

**ENHANCED RHIZODEGRADATION OF MUNITIONS
EXPLOSIVES AND DEGRADATES BY
SELECTED NATIVE GRASS SPECIES**

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Master of Science

By

HAN YANG

Drs. John Yang, Stephen Anderson and Chung-ho Lin, Thesis Supervisors

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

**ENHANCED RHIZODEGRADATION OF MUNITIONS
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Presented by

HAN YANG

a candidate for the degree of

MASTER OF SCIENCE

and hereby certify that in their opinion it is worthy of acceptance.

Dr. John Yang (major advisor) Soil, Environmental, and Atmospheric Sciences

Dr. Stephen Anderson (co-advisor) Soil, Environmental, and Atmospheric Sciences

Dr. Chung-ho Lin (co-advisor) Forestry

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Han Yang

Drs. John Yang, Chung-ho Lin, and Stephen Anderson, Thesis Advisors

ABSTRACT

TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) are the two most widespread explosives in the world. The manufacture, use and disposal of explosives as well as the demilitarization of military facilities can result in environmental contamination. The explosive contamination in soil and water is reported as a threat to human health and ecosystems. Compared to highly expensive and inefficiently conventional remediation methods, phytoremediation has been considered as a cost-effective way to clean up contaminated sites. The objectives of this research are to: (1) evaluate the degradation kinetics of TNT and RDX in the rhizospheres of two selected native grass species; (2) determine the environmental fate of TNT and RDX in rhizosphere ecosystems; (3) investigate the synergic effects of TNT and RDX degradation in both rhizospheres by inoculating with known explosive degraders; and (4) assess the quantitative expression of the degradative genes of inoculated degraders under rhizospheres using the real-time PCR method, an advanced technique based on

polymerase chain reaction to detect and quantify one or more DNA sequences in real time. Two native grass species were selected in this study including: eastern gamagrass (EG; *Tripsacum dactyloides*) and switchgrass (SW; *Panicum virgatum*). The rhizosphere soils of these two grasses were collected and either C¹⁴ labeled TNT or RDX were applied in soils, followed by the application of the explosive-degrading bacteria. Explosive degradation profiles and mineralization rates were monitored during an 8-week incubation period. Additional studies were conducted to monitor the TNT degradation profile and functional gene copy numbers with the presence of these two species. Results suggested that the TNT was rapidly degraded into its major metabolites in both rhizospheres and control soil; the degradation of the TNT metabolites was significantly enhanced in the rhizosphere soils as compared with the control. However, the mineralization of TNT in all the treatments was limited (< 5%). In contrast, the degradation of RDX and its metabolites in the rhizosphere soils were significantly enhanced over the control. More than 13% RDX was mineralized in rhizosphere soils as compared to 5% in the control. Overall, EG appeared to be more effective for RDX degradation, while SW to be more suitable for TNT degradation. Inoculation of TNT degrader *P. putida* KT2440 to SW could enhance TNT degradation as compared to use SW alone.

CHAPTER 1

INTRODUCTION

Significance of Study

An explosive is defined as a material that can be detonated by suddenly releasing huge amounts of energy in the form of an “explosion”. TNT (2,4,6-trinitrotoluene) and RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) are the two most widespread explosives in the world. The manufacture, use and disposal of the explosives as well as the demilitarization of military facilities can result in environmental contamination. The contamination of explosives in soil and water is reported to be a threat to human health and ecosystems. The potential adverse, even toxic, impacts on human health associated with the exposure to explosives and their degradates through drinking contaminated waters and/or ingesting air-borne soil particulates have been widely reported. TNT and RDX have been reported to be carcinogenic (Agency for Toxic Substances and Disease Registry, 1995a; Agency for Toxic Substances and Disease Registry, 1995b). Burdette (Burdette et al., 1988) found RDX affected human central nervous systems, being a potent convulsant. The studies by Yinon and Honeycutt (Honeycutt et al., 1996; Yinon, 1990) showed that TNT can cause anaemia, abnormal liver function, cataract development, and skin irritation in humans. Thus, in order to safeguard humans and the environment from contamination, studies of explosive behavior in the environment and development of novel, cost-

effective remediation methods that lead to decreasing explosive levels in the environment are needed.

Phytoremediation, an environmental-friendly remediation technology that uses plants to reduce human and ecological risks of environmental contaminants through degradation, uptake, and stabilization processes, has been reported to be a promising approach to enhance the degradation of organic pollutants, including chlorinated hydrocarbons, pesticides, petroleum, explosives, dyes and detergents in contaminated soils and waters (Anderson et al., 1993; Chaudhry et al., 2001; Haby and Crowley, 1996; Reilley et al., 1996; Schwitzgubel et al., 2001; Siciliano and Greer, 2000). These studies have proved phytoremediation to be an *in situ*, environment-friendly, cost-effective method to remediate explosive-contaminated soils. However, most of these studies were focused on using aquatic plants and terrestrial plants such as hybrid poplar trees and maize. According to the preference of Army Research Office and the governmental regulations, use of native grass species is required for remediating explosive-contaminated sites, in the context of ecosystems or environmental sustainability. The efficacy of native grass species on the degradation of explosives in soil, especially TNT and RDX, has not been studied or reported yet and remains largely unknown.

Objectives

This project was initiated to assess the efficacy of two Missouri native grass species, eastern gammagrass (EG) and switchgrass (SW), on enhancing the biodegradation of TNT and RDX in soil through laboratory studies. Two native grass species were selected based on their effectiveness resulting from previous screening tests. The overall goal of

this study is to determine the efficacy of two Missouri native grass species, EG and SW, for degrading TNT and RDX in soils. Specific objectives are:

- 1) Evaluate the degradation kinetics of TNT and RDX in the rhizospheres of EG and SW, in terms of degradation rates and capacity, by a radioactive tracing technique;
- 2) Determine the environmental fate of TNT and RDX in the EG or SW rhizosphere ecosystems through the mass balance approach;
- 3) Monitoring the degradation profiles of TNT and RDX in the rhizospheres of EG and SW by a High Performance Liquid Chromatography (HPLC) method.
- 4) Investigate the synergistic effects of TNT and RDX degradation in the rhizospheres inoculated with identified explosive degraders; and
- 5) Assess the quantitative expression of the degradative genes of inoculated degraders under the EG and SW rhizospheres using the real-time PCR method.

Results from these studies could substantially improve our understanding of environmental behaviors and plant-enhanced degradative mechanisms of TNT and RDX under soil ecosystems, which potentially lead to sustainable remedial technologies to support the restoration of contaminated sites in field scale and safeguard humans and the environment from such contamination.

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CHAPTER 2

LITERATURE REVIEW

Explosives

Explosives are a group of synthesized chemical compounds that store huge amounts of energy. When detonated by appropriate external initial stimulations, they will release energy rapidly in the form of “explosion” accompanied by a rapid production of heat and gas. Explosives are usually organic compounds consisting of four elements, hydrogen (H), oxygen (O), carbon (C), and nitrogen (N). Explosives have been mainly used in the military industry such as production of weapons, bombs and shells, and other industries like mining and road building.

The first explosive material, known as “gunpowder” was invented by the ancient Chinese in the 9th century or earlier. The explosive did not get much development until it was introduced to the west. After the 18th century, with the rapid development of chemistry as a discipline of science, chemists were able to synthesize more efficient and more powerful explosives. In 1863, TNT, 2,4,6-trinitrotoluene (Figure 2.1), was first synthesized by Joseph Wilbrand. It is a nitroaromatic-based, yellow and odorless solid explosive, with a solubility of 101.5 mg L⁻¹ at 25⁰C (Ro et al., 1996) and a symmetrical structure consisting of three nitroso groups attached to a toluene ring. TNT can be formulated industrially by nitration of toluene with a mixture of nitric and sulfuric acids followed by washing with aqueous sodium sulfite (Agency for Toxic Substances and

Disease Registry, 1995a; Lewis et al., 2004). RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine, was first synthesized by Hans Hemming in 1899. RDX stands for Royal Demolition Explosive or Research Department Explosive (Figure 2.2), which is a nitramine-based, white explosive with a solubility of 43.2 mg L⁻¹ at 20⁰C (Gorontzy et al., 1994) and a symmetrical structure containing three nitroso groups attached to 3 nitrogen atoms on the ring constructed by 3 nitrogen atoms and 3 methyl groups. RDX can be commonly produced via two methods: 1) Bachmann process that is the most common method used in the United States for RDX production, in which hexamine is used to react with nitric acid, ammonium nitrate, glacial acetic acid, and acetic anhydride, followed by filtering and recrystallization; and 2) another process that is not as productive as the Bachmann process, in which octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is directly nitrated to form RDX (Agency for Toxic Substances and Disease Registry, 1995b).

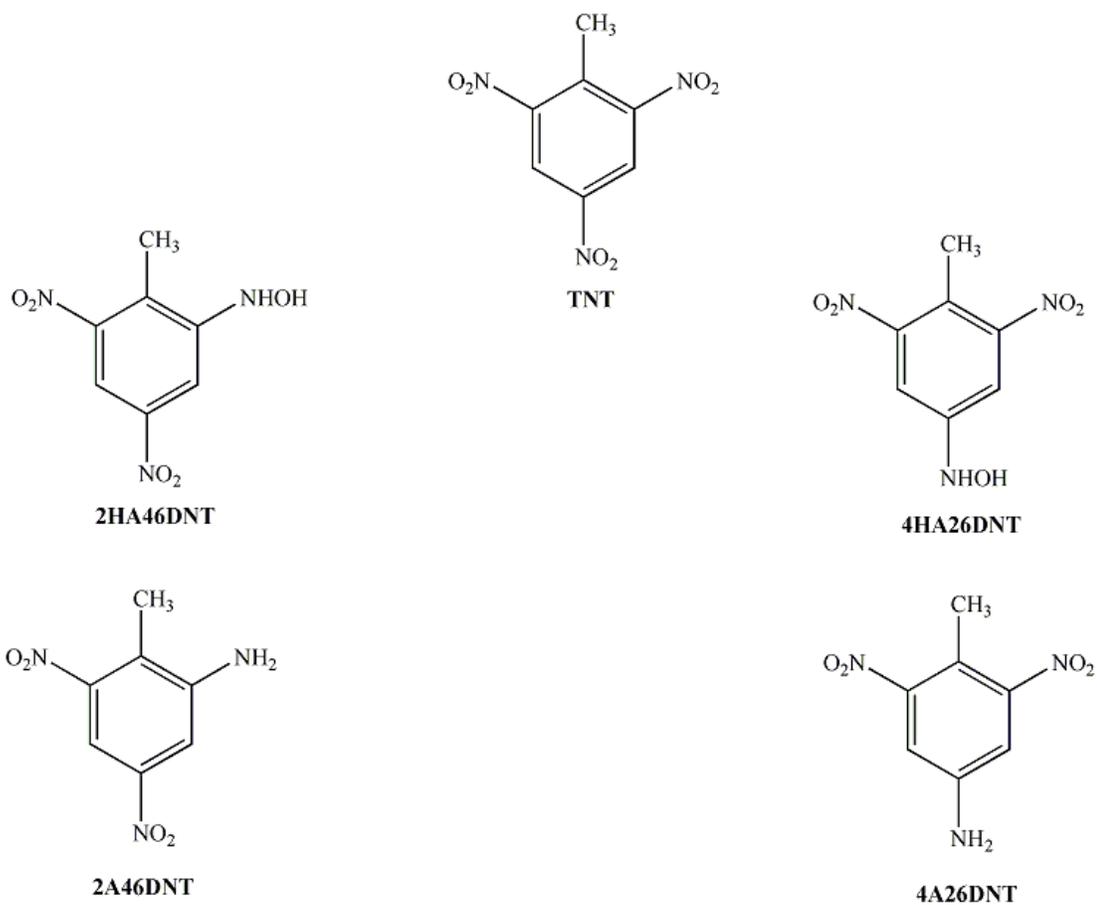


Figure 2.1. TNT and its major metabolites.

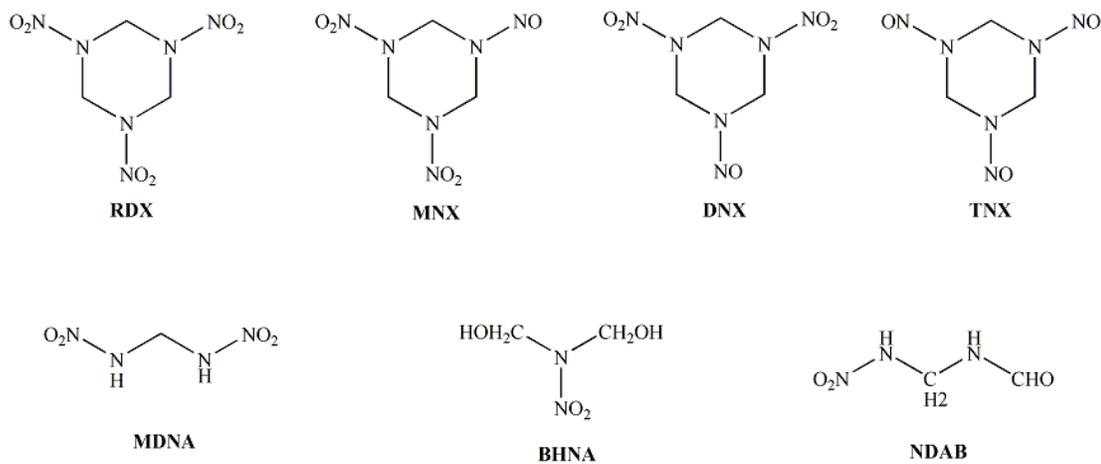


Figure 2.2. RDX and its major metabolites.

Contamination by Explosives

TNT and RDX are two of the major explosives used in military operations. During past decades, both compounds, especially TNT, had been massively produced world-wide in demands by wars, reaching its peak in the period of World War I & II (Nyanhongo et al., 2005). In the United States, TNT had become the major explosive in military ordnance during World War I (Lewis et al., 2004). It was reported that the US produces approximately 1,000,000 kg TNT per year (Harter, 1985). RDX production had reached its peak in the 1960s in the United States. From 1969 to 1971, RDX was estimated to be produced at 6,803,885 kg per month on an average volume basis; however, it was decreased to 7,257,477 kg per year in 1984 (Agency for Toxic Substances and Disease Registry, 1995b).

The presence of TNT and/or RDX in soil and water can cause environmental contamination, posing a threat to humans and ecosystems. The contamination is usually associated with manufacture, use and disposal of the explosives. The waste water from the processes of TNT production is called “pink water” or “red water”, which contains high levels of TNT and some co-products like dinitrotoluene isomers (Gilbert, 1980; Lindner, 1980). A large quantity of “pink water” generated by TNT production has been reported to result in contamination of the areas adjacent to military production facilities. A single TNT factory can generate up to annual 2×10^6 liters of “pink water” (Jenkins et al., 1986). The loading, assembling and packaging of the explosives can also elevate the TNT level at ammunition plant sites (Pennington and Brannon, 2002). In the US, there are 22 ammunition production and packaging sites that have been identified to be contaminated by explosives (Hooker and Skeen, 1999). Ordnance, bombs and projectiles

used during war time have caused widespread contamination of explosives. It is believed that widespread explosive contamination in soil and water in the US was attributed to military activities during World War II and the Korean War (Hawari et al., 2000a). Soil contamination can occur at incineration sites. Dust containing the explosive particles produced at incineration sites can emit into the atmosphere and then deposit on nearby areas. Leachate from the incineration sites and landfill sites can reach groundwater, threatening drinking water resources and human health. The contamination may also result from spills of wastewater lagoons (Esteve-Nunez and Ramos, 1998; Rosenblatt et al., 1991).

Elevated TNT levels has been identified in at least 20 of the 1,397 hazardous waste sites of the EPA National Priorities List while RDX was identified in 16 of the 1,397 hazardous waste sites (Agency for Toxic Substances and Disease Registry, 1995a; Agency for Toxic Substances and Disease Registry, 1995b). In the US, over 1,200,000 tons of soil has been estimated to be contaminated by explosives (Lewis et al., 2004). Concentrations of explosives in contaminated sites are incredibly high. For example, in Louisiana Army Ammunition Plant (LA, USA) and the Cornhusker Army Ammunition Plant (NE, USA), explosive concentrations have been determined at up to 87,000 mg/kg in soil, 711,000 mg/kg in sediment, and 3,375 mg/L in surface water (Steevens et al., 2002). However, the USEPA recommended clean up levels are 17.2 mg TNT and 5.2 mg RDX per kg soil (Hundal et al., 1997).

Toxicity of Explosives on Human Health

Man-made explosives such as TNT and RDX are usually recalcitrant in the environment, meaning that they have a long residence time and are hard to decompose naturally. TNT is reported to cause health problems when human are exposed to it, such as anemia, abnormal liver function, cataract development and skin irritations (Honeycutt et al., 1996; Yinon, 1990). The cases of anemia and fatal aplastic anemia were reported among workers in TNT production factories through inhalation exposure in England during World War I (Hathaway, 1985). The TNT exposure was also found to induce toxic hepatitis among the workers relevant to explosive manufacture (Agency for Toxic Substances and Disease Registry, 1995a). It is believed that the occurrence of cataracts was primarily due to the chronic exposure of TNT (Hathaway, 1985; Savolainen et al., 1985). Studies also showed that the workers in explosive manufacture factories would suffer from dermatitis through TNT inhalation and dermal exposure (Morton et al., 1976). The deaths of 475 people resulting from TNT exposure have been reported in the US during World War I (McConnell and Flinn, 1946). A study conducted in China indicated that TNT had adverse effects on males' reproductive systems by lowering semen volume and increasing sperm malformation (Li et al., 1993). Although there is insufficient evidence that TNT causes cancer, it has been listed as a carcinogenic compound (Agency for Toxic Substances and Disease Registry, 1995a).

RDX has been characterized as a potent convulsant and has adverse effects on the human central nervous system (Burdette et al., 1988). People inhaling RDX dusts and C4 fumes (91% RDX) have been found to suffer symptoms like convulsion, unconsciousness, headaches, dizziness, vomiting, muscle twitching and confusion

(Hollander and Colbach, 1969; Kaplan et al., 1965). Adverse effects on human's central nervous systems can occur with oral exposure to RDX. Children who accidentally consumed an unknown quantity of RDX were reported to suffer from seizure (Woody et al., 1986). The cases of human death caused by RDX exposure have not yet been reported. However, animal deaths, like rats and rabbits, have been documented by animal RDX dosing tests (Agency for Toxic Substances and Disease Registry, 1995b). Like TNT, RDX is also designated as a carcinogenic compound.

Remediation Methods

As documented above, explosives and their environmental contamination have shown a great risk to human health and a threat to ecosystems. Environmental cleanup or risk reduction of contaminated waters and soils would be of public and regulatory concern. Efforts to develop cost-effective, environmentally-friendly, and sustainable solutions or remedial technologies to impaired ecosystems would be critical and a top priority in the context of safeguarding human health and the environment from contamination as well as environmental restoration and sustainability. The current technologies that have been used for explosive waste treatment and cleanup of explosive-contaminated soils are summarized below:

Incineration

The most prevalent method to treat the soils contaminated by explosives is incineration. This method has been utilized in several contaminated sites in the US (Jerger and Woodhull, 2000). The processes of incineration involve the excavation of

contaminated soils, the transportation to incineration sites, and combustion that destroys the explosives by heating. However, this method may not be as efficient as anticipated. Dusts produced during the combustion process may emit into the atmosphere, resulting in air pollution and subsequently soil or water contamination due to deposition of dusts. Leaching of soils in contaminated sites may also cause contamination of surface or ground water (Kuiper et al., 2004). High costs associated with incineration could also be a problem. Costs include soil excavation, transportation, facilities building, equipment purchase, energy for incineration and labor (Widrig et al., 1997). It is reported that the estimated cost of incineration is \$800 per ton of soil (Funk et al., 1993). Additionally soil excavation would destroy the natural structure and texture of soil and disturb the ecosystem (Hannink et al., 2002). Thus, incineration might not be a cost-effective, environmentally-sustainable method nor acceptable by the public.

Composting

Composting is a biological process that uses bacteria to degrade explosives under aerated conditions, which involves supplementing organic matter, bulking agents, water and heat to contaminated soils. Addition of organic matter is to provide carbon and nutrients for microbial growth; bulk agents are to improve soil structure for optimal aeration. Composting can effectively reduce TNT and RDX to levels below the treatment goal (Lewis et al., 2004). Studies conducted by Thorn and Kennedy (Thorn and Kennedy, 2002) indicated that, with a tracer technique, TNT in composting was transformed rather than mineralized. Most TNT carbon had been found to covalently bond to humic substances in the compost. The leachability test has proven that TNT-humic substance

complexes were resistant to solvent extraction and relatively stable (Hampton and Sisk, 1997). Soils and leachates post composting showed nearly no toxicity and mutagenicity to tested bacterial strains, aquatic invertebrates, earthworms and rats. The composting costs were estimated to be \$206 to \$766 per ton of soil, which is a 40 to 50 percent savings as compared to incineration. Composting was one of the first methods that was tested, approved, and selected for restoring military sites in the US (Craig et al., 1995; EPA, 1993).

Bioslurry

Bioslurry is another biological method to treat explosive-contaminated soils, in which soil and water are mixed together at an appropriate ratio and amended with nutrients to enhance microbial activity in a created bioreactor under sequential anaerobic or aerobic conditions. Studies showed that bioslurry is the same as effective as composting for explosives degradation. For example, TNT has been found to form strong, irreversible bonds with humic substances and soil organic matter, which reduce their mobility and bioavailability. The Fermentative Anaerobic Soil Treatment (FAST) was the first field-scale bioslurry reactor to remediate explosive-contaminated soil in the US. The costs are estimated to be \$200 to \$600 per ton of soil (Lewis et al., 2004).

Phytoremediation

Despite the treatment or cleanup methods described above that have shown some degree of effectiveness for the breakdown of contaminated explosives, these methods usually have high costs and are destructive. Development of a novel, cost-effective, and

environmentally-sustainable remedial method is needed. Phytoremediation has recently been reported to be a promising technology to degrade or clean up organic pollutants, including: chlorinated hydrocarbons, pesticides, petroleum compounds, explosives, dyes and detergents (Anderson et al., 1993; Chaudhry et al., 2001; Haby and Crowley, 1996; Reilley et al., 1996; Schwitzguebel et al., 2001; Siciliano and Greer, 2000). Phytoremediation is defined as the use of green plants to remove, destroy and sequester hazardous materials in soil in an *in situ*, environmentally-friendly, and cost-effective manner. Phytoremediation consists of phytoextraction in which plants remove contaminants from soils by uptake through root systems and by concentrating them in above-ground tissues through translocation; rhizofiltration is the process in which plant root systems buffer contaminants in the root zone and prevent their leaching; phytostabilization is the process in which plants immobilize contaminants through the root system and reduce their mobility; phytovolatilization is the process in which plants absorb volatile compounds from soil and release them into the atmosphere; phytostimulation is the process in which plants stimulate microbial activities in the rhizosphere and enhance the breakdown of contaminants; and phytotransformation is the process in which plants absorb and degrade contaminants through biochemical transformation processes (Glick, 2003).

Phytoremediation has several advantages over other cleanup methods; these include: 1) non-destructive, preserving the natural structure of the soil; 2) low energy inputs, using sunlight as an energy source; 3) natural, elevating levels of soil biomass; 4) low costs; and 5) rapid cleanup potential (Glick, 2003). Despite these advantages, limitations should be taken into account when phytoremediation is to be applied efficiently to restore

contaminated sites. These limitations include: 1) the plant tolerance range of phytotoxicity caused by pollutants; 2) the rate of plant growth; 3) the depth of root systems; 4) the endurance of climate changes; and 5) the time required to reach desired results (Khan et al., 2000). In the Milan and Iowa Army Ammunition Plant, an aquatic phytoremediation system showed its capability to degrade >95% TNT and RDX in groundwater (Best et al., 1998), but high concentrations of TNT or RDX in water or soils are usually toxic to plants, lowering the treatment efficacy and preventing large-scale application. Some plant species are sensitive to pollutants. In a study conducted by Behrends (Behrends et al., 1997), plants can eliminate TNT at the concentration of 4 ppm, but when TNT concentration was up to 20 ppm, the efficacy of plants removing TNT diminished. Plants have developed complex endogenous detoxification systems to deal with a wide range of hazardous substances in the environment during thousands of years of evolution. Two groups of pollutants, elemental and organic pollutants, are the target of phytoremediation. A wide range of plants have been proven to possess a promising ability to degrade organic pollutants resulting in thorough mineralization, including: poplar trees, willow, alfalfa, and different grass species (Kuiper et al., 2004).

Rhizodegradation

In phytoremediation, microbial activities in the rhizosphere had been considered as a major contributor for degradation of pollutants, which could be referred to as rhizodegradation. In some cases, plants were protected against hazardous compounds by bacteria. Studies have shown that various grass species are more suitable for rhizodegradation because of their vigorous root systems harboring large populations of

bacteria. Plant roots are believed to penetrate the soil with highly developed branches to increase aeration in the soil and to enhance the contact of bacteria colonized on them with pollutants, eventually to achieve better rhizodegradation (Kuiper et al., 2004).

The rhizosphere is a dynamic zone between roots and soil, characterized by low redox potentials, abundant energy and nutrients, low pH, and high microbial activities due to root activities. Root exudates secreted by plants contain sugars, organic acids and amino acids (Vancura and Hovadik, 1965), which can be utilized by microbes in the rhizosphere as energy and nutrient sources. Organic acids can also solubilize soil minerals, providing nutrients for microbial growth. The plant species-specific chemical conditions in the rhizosphere could preferentially differentiate or promote the growth and activity of certain microbial communities that are capable of targeting explosive contaminants, thus enhancing the biodegradation of the contaminants and plant growth as well. Explosive degrading bacteria in the rhizosphere are able to eliminate plant toxicity of explosives through enhanced biodegradation. On the other hand, many microbes isolated from the rhizosphere have been shown to pose plant growth-stimulating properties (Campbell and Greaves, 1990). They may fix nitrogen, synthesize siderophores and phytohormones, like auxins and cytokinins, and solubilize soil minerals for plant growth (Glick, 2003). The mutually beneficial interaction between plants and microbes is based on the colonization of microbes onto plant roots. Efficient colonization can lead to successful rhizodegradation. Coating seeds with bacteria is the most successful approach to inoculate bacteria leading to their adaptation in rhizospheres. *Pseudomonas* spp, plant-growth promoting bacteria, were found to be predominant in the rhizosphere. Cells of *Pseudomona* were found to colonize along the junctions of epidermal plant cells where

nutrients are exuded. However, the ability of colonization varies strongly within different *Pseudomonas* strains (Kuiper et al., 2004). Some microbes, like fungi, can colonize plant roots to enhance plant uptake of nutrients. Thus, the synergistic interactions between explosive degrading microbes and species-specific plants would enhance the feasibility of the application of phytoremediation technology on large scales at relatively high explosive concentrations.

Detoxification of Explosives in Plants

Plants have developed complex endogenous detoxification systems to deal with a wide range of hazardous substances in the environment during thousands of years of evolution. Lots of studies have justified the plasticity of these systems in plants to detoxify synthesized xenobiotics like explosives, herbicides and pesticides. In general, plants can't use xenobiotics as an energy source. Xenobiotics can be taken up, sequestered and compartmentalized into plants' biomass resulting in detoxification of xenobiotics. Xenobiotic metabolism in plants consists of 3 phases, including Phase I, the addition of reactive functional groups to xenobiotics; Phase II, the conjugation of endogenous hydrophilic molecules to xenobiotics; and Phase III, the excretion of conjugates and/or integration of conjugates into plants' biomass (Hannink et al., 2002).

In Phase I, after the entrance of xenobiotics in plants, functional groups, such as hydroxyl (-OH), amino (-NH₂) and sulfhydryl (-SH), are added to xenobiotic compounds with the help of specific enzymes (Komoba et al., 1995). There are two reaction types involved in the attachment of functional groups to xenobiotics, including: oxidation and reduction. Oxidation is more frequently observed with pesticides (Komoba et al., 1995),

while reduction is preferred by nitroaromatics (Burken et al., 2000). The addition of functional groups in Phase I makes preparation for Phase II reactions by creating active sites on xenobiotics. In Phase II, modified xenobiotics react with endogenous hydrophilic molecules, glucose, malonate and glutathione, to form conjugates catalyzed by glucosyl-, malonyl- and glutathione transferases. Those conjugates can be divided into two groups: soluble and insoluble based on their extractability from plant tissue by commonly used solvents. Xenobiotics conjugated to glucoside, glutathione, amino acid and malonyl groups prefer to form soluble conjugates in plants (Hannink et al., 2002). In Phase III, those conjugates could be either secreted out, like wetland plants (Burken et al., 2000), or integrated into plants' biomass. Soluble conjugates are mainly stored in the vacuole of plant cells, while insoluble ones are incorporated into cell wall components, like lignin (Komoba et al., 1995).

Although the detailed metabolism of explosives in plants has yet to be elucidated, TNT always undergoes serial two-electron reduction of nitro groups in Phase I detoxification in plants producing hydroxylamino- and amino- TNT derivatives. In Phase II, these intermediates conjugate with sugars and glutathione in plants. These conjugated compounds would be deposited in vacuoles, integrated into cell wall of plant cells, and/or excreted out of plants (Rylott and Bruce, 2009). Oxidation of methyle group of TNT in Phase I was also observed in aquatic plants (Bhadra et al., 1999). UDP-glycosyltransferases were identified to be involved in conjugation of transformed TNT intermediates in Phase II (Gandia-Herrero et al., 2008). Studies showed most of transformed TNT conjugates (95%) were found predominately in plant roots (Sens et al., 1999; Sens et al., 1998).

RDX is taken up readily by plants and transported to the aerial tissues of plants. After the entrance of RDX into plants, RDX would be reduced to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-nitroso-5-dinitro-1,3,5-triazine (DNX). These transformed RDX intermediates would be mineralized by plant cells with the help of light producing formaldehyde and methanol. Formaldehyde and methanol could be further mineralized to carbon dioxide with the presence of light (Rylott and Bruce, 2009).

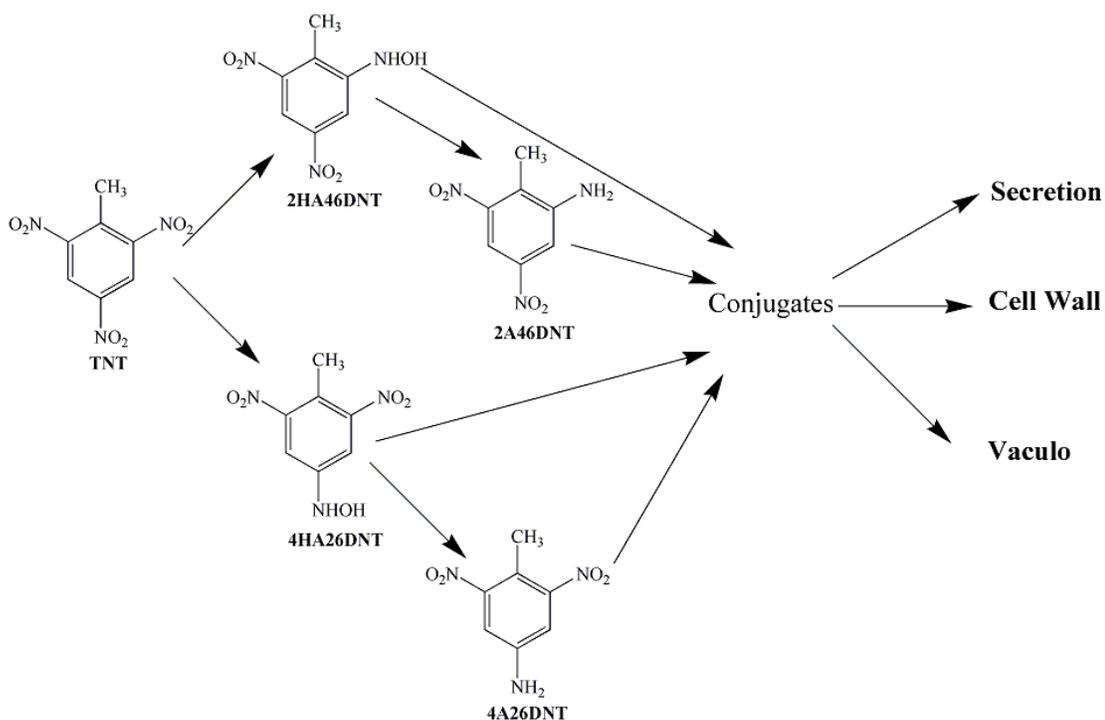


Figure 2.3. Proposed TNT degradation mechanism in plants.

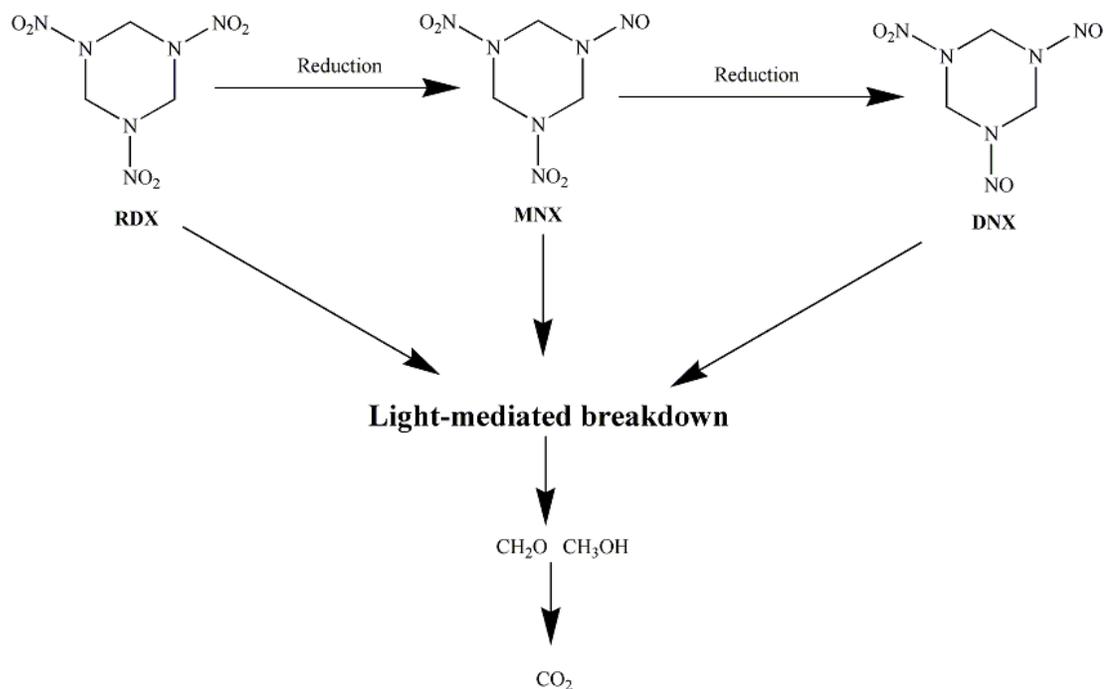


Figure 2.4. Proposed RDX degradation mechanism in plants (Rylott and Bruce, 2009).

RDX Biodegradative Mechanisms

RDX biodegradation has been documented under various environmental conditions, including: surface and subsurface soils, aquifers, sewage sludges, and cold marine sediments. There were three mechanisms that have been proposed for RDX degradation, including direct enzymatic cleavage, two-electron reduction, and single-electron reduction/denitration (Crocker et al., 2006).

Direct enzymatic cleavage involves the direct enzymatic attacks on C-H, C-C and C-N bonds of the RDX molecule (Crocker et al., 2006). In former studies (Halasz et al., 2002); (Hawari et al., 2000b), two direct ring cleavage metabolites, methylenedinitramine (MNDA) and bis-(hydroxymethyl)nitriamine (BHNA) were reported during the treatment of RDX with a domestic sludge under anaerobic conditions. And methylenedinitramine

(MNDA) and bis-(hydroxymethyl)nitriamine (BHNA) could spontaneously decompose in water to produce nitrous oxide and formaldehyde.

The two-electron reduction mechanism was originally proposed by McCormick et al. (McCormick et al., 1981). In McCormick's study, municipal sludge was utilized to degrade RDX under anaerobic conditions. Based on a series of RDX degradation products detected, McCormick postulated a pathway of RDX degradation involving: (i) the sequential reduction of RDX to mono-, di- and trinitroso-RDX derivatives (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, MNX; hexahydro-1,3-nitroso-5-dinitro-1,3,5-triazine, DNX; and hexahydro-1,3,5-nitroso-1,3,5-triazine, TNX) and (ii) further reduction through nitroso-RDX derivatives to hypothetical hydroxylamine-RDX derivatives and ring cleavage to yield hydrazines, methanol and formaldehyde. In other studies (Adrian and Chow, 2001; Adrian et al., 2003; Halasz et al., 2002; Hawari et al., 2000b; Oh et al., 2001), nitroso-RDX derivatives (MNX, DNX and TNX) were frequently observed as intermediates in RDX degradation as well as methanol and formaldehyde. Except for McCormick's study (reference??), no studies had identified hydrazines as RDX degradation products. In addition, no hydroxylamine-RDX derivatives were detected in the above studies, except for (Adrian and Chow, 2001) in which the hydroxylamine-DNT was reported.

The study conducted by Zhang and Hughes (Zhang and Hughes, 2003) showed RDX transformed through MNX, hydroxylamine-RDX and amino-RDX intermediates to 1,3,5-triamino-1,3,5-triazine as an end product in cell-free extracts of *Clostridium acetobutylicum* with H₂. The study by Bhushan (Bhushan et al., 2002) indicated that RDX was biodegraded through MNX, methylenedinitramine (MNDA) to ammonium, nitrous

oxide and formaldehyde by *Aspergillus niger*. The two proposed pathways above could serve as alternatives to the two-electron reduction mechanism.

The single-electron reduction/denitration mechanism refers to the loss of one nitro group on the RDX molecule through single electron transfer generating a free-radical anion ($\text{RDX}^{\cdot-}$). This mechanism occurs under both aerobic and anaerobic conditions and seems to be the major route of RDX biotransformation, because it is thermodynamically favorable (Crocker et al., 2006). Under anaerobic conditions, one nitro group of RDX molecule was released generating a free-radical anion ($\text{RDX}^{\cdot-}$). This free-radical anion ($\text{RDX}^{\cdot-}$) was unstable resulting in ring cleavage to yield methylenedinitramine (MNDA) as a transient intermediate. Then methylenedinitramine (MNDA) was further decomposed in water to produce nitrous oxide and formaldehyde (Halasz et al., 2002). While under anaerobic conditions, two nitro groups were removed from the RDX molecule resulting in the formation of 4-nitro-2,4-diazabutanal along with nitrous oxide, ammonium and formaldehyde (Bhushan et al., 2003; Fournier et al., 2002). In addition, an alternative pathway was identified that MNX could be denitrified under anaerobic conditions to produce methanol, nitrous oxide and carbon oxide as end-products after the reduction of RDX to MNX (Zhao et al., 2002).

RDX Degradation and Responsible Enzymes

Biodegradation of RDX has been intensively investigated for decades. Recently, the research interests have been shifted from the identification of RDX degradative metabolites to the identification of enzymes within RDX degrading bacteria, which are responsible for RDX degradation. 3 mechanisms of RDX biodegradation have been

proposed, including: denitration, two-electron reduction and direct enzymatic cleavage. Denitration appears to be a major RDX degradation pathway, which could occur under both aerobic and anaerobic conditions. Aerobic bacteria, including: *Rhodococcus rhodochrous* strain 11Y, *Rhodococcus* sp. Strain DN22, *Williamsia* sp. Strain KTR4 and *Gordonia* sp. Strain KTR9 degrade RDX through denitration. The functional gene, *xplA*, in *Rhodococcus rhodochrous* strain 11Y can encode a fused flavodoxin-cytochrome P450 enzyme which has been identified to be responsible for RDX degradation. Under anaerobic conditions, *K.pneumoniae* strain SCZ-1 and *C. bifermentans* strain HAW-1 use denitration to degrade RDX. Two-electron reduction can be initiated by oxygen-insensitive nitroreductase. Bacteria, including: *Klebsiella pneumonia* strain SCZ-1, *Clostridium bifermentans* strain HAW-1, *Shewanella halifaxensis* strain HAW-EB4, *Shewanella* sp. HAW-EB5, *Methylobacterium* spp., enterobacteria, *Shewanella* sp. HAW-EB2, can degrade RDX via two-electron reduction. Direct enzymatic cleavage of RDX was studied using a domestic sludge. Because of the complexity of microbe consortia, it is unclear what bacteria are involved in it (Caballero and Ramos, 2006).

TNT Biodegradative Mechanisms

The major mechanisms of TNT degradation were proposed including successive two-electron reduction and denitration (Nyanhongo et al., 2005; Smets et al., 2007). Successive two-electron reduction involves the sequential reduction of TNT to nitroso-TNT derivatives, hydroxylamino-TNT derivatives and amino-TNT derivatives. Frequently observed intermediates include: 2-hydroxylamino-4,6-dinitrotoluene (2HA46DNT), 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT), 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT) and 2,4-diamino-6-nitrotoluen. No nitroso derivatives were detected (Esteve-Nunez et al., 2001; Koder and Miller, 1998; Nyanhongo et al., 2005; Yin et al., 2004; Yin et al., 2005). These partially reduced TNT intermediates have been reported to react with each other in the presence of oxygen to form azoxytetranitrotoluene, which is more recalcitrant and mutagenic than TNT (George et al., 2001; Haidour and Ramos, 1996). Although oxygenolytic metabolism of TNT rarely occurs, a possible oxygenolytic attack of aminodinitrotoluene was observed. 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene have been reported to be oxidized to 3-amino-4-methyl-5-nitrocatechol and 3-amino-6methyl-5-nitrocatechol with the release of nitrite, respectively (Johnson et al., 2001). Hydroxylamino-TNT derivatives were observed to go through a Bamberger-like rearrangement to produce vicinal amino and hydroxyl substituents with the release of ammonium (Caballero and Ramos, 2006; Caballero et al., 2005a; Caballero et al., 2005b; Hughes et al., 1998).

Denitration refers to the direct hydride addition to the aromatic ring of TNT with the formation of TNT hydride-Meisenheimer complex (HTNT) (Esteve-Nunez et al., 2001; Nyanhongo et al., 2005; Smets et al., 2007). TNT hydride-Meisenheimer complex (H

TNT) would quickly convert to TNT dihydride-Meisenheimer complex (HTNT). TNT dihydride-Meisenheimer complex (HTNT) is an unstable orange product that could be further transformed resulting in nitrite release.

One electron reduction of TNT may also occur (Esteve-Nunez et al., 2001; Spain, 1995). One nitro group of TNT can be reduced to nitroso group with the formation of nitroanion radical. But this nitroanion radical can react with oxygen to form a superoxide and the original nitro aromatic compound through a futile cycle.

TNT Degraders and Responsible Enzymes

Due to toxicity of TNT to human beings, TNT degradation has been intensively investigated by researchers. Biodegradation of TNT has been investigated by scientists over decades. Frequently observed metabolites of TNT degradation have been identified, including: 2-hydroxylamino-4,6-dinitrotoluene (2HA46DNT), 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT), 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT) and 2,4-diamino-6-nitrotoluen (2ADA6NT) (Esteve-Nunez et al., 2001; Koder and Miller, 1998; Nyanhongo et al., 2005; Yin et al., 2004; Yin et al., 2005). The mechanisms of TNT biodegradation have been proposed as well, including: two-electron reduction, denitration and one-electron reduction (Nyanhongo et al., 2005; Smets et al., 2007). However, during the last decade, the investigative focus of TNT degradation has been changed from the identification of metabolites and degradation mechanisms to the identification of enzymes in degraders associated with the biodegradation of TNT. Since then, a great number of enzymes have been identified.

The predominant enzymes involved in TNT biodegradation are nitroreductases, which have a wide range of nitroaromatic substrates including: nitrofurazones, nitroarenes, nitrophenols, nitrobenzenes and explosives (Smets et al., 2007). Nitroreductases share similar properties, including: containing flavin mononucleotide (FMN) as a cofactor and consuming NADH/NADHP as the electron donor (Bryant and DeLuca, 1991; Bryant et al., 1991). There are two kinds of nitroreductases based on the sensitivity to the presence of oxygen: oxygen-insensitive nitroreductase (type I nitroreductase) and oxygen-sensitive nitroreductase (type II nitroreductase). Oxygen-insensitive nitroreductase performed successive two-electron reduction transforming TNT to hydroxylamino derivatives.

However, the reduction of TNT ended up to hydroxylamino derivatives and not beyond, even under excessively reductive conditions (Smets et al., 2007). Oxygen-insensitive nitroreductase reduced TNT via a ping-pong mechanism, in which the FMN of the enzyme was reduced by NADH/NADHP resulting in the reduction of nitro groups of TNT and regeneration of the enzyme. The activity of oxygen-insensitive nitroreductase remained functional even under the presence of oxygen (Nyanhongo et al., 2005). Oxygen-insensitive nitroreductase has been found in many bacteria including: PnrA and PnrB in *Pseudomonas putida*, NfsA and NfsB in *Escherichia coli*, SnrA and Cnr in *Salmonella enterica* serovar Typhimurium, NfsI in *Enterobacter cloacae*, RdxA in *Helicobacter pylori*, flavin reductase P in *Vibrio harveyi*, Frase I in *Vibrio fischeri*, NADH oxidase in *Thermus thermophilus*, and NitA and NitB in *Clostridium acetobutylicum* (Smets et al., 2007). Oxygen-sensitive nitroreductase catalyzed one electron reduction of TNT (Esteve-Nunez et al., 2001; Spain, 1995). One nitro group of TNT can be reduced to a nitroso group with the formation of nitroanion radical. But this nitroanion radical can react with oxygen to form a superoxide and the original nitro aromatic compound through a futile cycle. In our study, *P. putida* KT2440 was selected as a TNT degrader. *P. putida* KT2440 possesses the pnrA gene which locates on its chromosome. Studies have shown the pnrA gene demonstrated much more activity than the pnrB gene. The enzyme encoded by the pnrA gene catalyzes the transformation of TNT to 4-hydroxylamine-2,6-dinitrotoluene using the ping-pong mechanism described above (Caballero et al., 2005b).

Another important group of enzymes are called the old yellow enzyme family, including: XenA and XenB in *Pseudomonas* sp., PENT reductase in *E. cloacae*, NemaA in

E. coli, morphinone reductase in *P. putida* M10 and OYE in *Saccharomyces cerevisiae* (Smets et al., 2007). They not only possess the nitroreductive activities as nitroreductases, but also can reduce TNT via denitration. Denitration refers to the direct hydride addition to aromatic ring of TNT with the formation of TNT hydride-Meisenheimer complex (H⁻TNT) (Esteve-Nunez et al., 2001; Nyanhongo et al., 2005; Smets et al., 2007). TNT hydride-Meisenheimer complex (HTNT) would quickly convert to TNT dihydride-Meisenheimer complex (HTNT). The TNT dihydride-Meisenheimer complex (HTNT) is an unstable orange product. It could be further transformed resulting in nitrite release (Smets et al., 2007).

There are some other enzymes involved in biodegradation of TNT, including: respiration-associated nitroreductase, ring hydroxylating dioxygenases and Fe-only hydrogenase. Respiration-associated nitroreductase is a membrane-associated respiratory nitroreductase found in *P. putida* JLR11, which use TNT as the sole electron acceptor and acetate as the electron donor under anaerobic conditions. TNT can be reduced to 4-amino-2,6-dinitrotoluene along with the oxidation of acetate or H₂ (Smets et al., 2007). Ring hydroxylating dioxygenases, R34DDO in *Burkholderia cepacia* R34 and NBDO in *Comamonas* sp. Strain JS 765, are responsible for the oxygenolytic attack of reduced TNT metabolites, aminodinitrotoluene isomers (Smets et al., 2007). 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene have been reported to be oxidized to 3-amino-4-methyl-5-nitrocatechol and 3-amino-6methyl-5-nitrocatechol with the release of nitrite, respectively (Johnson et al., 2001). Fe-only hydrogenase in *C. acetobutylicum* catalyzes a H₂-dependent reduction TNT. TNT can be reduced to hydroxylamino

derivatives and eventually to dihydroxylamino derivatives under acidogenic conditions (Smets et al., 2007).

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CHAPTER 3

DEGRADATION OF RDX IN RHIZOSPHERE SOILS OF TWO NATIVE GRASS SPECIES

ABSTRACT

Soil contamination by munition explosive residues including TNT (2,4,6-trinitrotoluene), RDX (1,3,5-hexahydro -1,3,5-trinitrotriazine) and their metabolites is a potential threat to human health and ecosystems. Phytoremediation has been recognized as one of the most cost-effective and environmentally-safe strategies for reducing the risks of explosives through plant-enhanced biodegradation. The objective of this study was to investigate the rhizodegradation of RDX enhanced by two selected native grasses. Two selected Missouri native grasses, eastern gammagrass (EG, *Tripsacum dactyloides*) and switchgrass (SW, *Panicum virgatum* L.), were grown to maturity in a growth chamber. The rhizosphere soils were collected followed by the application with C¹⁴-labeled RDX, and incubated for 8 weeks. The RDX mineralization rates and degradation metabolites were analyzed by HPLC-UV coupled with an inline flow through scintillation analyzer, during the incubation period. Results indicated that the rhizodegradation of RDX and its metabolites was significantly stimulated by both SW and EG. More than 13% of applied RDX was converted into CO₂ as compared with 5% in the control. Eastern gammagrass appeared more effective in enhancing RDX

rhizodegradation. This study demonstrates that the two native grasses show a high potential for rhizodegradation of RDX explosives in soil, which may be incorporated into remediation designs to mitigate the adverse environmental impacts of RDX explosives and safeguard humans and ecosystems from contamination.

INTRODUCTION

RDX has been one of the most widely used explosives in the world. During past decades, RDX has been massively produced, reaching a peak in the 1960s in the United States. From 1969 to 1971, RDX production was estimated to be 180 million pounds per year; however, production decreased to 16 million pounds per year in 1984 (Agency for Toxic Substances and Disease Registry, 1995). The contamination of RDX explosives and its degradation products in land or soil is of environmental concern. The loading, assembling and packaging of the explosives can cause environmental contamination near ammunition plant sites (Pennington and Brannon, 2002). In the US, there are 22 ammunition production and packaging sites that have been identified to be contaminated by explosives (Hooker and Skeen, 1999). Use of ordnance, bombs and projectiles during war time had also caused the widespread contamination of the explosives in environment. It is believed that the widespread contamination in soil and water in the US could be attributed to military activities occurring in World War II and the Korean War (Hawari et al., 2002). Soil contamination can also result from spills from wastewater lagoons or dusts containing explosive particles released to the atmosphere at incineration sites (Esteve-Nunez and Ramos, 1998; Rosenblatt et al., 1991). Leaching from contaminated sites or landfill sites can contaminate groundwater, threatening drinking water resources.

RDX and its derivative compounds are recalcitrant or hard to decompose naturally and have a long residence time in the environment or ecosystems.

RDX has been characterized as a potent convulsant and classified as a carcinogenic compound. The oral exposure to RDX has negative effects on central nervous systems (L.J.Burdette et al., 1988). People who breath in RDX dusts and C4 fumes (91% RDX) have been observed to suffer symptoms like convulsion, unconsciousness, headache, dizziness, vomiting, muscle twitching and confusion (Hollander and Colbach, 1969; Kaplan et al., 1965). A child who accidentally consumed an unknown quantity of RDX has been reported to suffer from seizures (Woody et al., 1986). However, human deaths resulting from RDX exposure have rarely been observed. Nevertheless, cases of animal deaths, like rats and rabbits, have been documented due to direct RDX feeding (Agency for Toxic Substances and Disease Registry, 1995).

Phytoremediation has been reported to show a promise to degrade organic pollutants, including: chlorinated hydrocarbons, pesticides, petroleum compounds, explosives, dyes and detergents (Anderson et al., 1993; Chaudhry et al., 2001; Haby and Crowley, 1996; Reilley et al., 1996; Schwitzguebel et al., 2001; Siciliano and Greer, 2000). Phytoremediation has been recognized as an *in situ*, environmentally-friendly, cost-effective and efficient method to clean up soils contaminated with organic contaminants such as RDX, which is non-destructive, *in situ*, and natural (Glick, 2003).

The objectives of this study are to: 1) evaluate the biodegradation of RDX in the rhizosphere soils of two selected native grasses; 2) investigate the rhizodegradative kinetics by the two grasses; and 3) determine the metabolite profile of the RDX degradation pathway.

MATERIALS AND METHODS

Experimental Design

Two native grass species, EG (eastern gammagrass, *Tripsacum dactyloides*) and SW (switchgrass, *Panicum virgatum* L) were selected for this experiment, based on previous screening tests. The seeds of EG and SW were sterilized in 30% ethanol for 30 seconds and washed with distilled water for 1 minute. Then the seeds were grown in plastic pots containing a soil mixture of 50% sand and 50% silt-loam soil obtained from the greenhouse facility at the University of Missouri-Columbia in a growth chamber under conditions of light intensity: 1400 Einsteins·m⁻²·sec⁻¹; light/dark period: 15/9 hours; humidity: 50%; temperature: 25°C (light)/20°C (dark).

Rhizosphere soils of EG and SW were collected weekly until maturity (> 3 months) and stored at 4°C prior to use. The rhizosphere soil, 20 g, was spiked with 0.1 µCi C¹⁴-RDX (PerkinElmer Life and Analytical Sciences) and 17.6 µg 1000ug/mL RDX (Spex Certiprep, Inc) to achieve total concentration of 1 mg RDX kg⁻¹. Soil moisture content was gravimetricly adjusted to 15%. The treated soils were placed in a 15 mL erlenmeyer flask. Both flask and a 20 mL scintillation vial containing 10mL 1M NaOH (Fisher Scientific) as ¹⁴CO₂ trap were sealed in a mason jar with parafilm and duck tape. The mason jars were incubated at room temperature in the dark for 8 weeks. NaOH traps were replaced weekly during the whole incubation period. The collected NaOH traps were stored until analysis. The spiked soils were sampled at an interval of 0, 7, 14, 21, 42 and 56 days, then the RDX and its metabolites were extracted with 250 mL of 100% acetonitrile (Fisher Scientific). The extraction procedures used in this study were adapted from EPA method 8330, through which RDX and its major metabolites can be fully

recovered. The extracts were concentrated to 200 μ l by N₂ evaporator and stored at -20⁰C until analyses. After extraction, the soils were oven-dried overnight, ground and stored in 20 mL vials. A blank soil without the presence of the grasses was used as the control. Each treatment was arranged with triplicates.

Analytical Procedures

The applied C¹⁴-RDX that was transformed into ¹⁴CO₂ and trapped by 10 mL of 1M NaOH and was analyzed by a Beckman LS 6000SC liquid scintillation counter. One milliliter of 1M NaOH solution in CO₂ traps was transferred into a 7 mL scintillation vial and mixed with 4 mL cocktail (Ultima GoldTM AB, PerkinElmer). The mixtures were measured for radioactivity of ¹⁴CO₂, and the RDX mineralization rate was calculated. The concentrations of non-extractable C¹⁴-RDX metabolites in the soils were also determined by the Beckman LS 6000SC liquid scintillation counter. Briefly, 1 g (dry weight equivalent) of soil was placed in a 7 mL scintillation vial and mixed with 4 mL cocktail (Ultima GoldTM AB, PerkinElmer). The mixtures were vortexed, and non-extractable RDX C¹⁴radioactivity was quantified by the Beckman LS 6000SC liquid scintillation counter. Concentrations of acetonitrile-extractable C¹⁴-RDX and its metabolites were analyzed by a Shimadzu High Performance Liquid Chromatogram equipped with a SupelcosilTM LC-18 column and a IN/US radioactivity detector (IN/US Systems Inc, Brandon FL). The mobile phase consisted of 100% acetonitrile and 1g/L ammonium acetate (7:3, v:v) at a flow rate of 1ml/min. Standards of RDX (Spex Certiprep, Inc) and MNX (www.accustandard.com) were purchased and injected into HPLC under same conditions to determine retention time.

Statistical Analysis

The experimental design was a completely randomized design with two factors (rhizosphere and time) and three replicates. Three rhizosphere treatments (EG, SW, and control) and seven times (0, 7, 14, 21, 28, 42 and 56 days after initiation) were evaluated. Significant differences in treatments were set at $p < 0.05$. Data were found to be non-normally distributed. Data were analyzed using a non-parametric two-way ANOVA with SAS software.

RESULTS AND DISCUSSION

Enhanced Degradation

The degradation of RDX in the rhizosphere soils was significantly enhanced by the two native grass species. Results presented in Figure 3.1 showed that the dissipation rates of RDX in both rhizosphere soils were higher than in the control. At the end of incubation period, 60.41% of initial C^{14} -RDX concentration in EG soil and 50.21% in SW soil were degraded, as compared to 16.25% in the control. RDX degradation in both rhizosphere soils was significantly enhanced ($p=0.0379$). Results showed RDX degradation in EG was significantly higher compared to the control. While RDX degradation by SW soil was only a little lower than in EG soil as seen in Figure 3.1, analysis of variance results showed RDX degradation in SW was not significantly different either compared to EG nor compared to the control. The enhanced RDX degradation by grasses in the rhizosphere might be due to diverse microbial species and greater microbial activities. Rhizosphere is a dynamic zone between roots and soil, which is characterized by low redox potentials and pH, abundant energy and nutrients, and high microbial activities.

Root exudates secreted by plants contain many sugars, organic acids and amino acids that can be utilized by soil microbes as nutrients. Thus, a high microbial activity in the rhizosphere could stimulate RDX biodegradation in soil.

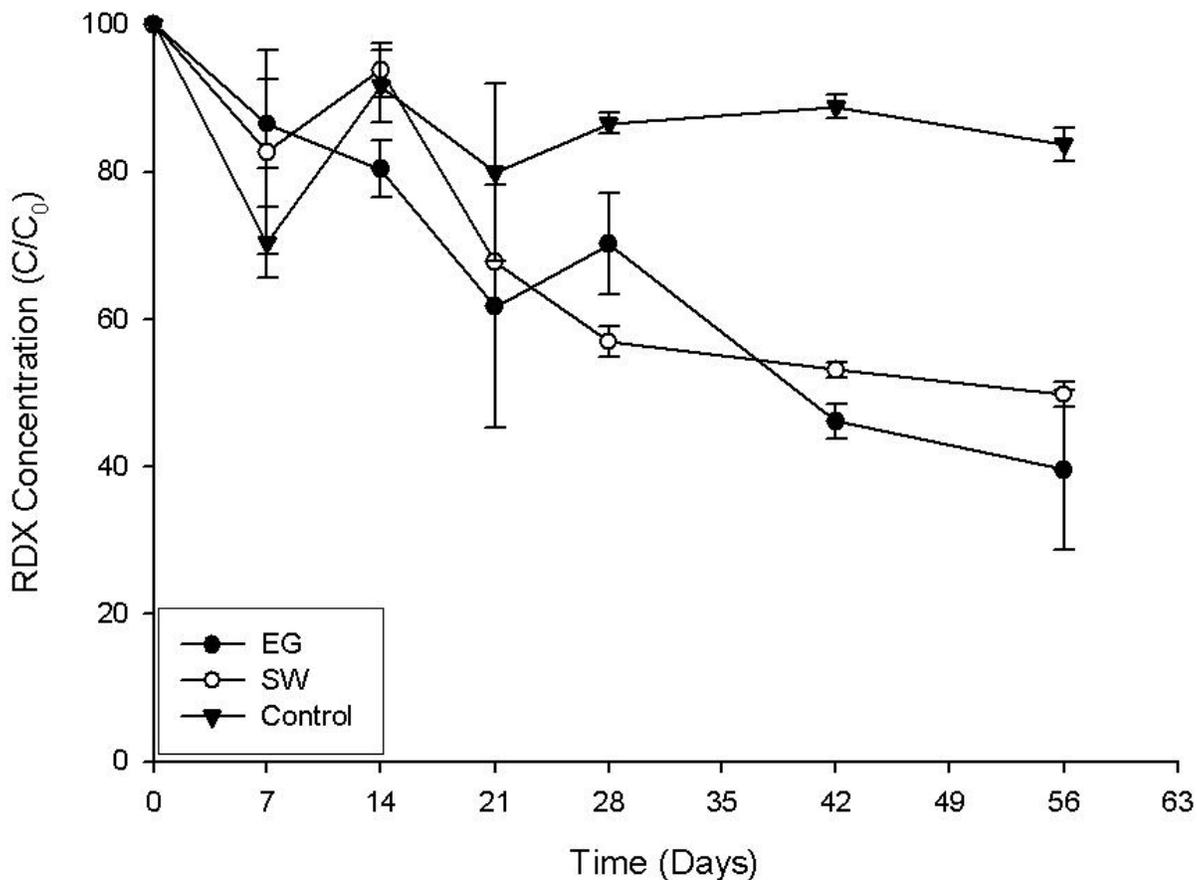


Figure 3.1 The percentage of applied C^{14} -RDX remained in the EG and SW rhizosphere soils and control during the 8-week incubation period.

Degradation Kinetics

Rhizodegradation of RDX in soil can be considered as an irreversible reaction. The rate law of RDX degradation can be expressed as:

RDX → Products

$$d[\text{RDX}]/d[t] = -k[\text{RDX}]^n$$

In this equation, [RDX] is the concentration of RDX, t is the time, k is the constant and n is the kinetic order. If $n=0$, the RDX degradation is zero order. The equation can be rewritten as:

$$d[\text{RDX}]/d[t] = -k[\text{RDX}]^0 = -k \quad [3.1]$$

Then, the equation can be integrated as:

$$[\text{RDX}] = [\text{RDX}]_0 - kt \quad [3.2]$$

If $n = 1$ or 2 , the RDX degradation is first or second order. Then, the equation can be integrated as:

$$\ln[\text{RDX}] = \ln[\text{RDX}]_0 - kt \quad [3.3]$$

$$1/[\text{RDX}] = 1/[\text{RDX}]_0 + kt \quad [3.4]$$

The half-life, $t_{1/2}$, is the time at which 50% of RDX can be reduced. We can obtain the equation of $t_{1/2}$ for all orders from the equations shown above.

$$n=0 \quad t_{1/2} = 0.5[A]_0/k \quad [3.5]$$

$$n=1 \quad t_{1/2} = 0.693/k \quad [3.6]$$

$$n=2 \quad t_{1/2} = 1/k[A]_0 \quad [3.7]$$

When [RDX], $\ln[\text{RDX}]$ and $1/[\text{RDX}]$ are plotted against time, a linear line indicates the kinetic order of RDX degradation.

RDX degradation took on different kinetic orders in different treatments. As presented in Figure 3.2, 3.3 and 3.4, the linear line indicated a first order of the RDX degradation in the EG rhizosphere soil (Figure 3.3), a second order in the SW rhizosphere soil (Figure

3.4), and a zero order in the control soil (Figure 3.2). All kinetic constants were calculated and shown in Table 3.1. The half-lives of RDX were significantly shorter in the EG and SW treatments than in the control. The shortest half life of RDX existed in the EG treatment, indicating RDX degraded fastest EG rhizospheres than in SW and control rhizospheres. This explains the different RDX degradation rates observed in Figure 3.1. The differences in root exudates and microbe consortium among the soils might account for the kinetic orders and half lives of RDX rhizodegradation in soil.

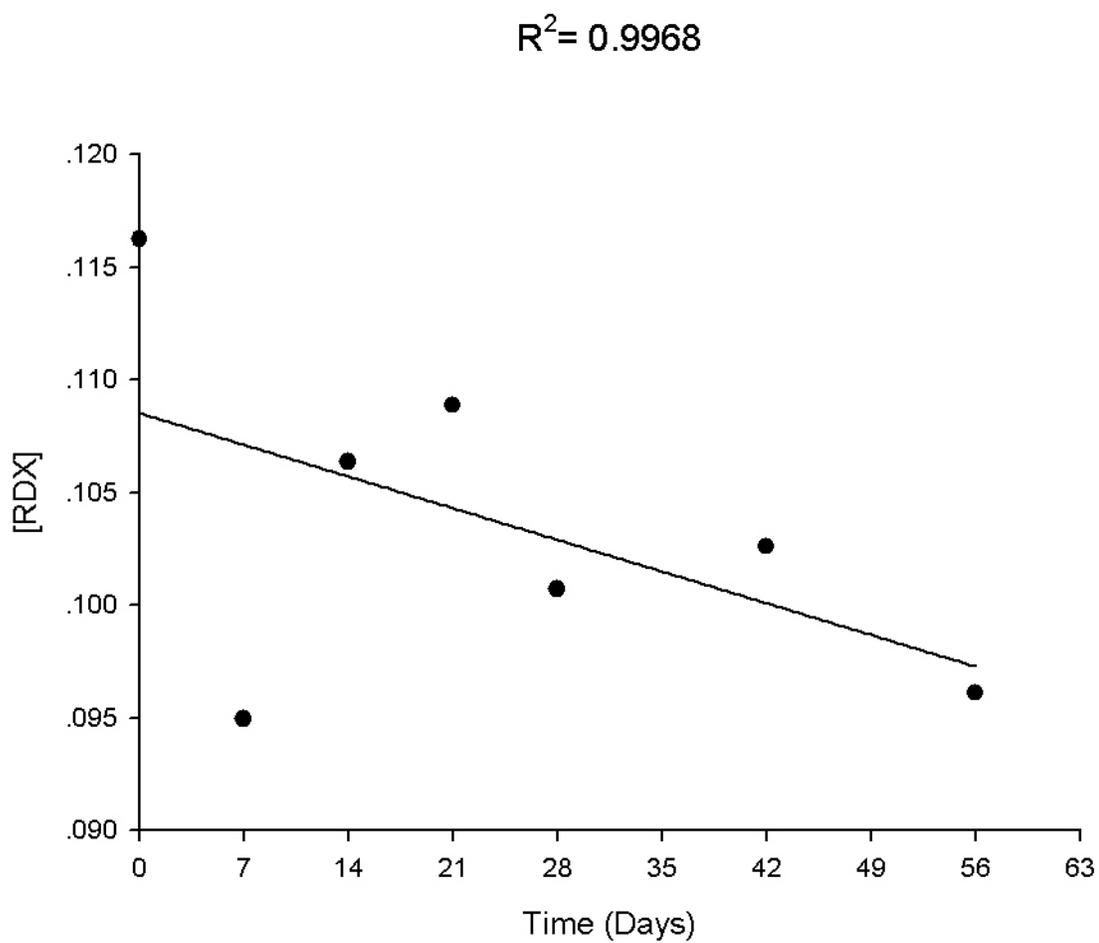


Figure 3. 2 The kinetics order of RDX rhizodegradation in the control treatment.

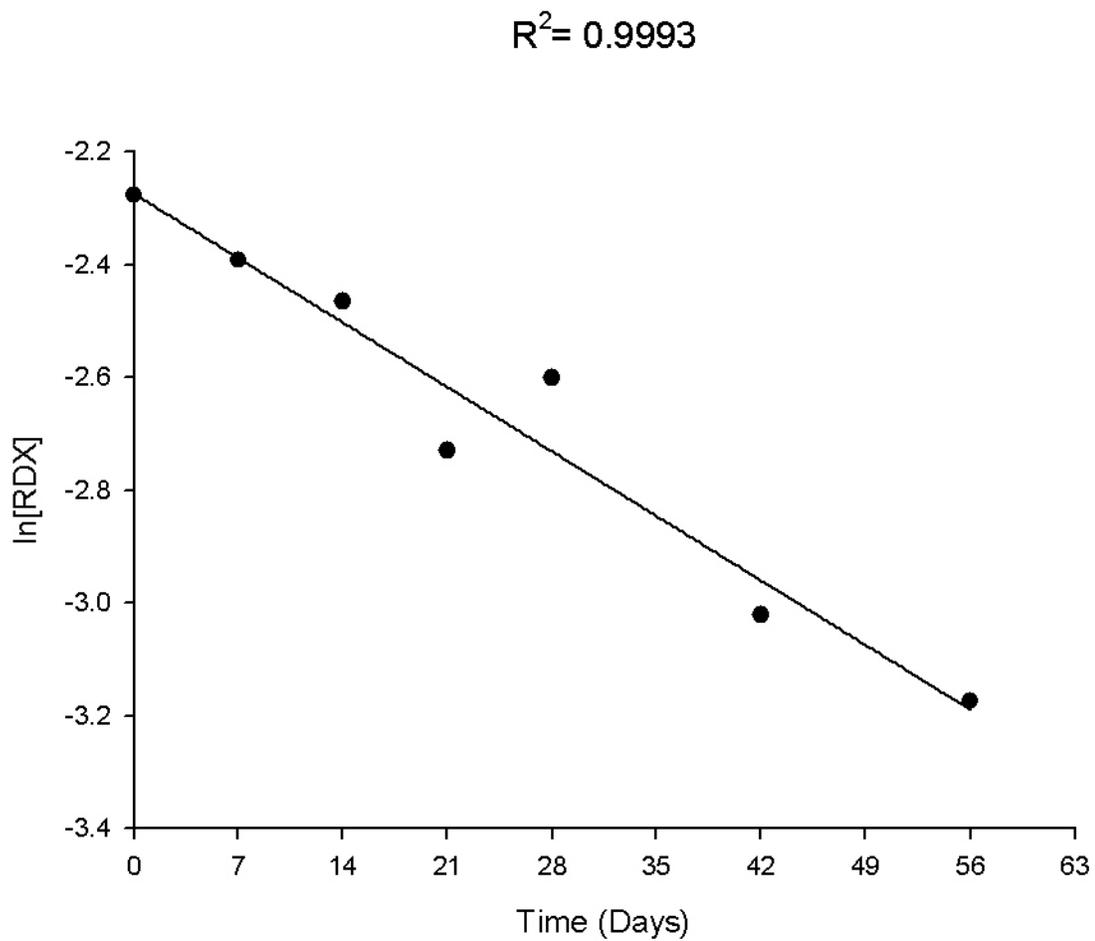


Figure 3. 3 The kinetics order of RDX rhizodegradation in the EG rhizosphere treatment.

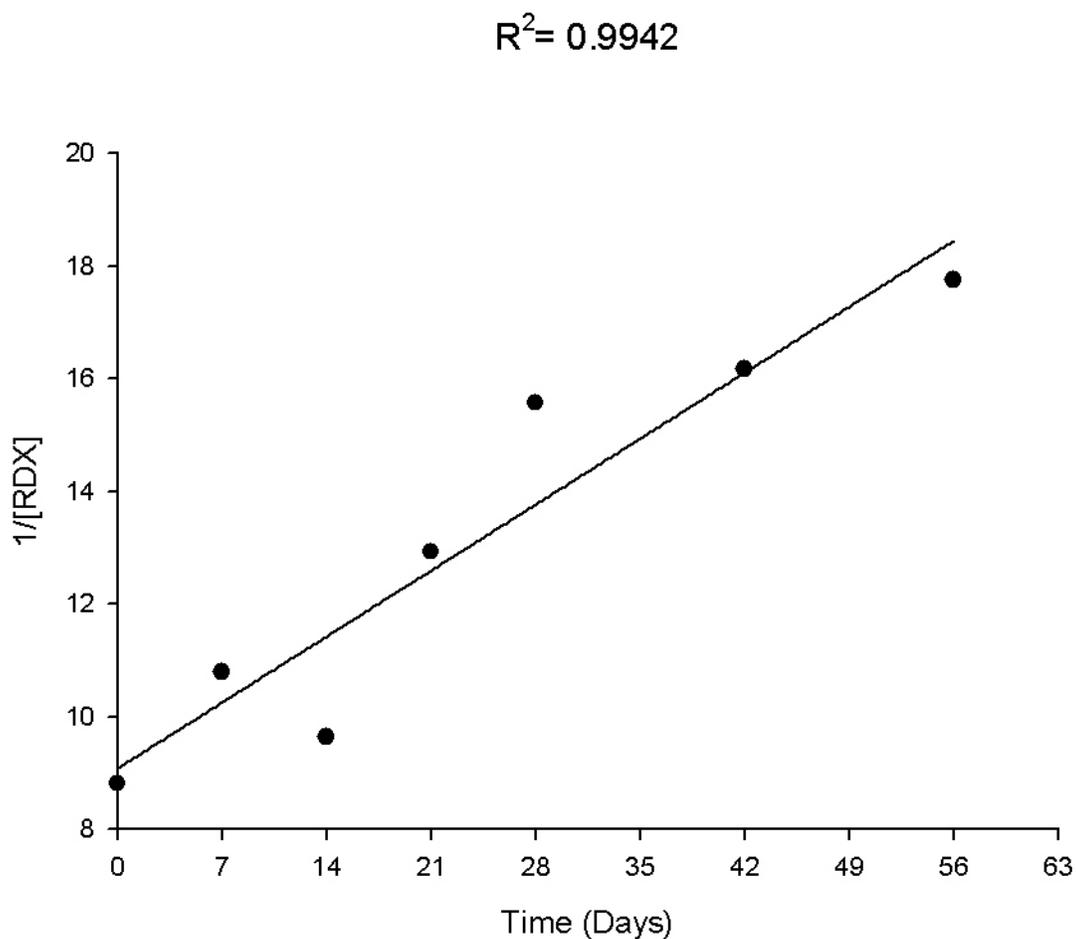


Figure 3. 4 The kinetics order of RDX rhizodegradation in SW the rhizosphere treatment.

Treatment	n	R^2	K	$T_{1/2}$
EG	1	0.9993	-0.0163	42.52
SW	2	0.9942	0.1671	52.79
Control	0	0.9968	-0.0002	290.63

Table 3. 1 Reaction constants during RDX degradation in different treatments.

Degradation Metabolites

There were four RDX degradation metabolites that were detected in the treatment soils, with a dominant MNX (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine) and small amounts of M1, M2, and M3, as illustrated in Figures 3.5, 3.6 and 3.7. MNX is a frequent RDX degradation product identified previously. The chemical structures of M1, M2 and M3 could not be determined. MNX appeared to be the major metabolite of RDX. Compared to MNX, concentrations of the other 3 metabolites were very low so that they can be regarded as negligible. The majority of the detected MNX in the control was accumulated in the first two weeks and started to attenuate until the end of the incubation period. In the EG treatment, the concentrations of MNX increased as function of time, while in the SW treatment, MNX became steady after the second week until the end.

The degradation of MNX was significantly enhanced by 57% and 80% in the rhizospheres of SW and EG, respectively, during the accumulation of MNX in 14 days. The amount of MNX was higher in SW than in EG during the first three weeks, whereas a higher concentration of MNX was observed in EG than in SW in the rest of the incubation period. Overall, much higher MNX concentrations were observed in the control than in EG and SW treatments in terms of the whole incubation period. This indicates metabolites of RDX as well as RDX could be degraded by rhizosphere soils. The RDX metabolites can be more toxic than the parent RDX (Agency for Toxic Substances and Disease Registry, 1995). Treatments of rhizosphere soils did enhance degradation of RDX metabolites significantly ($p=0.0030$). But between EG and SW, there was no significant difference.

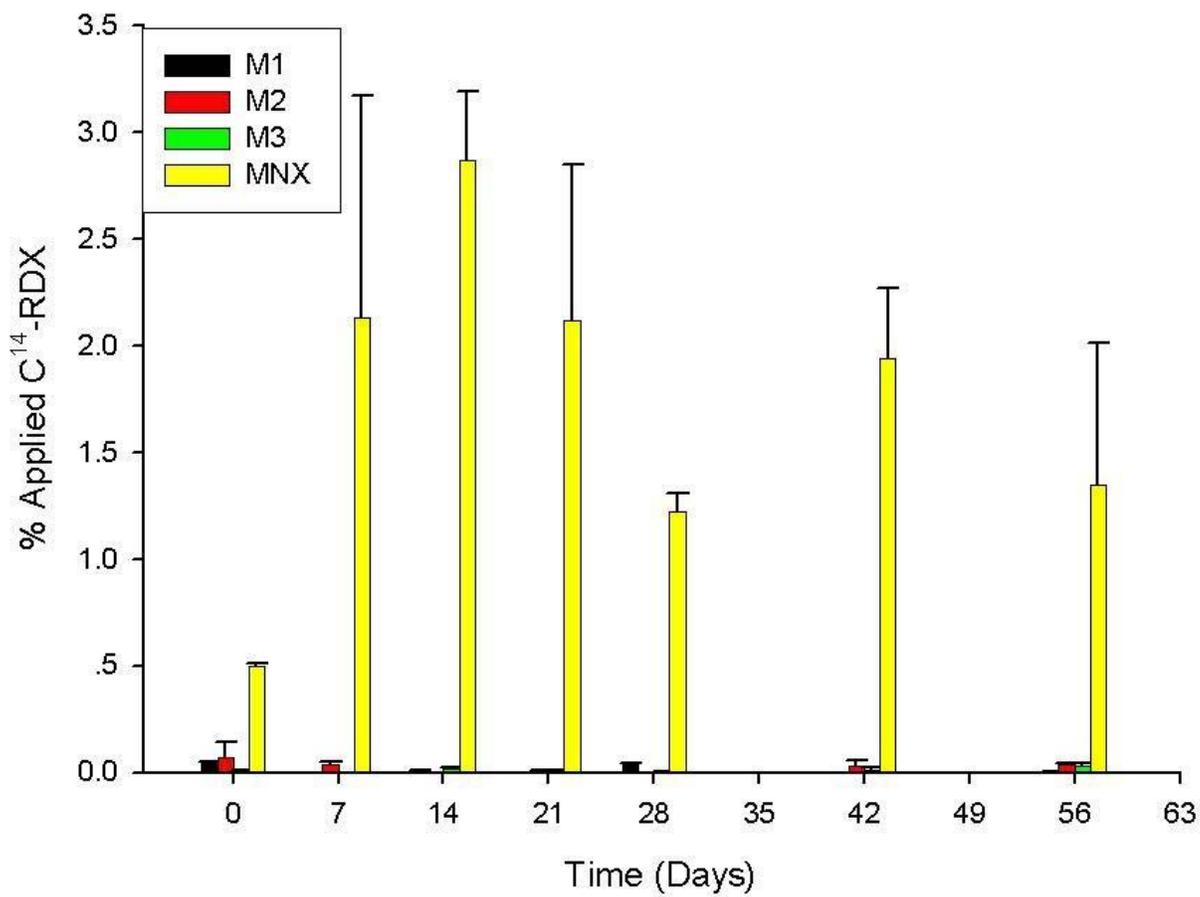


Figure 3. 5 Concentrations of RDX degradation metabolites in the control treatment.

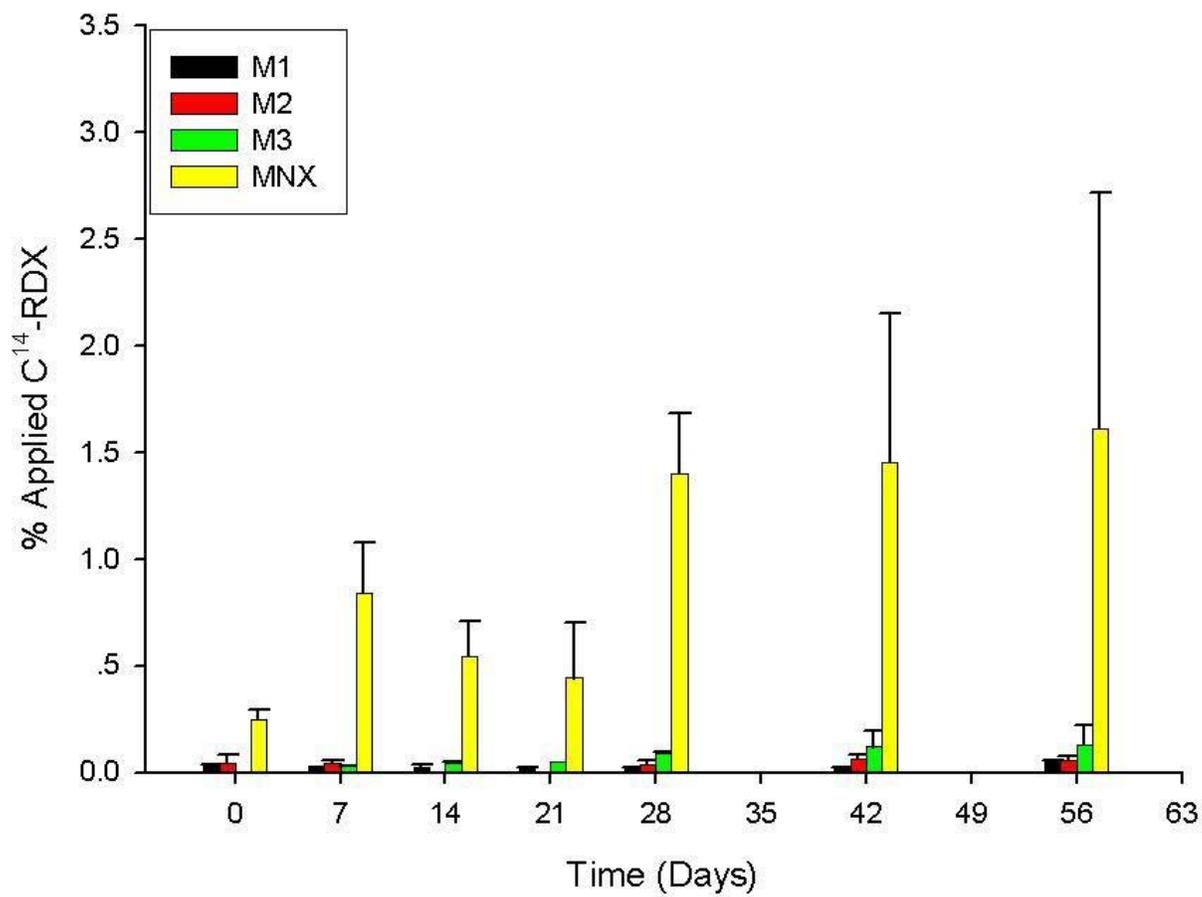


Figure 3. 6 Concentrations of RDX degradation metabolites in the EG rhizosphere soil treatment.

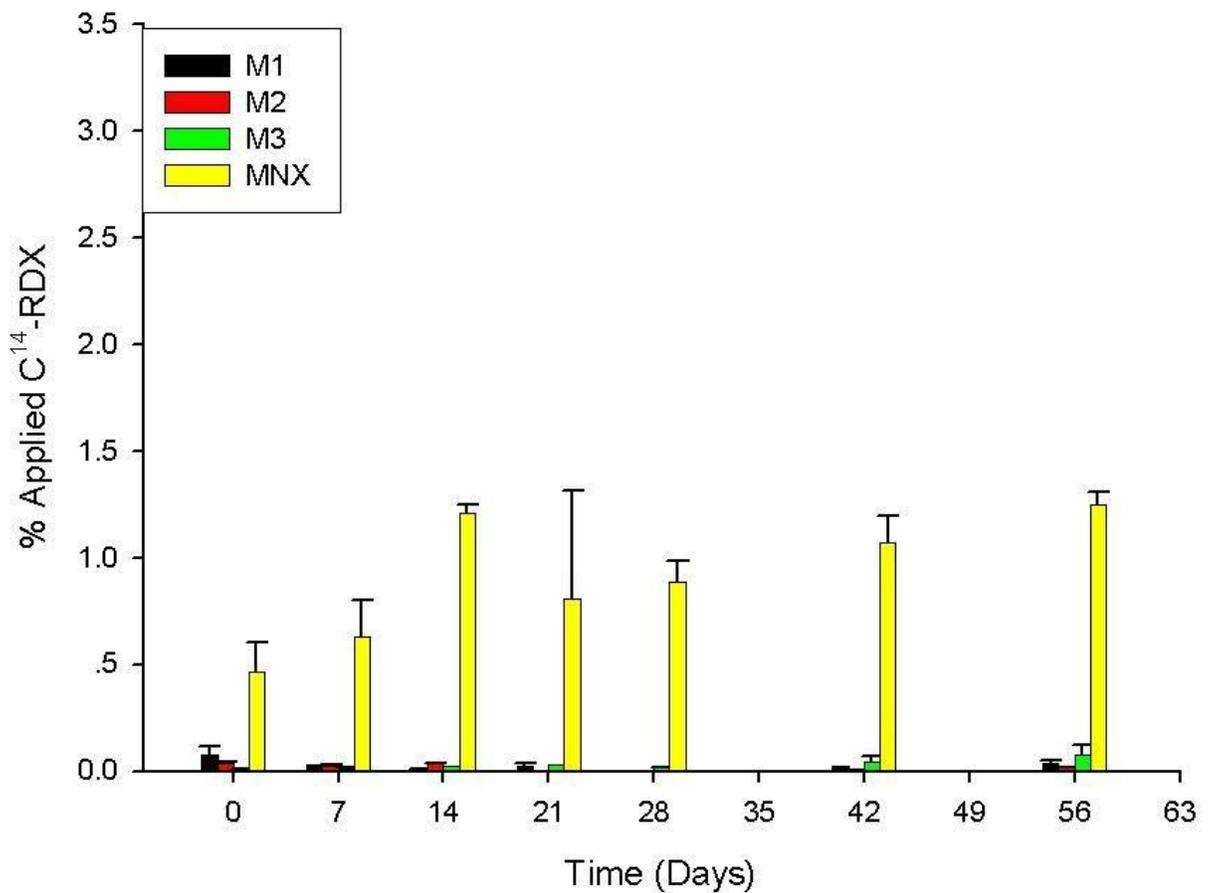


Figure 3.7 Concentrations of RDX degradation metabolites in the SW rhizosphere soil treatment.

Mineralization Rate

Figure 3.8 indicated that the mineralization rates of soil RDX were significantly higher in the rhizosphere soils than in the control. Compared to the control (5.44%), the RDX mineralization rates were 20.35% in EG rhizosphere soil and 13.37% in SW rhizosphere soil, increased by approximately 270% and 150%, respectively. The mineralization rates in all treatments were very similar in the first week of incubation. Differences started to emerge at the second week. Mineralization rates in treatments began to increase, while

mineralization rates in the control were fairly constant during the whole experimental period. Overall, the mineralization rates of RDX were significantly higher in both rhizosphere soils than in the control ($p < 0.0001$). Eastern gammagrass has shown a greater ability to sustain high mineralization rates of RDX compared to SW. The mineralization implied the complete ring cleavage during RDX degradation. The ring cleavage products were utilized by soil microbes to produce CO_2 .

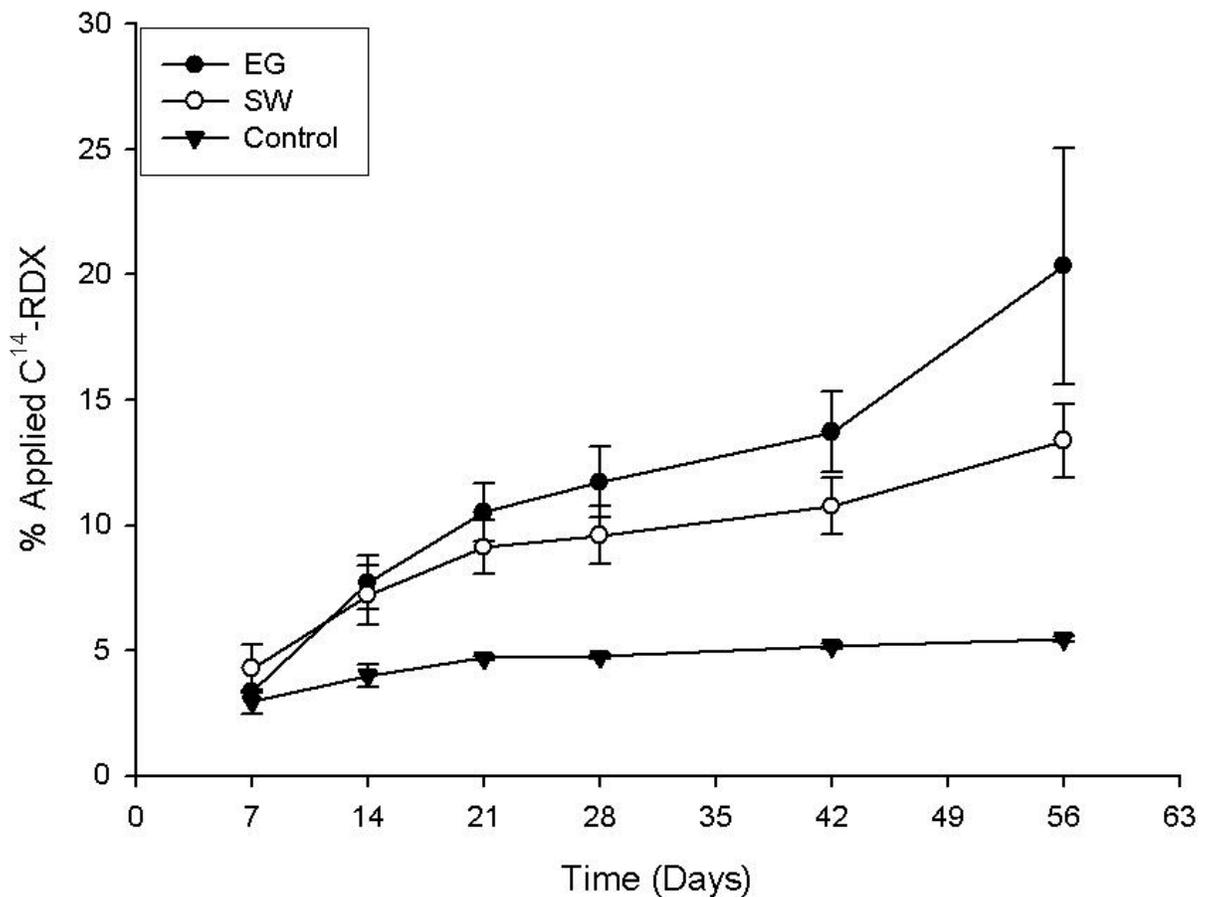


Figure 3. 8 The cumulative percentage of C^{14} -RDX mineralized in EG rhizosphere, SW rhizosphere, and control soils.

Non-extractable Residues

Several RDX degradation products are well known to irreversibly bind to clays and humic substances in soil through formation of covalent bonds (Pennington and Brannon, 2002), which make these degradation products resistant to solvent extraction. The non-extractable compounds in soil could be considered non-harmful to the environment and human health because of their very limited mobility, bioavailability and leachability. Figure 3.9 demonstrated that the EG rhizosphere soil contained a significantly higher non-extractable RDX fraction than the SW rhizosphere soil and the control ($p < 0.0001$). The relative concentrations of the non-extractable fraction in the SW rhizosphere were significantly higher than in the control during the whole incubation period.

Mass Balance

The mass balance of RDX degradation at the end of incubation period is listed in Table 3.2. It demonstrated the fate of RDX during the 8 week period. The poor mass balance was mainly attributed to the technique utilized for quantification of the non-extractable residues. Most of the applied RDX was degraded transforming into degradation metabolites, CO₂ and non-extractable residue in soil. In EG and SW rhizosphere soils, less RDX remained in soil and more RDX mineralized into harmless CO₂. Non-extractable residue in soil was supposed to account for a major portion of RDX dissipation. In our study, less than 4% of applied RDX was accounted for in soil as non-extractable residue. This value is much lower than has been reported in previous studies using oxidization approaches (Claus et al., 2007; Drzyzga et al., 1998). The results from this study have suggested that results derived from liquid scintillation counting should be

correlated and compared with results from conventional oxidization techniques before an estimate of non-extractable activity can be made. The use of the oxidization method or an improved liquid scintillation counting is recommended for future studies to achieve a better mass balance recovery.

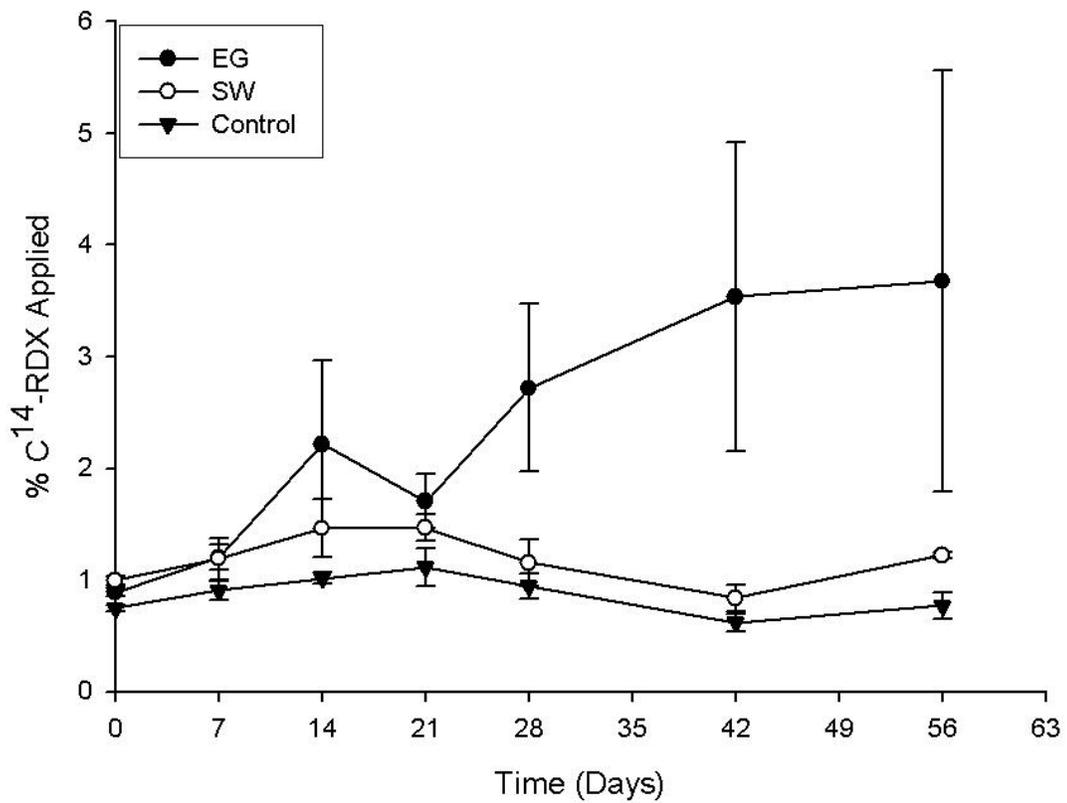


Figure 3. 9 The cumulative percentage of non-extractable RDX metabolites in EG rhizosphere, SW rhizosphere, and control soils.

	Metabolites(%)	RDX(%)	Mineralization(%)	Nonextractable Residue(%)	Sum(%)
EG	1.81	8.37	20.35	3.68	34.21
SW	1.37	11.27	13.37	1.22	27.22
Control	1.404	19.22	5.44	0.77	26.84

Table 3. 2 The mass balance of RDX in EG rhizosphere soil, SW rhizosphere soil, and control at the end of incubation period.

CONCLUSIONS

This study showed that both EG and SW rhizospheres appeared to be very effective for enhancing RDX degradation in soil. More than 13% of applied RDX was transformed into harmless CO₂. Enhanced degradation could be attributed to a grass species-specific stimulated microbial community and activity in the rhizospheres. Eastern gammagrass appeared to be more effective for RDX degradation in context of the mineralization and immobilization. The two selected native grass species showed a high rhizodegradation potential which could be incorporated into remediation designs to mitigate the adverse impacts of RDX in the environment.

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CHAPTER 4

DEGRADATION OF TNT IN TWO NATIVE GRASS SPECIES-SPECIFIC RHIZOSPHERE SOILS

ABSTRACT

Soil or water contamination by the munitions explosive residues of TNT (2,4,6-trinitrotoluene) and their metabolites has been identified a threat to human health and ecosystems. TNT and its metabolites are toxic compounds to humans. Compared to conventional remediation methods such as incineration, compost and bioslurry, phytoremediation, using plants to degrade, destroy or sequester hazardous compounds has been considered as one the most cost-effective and environmentally friendly methods for cleanup and restoration of contaminated sites. Objectives of this research were to investigate TNT degradation and kinetics in two native grass species-specific rhizosphere soils. The rhizosphere soils of eastern gammagrass (*Tripsacum dactyloides*) and switchgrass (*Panicum virgatum L.*) were spiked with C^{14} -TNT and incubated for eight weeks. The mineralization rates and degradation metabolite profiles of TNT were monitored over the experimental period. Results showed that both grass species rhizosphere soils had a greater capability to degrade TNT as compared to the control soil. More than 95% of applied TNT was degraded in the first 7 days, but less than 2% TNT was transformed into CO_2 . The degradation was found to be a second-order reaction. Six major degradation metabolites were identified. Overall, switchgrass appeared to be more

effective for degrading TNT than eastern gamma grass. This study suggests that native grass species especially switch grass, could have the potential to be used to mitigate the adverse human and environmental risks of TNT-contaminated sites.

INTRODUCTION

Contamination of munitions explosives such as TNT (2,4,6-trinitrotoluene) in soil and ground water is a threat to humans and ecosystems. TNT is one of two explosives which widely used in the world. TNT has been massively produced since 1902, reaching its peak during the World Wars (Nyanhongo et al., 2005). Since World War I, TNT had been used as a major explosive by the US Army (Lewis et al., 2004). Annual TNT production was reported to be approximately one million kg per year (Harter, 1985).

The use, manufacture and disposal of explosives would result in soil or water contamination, which is of concern for public health and the environment (Pennington and Brannon, 2002). Wastewater that is generated during TNT production, also called “pink water”, would result in land contamination adjacent to explosive production facilities (Gilbert, 1980; Lindner, 1980). It was reported that up to 2 million liters of “pink water” could be produced yearly by a single factory (Jenkins et al., 1986). At the Louisiana Army Ammunition Plant (LA) and the Cornhusker Army Ammunition Plant (NE), TNT concentrations in soils were determined at up to 87,000 mg/kg, in sediment up to 711,000 mg/kg, and in surface water up to 3,375 mg/L (Steevens et al., 2002). In the US, there are over 12 million tons of soils that have been contaminated by explosives (Lewis et al., 2004). On the EPA National Priorities List, at least 20 out of 1,397

hazardous waste sites have been identified to be contaminated by TNT (Agency for Toxic Substances and Disease Registry, 1995).

Human exposure to TNT can cause anemia, abnormal liver function, cataract development, and skin irritation (Honeycutt et al., 1996; Yinon, 1990). The death of 475 people who were exposed to TNT was reported in the US (McConnell and Flinn, 1946).

Biodegradation of TNT by bacteria has been extensively studied over several decades. However, outcomes were less than satisfactory. Results showed that TNT preferred to be biotransformed, rather than mineralized. Little TNT mineralization was observed (Hawari et al., 2000). This is probably due to the symmetric location of nitro groups on the toluene ring of TNT that limits enzymatic attack by classic dioxygenase during the microbial metabolism of aromatic compounds. The high electronegativity and partially positive charge of the N atom of the nitro group make TNT easily reducible (Esteve-Nunez et al., 2001).

Compared to conventional remediation methods such as incineration, compost and bioslurry, remedial technology using green plants to remove, destroy and sequester explosives could be the most cost-effective method for the cleanup of soil contaminated by explosives. It has been reported that phytoremediation has the promising ability to degrade organic pollutants, including chlorinated hydrocarbons, pesticides, petroleum compounds, explosives, dyes and detergents (Anderson et al., 1993; Caballero et al., 2005; Chaudhry et al., 2001; Haby and Crowley, 1996; Reilley et al., 1996; Schwitzguebel et al., 2001; Siciliano and Greer, 2000). Phytoremediation has several advantages over other remedial methods: 1) non-destructive, preserving the natural structure of soil; 2) using sunlight as an energy source; 3) elevating levels of biomass in

soil; 4) low maintenance cost; and 5) possessing the potential to clean up contaminants rapidly (Glick, 2003). Objectives of this study were to 1) investigate TNT degradation and mineralization in two selected native grass rhizosphere soils, 2) determine the kinetics of grass-specific TNT degradation; and 3) identify the profile of TNT degradation metabolites in the degradation pathways.

MATERIALS AND METHODS

Experimental Procedures

Two native grass species, EG (eastern gammagrass, *Tripsacum dactyloides*) and SW (switchgrass, *Panicum virgatum* L), were selected based upon previous screening results. The seeds of EG and SW were sterilized in 30% ethanol for 30 seconds and distilled water for 1 minute and then grown in soil pots in a walk-in growth chamber under the conditions of light intensity, 1400 Einsteins·m⁻²·sec⁻¹; light/dark period, 15/9 hours; humidity, 50%; temperature, 25°C (light)/20°C (dark). The soils were the mixture of 50% sand and 50% silt-loam obtained from the greenhouse facility at the University of Missouri-Columbia.

The rhizosphere soils of EG and SW were collected weekly until maturity (> 3 months) and stored at 4°C. Twenty g of the rhizosphere soil was applied with 0.5 µCi C¹⁴-TNT (PerkinElmer Life and Analytical Sciences) and 17.86 µg of 1000µg/mL TNT (Spex Certiprep, Inc) to achieve total TNT concentration of 1000 µg/kg in soil. Spiked soil was stored in a 15mL Erlenmeyer flask. The flask and a 20mL scintillation vial containing 10mL 1M NaOH (Fisher Scientific) as ¹⁴CO₂ trap were sealed in a mason jar with parafilm and duck tape. The jars were incubated at room temperature in the dark for

8 weeks. During the incubation period, moisture content in the soil was maintained gravimetricly at 15%, and NaOH traps were replaced every week with collected traps stored at room temperature in the dark. Spiked soils were sampled from the jars at day 0, 7, 14, 21, 42 and 56, and then extracted using 250mL 100% acetonitrile (Fisher Scientific). The extraction procedures used in our study were adapted from EPA method 8330, through which TNT and its major metabolites can be fully recovered. Extracts were concentrated to 200 μ l by N₂ evaporator and stored at -20⁰C until analyzed. After extraction, the soils were oven-dried overnight, ground and stored in 20mL scintillation vials at room temperature in the dark. In the experiment, blank soils were included as a control, and each treatment was replicated in triplicates.

Analysis Procedures

One milliliter of 1M NaOH solution in CO₂ traps was transferred into a 7mL scintillation vial and mixed with 4mL cocktail (Ultima GoldTM AB, PerkinElmer). Then, the ¹⁴CO₂ radioactivity was quantified by a Beckman LS 6000SC liquid scintillation counter, and the TNT mineralization rates were calculated. To determine the radioactivity of non-extractable ¹⁴C-TNT degradation products, 1g of soil (dry weight equivalent) was weighed in a 7mL scintillation vial and mixed with 4mL cocktail (Ultima GoldTM AB, PerkinElmer). The mixtures were vortexed, allowed to precipitate for 3 days, and the ¹⁴CO₂ radioactivity was determined by Beckman LS 6000SC liquid scintillation counter. Concentrations of acetonitrile extractable C¹⁴-TNT and its metabolites were analyzed by a Shimadzu High Performance Liquid Chromatogram equipped with a Phenomex Columbus C8 (5 μ x 110A) column and an in-line IN/US radioactivity detector (IN/US

Systems Inc, Brandon FL). The mobile phase consisted of 100% acetonitrile and 0.1% phosphoric acid (1:9, v:v) at a flow rate of 1ml/min. Standards of TNT (Spex Certiprep, Inc) and its metabolites (www.accustandard.com), including: 24DA6NT, 26DNT, 26DA4NT, 4HA26DNT, 4A26DNT, 2A46DNT, 24DNT, 2A4N4, and 2HA46DNT, were purchased and injected into HPLC under the same conditions to determine retention time.

Statistical Analysis

The experimental design was a completely randomized design with two factors (rhizosphere and time) and three replicates. Three rhizosphere treatments (EG, SW, and control) and seven times (0, 7, 14, 21, 28, 42 and 56 days after initiation) were evaluated. Significant differences in treatments were set at $p < 0.05$. Data were found to be non-normally distributed. Data were analyzed using a non-parametric two-way ANOVA with SAS software.

RESULTS AND DISCUSSION

TNT Degradation in the Soil

As data in Figure 4.1 show, the TNT was rapidly degraded within the first 7 days and gradually leveled off during the rest of the incubation period. More than 95% of applied TNT was degraded in the first 7 days. TNT degradation in EG and SW rhizosphere soils was significantly enhanced compared to the control soil ($p < 0.0001$). SW showed better ability to degrade TNT than EG. The rapid degradation of TNT has been observed in many other studies (Hawari et al., 2000). In addition, the low TNT mineralization rates across all the treatments suggest that the majority of the applied TNT was biotransformed

to nitroso-, hydroxylamino- and amino-TNT derivatives because of the easily reducible nitro groups, and the complete cleavage of the aromatic ring of these metabolites was limited.

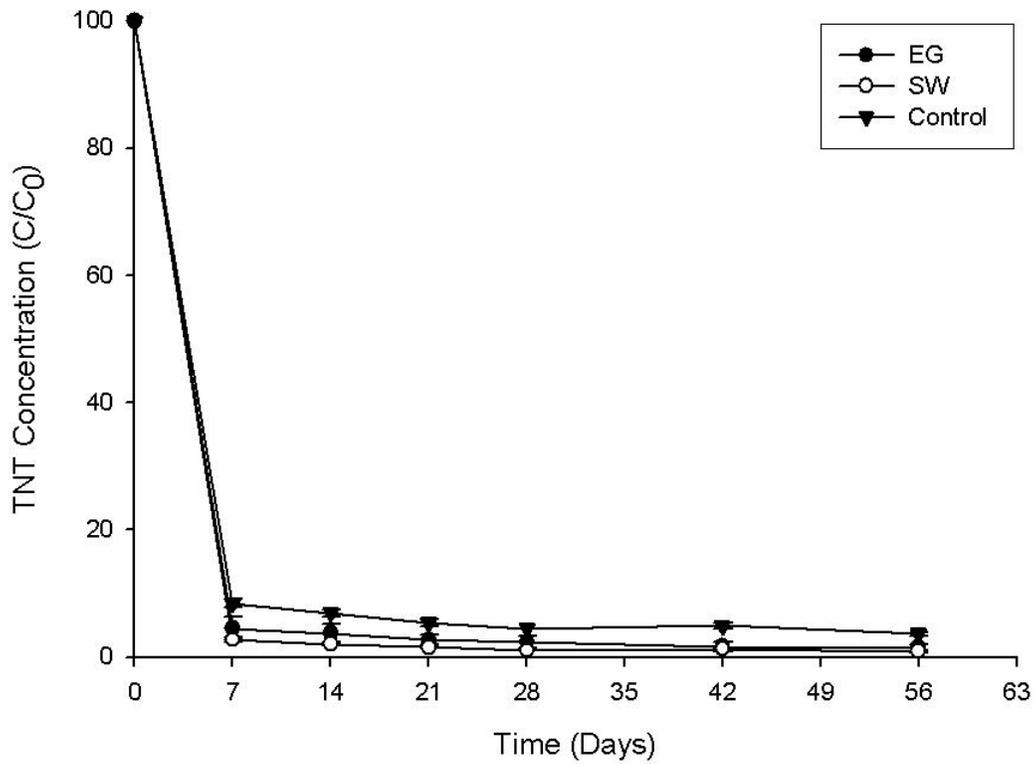
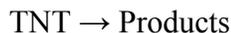


Figure 4.1. The percentage of applied C¹⁴-TNT remained in the EG rhizosphere soil, SW rhizosphere soil, and control soil during the 8-week incubation period.

Degradation Kinetic Order

TNT degradation in soil can be considered as an irreversible reaction. The rate law of TNT degradation can be expressed as:



$$d[\text{TNT}]/d[t] = -k[\text{TNT}]^n$$

In this equation, [TNT] is the concentration, t is the time, k is constant, and n is the kinetic order. If n=0, the TNT degradation is zero order, and the equation can be rewritten as:

$$d[\text{TNT}]/d[t] = -k[\text{TNT}]^0 = -k \quad [4.1]$$

Then, the equation can be integrated as:

$$[\text{TNT}] = [\text{TNT}]_0 - kt \quad [4.2]$$

If n=1 or 2, the TNT degradation is first or second order, then, the equation can be integrated as:

$$\ln[\text{TNT}] = \ln[\text{TNT}]_0 - kt \quad [4.3]$$

$$1/[\text{TNT}] = 1/[\text{TNT}]_0 + kt \quad [4.4]$$

The half-life, $t_{1/2}$, is the time in which 50% of TNT can be reduced. We can obtain the equation of $t_{1/2}$ for all orders from equations shown above.

$$n=0 \quad t_{1/2} = 0.5[A]_0/k \quad [4.5]$$

$$n=1 \quad t_{1/2} = 0.693/k \quad [4.6]$$

$$n=2 \quad t_{1/2} = 1/k[A]_0 \quad [4.7]$$

When [TNT], $\ln[\text{TNT}]$ or $1/[\text{TNT}]$ is plotted against time, a linear line would indicate the kinetic order for the TNT degradation.

Based on our results shown in Figures 4.2, 4.3, and 4.4, in all cases, the TNT degradation followed second order kinetics in the treatments. All kinetic constants were calculated and illustrated in Table 4.1. The half life of TNT was very short ranging from 0.54 to 2.6 days. This explains the rapid dissipation of TNT (> 95%) happened within the first 7 days. The shortest half life of TNT was found in SW, indicating RDX was degraded faster in SW than in the control.

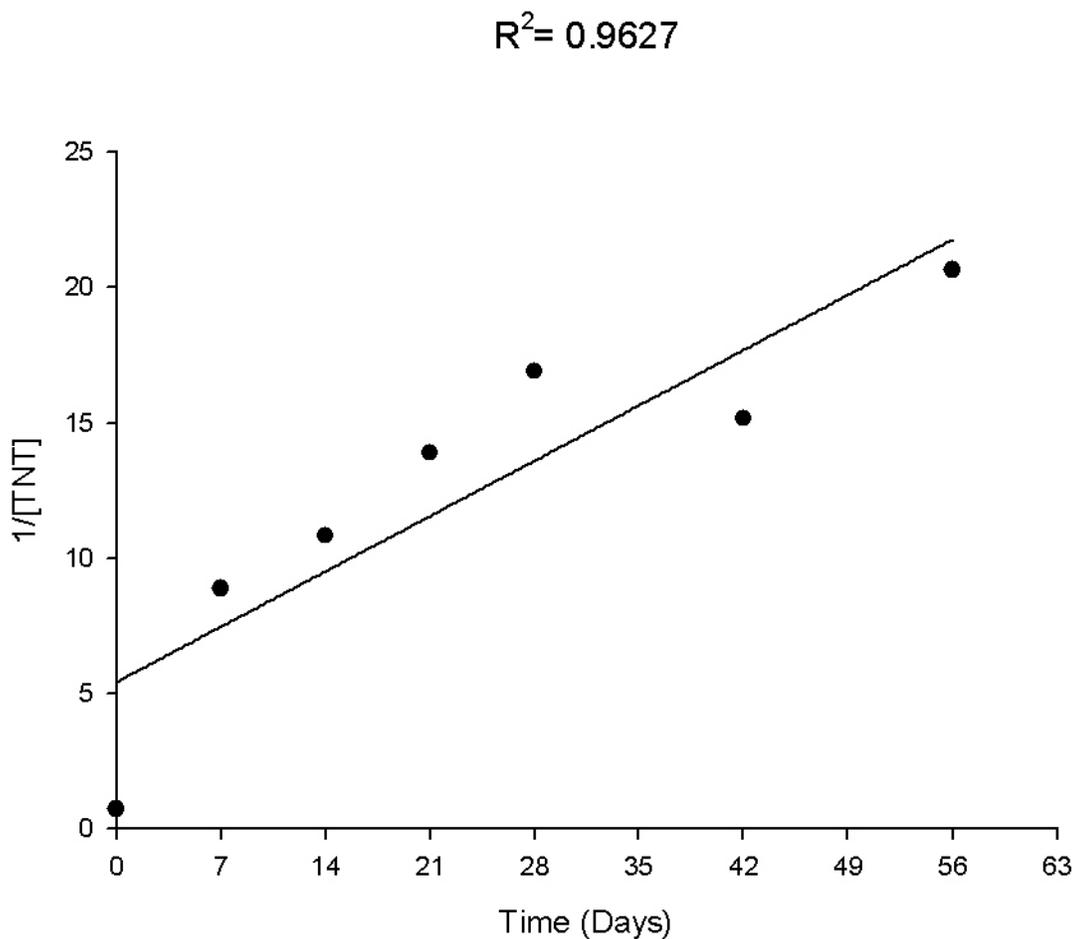


Figure 4.2. Kinetic order of TNT degradation in the control treatment.

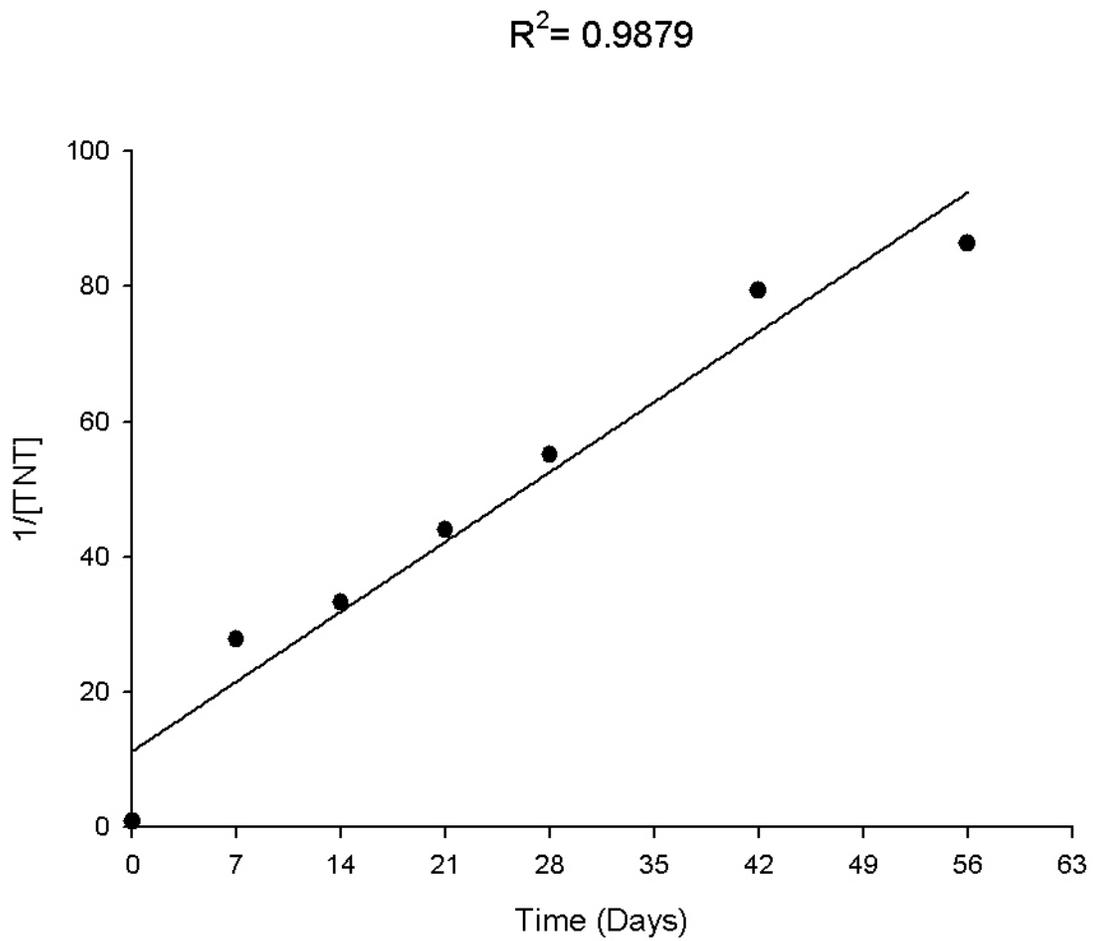


Figure 4.3. Kinetic order of TNT degradation in the EG rhizosphere soil treatment.

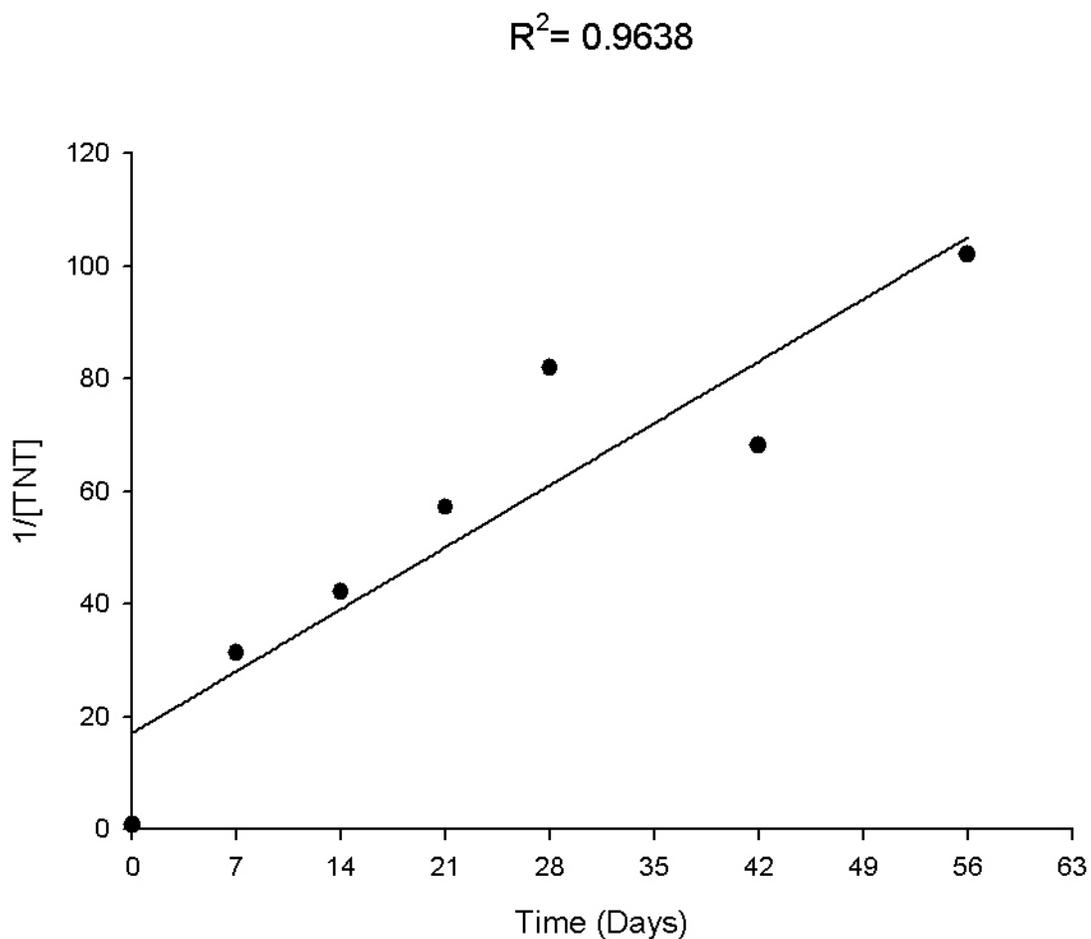


Figure 4.4. Kinetic order of TNT degradation in the SW rhizosphere soil treatment.

Treatment	n	R^2	K	$T_{1/2}$
EG	2	0.9879	1.4759	0.669
SW	2	0.9638	1.5704	0.5426
Control	2	0.9627	0.2918	2.559

Table 4. 1 Reaction constants during TNT degradation in different treatments.

Degradation Metabolites

There were six major degradation TNT metabolites identified (Figures 4.5, 4.6, and 4.7), including 4-nitrotoluene (4NT), 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT), and two unknowns, M1 and M2. Based on the degradation profile, TNT degradation pathways in soil seemed to undergo the sequential two-electron reduction mechanism. Hydroxylamino- and amino-TNT derivatives have been observed in this study, but no nitroso-TNT derivative were detected. This observation is in good agreement with many other studies in which hydroxylamino- and amino-TNT derivatives were frequently observed as TNT metabolites and no nitroso-TNT derivatives were observed (Esteve-Nunez et al., 2001; Koder and Miller, 1998; Nyanhongo et al., 2005; Yin et al., 2004; Yin et al., 2005).

Relatively higher concentrations of identified metabolites were observed across all the treatments during the first week, which corresponds to the rapid disappearance of TNT in early incubation. Overall, 2A46DNT and 4A26DNT appeared to be the major metabolites of TNT degradation. 4HA26DNT concentrations were very low in both soils. In comparison with the control, the concentrations of two major TNT metabolites were significantly lower in the rhizosphere soils during the incubation period ($p < 0.0001$), in which SW proved to degrade more than EG. It indicates that both native grasses not only enhanced TNT degradation but also the degradation of TNT metabolites. Several TNT metabolites have been reported to be more toxic than their TNT parent (Agency for Toxic Substances and Disease Registry, 1995; Esteve-Nunez et al., 2001). The nitroso and hydroxylamino groups were reported responsible for mutagenicity and carcinogenicity of

nitroaromatic compounds. Hydroxylaminodinitrotoluene can cause hemotoxic symptoms for people exposed to TNT (Esteve-Nunez et al., 2001). The reduction of nitro group to amino group may decrease the mutagenicity of the compound.

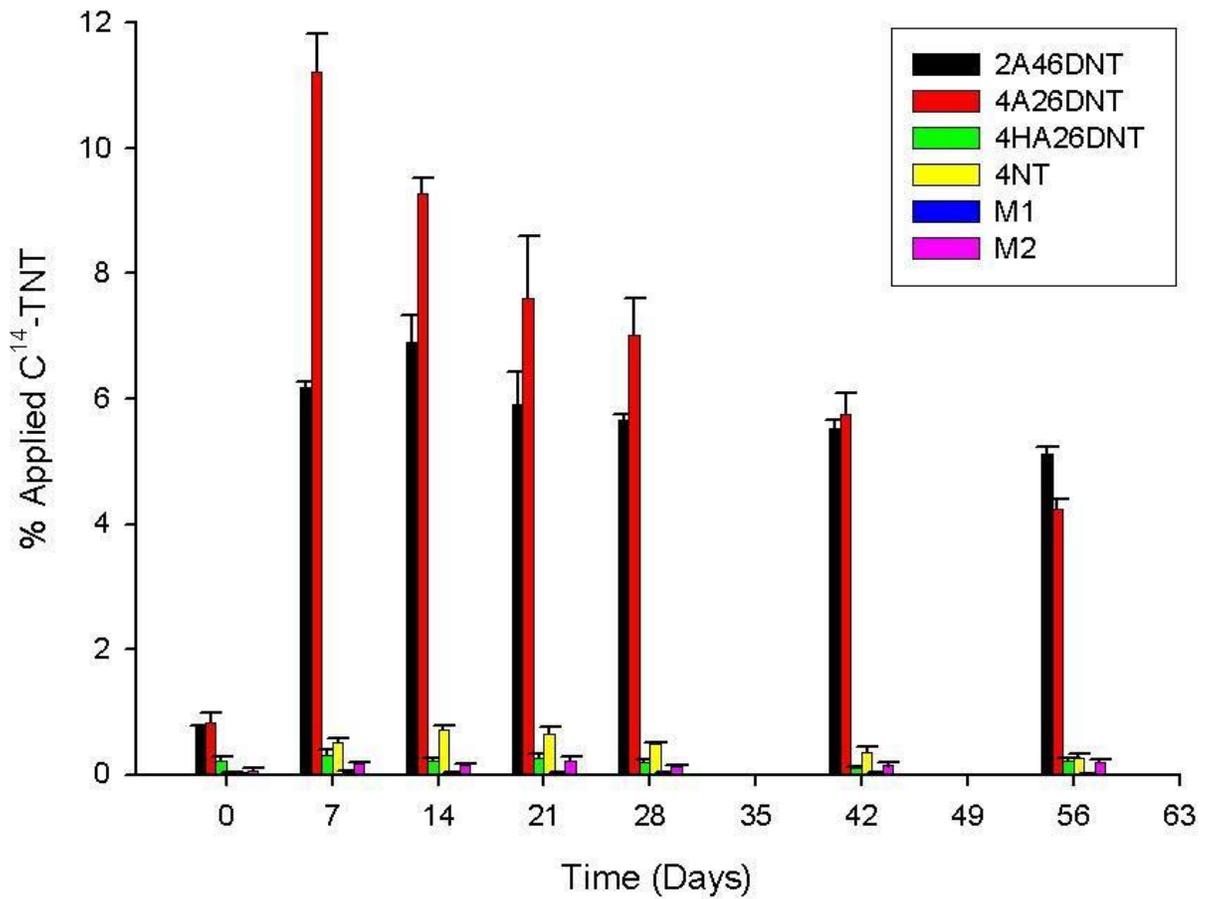


Figure 4.5. Concentrations of TNT degradative metabolites detected in the control treatment.

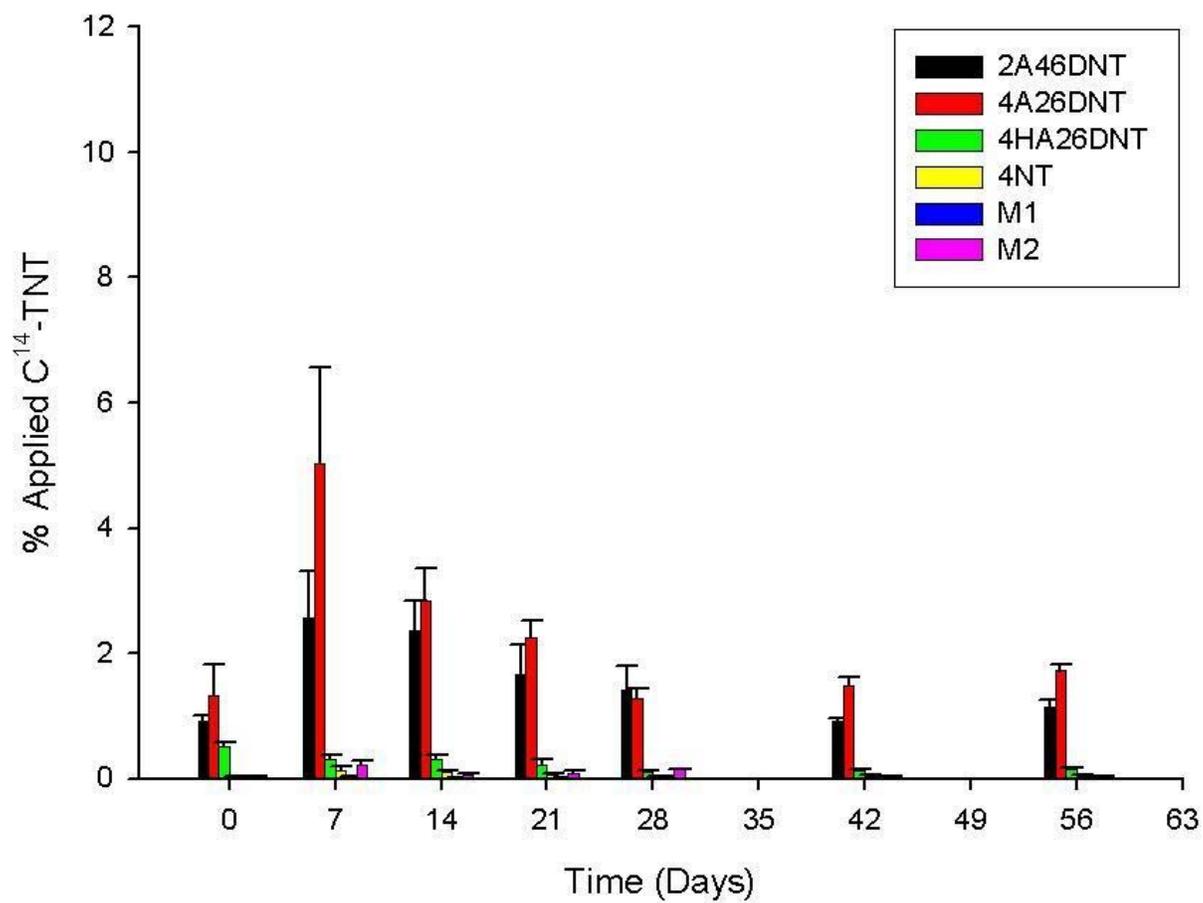


Figure 4.6. Concentrations of TNT degradative metabolites detected in the EG rhizosphere soil treatment.

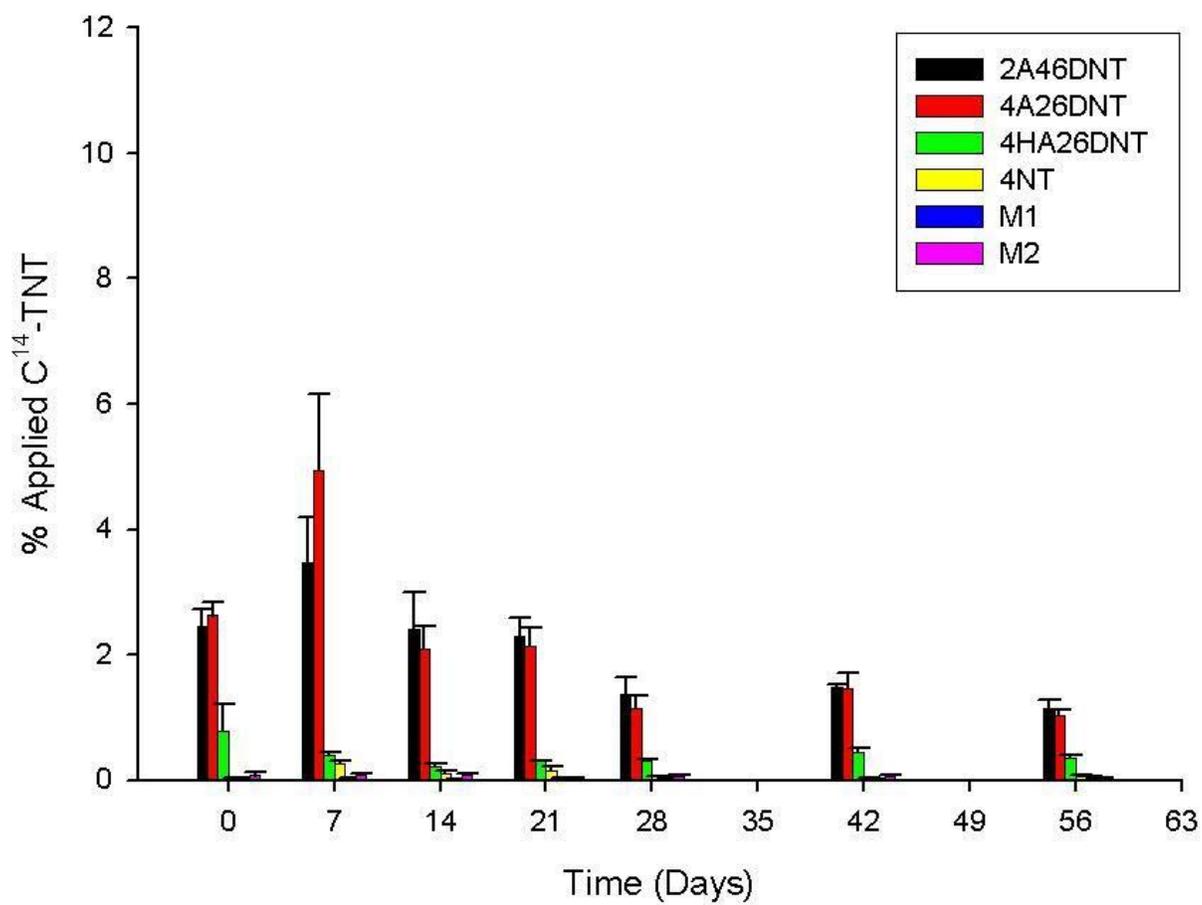


Figure 4.7. Concentrations of TNT degradative metabolites detected in the SW rhizosphere soil treatment.

Mineralization Rate

Both native grasses demonstrated better capacity to mineralize TNT degradation products into harmless CO₂ than the control, as illustrated in Figure 4.8. Approximately 2% of applied TNT in SW and 1.5% in EG were transformed to CO₂ as compared to 1% in the control. When the TNT mineralization rates were compared between the two rhizospheres treatments, SW has shown relatively better capacity to sustain the mineralization rates of TNT than EG ($p < 0.0001$). This result was still less than satisfactory, indicating that only a small fraction of TNT aromatic ring was completely cleaved off and the majority of the applied TNT was transformed to hydroxylamino- and amino-TNT. The properties of TNT make the aromatic ring intact which limits the mineralization of TNT. Low mineralization rates of TNT were also observed in many other studies (Hawari et al., 2000; Nyanhongo et al., 2005). The mineralization of TNT observed might also result from the TNT metabolites attacked by some specific species of soil microbes via unknown mechanisms (Hawari et al., 2000), resulting in ring cleavage and the formation of CO₂. The native grass species EG or SW may enhance the activity of species-specific soil microbes in the rhizosphere that stimulate more the degradation of the TNT metabolites into CO₂.

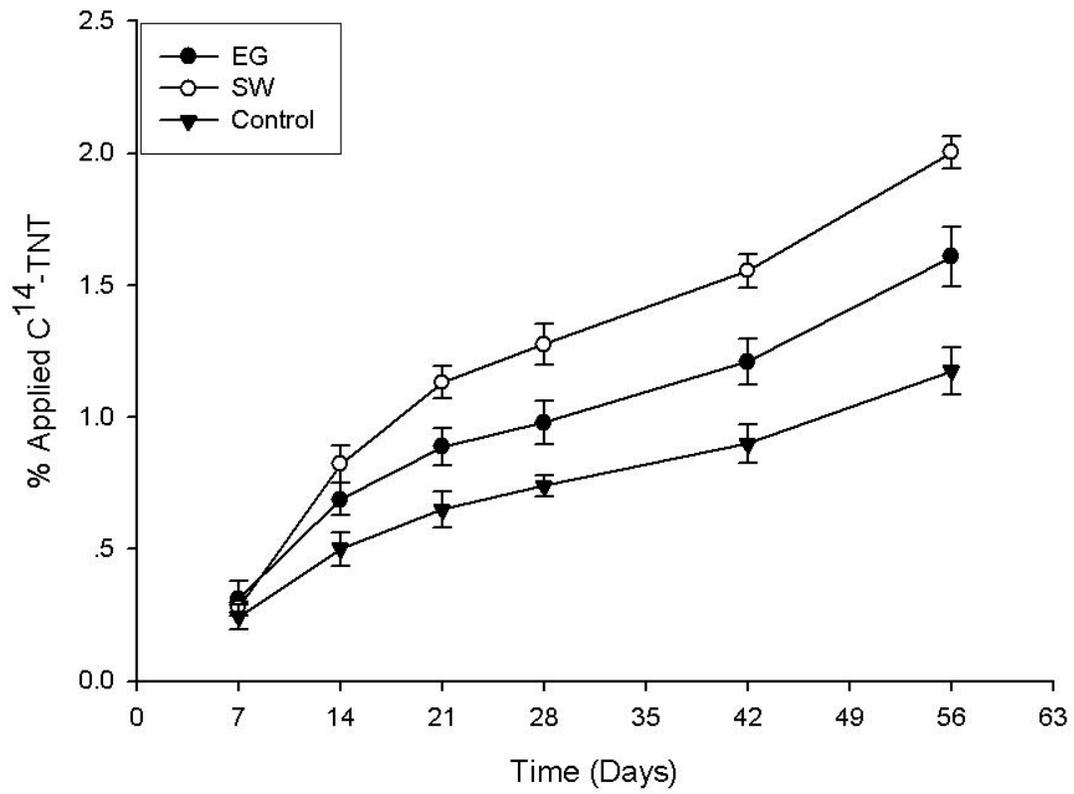


Figure 4.8. The cumulative percentage of C¹⁴-TNT mineralization in EG rhizosphere, SW rhizosphere, and control soils.

Non-extractable TNT Metabolites in Soil

TNT degradation products may be able to interact with clays and humic substances in soil, which form relatively stable compounds resistant to acetonitrile extraction. Thus, those non-extractable TNT compounds in soil could be considered as non-harmful fractions to the environment and human health, because of their very limited bioavailability and leachability. Therefore, the more non-extractable compounds in the soil, the less toxic they are to ecosystems. Data in Figure 4.9 indicated that there was no significant effect among the treatments in context of the amount of non-extractable TNT in the soils ($p=0.0666$). In all case, the non-extractable contents increased rapidly in the first 7 days and remained relatively stable during the rest of incubation period. This indicates that the rapid formation of relative stable TNT complexes may prevent TNT from further degradation in the late incubation period, as indicated in Figure 4.1.

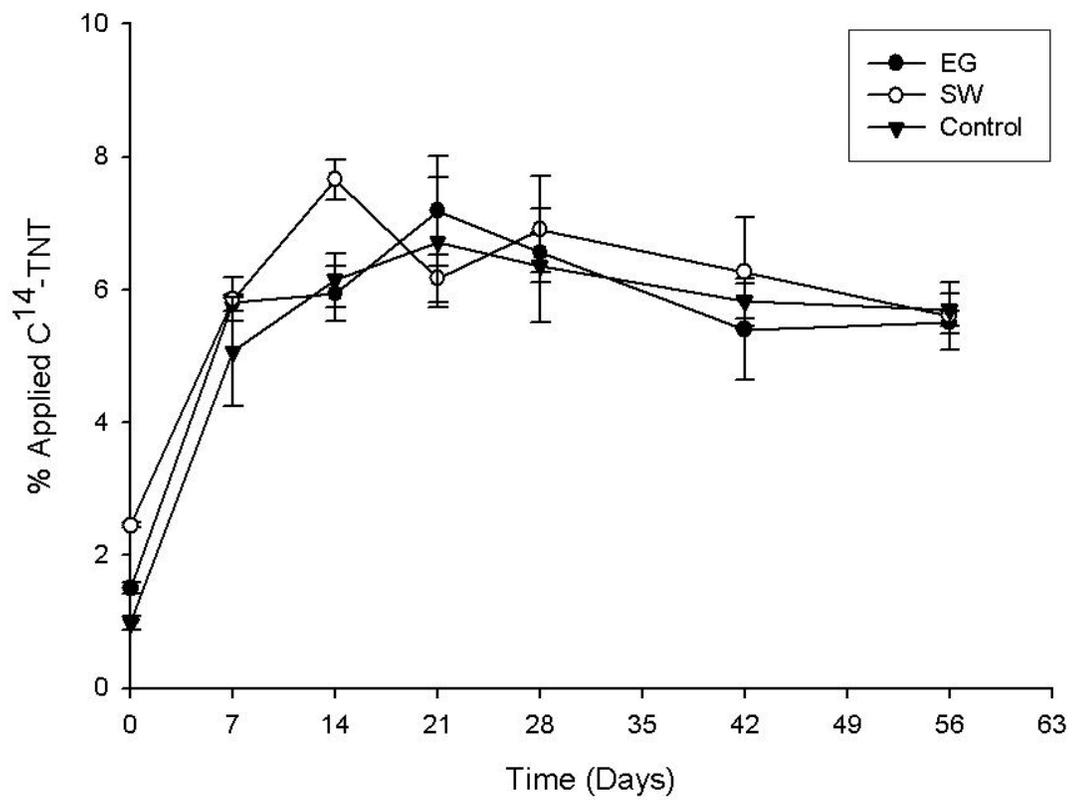


Figure 4.9. The cumulative percentage of non-extractable C^{14} -TNT in EG rhizosphere, SW rhizosphere, and control soils.

Mass Balance

The mass balance of TNT degradation at the end of incubation period is listed in Table 4.2. It demonstrated the fate of TNT during 8 weeks. Poor mass balance was achieved in this study primarily due to the technique utilized for determining the non-extractable fractions. Most of applied TNT was degraded transforming into degradation metabolites, CO₂ and non-extractable residues in the soil. In EG and SW rhizosphere soils, less TNT remained in soil and more TNT mineralized into harmless CO₂. Non-extractable residue in soil was supposed to account for more than 75% of the applied TNT in the system (Claus et al., 2007; Drzyzga et al., 1998). In this study, less than 6% of applied TNT was accounted for in soil as non-extractable residue. It is believed the non-conventional approach using liquid scintillation counting (instead of an oxidizer) for quantifying the non-extractable C¹⁴-TNT metabolites in this work was the major source for the discrepancies. The results from the study suggested that results derived from liquid scintillation counting should be correlated and compared with results from conventional oxidization techniques before estimates of non-extractable activities can be made. The use the oxidizer or improved liquid scintillation counting is recommended for future studies to achieve a better mass balance recovery.

	Metabolites(%)	TNT(%)	Mineralization(%)	Nonextractable Residue(%)	Sum(%)
EG	3.05	0.46	1.61	5.504	10.63
SW	2.61	0.39	2.004	5.61	10.62
Control	10.03	1.94	1.17	5.69	18.83

Table 4. 2 The mass balance of TNT in EG rhizosphere soil, SW rhizosphere soil and control soil at the end of incubation period.

CONCLUSIONS

This study showed that the TNT degradation in soil follows a second-order reaction in soils. More than 95% of TNT applied was transferred to its metabolic derivatives in the first 7 days. However, less than 2% of TNT has been transformed into harmless CO₂. Both native grass species appeared to enhance the degradation of TNT in terms of mineralization rates and its extractable metabolites in soils. The formation of relatively stable complexes with soil may limit further TNT degradation. Overall, SW appeared to be more effective than EG to enhance TNT degradation in soil.

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CHAPTER 5

INTRODUCTION OF DEGRADER *P. PUTIDA* KT2440 TO ENHANCE THE RHIZODEGRADATION OF TNT

ABSTRACT

Contamination by munitions explosive residues including TNT (2,4,6-trinitrotoluene) in soil is a potential threat to human health and ecosystems. TNT and its metabolites are toxic to humans. Compared to conventional remediation methods, phytoremediation has been considered as one of the most cost-effective mitigation approaches. Objectives of this research were to: 1) investigate the synergistic effects of inoculation of TNT degrader *P. putida* KT2440 on TNT degradation in rhizosphere soils, 2) investigate the fate of the introduced functional TNT degrading-genes in the rhizospheres, and 3) identify the species that could sustain the number of the functional genes and the synergistic degradation activities by comparing the degradation profiles with the molecular profiles. The rhizosphere soils of eastern gammagrass (*Tripsacum dactyloides*) and switchgrass (*Panicum virgatum* L.) were selected, spiked with C¹⁴-TNT, and incubated for 8 weeks. This allows us to monitor the degradation profiles and mineralization rates of TNT. Prior to application of C¹⁴-TNT, *P. putida* KT2440 culture was added to the soil. Results showed more than 90% TNT disappeared in the first 7 days. Less than 1.2% TNT was mineralized into harmless CO₂. TNT degradation

followed a second order reaction in soils. Inoculation of *P. putida* KT2440 appeared to suppress mineralization, but enhanced the concentrations of non-extractable residues. A sensitive quantitative real-time method was successfully developed to quantify the number of *pnrA*, a gene encoding a nitroreductase. The results from real-time-PCR analysis suggested that the population of *P. putida* KT2440 can be sustained to some extent in both rhizosphere soils in the first 7 days. Overall, switchgrass with *P. putida* KT2440 acted to possess the best capacity to degrade TNT in soil.

INTRODUCTION

2,4,6-trinitrotoluene (TNT) is an explosive widely used in the world. Production, use and disposal of explosives can cause environmental contamination in soil and water threatening public health. According to reports in the US, there are over 1,200,000 tons of soil contaminated by explosives (Lewis et al., 2004). Incredibly high levels of explosive concentrations have been identified near army ammunition plants (Stevens et al., 2002). For example, up to 87,000 mg/kg in soil, 711,000 mg/kg in sediment and 3,375 mg/L in surface water of explosive contamination have been detected near the Louisiana Army Ammunition Plant (LA, USA) and the Cornhusker Army Ammunition Plant (NE, USA). Twenty out of 1,397 hazardous waste sites on the EPA National Priorities List are contaminated by TNT (Agency for Toxic Substances and Disease Registry, 1995). It is reported that TNT can cause anemia, abnormal liver function, cataract development and skin irritation (Honeycutt et al., 1996; Yinon, 1990). Even, cases of people's death by exposure to TNT in production factories have been reported (McConnell and Flinn, 1946).

There are several remediation methods that have been developed to treat explosive contaminated soil, including incineration, compost and bioslurry. Compared to those conventional remediation methods, phytoremediation has been recognized as one of the most cost-effective mitigation approaches to clean up soil contaminated by explosives. Phytoremediation is defined as the use of green plants to remove, destroy and sequester hazardous materials, which has been reported to have a promising ability to degrade organic pollutants, including chlorinated hydrocarbons, pesticides, petroleum compounds, explosives, dyes and detergents (Anderson et al., 1993; Caballero et al., 2005a; Chaudhry et al., 2001; Haby and Crowley, 1996; Reilley et al., 1996; Schwitzguebel et al., 2001; Siciliano and Greer, 2000).

Explosive degrading bacteria in rhizospheres are able to eliminate plant toxicity of explosives through enhanced biodegradation, and several of them, such as *Pseudomonas* spp., are also well known for their plant growth-stimulating properties. *P. putida* KT2440, a plant growth-stimulating bacterium, possesses a pnrA gene which locates on its chromosome. Studies showed pnrA gene demonstrated much more activity than pnrB gene. The enzyme encoded by a pnrA gene catalyzes the transformation of TNT to 4-hydroxylamine-2,6-dinitrotoluene using the ping-pong mechanism described in former chapters (Caballero et al., 2005b). Several *P. putida* strains isolated from the rhizosphere have been shown to pose plant growth-stimulating properties (Campbell and Greaves, 1990). They may fix nitrogen, synthesize siderophores and phytohormones, like auxins and cytokinins, and solubilize soil minerals for plant growth (Glick, 2003). The mutually beneficial interaction between plants and microbes is based on the colonization of microbes onto plant roots. Efficient colonization can lead to successful rhizodegradation.

The plant-growth promoting *Pseudomonas* spp, are well adapted in rhizospheres. Cells of *Pseudomonas* were found to colonize along the junctions of epidermal plant cells where nutrients are exuded. However, the ability of colonization varies strongly within different *Pseudomonas* strains (Kuiper et al., 2004). Thus, the synergistic interactions between explosive degrading microbes and species-specific plants would enhance the feasibility of the application of phytoremediation technology on large scales at relatively high explosive concentrations.

The objectives of this research are to: 1) investigate the effects of inoculation of TNT degrader *P. putida* KT2440 on TNT degradation in rhizosphere soils, 2) investigate the fate of the introduced functional TNT degrading-genes in the rhizospheres using quantitative molecular techniques, and 3) identify the species that could sustain the number of the functional genes and the synergistic degradation activities by comparing the degradation profiles with the molecular profiles.

MATERIALS AND METHODS

Experimental Design

Two native grass species, EG (eastern gammagrass, *Tripsacum dactyloides*) and SW (switchgrass, *Panicum virgatum* L), were selected upon previous screening results. The seeds of EG and SW were sterilized in 30% ethanol for 30 seconds and washed with distilled water for 1 minute. The surface sterilized seeds were then grown in soil pots in a walk-in growth chamber under the conditions of light intensity, 1400 Einsteins·m⁻²·sec⁻¹; light/dark period, 15/9 hours; humidity, 50%; temperature, 25°C (light)/20°C (dark). The soils were a mixture of 50% sand and 50% silt-loam.

The rhizosphere soils of EG and SW were collected weekly until maturity (> 3 months) and stored in a 4⁰C condition. Twenty grams of the rhizosphere soil was collected and transferred into a flask. The rhizosphere soils and the control soil were applied with 0.5 μ Ci C¹⁴-TNT (PerkinElmer Life and Analytical Sciences) and 17.86 μ g of 1000ug/ml TNT (Spex Certiprep, Inc) to achieve total TNT concentration of 1000 μ g/kg in soil. Prior to application of C¹⁴-TNT, TNT degrader *P. putida* KT2440 was cultured and added to soil to achieve the concentration of 10⁸ cells per gram soil. Spiked soil was stored in a 15mL Erlenmeyer flask. The flask and a 20mL scintillation vial containing 10mL 1M NaOH (Fisher Scientific) as ¹⁴CO₂ trap were sealed in a mason jar with parafilm and duck tape. The jars were incubated at room temperature in the dark for 8 weeks. During the incubation period, moisture content of the soils was maintained gravimetricly at 15%, and NaOH traps were replaced every week with collected traps stored at room temperature in the dark. Spiked soils were sampled from the jars at day 0, 7, 14, 21, 42 and 56, and then extracted using 250mL 100% acetonitrile (Fisher Scientific). The extraction procedures used in our study were adapted from EPA method 8330, through which TNT and its major metabolites can be fully recovered. Extracts were concentrated to 200 μ L by N₂ evaporator and stored at -20⁰C until analyzed. After extraction, the remaining soils were oven-dried overnight, grounded and stored in 20mL scintillation vials at room temperature in the dark. In the experiment, blank soils were included as controls, and each treatment was in triplicate.

In a separate pot study, EG and SW were grown in nalgene bottles with 300g explosive-free and sterilized soil per one single plant in one bottle for over 3 months. 300 μ g non-radioactive TNT (cold TNT) was added to each bottle to make a total TNT

concentration of 1000 $\mu\text{g}/\text{kg}$ in the soil. Prior to application of cold TNT, TNT degrader *P. putida* KT2440 was cultured and added to each bottle as 2×10^8 cells per gram soil. Then plants were allowed to continue to grow for 8 weeks under growth chamber conditions as described above. At day 0, 7, 14, 21, 28, 42, and 56, rhizosphere soils were collected and extracted using the same procedure described above. In addition, the soil DNA extraction was performed with FastDNA SPIN Kit (MP Biomedicals, Solon OH). Both extracts were stored at -20°C until analyzed. In the experiment, bare soils were included as control, and each treatment was replicated in triplicate.

Analysis Procedures

One milliliter of 1M NaOH solution in CO_2 traps was transferred into a 7mL scintillation vial and mixed with 4mL cocktail (Ultima GoldTM AB, PerkinElmer). The $^{14}\text{CO}_2$ radioactivity was quantified by a Beckman LS 6000SC liquid scintillation counter, and the TNT mineralization rates were calculated. To determine the radioactivity of the non-extractable C^{14} -TNT degradation products, 1g of soil was transferred to a 7mL scintillation vial and mixed with 4mL cocktail (Ultima GoldTM AB, PerkinElmer). The mixtures were vortexed, allowed to precipitate for 3 days, and the $^{14}\text{CO}_2$ radioactivity was quantified by a Beckman LS 6000SC liquid scintillation counter. Concentrations of acetonitrile extractable C^{14} -TNT and its metabolites were analyzed by a Shimadzu High Performance Liquid Chromatogram equipped with a Phenomex Columbus C8 (5 μ , 110A) column and a IN/US in-line radioactivity detector. The mobile phase consisted of 100% acetonitrile and 0.1% phosphoric acid (1:9, v:v) at a flow rate of 1ml/min. Standards of TNT (Spex Certiprep, Inc) and its metabolites (www.accustandard.com), including:

24DA6NT, 26DNT, 26DA4NT, 4HA26DNT, 4A26DNT, 2A46DNT, 24DNT, 2A4N4, and 2HA46DNT, were purchased and injected into HPLC under the same conditions to determine retention time.

The non-radioactive TNT samples collected from the pot study were extracted with the same procedure described as above. The TNT and its six major metabolites, including 2A46DNT, 4A26DNT, 4HA26DNT and 4NT, were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). Standards of TNT (Spex Certiprep, Inc) and its metabolites (www.accustandard.com), including: 24DA6NT, 26DNT, 26DA4NT, 4HA26DNT, 4A26DNT, 2A46DNT, 24DNT, 2A4N4, and 2HA46DNT, were purchased and injected into the GC-MS under the same conditions to determine retention time. The copy number of *pnrA* gene in DNA extracts was quantified by an Applied Biosystems 7500 Fast Real-Time System using TaqMan probe. Both TaqMan probe, reverse and forward primers used for real-time quantitative PCR amplification were designed with Primer Express® Software (Applied Biosystems). The TaqMan Universal PCR Master Mix PCR kit containing deoxynucleoside triphosphate mix (dNTPs with dUTP), AmpliTaqGold® DNA polymerase, PCR buffer, AmpErase® UNG and 2.5 mM MgCl₂ were used for the RT-PCR mixture. The final PCR reaction mixture consists of 25 µL TaqMan Universal PCR Master Mix, 45 pmole of each primer, 12.5 pmole of the TaqMan probe, 1.0 µL of 1:10 diluted DNA extract, and RNase/DNase-free water to reach a final volume of 50 µL. Polymerase chain reaction was initiated by holding 50°C for 2 min followed by activation of AmpliTaqGold® polymerase at 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and extension at 60°C for 1 min. Standards for the quantitative RT-PCR were prepared using the constructed *E. coli* plasmid cloned with

the *pnrA* gene (StrataClone PCR Cloning Kit). The calibration was constructed with standards at concentrations of 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 gene copies/ μL . The copy number of the target gene in these standards was determined by UV absorbance at 260 nm. The copy number of the *pnrA* gene used for accessing the population of *P. putida* KT2440 in soil extracts was quantified by the RT-PCR Cycle threshold (Ct) measurements, the cycle number at which the fluorescence emission exceeds the fixed threshold.

The relative abundance of the introduced TNT degraders (expressed as number of gene copies per g soil) was determined for each grass treatment. The change in copy number of the *pnrA* was further compared with the degradation profiles and kinetics to characterize the potential efficiency of explosives degradation for each microcosm system. Real-time PCR analysis results were kindly provided by Drs. HsinYeh Hsieh and Brian Thompson in the Department of Veterinary Pathobiology of the University of Missouri-Columbia.

Statistical Analysis

The experimental design was a completely randomized design with two factors (rhizosphere and time) and three replicates. Three rhizosphere treatments (EG, SW, and control) and seven times (0, 7, 14, 21, 28, 42 and 56 days after initiation) were evaluated. Significant differences in treatments were set at $p < 0.05$. Data were found to be non-normally distributed. Data were analyzed using a non-parametric two-way ANOVA with SAS software.

RESULTS AND DISCUSSION

C¹⁴-TNT Degradation with Degradation in Soil

As the results in Figure 5.1 show, TNT degraded rapidly within the first 7 days of the 8 week incubation period after the inoculation of *P. putida* KT2440. Similar to the previous study without inoculation, more than 90% TNT degraded in SW rhizosphere soils and the control during the first 7 days, while approximately 70% of TNT degraded in the EG rhizosphere soils. The rate of TNT dissipation slowed down and became steady during the rest of incubation period. TNT concentration remained in SW rhizosphere soils at a lower than in the control. However, at the end of the incubation period, approximately 1.8% of applied TNT concentration was detected in the EG rhizosphere soils, compared to approximately 3.6% in SW rhizosphere soils and the control. In our previous non-inoculation study, 1.44% of applied TNT was detected in the EG treatment at the end of incubation period as well as 0.855% and 3.63% detected in the SW and control treatments, respectively. Overall, TNT degradation was significantly enhanced in SW as compared with the degradation rates in EG and the control ($p < 0.0001$). The differences in degradation rates between EG and the control were not significant.

The rapid dissipation of TNT during the first 7 days observed in this study is in agreement with our previous results of TNT degradation without inoculation of the TNT degrader. Approximately 70% of the initial TNT concentration was observed in all treatments in the first 7 days of the previous study. However, in contrast to our previous study without the TNT degrader, higher TNT degradation rates were found in EG and SW rhizosphere soils than in the control. More than 95% of the initial TNT concentration was degraded in the first week in all treatments without the degrader being added. The

results of the ANOVA analysis suggested that TNT degradation rates in all treatments with the inoculated degrader were significantly reduced during the whole incubation period, compared to those in the study without the degrader.

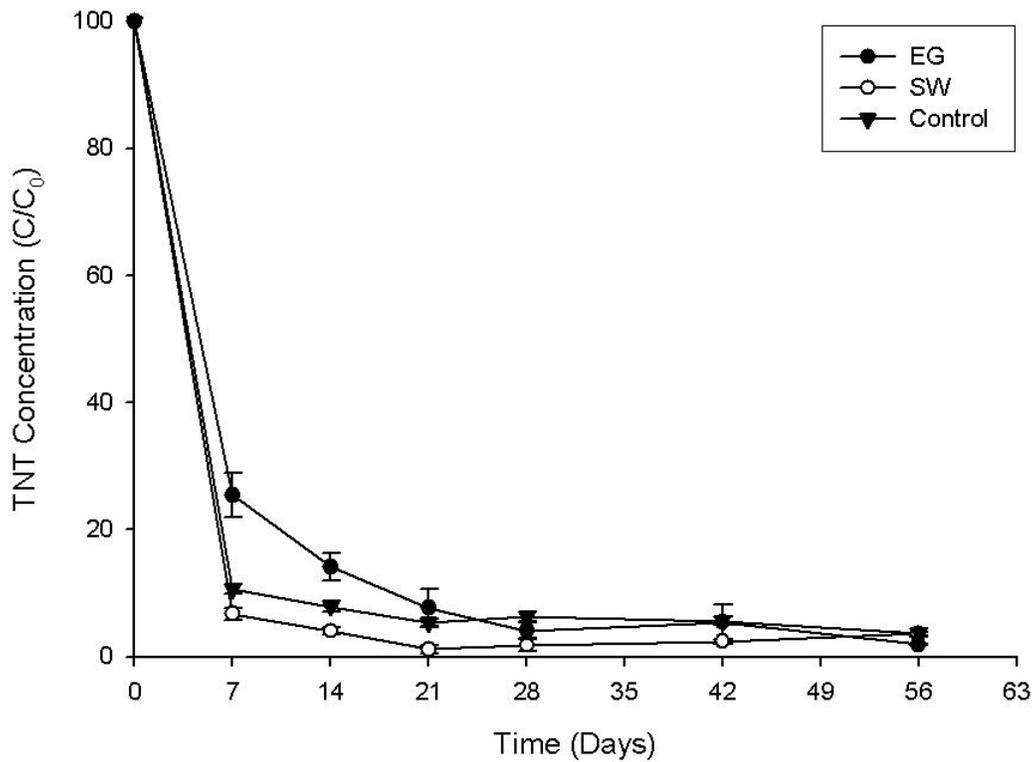


Figure 5.1. The percentage of C14-TNT remaining in EG rhizosphere soil, SW rhizosphere soil and control soil with degrader during the 8-week incubation period.

Degradation Kinetic Order of C¹⁴-TNT with Degradation

As results in Table 5.1 show, TNT degradation in all treatments with inoculation of the TNT degrader follow a second order degradation kinetic. The half-life ($T_{1/2}$) of TNT degradation for each treatment was calculated. A lower $T_{1/2}$ value indicates a higher degradation rate of TNT. During the whole incubation period, overall, TNT degraded faster in EG and SW rhizosphere soils than in the control, despite slower TNT degradation in EG than the control within the first 7 days. This means the rhizosphere treatments have had a positive effect on TNT degradation. The lowest value of $T_{1/2}$ for TNT degradation was in SW which indicates SW has the potential to possess the best capacity to degrade TNT.

Compared to results from our previous study of TNT degradation without a degrader, inoculation with a TNT degrader has slowed down TNT degradation rate in treatments (Table 5.2). It was speculated that the inoculated *P. putida* KT2440 has become a predominant bacteria in soil after inoculation. The oxygen-insensitive nitroreductase encoded by *pnrA* gene within the chromosome of *P. putida* KT2440 can only catalyze the transformation of TNT to hydroxylamino-TNT derivatives but does not cause further transformations. Therefore, the *P. putida* KT2440 competed with other native TNT degraders in the rhizospheres and completely redirected the degradation pathway. Eventually, the predominance of *P. putida* KT2440 minimized the activity of other degraders; as a result, it reduced the rate of TNT degradation. However, under both circumstances, with or without inoculation, TNT degraded faster in EG and SW than in the control. And the lowest $T_{1/2}$ value in SW suggests that SW can be a promising species for TNT mitigation.

Treatment	n	R ²	K	T _{1/2}
EG	2	0.9304	0.9739	1.185
SW	2	0.9255	1.2022	0.939
Control	2	0.9662	0.815	2.521

Table 5. 1 Reaction constants during TNT degradation in different treatments with inoculation of TNT degrader.

Treatment	n	R ²	K	T _{1/2}
EG	2	0.9879	1.4759	0.669
SW	2	0.9638	1.5704	0.5426
Control	2	0.9627	0.2918	2.559

Table 5. 2 Reaction constants during TNT degradation in different treatments without inoculation of TNT degrader.

Degradative Metabolites of C¹⁴-TNT with Degradar

There were nine TNT degradation metabolites identified and quantified in the inoculation pot study. The metabolites included M1, M2, M3, M4, M5, 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 4-hydroxylamino-2,6-dinitrotoluene (4HA26DNT) and 4-nitrotoluene (4NT). M1, M2, M3, M4 and M5 are degradation metabolites that have not been chemically characterized. Among these metabolites, 2A46DNT and 4A26DNT, were the major metabolites of TNT degradation (Figure 5.2, 5.3, and 5.4). The metabolite profiles suggested that TNT degradation underwent the sequential two-electron reduction mechanism, in which TNT was degraded to hydroxylamino derivatives and further to amino derivatives. This is in agreement with the proposed mechanism of pnrA gene. The major metabolite catalyzed

by *pnrA* gene should be 4HA26DNT. However, the findings did not show the trend of accumulation of 4HA26DNT. This might indicate the existence of other microbes which are responsible for facilitating further degradation of 4HA26DNT. Although no accumulation of 4HA26DNT was found, an accumulation of 4A26DNT was observed. The 4HA26DNT is the precursor of 4A26DNT. With the inoculation of TNT degrader, higher concentrations of 4A26DNT than 2A46DNT were found in all treatments. Among the three treatments, significantly much lower concentrations of TNT degradation metabolites were observed in SW ($p < 0.0001$) indicating SW possesses the best capacity to degrade not only TNT but also its metabolites.

When the metabolite profiles were compared *versus* those without inoculation, there were relatively more metabolites detected in inoculated samples. Under both circumstances, 2A46DNT and 4A26DNT were major metabolites of TNT degradation. We can speculate that TNT degradation in soil prefers to undergo the sequential two-electron reduction pathway. This might be due to the abundance of oxygen-insensitive nitroreductase possessed by soil microbes. However, inoculation of *P. putida* KT2440 did change the overall TNT degradation pathway in soil. Upon inoculation, the predominance of *P. putida* KT2440 reduces the number of other degraders in the system, in which thorough TNT degradation could be achieved. In the inoculation study, the degradation of TNT was significantly suppressed in all treatments, $p = 0.0002$ in EG, $p = 0.0002$ in SW and $p = 0.0226$ in the control compared to the non-inoculated study. The degradation of major TNT degradation metabolites, 2A46DNT and 4A26DNT, was also significantly suppressed in SW ($p < 0.0001$ and $p = 0.0062$) and the control ($p < 0.0001$ and $p < 0.0001$). While their degradation in EG was enhanced especially 4A26DNT ($p < 0.0001$).

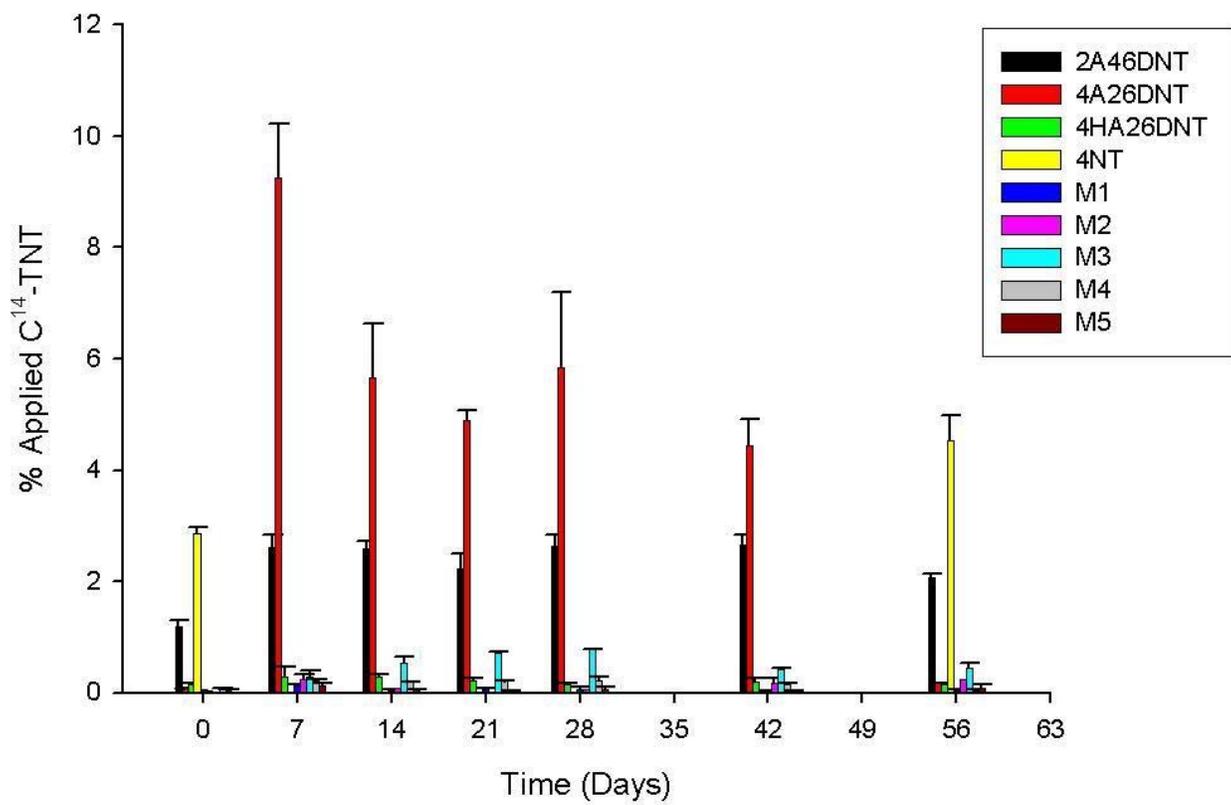


Figure 5. 2 Concentrations of TNT degradation metabolites in the control treatment with inoculation of degrade.

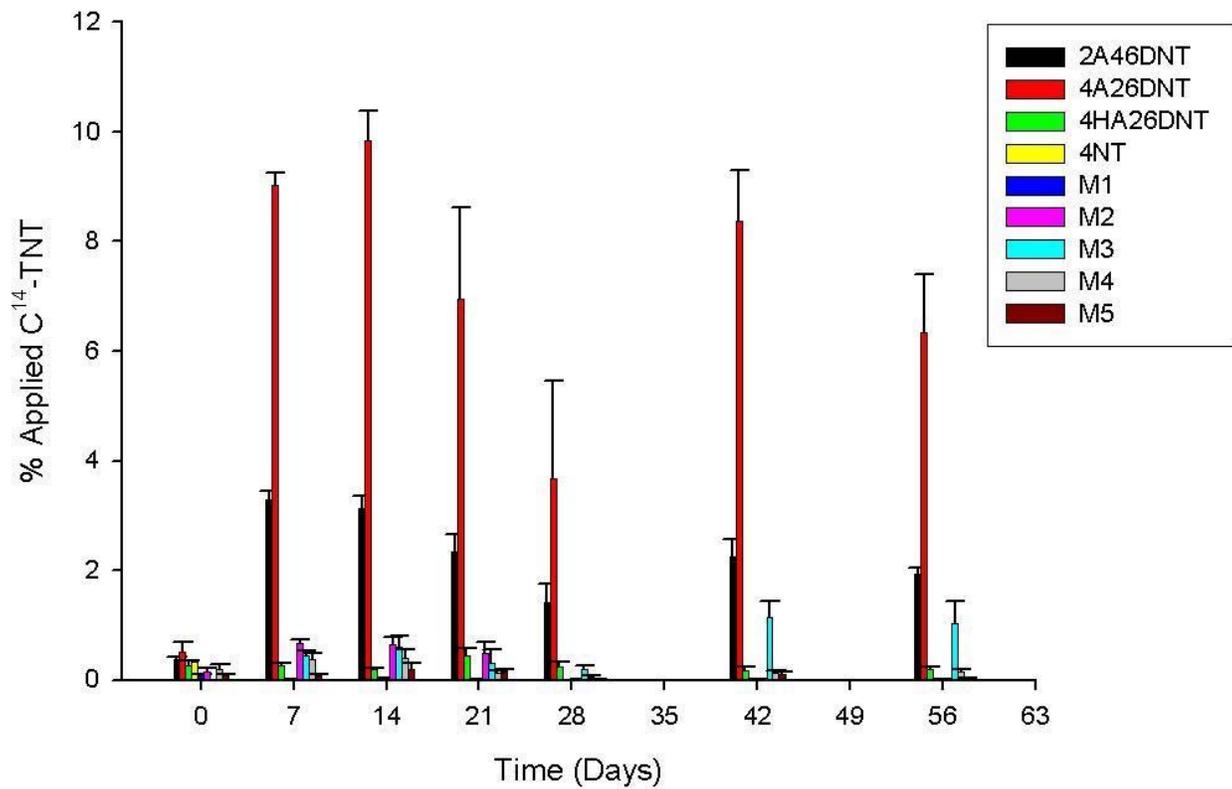


Figure 5. 3 Concentrations of TNT degradation metabolites in the EG rhizosphere soil treatment with inoculation of degrader.

SW

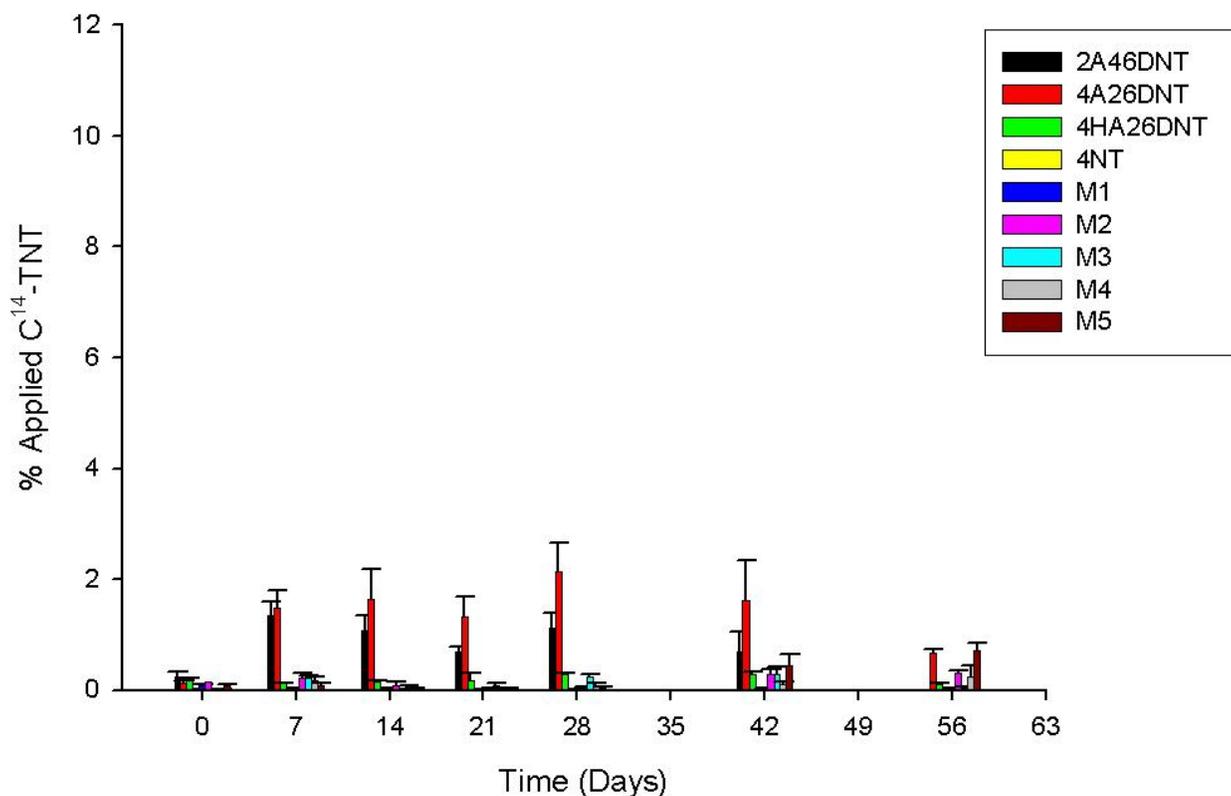


Figure 5. 4 Concentrations of TNT degradation metabolites in the SW rhizosphere soil treatment with inoculation of degrader.

Mineralization Rate of C¹⁴-TNT with Degradere

As the results in Figure 5.5 show, a very low mineralization rate of TNT, less than 1.2% of applied C¹⁴-TNT, was encountered. The formation of CO₂ indicates the complete cleavage of the aromatic ring. The chemical properties of TNT make the aromatic ring intact limiting the mineralization of TNT. Low mineralization of TNT were also observed in many other studies (Hawari et al., 2000; Nyanhongo et al., 2005). The mineralization rates of C¹⁴-TNT in SW were higher than in EG and the control (p<0.0001). The mineralization rate in EG was significantly lower than the control statistically.

Inoculation of the TNT degrader into rhizosphere soil has suppressed the mineralization of TNT. Compared to our previous data of TNT mineralization without the TNT degrader, mineralization rates in all treatment have been reduced ($p < 0.0001$). The mineralization of TNT might indicate TNT metabolites can be attacked by some specific microbes in soil via unknown mechanisms resulting ring cleavage and the formation of CO_2 . Inoculation of *P. putida* KT2440 has either redirected the reaction pathway or inhibited the growth of other TNT degraders in the systems resulting in the reduced mineralization of TNT. Switchgrass still possesses the best capacity to facilitate the TNT mineralization processes under both circumstances.

Non-extractable TNT in Soil of C^{14} -TNT with Degradation

TNT degradation products are well known for developing irreversible covalent bonds with soil minerals and humic substances, which make those compounds resistant to solvent extraction. In Figure 5.6, approximately 10% of non-extractable residue accumulated within the first 7 days. This may contribute to the rapid disappearance of TNT within the first 7 days observed in our study. During the rest of the incubation period, these residues stayed constant. Significant differences were observed in the control compared to EG and SW ($p < 0.0001$). More residues were detected in the control. Compared to our study without inoculation, a 2% higher residue mass was observed in this study with inoculation. All treatments followed a similar pattern as in inoculated study. The existence of those non-extractable compounds in soil could be considered as not harmful to the environment and human health because of their very limited availability. The more non-extractable compound in soil, the less toxic to ecosystems.

According to ANOVA analysis, inoculation with the TNT degrader did enhance the mass of non-extractable residue in EG and the control; while, there were no significant effects in SW.

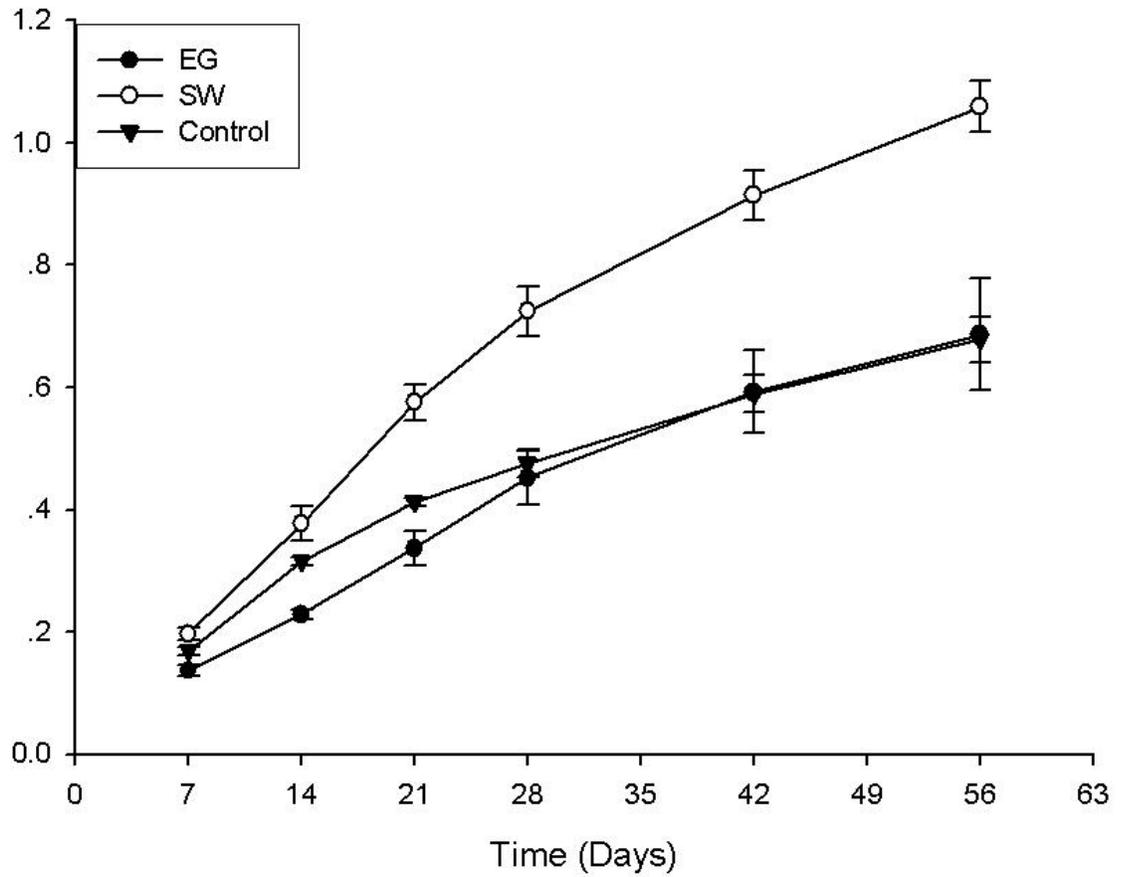


Figure 5. 5 The cumulative percentage of C^{14} -TNT mineralized in EG rhizosphere soil, SW rhizosphere soil and control soil with inoculation of degrader.

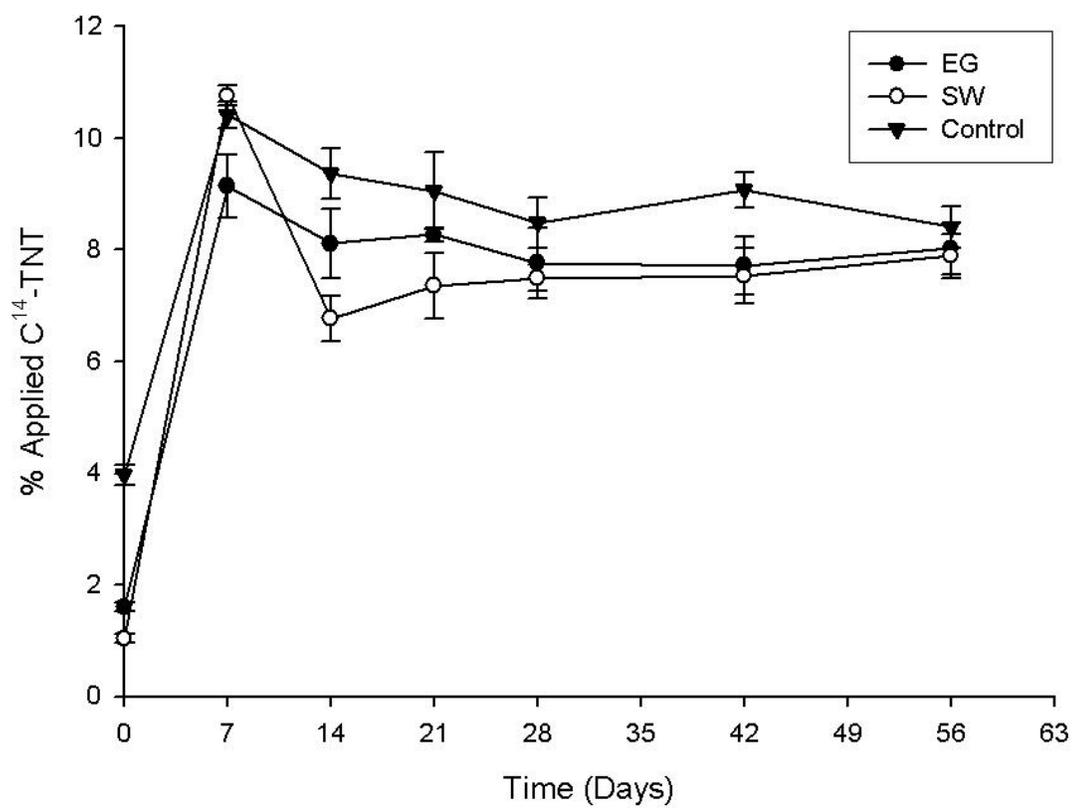


Figure 5. 6 The cumulative percentage of non-extractable C^{14} -TNT metabolites in EG rhizosphere soil, SW rhizosphere soil and control soil with inoculation of degrader.

	Metabolites(%)	TNT(%)	Mineralization(%)	Nonextractable Residue(%)	Sum(%)
EG	9.63	0.64	0.67	8.02	18.99
SW	1.97	1.27	1.06	7.89	12.19
Control	7.31	0.68	0.68	8.41	17.09

Table 5. 3 The mass balance of TNT in EG rhizosphere soil, SW rhizosphere soil and control soil at the end of incubation period with inoculation of degrader.

Mass Balance of C¹⁴-TNT with Degradation

Poor mass balance was achieved in all treatments at the end of incubation period. Most of TNT should be in the form of non-extractable residues. But less than 10% of applied C¹⁴-TNT was bonded in the soil. We used a liquid scintillation counter to measure radioactivity of residues in soil. Conventionally, an oxidizer should be used to measure these values. The results from our study have strongly suggested that results derived from liquid scintillation counting should be correlated and compared with results from conventional oxidization techniques before accurate estimate of non-extractable activities can be made. The use of the oxidizer or improved liquid scintillation counting is recommended for future studies to achieve a better mass balance.

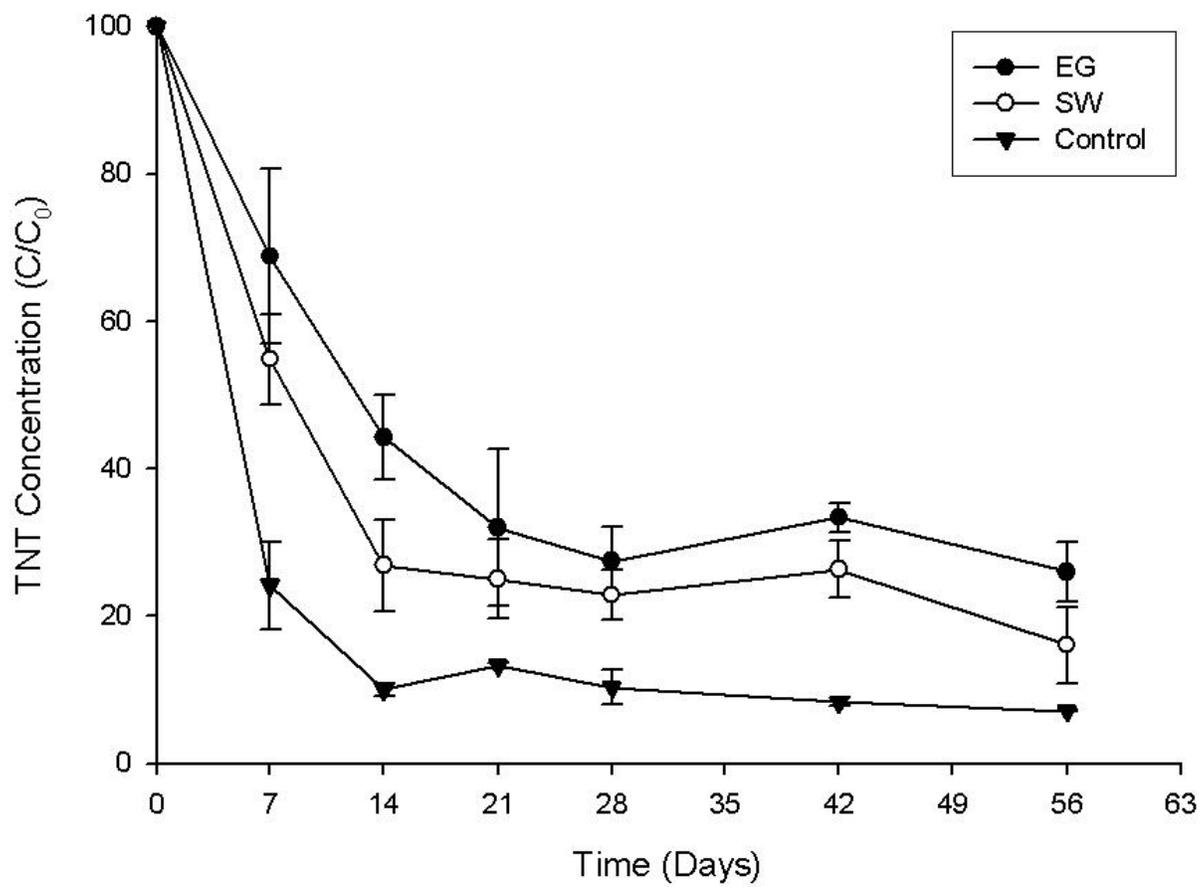


Figure 5. 7 The concentration of applied TNT (C/C_0) remaining in soil with living EG treatment and SW treatment along with the control over an 8-week incubation period.

TNT Degradation with Degradation in Soil (Pot Study with Living Plants)

Non-radio-labeled TNT and TNT degrader, *P. putida* KT2440, were applied to pots with soil and living plants. The results suggested that, most of TNT disappeared within the first 14 days (Figure 5.7). The TNT degradation rate in the control was significantly higher than in EG and SW, while degradation in SW was higher than in EG ($p < 0.0001$). The results are not consistent with our study using C^{14} -TNT and degrader. From my point of view, there might be two explanations. The first one is inactivation of pnrA gene in the rhizospheres. The functional gene, pnrA, was supposed to encode a nitroreductase which transforms TNT into hydroxylamino derivatives. But, somehow, in the rhizosphere consisting of many complicated constituents, pnrA was deactivated by some substances in the rhizosphere resulting in its "silence". The second explanation is that *P. putida* KT2440 chose an alternative pathway other than consuming TNT as a N-source. Fertilizers were applied to the plants in order to maintain vigorous plant growth. Because of the excessive existence of fertilizers, *P. putida* KT2440 would get nutrients very easily from soluble fertilizers rather than degrading TNT for N.

The functional gene, pnrA, is located on the chromosome of *P. putida* KT2440. The decrease of gene copy numbers indicates the death of *P. putida* KT2440. As we can see in Figure 5.8, a rapid decrease of *P. putida* KT2440 happened in the first 7 days. *P. putida* KT2440 kept dying during the rest of the study. The rate of decrease of *P. putida* KT2440 in the control was faster than in EG and SW indicating *P. putida* KT2440 could be maintained in rhizospheres to some extent. But this collides with the study hypothesis. In the hypothesis, the population of *P. putida* KT2440 should use TNT as a N source and keep growing, because, *P. putida* KT2440 does possess the pnrA gene which is

responsible for degrading TNT. In my opinion, *P. putida* KT2440 is not isolated from TNT-contaminated soil, like *P. putida* JLR11 which also has pnrA gene and is reported to use TNT as a nutrient source. The ability of colonizing plant roots varies strongly with different strains of *Pseudomonas* sp. Successful colonization onto plant roots, which is a key point leading to successful rhizodegradation, might not be achieved. So, *P. putida* KT2440 might not as competent as *P. putida* JLR11 in soil with TNT resulting in poor survival. Harsh environmental conditions might be another reason. Plants were grown in nalgene pots, in which soil was not well aerated. Water and fertilizer applied may not have been evenly distributed resulting in the death of *P. putida* KT2440. Additionally, *P. putida* KT2440 was inoculated into soil after plants were established instead of coating plant seeds with it. *Pseudomonas* sp. was found to colonize the cells of plant roots which exude nutrients. Those spots might be occupied by other microbes during the process of plant growth. Thus, *P. putida* KT2440 could not get an ideal habitat. It can't get enough nutrients to maintain its growth resulting in its death.

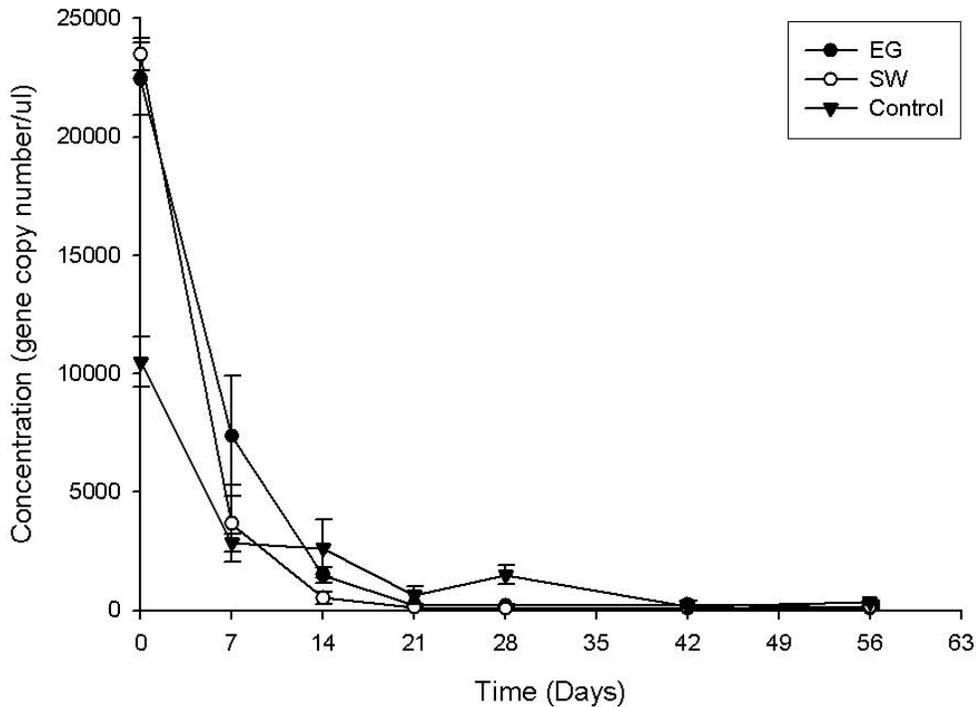


Figure 5. 8 The concentrations of pnrA gene remaining in the soil with living EG treatment and SW treatment along with the control over an 8-week incubation period.

TNT Degradation Kinetic Order (Pot Study with Living Plants)

TNT degradation followed a second order degradation. $T_{1/2}$ in the control was much lower than in EG and SW, indicating TNT degradation in the control is much faster than in the other two treatments (Table 5.4). This is contradictory to our results in the study using C^{14} -TNT and the degrader. This might be due to the poor survival of the degrader in soil. Noticeably, TNT degradation in SW was still faster than in EG.

Treatment	n	R ²	K	T _{1/2}
EG	2	0.9673	0.0008	21.2
SW	2	0.9659	0.0010	12.87
Control	2	0.9662	0.9652	4.82

Table 5. 4 Reaction constants during TNT degradation in different treatments with inoculation of TNT degrader in soil with living EG treatment and SW treatment along with the control.

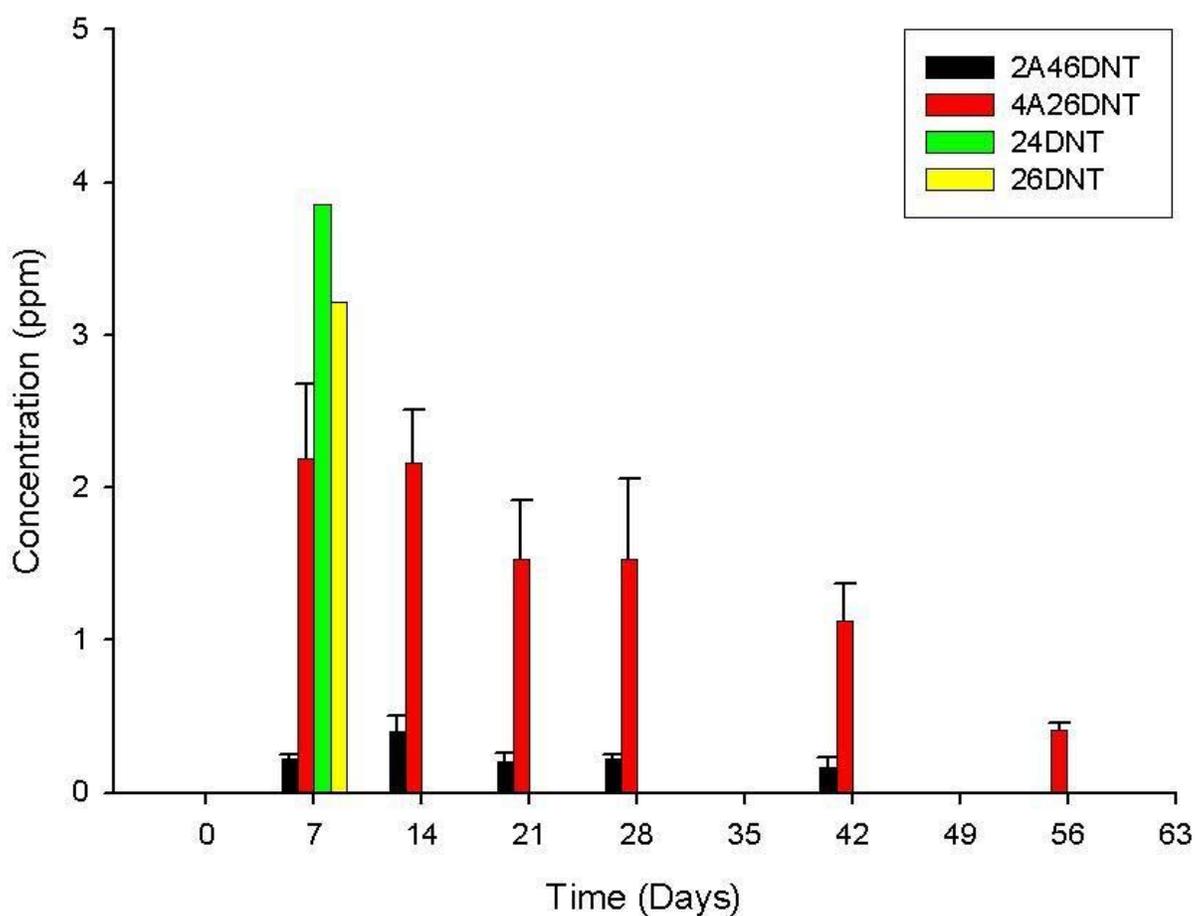


Figure 5. 9 Concentrations of TNT degradation metabolites in the control treatment.

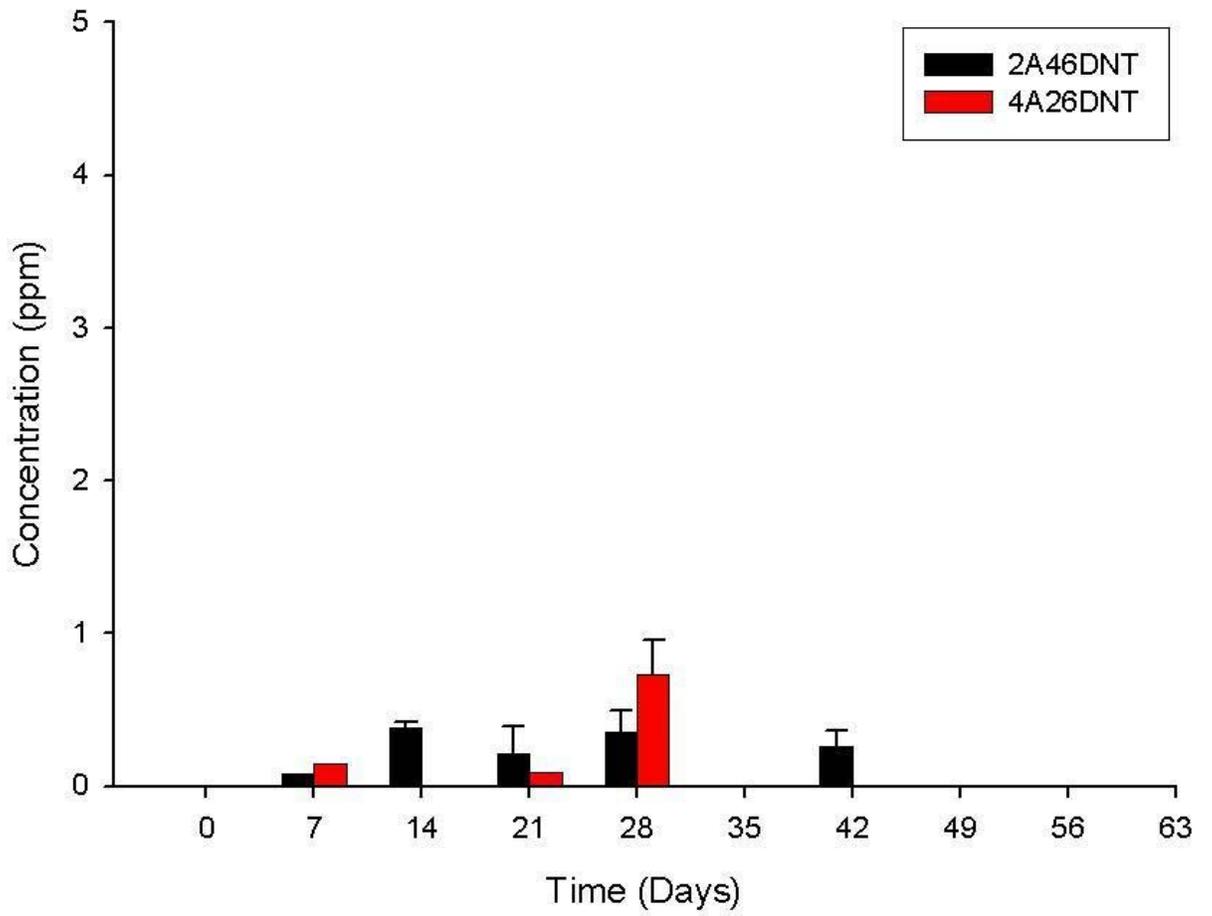


Figure 5. 10 Concentrations of TNT degradation metabolites in soil with living EG treatment.

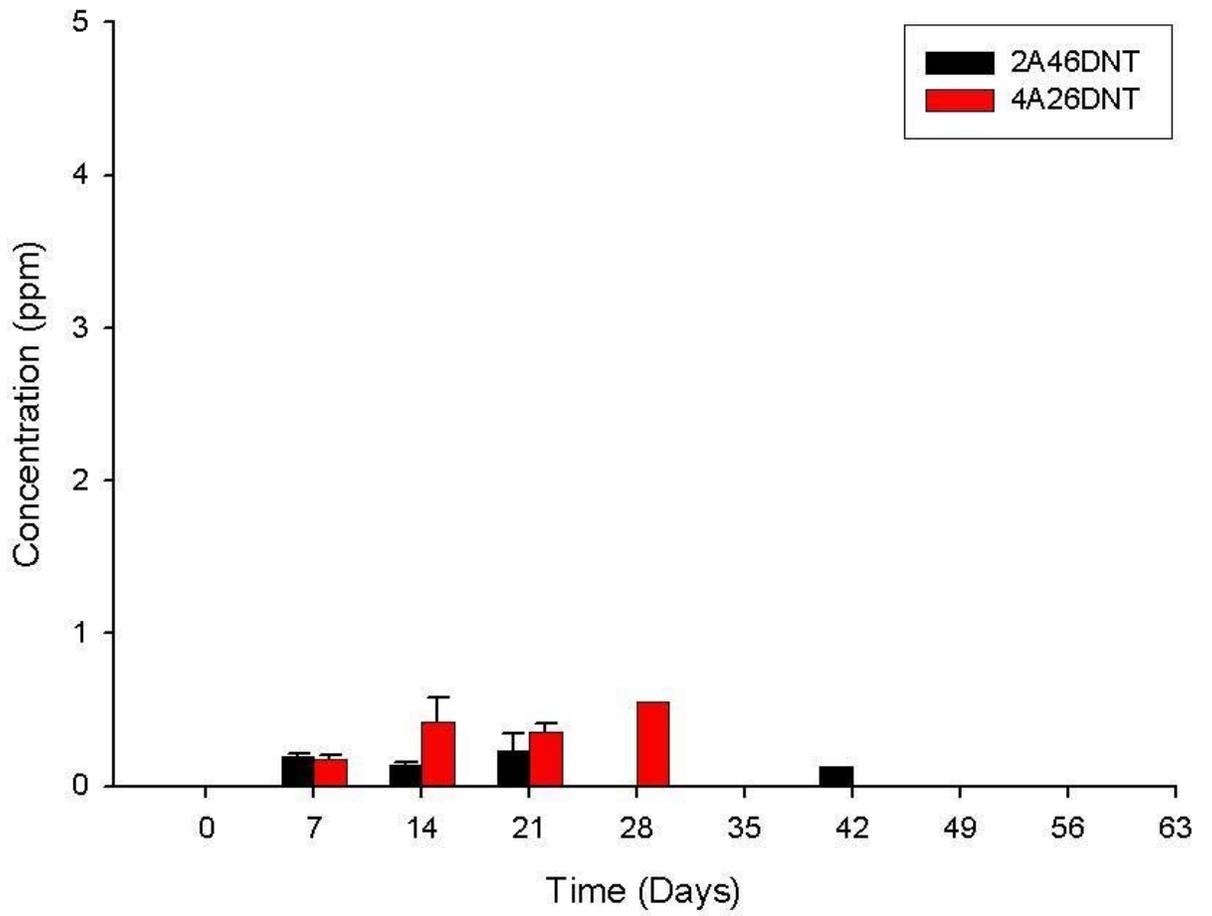


Figure 5. 11 Concentrations of TNT degradation metabolites in soil with living SW treatment.

Cold TNT Degradation Metabolites

Only four metabolites have been identified by GC. This is because many of the polar metabolites cannot be volatilized making them undetectable by GC (Figures 5.9, 5.10, and 5.11). The 2A46DNT and 4A26DNT were still the major metabolites of TNT. The degradation rates of TNT were higher in the control than in EG and SW ($p < 0.0001$). The concentrations of TNT metabolites were higher in the control than in EG and SW. Due to the application of non-radioactive TNT, we were unable to quantify the mineralization rates and non-extractable residues. There might be more CO₂ produced and more residues in soil to make it more sensible to be consistent to the previous results.

CONCLUSIONS

This study showed TNT degradation in soil with inoculation of a degrader follows a second order degradation. More than 90% of applied C¹⁴-TNT was degraded in the first 7 days. Less than 1.2% of applied C¹⁴-TNT was mineralized. Around 10% of applied C¹⁴-TNT was detected to be bonded with soil irreversibly. Overall, SW in combination with the degrader possesses the best capacity to degrade TNT, in terms of enhancing TNT degradation, reducing metabolite concentrations remaining in the soil and facilitating the mineralization process. Although mineralization rates were suppressed by inoculation with the degrader, more non-extractable residues were detected in soil. Additionally, concentrations of TNT degradation metabolites were lower. The inoculation of TNT degrader may have positive effects on TNT degradation. SW plus degrader *P. putida* KT2440 may have the potential to be integrated into remediation methods to treat TNT-contaminated soil.

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CHAPTER 6

CONCLUSIONS SUMMARY

The results of the studies described in this thesis were effective (1) to evaluate the degradation kinetics of TNT and RDX in the rhizospheres of EG and SW; (2) to determine the environmental fate of TNT and RDX in the EG or SW rhizosphere; (3) to monitor the degradation profiles of TNT and RDX in the rhizospheres of EG and SW; (4) to investigate the synergistic effects of TNT and RDX degradation in the rhizospheres inoculated with identified explosive degraders; and (5) assess the quantitative expression of the degradative genes of inoculated degraders under the EG and SW rhizospheres.

Results from Chapter 3 showed that both EG and SW rhizospheres appeared to be very effective for enhancing RDX degradation in soil. Approximately 30% of applied RDX was transformed into CO₂. Enhanced degradation could be attributed to grass species-specific stimulated microbial community and activity in the rhizospheres. EG appeared to be more effective for RDX degradation in context of enhancing mineralization and immobilization via transforming to less mobile metabolites. The two selected native grass species showing high rhizodegradation potential could be incorporated into remediation designs to mitigate the adverse impacts of RDX in the environment.

Studies in Chapter 4 showed the TNT degradation in soil follows a second-order reaction in soils. More than 95% applied TNT was transferred to its metabolic derivatives within the first 7 days. But less than 2% of TNT mineralized into CO₂. Both native grass

species enhanced the degradation of TNT in terms of mineralization rates and metabolite concentrations detected in soil. The formation of relatively stable complexes between metabolites and soil minerals or organic matter may limit further TNT degradation. Overall, SW appeared to be more effective than EG to enhance TNT degradation in soil.

Results from Chapter 5 showed the TNT degradation in soil with inoculation of a degrader follows a second order degradation. More than 90% of applied C¹⁴-TNT was degraded within the first 7 days. Less than 1.2% of applied C¹⁴-TNT was mineralized. Overall, SW in combination with the inoculated degrader *P. putida* KT2440 possesses the best capacity to degrade TNT and its degradation products. Although mineralization rates were suppressed by inoculation of degrader *P. putida* KT2440, more non-extractable residues were detected in soil. Additionally, concentrations of TNT degradation metabolites were lower. The inoculation of TNT degrader may have positive effects on TNT degradation. SW with inoculation of the degrader *P. putida* KT2440 may have the potential to be integrated into remediation methods to treat TNT-contaminated soil.

In summary, the degradation of the explosives TNT, RDX and their degradation products were significantly enhanced in rhizosphere soils compared to the control. The results from these studies also suggested higher concentrations of non-extractable explosive residues in rhizosphere soils than in the control. Eastern gammagrass has shown a promising potential for remediating RDX-contaminated sites. On the other hand, switchgrass has shown a great potential for remediating TNT-contaminated sites. Inoculation of TNT degrader *P. putida* KT2440 to SW could enhance TNT degradation compared to use SW along.