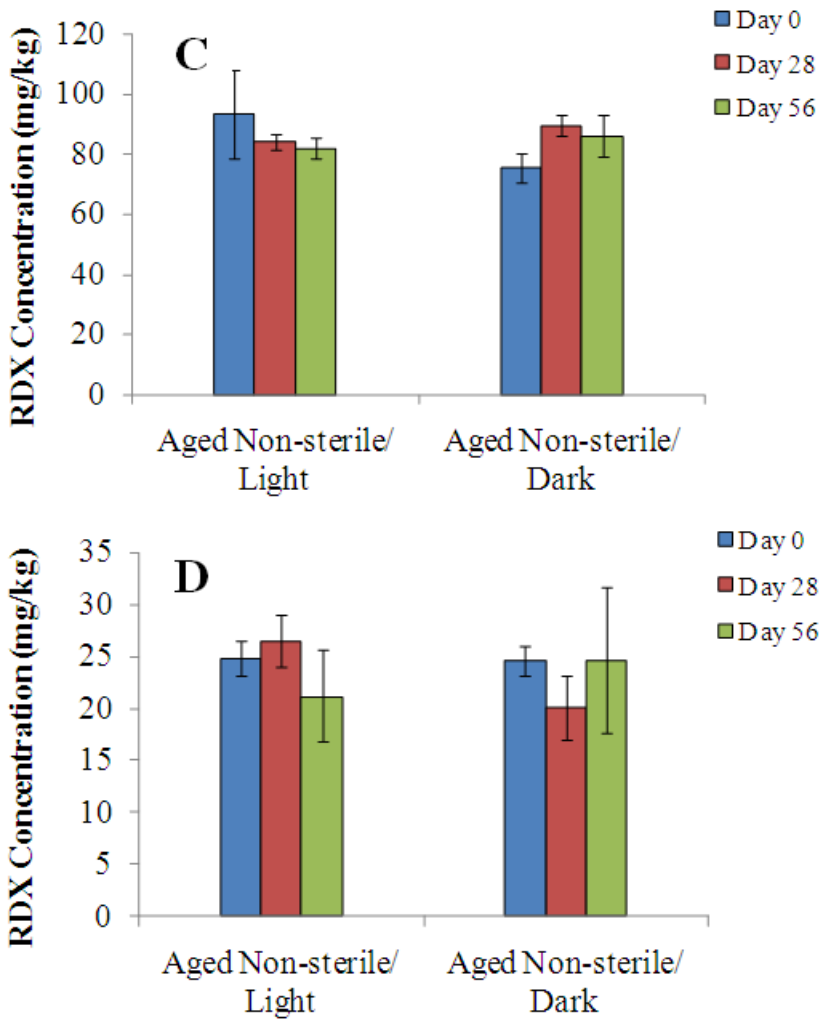


Figure 2-3. Degradation experiment of RDX incubated in (C) aged Dorovan Muck, (D) aged Lakeland Soil.

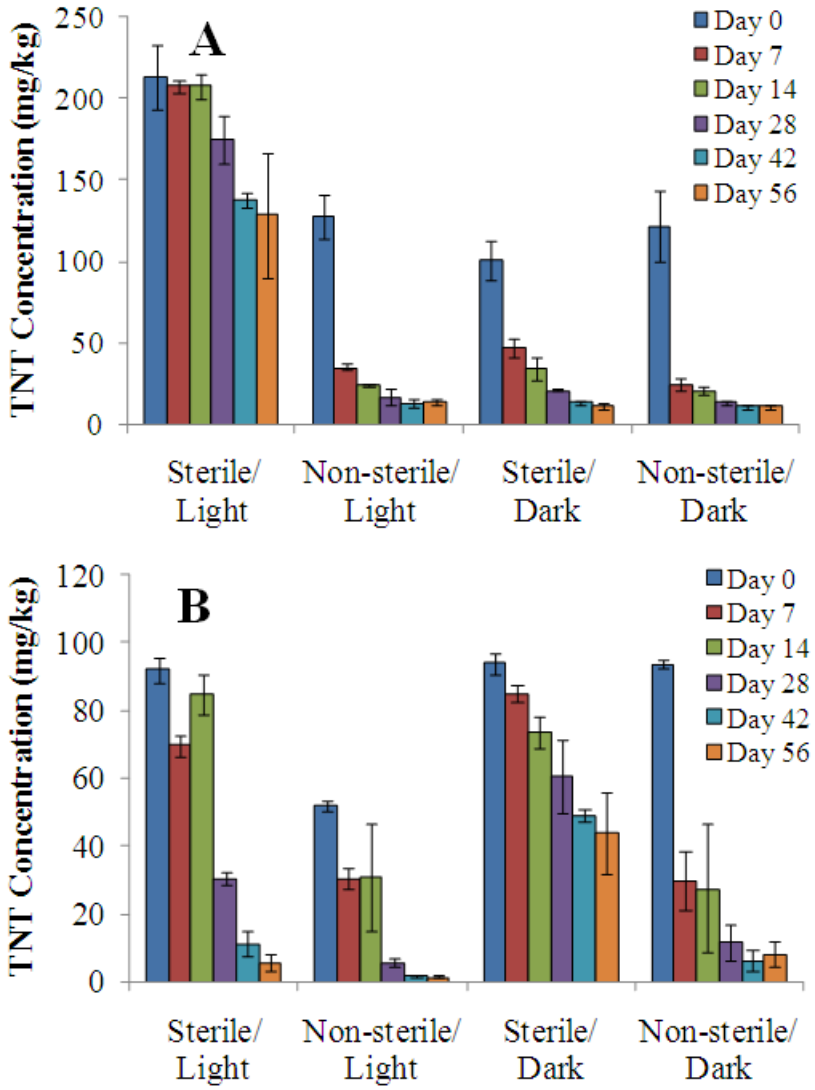


Figure 2-4. Degradation experiment of TNT incubated in (A) freshly contaminated Dorovan Muck and (B) freshly contaminated Lakeland Soil. Error bars represent one standard deviation.

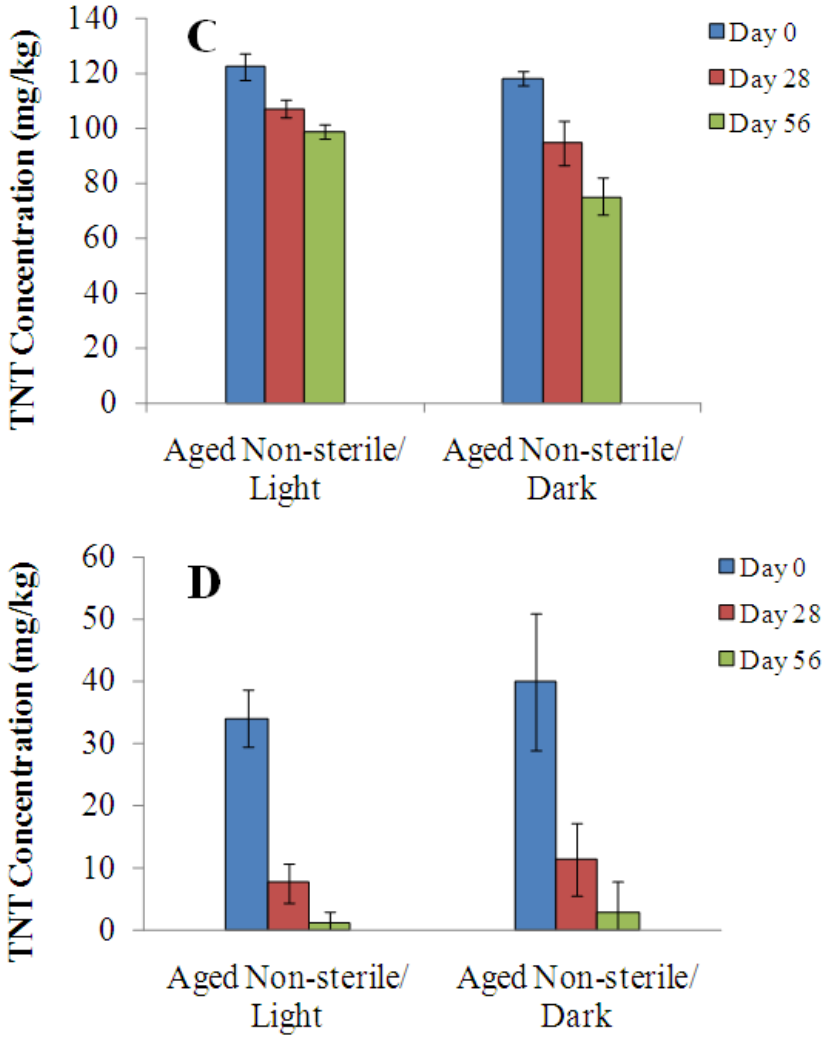


Figure 2-5. Degradation experiment of TNT incubated in (C) aged Dorovan Muck and (D) aged Lakeland Soil. Error bars represent one standard deviation.

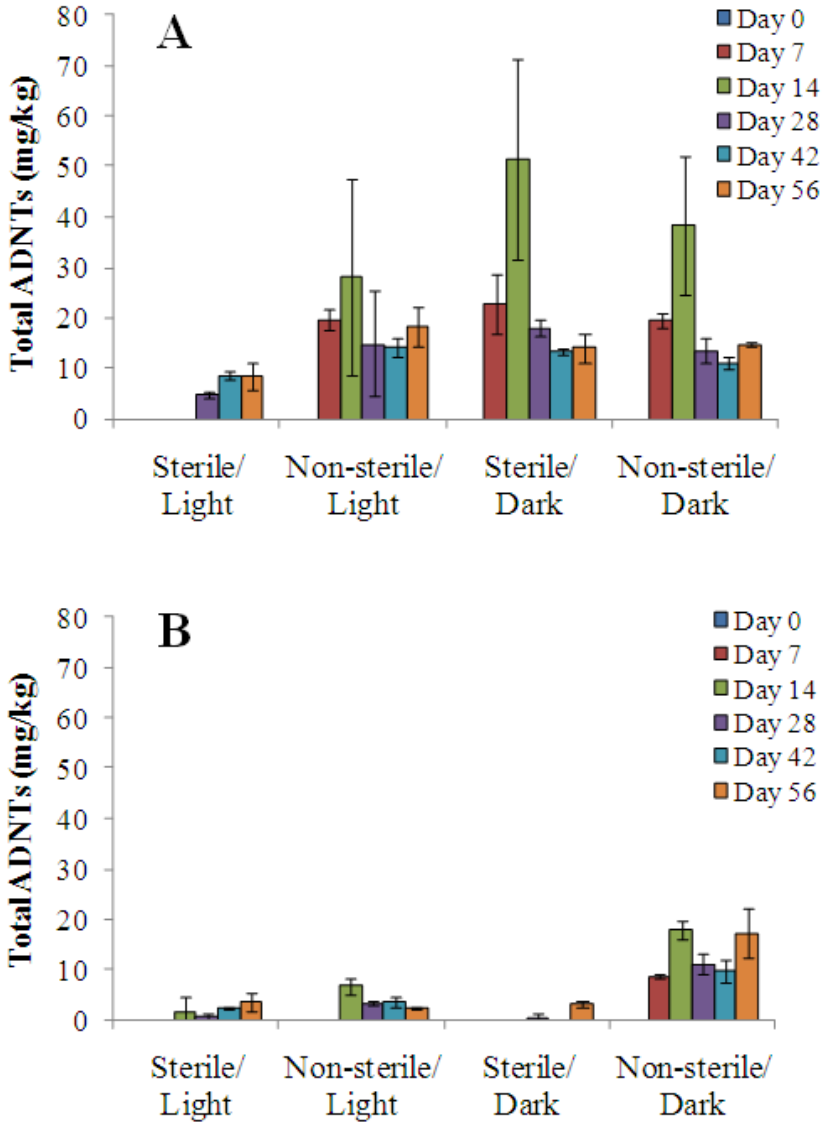


Figure 2-6. TNT metabolites 2-ADNT and 4-ADNT measured in (A) freshly contaminated Dorovan Muck and (B) freshly contaminated Lakeland Soil. Error bars represent one standard deviation.

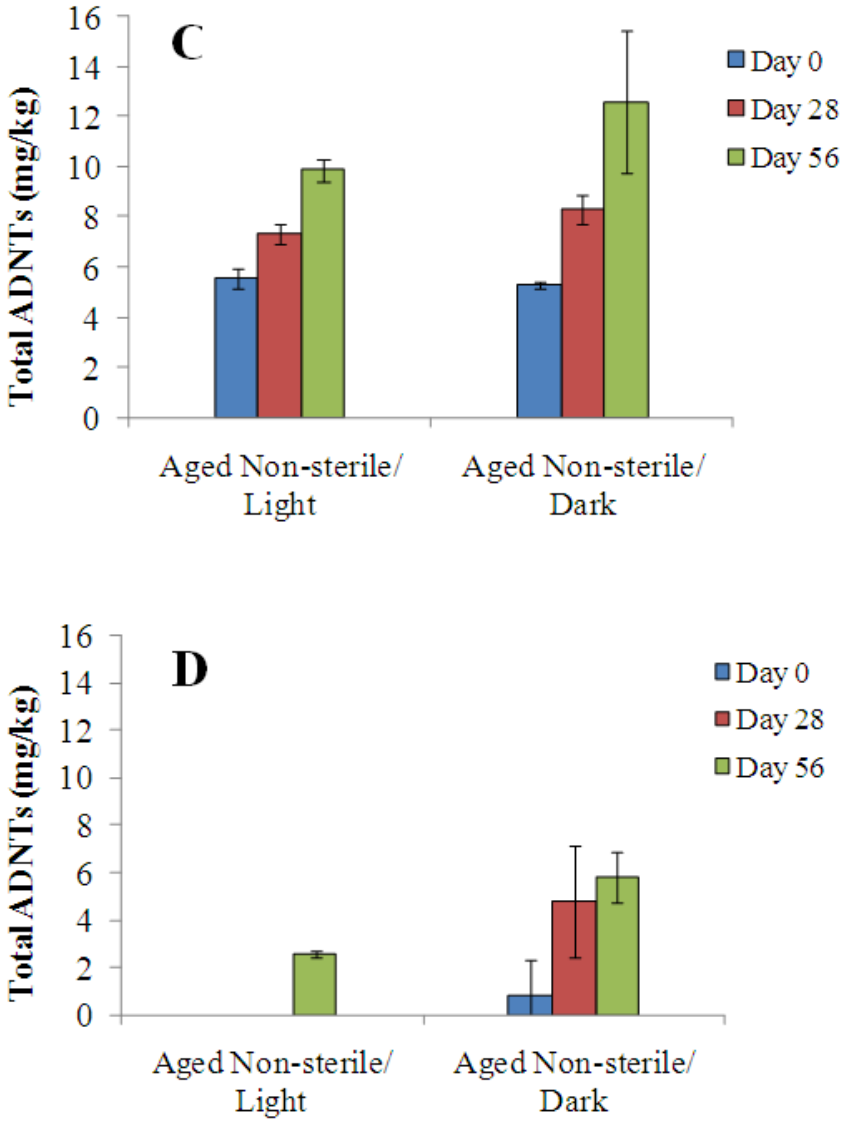


Figure 2-7. TNT metabolites 2-ADNT and 4-ADNT measured in (C) aged Dorovan Muck and (D) aged Lakeland Soil. Error bars represent one standard deviation.

CHAPTER 3
CHARACTERIZATION OF THE MICROBIAL COMMUNITY IN SOILS FROM EGLIN AIR FORCE BASE, FL

3.1 Introduction

The microbial ecology of soil is extremely important to the biogeochemical cycling of nutrients that is vital to life on our planet. Soil provides the largest reservoir of biodiversity on Earth, and sustains all other forms of terrestrial diversity while providing many ecosystem services (Hinsinger, et al., 2009). Therefore, determining the microbial community present in a particular soil is an important step toward understanding functional diversity that could lead to biodegradation of pollutants.

Understanding the microbial community structure is important for predicting the fate of contaminants in the environment. At the most basic level, characterizing the microbial community can give an indication of whether the desired microorganisms for bioremediation are present. If there is no population capable of degradative processes, inoculation with foreign organisms may be required (S. Liu & Suflita, 1993).

Bioremediation processes designed to favor specific groups of microorganisms must take into account microbial interactions such as competition for resources. Competition for nutritional resources can impede bioremediation efforts even though the relevant microorganisms are present. Understanding these microbial interactions are important for estimating the effectiveness inoculation may have in the environment (S. Liu & Suflita, 1993). Sufficiently characterizing the microbial community of an environmental sample is an important step to understanding microbial interactions critical to remediation efforts.

Microbial structure in soil can also be measured throughout a phytoremediation project to determine if critical changes in microbial community are occurring due to the presence of plants. Phytoremediation has been shown to result in changes to the microbial community and to improve the functional abilities of microorganisms compared to contaminated unplanted soil (Gremion, Chatzinotas, Kaufmann, Von Sigler, & Harms, 2004). Characterization of the microbial community throughout phytoremediation projects may give an indication of whether important microorganisms are becoming established in contaminated soil that has been planted. The symbiotic relationship which develops between microbial communities and plants may be vital to the degradation of otherwise recalcitrant contaminants.

The microbial community present in Lakeland Soil and Dorovan Muck samples was determined using Terminal Restriction Fragment Length Polymorphism (T-RFLP). T-RFLP has been shown to be a powerful tool for assessing the diversity of complex bacterial communities and for rapidly comparing the community diversity of different ecosystems (W. T. Liu, Marsh, Cheng, & Forney, 1997). T-RFLP is also a more appropriate microbial fingerprinting technique than Denaturing Gradient Gel Electrophoresis (DGGE) for large sample numbers due to its greater reproducibility (Smalla et al., 2007). The terminal restriction fragments of each 16S rRNA gene created during the T-RFLP method provides a quantitative basis for estimating diversity that is more sensitive than other techniques in microbial ecology (Tiedje, Asuming-Brempong, Nusslein, Marsh, & Flynn, 1999).

T-RFLP is a culture-independent method of obtaining the genetic fingerprint of a microbial community. During the T-RFLP method, extracted DNA is amplified through

Polymerase Chain Reaction (PCR) using a fluorescent primer. The fluorescent molecule attached to the primer is tagged to one end of the PCR amplicons during the PCR process. The amplified PCR product is then digested using restriction enzymes, producing terminal restriction fragments (fragments which are tagged with a fluorescent molecule at their terminal end). The terminal restriction fragments (T-RFs) are then separated by electrophoresis providing their size in base pairs and intensity of fluorescence. T-RF sizes can then be compared to known sequences in databases for phylogenetic assignment and analysis of microbial community (Blackwood, Marsh, Kim, & Paul, 2003).

T-RFLP has been used successfully for the comparison of bacterial diversity and composition in environmental soil samples (Hackl, Zechmeister-Boltenstern, Bodrossy, & Sessitsch, 2004). T-RFLP has also been implemented for monitoring the spatial and temporal variations in the microbial structure of agricultural soil (Lukow, Dunfield, & Liesack, 2000). This relatively new technique may be an important addition to more completely characterizing remediation efforts at contaminated sites.

3.2 Material and Methods

3.2.1 DNA Extraction

Soil DNA was extracted from 0.5 g of bulk soil for both Lakeland Soil and Dorovan Muck using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to kit instructions. DNA concentrations following extraction ranged from approximately 2 – 40 μg/ml.

3.2.2 PCR and Purification

The PCR method used is similar to the protocol described by Kent et al. (Kent, Smith, Benson, & Triplett, 2003). PCR amplification of soil DNA was performed using a HotStar Taq® Master Mix Kit (Qiagen) on a Mastercycler Gradient® thermocycler (Eppendorf, Hamburg, Germany) using the following program: a 15 minute start at 94 °C, followed by 35 cycles consisting of denaturation (35 s at 94 °C), annealing (45 s at 55 °C), and extension (90 s at 72 °C), and a final extension for 2 min at 72 °C. Reaction mixtures for PCR contained 50 µl HotStar Taq® Master Mix, 1 µl of each primer, 1 µl of DNA extract, and 2 µl of BSA buffer in a final volume of 100 µl. The primers used were 8F and 1492R. PCR products were then purified using a QIAquick® PCR Purification Kit (Qiagen) according to kit instructions.

3.2.3 T-RFLP

PCR products were digested separately with the restriction enzymes Hha1, Msp1, and Rsa1. Multiple digests using the three restriction enzymes were carried out to increase the specificity of the phylogenetic assignments. The lengths of the terminal restriction fragments were determined by electrophoresis with a Model 3730 DNA Analyzer (Applied Biosystems, Inc.). The mixture which was analyzed contained 1 µl of digested PCR product, 9.5 µl HiDi Formamide, and 0.5 µl of DNA fragment length standard (GeneScan 1200 LIZ). Negative controls were processed through the entire method and showed that there was no contamination during any of the procedures.

3.2.4 Analysis

Data from DNA fragment sequencing were analyzed using PeakScanner 1.0 software (Applied Biosystems, Inc.). Data tables containing the fragment size and abundance data for each digest were exported from PeakScanner as text files. The resulting text files were then uploaded to the T-RFLP phylogenetic assignment tool (PAT) provided by the University of Wisconsin Center for Limnology (Kent, et al., 2003). The PAT output data were then analyzed to determine the relative abundance for each phylogenetic assignment. The phylum, order, class, and family for each phylogenetic assignment were determined using the National Center for Biotechnology Information (NCBI) Nucleotide database.

3.3 Results

3.3.1 Lakeland Soil

The relative abundance of the microbial community present in Lakeland Soil at the family and phylum taxonomic level is provided in Figure 3-1 and Figure 3-2, respectively. There were 5 phyla, 8 classes, 13 orders, and 19 families classified in Lakeland Soil. As shown in Figure 3-1, the dominant family in Lakeland Soil is Burkholderiaceae, a Betaproteobacteria which represents 68% of the relative abundance at the family level. The dominant phylum present was proteobacteria which represented 97% of the relative abundance at the phylum level. Nearly 90% of the relative abundance was represented by only three families of bacteria, indicating that the microbial community lacks significant diversity in Lakeland Soil.

3.3.2 Dorovan Muck

The relative abundance of the microbial community present in Dorovan Muck at the family and phylum taxonomic level is provided in Figure 3-3 and Figure 3-4, respectively. Dorovan Muck contained nearly twice as many phylogenetic classifications as Lakeland Soil at every level, with 10 phyla, 12 classes, 26 orders, and 43 families. The relative abundance was also more evenly distributed among different taxonomic levels than in Lakeland Soil. As shown in Figure 3-3, the dominant family in Lakeland Soil is Rhodobacteraceae, an Alphaproteobacteria. Dorovan Muck was similar to Lakeland Soil in that proteobacteria heavily dominated the microbial diversity by representing 64% of the relative abundance at the phylum level. Cyanobacteria, also known as blue-green algae, were also found in Dorovan Muck and represented 6.6% of the relative abundance at the phylum level. The significant presence of cyanobacteria is likely due to the location from which the Dorovan Muck was sampled, which was a dried swamp bed.

3.4 Discussion

The microbial community of Dorovan Muck was much more diverse than that of Lakeland Soil. The higher levels of organic carbon and nutrients present in Dorovan Muck are likely the cause for greater diversity (see Table 2-2). Carbon resources have been shown to impact the microbial diversity of environmental samples. High organic carbon content plays a significant role in structuring microbial communities by allowing invading species (bacteria) to easily become established (Zhou et al., 2002).

Understanding microbial diversity and the mechanisms which control community structure is important for the management of bioremediation processes (Zhou, et al.,

2002). Microorganisms play an extremely important role in the global ecosystem by catalyzing many reactions important to life on earth. Contaminants such as explosive compounds can be incorporated into these reactions as an extension of normal microbial metabolism. The incorporation of contaminants into metabolic processes and overcoming the environmental constraints which govern these processes are major challenges in bioremediation efforts (S. Liu & Suflita, 1993).

Comparing the results between the two soil types indicates that Dorovan Muck may be more viable for bioremediation efforts due to its higher microbial diversity. The abundant diversity present in Dorovan Muck indicates that foreign bacteria are capable of becoming established, over time leading to increased microbial diversity. Bacteria which could be inoculated into Dorovan Muck would likely become established due the abundant nutrients and carbon sources in this soil type. Conversely, Lakeland Soil is dominated by one phylogenetic family, Burkholderiaceae, and may not be amenable to inoculation of explosives degrading bacteria. Competition for nutrients has led to the overwhelming dominance of proteobacteria in Lakeland Soil (97% of diversity), and foreign bacteria are likely unable to compete with the microorganisms already present in this soil. As Lakeland Soil is the dominant soil type present at EAFB, and due to the lack of inherent RDX and HMX degradative function in this soil type (see Chapter 2), bioremediation should be viewed as unsuitable for explosives treatment at EAFB.

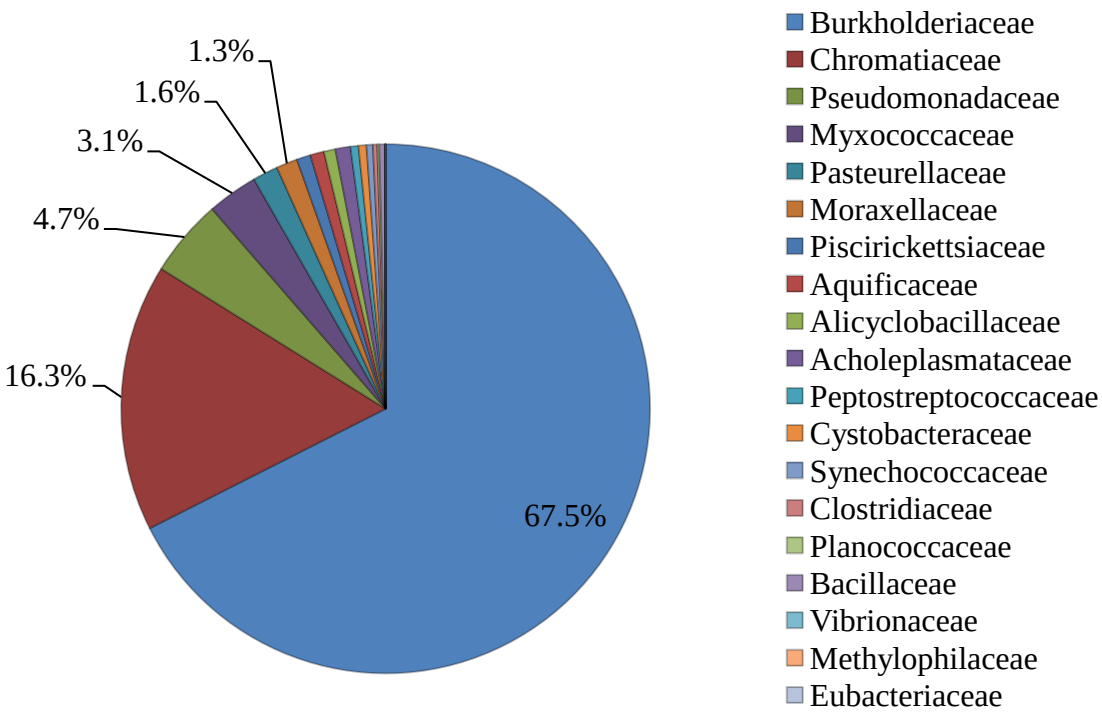


Figure 3-1. Microbial community of Lakeland Soil at the family classification level.

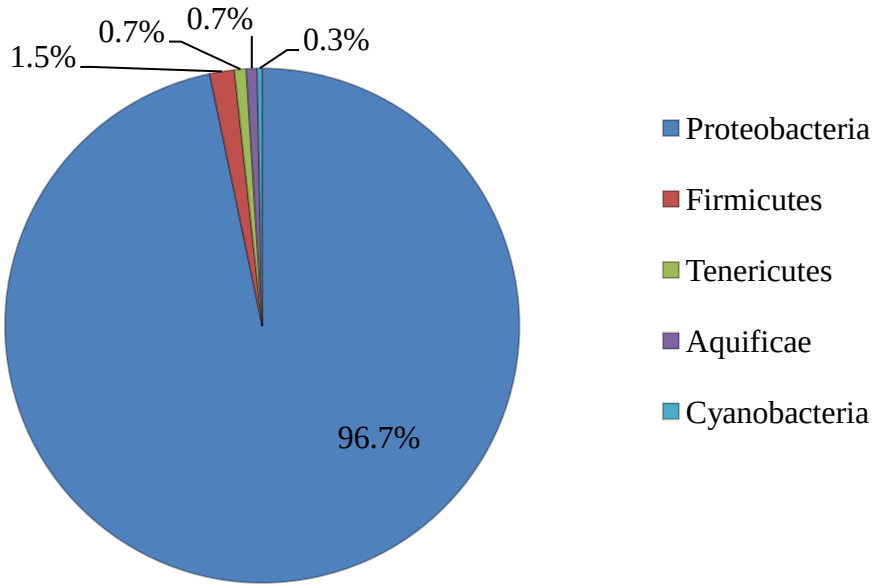


Figure 3-2. Microbial community of Lakeland Soil at the phylum classification level.

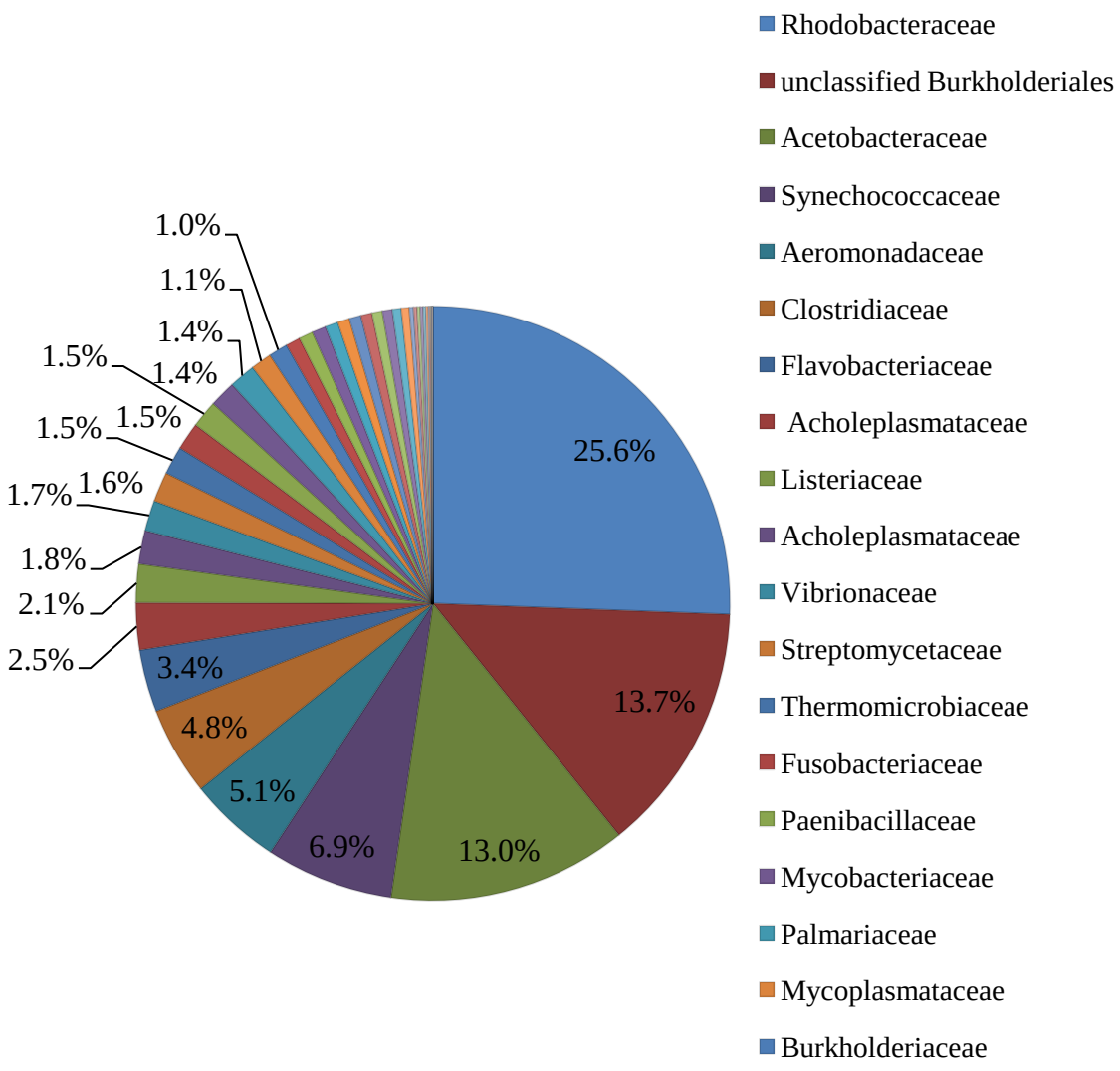


Figure 3-3. Microbial community of Dorovan Muck at the family classification level. Only families with 1% or higher relative abundance are shown in the legend.

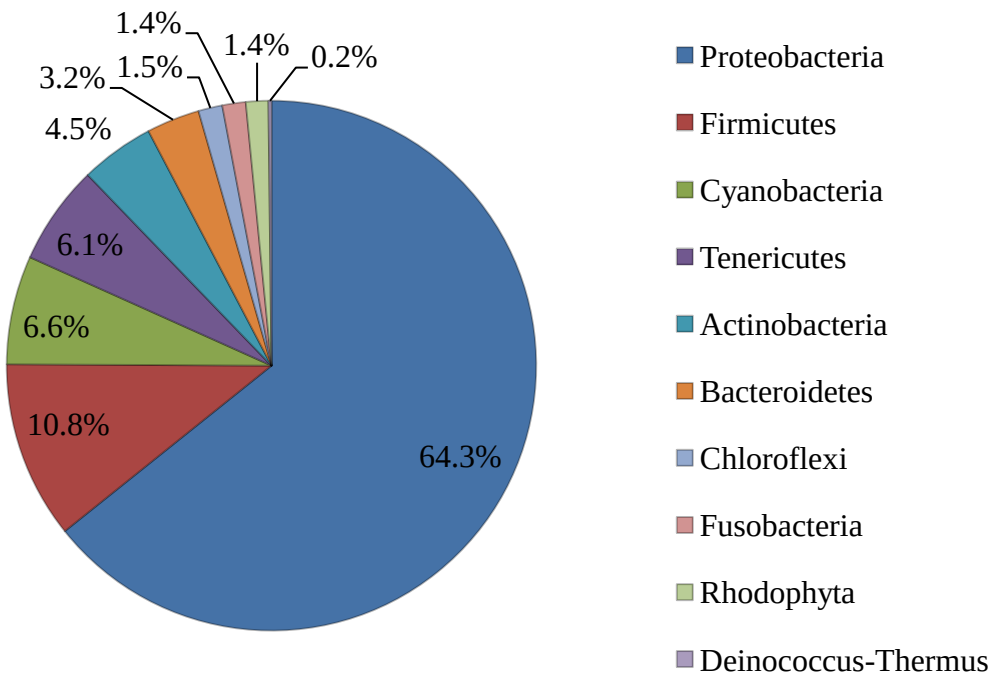


Figure 3-4. Microbial community of Dorovan Muck at the phylum classification level.

CHAPTER 4
PHYTOREMEDIATION FIELD STUDY FOR THE TREATMENT OF EXPLOSIVE COMPOUNDS AT EGLIN AIR FORCE BASE, FL

4.1 Introduction

Military training activities taking place at EAFB have led to extensive explosives contamination on the testing ranges of the installation. Explosives contamination resulting from military training exercises has been well documented and is inevitable due to the need for military preparedness (Alan D. Hewitt, et al., 2007; A. D. Hewitt, Jenkins, Walsh, Walsh, & Taylor, 2005; T. F. Jenkins, et al., 2006; Walsh et al., 2003). Phytoremediation is ideal for treating explosives contamination at military ranges due to the large land areas which are contaminated and the need for sustainable, long-term, cost effective treatment (Hannink, et al., 2002).

The purpose of this project is to determine whether phytoremediation can be used to prevent the leaching of energetics at EAFB, thus making the site more sustainable for range activities. The main area of interest for this research effort is the open burn/open detonation (OB/OD) area on Range C-62 of EAFB. The OB/OD site, which is used for demolition activities, is shown in Figure 4-1. Demolition ranges, such as the OB/OD site at EAFB, are used to destroy unexploded ordinance (UXO) of various munitions which are considered safe to move. A quantity of Composition 4 (C4) is placed on the UXO and detonated using a blasting cap, destroying the hazardous component of the UXO in the process (T. F. Jenkins, et al., 2006). Over time this practice leads to craters and pits in the OB/OD area of the demolition range which can contain high soil concentrations of energetic compounds. Substantial quantities of explosives can be released during

demolition practices, especially if the UXO being destroyed undergoes a low-order (incomplete) detonation (Alan D. Hewitt, et al., 2007). Further explosive residues are deposited if the C4 undergoes incomplete detonation (J. L. Clausen, Korte, Nic, et al., 2006). Initial soil samples collected in March 2008 near the OB/OD site showed contamination of several explosives such as TNT (2,4,6-trinitrotoluene), RDX (hexahydro-1,3,5-trinitro,-1,3,5-triazine), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), and TNB (1,3,5-trinitrobenzenze).

RDX is of particular concern as it is mobile in the environment and has caused extensive groundwater contamination at sites such as the Iowa Army Ammunition Plant and the Massachusetts Military Reservation (Best, et al., 1997; J. Clausen, et al., 2004). RDX is used as a high-explosive filler of munitions and is a main component of the compounds C4 (90% RDX) and Composition B (60% RDX) (Alan D. Hewitt, et al., 2007). RDX contamination of the drinking water aquifer at the Massachusetts Military Reservation caused a high amount of regulatory and public scrutiny (J. Clausen, et al., 2004).

4.2 Project Overview

Several plant species have been shown to uptake RDX into stems and leaves, and mineralization has been observed in plant tissues (Bhadra et al., 2001; Van Aken, et al., 2004). For this field study, Bahiagrass Pensacola *(Paspalum notatum)* was chosen to be planted in three plots near the vicinity of the OB/OD site. Bahiagrass Pensacola is a warm-season, drought tolerant grass that is relatively resistant to pests (Jia, Dukes, & Jacobs, 2007). The grass is also regularly used for erosion control practices at EAFB.

Bahiagrass Pensacola sod was planted in three 0.4 acre plots, two "impacted" plots adjacent to the OB/OD site and one "control" plot located up-gradient of the site. The locations of the three plots are shown in Figure 4-2. Plot #1 is located east of the OB/OD site and was expected to contain the highest concentration of explosive compounds. Plot #2 is located south of the OB/OD site and was expected to be an area of lesser contamination. Plot #3, the control plot, is located to the north and was not expected to contain significant contamination. Planting took place in May 2009 and biannual soil and vegetation sampling is scheduled to occur through November 2010 to determine the effectiveness of Bahiagrass Pensacola on degrading energetic compounds.

4.3 Site Work and Preparation

A site visit to EAFB was made on January 14, 2009 to meet with the Eglin Range Configuration Control Committee (RC3). The purpose of the RC3 meeting was to brief the committee on the scope of the phytoremediation project and to acquire clearance to plant vegetation on three plots near the OB/OD site. As a result of this meeting the use of phreatophytic tree species such as hybrid poplar was ruled out due to concerns with disturbing underlying UXO at the site. A site visit to Range C-62 was then conducted to finalize plans for field work with Three Rivers RC&D Council, Inc. (Milton, FL), the landscaping company which was chosen to perform the installation of vegetation. Planting Bahiagrass Pensacola in the form of sod offered a faster establishment period and a greater chance of survival over planting grass from seed.

Planting of the Bahiagrass Pensacola sod at the site occurred on May 26-27, 2009. Three Rivers RC&D performed the site preparation, which consisted of removing the top layer of vegetation from each plot, and then installing the sod as shown in Figure A-1 and

Figure A-2 in Appendix A. Initial watering to establish the sod was also provided by Three Rivers RC&D using a water truck. Unseasonably low precipitation occurred in the months of June and July 2009 at EAFB, delaying successful establishment and vigorous growth of the sod until August and September 2009. Photographs of Plot #1 throughout the first 6 months of the field study are provided in Figure A-3 through Figure A-6 in Appendix A.

4.4 Sampling Method

Initial soil samples were taken from each plot while planting took place in May 2009. The extreme spatial heterogeneity of explosives contamination on military training ranges makes characterization of these sites extremely difficult (Alan D. Hewitt, et al., 2007; T. F. Jenkins, et al., 2006). Therefore, a robust sampling method must be used to establish initial explosives concentrations and to adequately show that remediation is taking place over time.

The systematic random sampling method was chosen for use in the three plots to provide a large number of samples and increase the confidence in estimations of explosives concentrations (Alan D. Hewitt, et al., 2007). The systematic random sampling method which was used on each plot is shown in Figure 4-3. During the initial sampling in May 2009, a total of 100 discrete soil samples were taken from the planted portion of each plot, and 40 discrete soil samples were taken along the unplanted perimeter of each plot at 15 ft intervals. Fewer soil samples were taken in Plot #2 and #3 due to time constraints during the second sampling visit in November 2009. In Plot #2, 17 soil samples were taken from the planted portion and 14 soil samples were taken from the unplanted perimeter. In Plot #3, 34 and 14 soil samples were taken from the planted

portion and unplanted perimeter, respectively. The same number of samples was taken from Plot #1 in November 2009 as in May 2009 (100 samples from the planted portion and 40 samples from the unplanted perimeter).

4.5 Materials and Methods

4.5.1 Soil and Vegetation Sample Collection

Soil samples were collected using a 2 cm diameter soil corer at a depth of 5 cm and placed into a sealable plastic bag. The soil corer was wiped with a paper towel following each sample to minimize contamination between samples. The soil corer used during sampling is shown in Figure 4-4.

Vegetation samples were collected along the creeks down-gradient from the OB/OD area during the May 2009 site visit. Pruning shears were used to take leaf and grass samples at multiple locations along the creeks, and the location of each sample was recorded using a geographic positioning system (GPS) device. During the November 2009 sampling visit, samples of Bahiagrass Pensacola were taken using pruning shears at each location where soil samples were taken in all three plots.

4.5.2 Explosives Extraction from Soil

Extraction of explosives from soil was performed using a modified version of EPA Method 8330B (Agency, 2006). Soil samples were dried at room temperature until a constant weight was reached. Each sample was then ground with mortar and pestle, placed in a 15 ml vial, and extracted with 10 ml acetonitrile to 2 g soil in an ultrasonic bath for 18 hours. The samples were then filtered under 0.20 µm Durapore membrane filters. Sample filtrate was then collected for analysis by HPLC.

4.5.3 Explosives Extraction from Plants

Extraction of explosives from plants were performed using a modified version of EPA Method 8330B (Agency, 2006). Approximately 2 g of plant sample was ground under liquid nitrogen with mortar and pestle, placed in a 15 ml vial, and extracted with 10 ml acetonitrile in an ultrasonic bath for 18 hours. The samples were then filtered under 0.22 µm syringe filters for analysis by HPLC.

4.5.4 Explosives Extraction from Water Samples

Extraction of explosives from water samples were performed using a modified version of the EPA Method 8330B low-level aqueous method (Agency, 2006). Sodium chloride (25.1 g) was added to 77 ml of water sample and stirred using an electronic stir plate until dissolved. While the solution was being stirred, 25 ml of acetonitrile was added and stirred for 15 min. The stir plate was then turned off and the phases were allowed to separate for 10 min. The top layer of acetonitrile was removed by Pasteur pipette and transferred to a 100 ml volumetric flask. An additional 5 ml of acetonitrile was then added to the water-salt mixture and stirred for 15 min, followed by 10 min of phase separation. The top layer of acetonitrile was removed by Pasteur pipette and transferred to the 100 ml volumetric flask. The extracted acetonitrile was then mixed with 42 ml of salt water (32.5 g sodium chloride per 100 ml deionized water) and stirred for 15 min, followed by 10 min of phase separation. The top layer of acetonitrile was then transferred by Pasteur pipette to a graduated cylinder. An additional 1.0 ml of acetonitrile was added to the acetonitrile-salt water mixture and stirred for 15 min, followed by 10 min of phase separation. The final top layer of acetonitrile was then

transferred by Pasteur pipette to the graduated cylinder. The final volume of acetonitrile was recorded and used for analysis by HPLC.

4.5.5 Chemical Analyses

Analyses of extractions from soil and water samples were performed by HPLC (HP Series 1100; Hewlett-Packard, Palo Alto, CA) using an Acclaim® Explosives E1 column (Dionex Corporation). The mobile phase consisted of methanol:deionized water 43:57 v/v at a flow rate of 1.0 ml/min. Compounds were detected by UV absorbance at 230 nm and 254 nm using a UV visible photodiode array detector (HP series 1100).

Analyses of extractions from plant samples were performed using liquid chromatography-mass spectrometry (LC/MS). An Agilent 6140 Quadrupole LC/MS was used with an Acclaim 120 Å C_{18} column (2.1 x 150 mm, 5 µm; Dionex). The mass spectrometer was operated in negative-ion electrospray mode. The mobile phase consisted of acetonitrile:2mM ammonium acetate 50:50 v/v at a flow rate of 0.4 ml/min. Calibration standards and blanks were analysed before and and after sample runs to ensure quality control.

EPA 8330-R Explosives Mix was used as a standard for both HPLC and LC/MS analyses for the identification and quantification of explosive compounds. An HPLC chromatogram of the 14 compounds present in EPA 8330-R Explosives Mix is shown in Figure 4-5.

4.6 Results

4.6.1 May 2009 Results

All soil samples taken from Plots #1, #2, and #3 were analyzed discretely for detection of the 14 compounds present in EPA 8330-R Explosives Mix. Figure 4-6 shows the locations where explosive compounds were detected in Plot #1. Only Plot #1 had significant explosives contamination present. Plot #2 and Plot #3 had only one soil sample each which contained detectable levels of explosive compounds. As shown in Figure 4-6, RDX and HMX were the most common compounds detected in Plot #1, and were often found together in the same discrete sample. Several samples from Plot #1 also contained TNT and/or metabolites of TNT such as TNB (1,3,5-trinitrobenzene), 2-ADNT (2-amino-4,6-dinitrotoluene) 4-ADNT (4-amino-2,6-dinitrotoluene, 2,4-DNT (2,4-diamino-6-nitrotoluene) and 2,6 DNT (2,6-diamino-4-nitrotoluene). Explosives were not detected in any of the vegetation samples taken along the creeks down-gradient from the field site.

4.6.2 November 2009 Results

Soil samples taken during the November 2009 sampling visit were again analyzed discretely for the compounds present in EPA 8330-R Explosives Mix. As in May 2009, only Plot #1 had significant soil contamination present. As shown in Figure 4-7, RDX and HMX were the most common compounds detected in soil samples from Plot #1. TNT and TNT metabolites (TNB, 2,4-DNT, and NB (nitrobenzene)) were also present in some samples. Comparisons of detection frequency and soil concentration between May and November soil samples from the planted portion of Plot #1 are shown in Figure 4-8, Figure 4-10, and Figure 4-12 for HMX, RDX, and TNT/TNT metabolites, respectively.

Figure 4-9, Figure 4-11, and Figure 4-13 provide a comparison for the unplanted soil samples for HMX, RDX, and TNT/TNT metabolites, respectively. Plot #2 contained only one soil sample with a detectable level of explosives (HMX); Plot #3 contained three soil samples with detectable levels of explosives (all HMX).

Five soil samples were taken along a transect of the OB/OD site as shown in Figure 4-14. Explosives were detected in four of the five samples, with RDX and HMX being the most common contaminants present. There was also one detection each of TNT and 2,4-DNT. Contamination in each of the samples was approximately 0.1 mg/kg for each contaminant, with the exception of 2,4-DNT, which had one detection at 2.0 mg/kg.

Bahiagrass Pensacola samples were taken at each soil sampling point in the planted portion of Plot #1. Analysis of each plant sample showed RDX was present at concentrations ranging from 5 to 50 µg/kg. HMX was also detected in three plant samples, all with a concentration between 1 and 3 µg/kg. The locations where explosives detections occurred in plant samples are shown in Figure 4-15. A histogram showing the distribution of RDX concentrations present in plant samples from Plot #1 is provided in Figure 4-16.

Three water samples were taken during the November 2009 sampling visit. Water samples were taken from a nearby creek (Creek #1) not directly influenced by the OB/OD site, from the creek immediately down-gradient from the OB/OD site (Creek #2), and from standing water in the OB/OD crater. The concentrations of explosive compounds which were present in these water samples are shown in Figure 4-17. The highest concentration of explosives was present in the Creek #2 sample, which contained

RDX (1190 µg/L), HMX (95 µg/L), and TNT (8 µg/L). RDX was also detected in the Creek #1 at concentration of 45 µg/L. RDX (27 µg/L) and HMX (80 µg/L) were detected in the crater near the center of the OB/OD site which contained standing water (see Figure 4-1).

4.7 Discussion

4.7.1 Explosives in Soil Samples

Only Plot #1 contained significant levels of explosives contamination in soil in both May and November samples. There is a possibility of increased explosive residues due to continued activity on the range throughout this project. The low number of samples with explosives contamination in Plot #2 and Plot #3 in both May and November 2009 indicate that these areas have not received extensive contamination in the past and currently are not being affected to a great extent by use of the OB/OD site. In contrast, Plot #1 contained a variety of explosive compounds and a relatively large number of contaminated samples in both May and November 2009.

As shown in Figure 4-6 and Figure 4-7, the pattern of explosives contamination was similar for the May and November samplings. RDX and HMX are by far the most common compounds present, yet significant amounts of TNT and TNT metabolites were also detected. Frequency of detection appears to increase with increasing proximity to the OB/OD site along the western and southern edge of Plot #1. The frequency in detection of RDX and HMX is likely attributable to incomplete detonation of C4 used in demolition practices as discussed previously. Although 2,4-DNT is a known metabolite of TNT, the presence of this compound in soil samples is likely due to the demolition of propellants on the OB/OD site, which are known to contain 2,4-DNT .

To better characterize the explosives contamination present in Plot #1, Figure 4-8 through Figure 4-13 provide histograms detailing the frequency of explosives detection at various concentrations. As phytoremediation takes place in Plot #1, there is expected to be a decrease in both the concentration and frequency of detection for samples taken in the planted portion of the plot. Concentrations are expected to remain relatively stable in the unplanted perimeter of Plot #1 due to the anticipated lack of degradation in unplanted soil. A comparison of the May and November results show that in general the frequency of detections have remained relatively constant for planted and unplanted soil. There was a slight increase in detection frequency for both HMX and RDX from May to November, and a slight decrease in the detection frequency for TNT and TNT metabolites. Due to the extreme heterogeneity of explosives contamination on military ranges, such small changes in detection frequency cannot be used as a reliable indicator that conditions have changed.

One trend that is observed from the Plot #1 results is that concentrations have decreased slightly in both planted and unplanted soil. For all compounds, the histograms became more left-skewed from May to November. The likely cause for this is dissolution of explosives and infiltration to the groundwater, which would occur at approximately the same rate for both planted and unplanted soil. The lack of a clear decrease in explosives contamination (both frequency and concentration) in the planted portion of Plot #1 compared to the unplanted perimeter indicates that there has not yet been enough time for phytoremediation to occur at the site. The geometric mean, mean, and standard deviation for explosive compounds detected in Plot #1 are summarized in Table B-1 and Table B-2 in Appendix B.

4.7.2 Explosives in Plant Samples

RDX was present in 22 of the 100 Bahiagrass Pensacola samples which were taken in Plot #1 in November 2009. This provides evidence that translocation of RDX from range soil to plant leaves is occurring under field conditions. There were also three detections of HMX at lower concentrations than RDX, providing evidence for minimal HMX translocation. The concentrations of RDX in the grass samples were in the range of 5 – 50 µg/kg, approximately two orders of magnitude lower than the range detected in soil samples. This range of RDX concentrations present in vegetation is low considering the 10^{-6} risk based concentration of 1.3 mg/kg for soil (McCutcheon & Schnoor, 2003). The spatial distribution of RDX in plant tissues shown in Figure 4-15 matches well with the explosives detections in soil shown in Figure 4-6 and Figure 4-7. These results provide evidence for translocation of RDX and HMX under field conditions only 6 months after planting, an important finding of this research. Further phytotransformation of RDX is expected based on substantial evidence in the literature (Bhadra, et al., 2001; Harvey, Fellows, Cataldo, & Bean, 1991; Van Aken, et al., 2004; Yoon, et al., 2006).

4.7.3 Explosives in Water Samples

The relatively high concentrations of explosives in the water samples shown in Figure 4-17 are a major cause for concern with respect to the potential for contaminant migration off site. The RDX concentration of 1190 µg/L detected in Creek #2 is almost 600 times the EPA lifetime drinking water level of 2 µg/L. Although there are no public drinking water wells for miles from the site, the high concentration detected in the creek could pose a threat downstream even after considerable dilution. As the creeks in the area are groundwater fed, the high concentrations of explosives in the creek samples

point to massive shallow groundwater contamination in the area of the OB/OD site. This further illustrates the need for remediation strategies such as phytoremediation to be implemented at EAFB in the immediate future.

Figure 4-1. The open burn/open detonation (OB/OD) site at Eglin Air Force Base.

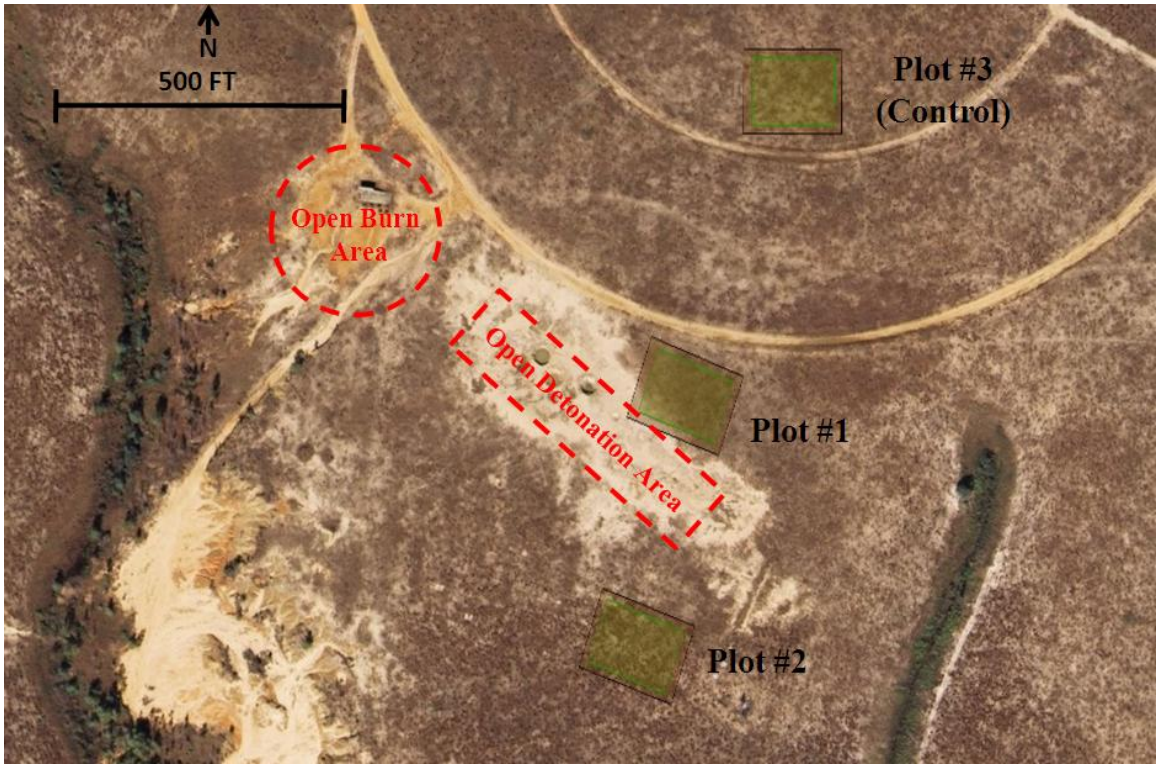


Figure 4-2. Area of phytoremediation field study at Eglin Air Force Base.

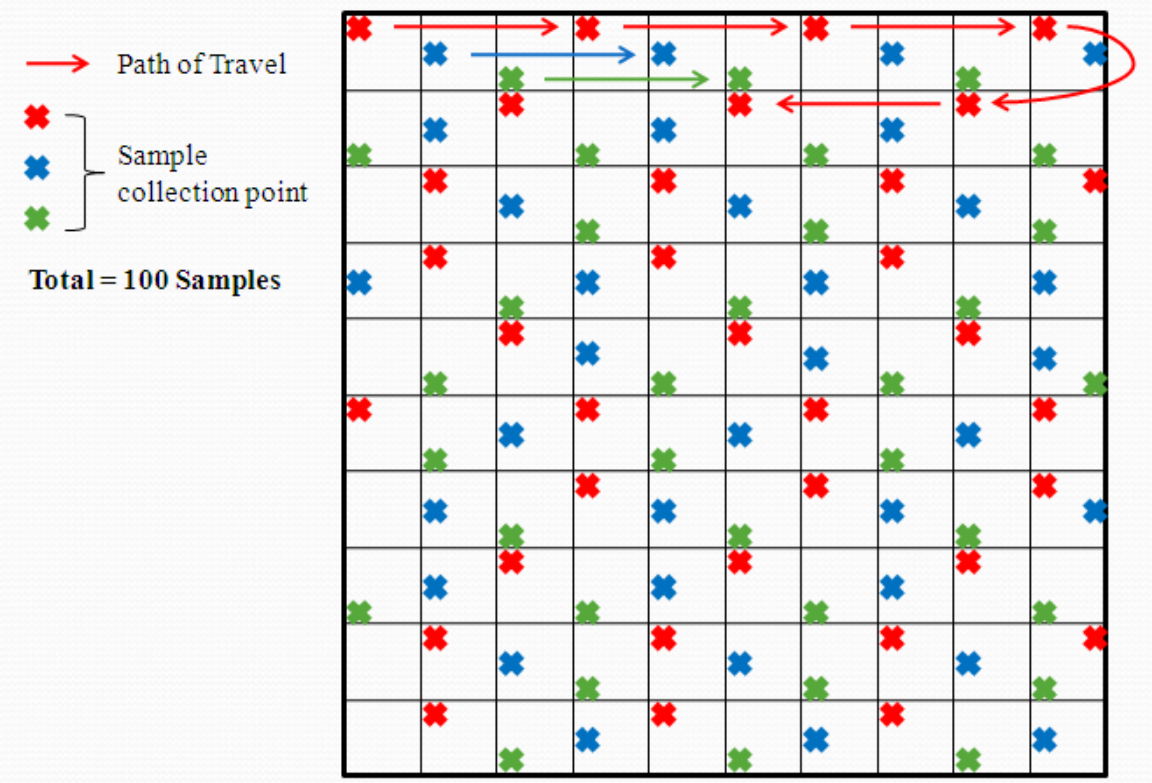
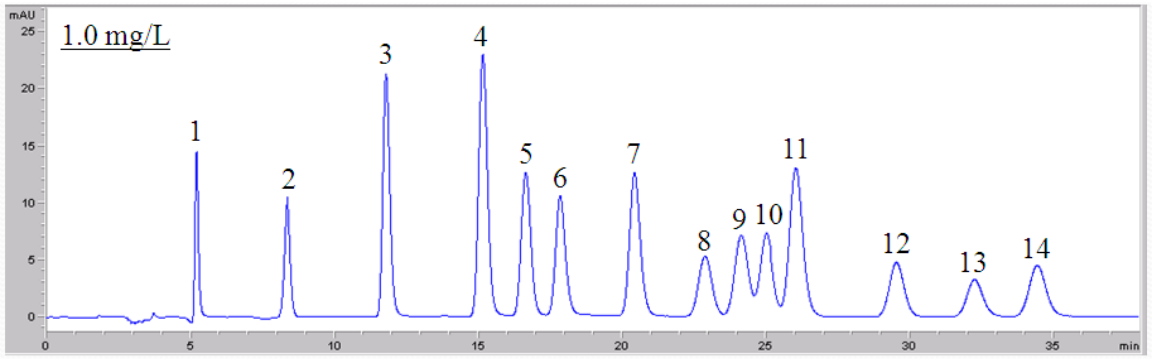


Figure 4-3. Systematic random sampling method used during soil sampling (Hewitt, 2007).

Figure 4-4. Soil corer (2 cm diameter) used to take soil samples at a depth of 5 cm.



1. HMX
2. RDX
3. TNB
4. DNB
5. NB
6. Tetryl
7. TNT
8. 4-ADNT
9. 2-ADNT
10. 2,6-DNT
11. 2,4-DNT
12. 2-NT
13. 4-NT
14. 3-NT

Figure 4-5. HPLC chromatogram showing EPA 8330 mix (1.0 mg/L) used as a standard for identification and quantification of explosive compounds.

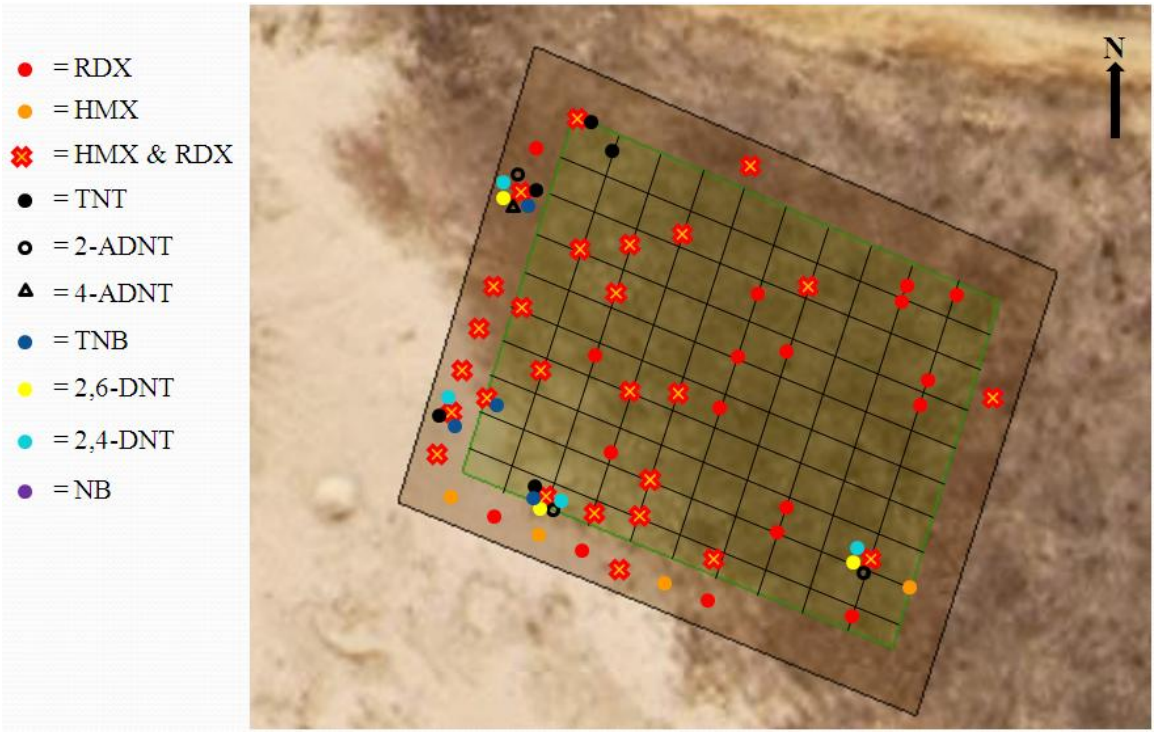


Figure 4-6. Results showing the detection of explosive residues from soil samples taken at Plot #1 in May 2009.

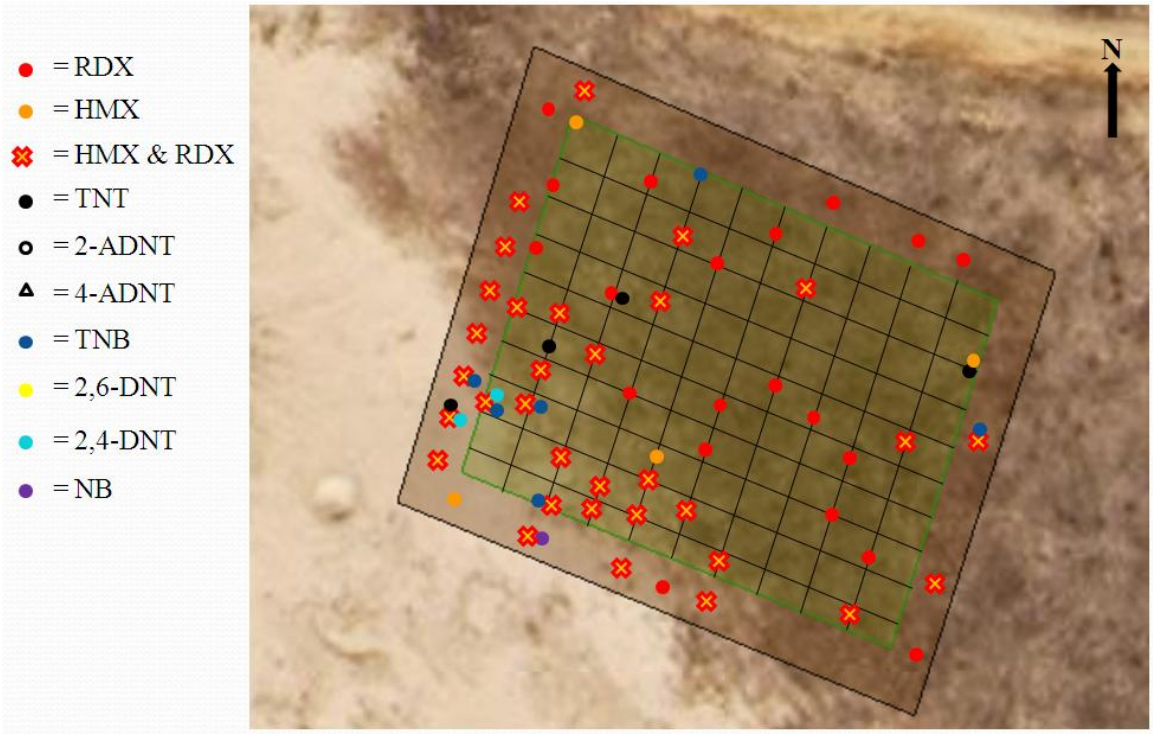


Figure 4-7. Results showing the detection of explosive residues from soil samples taken at Plot #1 in November 2009.

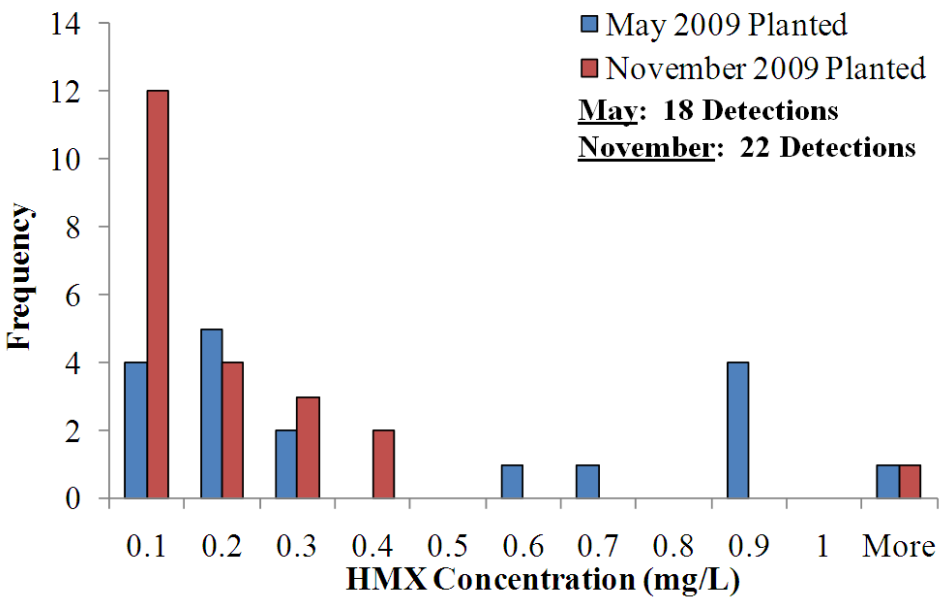


Figure 4-8. Histogram comparing the distribution of HMX concentrations from soil samples taken from the planted portion of Plot #1 in May and November 2009. Only soil samples with HMX detections are included in the histogram.

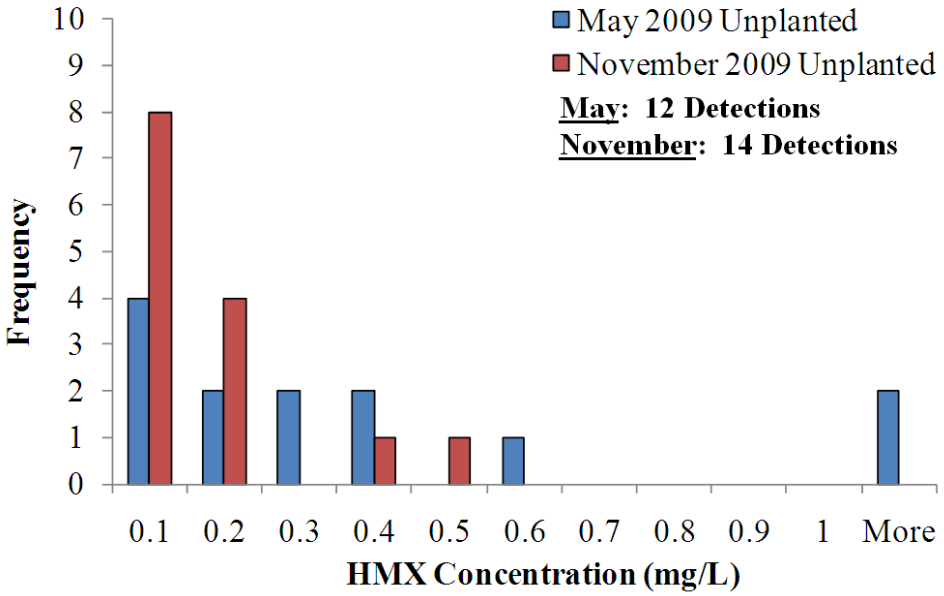


Figure 4-9. Histogram comparing the distribution of HMX concentrations from soil samples taken from the unplanted portion of Plot #1 in May and November 2009. Only soil samples with HMX detections are included in the histogram.

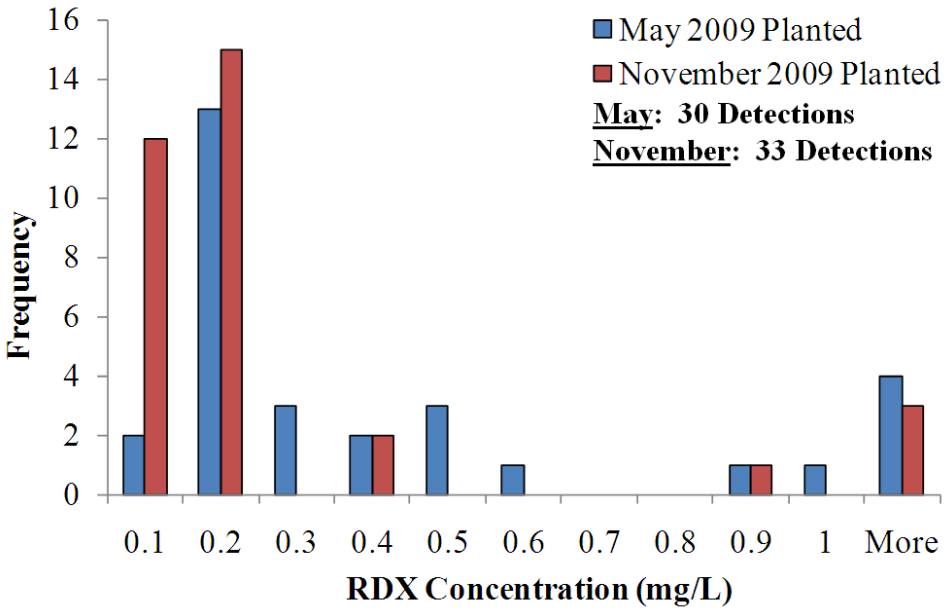


Figure 4-10. Histogram comparing the distribution of RDX concentrations from soil samples taken from the planted portion of Plot #1 in May and November 2009. Only soil samples with RDX detections are included in the histogram.

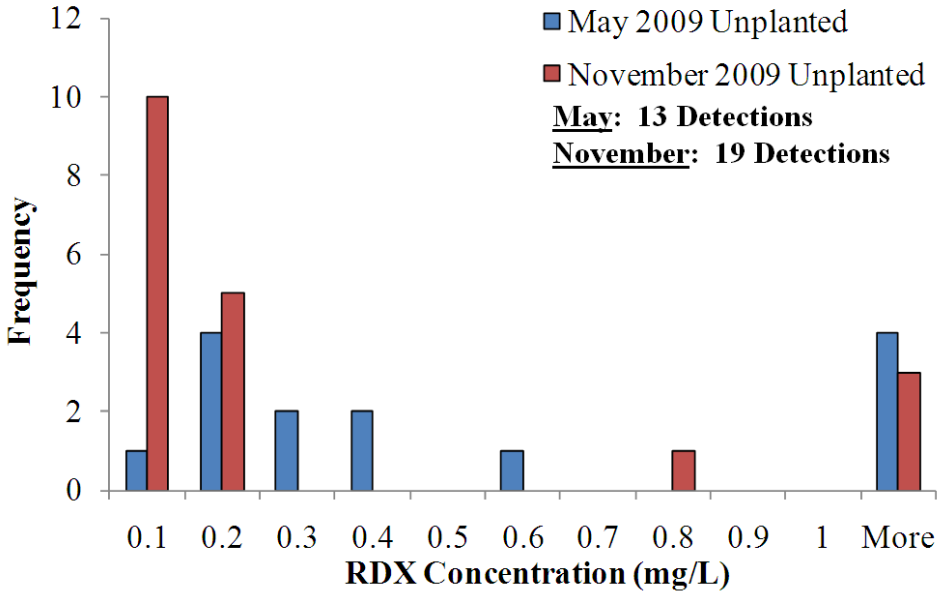


Figure 4-11. Histogram comparing the distribution of RDX concentrations from soil samples taken from the unplanted portion of Plot #1 in May and November 2009. Only soil samples with RDX detections are included in the histogram.

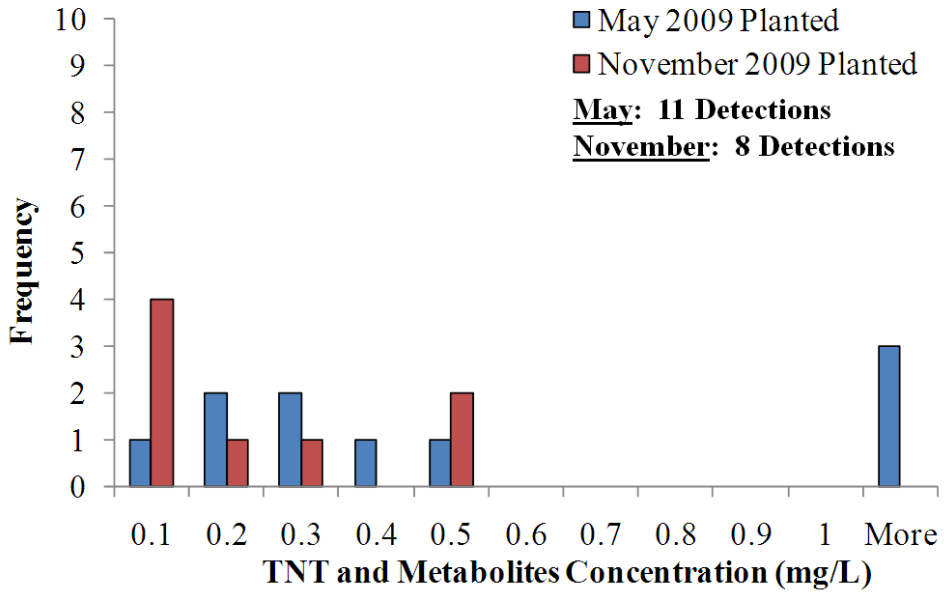


Figure 4-12. Histogram comparing the distribution of TNT and TNT metabolite concentrations from soil samples taken from the planted portion of Plot #1 in May and November 2009. Only soil samples with TNT and TNT metabolite detections are included in the histogram.

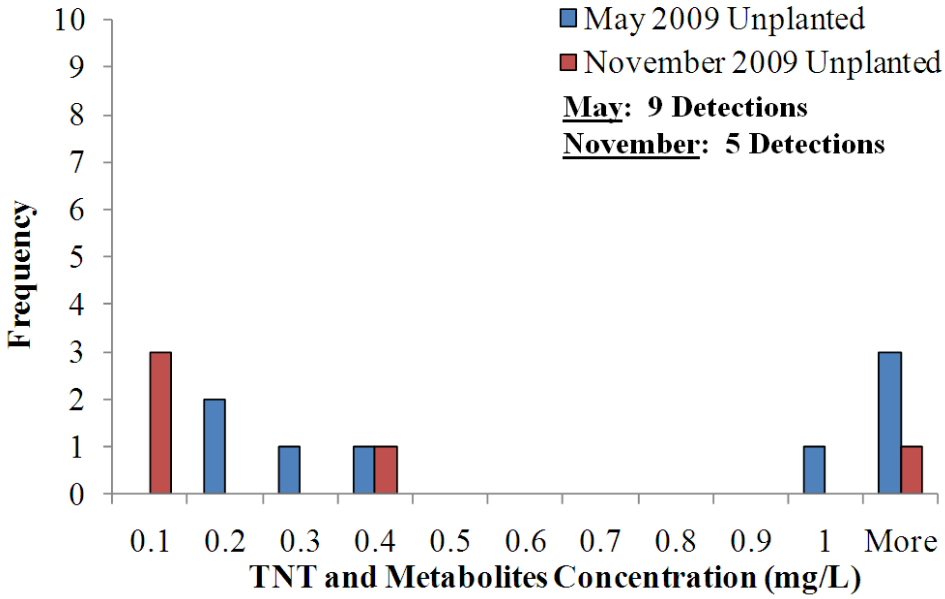


Figure 4-13. Histogram comparing the distribution of TNT and TNT metabolite concentrations from soil samples taken from the unplanted portion of Plot #1 in May and November 2009. Only soil samples with TNT and TNT metabolite detections are included in the histogram.

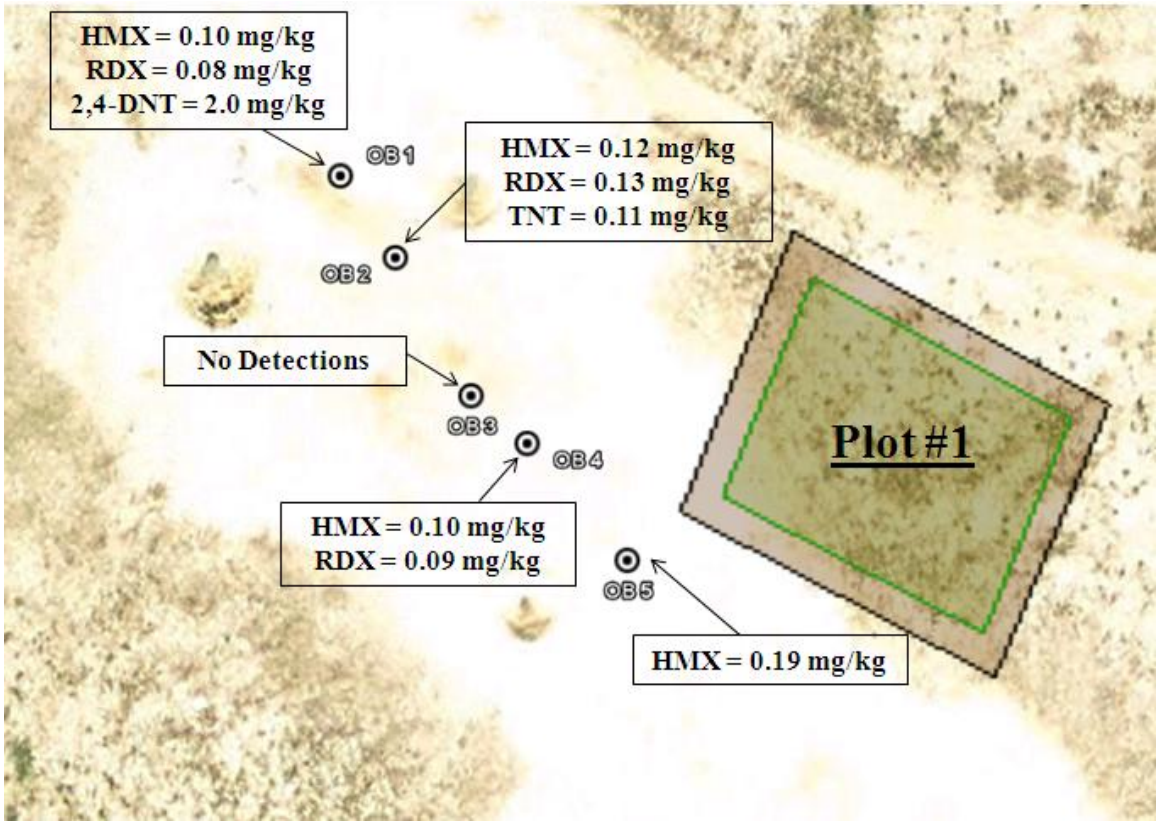


Figure 4-14. Results showing the detection of explosive compounds in soil samples taken in November 2009 at the OB/OD site.

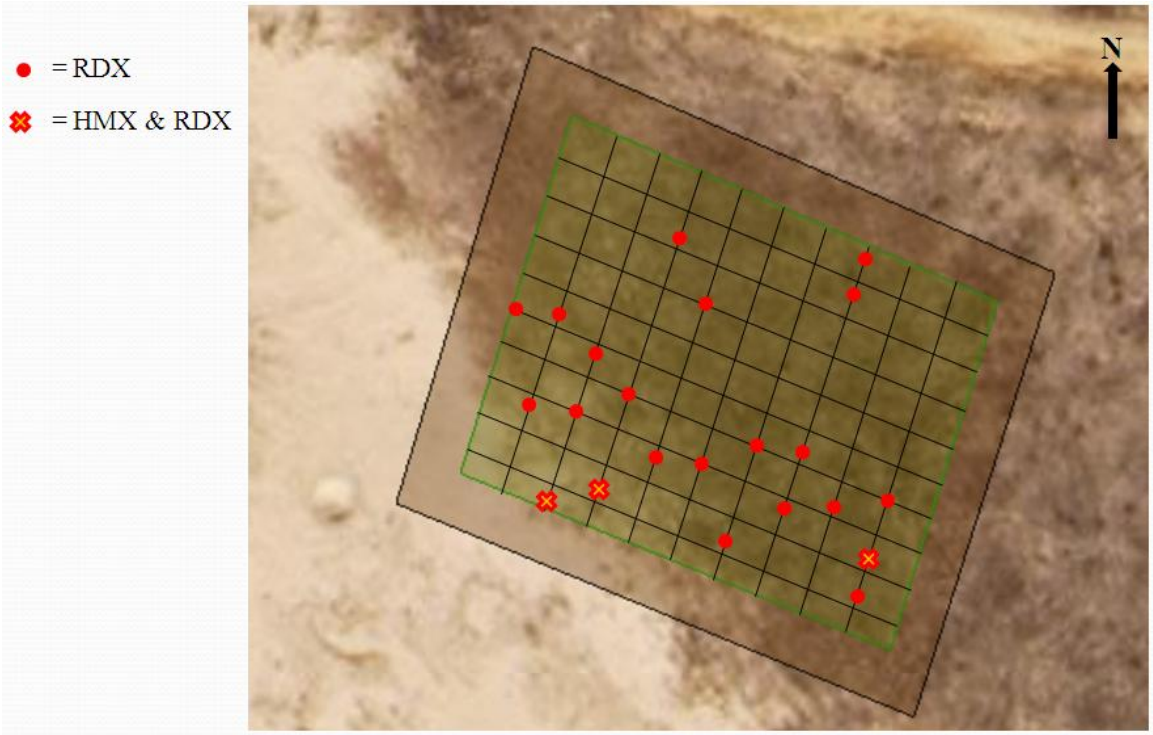


Figure 4-15. Results showing the detection of explosive residues from Bahiagrass Pensacola samples taken at Plot #1 in November 2009.

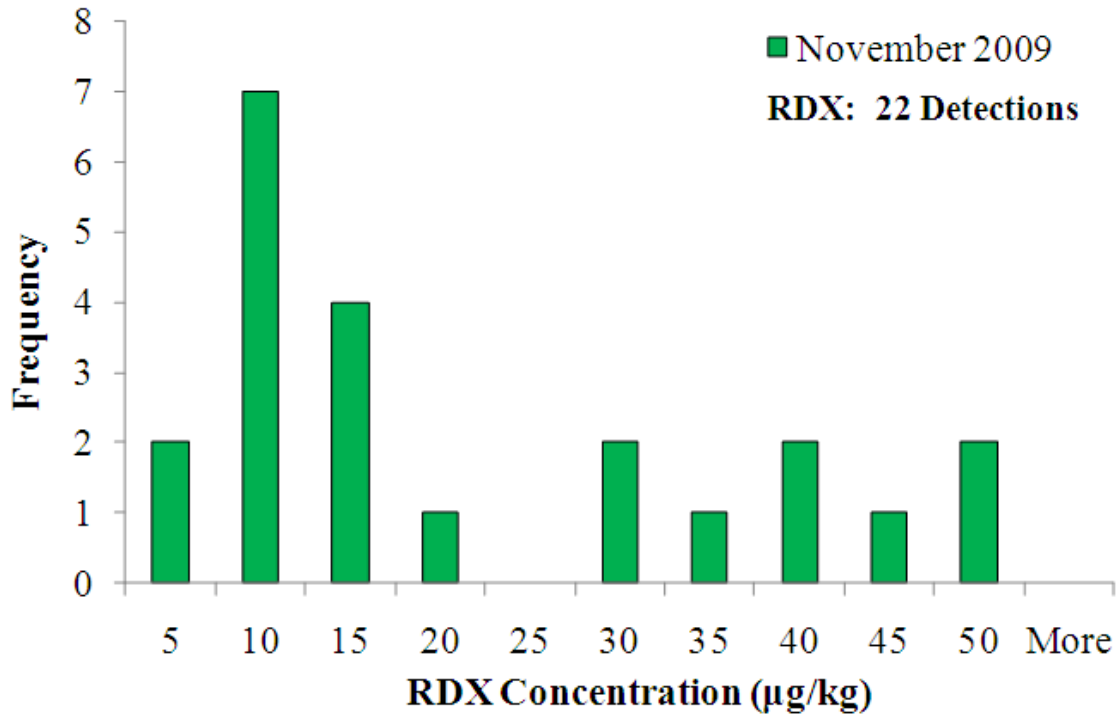


Figure 4-16. Histogram comparing the distribution of RDX concentrations from plant samples taken from the planted portion of Plot #1 in November 2009. Only plant samples with RDX detections are included in the histogram.

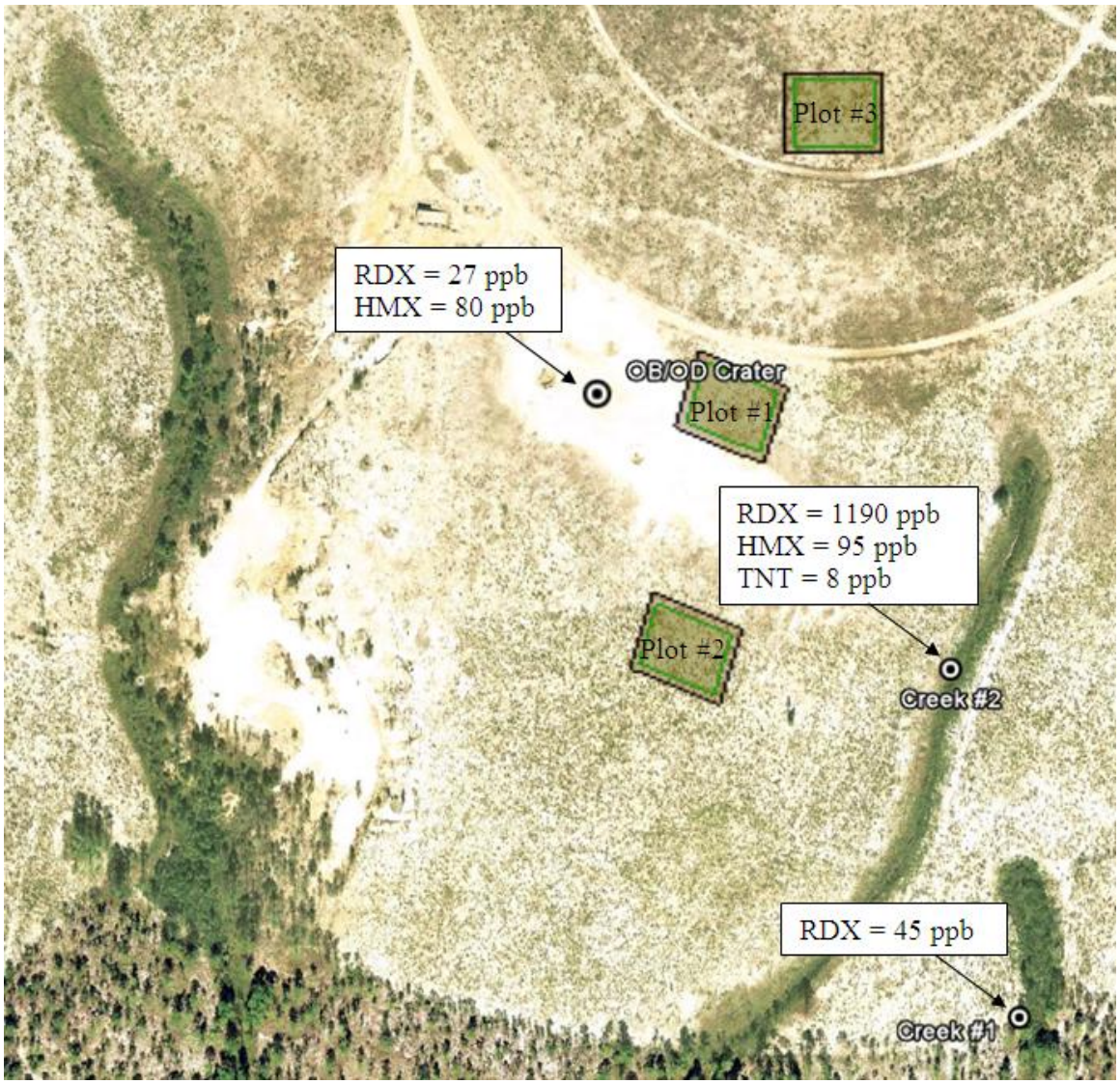


Figure 4-17. Results showing the detection of explosive compounds in water samples taken in November 2009.

CHAPTER 5
CONCLUSIONS

5.1 Research Objectives

The specific objectives and resulting conclusions for this research are shown below, with discussion in the following sections of this chapter:

1. Determine whether plants significantly improve biodegradation of explosives at a contaminated site near the Open Burn/Open Detonation area at Eglin Air Force Base

At this time a significant reduction in soil concentration has not been observed and future sampling is required to show the possible effectiveness for plant induced biodegradation at EAFB.

2. Determine whether plants can significantly uptake RDX and HMX at the site

Uptake and translocation of RDX and HMX in Bahiagrass Pensacola has been shown to occur under field conditions at EAFB.

3. Determine whether RDX, HMX, and TNT are bioavailable to microorganisms in rhizosphere soils at the contaminated area

RDX and HMX have been shown to be recalcitrant under moist aerobic conditions in soils from EAFB. Conversely, TNT disappears rapidly regardless of soil type, with slower rates occurring in aged Dorovan Muck. The metabolites 2-ADNT and 4-ADNT were detected but at approximately 10% of the reacted mass. This indicates that binding to clay particles may be playing a role in the removal of TNT.

5.2 Biodegradation of Explosives in Unplanted Soils from Eglin Air Force Base, FL

Biodegradation experiments performed in this research have addressed the degradation potential for RDX, HMX, and TNT in unplanted soils native to EAFB. Microcosm studies were performed using the two main soil types present at EAFB, Lakeland Soil and Dorovan Muck. Contaminated soil used in the experiments was kept at conditions similar to that of surface soil at EAFB. Soil was incubated at warm, moist, and aerobic conditions throughout the 56 day experiment. RDX and HMX were shown to be recalcitrant under these experimental conditions in both soil types. These results were expected due to similar observations presented in the literature for these compounds under aerobic conditions. TNT degraded relatively quickly to 2-ADNT and 4-ADNT in both soil types which were freshly contaminated. TNT degraded much more slowly in Dorovan Muck which had been contaminated and aged for 18 months, likely due to the high clay content of this soil. These results show that bioremediation or natural attenuation may be a viable treatment strategy for TNT contaminated soil without significant clay content. Conversely, RDX and HMX are recalcitrant under field conditions in EAFB soils and require alternative treatment strategies such as phytoremediation to ensure sustainable range management.

5.3 Characterization of the Microbial Community from Soils at Eglin Air Force Base, FL

The microbial communities of the two soil types present at EAFB were determined by Terminal Restriction Fragment Length Polymorphism (T-RFLP).

Dorovan Muck contained considerably more microbial diversity than Lakeland Soil, likely due to the higher organic content in Dorovan Muck. Dorovan Muck contained more phylogenetic classifications and relative abundance was more evenly distributed among different taxonomic levels than in Lakeland Soil. Proteobacteria was the dominant phylum type present in both soil types. The low microbial diversity present in Lakeland Soil indicates that bacteria which could be introduced for bioremediation purposes may not be able to compete with the current microbial population.

5.4 Phytoremediation Field Study for the Treatment of Explosive Compounds at Eglin Air Force Base, FL

The phytoremediation field study underway at EAFB is making great progress toward increasing sustainability on military testing and training ranges. The Bahiagrass Pensacola which was planted in May 2009 became well established and produced considerable biomass in just 6 months, even after near drought conditions in June and July of 2009. The sampling protocol used in the study has provided considerable background contamination data for both planted and unplanted areas near the OB/OD. RDX and HMX are the major contaminants present at the site along with lower levels of TNT and TNT metabolites. The explosives RDX, HMX, and TNT were also detected in the creek immediately down-gradient from the OB/OD site, raising concerns for off-site migration.

Although a significant reduction in soil concentration and explosives detection did not occur between May and November of 2009, there is considerable time left in this field study to show the possible effectiveness of phytoremediation at EAFB. Evidence

showing the uptake and translocation of RDX and HMX into Bahiagrass Pensacola provides proof of phytoremediation processes occurring. This is the first time uptake and translocation of RDX and HMX has been documented during a phytoremediation field study on military training ranges. Future monitoring of explosives and metabolites in soil and vegetation samples will provide the basis for recommendations regarding the implementation of phytoremediation as a viable treatment technique at EAFB.

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APPENDIX A
INSTALLATION OF BAHIAGRASS PENSACOLA SOD AT EGLIN AIR FORCE BASE

Figure A-1. Installation of Bahiagrass Pensacola sod on May 26, 2009.

Figure A-2. Installation of Bahiagrass Pensacola sod on May 26, 2009.

Figure A-3. Plot #1 on May 26, 2009.

Figure A-4. Plot #1 on June 24, 2009.

Figure A-5. Plot #1 on September 1, 2009.

Figure A-6. Plot #1 on November 17, 2009.

APPENDIX B

RESULTS OF EXPLOSIVES ANALYSIS

Table B-1. Results from soil samples taken at Plot #1 in May and November 2009.

		HMX		RDX	
	(mg/kg)	May	Nov	May	Nov
Planted n = 100	Mean	0.12	0.06	0.17	0.24
	St. Dev.	0.60	0.34	0.56	1.72
	Geomean	0.03	0.03	0.04	0.04
Unplanted n = 40	Mean	0.45	0.05	4.02	0.22
	St. Dev.	2.49	0.09	24.38	0.72
	Geomean	0.04	0.04	0.06	0.05

Table B-2. Results from soil samples taken at Plot #1 in May and November 2009.

		TNT		TNB		2,4-DNT	
	(mg/kg)	May	Nov	May	Nov	May	Nov
Planted n = 100	Mean	0.01	0.01	0.005	0.01	0.35	0.002
	St. Dev.	0.03	0.05	0.04	0.05	3.03	0.02
	Geomean	0.02	0.01	0.02	0.02	0.02	0.02
Unplanted n = 40	Mean	0.01	0.001	0.03	0.003	0.40	0.03
	St. Dev.	0.04	0.01	0.16	0.01	2.38	0.20
	Geomean	0.02	0.02	0.02	0.02	0.03	0.02