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Chemically Catalyzed Phytoremediation of 2,4,6-Trinitrotoluene (TNT) Contaminated Soil by Vetiver Grass (Chrysopogon zizanioides L.)

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CHEMICALLY CATALYZED PHYTOREMEDIATION OF 2,4,6-TRINITROTOLUENE (TNT) CONTAMINATED SOIL BY VETIVER GRASS (*Chrysopogon zizanioides* L.)

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We hereby approve the Dissertation

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2,4,6, TRINITROTOLUENE (TNT) CONTAMINATED SOIL BY VETIVER GRASS
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ABSTRACT

CHEMICALLY CATALYZED PHYTOREMEDIATION OF

2,4,6, TRINITROTOLUENE (TNT) CONTAMINATED SOIL BY VETIVER GRASS

(*Chrysopogon zizanioides L.*)

by Padmini Das

Urban sprawl in big cities often encroaches on military land, where residual toxic explosive compounds like 2, 4, 6-trinitrotoluene (TNT) in soil could pose a serious health risk. Additionally, in demilitarized areas, lack of sustainable remediation techniques hinder the much needed residential development.. Phytoremediation is an environmentally safe and cost effective solution; however, the characterized low aqueous solubility of nitroaromatic compounds resulting in limited availability of TNT for plant uptake is a major constraint. To overcome this limitation, we propose a new innovative phytoremediation technique using urea, a common nitrogen fertilizer, as a chaotropic agent, to enhance the solubility of TNT in the soil solutions and thus enhancing the TNT uptake by plant. A multi-process approach was used which included (1) sorption studies to understand the retention/release of TNT in soil solutions in the presence and absence of urea, followed by (2) a greenhouse study to fully characterize the urea catalyzed uptake of TNT using vetiver grass from TNT contaminated soils. This study also aimed to investigate the enzyme-mediated plant detoxification activities and changes in the plant-proteomic profile, to provide important clues to the mechanism of stress response and the TNT-tolerance in vetiver grass. Results showed that the extent of TNT sorption

and chaotropic effectiveness of urea varies with the soil properties, predominately with the soil organic matter. Urea significantly ($p<0.0001$) catalyzed TNT extraction from all soils, suggesting that it mobilizes soil-TNT by increasing its solubility at the solid/liquid interface. Vetiver grass showed high uptake (73%) and significant root-to-shoot translocation (38%) of TNT. Urea significantly enhanced ($p<0.0001$) the vetiver-TNT uptake and translocation. Within the limits of agronomic fertilizer N application rates, 125 mg kg^{-1} of urea was considered optimum for TNT uptake by vetiver grass (82%). However, increasing the urea rate to 1000 mg kg^{-1} further increased the TNT removal (91%). Three metabolites of TNT, such as 2-ADNT, 4-ADNT and 1,3,5-TNB were detected in the plant tissues. The enhanced nitroreductase (NR) enzyme activity in TNT treated vetiver grass suggests the role of NR mediated biochemical mechanism in transforming TNT. The optimum kinetic parameters of the NR enzymes were determined. To the best of our knowledge, this study is the first attempt to investigate the proteomic profiling of a plant under TNT stress. Root proteins showed a significant ($p<0.0001$) negative correlation ($r=-0.97$) with TNT. Proteomics technique with integrated bioinformatics approach revealed downregulation of growth-related proteins and key functional proteins involved in important cellular mechanisms like transcription, translation, ribosome biogenesis, nucleocytoplasmic transport, and protein glycosylation. Plant defense related proteins were upregulated at lower TNT treatments suggesting vetiver's innate defense mechanism against TNT stress. The highly encouraging results of the current study showed the potential of using chaotropically enhanced phytoremediation of TNT contaminated soils using vetiver grass.

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Padmini Das

DEDICATION

To my endearing parents,

Mr. Badal Kanti Das and Mrs. Manorama Das

Who not only brought me up with their unconditional love and enumerable sacrifices but also provided me with a great value system and passion to contribute meaningfully to the society

And

To my teacher, who became my friend, philosopher, and guide for lifetime

Late Sajal Kumar Ghosh

His lifelong dedicated commitment to meaningful education is the cradle of my inspiration and his uncompromised principles always direct me towards the right path…

TABLE OF CONTENTS

Content **Page**

Content **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF ABBREVATIONS

1,3,5-Trinitrobenzene (1,3,5-TNB)

2, 4, 6-trinitrotoluene (TNT)

2,4-Dinitrotoluene (2,4-DNT)

2,6-Dinitrotoluene (2,6-DNT)

2-Aminodinitrotoluene (2-ADNT)

4-Aminodinitrotoluene (4-ADNT)

Agency of Toxic Substance Disease Registry (ATSDR)

Aluminum (Al)

Aminodinitrotoluenes (ADNTs)

Ammonium thiocyanate (NH4SCN)

Base Realignment and Closure (BRAC)

Cation Exchange Capacity (CEC)

Dinitrotoluenes (DNTs)

Dissolved Organic Matter (DOM)

Effective Concentration 50 (EC50)

Electrical Conductivity (EC)

General Accounting Office (GAO, 2003)

High Performance Liquid Chromatography (HPLC)

Iron (Fe)

Lethal Dose 50 (LD50)

Mass Spectrometry (MS)

Matrix-Assisted Laser Desorption /Ionization-Time of Flight (MALDI-TOF)

Nitroreductase (NR)

Nuclear Magnetic Resonance (NMR)

Particulate Organic Matter (POM)

Quality Assurance and Quality Control (QA & QC)

Reference Dose (RfD)

Soil Organic Matter (SOM)

Solid Solution Ratio (SSR)

United States Army Center for Health Promotion and Preventive Medicine (USACHPPM)

United States Department of Agriculture (USDA)

United States Environmental Protection Agency (USEPA)

Vetiver System (VS)

CHAPTER 1

INTRODUCTION

1.1. Introduction

1.1.1. Statement of problem, and need for research

2, 4, 6-trinitrotoluene (TNT) is a group C human carcinogen and a potential mutatoxin (USEPA, 1993; Makris et al., 2007b). Sources of the worldwide environmental contamination of TNT include the war preparation activities (ammunition manufacturing, testing and training; Pennington et al., 2008), wartime activities (detonation; Walsh et al., 2010), and post war activities (sea dumping, dismantling, decommissioning; Stenuit and Agathos 2010). Civilian uses like mining and quarrying activities also majorly contribute to the environmental contamination of TNT. Severe landmine contamination is a major nonmilitary source of TNT in more than 70 countries (Hannam and Dearing 2008). In Africa only, 37 million landmines, which can be contaminated with TNT on their surfaces, are potential sources of TNT in soil, surface water, and groundwater through leaching (Stenuit and Agathos 2010). In United States, 15 million acres of land and over 2000 Department of Defense sites are either reported to be, or is suspected of being polluted with military contaminants like TNT and RDX (GAO, 2003). Potential migration of TNT to groundwater from these soils, as well as from waste disposal

lagoons, is of serious concern. Currently, several of these military sites are in the process of being transferred to non-military entities under the Base Realignment and Closure (BRAC) program. Following army base closures, military land may be offered to the public, but residual soil TNT concentrations may prohibit change of land use to residential development, unless appropriate remedial measures are taken.

TNT causes both ecotoxicological and adverse human health problems (Won et al., 1976; Styles and Cross, 1983; Nyanhongo et al., 2005). It enters the human system through the gastrointestinal tract, skin, and lungs; it is then distributed primarily to the liver, kidneys, lungs, and fat tissues, where it induces chronic diseases. Chronic exposure to TNT can cause aplastic anemia, abnormal liver function, hepatitis, cataract development, skin irritation (Yinon, 1990; Nyanhongo et al., 2005) and cancer in humans. USEPA has issued drinking water health advisory limit for TNT at 2 µg/L based on a lifetime exposure (Table 1, USEPA, 1995; Richardson and Bonmati, 2005). However, to the best of our knowledge, no such criteria still exist for TNT contaminated soil; clean-up levels are rather set on a case-by case manner based on the proximity to groundwater and the extent of soil-contamination. For instance, Adventus Americas (2004) reports that the soil clean up goal for TNT was set as 14 mg kg^{-1} at the Yorktown Naval Weapons Station in Virginia (as cited in Richardson and Bonmati 2005).

Table 1-1. Drinking water health advisories for TNT (USEPA, 1995; Medina et al., 2000; Richardson and Bonmati 2005)

Advisory	TNT concentration (μg L^{-1})
10 kg child (1 day, 10 days, long term)	20
70 kg adult (long term, drinking water equivalent level)	20
70 kg adult (life time)	2
Reference Dose (RfD)	0.5
Maximum contaminant level	2

Due to its persistence in the environment, ecotoxicity, and mutagenicity, the removal of TNT from contaminated military and nonmilitary sites or developing preventive strategies to reduce further damage became high priority for the environmental agencies worldwide (Stenuit and Agathos 2010). However, currently practiced expensive *ex situ* remediation techniques like landfilling and incineration that disrupts ecology by destroying the soil structure and migrate contaminants from one place to another (Peterson et al., 1998). Composting is not a preferred method for TNT contaminated soils as it results into incomplete degradation, which sometimes generates degradants that are more harmful than the parent compound (Larson et al., 2008).

Search for an ecologically viable, cost effective, and reliable method for self-cleaning explosive formulations has led to increased interest in the *in situ* bioremediation

techniques such as natural attenuation, bioaugmentation, and phytoremediation (Hannink et al., 2002; Stenuit and Agathos 2010; Makris et al., 2000c). Application of natural attenuation is not a preferred technique as TNT severely affects some of the naturally abundant soil organisms such as the oligotrophic slow bacteria (George et al., 2009; Stenuit and Agathos 2010). Bioaugmentation using TNT degrading bacteria has similar risk like that is discussed for composting; risk of incomplete remediation and synthesis of more harmful secondary metabolites prevails as TNT does not get completely mineralized by microbes. In comparison, phytoremediation is a sustainable alternative, which showed high promises in extracting and degrading TNT from both contaminated soil and aquatic systems (Hannink et al. 2002).

To develop an effective phytoremediation method for TNT contaminated soils is an immensely complex task whose success depends on a multitude of factors that includes (but are not limited to) the nature and extent of contamination, soil chemistry, binding of TNT to soil materials, and the ability of the target plant to uptake, tolerate the phytotoxic effects, translocate and detoxify the contaminant. Other researchers tested a variety of plant species and showed the effectiveness of some aquatic and terrestrial plants in removing TNT from both aqueous and soil media (Hannink et al., 2002). The effectiveness of phytoremediation is a function of bioavailability of TNT and the ability of the plant to uptake and to tolerate TNT stress (Burken et al., 2000 and Hannink et al., 2002). As evident from prior research, limited plant uptake and potent phytotoxic effects at high TNT concentrations are two major problems in developing an effective

phytoremediation system for TNT contaminated soil (Makris et al., 2007b, Makris et al., 2007c, Hannink et al., 2001, Pavlostathis et al., 1998 and French et al., 1999). Nitroaromatic compounds are characterized with low aqueous solubility. The solubility of TNT in water has been determined to be 101.5 mg L^{-1} at 25^0C and varies widely from 100 to 200 mg L^{-1} at room temperature (Ro et al., 1996; Makris et al., 2007b). Different investigators have reported different values of TNT solubility in water (from 100 to 200 mg L^{-1} at room temperature). The value reported by Ro et al. (1996) compared well with the values reported by the Merck Index and the Lange's Handbook of Chemistry. This study also reported that at higher pH TNT is transformed to other compounds and the solubility decreased. At neutral pH the aqueous solubility was found to be 101.5 mg L^{-1}. Lesser solubility of nitroaromatic compounds can limit plant uptake and hence reduce the effectiveness of phytoremediation. To overcome this problem innovative new techniques are necessary.

Moreover, TNT, like other explosives is a phytotoxic compound. Prior studies reported some plants that are characterized of inherent TNT-detoxification mechanisms, which they exhibit upon exposure to low TNT concentrations. However, at higher concentrations those are commonly found in contaminated sites, these plants exhibit many phytotoxic symptoms like suppressed growth, stunted root and shoot, and chlorosis of leaves. Some aquatic as well as terrestrial plants (Table 2 and 3) were found to have innate TNT detoxification systems through transforming TNT into other metabolites

(Hannink et al., 2002); however the precise enzyme mediated biochemical mechanisms have yet to be fully characterized.

Myriophyllum spicatum, an aquatic plant which was found to have the highest ability to uptake TNT from aqueous solutions (Makris et al., 2007a), showed phytotoxicity leading to chlorosis at initial TNT concentrations above 5.9 μM (Pavlostathis et al., 1998). At present there is no phytoremediation system which can overcome both these difficulties. To develop one, a comprehensive study taking into consideration soil chemical properties, plant physiology, and plant biochemistry is required.

1.1.2. Nature and Scope of Research

Soil properties play an important role in controlling TNT adsorption to soil particles. Thompson et al., (1998) used both hydroponic and soil systems for uptake of TNT by poplar trees. TNT was more bioavailable in the hydroponic system as expected, while 75% of the TNT remained in the soil (Hannink et al., 2002). Potential complexation of soluble TNT by soil organic matter renders the TNT-organic matter complex immobile and hence decreases the phytoavailability of TNT but it cannot prevent the potential migration of TNT to groundwater. Pennington and Patrick, 1990 showed that about 20% of adsorbed TNT was retained after three sequential desorption cycles of a soil which shows the highest ability to adsorb TNT and was most recalcitrant to desorption. This establishes the need of an innovative technology to improve plant TNT uptake.

1.1.3. The Use of Vetiver System as a Potential Phytoremediation Technique:

The Vetiver System (VS), is the application of a fast growing, perennial tropical/subtropical grass named vetiver (*Vetiveria zizanioides* L Nash, now reclassified for its "sunshine" variety as *Chrysopogon zizanioides* L Roberty), for soil and water conservation (Troung et al., 2008). The technology was first developed by the World Bank in India for agricultural land management. Researches during last two decades revealed that because of its exceptional characteristics, vetiver grass can be used as a very effective and sustainable bioengineering tool for environmental protection purposes such as wastewater disposal, prevent soil erosion, steep slope stabilization, and phytoremediation of contaminated land and water (Truong et al., 2008).

Noninvasiveness: Although vetiver grass originated in India and is considered a tropical or subtropical grass, it is not invasive in other parts of the world. The "sunshine" variety of vetiver is categorized as non-invasive by USDA, as it lacks the ability to produce viable seeds and to spread via stolons or rhizomes (Troung et al., 2008). (http://plants.usda.gov/plantguide/pdf/pg_chzi.pdf). For instance, in Fiji, non-native vetiver grass is being used over last 100 years for thatching and it did not show any sign of invasiveness (Troung et al., 2008). U.S. Department of Agriculture has declared it as a non-invasive species and safe to be used for bioengineering purposes.

Cold Tolerance: Vetiver grass has high tolerance to extreme temperature, ranging from -15ºC to +55ºC (Dalton et al., 1996; Truong et al., 2008). In spite of being tropical or subtropical in nature, it can thrive under cold conditions. The above ground growth of vetiver grass becomes dormant under severe winter, but its underground growing points can survive. Optimal temperature of soil is 25ºC was for root growth, but roots of vetiver grass can grow even at 13ºC. Root dormancy occurs at about 5ºC (Truong et al., 2008).

Massive root system: Vetiver grass has an enormous root system, which can go 3-4 m rooting depth in the very first year, making it an extremely efficient phytoremediation agent, as it can remove contaminants from a large area of contaminated land and water (Truong et al., 2008). Vetiver roots are also very fine (0.5-1.0 mm average diameter), which provides an enormous rhizospheric surface area for contaminant absorption and microbial breakdown processes in the root zone (Truong et al., 2008).

Other Advantages:

- The erect and stiff shoots of vetiver grass can grow to 3M (9 feet).
- It is a fast growing high biomass containing (dry matter production up to 100 t ha^{-1} $year^{-1}$) perennial grass. Thus it acquire high efficiency in removing a large volume of contaminants from contaminated lands than most hyperaccumulators (Truong, 2008).

- It can tolerate wide range of soil pH. No soil amendment is required from pH 3.3 to 12.5 (Troung et al., 2008). It can also grow in salinity, sodicity, and high magnesium conditions (Le van Du and Truong, 2010; Troung et al., 2008).
- It is highly resistant to pests as well as pesticides, several diseases, and fire (Troung et al., 2008).
- Another major advantage is its longevity and low cost (Troung et al., 2008). Long-term maintenance costs are low (Truong et al., 2008).

Disadvantages: The main disadvantage of the application of vetiver system is during the establishment phase, vetiver grass is vulnerable to shading that results in stunted growth in less shading and total loss in case of long term shading (Troung et al., 2008). A monitored initial phase (2-3 months in tropical weather and 4-6 months in temperate weather) is required for successful establishment of vetiver systems (Troung et al., 2008). However after the establishment phase, it does not need any maintenance (Troung et al., 2008; Troung et al., 2010).

It is evident that the significant advantages of using VS for bioengineering purposes like phytoremediation overshadow its minor limitations. In our earlier studies vetiver grass showed high effectiveness in removing TNT from aquatic systems (Makris et al., 2007b). Makris et al., (2007a) reported that vetiver grass was much more effective as compared to the majority of the plant species used so far for removing TNT in hydroponic systems, except for *Myriophyllum spicatum (*under similar plant concentrations and initial TNT

loads). Being a terrestrial fast growing perennial grass with high biomass, extensive root system, and potential ability to uptake and transform TNT, vetiver can be strongly recommended for phytoremediation of TNT contaminated soil.

1.1.4. The Use of Urea, a common N-Fertilizer, as a Chaotropic Agent

The use of urea as a chaotropic agent is a potential solution to the problem of limited phytoavailability of TNT (Makris et al., 2007b). Chaotropic agents have been conventionally used in increasing solubility of membrane proteins and dissociating the antigen-antibody complexes (Hatefi and Hanstein, 1969; and David and Hatefi, 1972). Chaotropic agents are specific anions (such as SCN^-) or polar carbamide derivatives (such as urea), which modify the water structure around aggregated proteins or sugars by increasing the solubility of their hydrophobic regions in aqueous environments (Farrah et al., 1981; Makris et al., 2007c). Exact chaotropic mechanism is not yet fully understood; however, Farrah et al. (1981) suggested that chaotropic agents increase the chaos or disorder of the structure of water (Makris et al., 2000c). This disorder helps to reduce the thermodynamic barrier that was raised with the introduction of a hydrophobic compound (like TNT) in water and hence, increase its solubility (Farrah et al., 1981; Makris et al., 2007c). Ammonium thiocyanate (NH_4SCN) is commonly used in gold mining operations to make gold soluble. In plant studies, Anderson et al. (1998) first used ammonium thiocyanate as a substrate amendment to increase the solubility of gold in a phytomining

study. Increasing concentrations of NH_4SCN significantly increased the uptake of gold in plants.

Our group has proposed a new innovative phytoremediation technique using urea which is a common N-fertilizer, as the chaotropic agent to increase the solubility and hence phytoavailability of TNT from aqueous solutions as well as soil. A previous hydroponic study conducted in our laboratory showed the effectiveness of urea as a chaotropic agent to enhance TNT uptake by vetiver grass from aqueous media (Makris et al., 2007b). However, this study has been conducted in aqueous system. Effective application of a new phytoremediation technique in hydroponic system does not promise success in soil, which is a much more dynamic and complex system. Soil properties play important roles in controlling TNT adsorption to soil particles. Thompson et al., (1998) used both hydroponic and soil systems for uptake of TNT by poplar trees. TNT was more bioavailable in the hydroponic system as expected, while 75% of TNT remained in the soil (Hannink et al., 2002). Potential complexation of soluble TNT by soil organic matter renders the TNT-organic matter complex immobile and hence decreases the phytoavailability of TNT (Hannink et al., 2002). Thus soil properties will influence the performance of urea as chaotropic agent in enhancing the solubility of TNT at soil solutions. Before applying urea as a chaotropic agent in phytoremediation system for TNT containing soil, the influences of soil properties on urea extractability are needed to be characterized in the absence of plants.

It is also important to evaluate the performance of urea as a chaotropic agent at the environmentally safe and agronomically recommended urea application rates. Urea application guideline for agricultural crops recommends use of more than 125 mg urea/kg (250 kg ha^{-1}) (EFMA 2000). A consistent yield depression of agricultural crop was found after 350 mg urea kg^{-1} (320 kg N ha^{-1}) (Trierweiler et al. 1983). Beyond 1000 mg kg^{-1} urea exhibited strong toxic effects on earthworms which are considered important indicators of soil health and environmental safety (Xiao et al 2004). Hence, 1000 mg kg^{-1} is the highest level of urea that could be used as a chaotropic agent in soil without affecting the soil health.

1.1.5. Enzyme-Mediated TNT Detoxification Mechanisms in Plant:

Unlike microorganisms, plants do not utilize TNT as an energy source (Hannink et al., 2002). However, numerous studies have reported that different aquatic and terrestrial plants have successfully taken up TNT from hydroponic or soil media and transformed it to other metabolites (Hannink et al. 2002).Table 1-2 and 1-3 enlists aquatic and terrestrial plants that that have been tested so far for their phytoremediation potentials. It is evident from these studies that plants produce similar TNT metabolites, mostly aminodinitrotoluenes (Table 1-2 and 1-3; Hannink et al., 2002). Transformation of TNT to these more polar metabolites are of utmost importance as they carry functional groups which are required for conjugation with plant macromolecules followed by sequestration

into cell vacuole. Direct conjugation is unlikely for TNT as it does not carry these required functional groups (Burken et al., 2000). Thus, as part of their detoxification mechanism plants must transform TNT to other metabolites that have the required functional groups for conjugation and transport. Earlier studies have reported both conjugation and sequestration with TNT metabolites (Hannink et al., 2002). Harvey et al. (1990) for the first time reported the presence of highly polar unextractable products in bush beans following exposure to ^{14}C TNT in a hydroponic system. This study has found 80% of the ^{14}C label was associated with plant biomass indicating most of the carbon associated to TNT was sequestered (Hannink et al., 2002). Thompson et al. (1998a) found 75% of the TNT label in root tissues and 10% in leaves of poplar trees. Bhadra et al. (1999) found four conjugates of TNT in the sterile root culture of *Catharanthus roseus*. Sens et al. (1998) have reported sequestration of TNT in bush bean tissues, 50% in the cytoplasm and the rest in the cell wall associated with lignin, pectin and hemicelluloses. Another study from the same research group demonstrated the compartmentalization of TNT and its metabolites in wheat as 43% in cytoplasm and 57% in cell wall constituents (Sens et al., 1999; Hannink et al., 2002). These notable reports have proved that plants can convert TNT to bound residues, and thus encourage the application of phytoremediation as bound residues are presumably less bioavailable.

Table 1-2: Transformation of TNT by Terrestrial Plants

Plant Type	Initial TNT Load	Experimental Conditions	Metabolites Produced	Reference
Yellow nutsedgej	up to 20 mg L^{-1}	Hydroponic	4-HADNT and ADNTs	Palazzo and Legget, 1986
Bush bean	l0 mg L^{-1}	Hydroponic	2 and 4ADNTs, acid-hydrolyzable conjugates (comprised partly of 2 and 4 ADNTs)	Harvey et al., 1990
Chive Alfalfa	0.1 to l0 mg L^{-1}	Hydroponic	2ADNT and 4ADNT	Gorge *et al.*, 1994
Bush bean, lupin, Purple fringe, Wheat, Rye, Meadow foxtail, Bromegrass, Turf grass, Alfalfa Cat's tail	10 mg kg^{-1}	Soil	2 and/or 4ADNTs	S'cheidemann etal., 1998
Carrots, Radishes Kale Lamb lettuce Bush bean	1 to 200 mg kg^{-1}	Soil	2 and 4 ADNTs, 2,4-DNT and/ or 2,6-DNT	Schneider et al., 1996
Bush bean	10 mg L^{-1}	Hydroponic	2-ADNT, 4-ADNT, 2,4-DNT, 2,6-DNT	Schneider et al, 1996
Madagascar periwinkle root cultures	100 g L^{-1}	Sterile tissue culture	2 and 4 ADNTs	Hughes et al., 1997
Madagascar periwinkle root cultures	25 to 3 1 mg L^{-1}	Hydroponic (sterile)	2 and 4 ADNTs, conjugates TNT-1 and 4-ADNT, TNT-2 and 2-ADNT	Bhadra et al., 1999b
Madagascar periwinkle	25 mg L^{-1}	Hydroponic (sterile)	2 and 4 ADNTs, conjugates TNT-1	Wayment et al, 1999

root cultures			and 4-ADNT- 1, conjugates TNT-1 and 4- ADNT- 1	
Hybrid poplar	32 mg L^{-1}	Hydroponic	2 and 4-ADNT, 2,4-DANT, unknown polar products	Thompson *et al,* 1998
Smooth bromegrass	36 mg L^{-1} (sand solution)	Sand culture system (sterile)	2 and 4-ADNT	Sun *et al,* 2000
Soybean, Barley Alfalfa Chickpea Pea Rye Sunflower Maize	23 mg L^{-1}	Hydroponic	2-ADNT, 4-ADNT, 2,6-DNT	Adamia et al, 2006
Vetiver Grass	40 mg L^{-1}	Hydroponic	Not Studied	Makris et al., 2006a
Vetiver Grass	0, 8, 15, 20, 40 mg L^{-1}	Hydroponic (With Urea)	1,3,5-trinitrobenzene, 2 and 4-ADNT	Makris et al., 2006b
Wheat	0, 8, 15, 20, 40 mg L^{-1}	Hydroponic (With Urea)	1,3,5-trinitrobenzene, 2 and 4-ADNT, Tetryl, Nitrobenzene	Makris et al., 2007
Yellow nutsedgej	up to 20 mg L^{-1}	Hydroponic	4-HADNT and ADNTs	Palazzo and Legget, 1986
Maize, Soybean, Wheat Rice	138 mg kg^{-1}	Soil	Polar metabolites Bound residues	Villa et al. 2007
Orchard grass Perennial ryegrass Tall fescue	11 mg kg^{-1}	Soil	2 and 4-ADNT Unextractable bound metabolites	Duringer et al., 2010

Table 1-3: Transformation of TNT by Aquatic Plants

Plant Type	**Initial TNT Concentration**	**Experimental Conditions**	**Metabolite s Produced**	**Reference**
Parrot feather	2 mg L^{-1}	Hydroponic	2- and 4-ADNTs	Larson *et al.,* 1999
Parrot feather, Arrowhead Pondweed Coontail Water plantain Fox sedge Wool-grass Blunt spikerush Reed canary grass Narrow leaf cat tail	lixplosives-contaminated groundwater containing 0.681 mg L^{-1} TNT , numerous TNT metabolites, and photolysis products	Non-sterile, hydroponic;	2 ADNT and/or 4ADNT and/or 2,4 DNT	Best *et al.,* 1997
Pondweed Reed canary grass Parrot feather	0.99 mg L^{-1}	Non-sterile, hydroponic;	2 and 4 ADNTs and polar metabolites	Best *et al.,* 1997
Parrot feather Eurasian water milfoil	50g L^{-1}	Hydroponic, sterile	2 and 4 ADNTs	Hughes *et al.,* 1997
Eurasian water milfoil	1.3 mg $L^{-1}$to 113.5 mg L^{-1}	Hydroponic, non-sterile	ADNTs, HADNTs, DANTs, 2-2' azoxy tetranitrotoluenes	Pavlostathis *et al,* 1998
Parrot feather	1 to 10 mg L^{-1}	Hydroponic, non-sterile	ADNTs, DANTs, trinitrobenzene and dinitroaniline (photolytic TNT degradation products)	Rivera *et al.,* 1998

Predominance of mono- and di- nitrotoluenes in the environment (Table 1-2 and 1-3) suggests that the reduction of nitro groups are the most preferred mechanisms of TNT degradation in nature. This preference can be well explained analyzing the chemical structure of TNT. TNT contains three nitro functional groups, each of which carries two electro negative elements: nitrogen and oxygen, (Preuss and Rieger, 1995 and Esteve-Nu´n˜ ez et al., 2001). As the electronegativity of oxygen is even more than that of the N atom, the N-O bond gets polarized, with partially positive charge remaining on the N atom (Preuss and Rieger, 1995 and Esteve-Nu´n˜ ez et al., 2001). As a result, the nitro groups tend to remove electron from the aromatic ring and thus become easily reducible (Preuss and Rieger, 1995 and Esteve-Nu´n˜ ez et al., 2001). Other common transformation pathways such as by microbial or plant dioxygenase enzymes are limited because of the symmetric arrangements of TNT's three nitro groups on its aromatic ring (Rieger et al. 1999).

Nitroreductase (NR) enzymes, which are responsible for reducing nitro groups to amines, has been widely found in several plant species (Trombly, 1995). The nitroreductases are found to be involved in the degradation of TNT by bacteria (Kitts et al., 2000), fungi (Rieble, 1994), as well as plants (Adamia et al., 2006; Richardson and Bonmati 2005). NR enzymes are classified into two types based on their sensitivity to oxygen: type I NR, which is insensitive to O_2 as it reduces nitro groups even in aerobic condition using a two electron reduction mechanism, forming hydroxyl-amino and amino derivatives (Peterson et al. 1979; Kitts et al., 2000); and O_2 sensitive type II NR, which uses a single electron reduction mechanism to transform nitro group to a nitro anion radical in strictly anaerobic

conditions (Kitts et al., 2000). If oxygen is present, this nitro anion radical gets oxidized back to a nitro group, also forming a superoxide radical (Peterson et al. 1979; Kitts et al., 2000).

There are relatively very fewer publications on biochemical pathways of TNT detoxifying enzymes isolated from plants. Few researchers have isolated nitroreductase enzyme from plants and used the enzyme extract as the phytoremediation agent without using the whole plant (Medina at al., 2004 and Richardson and Bonmati, 2005). Adamia et al., 2006 has determined nitroreductase activity in plants following an indirect method by measuring the untransformed TNT and thus calculating the rate of TNT reduction.

Plants used so far for the phytoremediation of TNT from both soil and aquatic media, have been found to exhibit TNT detoxifying mechanisms through transformation; however, the enzymes responsible for these processes are yet to be fully characterized (Hannink et al., 2002). Researchers have used an indirect method to determine kinetic parameters of TNT removal without isolating the actual TNT degrading enzyme from the plant tissue (Pavlostathis et al., 1998 and Medina et al., 2002). Pavlostathis et al. (1998) has derived the kinetic parameters assuming that enzymatic activity is proportional to plant concentrations, which is defined as the mass of plant material per unit volume of solution (Medina et al., 2000). The major limitation of this approach is that it assumes that there is only one enzyme responsible for TNT degradation. Moreover, uptake and the sorption on the plant material may complicate the use of this indirect approach (Medina et al., 2000). Other researchers prepared crude enzyme extracts from control plants not

exposed to TNT, and after assaying nitroreductase activity, used that crude enzyme extract as phytoremediation agent instead of using the whole plant (Medina at al., 2004 and Richardson and Bonmati, 2005). However, as the indirect approach suggested increased enzyme activity following TNT exposure (Adamia et al, 2006), it is important to directly assay the NR activity in the plant tissues after being exposed to TNT containing systems. Saturation kinetics of the nitroreductase enzyme as functions of important controlling factors like temperature, and initial substrate concentration need to be normalized for designing an effective phytoremediation system. Specific knowledge on TNT transformation rates in both soil and aquatic systems is limited (Richardson and Bonmati, 2005). Makris et al., (2007b) found two metabolites of TNT such as 2 amino dinitrotoluene and 4 amino dinitrotoluene in the root of vetiver grass suggesting a possible reduction of nitro group had taken place in vetiver root. This indicates a probable activity of a nitroreductase enzyme present in vetiver tissue which needs to be isolated and assayed to obtain the information on enzyme kinetics of the TNT detoxification pathway.

1.1.6. Changes in the plant proteome in response to the TNT stress:

Phytotoxic effect is a strong limitation to the use of plants for remediation purposes of TNT contaminated soil (Hannink et al., 2002). Phytotoxicity at higher TNT concentrations was a common problem faced by almost all researchers working with plants and TNT. This could explain the fact that after having so many successful

laboratory experiments on TNT uptake and transformation, yet phytoremediation technique could not be applied on a large scale to remediate contaminated military sites with high TNT concentrations. Researchers suggested one proposed solution to overcome this phytotoxicity problem is to create transgenic plants which will tolerate the stress associated with the higher TNT concentrations. However, few plants like parrot feather and vetiver grass show much higher tolerance compared to other plants; therefore it is important to understand the mechanism of TNT tolerance in these plants to investigate the innate detoxification systems present in plants which have higher TNT accumulating capacity. One way to do that at the systems level is to study proteomics.

Earlier researchers have successfully developed transgenic plants with enhanced TNT tolerance without investigating the changes in the plant proteomic profiles due to TNT exposure. Two pioneer studies paved the way of genetic engineering as a potential solution of this problem. French et al., 1998 and Hannink et al., 2001 have encouraged the future of phytoremediation of TNT by transferring bacterial nitroreductase into tobacco plants. These studies showed that transgenic plants are much more tolerant to the potent phytotoxic effects of TNT than the wild plants. French et al., (1999) expressed a bacterial, TNT reducing enzyme PETN reductase to construct transgenic tobacco plants. Transgenic tobacco plants tolerated TNT concentrations which produced deadly effects in wild type plants. Hannink et al., (2001) expressed a bacterial nitroreductase isolated from the soil organism *Enterobactor cloacae* into tobacco plants (Hannink et al., 2002). Transgenic tobacco plants expressing bacterial nitroreductase, showed a dramatically

enhanced ability compared to the wild plants, to tolerate, take up and detoxify TNT (Hannink et al., 2001 and 2002). The TNT tolerance of the transgenic tobacco plants were reported to be enhanced to such an extent that they tolerated up to 0.5 mM (114.3 mg L^{-1}) TNT, which is the aqueous solubility limit of TNT (Hannink et al., 2002). These studies are extremely encouraging to the future of phytoremediation of TNT contaminated systems. The enhanced TNT metabolism of transgenic tobacco indicates that introducing bacterial nitroreductase into fast growing, deep rooted plants like vetiver grass which is more suitable for phytoremediation of TNT, would significantly increase TNT removal in the field. In our previous preliminary hydroponic experiments vetiver grass exhibited minimal phytotoxic effects followed by the exposures to different TNT concentrations. However, upon increasing the initial TNT loads the phytotoxic effect may increase. It is necessary to find out what are the phytotoxic effects of TNT concentrations on vetiver grass and its ability and extent of tolerating the TNT toxicity. Our long term goal is to develop a transgenic vetiver grass by transferring a bacterial TNT detoxifying gene to the wild vetiver grass. We assume that the transgenic vetiver will show more tolerance to the higher concentrations of TNT found in the contaminated military sites. But before proceeding to that we need to understand vetiver's natural detoxifying mechanism for TNT.

Plant proteins play major roles in controlling the stress related mechanisms followed by exposure to contaminants (Ahsan et al., 2009). Loss of some functional proteins interrupts the biological processes of the plant and produce phytotoxic effects whereas

some plants generate proteins which take part in detoxification pathways and give the plant tolerance to the contaminants. Proteomics is a new approach for studying complex biological functions of proteins which are helpful to identifying the molecular mechanisms those play key roles in plant-contaminants interactions (Ahsan et al., 2009). For example, Gillet et al., 2006 found that in algae, the abundance of proteins involved in photosynthesis were significantly decreased on exposure to cadmium stress, whereas proteins related to the defense mechanisms such as GSH biosynthesis, ATP metabolism, and the response to the oxidative stress were significantly increased.

Most of the proteomics studies conducted so far investigated the changes in plant proteome following exposure to the toxic metals. However, similar phytotoxic effects caused by TNT indicate that studying the changes in the abundances of protein will help in understanding the stress related mechanisms caused by TNT exposure. The uptake of increasing levels of TNT by plant cells severely interrupts various physiological and biochemical pathways leading to a restriction of plant growth and ultimately cell death. The identification of the functional proteins that are involved in responses to TNT stress is a fundamental step in understanding the molecular mechanisms of stress response. Such an understanding could lead to the development of transgenic plants that have an enhanced tolerance to the stress associated with high TNT concentrations.

1.2. Research Objectives

The ultimate goal of this study was to develop a cost effective, in-situ phytoremediation technique to overcome a major limitation of the phytoremediation of TNT contaminated soils, i.e. limited plant uptake due to low aqueous solubility of TNT. This study also aimed to investigate the biochemical mechanisms in vetiver grass to detoxify TNT and determine the changes in the plant proteome as consequences of exposures to different TNT concentrations.

1.2.1. Central hypotheses

This project was based on three central hypotheses.

1. Use of urea as a chaotropic agent will enhance the plant TNT uptake. TNT uptake and the effect of urea will be functions of soil properties, TNT loads and urea application rates.

2. Biochemical mechanism of TNT tolerance and detoxification in vetiver grass is mediated by the TNT degrading enzyme(s) synthesized in the vetiver tissues. Rate of TNT degradation is influenced by the factors such as enzyme activity, temperature, and initial TNT concentrations.

3. Exposure to increasing TNT concentrations will cause significant changes in the plant proteome which will include increase in the proteins associated with the

detoxification mechanism and loss of some functional proteins which will result into phytotoxic effects.

1.2.2. Specific Aims

By performing the proposed research, these hypotheses were tested and relevant research questions were answered. These questions were answered by pursuing the following specific aims.

Specific Aim 1: Characterize retention or release of TNT as functions of soil properties, exposure time and initial TNT concentrations.

Specific Aim 2: Evaluate urea catalyzed extractability of TNT from contaminated soil as function of soil properties, initial TNT and urea load, and reaction time.

Specific Aim 3: Evaluate the use of a common agrochemical urea as a chaotropic agent, to enhance TNT phytoremediation by vetiver grass in soil systems.

Specific Aim 4: Identify the biochemical mechanisms behind detoxification of TNT by isolating, identifying, and quantifying TNT degrading enzyme from vetiver grass.

Specific Aim 5: Optimize factors influencing the kinetics of TNT removal and saturation kinetics of the TNT detoxifying enzyme isolated from the vetiver grass.

Specific Aim 6: Study the effects of TNT exposure on vetiver grass in a controlled environment using morphological, physiological and proteomic approaches.

1.3. Organization of Thesis

These specific objectives were accomplished and the research findings were written and discussed in this dissertation, in various chapters as organized bellow.

Chapter 2 entitled "Vetiver grass is capable of removing TNT from soil in the presence of urea" documented the preliminary findings, showing the ability of urea-vetiver system in TNT removal from a soil containing minimal TNT retention capacity. This chapter was published in Environmental Pollution (158 (2010) 1980–1983. DOI: 10.1016/j.envpol.2009.12.011).

Chapter 3 entitled "Effectiveness of Urea in Enhancing the Extractability of 2,4,6 Trinitrotoluene from Chemically Variant Soils" documented the retention and release of TNT in chemically variant soils and characterized the urea catalyzed TNT extraction as functions of soil properties and agriculturally recommended and environmentally safe

urea application rates (in absence of plants). This chapter was published in Chemosphere, 93:9: 1811-1817. (http://dx.doi.org/10.1016/j.chemosphere.2013.06.028).

Chapter 4 entitled "Urea-Catalyzed Uptake and Nitroreductase Enzyme-Mediated Transformation of 2,4,6-Trinitrotoluene in Soil using Vetiver Grass: A Greenhouse Study" fully characterized this stimulative phytoremediation technique using urea-vetiver system and reported the kinetics of TNT removal, uptake, translocation, enzyme mediated biotransformation of TNT by vetiver grass and potential leaching of TNT and metabolites in presence/absence of urea. Two papers will be submitted to different journals for publication. Part of this chapter was published in Journal of Environmental and Chemical Engineering, 3: 1: 445 – 452 (DOI:10.1016/j.jece.2015.01.008). The other part will be submitted to another appropriate journal for publication.

Chapter 5 entitled "Optimization of Kinetic Factors Influencing the Nitroreductase Enzyme Mediated Phyto-transformation of 2,4,6-Trinitrotoluene (TNT) by Vetiver Grass" reported the saturation kinetics of the nitroreductase enzyme mediated TNT transformation as functions of plant concentrations in the crude enzyme extracts, temperature, and substrate concentration, three major factors that significantly influence transformation of TNT to amines mediated by nitroreductase enzyme. One paper will be submitted to a journal for publication.

Chapter 6 entitled “Proteomic Profiling of Vetiver grass under 2,4,6 Trinitrotoluene (TNT) stress” documented the effect of increasing TNT concentrations on growth, chlorophyll content, total protein content of vetiver grass and reported the changes in vetiver’s proteomic profile following TNT stress. One paper will be submitted to a journal for publication.

In conclusion, the section entitled “Environmental Implications” summarized the significant findings of this research project; explained how these findings contribute to the body of knowledge; and why this sustainable innovative green technology could be an effective solution for wide range of TNT contaminations in military sites.

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CHAPTER 2

VETIVER GRASS IS CAPABLE OF REMOVING TNT FROM SOIL IN THE PRESENCE OF UREA

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Abstract

The high affinity of vetiver grass for TNT and the catalytic effectiveness of urea in enhancing plant uptake of TNT in hydroponic media we demonstrated earlier were further illustrated in this soil-pot experiment. Complete removal of TNT in urea-treated soil was accomplished by vetiver at the low initial soil TNT concentration (40 mg kg^{-1}), masking the effect of urea. Doubling the initial TNT concentration (80 mg kg^{-1}) resulted in significantly ($p<0.002$) increased TNT removal by vetiver, in the presence of urea. Without vetiver grass, no significant ($p=0.475$) change in the soil-TNT concentrations was observed over a period of 48 days, suggesting that biological degradation of soil TNT was not responsible for the documented TNT disappearance from soil.

2.1. Introduction

The U.S. Environmental Protection Agency (U.S. EPA) has classified 2,4,6 trinitrotoluene (TNT) as a group C human carcinogen (U.S. EPA, 1991). Downward migration of TNT to groundwater from explosive-contaminated sites and related wastewater lagoons is of serious concern. Numerous military sites in the U.S. are in the process of being transferred to non-military entities under the base realignment and closure (BRAC) program. Following army base closures, military land may be offered to the public, but residual soil TNT concentrations prohibit change of land use, unless appropriate remedial measures are taken. High costs and environmental concerns associated with most *ex-situ* remedial practices for TNT-contaminated soils have built interest in *in-situ* bioremediation practices (Makris et al., 2009). Our group has been investigating novel *in-situ* bioremediation methods for the restoration of TNT-contaminated sites. In a previous hydroponic study, we showed that vetiver grass exhibited high uptake capacity for soluble TNT (Makris et al., 2007a). The current study performed in a greenhouse setting showed that vetiver can remove TNT from soil as well, by utilizing the stimulative phytoremediation method. Stimulative phytoremediation is an *in-situ* bioremediation method for nitroaromatics that stems from the synergistic combination of phytoremediation and biostimulation via the use of nutrient/chaotropic agent amendments. The limited phytoavailability of soil-TNT prompted us to test the stimulative phytoremediation method, using urea as a chaotropic agent, to enhance the solubility and plant uptake of TNT. Addition of urea altered the water structure, reducing

the thermodynamic barrier associated with the introduction of a hydrophobic compound (TNT), thus increasing TNT solubility and plant uptake in a hydroponic setup (Makris et al., 2007a).

Under conditions of similar initial TNT concentration, vetiver grass was superior to other plant species in removing TNT from aqueous media (Makris et al., 2007a, b), but its ability to take up TNT from soil is yet to be evaluated. Soil properties play an important role in controlling soil particle-bound TNT availability to plants/trees and soil biological organisms (Pennington and Patrick 1990). Eriksson et al. (2001) showed that mobility of TNT in soil primarily depended upon the soil organic matter (SOM) content. This short study was conducted to test the effectiveness of stimulative phytoremediation using the urea-vetiver system in enhancing TNT removal from Immokalee soil which has low SOM. The objective of this study was to evaluate the effectiveness of urea as a chaotropic agent in enhancing TNT removal by vetiver grass from TNT-contaminated soil.

2.2. Materials and Methods

The soil-pot study was conducted in a greenhouse setting with the following treatments: i) three TNT concentrations (0, 40, 80 mg kg^{-1}); and ii) two chaotropic agent (urea) concentrations (0 and 1000 mg urea kg^{-1}). Assuming that TNT would be less available for plant uptake from soil when compared to the hydroponic system (Thompson et al., 1998), 1000 mg urea kg^{-1} (1045 kg urea-N ha^{-1}assuming a 15cm soil depth) rate was used, which

was the highest urea concentration tested during the hydroponic study (Makris et al., 2007b, c). This is also the highest concentration of applied urea complies with current agronomic and environmental guidelines (Xiao et al., 2004).

The Immokalee soil (pH 6, >90% sand, and 0.8% soil organic matter) (Sarkar et al., 2005) was collected from the surface horizon in the Southwest Florida Research and Education Center, Immokalee, Florida. Vetiver plants were allowed to acclimatize for 2 weeks in uncontaminated (no TNT) Immokalee soil. After two weeks, plants were transferred to the TNT-spiked soil pots, reaching uniform plant concentrations of 30 ± 0.5 g kg^{-1}.

Three TNT-free control soil pots were set up with vetiver grass to compare the potential toxic effects of TNT on TNT amended plants. Six plant- and urea-free, TNT-amended soil pots (40 and 80 mg kg^{-1}TNT) were also included to investigate any TNT losses due to biodegradation. All treatments were performed in triplicates. Pots were wrapped with aluminum foil to prevent potential TNT photodegradation.

Experiments were carried out until near complete removal of TNT (12 days) from the spiked soil with 40 mg kg^{-1} TNT. Soil samples were collected after 3 days to monitor soil TNT removal kinetics by vetiver grass. Periodic soil samples were collected to evaluate the kinetics of TNT removal from soil by using a nondestructive soil sampling approach. Soil samples were collected from the same soil pot for a treatment at different sampling

intervals. Soil samples were collected at different depths randomly and were mixed to make a representative composite sample. Previous phytoremediation studies on TNT contaminated studies reported that microbes present in the system play major roles in controlling the removal of TNT (Hannink et al., 2002). The microbes present in the root zone and in the bulk soil play different and significant roles in TNT removal from soils (Scheidemann et al., 1998). As in the scope of current study, the soil-microbial population was not controlled; a destructive soil sampling approach would have added more variation within the treatments. Thus, nondestructive sampling approach was used for collecting periodic soil samples.

Soil microbial community can play major role in decreasing soil-TNT by transforming TNT to metabolites (Hughes et al. 1997). The biological augmentation of TNT in soil was investigated by including plant-free, TNT-amended soil pots in the greenhouse for 48 days; soil samples were collected after 0, 12, 22, 32, 41, and 48 days for TNT estimation.

Residual TNT in soil was extracted using the USEPA 8330 method, and analyzed using HPLC system (Prostar, Varian inc., USA) equipped with a UV/VIS absorbance detector (U.S. EPA, 1997, Makris et al., 2007b). Reaction rates of TNT removal by vetiver grass from soil were calculated as described by Pavlostathis et al. (1998), and Makris et al. (2007b). Statistical analyses were performed using the JMP IN version 5.1 (Sall et al., 2005).

2.3. Results and Discussion

After 12 days of exposure to soil-TNT, vetiver plants did not show any phytotoxic symptoms for the 40 mg kg^{-1} TNT load. For the 80 mg kg^{-1} TNT load, vetiver developed yellow coloration on leaves after 7 days, but there was no diminishing effect on root and shoot growth. Control (no TNT) plants were used to study the effect of TNT on growth. After 12 days, plant-, and urea-free soil pots treated with 40 and 80 mg kg^{-1} initial TNT loads showed 27% and 7.5% decrease in TNT respectively (Figure 1). After the completion of the phytoextraction experiment, no significant ($p>0.05$) difference was observed for the soil TNT concentrations between 12 and 48 days. The small decrease in the soil TNT concentrations observed in the absence of vetiver grass and urea could be ascribed to the indigenous microbial population.

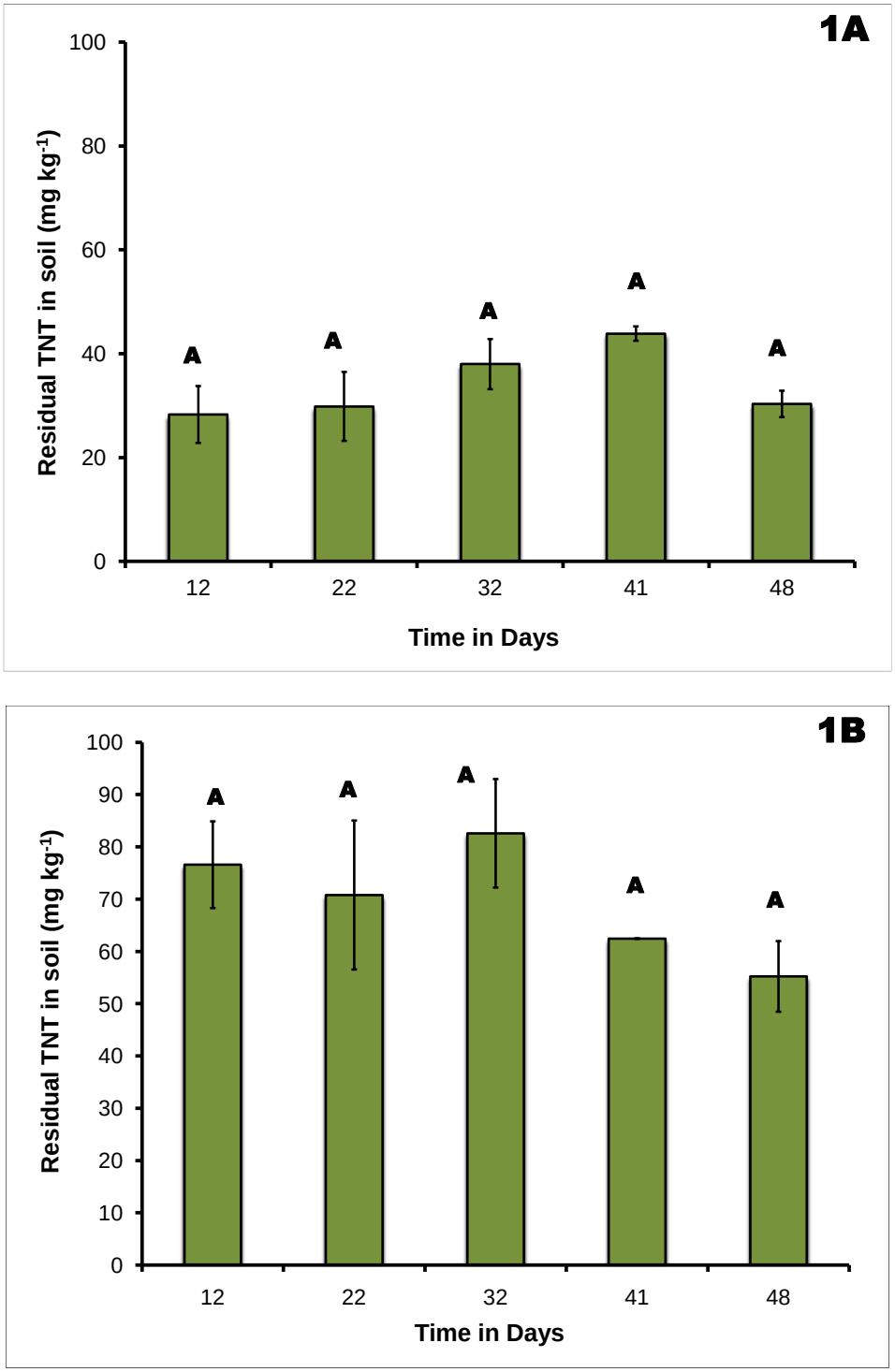

Figure 2-1. Residual TNT in soils (mg kg^{-1}) initially treated with 40 mg kg^{-1} (1A) and 80 mg kg^{-1} (1B) TNT in plant-free, TNT-amended controls. Data are expressed as mean (n = 3) $\pm$ 1 standard deviation.

Vetiver grass significantly ($p<0.001$) decreased soil TNT concentrations (both in presence and absence of urea) compared to the TNT amended-no-plant controls (Figure 2-2). After 3 days, TNT reduction by vetiver grass from soil treated with 40 mg kg^{-1}TNT reached 97% (Figure 2-2A) and remained unchanged until the 12^{th} day (Figure 2-2B). Doubling the initial TNT concentration (80 mg kg^{-1}), resulted in 39% and 88% TNT removal by vetiver grass after 3 and 12 days, respectively (Figure 2A, B). Pavlostathis et al. (1998) reported that TNT disappearance from soil is a function of both plant concentration and initial TNT concentrations. TNT removal by different plants in hydroponic media (Adamia et al., 2006; Makris et al., 2007b,c) as well as from soil (Scheidemann et al., 1998) decreased with increasing TNT concentrations. In accordance with our hydroponic results (Makris et al., 2007b), this soil-pot-experiment suggests gradual saturation of vetiver's TNT adsorption capacity with increasing initial TNT loads.

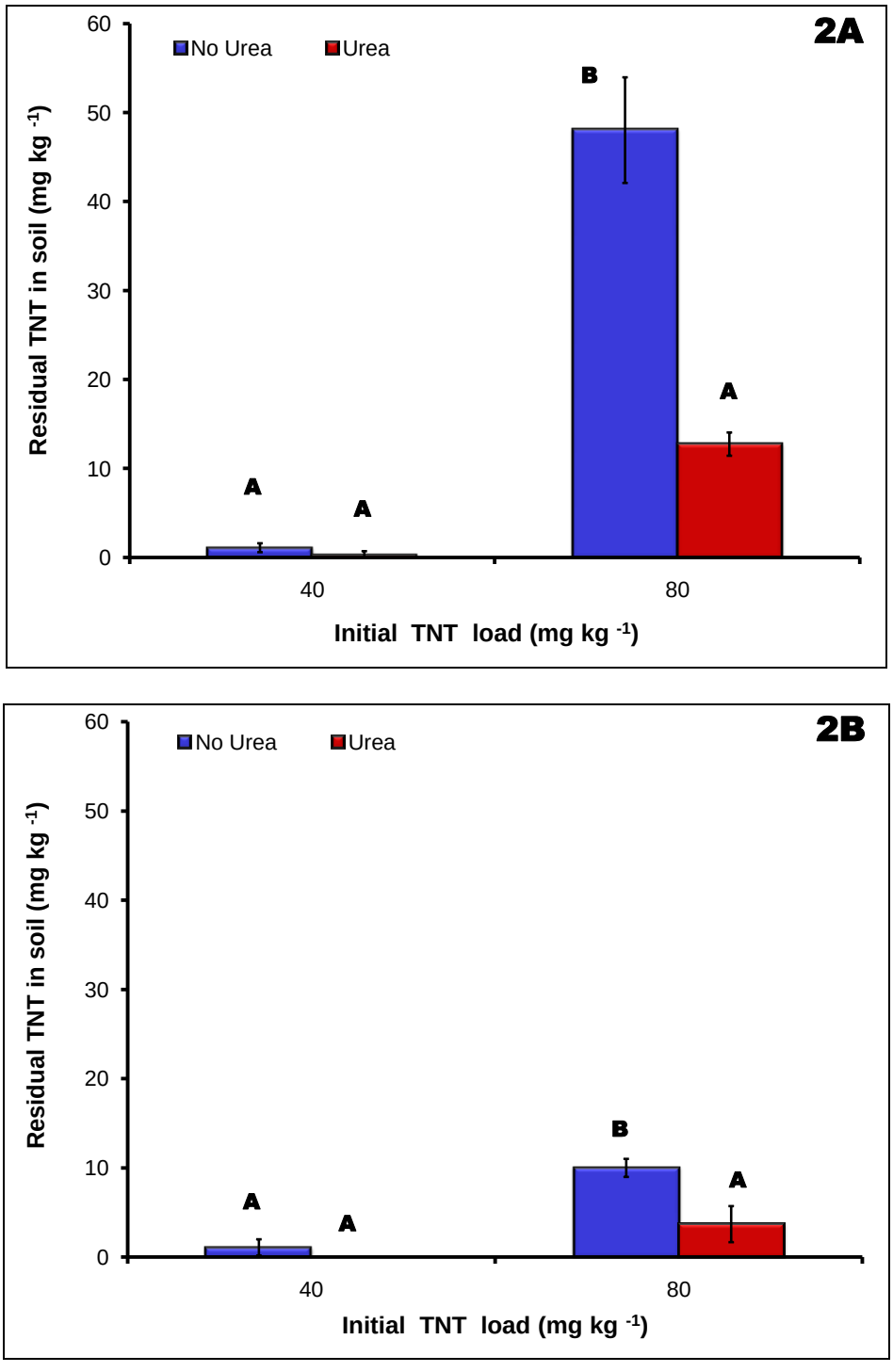

Figure 2-2. Residual TNT in soils (mg kg^{-1}) initially treated with 40 mg kg^{-1} and 80 mg kg^{-1} TNT with two urea concentrations (0 and 1000 mg kg^{-1}) in presence of vetiver grass after 3 days (2A) and 12 days (2B). Data are expressed as mean (n = 3) $\pm$ 1 standard deviation.

Addition of urea significantly ($p<0.001$) enhanced the soil-TNT removal by vetiver grass. After 12 days, complete removal of TNT was observed in soils treated with 40 mg kg^{-1} TNT. However, at this TNT load, no significant difference was observed in soil TNT concentrations between the plant treatments with urea (100% TNT removal) or without (97% TNT removal), masking any urea effect. At 80 mg kg^{-1} TNT load, soil TNT concentration decreased by 84% in the presence of urea within 3 days, while in the absence of urea only 39% was removed by vetiver (Figure 2). After 12 days, urea-vetiver system achieved 95% TNT removal, which was significantly higher than the untreated (no urea) vetiver treatment (84% removal).

Pseudo first order (k_1) and plant-normalized second order (k_p) reaction rate constants were calculated to describe TNT removal kinetics by vetiver grass in the presence and absence of urea (Table 1). Results show, k_1 and k_p values were higher in urea treatments when compared to the untreated (no urea) controls. However, after 3 days, the differences in these rate constants between urea treated and untreated pots at the lower TNT treatments were not significant, suggesting that urea effect was masked by the high affinity of vetiver grass for TNT at lower initial load. Similar rate constants in higher concentration after 12 days can be explained by the phytotoxic effects that were observed after 7 days in vetiver grass exposed to 80 mg kg^{-1} TNT. k_1 values at 40 mg kg^{-1} TNT treatments were lower than those reported by Makris et al., 2007b in the hydroponic systems with 40 mg l^{-1} initial aqueous TNT concentrations. In the absence of urea, the k_1 value obtained in the present soil study (k_1 = 0.014 h^{-1}) is significantly lower than the k_1

(0.029 h^{-1}) reported in the hydroponic study. In hydroponic system, TNT may be more readily available to plants whereas soil-bound TNT was less available for plant uptake. In the presence of urea, these values are not significantly different from each other (k_{1soil} =0.022h^{-1}; $k_{1hydroponic}$=0.026h^{-1}). This dataset indicated that the presence of urea helps to release the soil-bound TNT to solution and hence enhanced its phytoavailibility. Chaotropic effects of urea catalyzed the TNT removal capacity by vetiver grass from soil due to the water structure modifications around soil particle surfaces that increased TNT solubility at particle/solution interface and thus enhanced potential for adsorption by root hair.

Table 2-1. Reaction rate constants during TNT removal from soil using vetiver grass. Plant concentrations were 30 g kg^{-1}. The k_p values were calculated by dividing k_1 by the plant concentrations. Mean separation was conducted for each initial TNT concentrations for each day separately. Treatments with different superscript letters are significantly different at the 95% confidence interval.

Time(day)	Urea (mg kg^{-1})	Initial TNT (mg kg^{-1})	Pseudo first order rate constant k_1(h^{-1})	Plant-normalized second order rate constant k_p (kg d^{-1} g^{-1})
3	0	40	0.051± 0.01[a]	0.041± 0.01 [a]
3	1000	40	0.062 ± 0.01[a]	0.050± 0.01 [a]
3	0	80	0.007± 0.00 [a]	0.006± 0.00 [a]
3	1000	80	0.026± 0.00 [b]	0.020± 0.00 [b]
12	0	40	0.014± 0.00 [a]	0.011± 0.00 [a]
12	1000	40	0.022± 0.00 [b]	0.017± 0.00 [b]
12	0	80	0.007± 0.00 [a]	0.006± 0.00 [a]
12	1000	80	0.015± 0.01 [a]	0.012± 0.01 [a]

This preliminary soil-pot experiment validates the encouraging results obtained in the hydroponic studies (Makris et al., 2007a,b,c). The urea-stimulated phytoremediation method for a TNT-contaminated soil was effective in enhancing TNT phytoextraction from soil. The enhanced rate of phytoextraction of TNT in the urea treatment suggested that urea facilitated the release of soil-bound TNT into soil solution, making it more phytoavailable. However, the processes governing urea-catalyzed release of previously sorbed TNT from soil need to be investigated in both the presence/absence of plants. The present study has evaluated the effectiveness of the highest possible urea application rate (1000 mg kg^{-1}) in soil to enhance the TNT removal by vetiver grass from soil. Recommended agronomic urea application rates for agricultural crops (125 to 350 mg kg^{-1}) were lower than that used in this study (EFMA, 2000, Fenn et al., 1987, Trierweiler et

al., 1983). Soil pot experiments are underway to evaluate the effect of urea as a chaotropic agent using various urea application rates. Further studies on the proposed stimulative phytoremediation method are necessary to ascertain the extent of TNT sequestration by vetiver grass as well as its transformation within the plant tissue.

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CHAPTER 3

Effectiveness of Urea in Enhancing the Extractability of 2,4,6 Trinitrotoluene from Chemically Variant Soils

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Abstract

One of the major challenges in developing an effective phytoremediation technology for 2, 4, 6-trinitrotoluene (TNT) contaminated soils is limited plant uptake resulting from low solubility of TNT. The effectiveness of urea as a solubilizing agent in increasing plant uptake of TNT in hydroponic systems has been documented. Our preliminary greenhouse experiments using urea were also very promising, but further characterization of the performance of urea in highly-complex soil-solution was necessary. The present study investigated the natural retention capacity of four chemically variant soils and optimized the factors influencing the effectiveness of urea in enhancing TNT solubility in the soil solutions. Results show that the extent of TNT sorption and desorption varies with the soil properties, and is mainly dependent on soil organic matter (SOM) content. Hysteretic desorption of TNT in all tested soils suggests irreversible sorption of TNT and indicates the need of using an extractant to increase the release of TNT in soil solutions. Urea significantly ($p<0.0001$) enhanced TNT extraction from all soils, by increasing its solubility at the solid/liquid interface. Soil organic matter content and urea application

rates showed significant effects, whereas pH did not exert any significant effect on urea catalysis of TNT extraction from soil. The optimum urea application rates (125 or 350 mg kg^{-1}) for maximizing TNT extraction were within the limits set by the agronomic fertilizer-N rates used for major agricultural crops. The data obtained from this batch study will facilitate the optimization of a chemically-catalyzed phytoremediation model for cleaning up TNT-contaminated soils.

3.1. Introduction

2,4,6 trinitrotoluene (TNT) is a major component of composition B (Comp B), a commonly used military formulation composed of toxic explosive compounds (Douglas et al., 2012). Due to its long persistence in the environment and its toxic and mutagenic effects on organisms, including humans, substantial efforts have been put into developing effective remediation techniques for TNT contaminated soils. Most of the contaminated sites use traditional *ex situ* remediation methods like incineration - which destroys soil structure and disrupts ecology - and dumping which displaces untreated contamination to another site with potential leaching into groundwater (Peterson et al., 1998). However, over the past two decades, the search for a cost-effective, ecologically safe and environmentally sound remediation technique has led to the development of *in situ* remediation processes like immobilization of TNT using surface amendments (Hatzinger et al., 2004; Fuller et al., 2005; Douglas et al., 2012), bioremediation using TNT degrading bacteria and fungi (Nyanhongo et al., 2005), and phytoremediation using TNT accumulating plants (Hannink et al., 2002).

Bioremediation of TNT has met with mixed success due to the variability in binding of TNT to various soil types (Larson et al., 2008). Limited bioavailability resulting from the low aqueous solubility of TNT (100 to 200 mg L^{-1} at room temperature; Ro at al., 1996) restricts plant uptake and reduces the effectiveness of phytoremediation. To overcome this problem, we propose using a solubilizing agent as an amendment to increase the solubility of TNT in soil solutions, thereby enhancing the uptake of TNT by plants. Our

group has proposed a new innovative phytoremediation technique using urea - a common N-fertilizer - as the solubilizing agent to increase solubility and phytoavailability of TNT from aqueous solutions and soil. Urea has long been used as a chaotropic agent in increasing solubility of membrane proteins and dissociating antigen-antibody complexes (Hatefi and Hanstein, 1969). Chaotropic agents are specific anions (SCN^-) or polar carbamide derivatives (urea) which modify the water structure around aggregated proteins or sugars, increasing the solubility of their hydrophobic regions in aqueous environments (Farrah et al., 1981). Ammonium thiocyanate (NH_4SCN), a commonly used chemical in gold mining operations to make gold soluble, was successfully used in phytomining studies, enhancing the uptake of gold by plants from aqueous media (Anderson et al., 1998). Although the effectiveness of urea versus thiocyanate in enhancing plant uptake of TNT from hydroponic systems was not compared, urea has much lower toxicity as compared to thiocyanate and its extensive use in agriculture over decades makes it a better choice for as a solubilizing agent in TNT contaminated sites (Makris et al., 2007a).

Our initial attempts of using urea to enhance the plant-TNT uptake were highly encouraging. Makris et al. (2007a, b) showed the effectiveness of urea as a solubilizing agent to enhance TNT uptake by vetiver grass and wheat from aqueous media. Presence of urea significantly increased the solubility of TNT at the root-hair/solution interface and hence increasing the TNT removal capacity and kinetics by different plants, irrespective of their varied affinity for TNT (Makris et al., 2007a). To investigate the applicability of

this technique in soil, a preliminary soil-pot experiment was conducted using an acidic sandy soil which contains minimal TNT retaining capacity (Das et al., 2010). Significant ($p<0.001$) urea enhancement of TNT removal by vetiver grass was achieved, allowing for complete removal of TNT from soil treated with 40 mg kg^{-1} TNT and 1000 mg kg^{-1} urea within 12 days (Das et al., 2010). However, the successful application of this technique in hydroponic systems and one soil which contains minimal TNT retention capacity does not promise its success in all soil environments.

Prior research has shown that retention and release of TNT at soil solutions highly varies with soil properties (Pennington and Patrick, 1990; Eriksson and Skyllberg, 2001). Hassett et al. (1983) suggested that nonpolar organic compounds such as TNT are distributed between water and SOM through hydrophobic partitioning. Eriksson and Skyllberg (2001) showed that the retention of TNT in soil is dependent on the ability of solid phase particulate organic matter (POM) to adsorb TNT. On the other hand, binding of TNT metabolites to dissolved organic matter (DOM) increases the mobility and possible transportation of TNT and its metabolites into soil solutions. The association between hydrophobic contaminants like TNT and SOM strongly limits its bioavailability and hence causes contaminant stability and prolonged persistence in the soils (Singh et al., 2010).

Another major challenge in developing the urea catalyzed phytoremediation technique for TNT is to optimize the solubilizing effect of urea within the environmentally safe and

agro-recommended urea application rates. Urea application guideline for agricultural crops recommends use of more than 125 mg urea kg^{-1} (250 kg ha^{-1}) (EFMA 2000). A consistent yield depression of agricultural crop was found after 350 mg urea kg^{-1} (320 kg N ha^{-1}) (Trierweiler and Omar, 1983). Makris et al., 2007b suggested that solubilizing effectiveness of urea may or may not be achieved unless urea application rates greater than those used in agriculture are applied. However, beyond 1000 mg kg^{-1} urea exhibited strong toxic effects on earthworms, which are considered as important indicators of soil health and environmental safety (Xiao et al., 2004). Hence, 1000 mg kg^{-1} is the highest level of urea that can be used in soil without affecting the soil health. Another concern regarding the use of urea lies in the stability of urea in different soil environments (Makris et al., 2007a). Abundance of urease enzyme in soil and variation in soil pH may cause instability of urea and hence undermine its effectiveness in increasing the solubility of TNT in soil solutions.

Therefore, optimizing this remediation technique as a function of SOM and different urea application rates is of utmost importance to understanding its applicability in different soils environments. In the present study, batch adsorption, desorption, and extraction experiments were conducted to optimize the factors that may influence the effectiveness of urea in enhancing the TNT solubility in soil solutions. The specific objectives of the studies were i) characterizing adsorption and desorption of TNT, in absence of urea, as functions of soil properties; ii) characterizing the urea-catalyzed extraction of pre-adsorbed TNT in soil solutions as functions of soil properties and urea application rates;

iii) examining the stability of urea in different soils during the experiment; and iv) investigating the effects of reaction time and pH on the urea-catalyzed extraction of TNT.

3.2. Materials and Methods

Soils: Four soils were chosen based on their widely varied physico-chemical properties primarily focusing on their soil organic matter content: (1) Immokalee, an acid sand which contains minimal SOM (0.84%). (2) Millhopper, an acidic sandy loam with low pH and relatively low SOM content (4.38%), which is higher than that of Immokalee. (3) Orelia is an alkaline soil with moderate soil organic matter (23.9%). And (4) Belleglade is an acidic sandy soil but has very high organic matter content (85.4%). The Immokalee series soils were collected from surface horizons in the Southwest Florida Research and Education Center, Immokalee, Florida. Millhopper series soils were collected from the surface horizons in the University of Florida campus at Gainesville, FL. Orelia series soil was collected from Corpus Christi, Texas. Belleglade soil was collected from Everglades Research and Education Center at Belle Glade, Florida. Soil properties are summarized in Table 3-1.

Table 3-1. Physico-chemical properties of soils. (reproduced from Datta and Sarkar, 2005)

Properties	Immokalee	Millhopper	Orelia	Belleglade
pH	6.0	6.4	8.2	5.9
EC††† (μs cm^{-1})	59	145	203	558
CEC†† (C mol kg^{-1})	777	2356	3810	18,908
SOM† (%)	0.84	4.38	23.9	85.4
Clay Content (%)	0.57	1.62	21.91	4.67
Oxalate extractable Fe+Al (mg kg^{-1})	66	704	380	1957
Total Fe+Al (mg kg^{-1})	212	4745	6100	6010

††† Electrical Conductivity, ††Cation Exchange Capacity, † Soil Organic Matter

3.2.1. TNT

2,4,6-trinitrotoluene (TNT) was purchased from Chem Service (West Chester, PA, USA) in an aqueous slurry form. TNT was air-dried, dissolved in acetonitrile, and stored in the dark at 4°C. HPLC-grade standards of TNT and its eleven metabolites, including 1,3,5-trinitrobenzene (1,3,5-TNB), 4-amino 2,6-dinitrotoluene (4-ADNT), and 2-amino 4,6-dinitrotoluene (2-ADNT), 1,3-dinitrobenzene (1,3-DNB) were purchased from AccuStandard (New Haven, CT, USA).

3.2.2. Sorption and Desorption Studies

Kinetic adsorption and desorption experiments were carried out at two initial TNT concentrations (5 mg L^{-1} and 25 mg L^{-1}) for 0, 1, 1.5, 2, 2.5, 5, 10 and 24 h, to determine the effect of contact time on TNT adsorption and desorption in the soils. To determine the effects of initial TNT concentrations on retention/release of TNT in these soils, equilibrium adsorption and desorption experiments were conducted on each soil, using six initial concentrations of TNT (1, 5, 10, 25, 50 and 100 mg L^{-1}) for 24 h. Two grams of

soil samples were equilibrated with 40 mL TNT solution on a shaker at maximum speed of 250 rpm for their respective duration. Each sample was centrifuged; the supernatant was filtered through 0.2 μm filter and analyzed for TNT and its metabolites. After the adsorption experiments, soils were air dried in the dark and used for desorption experiments. Two grams of soil samples were equilibrated with water on a shaker for various time periods as described above. The supernatant was filtered and analyzed for TNT and its metabolites.

3.2.3. Urea extractability studies

Each soil was equilibrated with TNT containing solutions, allowing for reaching 100 $\pm$ 5 mg of adsorbed TNT kg^{-1} of soil. The soils were then separated from the solution phase, air dried in the dark, and used as TNT contaminated soils for the following batch extraction experiments in the presence or absence of urea. For this part of the study, tap water is used as a comparatve extractant of TNT in the batch urea extraction experiments. The tap water was analyzed for background TNT and urea concentrations. Both were below detection limit.

Effect of equilibration time: Kinetics of TNT desorption using two extractants, urea at its highest concentration (1000 mg kg^{-1}) and water, were investigated for understanding their comparative ability to extract TNT from all four contaminated soils. 1.5 grams of each contaminated soil sample was equilibrated with 30 mL of urea or water for 0, 1, 2, 5, 10, 24 and 48 h with constant shaking. The samples were centrifuged and the

supernatants were analyzed for TNT and its metabolites. Urea was also analyzed in samples collected at different sampling intervals to investigate the stability of urea in all soil solutions.

Effect of urea application rates: Four urea concentrations (0, 125, 350, and 1000 mg kg^{-1}) were chosen to investigate the effectiveness of urea within the agronomically recommended and environmentally safe urea application range. Two grams of contaminated soil samples were mixed with 30 mL solution through end over end mixing on a shaker at maximum speed of 250 rpm for 10 h. Soils and solutions were separated by centrifugation and the supernatants were removed, filtered through 0.2 µm filter, and analyzed for TNT and its metabolites.

Effect of pH: TNT-spiked Millhopper (soil pH 6.4) and Orelia (soil pH 8.1) soils were tested to determine the effects of pH on the effectiveness of urea in catalyzing TNT extractability. One gram of contaminated soil samples were equilibrated with 10 mL of solutions of all four urea concentrations (0, 125, 350, and 1000 mg kg^{-1}) for 0, 24, 48, and 96 h. All tubes were kept on a shaker at maximum speed of 250 rpm for end over end mixing. The pH of the solutions was maintained as 3, 5, 7, and 8 by adding 0.1 M HCl or NaOH. All samples were centrifuged and supernatants were removed, filtered, and analyzed for TNT and its metabolites.

Competing effects of urea on adsorption of TNT: There is a possibility that urea may compete with TNT for the binding sites present in the soils, rather than acting as a solubilizing agent to release it more in the solutions. To understand the interaction of urea and TNT in the soil surface, three soils (Immokalee, Millhopper, and Belleglade) and a pure mineral kaolinite was used. Equilibrium adsorption experiments were conducted in presence of urea (1000 mg kg^{-1}) to determine the competing effect of urea on adsorption of TNT. Batch adsorption tests were conducted on soils and kaolinite using one initial aqueous concentration of TNT (100 mg L^{-1}) and two urea concentrations (0, 1000 mg kg^{-1}). Two g of soils were equilibrated with 40 mL TNT and urea solutions on a shaker at maximum speed of 250 rpm for 24 h. The samples were centrifuged and supernatants were removed, filtered, and analyzed for TNT.

3.2.4. Analyses

Aqueous samples were analyzed for TNT and it's eleven metabolites on a HPLC system (ProStar, Varian Inc., USA) using the USEPA 8330 method (USEPA, 1997) at a wavelength of 254 nm. A C-18 column with corresponding guard column (250 x 4.6 mm, 5 mm silica-based column; Chromstar, Varian Inc., CA, USA) with a mobile phase of a 1:1 methanol (HPLC grade) and d-H_2O solution was used after degassing (20 min). The flow rate, sample injection volume, and run time of the chromatograph were 1.5 ml/min, 100 µl, and 12 min, respectively. A five level calibration curve was obtained for TNT and its eleven metabolites ($R^2 > 0.99$ for each compound). Calibration verification standards

for all compounds were analyzed after each set of 10 samples. Colorimetric determination of urea was carried out using Bio-Rad benchmark microplate reader at 527nm using the method described by Greenman et al. (1995).

3.2.5. Statistical analyses and Modeling

All data were expressed as mean (n=2) along with standard deviation. As the batch experiments were conducted in a controlled set up, we expected that variations within the treatments would not be high and thus used two replicates. Measured data supports our assumption as the standard deviations are low throughout the study. Large F ratios and small p values, found in all different data set, suggest variation among the treatments are much higher than variation within the treatments. Two-way ANOVA was carried out using statistical software JMP IN version 8.0 (Sall et al., 2005). Significant differences among treatment means were calculated using a Tukey-Kramer honest significant difference (HSD) test. Adsorption data were fit to a linear and two non-linear models namely Freundlich and Langmuir Isotherm models. Correlation analyses of % adsorption and % extraction of TNT by both water and urea were performed with soil properties using JMP IN version 8.0 (Sall et al., 2005). Adsorption data were correlated with soil pH, electrical conductivity, cation exchange capacity, percent soil organic matter, total and extractable iron and aluminum, and percent clay.

3.3. Results and Discussion

3.3.1. Adsorption and desorption of TNT in absence of urea

Adsorption of TNT in all four tested soils followed the characteristic biphasic kinetics: a rapid, reversible initial phase followed by a much slower, irreversible stage (Essington, 2004). The kinetics of TNT adsorption (Appendix, Fig A1) was influenced by the availability of the TNT binding sites on the soil surface. In low to moderate organic matter containing soils like Immokalee, Millhopper, and Orelia, TNT reached the adsorption steady state within 1.5 to 2 h at both initial TNT concentrations. Whereas, in highly organic soils such as Belleglade, TNT reached the adsorption equilibrium within 5 h and 10 h at 5 and 25 mg L^{-1} initial TNT concentrations, respectively. Desorption occurred almost as rapidly as adsorption, reaching desorption equilibrium within 2 h in all soils except Immokalee, where the steady state was reached in 10 h.

The extent of sorption and desorption increased with increasing TNT load for all soils (Figure 3-1). However, percent adsorption was higher at lower initial TNT load, and decreased with increasing initial TNT concentration in solution. Immokalee showed the least affinity to TNT resulting in approximately 10% average sorption (Fig 3-1a). An average of 23.7% TNT was adsorbed in Millhopper soil, whereas desorption increased with increasing initial load, subsequently reaching a desorption plateau (Fig 3-1b). L-type adsorption and desorption curves for Immokalee and Millhopper soils showed best fit (R^2 > 0.98) to the linearized Freundlich equation.

An average of 32.8% TNT was adsorbed by Orelia (Fig 3-1c). Belleglade soil showed the highest TNT sorption capacity with a mean percentage of 80.7 sorbed (Fig 3-1d). C-Type isotherm obtained from the adsorption data for Belleglade and Orelia soil suggest hydrophobic partitioning of TNT with SOM. Adsorption data for Orelia and Belleglade soil best fit the linear model ($R^2 = 0.99$) followed by Freundlich model ($R^2 > 0.81$). The linearity of the sorption data, especially in moderate to high SOM containing soils suggests 1:1 partitioning between TNT and SOM. The hydrophobic partitioning occurs between non-polar organic compound like TNT and non-polar moieties of SOM and gives linear isotherm (Singh et al., 2010).

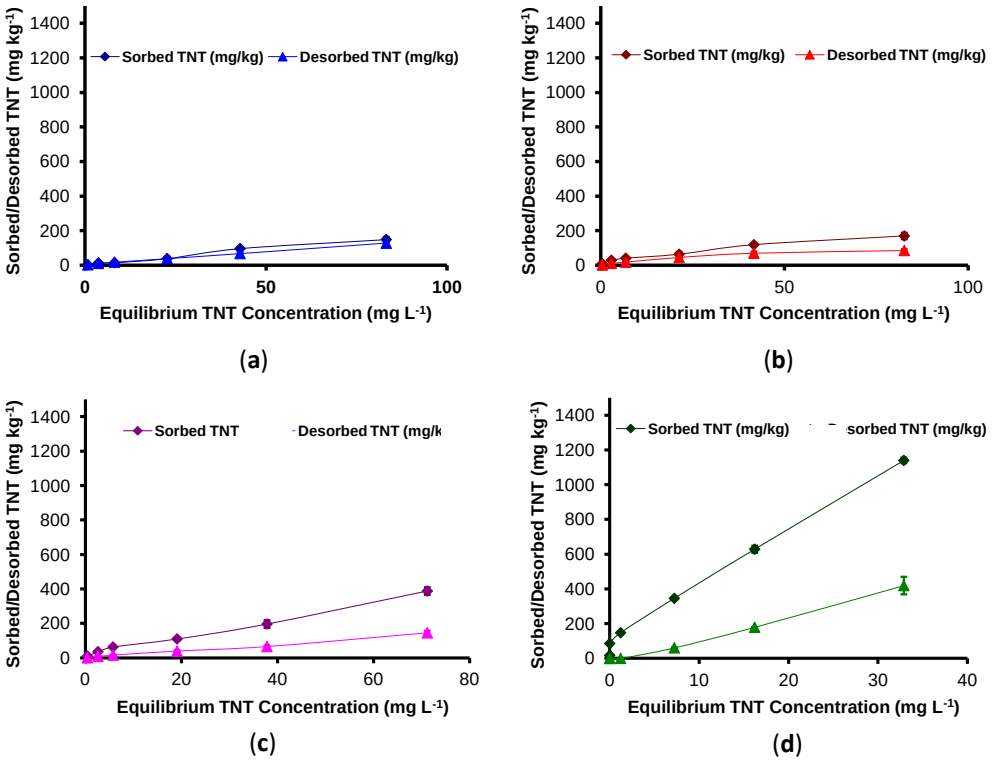

Figure 3-1. Equilibrium sorption and desorption of TNT at varied initial TNT load in Immokalee (a), Millhopper (b), Orelia (c), and Belleglade (d) soils . Data are expressed as mean (n=2) and one standard deviation.

In Belleglade, Orelia and Millhopper soils, the slope of the Freundlich adsorption isotherms ($1/n_{ads}$) were close to 1. This type of isotherm indicates hydrophobic partitioning between TNT and SOM (Evangelou, 1998). The isotherms suggest no single specific interaction took place between TNT and the SOM, and thus no saturation was attained. Eriksson and Skyllberg (2001) reported that binding of TNT in particulate organic matter (POM) is due to more linear hydrophobic partitioning, which is non-specific and independent of pH. They also suggested a slower, specific nonlinear binding of TNT with DOM through formation of TNT metabolites. Studies using ^{15}N Nuclear Magnetic Resonance (NMR) spectroscopy showed that reduced degradation products of

TNT, TNT amines and their isomers (ADNT, DANT, and TAT) undergo nucleophilic addition with ketone and quinone groups, resulting in covalent bonding to SOM (Thorn and Kennedy, 2002). This specific interaction between TNT metabolite and SOM results in non-linear isotherm. In the current study, as no TNT metabolite was found, it is evident that the binding mechanisms of TNT in all four soils were nonspecific hydrophobic partitioning with POM in the soils.

The slopes of the Freundlich desorption isotherms ($1/n_{des}$), which express the intensity of desorption (Singh et al., 2010), suggest that the intensity of TNT desorption is highest in Immokalee (1.11), the soil containing the least organic matter, followed by Millhopper (0.67), Orelia (0.56), and Belleglade (0.27). The hysteretic behaviors of TNT in all soils are illustrated in Fig 2 and table 3-2. Desorption hysteresis is the apparent increase in the distribution coefficient (K_f) when equilibrium is approached from a desorption direction (Essington, 2004). TNT showed hysteretic desorption in all soils as the desorption data points did not fall on the adsorption isotherms (Appendix, Fig A2) and the measured $(K_f)_{des}$ values were higher than the $(K_f)_{ads}$ values for all soils (Table 3-2). Table 3-2 also showed another parameter $(K_f)_H$ which is the distribution coefficient that represents the complete hysteresis or complete irreversibility. $(K_f)_H$ values were calculated assuming that desorption did not occur and q, the sorbed TNT concentrations at adsorption equilibriums, remained constant throughout desorption (Essington, 2004). In all four soils, the $(K_f)_{des}$ values were higher than the corresponding $(K_f)_{ads}$ values, but lower than those expected for the complete irreversibility, indicated by the $(K_f)_H$ values. This

suggests that dilution of the equilibrium solution did lead to desorption of some of the adsorbed TNT. However, considerable amounts of adsorbed TNT were retained in the matrix showing irreversible adsorption of TNT in all four tested soils (Essington, 2004). This establishes the need of using an extractant to catalyze the release of pre-adsorbed TNT in these soils.

Table 3-2. The hysteretic behavior of TNT in all tested soils, as qualified by measured Freundlich distribution coefficients (K_f).

Soils	Adsorption		Desorption		Complete Hysteresis	
	R^2_{ads}	$(K_f)_{ads}$	R^2_{des}	$(K_f)_{des}$	R^2_H	$(K_f)_H$
Immokalee	0.98	2.63	0.90	3.09	0.99	20.57
Millhopper	0.99	14.13	0.80	24.55	0.97	48.7
Orelia	0.99	18.2	0.92	61.66	0.94	88.02
Belleglade	0.81	56.23	0.89	269.15	0.97	388.15

3.3.2. Urea-catalyzed extraction of TNT

Effect of reaction time: Urea at its highest concentration (1000 mg kg^{-1}) and water were compared for their ability to extract TNT from soils as a function of reaction time. Urea significantly ($p<0.05$) influenced extraction from all soils, and 10 h was adequate to reach TNT desorption equilibrium during extraction (Figure 3-2). TNT extraction reached steady state in Immokalee soil within 1 h in the absence of urea, whereas 10 h was needed in the presence of urea. Immokalee soil showed the highest ability to release TNT followed by Millhopper, Orelia, and Belleglade in presence or absence of urea. Urea extracted a maximum of 94% sorbed TNT, whereas, water could extract 60% of the previously sorbed TNT from Immokalee soil. TNT extraction reached the equilibrium in

Millhopper soil within 1 h with water but needed 10 h to reach the equilibrium with urea. Water extracted a maximum of 31% of sorbed TNT from Millhopper soil. Urea significantly enhanced TNT extraction, reaching maximum of 49% of pre-adsorbed TNT. TNT extraction reached the equilibrium almost instantly in Orelia, within 1 h, both in presence and absence of urea. In Orelia soil, urea extracted maximum 18% of sorbed TNT, whereas, in the absence of urea, maximum 13% of the previously adsorbed TNT was released in solution. Although urea significantly enhanced the solubility of TNT in Orelia soil solution, lower TNT extraction from this soil compared to Immokalee and Millhopper can be explained by the stability of urea in these soils (Figure 3a). During the desorption experiment, urea remained most stable in Immokalee soil followed by Millhopper and Belleglade. However, in Orelia soil, urea was unstable because it dissolved at high pH with the formation of ammonia. Dissolved urea showed a significant ($p<0.0001$) negative correlation ($R^2 = - 0.59$) with the solution pH. Belleglade showed minimal capacity to release TNT, and reached desorption equilibrium within 1 h.

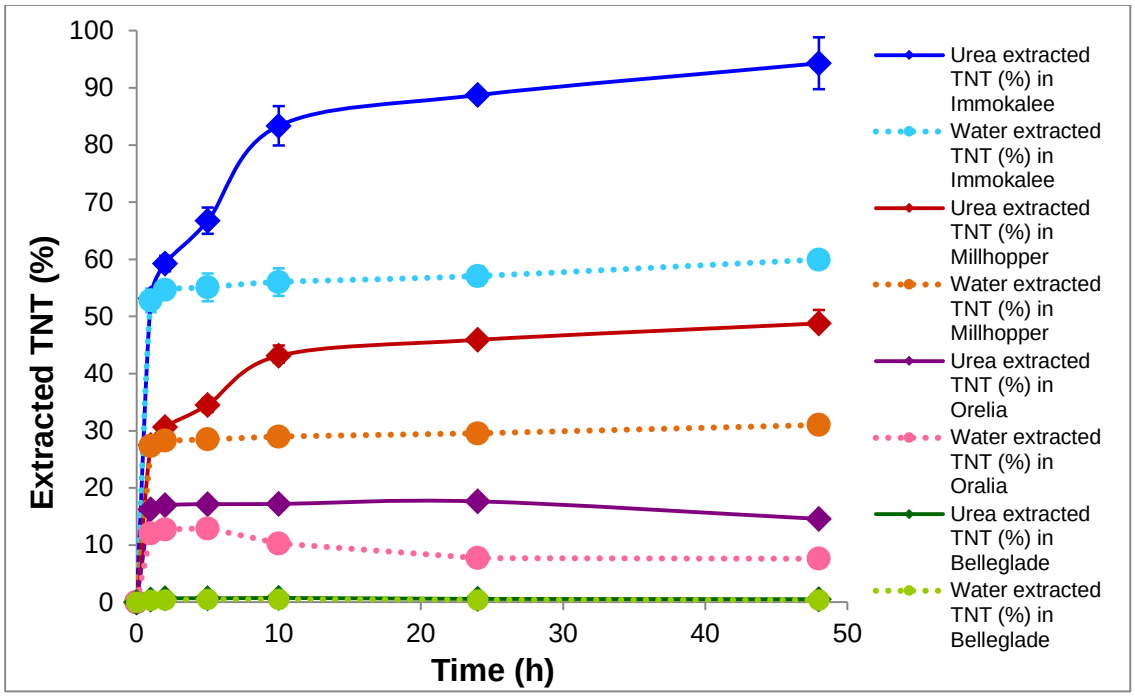

Figure 3-2. Kinetics of TNT extraction (expressed as % of initial TNT in soil) from all soils by two extractants, urea (1000 mg kg^{-1}) and water. Data are expressed as the mean (n=2) and one standard deviation.

Effect of urea application rates: TNT extraction from all four soils was significantly enhanced with increasing urea load (Figure 3b). The maximum effect of urea as a solubilizing agent was found in the acidic soils with low SOM. In Immokalee soil, there was no significant difference using all three urea concentrations (125, 350 and 1000 mg kg^{-1}). The lowest urea application rate (125 mg kg^{-1}) was enough to achieve the maximum TNT extraction from both Immokalee and Millhopper soils. This suggests that for low organic matter containing acidic soils the optimum urea-catalyzed TNT extraction can be achieved within the agronomically recommended urea application range. Although significant ($p<0.01$) enhancement in TNT extraction was seen at lower urea rates (125 and 350 mg kg^{-1}) in Orelia soil, the maximum TNT extraction was

achieved at 1000 mg kg^{-1}. The instability of urea at high pH soil like Orelia (Figure 3a) resulted in lower extraction of previously sorbed TNT from this soil. Instability of urea at high pH soil like Orelia can explain the need of higher urea application rate to maximize TNT extraction. The extremely high organic matter content (84 % SOM) of Belleglade and hence its high TNT retention capacity prevents urea from extracting any appreciable amount of TNT from this soil. The minimum concentrations of urea which maximize the urea catalysis of TNT extraction were found to be within the urea application rates recommended for agricultural crops for all soils.

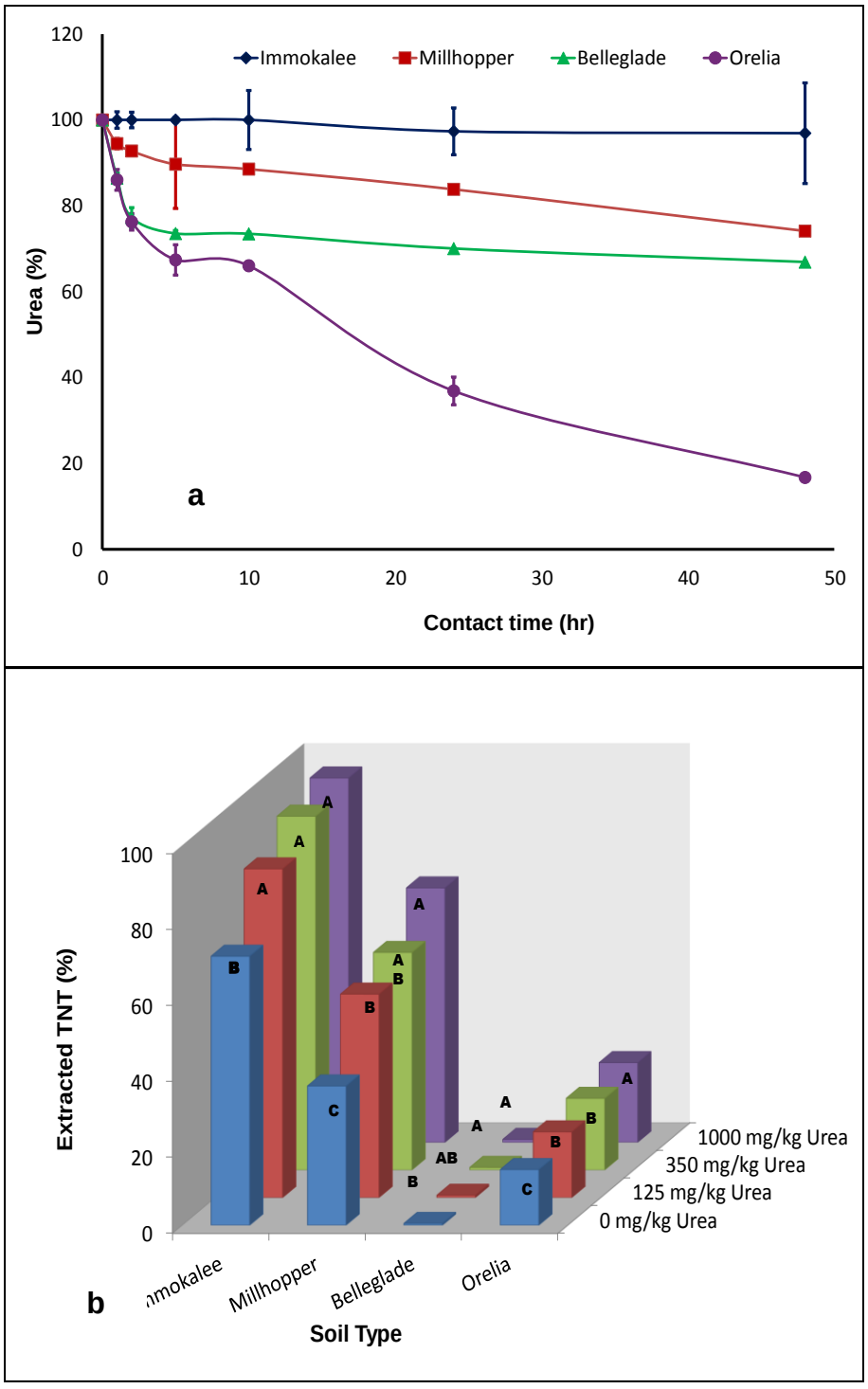

Figure 3-3. Urea concentrations (expressed as % of initial urea load) during TNT extraction (a) and Effect of four different urea loads (0, 125, 350 and 1000 mg kg^{-1}) on extraction of TNT (expressed as % of initial TNT in soil) from four soils after 10 hrs.

Statistical analysis was conducted separately for each soil (b). Data are expressed as the mean (n=2) and one standard deviation.

Effect of pH: Solution pH did not exert any significant effect ($p>0.05$) on TNT extraction from Millhopper ($p=0.5$) and Orelia ($p=0.06$) soil series (Appendix, A3). Although pH significantly influences the stability of urea in solution, it did not significantly affect TNT extraction from soils. This could be explained by the pH independent binding of TNT to soil as evident from the current and previously reported sorption/desorption studies (Pennington and Patrick, 1990). The pH independence of TNT sorption can be explained by the study of Haderleln and Schwarzenbach (1993), which found that non-ionizable nitroaromatic compounds showed no systemic variations in adsorption between pH 4 and 8.2. According to this finding, adsorption of TNT, being a nonionizable nitroaromatic compound should be independent of pH variations.

Competing effect of urea on TNT sorption: Competing effect of urea on TNT adsorption was investigated to examine whether presence of urea is competing with TNT for binding sites in the soil surface and hence restricting the extent of TNT sorption. The results showed that presence of urea did not cause significant differences ($p=0.36$; Appendix, A4) in the adsorption of TNT on these soils and kaolinite indicating that competitive effect of urea for the TNT binding sites was minimal.

Correlation with soil properties: Results of correlation analysis (Table 3-3) of % TNT adsorption with soil properties showed that adsorption was significantly correlated with percent SOM, cation exchange capacity, electrical conductivity (EC) and extractable Fe + Al, but poorly correlated with the clay content and pH. Influence of SOM on TNT retention and release is well documented in literature (Singh et al., 2010). Pennington and Patrick (1990) reported good correlation of TNT sorption with cation exchange capacity, Fe content and % clay. The % adsorption showed lowest correlation with soil pH, which suggests pH independent hydrophobic partitioning with the POM (Erikson and Skyllberg, 2001). The lack of correlation with clay in the current study could be explained as three out of four tested soils (Immokalee, Millhopper, and Belleglade) are sandy soils with minimal clay content. Cation exchange capacity is generally influenced by the clay content as clay provides negatively charged surfaces and thus acts as the cation exchanger. However, CEC is not solely restricted to the clay content and also depends on the other charged ions present in the soils. Haderleln and Schwarzenbach (1993) reported that the nature of the cations bound to soil particles and clay minerals showed dramatic effects on the adsorption coefficients of nitro aromatic compounds in soils. This study suggested that sites at which cation exchange takes place in soils and the nature of the charged ions present in these sites contributes to the overall cation exchange capacity and thus play an important role for the adsorption of nitro aromatic compounds in soils. Thus the good correlation of TNT adsorption with CEC and lack of correlation with clay, which is observed in the current study, could be explained.

The present study also found that solubilizing effectiveness of urea was influenced by the TNT adsorption capacity controlled by the soil properties. Percent desorption of pre-adsorbed TNT by both urea and water showed good negative correlation ($r > -0.80$) with SOM, total Fe + Al, and EC.

Table 3-3. Correlation coefficients (r) representing the correlation of soil properties with % TNT adsorption, % TNT desorption by water, and % TNT desorption by urea.

Soil Properties	% TNT adsorption	% TNT extraction by water	% TNT extraction by urea
pH	-0.23	-0.38	-0.32
EC††† ($\mu s\ cm^{-1}$)	0.98*	-0.81	-0.85
CEC†† (C mol kg^{-1})	0.99*	-0.74	-0.78
SOM† (%)	1.00**	-0.80	-0.83
Fe+Al (mg kg^{-1}) Total	0.56	-0.93	-0.92
Oxalate extractable Fe+Al (mg kg^{-1})	0.93	-0.71	-0.75
% Clay	0.06	-0.59	-0.54

** $p < 0.01$, * $p < 0.05$; ††† Electrical Conductivity, ††Cation Exchange Capacity, † Soil Organic Matter

3.4. Conclusion

The current study has characterized the major factors influencing the effectiveness of urea in enhancing TNT solubility in soil solutions. TNT showed high leachability in low organic matter containing soils while highly organic soil retained most of the sorbed TNT suggesting that mobility of TNT in soil varies widely depending on the adsorption sites

present in the soil. Hysteretic desorption of TNT in all four tested soils suggests irreversible sorption of TNT in these soils and thus establishes the need of using an extractant to facilitate the release of TNT in soil solutions. This study showed the beneficial effect of urea, as an extractant, over that of the water in significantly ($p<0.0001$) catalyzing TNT extraction from all the soils examined. However, urea may not prove very useful in extracting TNT from very high organic soils like Belleglade. Solution pH did not exert any significant effect on soil TNT extractability by urea, although use of urea in alkaline soils may result in urea loss via the formation of ammonia. The effective urea concentrations (125 or 350 mg kg^{-1} urea) required to extract TNT from soils are within the urea application limits set by the agronomic fertilizer-N rates used for major agricultural crops.

The present study showed the significant role of urea in enhancing TNT extractability at the soil/solution interface. One concern may arise that as urea amendment in TNT contaminated soils enhanced TNT concentrations in soil solutions, it may increase the risk of migration of TNT to groundwater or downstream water bodies. However, we speculate that the presence of a high TNT accumulator like vetiver grass (Makris et al., 2007b and Das et al., 2010) will effectively remove the soluble TNT from soil solutions and thus decrease the potential risk of migration of TNT through surface water runoff and leaching to groundwater. Data from the current study enabled us to design greenhouse experiments that are underway to optimize the effectiveness of urea in the presence of vetiver grass as a function of agriculturally recommended urea application rates and

initial TNT concentrations. The results obtained will pave the way in achieving our long term goal of developing a urea-catalyzed phytoremediation technology using vetiver grass to remediate TNT contaminated soil.

3.5.**References**

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CHAPTER 4

Urea-Catalyzed Uptake and Nitroreductase Mediated Transformation of 2,4,6-Trinitrotoluene in Soil using Vetiver Grass

Abstract

Limited bioavailability of hydrophobic nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT) is a major challenge toward developing an effective *in situ* bioremediation method for active or former military sites. A greenhouse-scale study evaluated the efficiency of a stimulative phytoremediation method using urea, a common nitrogen fertilizer, as a solubilizing agent that catalyzed TNT uptake by vetiver grass (*Chrysopogon zizanioides L.*). Kinetics of TNT removal by vetiver from the TNT-spiked soil (100 mg kg^{-1}) was fast (up to 0.004 kg d^{-1} g^{-1}), following a pseudo first-order reaction rate. Vetiver showed high affinity for TNT (> 80% removal within 22 days), and significant root-to-shoot TNT translocation (average 37%). Soil TNT removal rates by vetiver were significantly ($p<0.0001$) enhanced by urea. Urea application at agronomically-recommended nitrogen rates (~125 mg kg^{-1}) was optimum for TNT uptake by vetiver grass. Monoaminodinitrotoluenes and 1,3,5-trinitrobenzene were the main TNT metabolites detected in plant tissues, posing little, if any, influence on plant health. The enhanced activity of nitroreductase enzyme (NR) in TNT treated vetiver plants helps in elucidating the prevalence of amino-based TNT metabolites within plant tissues, indicating an effective biochemical defense mechanism against TNT toxicity. Results of a

long term soil column experiment showed that 80% of TNT and monoaminodinitrotoluenes (ADNTs) were retained in soil after 6 months in the plant-free TNT amended control soil columns. Complete removal of TNT was achieved in the vetiver-urea treatments within 6m up to 100 mg kg-1 initial TNT concentrations. Urea (325 mg kg-1) significantly enhances the TNT removal at all TNT concentrations. Significantly higher TNT and its metabolites were found in the leachate in plant-free-controls than that was found in the leachate in presence of both plant and urea. Along with TNT and ADNTs, dinitrotoluenes (2,4-DNT and 2,6-DNT), were found in the plant-free TNT amended controls which raises high concern as both of these compounds are found to be more toxic than TNT itself in in-vivo studies (LD_{50} in rat = 270 and 180 mg kg^{-1}, respectively). According to USEPA classification DNTs are listed as group B human carcinogens. These compounds were not detected in leachate in presence of vetiver, because of the fast removal of TNT and its metabolites by vetiver grass. The highly encouraging results of the current study showed the potentials of using stimulative phytoremediation of TNT contaminated soils using vetiver grass and urea.

Keywords: Vetiver, TNT, Phytoremediation, Urea, Nitroreductase.

4.1. Introduction

2,4,6, Trinitrotoluene has been historically the most widely used secondary explosives. Secondary explosives like TNT and RDX are more widely used than the primary explosives, which are extremely sensitive to stimuli such as impact, friction, or heat and thus difficult to handle and store. In comparison, secondary explosives are less sensitive as they require substantially more energy to be initiated, safer to handle and store as compared to the primary explosives, thus more widely used. It is a potential mutatoxin and a group C human carcinogen (Stenuit and Agathos, 2010 and USEPA, 1993). Due to its persistence in the environment, the removal of TNT from contaminated military and non-military sites became high priority for environmental agencies worldwide (Stenuit and Agathos, 2010). Search for ecologically-viable and cost effective environmental remediation/restoration methods has identified novel *in situ* bioremediation techniques, such as bioaugmentation, and phytoremediation (Hannink et al., 2002). Limited bioavailability of hydrophobic nitroaromatics like TNT is one of the primary challenges that needs to be overcome for implementing a successful *in situ* biological remediation technique (George et al., 2009). To address this problem, our group has proposed a novel TNT remediation method called *in situ* stimulative phytoremediation, which uses the synergistic combination of phytoremediation using both vetiver grass (*Chrysopogon zizanioides L.*) and a solubilizing agent, i.e., urea, which is commonly used as a crop fertilizer (Makris et al., 2010).

Urea has long been used as chaotropic agents in solubilizing membrane proteins and dissociating antigen-antibody complexes (Hatefi and Hanstein, 1969). They are specific anions (SCN^-) or polar carbamide derivatives (urea) that modify the water structure around aggregated proteins or sugars, thereby increasing the solubility of their hydrophobic regions in aqueous environments (Farrah et al., 1981). Ammonium thiocyanate (NH_4SCN), which is commonly used in gold mining operations to make gold soluble, has been successfully used by Anderson et al. (1998) in a phytorestoration study, enhancing gold uptake by plants from aqueous media. Our previous studies using urea as a TNT-extractant were encouraging; urea enhanced TNT solubility in aqueous media, significantly increasing the phytoextraction of TNT by vetiver and wheat in hydroponic settings (Makris et al., 2007c and b). A pilot experiment using a soil with minimal TNT retention capacity demonstrated a significant ($p<0.001$) increase in TNT removal rates by vetiver grass in the presence of a high urea application rate (1000 mg kg^{-1}) (Das et al., 2010).

However, the performance of urea at agronomically-recommended application rates (<1000 mg kg^{-1}) in enhancing soil residual TNT uptake is yet to be evaluated. Further, transformation of TNT to more polar metabolites are of utmost importance as direct conjugation is unlikely for TNT as it does not carry these required functional groups (Burken et al., 2000, Hannink et al. 2002). The assessment of the enzyme mediated detoxification pathway, which transforms TNT to metabolites containing appropriate functional groups for conjugation, is required to evaluate the effectiveness of

our phytoremediation technique, as the bound residues are presumably less bioavailable (Burken et al., 2000).

The objectives of this study were to: i) determine the kinetics of TNT removal from soil by vetiver grass in the presence of added urea, ii) evaluate the effectiveness of urea, as a solubilizing agent, within the range of environmentally-relevant and agronomically-recommended fertilizer N rates in catalyzing soil TNT uptake by vetiver grass, iii) measure the magnitude of plant TNT uptake and monitor both TNT and its metabolites in root and shoot tissues, while measuring the activity of nitroreductase (NR) enzyme responsible for the transformation of TNT to amino-based metabolites within vetiver grass, which is required for detoxification, iv) investigate the risk of potential migration of urea-mobilized TNT to groundwater.

4.2. Materials and Method

Chemicals: 2,4,6-trinitrotoluene (TNT) was purchased from Chem Service (West Chester, PA, USA) in an aqueous slurry form. It was air-dried, dissolved in acetonitrile, and stored in dark at 4^{o}C. HPLC-grade standards of TNT and its ten metabolites, 1, 3 – Dinitrobenzene, 2, 4 – Dinitrotoluene, 2, 6 – Dinitrotoluene, Nitrobenzene, 3 – Nitrotoluene, 4 – Nitrotoluene, Tetryl, 1, 3, 5 – Trinitrobenzene, 2 –Amino – 4, 6 – Dinitrotoluene, 4 –Amino – 2, 6 - Dinitrotoluene were purchased from AccuStandard

(New Haven, CT, USA). Urea was purchased from Fisher Scientific. HPLC grade solvents and nano-pure quality water was used for preparing solutions.

Experimental Units: This study was conducted in two phases. Firstly, an acute exposure study was conducted to investigate the performance of urea as a solubilizing agent, within the environmentally safe and agronomically recommended urea application guideline (phase I) and secondly, a soil column study was conducted to investigate the solubilizing effectiveness of optimum urea application rate as functions of varied initial TNT concentrations (phase II). Phase I of this study fully characterized the phytoremediation potential of vetiver-urea system, at varying urea application rates, by evaluating the kinetics of TNT removal, urea enhanced rhizospheric mobilization of soil TNT to plant system, TNT accumulation into vetiver root, translocation to above ground tissue, transformation of TNT in the root and shoot tissues of vetiver grass, and quantification of the activity of plant enzyme responsible for TNT transformation in vetiver grass. Whereas, phase II of this study investigated the long term fate, and potential migration of urea-mobilized TNT and its metabolites in presence and absence of vetiver grass at varying initial TNT treatments.

Soil selection: The Millhopper soil, which is a sandy loam with low pH (6.4) and relatively low organic matter content (4.38%), was chosen based on our previous batch experiments conducted in the absence of plants to investigate its TNT-sorption characteristics (Das et al., 2013). Hysteretic sorption of TNT by Millhopper soil

suggested irreversible adsorption of TNT in soils and thus indicated that the adoption of an extractant to increase soil-bound TNT bioaccessibility would be beneficial (Das et al., 2013). Effective enhancement of bioaccessibility was observed within the agronomically-recommended urea application rates in Millhopper soil, allowing for a significant *($p<0.001$)* increase in the extraction of pre-adsorbed TNT by urea (56%), when compared with that of tap water-based TNT extraction (36%) (Das et al., 2013).

TNT treatments: For phase I, this study used 100 mg kg^{-1} soil TNT concentrations being much higher than the benchmark of 30 mg kg^{-1} TNT toxicity limit for terrestrial plants (Talmage et al., 1999). Duringer et al. (2010) reported that low initial concentration of TNT and soil aging results into low uptake by plant and recommended using higher doses of TNT and exposing the plant immediately after the soil amendment to fully evaluate the phytoremediation potential of a plant species.

For phase II, four different concentrations of TNT (0, 50, 100, and 200 mg kg-1) were chosen to investigate the chaotropic effectiveness of the optimum urea concentration at different initial TNT loads. All these concentrations are higher than the benchmark (30 mg kg^{-1}) of TNT for toxicity to terrestrial plants (Talmage et al., 1999). These concentrations are also commonly found in the TNT contaminated military sites (Dillewijn et al., 2007). The solubility of TNT in water has been determined to be 101.5 mg l^{-1} at 25^0C (Ro et al., 1996). As urea increased the TNT extraction by 56% in

Millhopper soil (Chapter 3), up to 200 mg kg-1 initial TNT concentration was chosen to determine the ability of the optimum urea concentration as a chaotropic agent.

Urea treatments: For phase I, four urea concentrations (0, 125, 350, 1000 mg kg^{-1}) were chosen to evaluate the performance of urea as a solubilizing agent at environmentally-relevant (0 to 1000 mg kg^{-1}) and agronomically-recommended (125-350 mg kg^{-1}) application rates to a TNT-contaminated soil. Optimum agricultural crop guidelines recommend use of urea at > 125 mg urea kg^{-1} (250 kg ha^{-1}) (EFMA, 2000). A consistent yield depression of agricultural crop like maize was observed after a single urea application rate of 350 mg kg^{-1} (320 kg N ha^{-1}) (Trierweiler et al., 1983). Higher than 1000 mg kg^{-1} urea application rates exhibited strong toxic effects on earthworms, often considered soil ecotoxicological indicators (Xiao et al., 2004). Hence, 1000 mg kg^{-1} is the highest level of urea that can be used in soil without affecting the soil health. The optimum urea application rate found in phase I was chosen for phase II.

Soil Preparation: Millhopper soil samples were collected from the surface horizon (0-30 cm) at the University of Florida campus at Gainesville, FL, USA. The soil was spiked with TNT stock solution, reaching desired soil-TNT concentrations. For phase I, TNT-spiked soil was poured in polyethylene plastic bags, placed in three pots for each treatment and kept seven days for equilibration before planting vetiver. For phase II, TNT contaminated soils were loaded in PVC columns (15” high x 6” diameter) as shown in the figure 4-1.

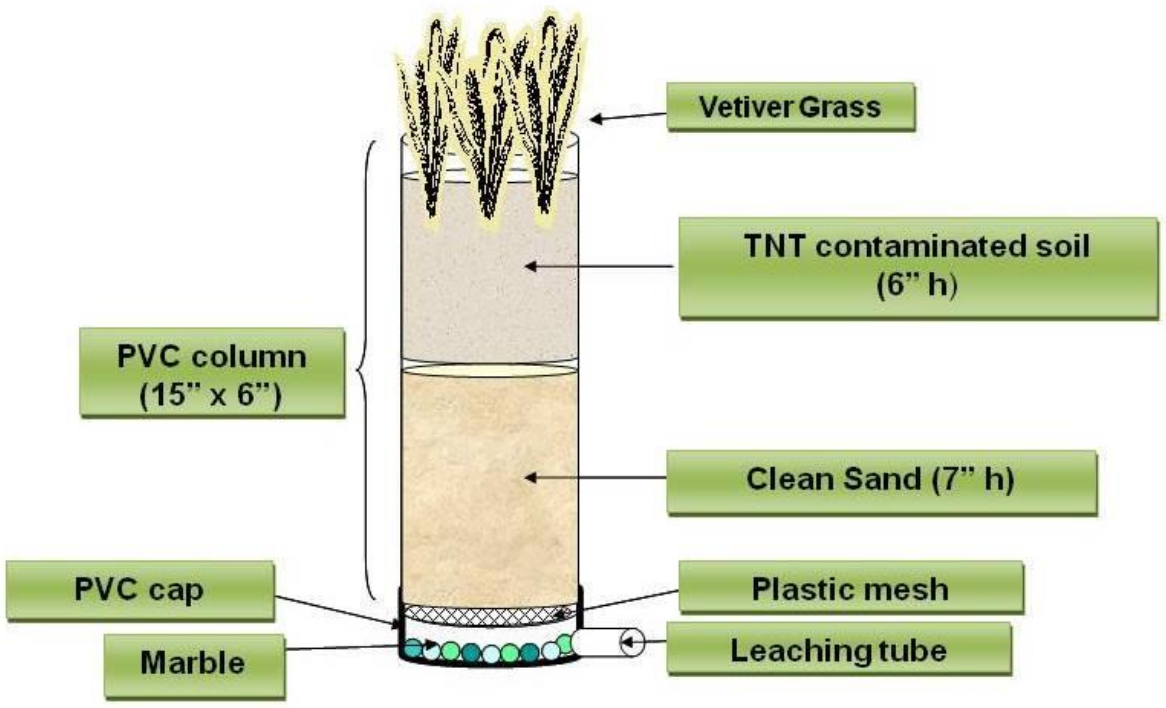

Figure 4-1. Experimental unit for greenhouse column study (Phase II).

Greenhouse Set-up: Vetiver (*Chrysopogon zizanioides L.*) plants were purchased from Florida Farms and Nursery, Florida. Plants were selected to obtain uniform distribution of biomass (both root and shoot) for all experimental units (following Makris et al. 2007a, b, c) and were allowed to acclimatize in potting soil for two weeks at 25^0C and 16 h photoperiod within a state-of-the-art greenhouse located within the premises of Montclair State University. At the beginning of the experiments (day 0), plants were washed with tap water, weighed and placed in the pots, containing 2 kg TNT-spiked soil. Uniformly weighing vetiver plants were placed in each pot or column. On day 1, urea solution was prepared in half-strength Hoagland solution (Hoagland, 1950) and added to the pots or columns. The volumes of tap water-based solutions added to the pots were frequently

adjusted to maintain soil at 70% water holding capacity throughout the experimental period. The pots were covered with aluminum foil to avoid possible photo-degradation reactions with TNT. All treatments were performed in triplicates. Three TNT-free (control) soil pots or columns were set up with vetiver grass. Three plant-, and urea-free, TNT-amended soil pots were also kept as controls to capture TNT losses due to indigenous soil biodegradation processes.

Sampling and Extraction: For phase I, soil samples were collected after 2, 5, 9, 14, and 22 days to evaluate TNT removal kinetics from soil. Three grams of soil was collected from different parts of the pot and thoroughly mixed to create a composite soil sample. After 22 days, plants were harvested and two types of soil samples were collected: Soil adhering to the roots (rhizospheric soil) and the remaining soil (bulk soil). Total TNT and metabolites were extracted from soil using acetonitrile per the USEPA 8330 method. The harvested plants were separated into shoot and root. The length and biomass of the plants were measured to investigate possible phytotoxic effects of TNT on vetiver growth. Root and shoot tissues of vetiver grass washed with tap water followed by deionized water. After the excess water was removed, plants and chopped with scissors to pieces. The vetiver tissues were finely ground in liquid nitrogen to minimize thermally-induced TNT transformations (Makris et al., 2007c). For phase II, the experiment was continued until complete removal of TNT was achieved in one treatment. Periodic soil and leachate samples were collected after 1, 2, 4, and 6 months to evaluate the fate and potential

migration of mobilized TNT in presence and absence of vetiver grass. Similar sampling and extraction procedures were followed as applied for phase I.

HPLC analyses of TNT and metabolites: Samples were analyzed for TNT and its eleven metabolites using the USEPA 8330 method (USEPA, 2007) with an HPLC system (Finnigan Surveyor plus, Thermo Scientific, USA). A C-18 column (250 x 4.6 mm, 5 mm silica-based column; Chromstar, Varian Inc., CA, USA) with a guard column with a mobile phase of a 1:1 methanol (HPLC grade) and d-H_2O solution were used after degassing (20 min). The flow rate, sample injection volume, and run time of the chromatograph were 1.5 ml min^{-1}, 100 µl, and 12 min, respectively. A five level calibration curve was obtained for TNT and its eleven metabolites ($R^2 > 0.99$ for each compound). Quality control using spiked TNT soil samples and appropriate blanks was performed every 10 samples.

TNT-degrading enzyme assay: Root and shoot tissues were ground in liquid nitrogen and mixed with pre-chilled, buffered protease inhibitor cocktail (0-4°C). The extraction cocktail was modified from Nakagawa et al. (1985) (Richardson and Bonmati, 2005), consisting of phenylmethylsulfonyl fluoride (1 mM), isopropyl alcohol (5%), EDTA (1 mM), and dithiothreitol (0.1 mM). Two milliliters of the extraction buffer was added to 1.5 g plant tissue and the mixture was sonicated using 0.5 sec pulses at a power of 400Wfor 2 min in a 40 kHz Branson 2510 (Dambury, CT, USA) in a sonication bath. The plant homogenate was filtered and centrifuged for 15 min. Enzyme assay mixture was

prepared following Nakagawa et al. (1985) and Richardson and Bonmati (2005) with potassium phosphate buffer (0.1M), isopropyl alcohol (5%), potassium nitrate (10 mM), and NADH (200 µM). Combining 1:1 ratio of crude extract assay mixture together and allowing contact for 15 min at 20°C initiated the reaction (Richardson and Bonmati 2005; Harley 1993). Equal amount of HCl (2.5N) containing sulfanilamide (58.1 mM) and same amount of N-1-naphthylethylenediamine dihydrochloride (0.77 mM) were added subsequently to stop the reaction (Richardson and Bonmati, 2005; Harley 1993). Absorbance of the resultant red color was measured after 10 min at 540 nm and the amount of formed nitrite was colorimetrically determined. Absorbance was measured after 10 min at 540 nM using a Bio-Rad benchmark microplate reader.

Data analyses: All data were expressed as mean (n=3) along with standard deviation. Two-way ANOVA was carried out using statistical software JMP IN version 8.0 (Sall et al., 2005). Significant differences among treatment means were calculated using a Tukey-Kramer honest significant difference (HSD) test. Reaction rates of soil TNT removal by vetiver grass were calculated according to Pavlostathis et al. (1998) and Makris et al. (2007a).

4.3. Results and Discussion

4.3.1. Phase I: Full characterization of the vetiver-urea system – Effect of urea application rates

TNT Phytotoxicity: Tolerance to residual soil TNT may considerably vary among plant species (Hannink et al., 2002). For example, alfalfa was unable to grow in soil contaminated with an average 100 mg TNT kg^{-1} concentration, while wheat and bush bean growth proceeded well in a soil contaminated with 500 mg kg^{-1} TNT concentration (Scheidemann et al., 1998). A phytotoxicity threshold value in soils of 30 mg TNT kg^{-1} has been proposed for terrestrial plants (Talmage et. Al 1999). Out of the 10 possible TNT metabolites, only two were detected in soil (2-ADNT and 4-ADNT) in our study. Up to 9 mg kg^{-1} 4-ADNT, and 2 mg kg^{-1} 2-ADNT were found in the bulk soil on the day plants were harvested (day 22); however, the root and shoot growth of the vetiver grass was unaffected by the presence of TNT and ADNTs, and no significant change was observed for total plant biomass. Partial chlorosis of the leaves was observed in all TNT-treated vetiver plants after 14 days of exposure, but did not increase by the end of day 22.

Figure 4-2. Vetiver grass grown in soil pots in greenhouse (Phase I).

Kinetics of TNT removal by vetiver grass: TNT was below limit of quantification (1.1 μg L^{-1}; standard deviation of 0.1 μg L^{-1}) in tap water or Hoagland's solution used in all experimental runs. In the absence of vetiver grass and added urea, no more than 25% of the initial TNT concentration was degraded by indigenous soil microorganisms (Makris et al., 2010) after 22 days of the experiment (Figure 4-3). When vetiver was grown in TNT without added urea, a significant reduction in soil TNT concentrations occurred, leaving < 30% of initial TNT added to the soil after 22 days. Rapid TNT removal during the first two weeks by vetiver led us to harvest the plants after 22 days, so that a balanced partitioning of TNT between soil and plant tissues was depicted. Typically, soil TNT removal by plants in the absence of added chemical agents to improve TNT extractability or solubility may not be satisfactory; less than 25% of the initial soil TNT concentration (11.5 mg kg^{-1}) was taken up by three cool season grasses (orchard grass, perennial

ryegrass, and tall fescue (Duringer et al., 2010). Our results illustrated faster removal of TNT by vetiver grass when compared with other terrestrial grasses used in TNT remediation schemes.

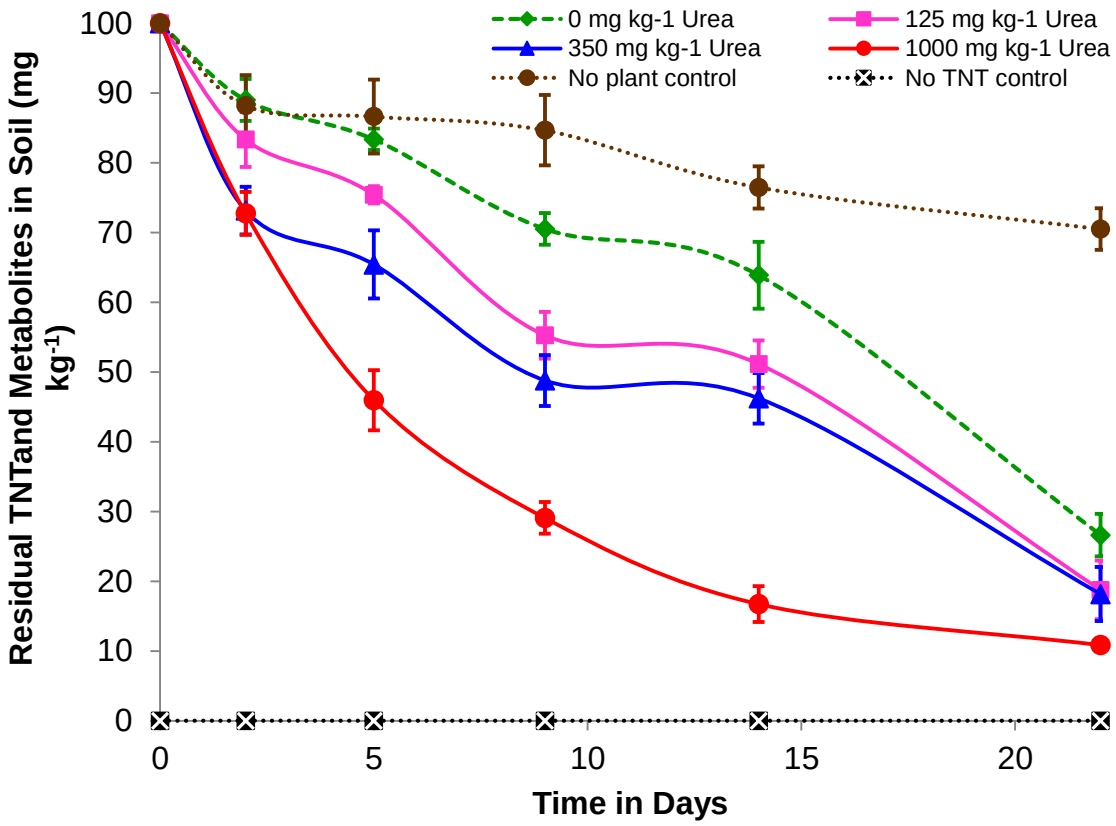

Figure 4-3. Kinetics of removal of TNT and its metabolites from soil by vetiver grass. Data are expressed as mean (n=3) ± one standard deviation.

Table 4-1. Reaction rate constants during TNT removal from Millhopper soil using vetiver grass. Data are represented as the mean of three replicates. Plant concentrations in the crude enzyme extracts was 50 g kg^{-1}. The k_P was calculated (as shown by Makris et al., 2007b) by dividing k_1 by the plant concentrations. Treatments with the different superscript letters are significantly different at the 95% confidence interval. Means separation was conducted separately for each day.

Time (Day)	Urea application rates (mg kg^{-1})	1st order reaction rate k_1 (h^{-1})	Plant normalized 2nd order reaction rate k_p ($Kg\ d^{-1}g^{-1}$)
2	0	0.0018	0.0009^{c}
	125	0.0038	0.0018^{bc}
	350	0.0049	0.0024^{b}
	1000	0.0066	0.0032^{a}
5	0	0.0015	0.0007^{c}
	125	0.0024	0.0011^{bc}
	350	0.0036	0.0017^{b}
	1000	0.0065	0.0031^{a}
9	0	0.0016	0.0008^{c}
	125	0.0028	0.0013^{bc}
	350	0.0034	0.0016^{b}
	1000	0.0058	0.0028^{a}
14	0	0.0013	0.0006^{c}
	125	0.0020	0.0010^{b}
	350	0.0023	0.0011^{b}
	1000	0.0053	0.0026^{a}
22	0	0.0030	0.0014^{c}
	125	0.0041	0.0020^{b}
	350	0.0043	0.0021^{b}
	1000	0.0079	0.0038^{a}

Presence of urea significantly ($p<0.001$) enhanced soil TNT removal kinetics by vetiver grass (Figure 4-3). By the end of the equilibration period (22 days), ~ 81%, 82% and 90% of the initial soil TNT concentration was removed by vetiver grass at 125, 350

and 1000 mg kg^{-1} urea application rates, respectively, suggesting plant uptake by vetiver grass due to the urea-induced higher TNT solubility in soil solution (Figure 4-3). The effect of urea on increasing TNT solubility, and thus, its phytoavailability was corroborated by the nearly linear removal of TNT from soil with increasing urea application rates (Figure 4-3). First-order and second-order reaction rate constants were calculated for soil TNT removal kinetics using vetiver grass at various urea application rates (Table 4-1). As plant weight remained practically unchanged, due to the short experimental period, we expressed the 2nd-order reaction rate constants as the pseudo-1st-order rate constants (k_P) (9). The k_P values significantly ($p < 0.001$) increased with urea application rates. The high affinity of vetiver grass for soil-TNT masked the significant effect of the lowest urea treatment (125 mg kg^{-1}) for the first three sampling periods. However, after 14 days, the second order reaction rate constants were significantly ($p < 0.001$) enhanced in the presence of 125 mg kg^{-1} urea, over unamended control plants (Table 4-1). The k_P values were not significantly ($p>0.05$) different between the 125 and 350 mg kg^{-1} urea treatments for all sampling periods, which is urea's agronomically recommended application rate to crops. This suggests that the 125 mg urea kg^{-1} rate would be adequate to provide synergy for maximum TNT removal by vetiver grass, minimizing environmental risks from over-application of nitrogen fertilizers. However, at 1000 mg kg^{-1} urea application rate, the k_P values were significantly higher than those of the lower urea treatments after the second day. The observed k_P values at all sampling periods were significantly ($p<0.0001$) correlated with all tested urea application rates for all sampling periods (Table 4-2).

Table 4-2. Pairwise correlation between 2nd order reaction rate k_p for all sampling time intervals and the urea application rates. $p < 0.05$ shows significant correlations.

Variable	by Variable	Correlation	p value
k_p at Day 2	Urea application rates	0.85	0.0005
k_p at Day 5	Urea application rates	0.97	< 0.0001
k_p at Day 9	Urea application rates	0.95	< 0.0001
k_p at Day 14	Urea application rates	0.97	< 0.0001
k_p at Day 22	Urea application rates	0.95	< 0.0001

As expected, plant-normalized reaction rates were much lower than those reported in our previous hydroponic (Makris et al., 2007a) and soil batch (Das et al., 2010) studies in the laboratory. We noted that the k_P values found in this study were significantly lower than those reported in our earlier soil study where after 12 days of interaction with 1000 mg kg^{-1} urea and 80 mg kg^{-1} soil TNT, second order TNT removal rates for Immokalee soil was 0.012 $kg\ d^{-1}g^{-1}$(Das et al., 2010). However, in the current study, the plant normalized second order TNT removal rates for Millhopper soil was as low as 0.0026 $kg\ d^{-1}g^{-1}$. This difference in soil TNT removal rates between this study and the earlier report by Das et al. (2010) could be partially ascribed to: i) higher TNT extractability in Immokalee, containing lower amounts of organic matter (Das et al., 2013) and ii) decreasing TNT removal rates with increasing initial TNT soil loads (> 80 mg kg^{-1}) (Das et al., 2010).

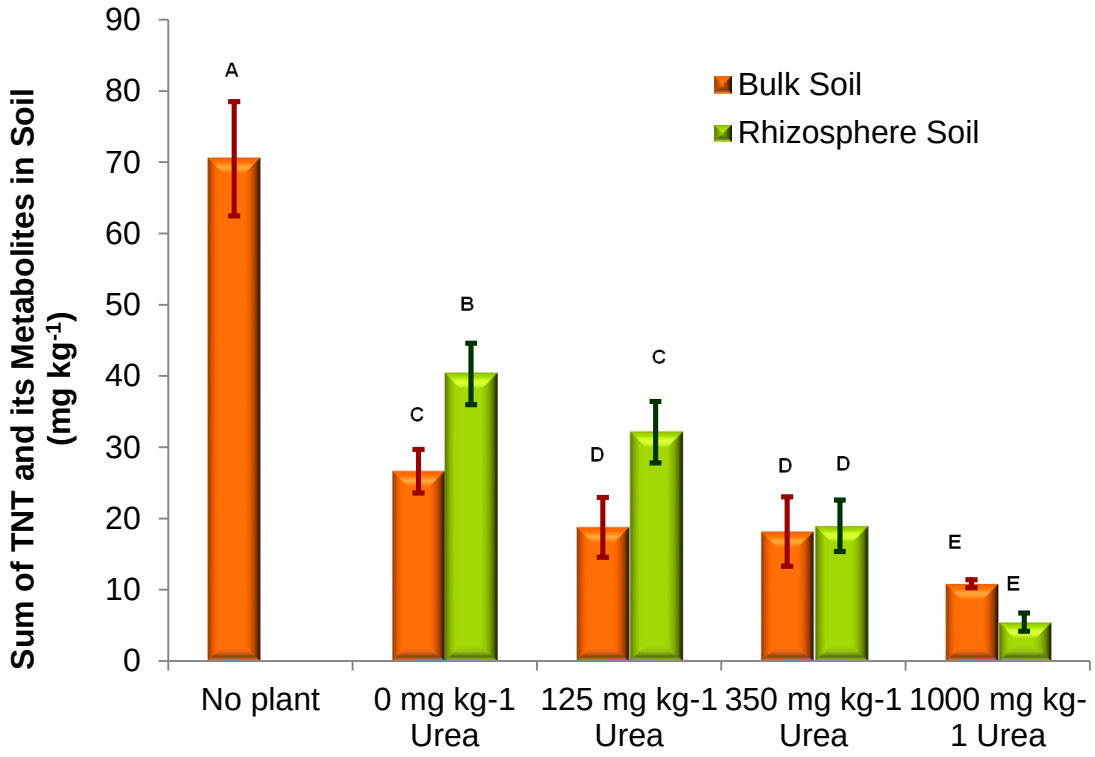

Figure 4-4. Sum of residual TNT and its metabolites in bulk and rhizospheric soil after 22 days. Data are expressed as mean (n=3) ± one standard deviation.

Rhizospheric interactions of TNT and urea: No TNT metabolite was detected in soil up to 14 days. On the 22nd day, ADNT was observed in all TNT-spiked soil treatments in the presence and absence of vetiver grass, suggesting the microbial reduction of a nitro- to an amino- functional group in TNT. Rhizospheric TNT concentrations and two metabolites (4-ADNT and 2-ADNT) that were consistently detected in most treatments significantly ($p< 0.001$) decreased with increasing urea application rates (Figure 4-4). Similar result was observed in bulk soil samples, except that no significant ($p> 0.05$) difference was observed for soil TNT concentrations in the 125 and 350 mg kg^{-1} urea treatments (Figure 4-2). Klunk et al. (1996) found significantly lowered TNT

concentration in rhizosphere when compared with that in bulk soil. However, in the current study, concentration of TNT and certain TNT metabolites in the rhizosphere were significantly higher than those of the bulk soil in the absence of urea and even at the 125 mg kg^{-1} urea load. At 350 mg kg^{-1} urea, there was no significant difference in the total concentrations of TNT and its metabolites between rhizosphere and bulk soil. Whereas, at 1000 mg kg^{-1} urea load, the total TNT and its metabolites in the rhizosphere was lower than that in the bulk soil, although the difference was not significant. This difference from the previously reported findings can be explained by the distribution of TNT and its metabolites in the bulk and rhizosphere soil (Table 4-3). Scheidemann et al. (1998) found more TNT metabolites in the rhizospheric soil (20-42% 4-ADNT and 10-21% 2-ADNT) as compared with the bulk soil (6-13% 4-ADNT and 3-7% 2-ADNT). This result suggests greater extent of TNT degradation in the rhizosphere due to enhanced microbial activity in the root zone, or due to the presence of TNT-degrading plant enzymes exuded by the root. In the current study, concentrations of TNT metabolites in the rhizosphere were not significantly different than those of the bulk soil which can be explained by limited rhizospheric degradation of TNT and the urea catalyzed mechanism of the transport of TNT and its metabolites from soil to the plant roots. Presence of these metabolites (14% 4-ADNT and no 2-ADNT) in plant-free TNT-amended controls (no urea added) in the absence of vetiver grass also supports this hypothesis. The higher concentration of nitroaromatics (NACs) in the rhizosphere is likely caused by the difference between movement of NACs into the rhizosphere through advective mass flow of soil water and its uptake into roots. Similar mechanism of TNT transport was reported

by Kim et al. (2004a and 2004b), who also found higher NACs concentrations in the rhizosphere than those of the bulk soil. It could be partially ascribed to the greater mass transfer coefficient of TNT transport from the surrounding soil to the rhizosphere than that for root uptake, as roots do not readily take up hydrophobic organic molecules. Organic contaminants with log K_{ow} values of 0.5 – 3 can be easily taken up by plant roots. The log K_{ow} value of TNT is 1.9 and those of ADNTs range between 1.85-2.1 (Kim et al., 2004a).

The present data shows the effectiveness of urea, as a solubilizing agent, at the hydrophobic root zone, in enhancing TNT uptake by the plant root. With increasing urea application rates, the accumulation of NACs at the rhizosphere significantly decreases, which supports our hypothesis that urea increased TNT solubility in soil solution, thereby, stimulating absorption rates of TNT and its metabolites by the root.

Table 4-3. Distribution of nitroaromatic compounds in the bulk and rhizosphere soil after 22 days. Data are expressed as mean (n=3) ± one standard deviation.

	Bulk Soil (mg kg^{-1})			Rhizospheric Soil (mg kg^{-1})		
Treatments	TNT	4 ADNT	2 ADNT	TNT	4 ADNT	2 ADNT
No plant	60.37±8.02	10.12±1.55	0	NA	NA	NA
0 mg kg^{-1} Urea	19.68±3.05	5.60±0.93	1.33±0.03	39.26±4.31	1.03±0.02	0
125 mg kg^{-1} Urea	14.09±4.19	3.76±0.32	0.90±0.03	32.11±4.32	0.00±0.00	0
350 mg kg^{-1} Urea	13.86±4.87	3.13±0.03	1.17±0.01	15.79±3.61	3.17±0.59	0
1000 mg kg^{-1} Urea	1.58±0.55	8.88±1.02	0.38±0.00	3.21±0.29	2.24±0.04	0

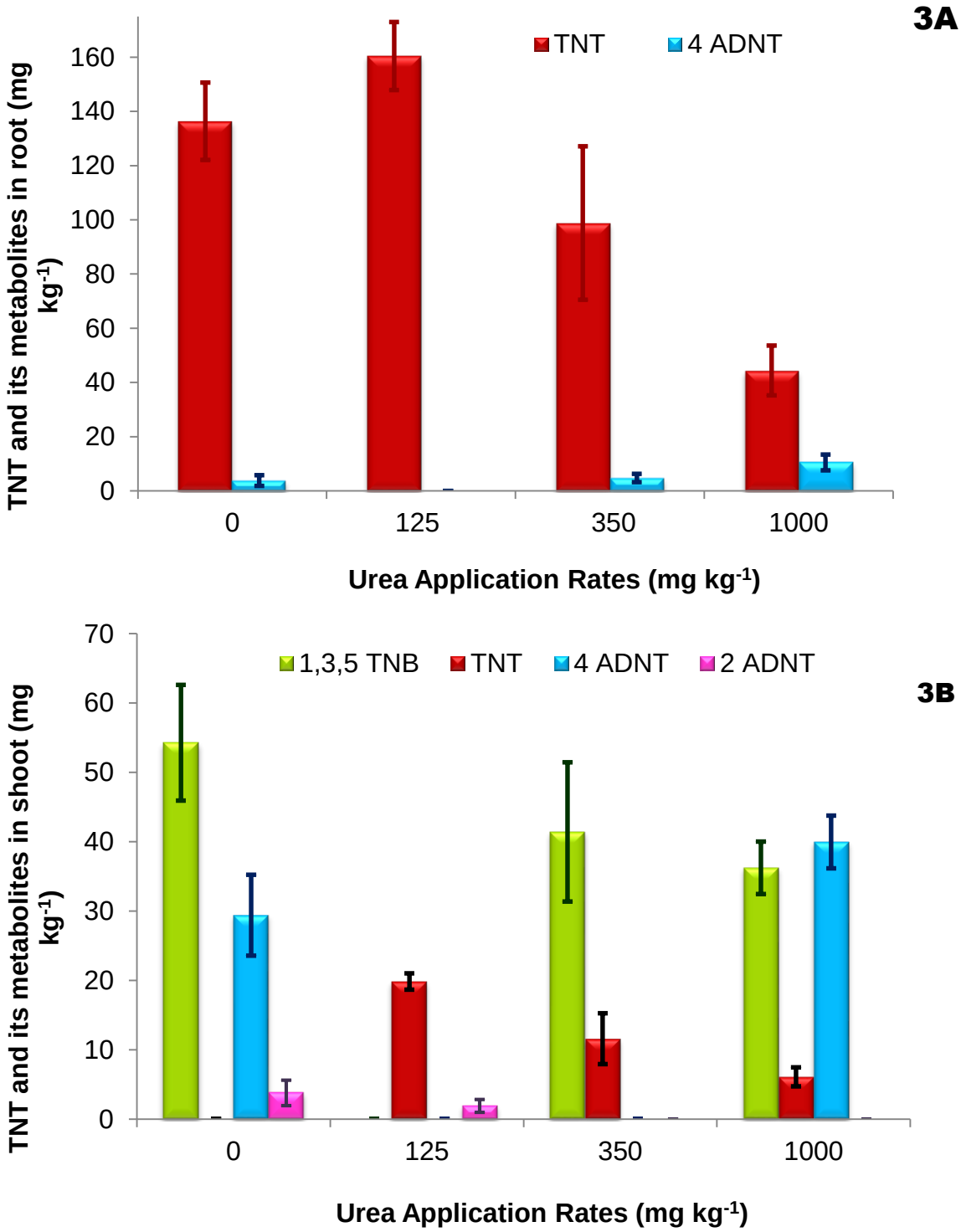

Figure 4-5. TNT and its metabolites (mg kg^{-1}) in the root (3A) and shoot (3B) tissues of vetiver grass. Data are expressed as mean (n=3) ± one standard deviation.

TNT accumulation, transformation, and translocation in vetiver grass: High concentrations of TNT and traces of ADNTs were found in the roots of vetiver grass (Figure 4-5). Although TNT removal from soil increased with urea application rates, a corresponding increasing trend was not observed for the root TNT concentrations. Once TNT enters into the plant system, it can be distributed in many possible ways. TNT can either translocate to the above-ground parts of the plant, enzymatically transformed to other metabolites, or conjugated with various biomolecules in the plant and sequestered to cell wall or vacuole (Hannink et al., 2002). The metabolite 4-ADNT was also found in bulk and rhizosphere soil, implying that it was either taken up by vetiver grass from soil, or TNT degradation to 4-ADNT was mediated by the NR enzyme activity in root. At similar initially added TNT concentrations to soil, vetiver grass performance in removing soil TNT and its metabolites was superior compared to other plant species (Table 4-4). After 8 weeks, *Triticum aestivum* and *Phaseolus vulgaris* contained the largest quantities (91 and 99 mg kg^{-1} plant weight) of TNT and its metabolites from a soil contaminated with 100 mg kg^{-1} TNT (Scheidemann et al., 1998; Hannink et al., 2002). In the present study, we detected 160 mg kg^{-1} TNT and its metabolites in vetiver root (in the absence of urea) after only 22 days' exposure in soil with the same initial TNT treatment.

Table 4-4. Concentrations of TNT and its metabolites detected in plant roots by previous and present studies under similar initial TNT treatment.

Plant	Initial soil - TNT (mg/kg)	Exposure time in soil	TNT + Metabolites (µg/g)detected in root	Reference
Phaseolus	100	8 weeks	91.0 ± 37.1	Scheidemann et al., 1998
Lupinus	100	8 weeks	14.9 ± 3.45	Scheidemann et al., 1998
Trifolium	100	8 weeks	33.5 ± 21.4	Scheidemann et al., 1998
Phacelia	100	8 weeks	23.2 ± 3.38	Scheidemann et al., 1998
Triticum	100	8 weeks	98.6 ± 60.8	Scheidemann et al., 1998
Alopecurcurus	100	8 weeks	55.8 ± 42.5	Scheidemann et al., 1998
Bromus	100	8 weeks	37.8 ± 12.1	Scheidemann et al., 1998
Festuca	100	8 weeks	46.4 ± 10.6	Scheidemann et al., 1998
Lolium	100	8 weeks	34.0 ± 14.6	Scheidemann et al., 1998
Phleum	100	8 weeks	30.7 ± 21.8	Scheidemann et al., 1998
Vetiver	**100**	**3 weeks**	**160.43 ± 12.57**	**Present Study**

Significant TNT translocation from root to shoot was observed (Figure 4-5). Vetiver showed higher translocation capability (average of 37%) of TNT and its metabolites into the shoot. At 1000 mg kg^{-1} urea treatment, 59% TNT translocation was reported, which, to our knowledge, is much higher than that reported in literature. Among the 11 tested metabolites of TNT, three metabolites, i.e., 2-ADNT, 4-ADNT and 1,3,5-TNB were

detected in shoot tissues, suggesting TNT phytodegradation by vetiver grass (Figure 4-5). These three metabolites were also found by Makris et al. (2007a) in the root of vetiver grass in our previous hydroponic study, but none of them were detected in the shoot. Most of the past studies have reported limited TNT translocation to the shoot (Hannink et al., 2002); Vila et al. (2007) reported less than 25% of soil TNT translocation.

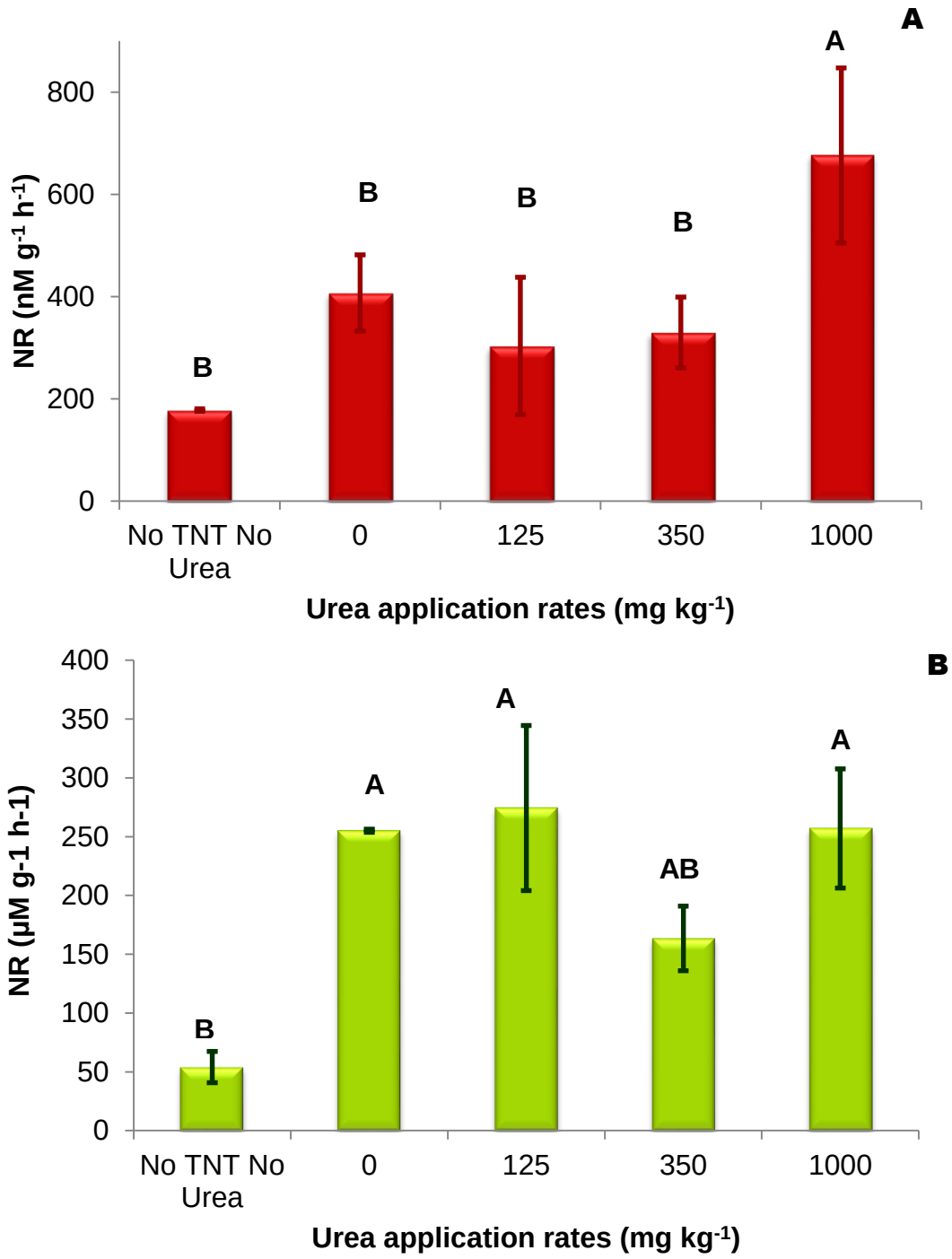

Figure 4-6. Nitroreductase activity in the root (4-6A) and shoot (4-6B) tissues of vetiver grass. Data are expressed as mean (n=3) ± one standard deviation. Please note the difference at the Y axis scale between figure 4-4A and 4-4B. The y-axis scale in Figure 4-6B is 1000x higher than that of Figure 4-6A.

Nitroreductase activity in vetiver plants: Our nitroreductase enzyme assay revealed that the NR-activity ($nMg^{-1}h^{-1}$) was higher ($p<0.007$) in both root and shoot tissues of the TNT-treated vetiver plants as compared to the TNT-free control plants (Figure 4-6). Similar results were found in maize and soybean by Adamia et al. (2006) where NR activities were significantly enhanced during the plant cultivation on TNT-containing hydroponic media. Transformation of TNT to more polar metabolites are of utmost importance as direct conjugation is unlikely for TNT as it does not carry these required functional groups (Burken et al., 2000, Hannink et al. 2002). Thus, as a part of their detoxification mechanism, plants need to transform TNT to metabolites like ADNTs in possession of prerequisite functional groups for conjugation and transport (such as $–NH_2$). Earlier studies have reported that conjugated residues of TNT were non-extractable with 80% of the ^{14}C label in bush been (Harvey et al., 1990) and 85% of the ^{14}C label in poplar tree (Thompson et al., 1998), indicating most of the carbon associated to TNT being conjugated and sequestered. As evident from the NR activity and mass balance data (Figure 4-5), the urea treatment increased the total plant TNT uptake, activating enzyme-mediated (NR) transformation to metabolites like ADNTs. The NR-activity in the shoot was >100x higher than that of the root, indicating elevated TNT phytodegradation activity in the shoot. This was further corroborated by the significantly ($p<0.001$) higher levels of TNT metabolites in shoot when compared with those in the root.

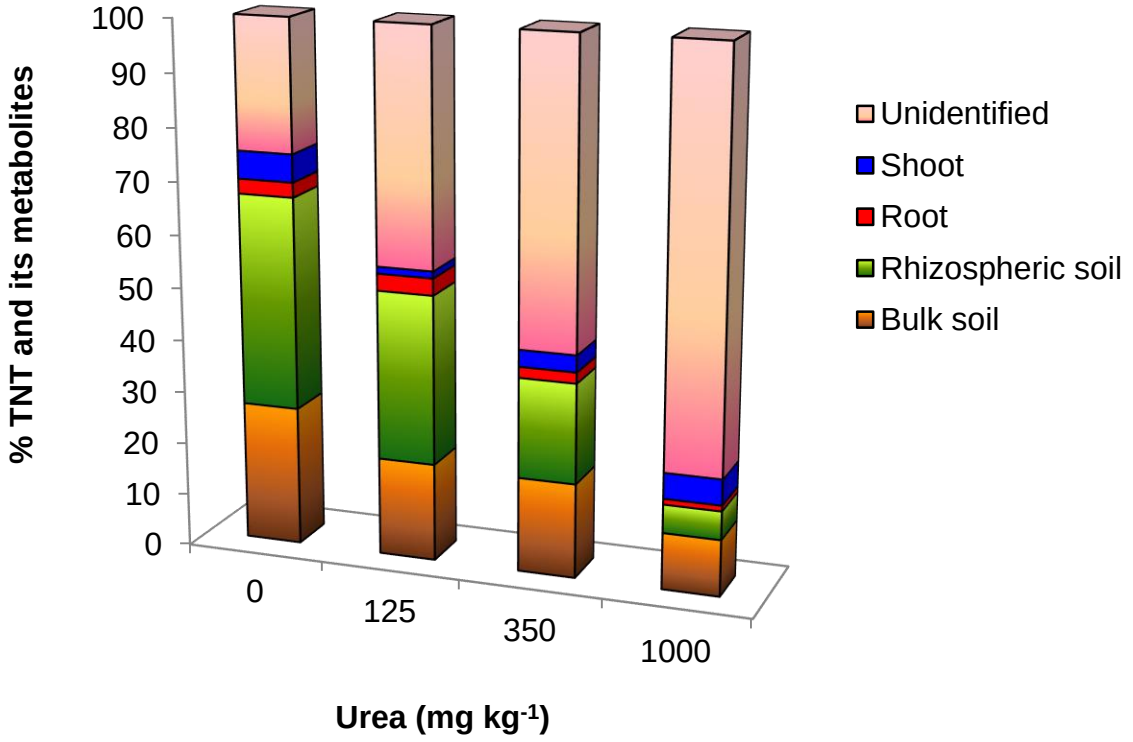

Figure 4-7. Mass balance of the mean (n=3) TNT and its metabolites (%) in soil and plant samples.

The biochemical pathway behind the transformation of TNT to 1,3,5 TNB, the other metabolite found in the shoot tissues, is yet unknown. As it was not found in the soil, we assume that it is another plant TNT-metabolite. Although it is not a commonly found plant-TNT metabolite, Rivera et al. (1998, see ref 32) reported presence of 1,3,5 TNB in parrot feather. It was also found in TNT-treated vetiver grass in our earlier hydroponic study (Makris et al., 2007a). In animal model experiments, 1,3,5 TNB toxicity was higher (LD_{50} in rat = 284 mg kg^{-1}, (ATSDR, 1995a) than that of TNT (LD_{50} in rat = 795 mg kg^{-1}) (ATSDR, 1995b). However, in terrestrial plants, it exhibited lesser toxicity (EC_{50} =129 mg kg^{-1}) than that of TNT (EC_{50} =93 mg kg^{-1}) (Rocheleau et al.,

2006). Ongoing studies in our laboratory aim at exploring the biochemical mechanism behind the transformation of TNT to 1,3,5 TNB in vetiver grass.

4.3.2. Phase II: Long term fate of TNT in soil-column set up with vetiver-urea system at the optimum urea application rate

A commonly expressed concern with soil extractants mobilizing metals (like Pb) and organics (TNT) from soils refers to an increased risk of solute migration to groundwater or downstream water bodies. The data obtained in the phase I experiment showed the effectiveness of urea application in the agronomically recommended range (125 to 350 mg kg^{-1}) in catalyzing TNT uptake by vetiver grass. The highest urea application rate in the agronomically recommended window (350 mg kg^{-1}) was chosen to investigate the potential migration of TNT to groundwater in presence and absence of vetiver grass. Figure 4-8 shows the vetiver grass grown in greenhouse column set up. As evident from the picture vetiver showed high tolerance to TNT stress even at concentrations as high as 200 mg kg^{-1}. However, biomass reduction and partial chlorosis of leaves were observed in higher TNT concentrations (100 to 200 mg kg^{-1}).

Figure 4-8. Vetiver grass grown in greenhouse column set up (Phase II).

Figure 4-9 shows residual TNT and its metabolites in soil after six months. Complete removal of TNT was achieved within six months at 50 and 100 mg kg^{-1} initial TNT concentration in presence of vetiver grass and in presence and absence of urea (figure 4-9A). At 200 mg kg^{-1} initial TNT concentration, 81% TNT was removed by vetiver grass and 95% TNT removal was achieved in urea-vetiver system. In absence of vetiver grass, only 30 to 35% TNT was removed from control soil columns at all initial TNT treatments. This loss of TNT in control columns is probably attributed to the combination of photodegradation and microbial degradation of TNT. 4 ADNT, the most commonly found metabolite of TNT, was found in all treatments including plant-free control

columns, indicating the transformation of TNT to ADNT by soil microbial community (figure 4-9B).

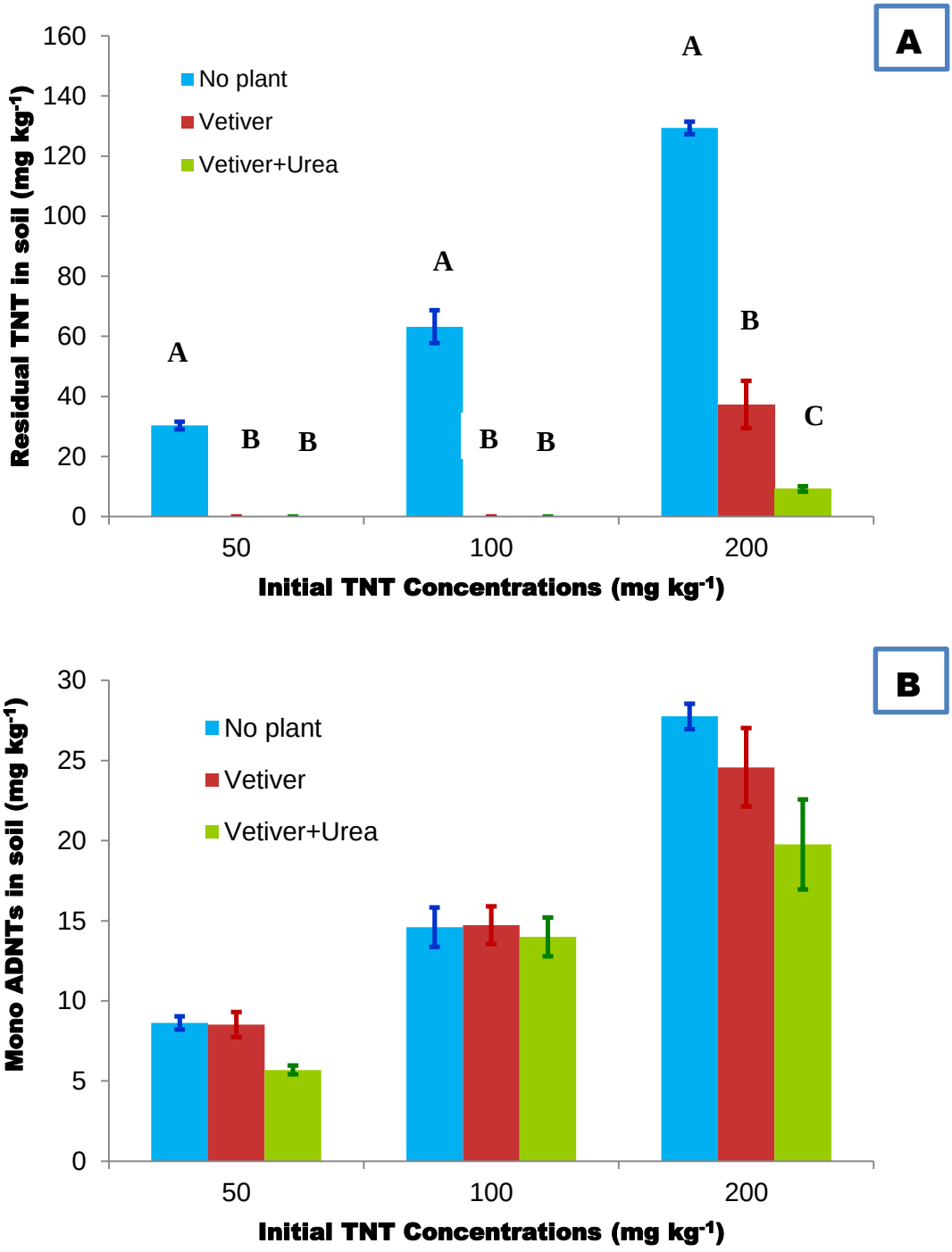

Figure 4-9. Residual TNT (A) and ADNT (B) (mg kg^{-1}) in soil after 6 months. Data are expressed as mean (n=3) $\pm$ one standard deviation. Mean comparison in figure A was conducted separately for each initial TNT treatments.

Figure 4-10 presents the sum of TNT and its metabolites in leachate after four and six months. At both sampling period, it is noted that in absence of vetiver and urea, high concentrations of TNT and its metabolites were found in the leachate. As urea makes TNT more soluble, in absence of vetiver, significantly higher TNT was found in the leachate in urea treated controls. However, in presence of both plant and urea, least amount of TNT was found in the leachate as a result of urea catalyzed plant-uptake of TNT. Similar trend was observed in leachates collected at other sampling periods (after one and two months; data not shown), negating the concern of enhanced migration of urea-mobilized TNT to groundwater in presence of vetiver grass.

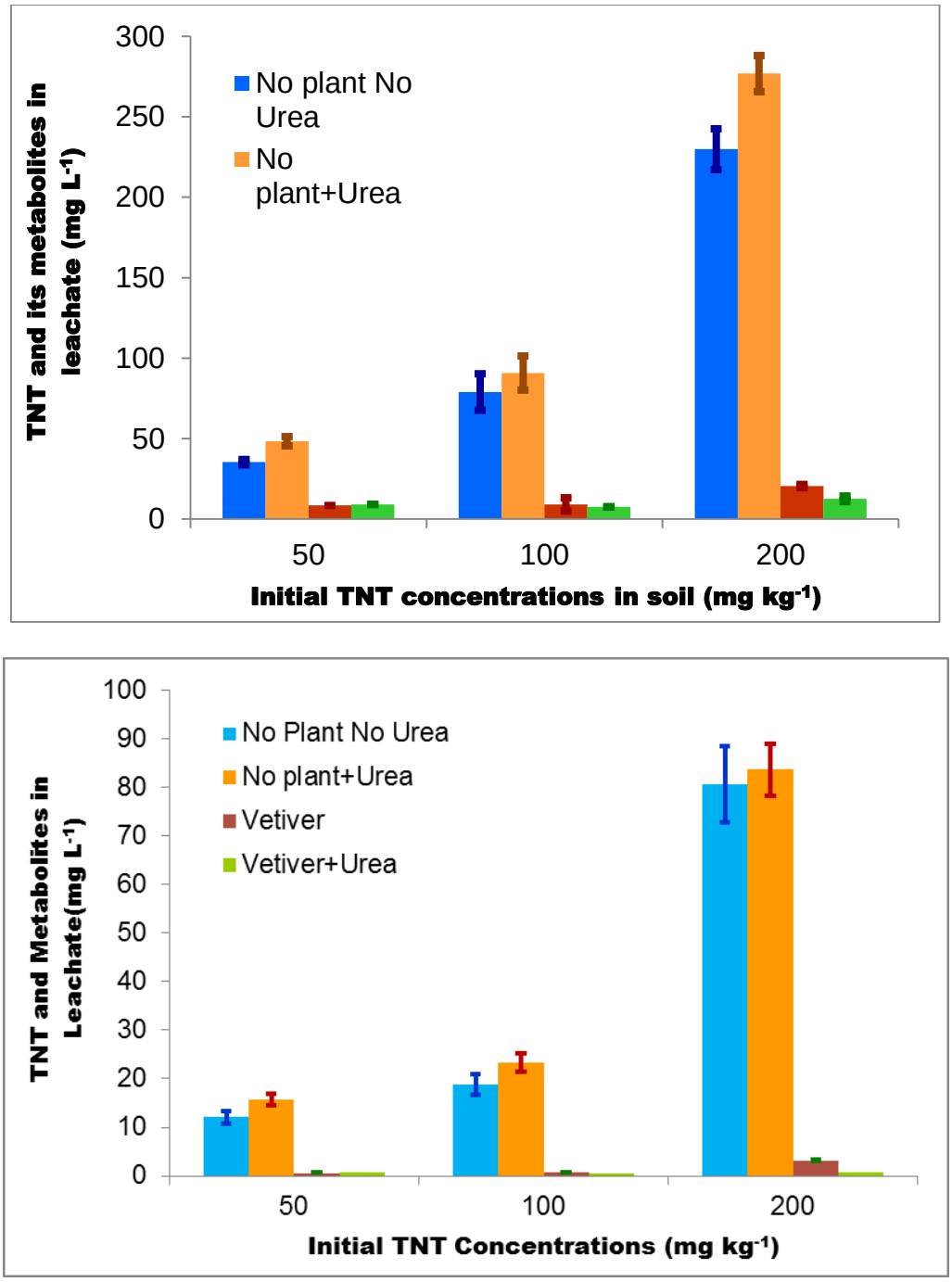

Figure 4-10. Sum of TNT and its metabolites in leachate after four (A) and six (B) months. Data are expressed as mean (n=3) ± one standard deviation.

Table 4-5. Toxicity of TNT and its metabolites in mammalian system and terrestrial plants.

TNT and Metabolites	LD_{50} in rats (oral) (mg kg^{-1})	EC_{50} (Terrestrial Plants) (mg kg^{-1})	Solubility (mg L^{-1})
2, 6, DNT	180 (USEPA 2008)	9.5 (Rocheleau et al., 2006)	180 at 22^0C (USEPA 2008)
2,4, DNT	270 (USEPA 2008)	56 (Rocheleau et al., 2006)	300 at 22^0C (USEPA 2008)
1,3,5 TNB	284 (ATSDR 1995)	129 (Rocheleau et al., 2006)	340 at 20^0C (ATSDR 1995)
TNT	795 (ATSDR 1995)	93 (Rocheleau et al., 2006)	101.5 at 25^0C (Ro et al., 1996)
4 ADNT	959 (Talmage et al., 1999)	Not available	43 at 20^0C (USCHPPM 2005)
2 ADNT	1522 (Talmage et al., 1999)	Not available	35 at 20^0C (USCHPPM 2005)

On the contrary, the finding of this study challenges the assumption that soil-bound TNT always decrease the threat of exposure to TNT and its harmful metabolites. It is evident in literature that soil microbial community can transform TNT to numerous metabolites which could be more or less harmful than the parent compound. One of the metabolites that was found in the leachates of plant-free control columns, was dinitrotoluene (both isomers, 2,4-DNT and 2,6-DNT) (figure 4-11). Both of these isomers of dinitrotoluenes are found to be more toxic than TNT itself in in-vivo studies (LD50 in rat = 270 and 180 mg kg-1, respectively). According to USEPA classification DNTs are listed as group B human carcinogens (USEPA, 2008). After four months onwards, DNTs were found in the

leachates of all plant free control columns and were not present in presence of vetiver grass, irrespective of presence or absence of urea. Absence of DNT in leachate samples collected earlier in the experimental duration indicates a slow microbial transformation of residual TNT or ADNT to DNTs. As DNTs are more soluble than TNT and ADNT, they were found in the leachates and not in the soils. Whereas, in present of vetiver-urea systems, the faster kinetics of TNT removal allowed the plant to take up the TNT and its metabolites from soils, allowing minimal TNT to retain in the soil to be subjected of slow microbial transformations. This unexpected finding raised the concern of not removing TNT from the contaminated system, which could lead to TNT transformation to more harmful and soluble metabolites, which could migrate to ground water easily and cause more risk than the parent compound itself.

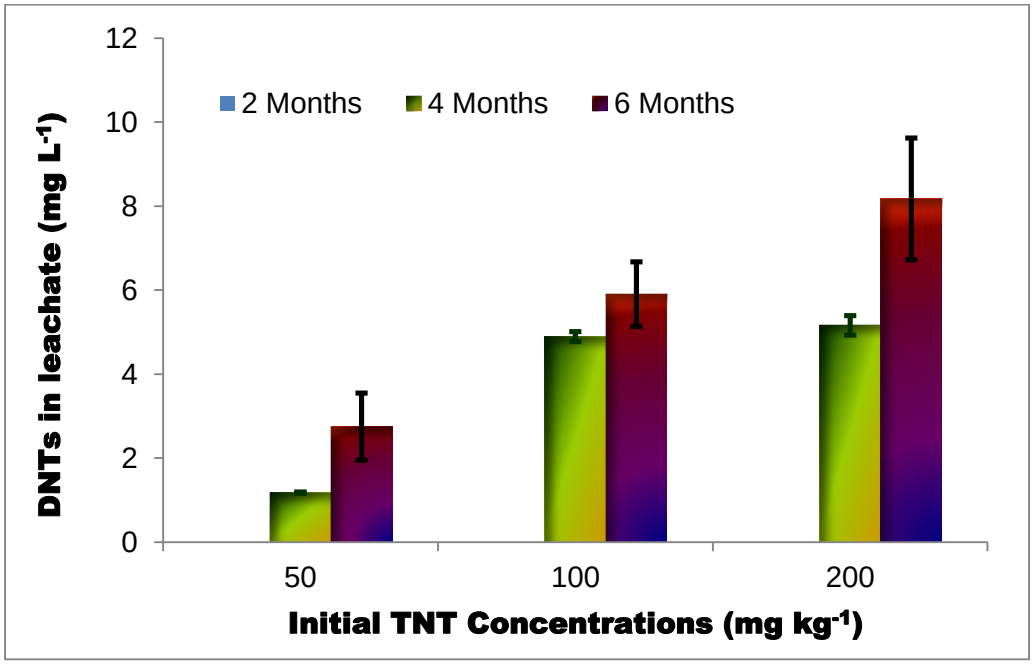

Figure 4-11. Dinitrotoluenes in the leachates of plant-free control columns. Data are expressed as mean (n=3) ± one standard deviation.

4.4. **Conclusions**

This study showed that vetiver grass has high potential to remediate TNT contaminated soils. Vetiver grass exhibited very high affinity for TNT irrespective of the presence or absence of urea. Presence of urea significantly ($p<0.001$) enhanced the kinetics of TNT removal from soil. One of the major findings of the current study is, although the agronomically recommended urea application rates are much lower than the effective chaotropic doses reported in the previous studies, the use of urea at agronomically-recommended rate successfully enhances the phytoavailable TNT in soil solutions and hence cause significant increase in the TNT uptake by vetiver grass from soil. The minimum agronomically recommended urea application rate (125 mg kg^{-1}) resulted in significant TNT uptake. TNT concentrations in the root of vetiver grass were higher compared to the reported values in other potential TNT accumulators. Significant TNT translocation from root to shoot was observed. Three metabolites of TNT, like 2-ADNT, 4-ADNT and 1,3,5-TNB were detected in shoot, suggesting translocation followed by phytodegradation of TNT by vetiver grass. Presence of ADNTs in the root and shoot tissues of the plant suggests the biochemical pathway of TNT is mediated by the nitroreductase enzyme. Nitroreductase activities are identified in both root and shoot tissues of the vetiver grass. NR activity is much higher in the shoot than that of the root suggesting more phytotransformation of TNT in the shoot tissues of the vetiver grass.

Phase I determined that the optimum chaotropic effectiveness of urea in enhancing the TNT uptake by vetiver grass falls within the agronomically-recommended urea application rates. However, the effectiveness of this process can change at different TNT loads. Phase II evaluated the effectiveness of the optimum urea concentrations at different initial soil TNT concentrations in a greenhouse soil column set up. This part of the study also investigated the concern of probable migration of urea-mobilized TNT into groundwater. Optimum urea concentration significantly enhanced the TNT removal at all TNT concentrations. The effectiveness of urea-vetiver system in phytoextraction of TNT was evident in complete removal of TNT up to 100 mg kg^{-1} initial TNT loads and 95% removal in 200 mg kg^{-1} initial TNT concentrations within six months. Significantly higher TNT and its metabolites were found in the leachate in plant-free-controls than that was found in the leachate in presence of both plant and urea. Along with TNT and ADNTs, dinitrotoluenes (2,4-DNT and 2,6-DNT), were found in the plant-free TNT amended controls which raises high concern as both of these compounds are found to be more toxic than TNT itself in in-vivo studies (LD_{50} in rat = 270 and 180 mg kg^{-1}, respectively). According to USEPA classification DNTs are listed as group B human carcinogens (USEPA 2008). These compounds were not detected in leachate in presence of vetiver, because of the fast removal of TNT and its metabolites by vetiver grass.

The findings of the current study are highly encouraging and will pave our way to the next step of achieving our long term goal of developing a urea-catalyzed phytoremediation technique for TNT contaminated soils using vetiver grass.

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CHAPTER 5

Optimization of Kinetic Factors Influencing the Nitroreductase Mediated Phyto-transformation of 2,4,6-Trinitrotoluene (TNT) by Vetiver Grass

Abstract

The search for a cost-effective and environmentally safe remediation technique for military contaminants such as 2,4,6-Trinitrotoluene (TNT) has generated interest in developing innovative phytoremediation systems. In our earlier studies, we found that vetiver grass, in the presence of urea used as a chaotropic agent, was highly effective in removing TNT from both soil and aqueous media. The present study aims at optimizing the parameters influencing TNT degradation by vetiver grass, which is the key step in designing an effective phytoremediation system. Nitroreductase (NR) is the most important enzyme identified so far as involved in the transformation of TNT, by catalyzing the reduction the nitro groups to amino groups. Saturation kinetics of NR were determined by using two different approaches; using whole vetiver plants grown in different TNT containing nutrient solutions and using crude enzyme extract isolated from vetiver shoots. The results show that NR-activity was significantly ($p<0.001$) higher in both root and shoot tissues of the TNT-treated plants as compared to the control plants at all sampling intervals. TNT transformation by NR enzyme in the shoot was much higher than that of the root. Pseudo first order rates (k_1 h^{-1}) of TNT transformation by NR

enzyme increased with plant concentration in the crude extract (0-500 g L^{-1}) and showed an initial increase, followed by decrease as functions of temperature (5 - 45^0C) and initial TNT concentrations (0-100 mg L^{-1}). Important kinetic parameters like maximum reaction rate constant, half saturation constant, and enzyme activation energy were determined by fitting the kinetic data to Michaelis-Menten and Arrhenius equations. The optimum range of the factors influencing NR mediated TNT transformation and the kinetic parameters will be very helpful for applying the phytoremediation technique for TNT contaminated systems using vetiver grass.

5.1. Introduction

Conjugation and sequestration of xenobiotic compounds to cell wall and cell vacuole are the key processes responsible for the innate defense mechanism of plants against phytotoxic compounds. 2,4,6-Trinitrotoluene (TNT), which was classified as a group C human carcinogen exhibited potent phytotoxic effects to a wide variety of terrestrial and aquatic plants. Our earlier studies reported vetiver grass to be able to uptake and tolerate TNT stress at concentrations much higher than the benchmark toxicity levels for plants. The effectiveness of vetiver grass as a phytoremediation agent suggests presence of a potent detoxification pathway to tolerate TNT stress. transformation of TNT to more polar metabolites are of utmost importance as direct conjugation is unlikely for TNT as it does not carry these required functional groups (Burken et al., 2000; Hannink et al., 2002). Thus, as a part of their detoxification mechanism, plants must transform TNT to metabolites that contain the required functional groups for conjugation and transport (Hannink et al., 2002), as bound residues can be sequestered and rendered less toxic. Numerous studies have reported that different aquatic and terrestrial plants successfully take up TNT from hydroponic or soil media and transform it to various metabolites (Hannink et al., 2002). Isomers of mono-amino di-nitrotoluenes (ADNTs) were the most frequently found TNT metabolites, suggesting that the nitroreductase (NR) enzyme plays a strong role in the TNT transformation process in plants. Reduction of the nitro groups is the preferred transformation pathway for TNT as each of its three nitro groups consists of two electronegative elements, nitrogen and oxygen; The N-O bond becomes polarized as

oxygen is more electronegative than the nitrogen atom. Due to the higher electronegativity of oxygen, N atom retains the partial positive charge that makes the nitro- group easily reducible (Esteve-Nu´n˜ ez et al., 2001).

Researchers also showed that plants like soybean that demonstrated high NR activity exhibited high potential to uptake and tolerate TNT stress (Adamia et al., 2006). Our earlier studies using vetiver grass in both hydroponic systems (Makris et al., 2007) and soil (Chapter 4) reported the presence of TNT-metabolites such as 2 amino di-nitrotoluene (2 ADNT) and 4 amino di-nitrotoluene (4 ADNT) in the root and shoot tissues of vetiver grass, strongly suggesting a possible reduction of nitro group in vetiver tissues. Probable involvement of NR enzyme in the transformation of TNT in vetiver tissue was confirmed after observing much higher NR activity in the vetiver grass treated with TNT as compared to the TNT free control vetiver plants (Chapter 4).

Vetiver grass has been shown to possess NR mediated detoxification systems resulting in TNT transformation, but the kinetic parameters responsible for influencing the NR activity have yet to be optimized. As evident from literature, three major factors influencing the saturation kinetics of TNT detoxifying enzymes are initial TNT treatments as substrate concentrations, plant concentrations in the crude enzyme extracts, and temperature (Medina at al., 2004 and Richardson and Bonmati, 2005). To design a successful plant based remedial system for TNT contaminated systems the factors influencing the kinetics of plant enzymes must be characterized. Researchers have used an indirect method to find out the kinetic parameters of TNT removal without assaying

the actual TNT degrading enzyme activity in the plant tissue (Pavlostathis et al., 1998 and Medina et al., 2002). Pavlostathis et al. (1998) has derived the kinetic parameters assuming that enzymatic activity is proportional to plant concentrations in the crude enzyme extracts, which was defined as the mass of plant material per unit volume of solution (Medina et al., 2000). The major limitation of this approach was the assumption that there is only one enzyme responsible for TNT degradation. Other researchers assayed background NR enzyme activity of the crude enzyme extract prior to exposure of TNT containing system and used that crude enzyme extract as the phytoremediation agent instead of using the whole plant (Medina at al., 2004 and Richardson and Bonmati, 2005). However, as the indirect approach suggested, increased enzyme activity followed by TNT exposure (Adamia et al, 2006), it is important to directly assay the NR activity in the plant tissues following TNT exposures to varying levels. The present study specifically determined the saturation kinetics of NRenzyme mediated TNT transformation as functions of three major factors; plant concentrations in the crude enzyme extracts, temperature, and initial TNT concentrations. This study determined the optimum range of these factors, where maximum NR mediated TNT transformations were achieved.

The specific objectives of the current study were to i) investigate the kinetics of NR enzyme activity in root and shoot tissues of vetiver grass as function of exposure to varied initial TNT concentrations, and ii) determine the saturation kinetics of NR enzyme

extracted from vetiver grass as functions of three major factors; plant concentration in the crude extract, temperature, and initial TNT loads.

5.2. Materials and Method

Experimental unit: The experiments were conducted in two phases. In phase I, whole vetiver plants were grown in hydroponic solution containing TNT in varying concentrations (0, 25, 50, 100, and 200 mg L^{-1}) for 30 days. Triplicates of plants were kept for each sampling time to analyze the NR enzyme activity in the root and shoot tissues of vetiver grass at various exposure times (0, 5, 10, 15, 30 days). TNT free control plants and plant free TNT amended controls were set up.

In phase II, the saturation kinetics of NR enzyme was investigated using experimental units as aqueous phase microcosms which were created by mixing TNT solutions with the crude nitrate reductase enzyme extracted from vetiver grass. Deionized water in place of crude enzyme extracts was used as controls. As NR enzyme activity was found to be much higher in the shoot tissues than that of the root (Chapter 4), only shoot tissues were used to investigate the saturation kinetics of the NR as functions of plant concentrations in the crude enzyme extract, temperature, and initial TNT treatments. To investigate the effect of each of these three factors, the enzyme kinetics was determined

at each factor's varying levels, keeping the other two factors constant. Table 5-1 lists the details of the experimental conditions for each kinetic factor variation study.

Table 5-1. Experimental conditions for kinetic factors variation studies

Parameters	Plant Concentrations (in the crude enzyme extract) Variation Study	Initial Substrate Concentrations Variation Study	Temperature Variation Study
Plant Mass	50,100,200,250,500 g L^{-1}	250 g L^{-1}	250 g L^{-1}
TNT	20 mg L^{-1}	5, 10, 20, 30, 40, 50,100 mg L^{-1}	20 mg L^{-1}
Sampling Intervals	0,2,5,10,16,20,24, 32, 36, 40, 48 h	0,2,5,10,16,20,24, 32, 36, 40, 48 h	0,2,5,10,16,20,24, 32, 36, 40, 48 h
Temperature	20^0C	20^0C	5,15,20,25,30,35,45^{0}C
Aqueous Phase Microcosm	10 mL crude extract + 10 mL Enzyme Assay buffer + 5 mL of 20 mg/L TNT solution	10 mL crude extract + 10 mL Enzyme Assay buffer + 5 mL of 20 mg/L TNT solution	10 mL crude extract + 10 mL Enzyme Assay buffer + 5 mL of 20 mg/L TNT solution
Control	10 mL D.I. water + 10 mL Enzyme Assay + 5 mL of 20 mg/L TNT solution	10 mL D.I. water + 10 mL Enzyme Assay + 5 mL of 20 mg/L TNT solution	10 mL D.I. water + 10 mL Enzyme Assay + 5 mL of 20 mg/L TNT solution
Replicates	3	3	3

Preparation of the Crude Enzyme Extract and Enzyme Assay: Crude enzyme extracts were prepared following the protocol described in Chapter 4.

Data analyses: All data were expressed as mean (n=3) along with standard deviation. Two-way ANOVA was carried out using statistical software JMP IN version 8.0 (Sall et

al., 2005). Significant differences among treatment means were calculated using a Tukey-Kramer honest significant difference (HSD) test. Nitroreductase activity (μmol-NO_2 min^{-1} or U) was measured for each factor at different time intervals and dimensionless NR mediated TNT transformation was calculated as A/A_0, where A and A_0 are the final and the initial NR activities in the aqueous phase microcosm respectively. NR mediated TNT transformation (A/A_0) was plotted over time and was fit to the pseudo first order model to estimate the kinetic rate constant (k_1). These reaction rate constants were estimated to understand the trend with respect to initial substrate (TNT) concentrations, plant concentrations in the crude enzyme extract, and temperature.

5.3. 5.3. Results and Discussion

5.3.1. Effect of TNT exposure on growth of whole vetiver plants

Our earlier studies showed that vetiver could tolerate higher TNT treatments than the benchmark of 30 mg kg^{-1} TNT toxicity limit (Talmage et al., 1999) for terrestrial plants in soil (Chapter 4). The present study evaluated the phytotoxic effects of TNT as functions of increasing TNT loads and exposure time in hydroponic media, where TNT was completely available for the plants to take up. The results showed that increasing concentrations of TNT affected the growth of vetiver grass. At lower concentrations (25 and 50 mg L^{-1}), no significant effect on growth was observed. In higher initial TNT loads (100 and 200 mg L^{-1}), significant biomass reduction was noted after 10 days (Figure 5-1).

However, no visible signs of toxicity like chlorosis or stunted root growth were noted in any of the TNT treatments over the 30 day experimental period. The length of the root as well as shoot also remained unaffected, showing vetiver's innate ability to tolerate TNT stress up to 200 mg L^{-1} aqueous TNT concentrations.

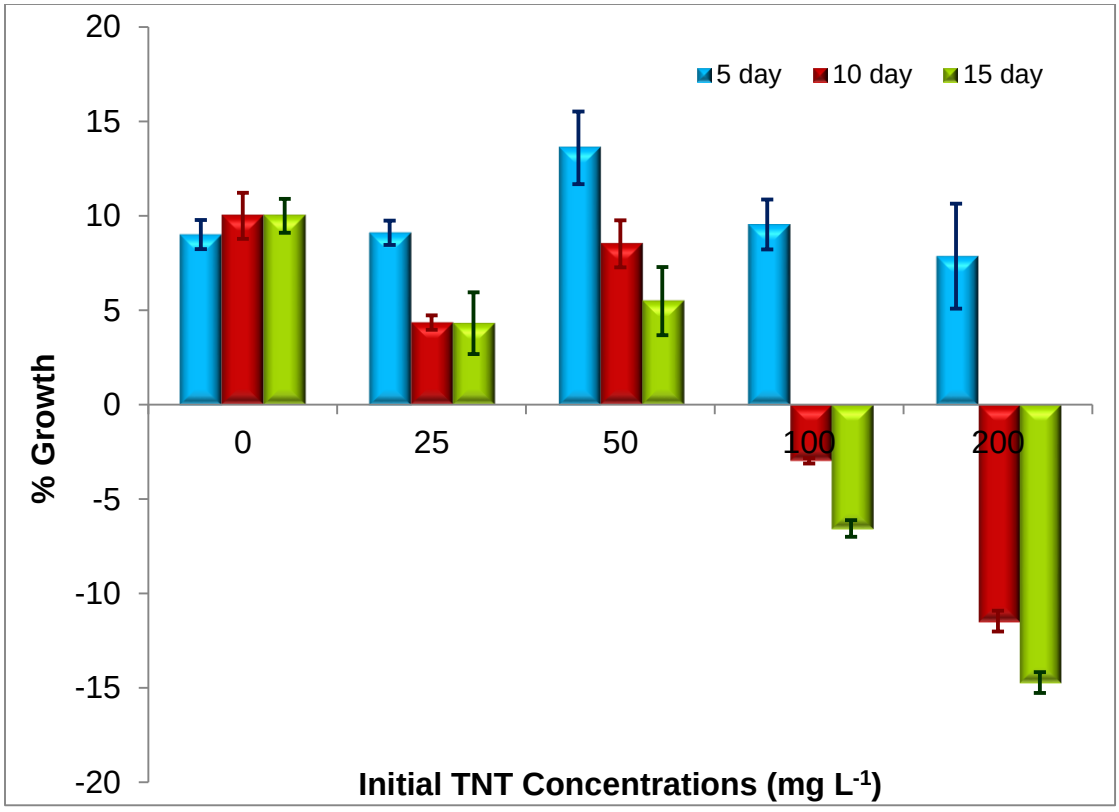

Figure 5-1. % Growth of vetiver grass following varying TNT exposures. Negative values express the reduction in biomass. Data are expressed as mean (n=3) and one standard deviation.

NR activity in whole vetiver plant as functions of initial TNT concentrations and exposure time: Nitroreductase enzyme activity in the root of vetiver grass was significantly influenced by initial TNT concentrations ($p<0.0001$) (Fig 5-2). Adamia et

al., 2006 also reported that NR activities were significantly enhanced during the plant cultivation of TNT-containing hydroponic media. In our earlier greenhouse soil pot study, as noted in chapter 4, vetiver grass harvested after 22 days exposure in TNT containing soils showed significant increase in the NR activity in TNT-treated plants compared to TNT-free control plants. Additionally, the current hydroponic study found that at each initial TNT treatment, NR activity in the root of vetiver grass was significantly ($p<0.01$) enhanced by the exposure time whereas the root of the TNT-free control plants did not show any significant change in the NR activity over time (Fig 5-2). The kinetics of NR enzyme activity followed first order reactions in lower TNT concentrations (25, 50, and 100 mg L^{-1}; R^2 = 0.98, 0.84, 0.94 respectively), whereas, the increase in the enzyme activity at 200 ppm initial TNT concentration followed a second order reaction (R^2 = 0.92). The second order reaction rate constant was calculated using the slope ($(n-1)*kn*A_0$) of the second order fit. The second order reaction rate constant (k_s) was found as 1.51 $U^{-1}mL\ h^{-1}$.

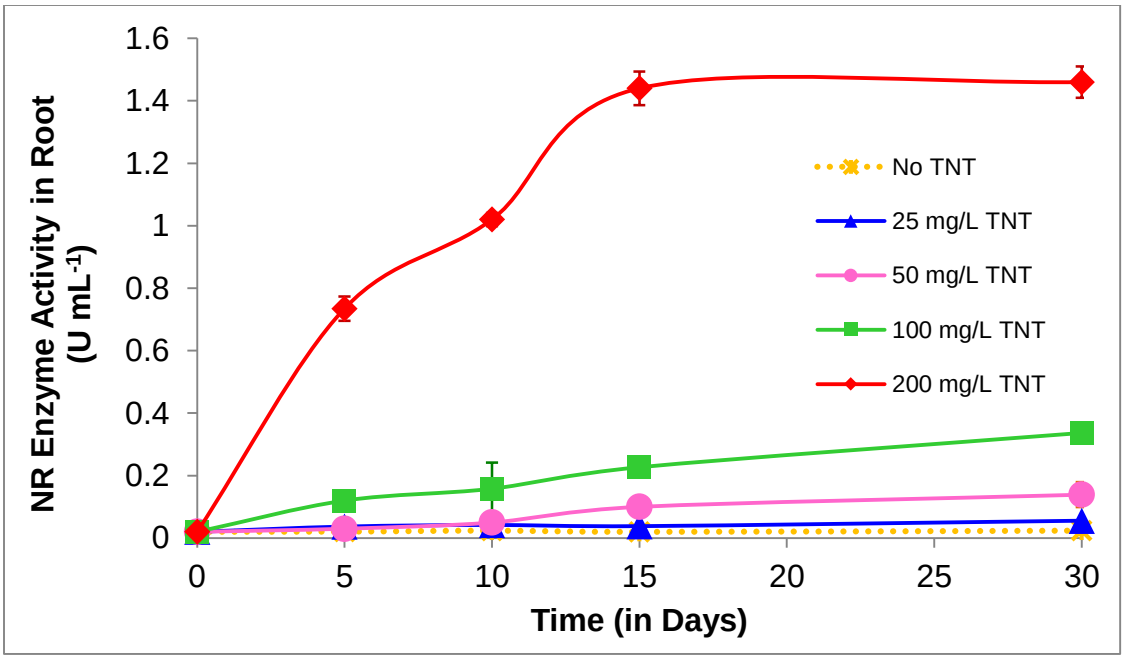

Figure 5-2. Kinetics of Nitroreductase enzyme activity in the root of vetiver grass following exposure to various concentrations of TNT. NR enzyme activity is expressed in U mL^{-1}. Data expressed as mean (n=3) and one standard deviation.

NR activity was higher in shoot than that of the root in all TNT treatments, suggesting more transformation of TNT in the shoot tissues of vetiver grass. This supports our current and previously reported results showing more TNT metabolites in shoot as compared to root (Chapter 4). As expressed in figure 3, increasing initial TNT treatments resulted in a significant increase in the NR activity in the shoot; however, this trend was not as evident in higher exposure time. This could be explained by the suggested conjugation of the TNT metabolites over time, followed by the sequestration of the bound residues in the cell wall or cell vacuole (Harvey et al., 1990).

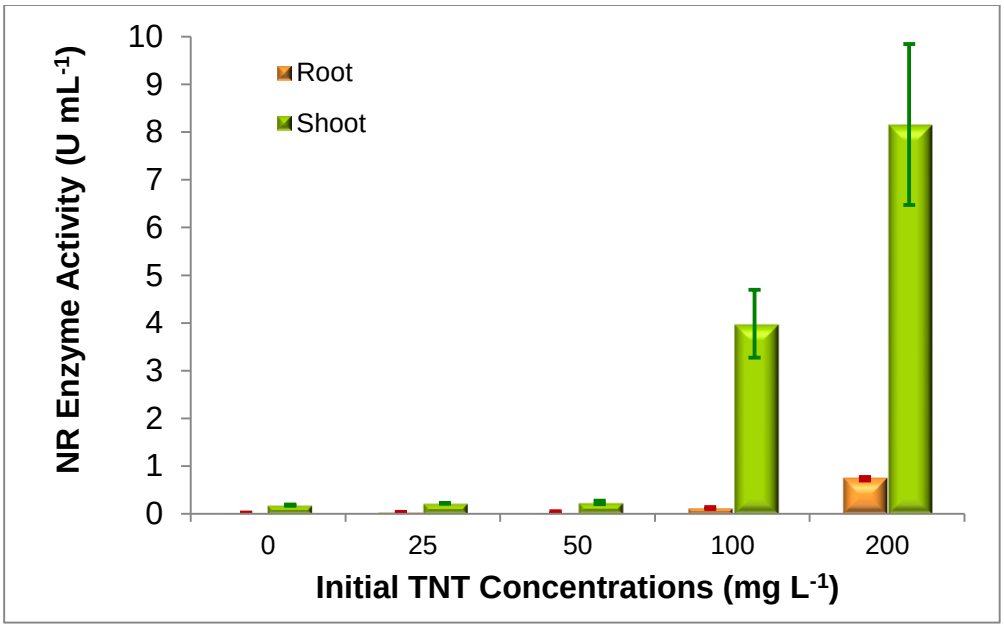

Figure 5-3. Relative NR activity in the root and shoot tissues of vetiver grass after 5 days. Data expressed as mean (n=3) and one standard deviation.

5.3.2. Saturation kinetics of NR in aqueous phase microcosm

Effect of Varying Plant Concentrations in the Crude Enzyme Extract: This part of the experiments was conducted to determine the optimum plant concentrations in the crude enzyme extract, which would exhibit the maximum NR activity. NR enzyme activity significantly ($p<0.0001$) increased with increasing plant concentrations in the crude enzyme extract. Initial kinetics was slow irrespective of plant concentrations in the crude enzyme extracts, resulting in minimal enzyme activity until 20 h. Optimum NR activity was observed between 20 to 40 h in all plant concentrations in the crude enzyme extracts tested. Kinetic rates of NR mediated TNT transformations in terms of the enzyme activity (where initial TNT concentration and temperature are constant) were

determined. The kinetics of NR enzyme activity followed first order reactions at higher plant concentrations in the crude enzyme extracts (100,150, 200, 250, and 500 g L^{-1}). Pseudo first order reaction rate constants (k_1) increased with increasing plant concentrations in the crude enzyme extracts (Fig 5-4). As exhibited in the figure 4, 250 g L^{-1} plant concentration exhibited the maximum NR activity. Further increase in plant concentrations did not cause any significant increase in the NR activity. Hence, the effects of initial TNT concentrations and temperature were evaluated using 250 g L^{-1} plant concentration in the crude enzyme extract.

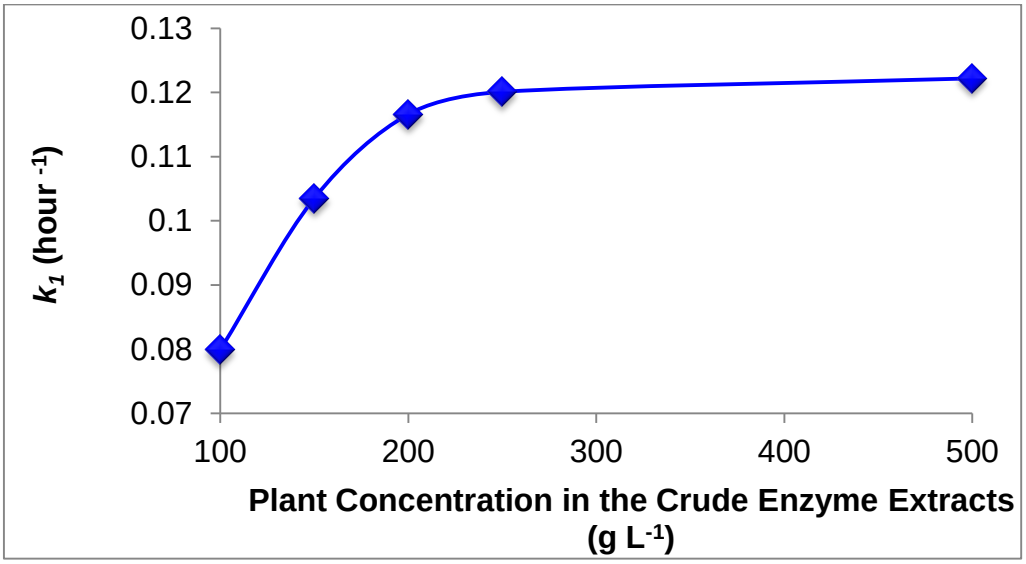

Figure 5-4. Saturation kinetics of NR expressed as pseudo first order rate constant (k_1)of NR mediated TNT transformation reaction as functions of plant concentration in the crude enzyme extract, at constant temperature (30^0C) and TNT load (20 mg L^{-1}). Data are expressed as mean (n=3).

Effect of Initial TNT Concentrations: Initial substrate (TNT) concentrations showed significant effects ($p<0.001$) on the kinetics of NR enzyme activity (Figure 5-4). NR

activity increased with increasing initial TNT loads up to 40 mg L^{-1}. Further increase in TNT loads resulted in decreased enzyme activity. The kinetics of NR enzyme activity followed first order reaction within 10 to 100 mg L^{-1}. Pseudo first order reaction rate constants (k_1) increased with increasing initial TNT concentrations up to 40 mg L^{-1}, followed by a decrease with further increase in initial substrate concentrations.

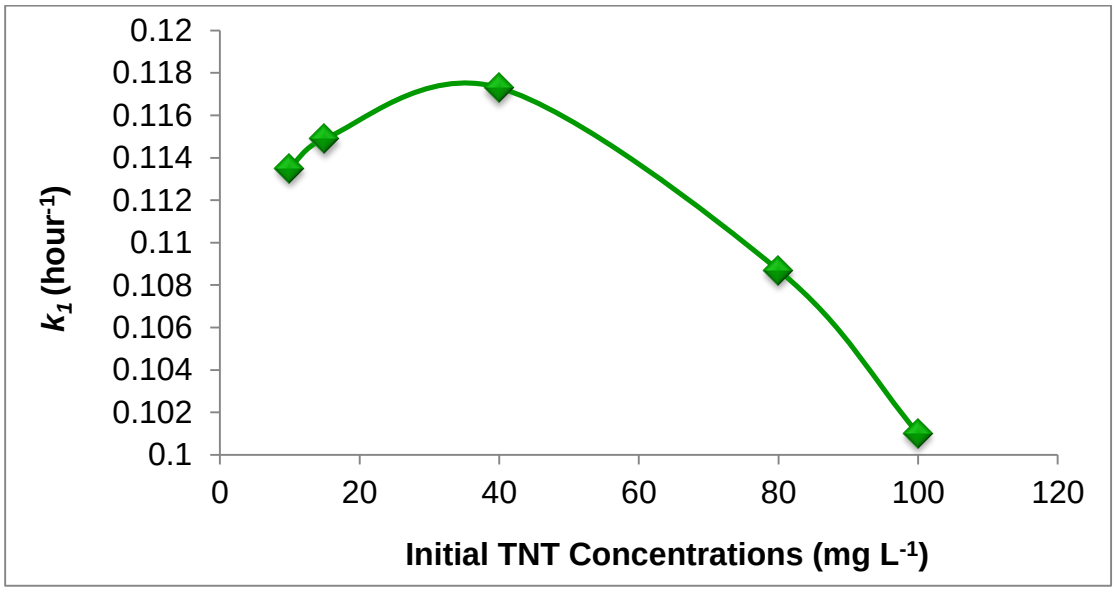

Figure 5-5. Saturation kinetics of NR expressed as pseudo first order rate constant (k^1) of TNT transformation reaction as function of initial TNT concentrations, at constant plant concentration in the crude enzyme extract (250 g L^{-1}) and temperature (30^0C). Data expressed as mean (n=3).

Modified Michaelis-Menten equation for enzyme saturation. TNT transformation kinetics were evaluated in terms of enzyme saturation by applying an alternate form of

the Michaelis-Menten equation written in terms of enzyme activity (Richardson and Bonmati, 2005).

$$k = kmax\{1/(Ksat + A) \qquad (1)$$

Where, k_{max} = maximum rate constant under excess enzyme activity at a given TNT concentration, and K_{sat} = half-saturation constant.

This rectangular hyperbola functions was linearized using the Hanes-Woolf linear transformation to determine the k_{max} and K_{sat} (Richardson and Bonmati, 2005).

$$\left(\frac{A}{k}\right) = \left(\frac{A}{kmax}\right) + \left(\frac{Ksat}{kmax}\right) \qquad (2)$$

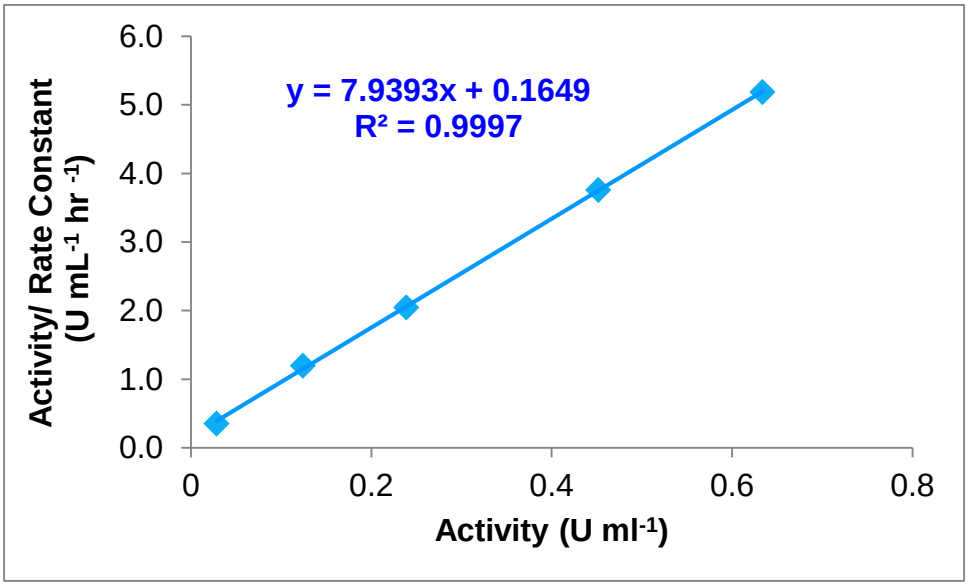

Figure 5-6. Hanes-Woolf type of linear transformation plot of Modified Michaelis-Menten equation for enzyme saturation.

The ratio of activity and pseudo-first-order rate constant (*A/k*) was plotted over the enzyme activity (*A*) as the Hanes Woolf type linear transformation (2) of the modified Michaelis –Menten equation (4) (Figure 5-6). The maximum rate of reaction (k_{max}) was 0.13 h^{-1}, as calculated from the inverse slope. The half saturation constant K_{sat}, which was determined using the intercept and the k_{max}, was found to be 0.02 U mL^{-1}. These values are much lower compared to k_{max} and K_{sat} values found by Richardson and Bonmati (2005) in spinach (0.50 $h^{-1}$and 0.17 U mL^{-1} respectively). This difference between the current study and the previously reported literature happened because the reaction rate constants in earlier studies were calculated by measuring the total TNT removal from the solution, which is possibly caused not only by NR but also other enzymes present in the crude enzyme extracts; whereas, our study has specifically calculated the NR mediated TNT transformation reaction rate constants by measuring the changes in the NRactivity as functions of various plant concentrations in the crude enzyme extracts and exposure time. Moreover, the enzyme activity measured by these researchers were the background NR activity; whereas the activity measured in this study is followed by TNT exposure which caused a significant increase in the NR activity.

Effect of Temperature: As expected, temperature showed a pronounced effect on the kinetics of NR enzyme activity (Fig 7). NR activity significantly ($p<0.001$) increased with increasing temperatures up to 35^0C. Further increase in temperature denatured the

enzyme, resulting in minimal activity at 45^{0}C. The optimum range of temperature was 30 to 35^{0}C. The most consistent NR activity was observed at 30^{0}C.

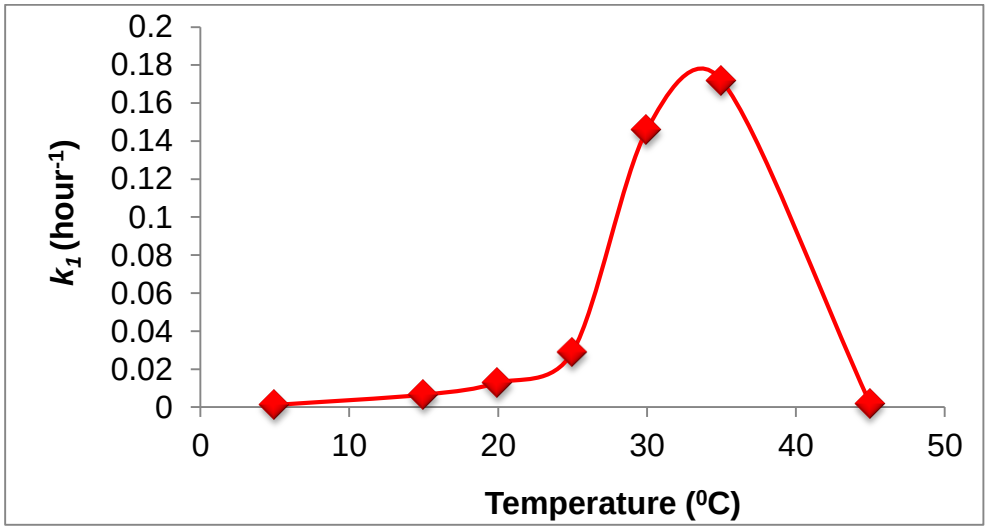

Figure 5-7. Saturation kinetics of NR expressed as pseudo first order rate constant (k_1) of NR mediated TNT transformation reaction as a function of temperature, at constant plant concentrations in the crude enzyme extracts (250 g L^{-1}) and TNT load (20 mg L^{-1}). Data expressed as mean (n=3).

The kinetics of NR enzyme activity followed first order reaction within 5 to 35^{0}C. Pseudo first order reaction rate constants (k_1) increased with increasing temperature up to 35^{0}C, followed by a sharp decrease at 45^{0}C. The pseudo first order rate constants of TNT transformation were fit to the Arrhenius relationship to understand the effect of temperature on the saturation kinetics of the NR enzyme.

$$k_1 = A \exp^{-\frac{E_a}{RT}} \qquad (3)$$

Where A= Pre exponential Constant, E_a = Enzyme activation energy, R is the ideal gas constant = 8.31joules/^{0}K/mole. The data were fit to the linearized form of this equation that is:

$$\ln k_1 = \ln A + (\frac{E_a}{RT}) \quad (4)$$

ln k_1 was plotted over 1/T to determine the enzyme activation energy (E_a) from the slope (E_a/R = slope) of the plotted line. Pseudo first order reaction rate constants of NR mediated TNT transformation showed good fit (R^2=0.98) to the Arrhenius equation between 5 to 35^{0}C. Enzyme activation energy was calculated as 123.74 KJ Mole^{-1} (Fig 5-8).

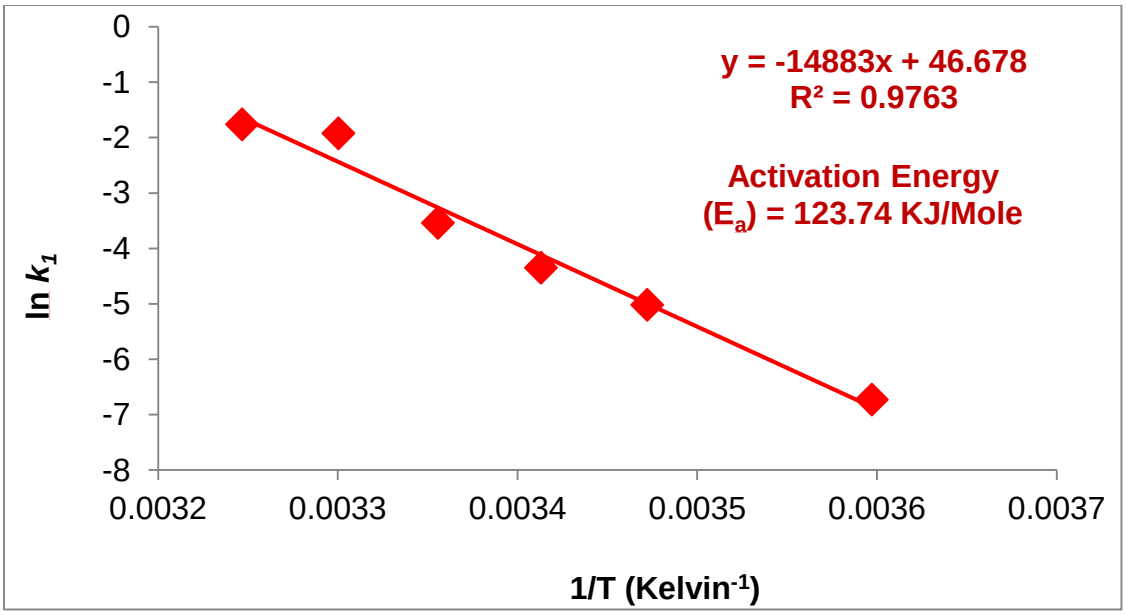

Figure 5-8. Arrhenius relationship of pseudo first order reaction rate constants between 5 to 35^0C.

Medina et al. (2000) documented an activation energy of 62.3 kJ/mol for TNT transformation in *Myriophyllum aquaticum* (between 2 to 34^0C) and Richardson and Bonmati (2005) reported an an activation energy of 54.7 kJ/mol in spinach (between 5 to 30^0C). The enzyme activation energy found in vetiver grass (between 5 to 35^0C) was much higher than those reported by the earlier researchers, probably because in the current study, the NR mediated TNT transformation rates were calculated directly through measuring the NR activity in the crude enzyme extract and not from the overall TNT transformation, whereas, the other studies attributed overall TNT transformation by the crude enzyme extracts, which could be caused by more than one enzyme.

5.4. Conclusions

Nitroreductase, the major TNT degrading enzyme, was assayed in vetiver grass and characterized as functions of three controlling factors; initial TNT load, plant concentrations in the crude enzyme extracts, and temperature. This study determined the kinetics of NR mediated TNT transformation by directly measuring the NR activity under different conditions, and not by the removal of TNT from the media, to avoid measuring additional possible TNT transformation reactions by other plant enzymes. Nitrate reductase enzyme activity in both root and shoot tissues of vetiver grass significantly ($p<0.0001$) increased with increasing levels of TNT, suggesting a role for the NR enzyme in TNT degradation in vetiver grass. Higher NR activity in the shoot suggests more TNT-degradation potential in shoots than that of the root tissues of vetiver grass.

Pseudo first order rates of NR mediated TNT transformation reaction increased with increasing plant concentrations in the crude enzyme extracts, up to 35^0C and 40 mg L^{-1} initial TNT concentrations. Further increase in temperature or initial TNT loads resulted in a decrease in NR enzyme activity. Rate constants as function of plant concentrations in the crude enzyme extracts continued increasing with increasing plant concentrations, reaching a plateau at 250 g L^{-1}. No significant increase in the enzyme activity was noted with further increase in plant concentrations in the crude enzyme extracts. This study determined the important kinetic parameters of the NR mediated TNT transformation reaction in vetiver grass, which will help to optimize the factors influencing

phytodegradation of TNT and designing a successful plant based remediation system for TNT contaminated soil/water using vetiver grass. The difference in values of these kinetic parameters from the previously reported values for TNT transformation in literature suggests the presence of other TNT transforming plant enzymes in the crude enzyme extracts, which, in addition to NR, could probably contribute to the overall rates of TNT transformation. The current study specifically reported the kinetic parameters of NR mediated TNT transformations which differ from those reported for the overall TNT transformation reactions. Based on the optimum enzyme conditions found in the current study, experiments are underway in our laboratory to design a phytoreactor to remediate TNT contaminated aqueous media, using the enzyme extract, isolated from the shoot tissues of vetiver grass.

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CHAPTER 6

Proteomic profiling of Vetiver grass (Chrysopogon zizanoides) under 2,4,6 Trinitrotoluene (TNT) stress

Abstract

One of the major challenges in successful application of phytotechnology to remediate 2,4,6-trinitrotoluene (TNT) is its phytotoxicity, as TNT is commonly found in high concentrations in contaminated military sites. Our earlier studies have shown that vetiver grass is an ideal plant for TNT phytoremediation. The current study is the first attempt to investigate the changes in the proteomic profile of a plant under TNT stress. Vetiver plants were grown in a plant growth chamber in nutrient media with varying concentrations of TNT (0, 25, 50, 100 mgL-1) for 10 days. Although the plants appeared healthy, significant biomass reductions ($p<0.001$) were found in all the TNT treated plants. However, a significant ($p=0.03$) reduction in total chlorophyll content was observed only in 100 mg L-1 TNT treatment. Total proteins in the root decreased significantly ($p=0.0003$), but no significant ($p>0.05$) change was noted in the shoot Classical 2-DE-gel-electrophoresis was conducted to separate the proteins. Gel analyses using the Image Master Platinum 6.0 software (GE healthcare Lifesciences) showed that 20 protein spots had a minimum of two fold change in their intensities (6 upregulated and 14 downregulated), compared to the control gel. Protein spots with a minimum two fold

change were excised from the gel for MALDI-TOF analysis. Functional annotation of these proteins identified downregulation of functional proteins which are involved in key cellular mechanisms like transcription of DNA, ribosome ribosome biogenesis, nucleocytoplasmic transport of protein, protein glycosylation, and translation. Growth related proteins were downregulated which supports our biomass reduction data. Plant defense proteins were upregulated at lower TNT concentrations suggesting enhanced defense mechanism; however, at higher TNT concentrations these proteins also downregulated because of TNT stress. Comprehensive understanding of changes in the proteomic profile provides important clues to the mechanism of stress response and the tolerance in vetiver grass.

6.1. Introduction

Phytotoxicity associated with 2,4,6-trinitrotoluene (TNT) is a strong limitation to the use of plants for remediation of TNT contaminated soil and aqueous media. It is a common problem faced by almost all researchers working with plants and TNT (Hannink et al., 2002). This could explain the fact that after having so many successful laboratory experiments on TNT uptake and transformation, yet phytoremediation technique could not be applied on a large scale to remediate contaminated military sites with high TNT concentrations. One of the proposed solutions is creating transgenic plants which will tolerate the stress associated with the higher TNT concentrations (French et al. 1999 and Hannink et al. 2001). Researchers have successfully developed transgenic plants with enhanced TNT tolerance without looking into much detail on the exact biochemical mechanisms, which provide wild plants with its innate tolerance to TNT stress. Although most plants exhibit a range of adverse effects including impaired growth and chlorosis, few plants like parrot feather and vetiver grass exhibit high TNT tolerance, suggesting presence of innate detoxification mechanisms in high TNT accumulating plants (Hannink et al., 2002). One way to understand these plants' biochemical mechanism of TNT tolerance is through using modern proteomics techniques with integrated bioinformatics, which recently opened novel avenues to investigate plants' response to the environment proteomic profiles under various conditions.

Plant proteins play major roles in controlling the stress related mechanisms followinged by exposure to contaminants (Ahsan et al., 2009). Loss of some functional proteins interrupts the biological processes of the plant and produce phytotoxic effects whereas some plants generate proteins which take part in detoxification pathways and give the plant tolerance to the contaminants. Proteomics is a new approach for studying complex biological functions of proteins which is helpful are helpful to identify the molecular mechanisms those play key roles in plant-contaminants interactions (Ahsan et al., 2009). For example, Gillet et al., 2006 found that in algae, the abundance of proteins involved in photosynthesis were significantly decreased on exposure to cadmium stress, whereas proteins related to the defense mechanisms such as GSH biosynthesis, ATP metabolism, and the response to the oxidative stress were significantly increased.

Most of the proteomics studies conducted so far investigated the changes in plant proteome following exposure to the toxic metals. However, similar phytotoxic effects caused by TNT indicate that studying the changes in the abundances of protein will help in understanding the stress related mechanisms caused by TNT exposure. The uptake of increasing levels of TNT by plant cells severely interrupts various physiological and biochemical pathways leading to a restriction of plant growth and ultimately cell death. The identification of the functional proteins that are involved in responses to TNT stress is a fundamental step in understanding the molecular mechanisms of stress response. Our earlier experiments reported vetiver grass to exhibit minimal phytotoxic effects followed by the exposures to varying TNT concentrations (Chapter 2, 3, and 4). However, upon increasing the initial TNT loads the phytotoxic effect may increase. It is

necessary to find out what are the phytotoxic effects of TNT concentrations on vetiver grass and its ability and extent of tolerating TNT toxicity. The present study conducted proteomic profiling of vetiver grass to identify candidate proteins that are likely to play major roles in regulating biochemical, molecular, and physiological responses under varying levels of TNT stress.

6.2. Materials and Method

Experimental set up: The effect of TNT on the vetiver proteome was investigated in hydroponic media containing four varying TNT concentrations (0, 25, 50, 100 mg L^{-1}). The highest TNT load was chosen as 100 mg L^{-1} to check the effect of TNT stress close to its maximum solubility level as the aqueous solubility of TNT is 101.5 mg L^{-1} at room temperature (Ro et al., 1996; Makris et al., 2007b). Uniform distribution of vetiver plants were grown in plant growth chamber with a 16/8h day/night photoperiod. After 10 days, vetiver plants were removed and washed with deionized water. Final weights of the plants were measured to determine the growth or biomass reduction. Root and shoot parts were separated and plant materials were flash frozen in liquid nitrogen. Both root and shoot samples were stored at -80 ºC for further analyses.

Total chlorophyll content: The chlorophyll pigments were extracted using 80% acetone. The absorbance was measured at 663 nm and 645 nm. The total chlorophyll content was determined using the equation reported by Sunkar, 2010.

Total soluble protein: The total soluble proteins from both the root and shoot tissues of vetiver grass were extracted twice using ice-cold phosphate buffer (50mM, pH 7.8). The protein concentration was quantified by using ReadyPrepTM protein extraction kit (Bio-Rad, CA) and quantified using BCA protein assay kit (Bio-Rad, CA).

Analysis of plant proteome: Two-dimensional (2-D) gel electrophoresis was conducted (according to the manufacturer's instructions, Bio-Rad, CA) to separate the proteins and Gels were stained by Coomassie Blue G-250 and scanned using GS-800TM densitometer (Bio-Rad, CA). ImageMaster™ 2D Platinum (version 7.0, GE Healthcare, WI) was used to determine the differentially expressed proteins. Significantly differential protein spots those exhibited fold change ≥2 were selected for mass spectrometric analysis. Protein spots of interest were excised from gels, digested with trypsin, and analyzed through matrix-assisted laser desorption /ionization-time of flight (MALDI-TOF) (Bruker, WI) following the method described by Shevchenko et al. (2006). The mass spectra were processed by flex analysis software (Bruker, WI). As vetiver grass is not sequenced yet, the mass lists were searched against NCBI_nr for green plants. The identification of proteins was conducted carefully based on the top match score and by comparing the molecular weight and pI (calculated based on amino acid sequence) with the gel locations. Functional annotations of the identified proteins were carried out according to Uniprot database and predicted functional partners of the identified proteins were searched using String database (String 9.0).

Data analyses: Data were expressed as mean (n=2) along with standard deviation. Two-way ANOVA was carried out using statistical software JMP IN version 8.0 (Sall et al., 2005). Significant differences among treatment means were calculated using a Tukey-Kramer honest significant difference (HSD) test. Statistical significance of protein spots' intensities was calculated using Student's t-test using ImageMaster™ 2D Platinum software.

6.3. Results and Discussion

Although vetiver grass showed much more tolerance than the other grasses studied for TNT phytoremediation, in our earlier experiments biomass reduction wasis noted in our earlier experiments at higher TNT concentrations (Chapter 5). The current study again evaluated the effect of TNT exposure on growth of vetiver plants to corroborate the biomass reduction with any potential loss of functional proteins determined by the proteomics approach. After 10 days of exposure to varying TNT containing solutions, the plants appeared healthy with no visible sign of toxicity like chlorosis of leaves or stunned root growth, which are common phytotoxicity symptoms associated with TNT stress (figure 1) (Hannink et al., 2002). However, significant biomass reduction ($p<0.001$) was found with each increasing TNT treatments (figure 2).

Figure 6-1. Vetiver grass grown in solutions containing varying TNT concentrations after 10 days.

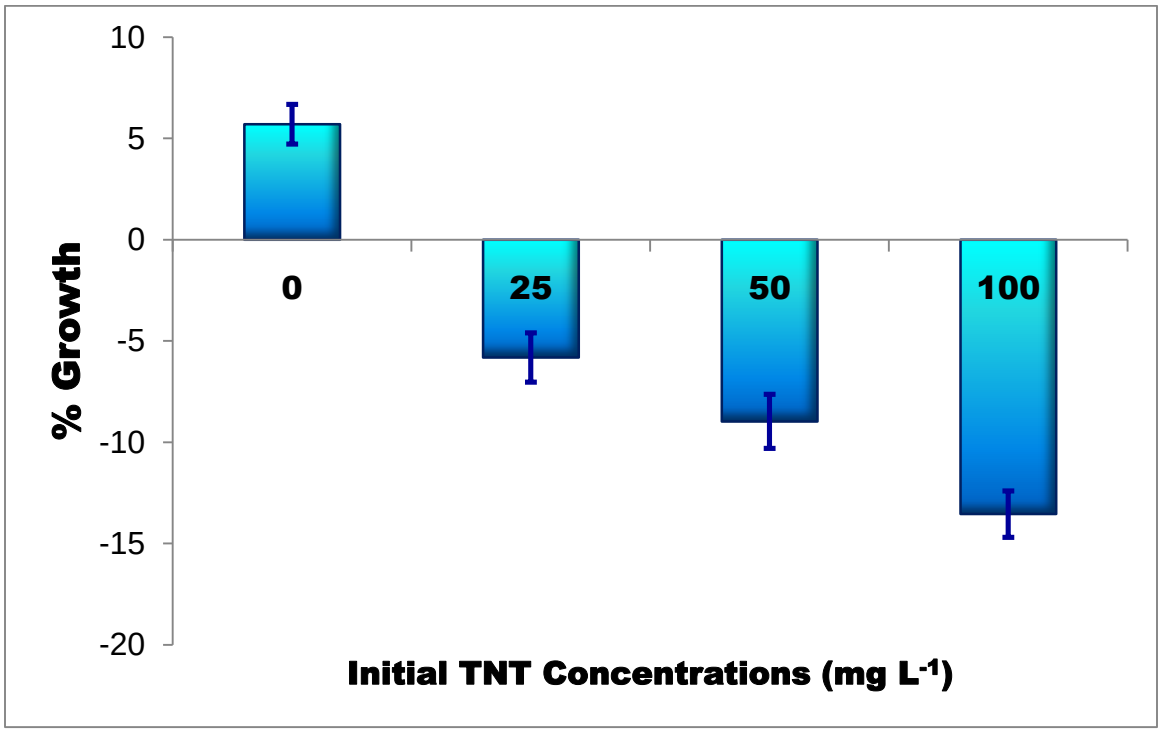


Figure 6-2. Effect of TNT on % growth ((initial biomass-final biomass)*100/initial biomass) of vetiver grass after 10 days. Negative values indicated the reduction of biomass. Data are expressed as mean (n=2) ± one standard deviation.

Figure 6-3 expresses the effect of TNT exposure at total chlorophyll content. Loss of chlorophyll as functions of TNT treatment was determined as chlorosis of leaves is one of the common TNT stress symptom. Significant ($p=0.03$) decrease in total chlorophyll was noted in TNT treated plants (25 mg L^{-1}) as compared to the TNT-free control plants; however, the chlorophyll did not continue to decrease significantly upon increasing the TNT load up to as high as 100 mg L^{-1}. This result also suggests vetiver's innate defense mechanism to fight TNT stress up to a considerably high TNT concentration for plant tolerance.

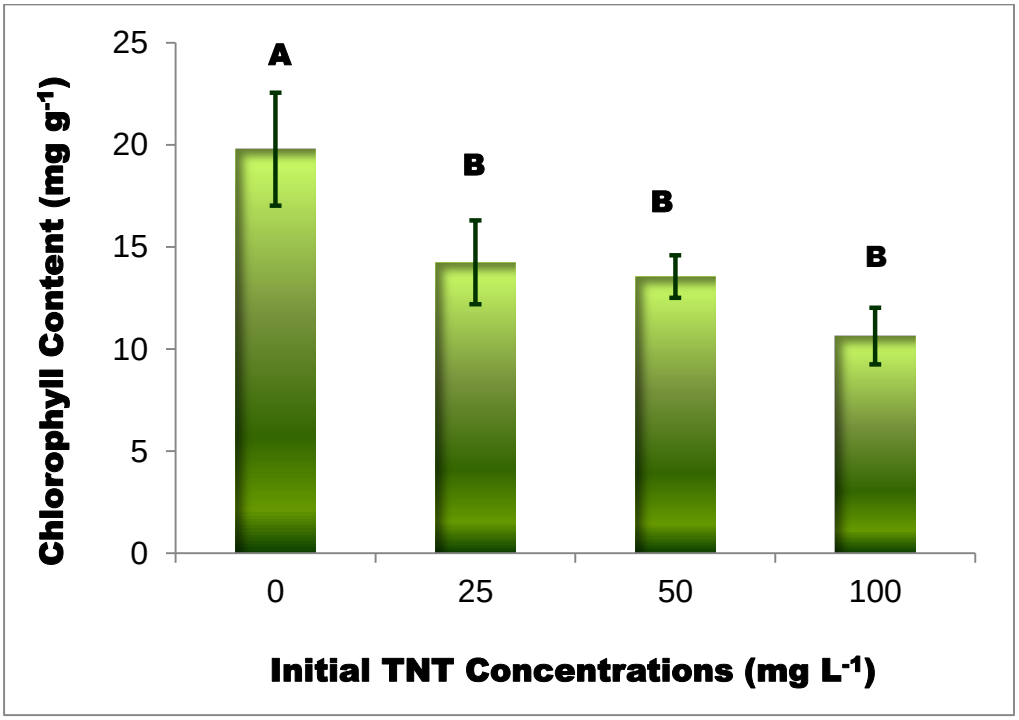


Figure 6-3. Effect of TNT on total chlorophyll content in vetiver shoots after 10 days. Data are expressed as mean (n=2) ± one standard deviation.

The total soluble protein content in the vetiver root showed significant ($p=0.0003$) continual decrease as consequence of increasing TNT concentrations (Figure 6-4). In the root tissues of vetiver grass, the total soluble protein content decreased by 15%, 42%, and 59% inat plants grown in solutions containing 25, 50, and 100 mg L^{-1} initial TNT concentrations respectively. However, similar results wereas not observed in the shoot tissue of the vetiver grass. The total soluble protein content in shoot did not show any significant change ($p>0.05$) (data not shown). It is unclearstill not sure whether the shoot data are the true representation of the effect of TNT on shoot, or an artifact of the extraction procedure, as shoot has lot more proteins and other pigments which normally do could interfere with the protein extraction and estimation purification process. Ongoing experiments in our laboratory are focusinges on optimizing the extraction procedure for shoot. In the current study, we continued with the root samples and studied the proteomic profiling of vetiver root as functions of TNT stress. Root proteins showed a significant ($p<0.0001$) negative correlation ($r=-0.97$) with TNT and followed a linear ($R^2=0.94$) decrease with increasing TNT concentrations in solution (table 1), suggesting significant loss of functional proteins in the root tissues of vetiver grass as results of TNT stress.

Table 6-1. Bivariate correlation and regression parameters of initial TNT treatments with the growth of vetiver grass, total chlorophyll content of leaves, and total soluble protein content of the vetiver root.

Parameters	Correlation	Regression	
	r	R^2	p value
Growth	-0.91	0.82	0.0019
Total Chlorophyll	-0.85	0.72	0.0076
Total Protein Content in Root	-0.97	0.94	<0.0001

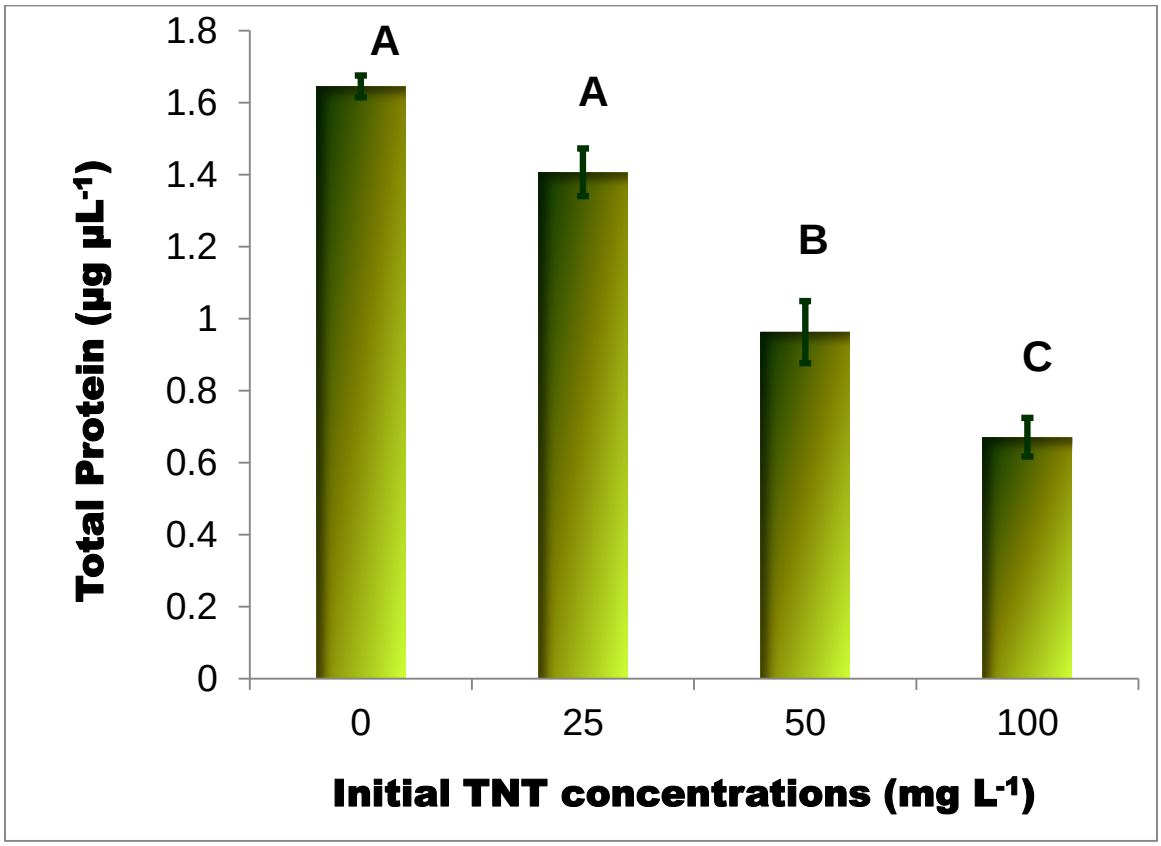


Figure 6-4. Effect of TNT on the total proteins in root. Data are expressed as mean (n=2) ± one standard deviation.

Figure 6-5 shows the gel images of the root samples after 2D classical gel electrophoresis. Twenty protein spots were found to have a minimum two fold changes

in their intensities compared to the control gel (root tissue of the vetiver plant grown in TNT-free solution). Among them, 14 protein spots were significantly ($p<0.05$) downregulated with each increasing initial TNT treatments. Total 6 protein spots were found to be upregulated at lower initial TNT treatments but downregulated at higher initial TNT loads. The proteins exhibiting these trends in response to TNT exposure were identified using MALDI-TOF-MS and functional annotation analyses were carried out.

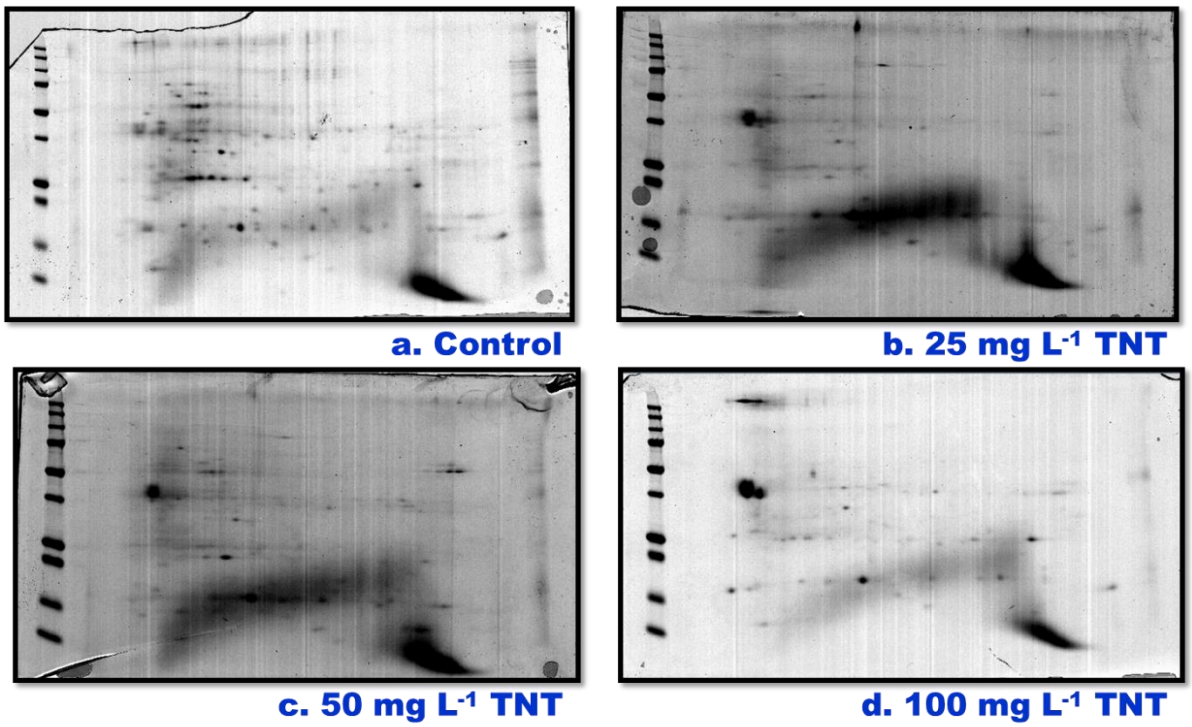


Figure 6-5. Gel images showing the protein spots in root tissues treated with different initial TNT concentrations.

Nine out of fourteen root proteins, which showed continuous downregulation in response to the exposure to increasing levels of TNT, were identified and presented in figure 6-6. Functional annotation analysis using UniProt database revealed the major functions of

these proteins and the biochemical pathways they are involved in. Figure 6-7 shows the predicted functional partners of these downregulated proteins (String 9.0).

The results showed TNT stress majorly affects the key functional cellular mechanisms such as transcription of DNA, ribosome biogenesis, nucleocytoplasmic transport of protein and, protein glycosylation pathway. Histone H24A is a subunit of histone protein, a core component of nucleosome which wrap and compact DNA into chromatin. Thus histone plays a principal role in transcription regulation, DNA repair mechanism, DNA replication and chromosomal stability by limiting DNA accessibility to the cellular machineries that need DNA as a template (www.systembiology.org). DNA-dependent RNA polymerase was found to be another downregulated protein that plays a major role in transcription by catalyzing the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates. It also helps in DNA binding. Dead box ATP-dependent RNA helicase is ubiquitous, preferentially expressed in the root (Mingam et al., 2004). It is involved in ribosome biogenesis through rRNA processing and decaying nonsense-mediated mRNA (Mingam et al., 2004). TNT stress also resulted in downregulation of GTP-binding protein which is plays an important role in controlling cell cycle and condensation of chromatin (www.systembiology.org). It is also necessary for transporting RNA and importing proteins to nucleus and thus plays major role in nucleocytoplasmic transport. (www.systembiology.org). Beta-1,3-galactosyltransferase is involved in protein glycosylation pathway. It transfers galactose from UDP galactose to substrates with a terminal glycosyl residue. The current study revealed a continuous, significant downregulation of these proteins in response to TNT exposure. To our

knowledge, our study is the first attempt to investigate the proteomic profiling of a plant in response to TNT stress. However, it was reported in the literature that various environmental stresses caused changes in chromatin structure, gene expression, and protein pattern (Pawlak and Deckert, 2007).

Downregulation of growth related proteins were also noted due to increasing TNT stress. Glutamine synthetase cytosolic isozyme 2 is involved in glutamine biosynthesis process, through which ammonium assimilation into glutamine and glutamate occurs, which are precursors for almost all N-compounds and thus plays important role in plant growth (Teixeira and Fidalgo, 2009). RNA pseudouridine synthase 6 is another protein which was majorly affected by TNT exposure. This enzyme catalyzes the synthesis of pseudouridine, the most abundant, ubiquitous yet enigmatic constituent of structural RNAs (Charette and Gray, 2000). Normal growth is severely compromised in absence of pseudouridine synthase. Earlier researches also showed that genetic mutants lacking specific psi residues in tRNA or rRNA exhibited difficulties in translation, displayed slow growth rates in an Escherichia coli mutant deficient in a pseudouridine synthase (Charette and Gray, 2000).

As expected, exposure to TNT also influenced the plant defense mechanism. Ent-pimara-8(14),15-diene synthase, a plant defense protein that is reported to be highly expressed in plant root (Margis-Pinheiro et al., 2005) was also significantly downregulated as consequence of TNT treatments, which probably would contribute to cause phytotoxicity

symptoms in to the plant. Ethylene receptor 1 is a membrane component which binds ethylene. It acts in the ethylene signal transduction pathway, as an ethylene receptor, or as a redundant negative regulator of ethylene signaling. Downregulation affect ethylene binding and metabolism of other associated plant hormones such as auxin, cytokinins, ABA and gibberellic acid (String 9.0). As it is a negative regulator of ethylene response, downregulation of this protein will result in increased response of ethylene, which is a known plant defense hormone.

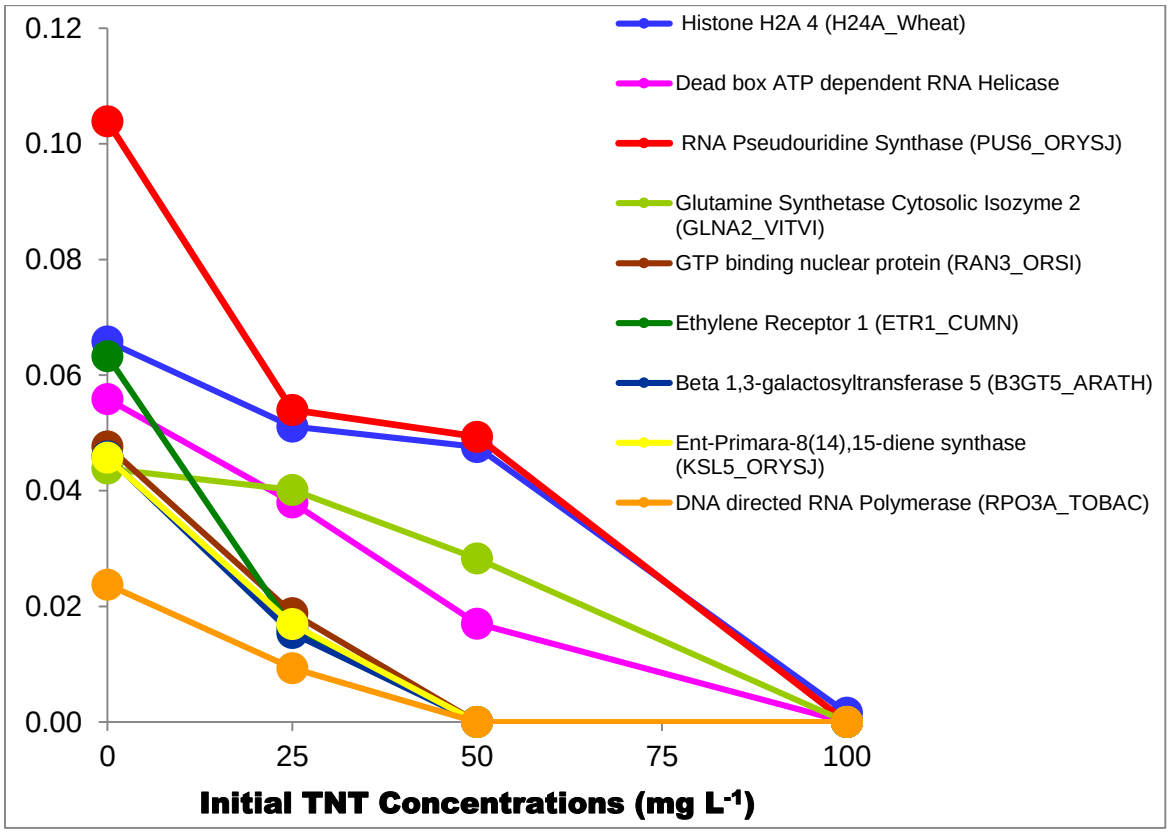
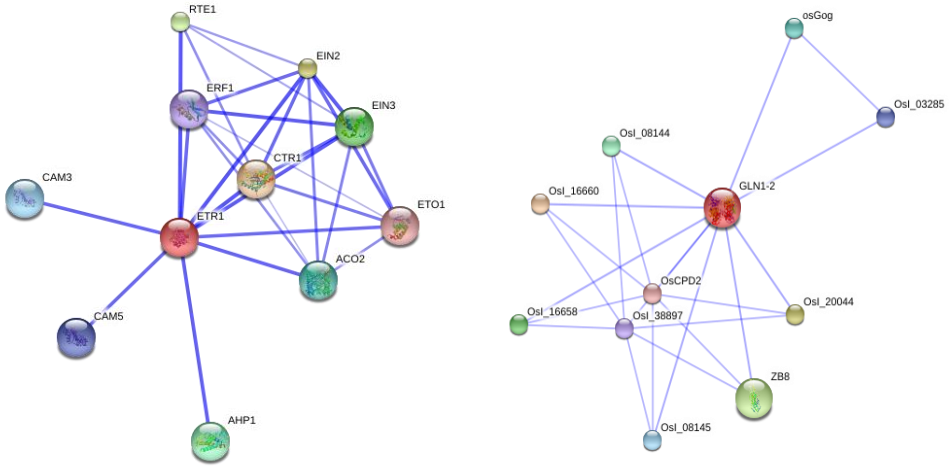


Figure 6-6. Identified proteins that showed continued downregulation with each increasing TNT treatments.



a. Ethylene Receptor 1 **b. Glutamine Synthetase Cytosolic Isozyme 2**

Figure 6-7. Predicted functional partners of downregulated proteins; Ethylene Receptor 1 (ETR1; a) and Glutamine Synthetase Cytosolic Isozyme 2 (GLN1-2; b). Stronger associations with functional partners are exhibited with darker blue lines (STRING 9.5 database).

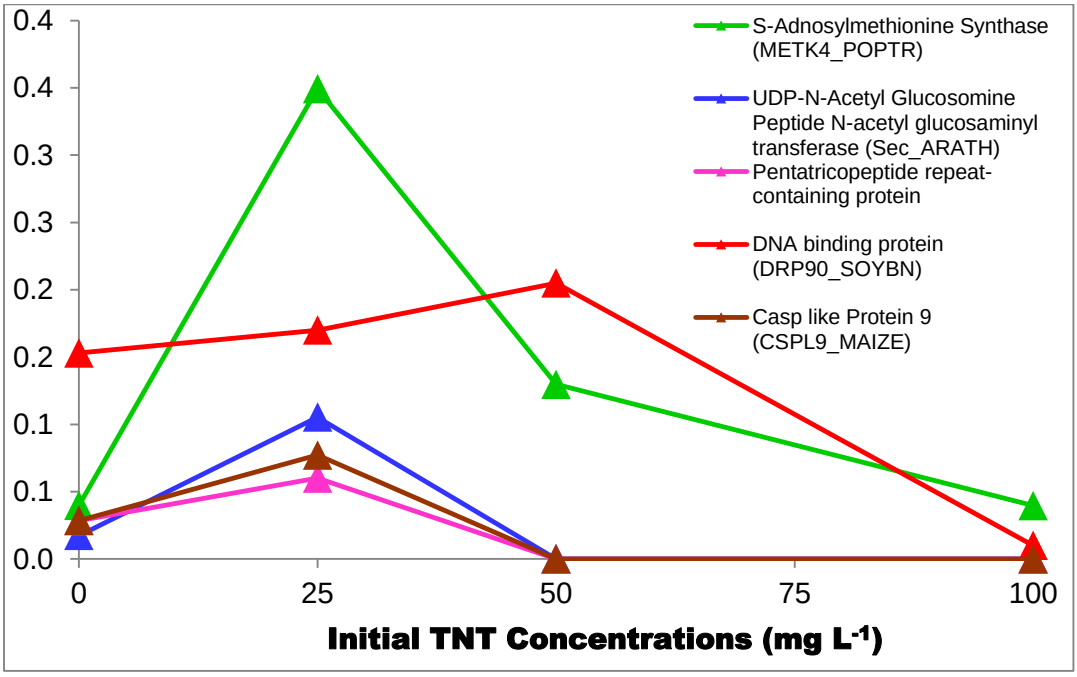
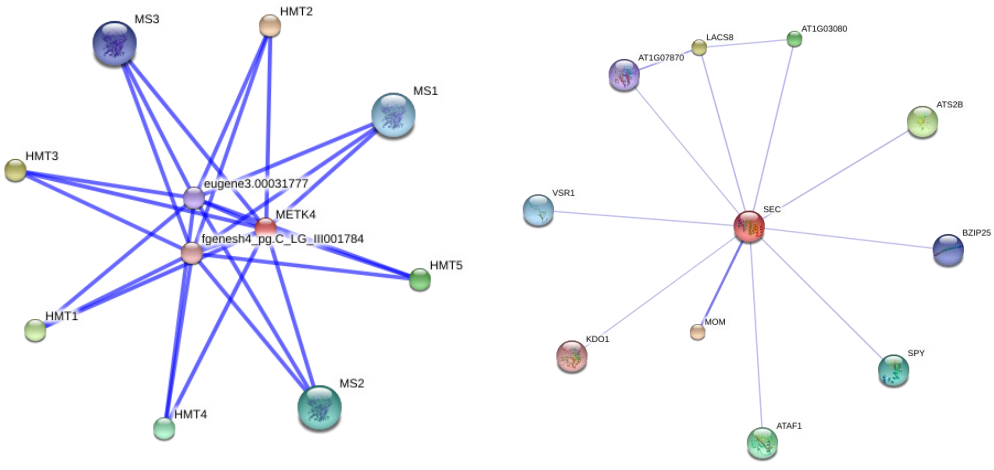


Figure 6-8. Identified proteins that upregulated at lower TNT treatments but downregulated at further increase in TNT concentrations.

Figure 6-8 expressed the proteins that were initially upregulated; probably causing vetiver's enhanced defense mechanisms against TNT stress at lower concentrations, but ultimatelyfinally downregulated at higher TNT treatments. One of such protein named S-adenosylmethionine synthase 4 again establishes the role of ethylene biosynthesis pathway as one of the biochemical defense mechanisms against TNT stress. This enzyme catalyzes the reaction of methionine and ATP to form of S-adenosylmethionine, which is also called AdoMet. AdoMet is a precursor in ethylene biosynthesis. It is also required for biosynthesis of the phenylpropanoid constituents of the cell wall, which is also produced as a response to stress.



a. S-Adenosylmethionine Synthase **b. UDP-N-Acetyl Glucosomine Peptide N-acetyl glucosaminyl transferase**

Figure 6-9. Predicted functional partners of upregulated and then downregulated proteins; S-Adenosylmethionine Synthase (a) and UDP-N-Acetyl Glucosomine Peptide

N-acetyl glucosaminyl transferase (SEC; b). Stronger associations with functional partners are exhibited with darker blue lines (STRING 9.5 database).

UDP-N-acetylglucosamine--peptide Nacetylglucosaminyltransferase (also called SEC for secret agent), a protein associated with plant's defense mechanism, also showed initial upregulation at lower TNT concentrations, followed by downregulation at higher TNT loads. It is known to act in plant's defense mechanism against viral infection by mediating O-glycosylation of capsid protein (CP) of virus in case of infection by Plum pox virus. It is also involved in protein glycosylation pathway. It also shows strongest association with Morpheus Molecule (MOM), which is involved in chromatin silencing (Figure 6-9). Similar trend is shown by two other proteins; DNA-binding protein DRP90 is involved in DNA dependent transcription regulation and DNA binding. CASP-like protein 9 is a cell membrane protein whose exact function is yet unknown.

6.4. Conclusions

To the best of our knowledge, the current study is the first attempt that reported the proteomic profiling of plant system under TNT stress. Exposure to varying levels of TNT affected the growth, total chlorophyll content of leaves, and total soluble protein content in the root of vetiver grass. Proteomic profiling and functional annotation analysis of the root proteins that showed minimum two folds changes revealed that TNT stress majorly affect the key cellular pathways such as, transcription of DNA, ribosome biogenesis, nucleocytoplasmic transport of protein, protein glycosylation pathway. Downregulation

of growth related proteins corroborates with our data that showed strong and significant negative correlation of growth with initial TNT concentrations. Ethylene biosynthesis pathway was found to play an active role in vetiver's defense mechanism against TNT stress. Proteins associated with plant defense initially upregulated at lower TNT treatments providing the plant with its tolerance to TNT stress; however at higher concentrations, downregulation of these proteins probably contributes in developing phytotoxicity symptoms in response to TNT.

This study provides pioneering findings of plant proteomics under stress fromof a known phytotoxic compound like TNT. The chlorophyll data obtained from current study suggested potential loss of functional proteins involved in key functions like photosynthesis. Ongoing experiments in our laboratory are aiming to study the proteomic profiling of shoot tissues of vetiver grass under TNT stress.

6.5. References

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ENVIRONMENTAL IMPLICATIONS

Increasing numbers of urban dwellers has been driving the unprecedented sprawling of cities toward peripheral areas, sometimes close to former military sites. Residential expansion toward such military land is often impeded by the high remediation cost of large military areas contaminated with relatively low residual TNT concentrations (< 150 mg kg^{-1}). This study highlighted a cost-effective stimulative phytoremediation method using a solubilizing agent (urea) that catalyzed TNT uptake by vetiver grass, confirming our earlier results obtained in laboratory and hydroponic set-ups. Hysteretic desorption of TNT in chemically variant soils, containing wide range of potential TNT binding sites suggests irreversible sorption of TNT in all soils and thus establishes the need for using an extractant to facilitate the release of TNT in soil solutions to enhance plant uptake of TNT. This study showed the beneficial effect of urea as an extractant, over that of water in significantly ($p<0.0001$) catalyzing TNT extraction from all the soils examined; however, low organic matter containing acidic soils were found to be ideal to apply this technology. The effectiveness of vetiver grass in removing soil residual TNT was further enhanced by the application of urea under more realistic greenhouse conditions. Vetiver is characterized by a massive (2-3m), very fine root system (average diameter 0.5-1.0 mm) and it is easily adapted in various geographic regions because it exhibits tolerance to a wide range of climatic conditions, such as extreme air temperatures (-15ºC to +55ºC) and soil pH (3.3 to 12.5) (Dalton et al., 1996).

Agronomically-recommended and environmentally-relevant urea application rates successfully enhanced soil TNT phytoavailability and facilitated its uptake by vetiver grass. Significant TNT translocation from root to shoot was observed, while mass balance data showed that the non-extractable bound TNT fraction increased with urea application rates, suggesting the expression of vetiver's biochemical defense mechanism against TNT.

The long term greenhouse column study showed the concern for increased risk of urea-mobilized TNT migration to groundwater and downstream water bodies is not applicable in this case, because of the faster kinetics of TNT uptake by vetiver in the presence of urea, when compared with those of other phytoremediation methods. Urea's solubilizing effect on TNT may be only warranted, if environmental conditions, such as soil pH and minimal rainfall favor urea stability in soil. This study demonstrated the stability of urea in acidic soils; no significant change in soil pH of our samples was observed (average pH 6, data not shown). Special attention was paid to ensure urea application rates falling within agronomic recommendations (~ 125 mg kg^{-1}) that safeguard surrounding environments against over-application of nitrogen species with detrimental environmental consequences.

Presence of both isomers of dinitrotoluenes (2,4-DNT and 2,6-DNT), both expressing higher toxicity than TNT, in leachates of the plant-free control columns raises the concern of microbial transformation of unremoved TNT to more toxic and soluble metabolites and their high risk of migration to groundwater. Whereas, advantage of this technique was demonstrated in the absence of formation of the dinitrotoluenes,

monoaminodinitrotoluenes were formed in soils and leachates of experimental units with vetiver grass, and also in the root and shoot tissues of vetiver, exhibiting lower toxicity than the parent TNT compound (LD50 in rat = 959 and 1522 mg kg^{-1} for 4-ADNT and 2-ADNT, respectively) (Table 4-5) (USACHPPM, 2005). Enhanced nitroreductase activity in TNT treated vetiver grass showed a major role of NR enzyme in transforming TNT to other metabolites which probably contains the functional groups required for conjugation and sequestration of these xenobiotics in cell was or cell vacuole of vetiver grass. The kinetic parameters of the NR enzyme were determined which will be useful for designing field based application of this technique.

This study reported the proteomic profiling of vetiver root treated with varying levels of TNT, which is probably the first documentation of the changes in any plant proteome under TNT stress. This study reveals significant finding of loss of functional proteins which are involved in vital cellular mechanisms like transcription, translation, protein glycosylation, nucleocytoplasmic transport, and ribosome biogenesis. Downregulation of growth related proteins supported the biomass reduction data; whereas, upregulation of plant defense related proteins demonstrated vetiver's innate detoxification system at lower TNT concentrations which is evident from vetiver's higher tolerance for TNT compared to the other reported grass for TNT phytoremediation.

Quantile probability plots of soil TNT concentrations in contaminated military sites may be typically non-linear and highly skewed. In Joliet army ammunition site, soil TNT

concentrations ranged from < detection level to as high as 87,000 mg kg^{-1} (Talmage et al., 1999). A considerable fraction of USA military sites contain relatively low soil residual concentrations ranging from 0.1 to 115 mg kg^{-1} TNT (Talmage et al., 1999), falling within the range of applicability of our proposed TNT remediation technology. This technology needs to be further tested under field conditions in pilot studies within TNT-contaminated military locations. It would also be critical to evaluate the performance of stimulative phytoremediation technique in a mixture of nitroaromatics that are likely to be present in such military sites.

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LIST OF APPENDICIES

Appendix A

Supplementory Information

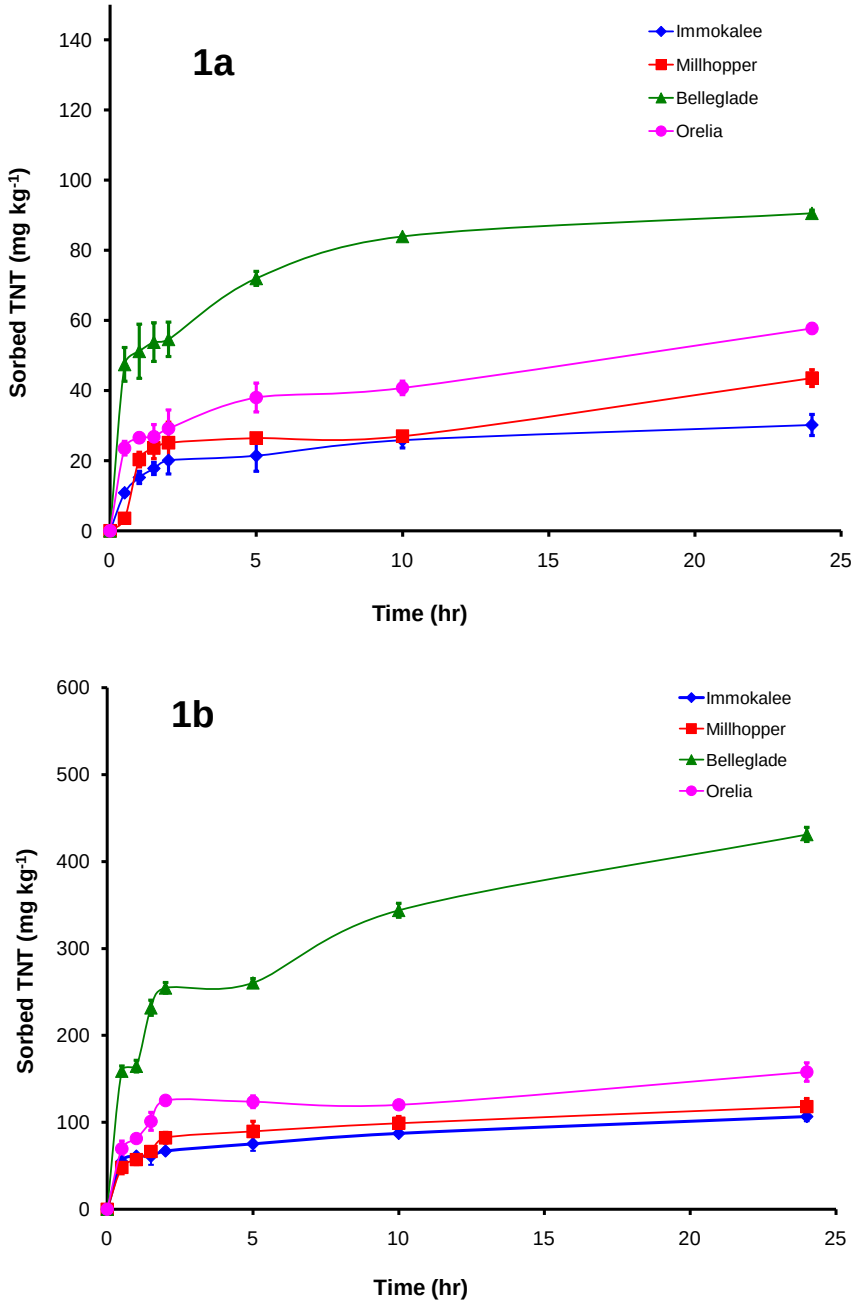

Figure A1. Kinetic adsorption of TNT at 5 mg L^{-1} (a) and 25 mg L^{-1} (b) initial aqueous TNT load. Data are eas mean (n=2) and one standard deviation (Chapter 2).

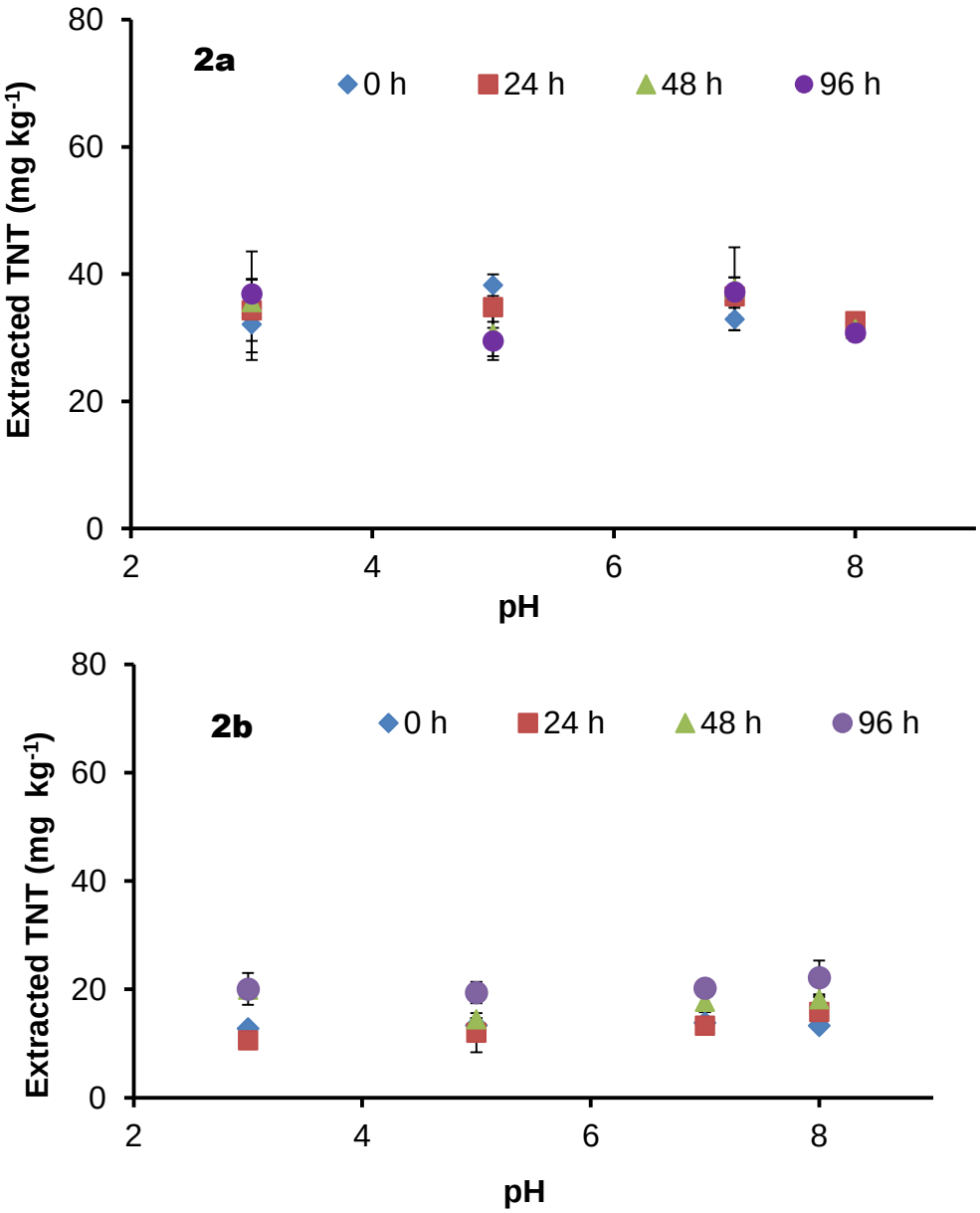

Figure A2. Effect of pH on TNT extraction from Millhopper (a) and Orelia (b) soils at 1000 mg kg^{-1} urea concentrations. Data are expressed as the mean (n=2) and one standard deviation (Chapter 2).

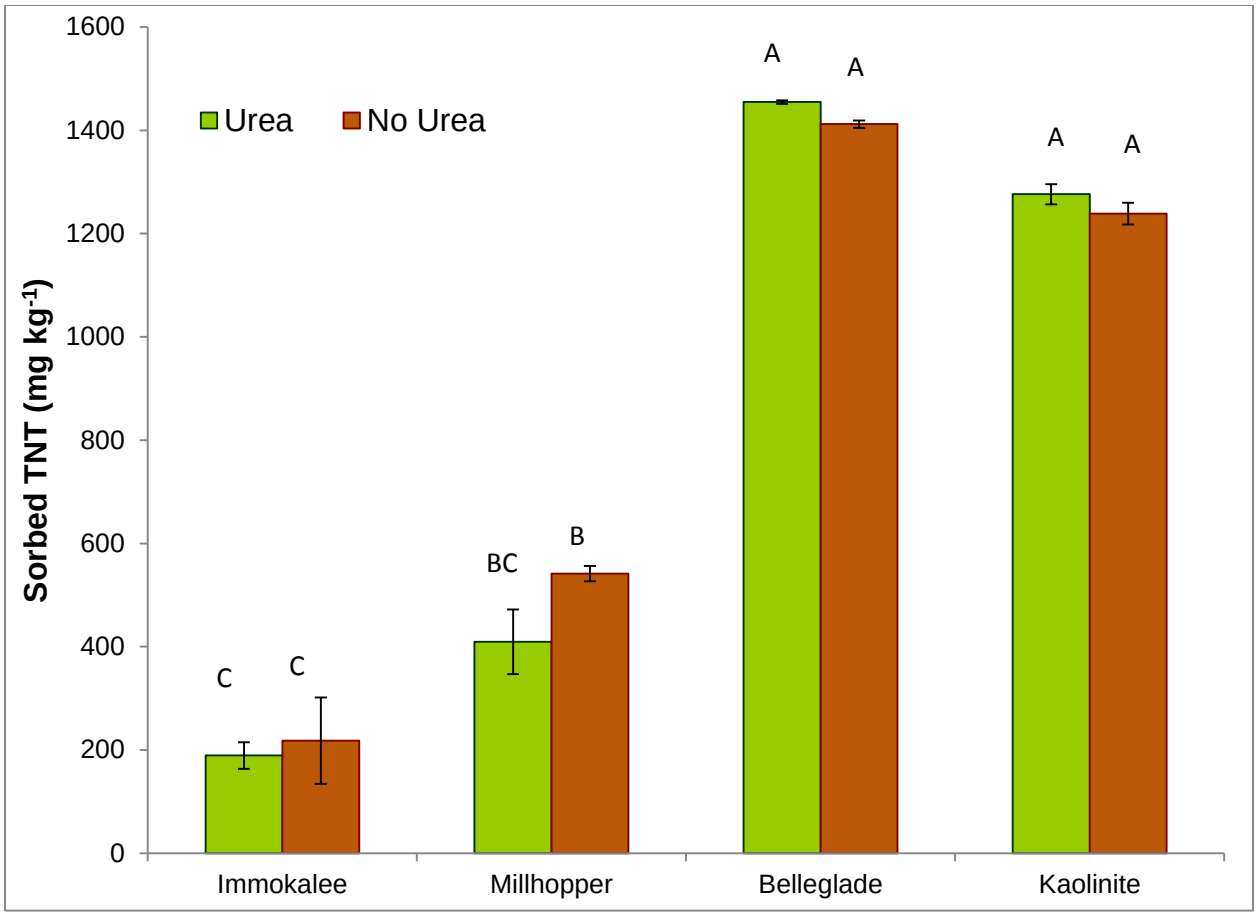

Figure A3. Competing effects of urea (1000 mg kg^{-1}) on TNT adsorption. Data are expressed as mean (n=3) and one standard deviation (Chapter 2).

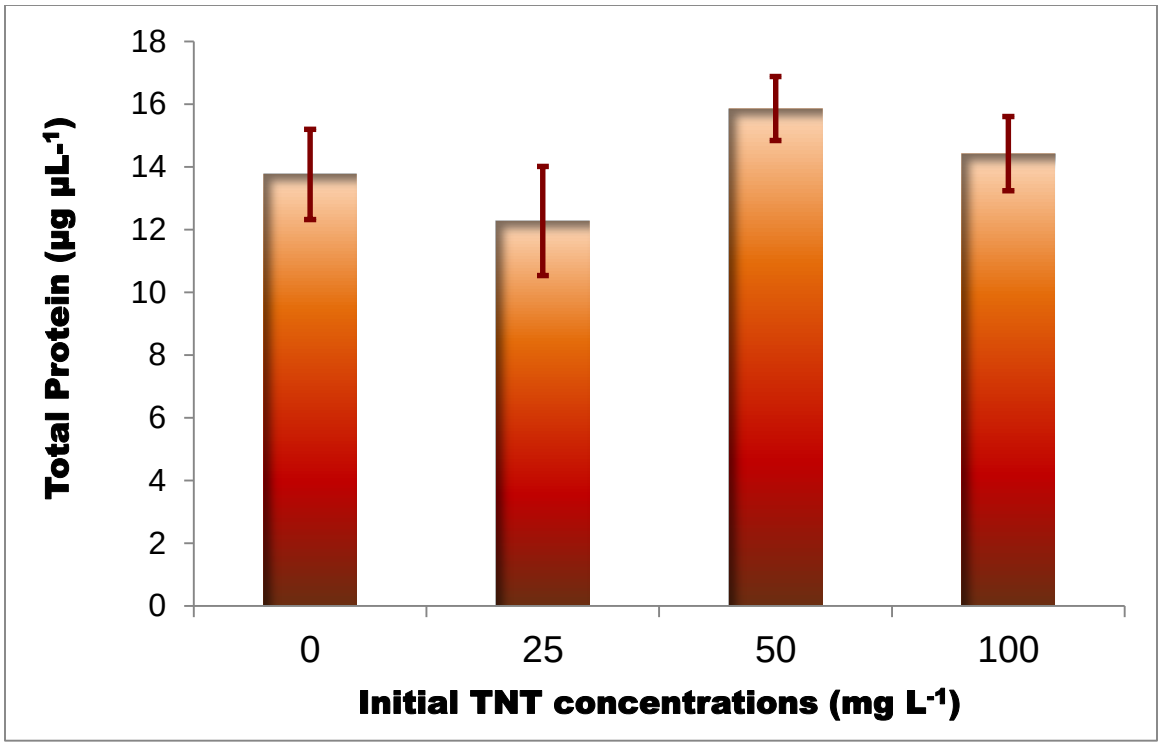

Figure A4. Total proteins in the shoot. Data are expressed as mean (n=2) and one standard deviation (Chapter 6).

Appendix B

Experimental Data for Chapter 2

Table B-1. Residual TNT in soils (mg kg^{-1}) initially treated with 40 mg kg^{-1} and 80 mg kg^{-1} TNT in plant-free, TNT-amended controls. Data are expressed as mean (n = 3) $\pm$ 1 standard deviation.

(a) Initial TNT concentrations (40 mg kg^{-1})

Time (Days)	Residual TNT in soil (mg kg^{-1}) Mean	Residual TNT in soil (mg kg^{-1}) Standard Deviation
9	25.28	5.48
22	24.83	6.65
32	32.99	4.83
41	43.86	1.37
48	30.34	2.54

(b)Initial TNT concentrations (80 mg kg^{-1})

Time (Days)	Residual TNT in soil (mg kg^{-1}) Mean	Residual TNT in soil (mg kg^{-1}) Standard Deviation
9	73.02	7.91
22	67.49	13.58
32	78.75	9.89
41	59.54	0.00
48	52.65	6.44

Table B-2. Residual TNT in soils (mg kg^{-1}) initially treated with 40 mg kg^{-1} and 80 mg kg^{-1} TNT with two urea concentrations (0 and 1000 mg kg^{-1}) in presence of vetiver grass after 3 days and 12 days. Data are expressed as mean (n = 3) $\pm$ 1 standard deviation.

Initial TNT concentration (mg kg^{-1})	Initial Urea Concentration (mg kg^{-1})	Time (Days)	Residual TNT in soil Mean (mg kg^{-1})	Residual TNT in soil Standard Deviation (mg kg^{-1})
0	0	3	0.00	0.00
40	0	3	1.09	0.21
40	1000	3	0.00	0.00
80	0	3	10.00	1.01
80	1000	3	3.70	0.82
0	0	12	0.00	0.00
40	0	12	0.04	0.02
40	1000	12	0.00	0.02
80	0	12	0.33	0.03
80	1000	12	0.12	0.04

Appendix C

Experimental Data for Chapter 3

Table C-1. Equilibrium sorption and desorption of TNT at varied initial TNT load in Immokalee (a), Millhopper (b), Orelia (c), and Belleglade (d) soils . Data are expressed as mean (n=2) and one standard deviation.

(a) Immokalee

Residual TNT in solution (mg L^{-1})	Sorbed TNT (mg kg^{-1}) Mean	Sorbed TNT (mg kg^{-1}) Standard Deviation	Desorbed TNT (mg kg^{-1}) Mean	Desorbed TNT (mg kg^{-1}) Standard Deviation
0.78	1.91	0.85	2.49	0.03
3.73	13.09	1.35	10.50	0.88
8.16	14.61	3.17	18.04	0.48
22.68	37.89	11.80	38.15	1.98
42.82	96.21	9.18	67.23	6.94
83.20	148.53	14.97	127.61	12.42

(b) Milhopper

Residual TNT in solution (mg L^{-1})	Sorbed TNT (mg kg^{-1}) Mean	Sorbed TNT (mg kg^{-1}) Standard Deviation	Desorbed TNT (mg kg^{-1}) Mean	Desorbed TNT (mg kg^{-1}) Standard Deviation
0.43	8.976	0.17	1.32	0.19
2.93	28.895	0.18	10.59	0.36
6.85	40.675	3.75	17.57	0.01
21.37	63.709	13.63	44.85	0.40
41.71	118.513	0.48	69.34	9.43
82.66	169.312	18.49	85.08	7.59

(c) Orelia

Residual TNT in solution (mg L^{-1})	Sorbed TNT (mg kg^{-1}) Mean	Sorbed TNT (mg kg^{-1}) Standard Deviation	Desorbed TNT (mg kg^{-1}) Mean	Desorbed TNT (mg kg^{-1}) Standard Deviation
0.38	9.87	0.33	0.25	0.06
2.60	35.57	0.82	8.54	0.31
5.73	63.078	4.24	15.81	1.19
19.08	109.758	9.99	39.25	5.49
37.81	196.27	22.17	65.46	7.06
71.21	387.798	21.92	145.13	12.07

(d) Belleglade

Residual TNT in solution (mg L^{-1})	Sorbed TNT (mg kg^{-1}) Mean	Sorbed TNT (mg kg^{-1}) Standard Deviation	Desorbed TNT (mg kg^{-1}) Mean	Desorbed TNT (mg kg^{-1}) Standard Deviation
0.00	17.52	0.00	0	0
0.00	85.39	1.78	0	0
1.21	147.67	0.69	0	0
7.22	345.52	3.69	60.79	2.90
16.21	628.32	20.18	179.25	0.73
32.89	1139.55	15.61	419.15	50.45

Table C-2. Kinetics of TNT extraction (expressed as % of initial TNT in soil) from all soils by two extractants, urea (1000 mg kg^{-1}) and water. Data are expressed as the mean (n=2) and one standard deviation.

(a) Immokalee

Time (hr)	Urea extracted TNT (%) Mean	Urea extracted TNT (%) Standard Deviation	Water extracted TNT (%) Mean	Water extracted TNT (%) Standard Deviation
0	0	0	0	0
1	53.16	0.77	52.82	2.08
2	59.24	1.34	54.65	0.64
5	66.76	2.30	55.11	2.44
10	83.36	3.44	56.03	2.40
24	88.74	0.69	57.08	1.69
48	94.30	4.54	59.95	0.01

(b) Millhopper

Time (hr)	Urea extracted TNT (%) Mean	Urea extracted TNT (%) Standard Deviation	Water extracted TNT (%) Mean	Water extracted TNT (%) Standard Deviation
0	0	0	0	0
1	27.50	0.40	27.33	1.08
2	30.65	0.69	28.27	0.33
5	34.54	1.19	28.51	1.26
10	43.12	1.78	28.99	1.24
24	45.91	0.36	29.53	0.87
48	48.78	2.35	31.01	0.00

(a) Orelia

Time (hr)	Urea extracted TNT (%) Mean	Urea extracted TNT (%) Standard Deviation	Water extracted TNT (%) Mean	Water extracted TNT (%) Standard Deviation
0	0	0	0	0
1	16.24	0.98	12.07	1.09
2	16.97	0.39	12.73	0.07
5	17.17	0.23	12.90	0.25
10	17.19	0.38	10.29	0.01
24	17.65	1.00	7.74	0.38
48	14.58	0.28	7.63	0.27

(a) Belleglade

Time (hr)	Urea extracted TNT (%) Mean	Urea extracted TNT (%) Standard Deviation	Water extracted TNT (%) Mean	Water extracted TNT (%) Standard Deviation
0	0	0	0	0
1	0.46	0.05	0.36	0.06
2	0.69	0.04	0.40	0.01
5	0.70	0.03	0.49	0.03
10	0.72	0.02	0.52	0.02
24	0.59	0.10	0.41	0.02
48	0.50	0.02	0.39	0.00

Table C-3. Urea concentrations (expressed as % of initial urea load) during TNT extraction. Data are expressed as the mean (n=2) and one standard deviation.

(a) Immokalee and Millhopper

Time (hr)	Residual Urea (%) in Immokalee Mean	Residual Urea (%) in Immokalee Standard Deviation	Residual Urea (%) in Millhopper Mean	Residual Urea (%) in Millhopper Standard Deviation
0	100	0	100	0
1	100	1.92	94.49	1.21
2	100	1.82	92.78	0.51
5	100	0.61	89.70	10.31
10	100	6.87	88.56	0.10
24	97.35	5.46	83.84	0.10
48	96.92	11.73	74.12	0.91

(b) Orelia and Belleglade

Time (hr)	Residual Urea (%) in Orelia Mean	Residual Urea (%) in Orelia Standard Deviation	Residual Urea (%) in Belleglade Mean	Residual Urea (%) in Belleglade Standard Deviation
0	100	0	100	0
1	86.06	2.43	86.49	1.21
2	76.27	1.92	77.27	2.32
5	67.40	3.54	73.55	0.71
10	66.04	1.01	73.48	0.40
24	36.88	3.23	70.05	0.00
48	16.73	0.20	66.90	0.00

Table C-4. Effect of four different urea loads (0, 125, 350 and 1000 mg kg^{-1}) on extraction of TNT (expressed as % of initial TNT in soil) from four soils after 10 hrs. Statistical analysis was conducted separately for each soil. Data are expressed as the mean (n=2) and one standard deviation.

(a) Immokalee and Millhopper

Urea Load (mg kg^{-1})	Extracted TNT (%) in Immokalee Mean	Extracted TNT (%) in Immokalee Standard Deviation	Extracted TNT (%) in Millhopper Mean	Extracted TNT (%) in Millhopper Standard Deviation
0	70.82	8.60	36.66	3.30
125	86.54	6.38	53.57	3.63
350	93.13	1.36	57.27	0.91
1000	95.93	3.14	66.99	2.71

(b) Orelia and Belleglade

Urea Load (mg kg^{-1})	Extracted TNT (%) in Orelia Mean	Extracted TNT (%) in Orelia Standard Deviation	Extracted TNT (%) in Belleglade Mean	Extracted TNT (%) in Belleglade Standard Deviation
0	14.65	0.34	0.47	0.05
125	17.30	0.39	0.61	0.03
350	18.87	0.51	0.68	0.02
1000	20.99	0.51	0.78	0.06

Appendix D

Experimental Data for Chapter 4

Table D-1. Kinetics of removal of TNT and its metabolites from soil by vetiver grass. Data are expressed as mean (n=3) ± one standard deviation.

Mean

Treatments	Residual TNT in soil after 0 Days	Residual TNT in soil after 2 Days	Residual TNT in soil after 5 Days	Residual TNT in soil after 9 Days	Residual TNT in soil after 14 Days	Residual TNT in soil after 22 Days
0 mg kg^{-1} Urea	100	89.01	83.36	70.52	63.88	26.61
125 mg kg^{-1} Urea	100	83.34	75.41	55.25	51.15	18.76
350 mg kg^{-1} Urea	100	73.14	65.42	48.79	46.23	18.15
1000 mg kg^{-1} Urea	100	72.75	45.96	29.07	16.72	10.84
No plant control	100	88.19	86.63	84.66	76.48	70.50
No TNT control	0	0	0	0	0	0

Standard Deviation

Treatments	Residual TNT in soil after 0 Days	Residual TNT in soil after 2 Days	Residual TNT in soil after 5 Days	Residual TNT in soil after 9 Days	Residual TNT in soil after 14 Days	Residual TNT in soil after 22 Days
0 mg kg^{-1} Urea	0	2.99	1.53	2.29	4.80	3.05
125 mg kg^{-1} Urea	0	3.92	1.22	3.35	3.39	4.19
350 mg kg^{-1} Urea	0	3.44	4.90	3.65	3.62	3.87
1000 mg kg^{-1} Urea	0	3.05	4.31	2.27	2.57	0.55
No plant control	0	4.36	5.30	5.05	3.03	2.98
No TNT control	0	0	0	0	0	0

Table D-2. Sum of residual TNT and its metabolites in bulk and rhizospheric soil after 22 days. Data are expressed as mean (n=3) ± one standard deviation.

Treatment	Residual TNT in Bulk Soil (mg kg^{-1}) Mean	Residual TNT in Bulk Soil (mg kg^{-1}) Standard Deviation	Residual TNT in Rhizospheric Soil (mg kg^{-1}) Mean	Residual TNT in Rhizospheric Soil (mg kg^{-1}) Standard Deviation
No plant	60.37	8.02	0.00	0.00
0 mg kg^{-1} Urea	19.68	3.05	39.26	4.31
125 mg kg^{-1} Urea	14.09	4.19	32.11	4.32
350 mg kg^{-1} Urea	13.86	4.87	15.79	3.61
1000 mg kg^{-1} Urea	1.58	0.55	5.21	1.29

Table D-3. TNT and its metabolites (mg kg^{-1}) in the root and shoot tissues of vetiver grass. Data are expressed as mean (n=3) ± one standard deviation.

(a) Root

Initial Urea Concentrations in soil (mg kg^{-1})	TNT (mg kg^{-1}) Mean	TNT in root (mg kg^{-1}) Standard Deviation	4 ADNT in root (mg kg^{-1}) Mean	4 ADNT in root(mg kg^{-1}) Standard Deviation
0	136.36	14.31	3.83	1.96
125	160.43	12.57	0.00	0.00
350	98.83	28.31	4.77	1.53
1000	44.45	9.18	10.50	2.89

(b) Shoot

Initial Urea Concentrations in soil (mg kg^{-1})	1,3,5 TNB (mg kg^{-1}) Mean	1,3,5 TNB (mg kg^{-1}) Standard Deviation	TNT (mg kg^{-1}) Mean	TNT (mg kg^{-1}) Standard Deviation
0	54.28	8.36	0	0
125	0	0	19.84	1.17
350	41.42	10.05	11.60	3.68
1000	36.26	3.79	6.09	1.37

(c) Shoot

Initial Urea Concentrations in soil (mg kg^{-1})	4 ADNT (mg kg^{-1}) Mean	4 ADNT (mg kg^{-1}) Standard Deviation	2 ADNT (mg kg^{-1}) Mean	2 ADNT (mg kg^{-1}) Standard Deviation
0	29.42	5.84	3.79	1.85
125	0	0	1.91	0.93
350	0	0	0	0
1000	39.97	3.82	0	0

Table D-4. Nitroreductase activity in the root and shoot tissues of vetiver grass. Data are expressed as mean (n=3) $\pm$ one standard deviation.

(a) Root

Treatment	NR Activity (nM g^{-1} h^{-1}) Mean	NR Activity (nM g^{-1} h^{-1}) Standard Deviation
No TNT No Urea	178.31	2.51
0 mg kg^{-1} Urea	406.84	74.78
125 mg kg^{-1} Urea	303.54	134.55
350 mg kg^{-1} Urea	329.91	69.33
1000 mg kg^{-1} Urea	676.02	171.09

(a) Shoot

Treatment	NR Activity (μM g^{-1} h^{-1}) Mean	NR Activity (μM g^{-1} h^{-1}) Standard Deviation
No TNT No Urea	54.11	13.27
0 mg kg^{-1} Urea	255.08	1.30
125 mg kg^{-1} Urea	274.29	70.20
350 mg kg^{-1} Urea	163.42	27.49
1000 mg kg^{-1} Urea	256.98	50.68

Table D-5. Mass balance of the mean (n=3) TNT and its metabolites (%) in soil and plant samples.

Urea Treatments	Bulk soil (%)	Rhizospheric soil (%)	Root (%)	Shoot (%)	Unidentified (%)
0 mg kg^{-1} Urea	26.61	40.29	7.01	8.75	17.34
125 mg kg^{-1} Urea	18.76	32.11	8.02	2.18	38.93
350 mg kg^{-1} Urea	18.15	18.97	5.18	5.30	52.40
1000 mg kg^{-1} Urea	10.84	5.45	2.75	8.23	72.73

Table D-6. Residual TNT and ADNT (mg kg^{-1}) in soil after 6 months. Data are expressed as mean (n=3) $\pm$ one standard deviation.

(a)TNT (Mean)

Initial TNT treatments (mg kg^{-1})	Residual TNT in no plant control column (mg kg^{-1})	Residual TNT in columns with vetiver grass (mg kg^{-1})	Residual TNT in columns with vetiver grass and urea (mg kg^{-1})
50	30.34	0.00	0.00
100	63.19	0.00	0.00
200	129.36	37.31	9.22

(b) TNT (Standard Deviation)

Initial TNT treatments (mg kg^{-1})	Residual TNT in no plant control column (mg kg^{-1})	Residual TNT in columns with vetiver grass (mg kg^{-1})	Residual TNT in columns with vetiver grass and urea (mg kg^{-1})
50	1.27	0	0
100	5.46	0	0
200	2.09	7.88	0.90

(c)ADNT (Mean)

Initial TNT treatments (mg kg^{-1})	ADNT in no plant control column (mg kg^{-1})	ADNT in columns with vetiver grass (mg kg^{-1})	ADNT in columns with vetiver grass and urea (mg kg^{-1})
50	8.62	8.51	5.68
100	14.60	14.73	13.99
200	27.76	24.57	19.76

(d) ADNT (Standard Deviation)

Initial TNT treatments (mg kg^{-1})	ADNT in no plant control column (mg kg^{-1})	ADNT in columns with vetiver grass (mg kg^{-1})	ADNT in columns with vetiver grass and urea (mg kg^{-1})
50	0.41	0.78	0.28
100	1.23	1.18	1.21
200	0.80	2.45	2.81

Table D-7. Dinitrotoluenes in the leachates of plant-free control columns. Data are expressed as mean (n=3) ± one standard deviation.

(a)Mean

Initial TNT Concentrations (mg L^{-1})	DNT in leachate after 2 Months (mg L^{-1})	DNT in leachate after 4 Months (mg L^{-1})	DNT in leachate after 6 Months (mg L^{-1})
50	0	1.18	2.75
100	0	4.90	5.90
200	0	5.16	8.17

(a)Standard Deviation

Initial TNT Concentrations (mg L^{-1})	DNT in leachate after 2 Months (mg L^{-1})	DNT in leachate after 4 Months (mg L^{-1})	DNT in leachate after 6 Months (mg L^{-1})
50	0	0.014	0.8
100	0	0.12	0.77
200	0	0.23	1.45

Appendix E

Experimental Data for Chapter 5

Table E-1. % Growth of vetiver grass following varying TNT exposures. Negative values express the reduction in biomass. Data are expressed as mean (n=3) and one standard deviation.

(a) Mean

Initial TNT concentrations (mg L^{-1})	Growth of vetiver grass after 5 days (%)	Growth of vetiver grass after 10 days (%)	Growth of vetiver grass after 15 days (%)
0	9	10	10
25	9.10	4.35	4.31
50	13.60	8.51	5.48
100	9.54	-2.97	-6.57
200	7.86	-11.46	-14.71

(a) Standard Deviation

Initial TNT concentrations (mg L^{-1})	Growth of vetiver grass after 5 days (%)	Growth of vetiver grass after 10 days (%)	Growth of vetiver grass after 15 days (%)
0	0.77	1.22	0.9
25	0.64	0.38	1.64
50	1.92	1.25	1.8
100	1.32	0.14	0.44
200	2.78	0.55	0.55

Table E-2. Kinetics of Nitroreductase enzyme activity in the root of vetiver grass following exposure to various concentrations of TNT. NR enzyme activity is expressed in U mL^{-1}. Data expressed as mean (n=3) and one standard deviation.

(a) Mean

Treatments	NR activity after 0 days (U mL^{-1})	NR activity after 5 days (U mL^{-1})	NR activity after 10 days (U mL^{-1})	NR activity after 15 days (U mL^{-1})	NR activity after 30 days (U mL^{-1})
No TNT	0.02	0.02	0.02	0.02	0.02
25 mg L^{-1} TNT	0.02	0.04	0.04	0.04	0.06
50 mg L^{-1} TNT	0.02	0.03	0.05	0.10	0.14
100 mg L^{-1} TNT	0.02	0.12	0.16	0.23	0.34
200 mg L^{-1} TNT	0.02	0.73	1.02	1.44	1.46

(a) Standard Deviation

Treatments	NR activity after 0 days (U mL^{-1})	NR activity after 5 days (U mL^{-1})	NR activity after 10 days (U mL^{-1})	NR activity after 15 days (U mL^{-1})	NR activity after 30 days (U mL^{-1})
No TNT	0.00	0.00	0.00	0.00	0.01
25 mg L^{-1} TNT	0.00	0.00	0.00	0.00	0.00
50 mg L^{-1} TNT	0.00	0.02	0.01	0.00	0.04
100 mg L^{-1} TNT	0.00	0.02	0.08	0.02	0.02
200 mg L^{-1} TNT	0.00	0.04	0.02	0.05	0.05

Table E-3. Relative NR activity in the root and shoot tissues of vetiver grass after 5 days. Data expressed as mean (n=3) and one standard deviation.

Treatments	NR activity in the root (U mL^{-1}) Mean	NR activity in the root (U mL^{-1}) Standard Deviation	NR activity in the shoot (U mL^{-1}) Mean	NR activity in the shoot (U mL^{-1}) Standard Deviation
No TNT	0.02	0.00	0.1825	0.0143
25 mg L^{-1} TNT	0.04	0.00	0.2183	0.0019
50 mg L^{-1} TNT	0.03	0.02	0.2365	0.037
100 mg L^{-1} TNT	0.12	0.02	3.9832	0.7116
200 mg L^{-1} TNT	0.73	0.04	8.1593	1.69

Table E-4. Saturation kinetics of NR expressed as pseudo first order rate constant (k_1)of NR mediated TNT transformation reaction as functions of plant concentration in the crude enzyme extract, at constant temperature (30^0C) and TNT load (20 mg L^{-1}).

Plant concentrations in the crude enzyme extract (g L^{-1})	Pseudo first order reaction rate constant (k_1)	R^2
100	0.08	0.85
150	0.10	0.94
200	0.12	0.95
250	0.12	0.91
500	0.12	0.91

Table E-5. Saturation kinetics of NR expressed as pseudo first order rate constant (k_1) of TNT transformation reaction as function of initial TNT concentrations, at constant plant concentration in the crude enzyme extract (250 g L^{-1}) and temperature (30^0C).

Initial TNT concentrations (mg L^{-1})	Pseudo first order reaction rate constant (k_1)	R^2
10	0.11	0.85
15	0.11	0.83
40	0.12	0.85
80	0.11	0.83
100	0.10	0.89

Table E-6. Hanes-Woolf type of linear transformation plot of Modified Michaelis-Menten equation for enzyme saturation.

NR Activity (A) (U mL^{-1})	Pseudo first order reaction rate constant (k_1)	A/ k_1
0.03	0.08	0.33
0.26	0.10	2.51
0.41	0.12	3.50
0.69	0.12	5.76
0.76	0.12	6.23

Table E-7. Saturation kinetics of NR expressed as pseudo first order rate constant (k_1) of NR mediated TNT transformation reaction as a function of temperature, at constant plant concentrations in the crude enzyme extracts (250 g L^{-1}) and TNT load (20 mg L^{-1}).

Temperature (^{0}C)	Pseudo first order reaction rate constant (k_1)	R^2
5	0.001	0.89
15	0.007	0.89
20	0.013	0.86
25	0.029	0.90
30	0.146	0.96
35	0.172	0.99
45	0.002	0.65

Table E-8. Arrhenius relationship of pseudo first order reaction rate constants between 5 to 35^{0}C.

Temperature (^{0}C)	Temperature (^{0}kelvin)T	k_1	1/T	ln k_1
5	278	0.001	0.004	-6.73
15	288	0.007	0.003	-5.02
20	293	0.013	0.003	-4.35
25	298	0.029	0.003	-3.54
30	303	0.146	0.003	-1.92
35	308	0.172	0.003	-1.76
45	318	0.002	0.003	-6.35

Appendix F

Experimental Data for Chapter 6

Table F-1. Effect of TNT on % growth ((initial biomass-final biomass)*100/initial biomass) of vetiver grass after 10 days. Negative values indicated the reduction of biomass. Data are expressed as mean (n=2) ± one standard deviation.

Initial TNT concentrations ($mg\ L^{-1}$)	% Growth Mean	% Growth Standard Deviation
0	3.5	0.98
25	-3.72	1.22
50	-5.75	1.33
100	-8.46	1.15

Table F-2. Effect of TNT on total chlorophyll content in vetiver shoots after 10 days. Data are expressed as mean (n=2) ± one standard deviation.

Initial TNT concentrations ($mg\ L^{-1}$)	Total Chlorophyll ($mg\ g^{-1}$) Mean	Total Chlorophyll ($mg\ g^{-1}$) Standard Deviation
0	19.79	2.77
25	14.25	2.06
50	13.55	1.04
100	10.64	1.39

Table F-3. Effect of TNT on the total proteins in root. Data are expressed as mean (n=2) ± one standard deviation.

Initial TNT concentrations ($mg\ L^{-1}$)	Total Proteins in root ($\mu g\ L^{-1}$) Mean	Total Proteins in root ($\mu g\ L^{-1}$) Standard Deviation
0	1.65	0.03
25	1.41	0.07
50	0.96	0.09
100	0.67	0.05

Table F-4. Identified proteins that showed continued downregulation with each increasing TNT treatments.

Identified Proteins	0 mg L^{-1} Initial TNT concentration	25 mg L^{-1} Initial TNT concentration	50 mg L^{-1} Initial TNT concentration	100 mg L^{-1} Initial TNT concentration
Histone H2A 4 (H24A_Wheat)	0.066	0.051	0.048	0.002
Dead box ATP dependent RNA Helicase	0.056	0.038	0.017	0.000
RNA Pseudouridine Synthase (PUS6_ORYSJ)	0.104	0.054	0.049	0.000
Glutamine Synthetase Cytosolic Isozyme 2 (GLNA2_VITVI)	0.044	0.040	0.028	0.000
GTP binding nuclear protein (RAN3_ORSI)	0.048	0.019	0.000	0.000
Ethylene Receptor 1 (ETR1_CUMN)	0.063	0.016	0.000	0.000
Beta 1,3-galactosyltransferase 5 (B3GT5_ARATH)	0.046	0.015	0.000	0.000
Ent-Primara-8(14),15-diene synthase (KSL5_ORYSJ)	0.046	0.017	0.000	0.000
DNA directed RNA Polymerase (RPO3A_TOBAC)	0.024	0.009	0.000	0.000

Table F-5. Identified proteins that upregulated at lower TNT treatments but downregulated at further increase in TNT concentrations.

Identified Proteins	0 mg L^{-1} Initial TNT concentration	25 mg L^{-1} Initial TNT concentration	50 mg L^{-1} Initial TNT concentration	100 mg L^{-1} Initial TNT concentration
S-Adnosylmethionine Synthase (METK4_POPTR)	0.040	0.349	0.130	0.040
UDP-N-Acetyl Glucosomine Peptide N-acetyl glucosaminyl transferase (Sec_ARATH)	0.017	0.105	0.000	0.000
Pentatricopeptide repeat-containing protein	0.028	0.060	0.000	0.000
DNA binding protein (DRP90_SOYBN)	0.153	0.170	0.205	0.010
Casp like Protein 9 (CSPL9_MAIZE)	0.03	0.08	0.00	0.00

APPENDIX G

PREFACE

"This Doctoral Dissertation was produced in accordance with guidelines which permit the inclusion as part of the Doctoral Dissertation the text of an original paper, or papers, submitted for publication. Doctoral Dissertation must still conform to all other requirements explained in the "Guide for the Preparation of the Doctoral Dissertation at The Montclair State University." It must include a comprehensive abstract, a full introduction and literature review, and a final overall conclusion. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported.

It is acceptable for this Doctoral Dissertation to include as chapters authentic copies of papers already published, provided these meet type size, margin, and legibility requirements. In such cases, connecting texts, which provide logical bridges between different manuscripts, are mandatory. Where the student is not the sole author of a manuscript, the student is required to make an explicit statement in the introductory material to that manuscript describing the student's contribution to the work and acknowledging the contribution of the other author(s). The signatures of the Supervising Committee which precede all other material in the Doctoral Dissertation attest to the accuracy of this statement."

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