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Ecotoxicogenomic evaluation of hybrid poplar tree phytoremediation of nitro-substituted explosives

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ECOTOXICOGENOMIC EVALUATION OF HYBRID POPLAR TREE PHYTOREMEDIATION OF NITRO-SUBSTITUTED EXPLOSIVES

by

Brittany Renee Flokstra

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Civil and Environmental
Engineering in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Professor Jerald L. Schnoor

ABSTRACT

Poplar (*Populus deltoides x nigra* DN34) tissue cultures removed 2,4,6-trinitrotoluene (TNT) from an aqueous solution in five days, reducing the toxicity of the solution from highly toxic Microtox® EC value to that of the control. 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) was taken up by the plant tissue cultures more slowly, but toxicity reduction of the solution was evident. The measurement of toxicity reduction of aqueous solutions containing TNT and RDX was performed using a novel methodology developed for use with the Microtox® testing system. Radiolabeled TNT and RDX were used to confirm removal of explosives from hydroponic solutions containing plant tissue cultures and to verify that toxicity did not change in solutions where no plant cultures were present (positive controls). High Performance Liquid Chromatography (HPLC) and Liquid Scintillation Counter (LSC) measurements confirmed removal of TNT and RDX from solutions containing poplar plant tissue cultures and constancy of the plant-free controls. In addition, metabolites were identified in remediated solutions by HPLC, confirming the mechanism by which plants can remediate groundwater, surface water, and soil solutions.

Using an Affymetrix® microarray, poplar trees were exposed to TNT over 48 hours. General patterns, as well as significant downregulated and upregulated genes were studied. We identified several new genes that were implicated in the detoxification and metabolism of TNT by *Populus*. In particular, our results support the "green liver" model of different gene families being expressed during the time course experiments. This suggests an alignment with Phase I transformation, Phase II conjugation, and Phase III compartmentation processes. Many of the genes identified in this study were related to

those significantly expressed in previous *Arabidopsis* studies, supporting the comparison between these two plants. Gene families represented as significant in this study were glucosyltransferases, glycosyltransferases, cytochromes, and phosphofructokinases. We saw patterns in the areas of respiration, citric acid cycle, shikimatic pathway, and toxic responses.

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Thesis Supervisor

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To my supportive spouse, Susan Hill, and our son, Colton Questra
for all of the love that you both give me every single day.
To my parents Dr. Gerard J. Flokstra, Jr. and Ruth M. Barney Flokstra
for their patience and belief in me.
To Diana's Grove (Cynthea & Patricia) for your relentless support, for daring me to grow, and for forming the small groups that will, indeed, change the world.
Dedicated to the memory of Fizban Fistandantalus Flokstra the First.

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TABLE OF CONTENTS

LIST OF TABLES vi

LIST OF FIGURES vii

CHAPTER I INTRODUCTION 1

1.1 Background 1
1.2 Objectives 2

CHAPTER II LITERATURE REVIEW 3

2.1 History and Use of Nitro-Substituted Explosives 3
2.2 Physical and Chemical Properties of TNT, RDX, and HMX 4
2.3 Toxicity of TNT, RDX, and HMX 5
2.3.1 Toxicity to Plants (Phytotoxicity) 7
2.3.2 Toxicity to humans 7
2.4 Removal and Remediation of TNT, RDX, and HMX 8
2.4.1 Traditional Removal Methods 8
2.4.2 Bioremediation 8
2.4.3 Phytoremediation 9
2.5 Phytoremediation Model Plant Systems 10
2.5.1 Poplar Trees 10
2.5.2 Plant Tissue Cultures 12
2.5.3 Arabidopsis thaliana 12
2.6 Ecotoxicogenomics 14
2.6.1 Background 14
2.6.2 Microarray Platforms 15
2.6.3 Basic Overview of Microarray Process: 17
2.6.4 Microarray Toxicology and Phytotoxicity Studies 18
2.6.5 Model Species and Microarrays 20

CHAPTER III PLANT TISSUE CULTURES AS MODEL SYSTEMS FOR DETERMINING TOXICITY REDUCTION IN A PHYTOREMEDIATION SCHEME 22

3.1 Introduction 22
3.2 Materials and Methods 24
3.2.1 Chemicals and Supplies 24
3.2.2 Cultivation and Growth 24
3.2.3 Experimental Procedure and Sampling 25
3.2.4 Analysis 26
3.3 Results and Discussion 27
3.3.1 TNT 27
3.3.2 RDX 29
3.3.3 Comparison of TNT and RDX 30
3.3.4 HMX 30
3.4 Conclusions 31

CHAPTER IV POPLAR TREE PHYTOTOXICITY AND GENETIC RESPONSES 39

4.1 Introduction and Background 39
4.2. Methods: 40
4.2.1 MIAME for Affymetrix® Experiments 40
4.2.2 Plant Growth and Treatment 40
4.2.3 RNA Extraction and Microarray Analysis 41
4.2.4 Data Analysis: 42
4.3. Results and Discussion: 43
4.3.1 Phase 1: Transformation....43
4.3.2 Phase 2: Conjugation....44
4.3.3 Phase 3: Transport and Sequestration....45
4.3.4 Other Upregulated Genes 46
4.3.5 Downregulated Genes 47
4.4 Conclusions....48

CHAPTER V CONCLUSIONS 61

APPENDIX A: PLANT MEDIA PREPARATION 64

A-1. Plant Tissue Culture MS Media Information from Caisson Labs 64
A-2. Poplar Tree Growth Solution (modified Hoagland's) 66

APPENDIX B: RNA PROTOCOLS 68

B-1 RNA Extraction using RNeasy Qiagen extraction kit 68

APPENDIX C: ATTEMPTED EXPERIMENTS....71

C-1 *Eisenia fetida* Studies 71
C-2 *Caenorhabditis elegans* Studies 71

REFERENCES 73

LIST OF TABLES

Table 3-1 Microtox® effective concentration levels of toxicity 32

Table 4-1 List of Phase 1 Cytochrome P450 genes (by Affymetrix ID) upregulated at 8, 24, and 48 hours after exposure to TNT. 50

Table 4-2 Upregulation of Cupin during Phase 1 50

Table 4-3 List of Phase 2 gene upregulation of glutathione S-transferases, glycosyl- and glucosyltransferases. 55

Table 4-4 List of Phase 3 upregulated genes. 57

Table 4-5 List of downregulated genes in response to oxidative stress.....58

LIST OF FIGURES

Figure 2-1 Chemical Ring Structure Representations of TNT (top left), RDX (top right), and HMX (bottom) 5

Figure 2-2 Hybrid poplar cuttings with increasing concentrations of TNT from left to right (in units of mg L^{-1}): 0, 4, 6, 8, 10, and 20. 11

Figure 2-3 Poplar Tree Plant Tissue Cultures in 250 mL flask with growth solution. 13

Figure 2-4 Simple Diagrams of a) A Spotted Microarray and b) An Oligonucleotide array (Mount, 2004) 16

Figure 2-5 Schematic of Affymetrix® GeneChip application of samples (Mount, 2004) 19

Figure 3-1Microtox® 5-min test EC_{50} percent concentrations over the duration of the experiment beginning with 20 mg TNT L^{-1} in solution with poplar tree plant tissue cultures in sterile 250 mL flasks. 33

Figure 3-2 LSC results from experimental and control samples for the duration of the experiment. Concentrations of TNT are expressed as a percentage of the initial TNT radioactivity. HPLC results from experimental flask samples for the duration of the experiment. Concentrations of TNT are expressed as a normalized percentage of the initial TNT concentration of 20 mg TNT L^{-1}. 34

Figure 3-3 HPLC radiochemical results from ^{14}C-TNT standard degradation by poplar plant tissue cultures including metabolite and mass balance studies. Unknown was recovered as $^{14}CO_2$ by biovidizing radiolabeld TNT metabolites in plant tissue cultures. (Courtesy of B. VanAken, previously unpublished data.) 35

Figure 3-4 Microtox® 5-min test EC_{50} percent concentrations over the duration of the experiment beginning with 20 mg RDX L^{-1} in solution with poplar tree plant tissue cultures in sterile 250 mL flasks. 36

Figure 3-5 Microtox® EC_{50} of various growth solutions constituents at both the 5- and 15-min acute toxicity test times. MS, Murashige and Skoog medium with everything added; K, kinetin; D, 2-4, D; B, benomyl; Glu, glucose; MSO, MS medium only (no additions); Gly, glycine; and DI, de-ionized water. 37

Figure 3-6 LSC results from experimental and control samples for the duration of the experiment. Concentrations of RDX are expressed as a percentage of the initial RDX radioactivity. 38

Figure 4-1 Upregulation of Cytochrome P450s at 8, 24, and 48 hours. The average of all CytP450 genes is plotted as a line and standard deviation error bars are shown. Cytochrome P450 genese are identified by Affymetrix® ID in Table 4-1 51

Figure 4-2 Phase 2 glutathione S-transferase (GST) upregulation over time, with standard deviation error bars.....52

Figure 4-3 Phase 2 glycosyltransferase upregulation over time, with standard deviation error bars. 53

Figure 4-4 Phase 2 glucosyltransferase upregulation over time, with standard deviation error bars. 54

Figure 4-5 Chart of gene categories for other significantly upregulated genes over the course of 48 hours when exposed to TNT. 56

Figure 4-6 Chart of gene functions of 73 respiration related downregulated genes. 59

Figure 4-7 Chart of gene functions of 37 DNA Binding related downregulated genes. 60

CHAPTER I

INTRODUCTION

1.1 Background

Soil and water contaminated with nitro-substituted explosives, 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX, Royal Demolition eXplosive) and1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane (HMX, High Melting eXplosive), are located on military properties, including bases, ranges, and munitions manufacturing centers, throughout the world. Remediation of these extensively contaminated sites is a serious problem facing the military and regulatory agencies (Van Aken et al., 1999; Hawari et al., 2000b; Van Aken and Schnoor, 2002; Jenkins, 2006; Ryu, 2007).

Phytoremediation, the use of plants to remove contaminants from the environment, is one proposed method for site clean-up of these explosives. Transformation in plant tissues is based on the "green liver" model, which describes the fate of organic contaminants within plant tissues(Sandermann, 1994; Burken and Schnoor, 1998; Salt, 1998; Hannink, 2002). Hybrid poplars, such as *Populus deltoides x nigra*, DN34, are considered ideal plants for phytoremediation of large quantities of explosives in soil and water. Hybrid poplars have been shown to take up and biotransform explosives in both laboratory and field studies (Thompson et al., 1998b; Thompson et al., 1998a, 1999; Yoon et al., 2002; Mezzari et al., 2004; Van Aken et al., 2004).

In addition to traditional plant species, like the poplar tree, plant tissue cultures can also be used in laboratory studies. Plant tissue cultures include undifferentiated callus growths taken from whole plant species. They have the advantage of large quantities of genetically identical plant material that can be quickly and easily

reproduced. Previous studies of plant tissue cultures report that degradation and mineralization by the tissue cultures mimics plant degradation in a controlled environment. (Hughes et al, 1997, Nepovim et al., 2004).

Recent technological advances in genomic research have changed the face of toxicity testing. In addition to standard toxicity methods, new methodologies based on whole genome microarrays are being developed (Goffeau et al., 1996b; Goffeau et al., 1996a; Nuwaysir et al., 1999; Snape et al., 2004). Gene expression studies focusing on the phytoremediation of explosives has primarily been limited to the model plant *Arabidopsis thaliana*, as its genome has already been sequenced and annotated.

The *Populus* genome is currently available on Affymetrix® microarrays and, even though it is not fully documented and annotated, it can provide useful information regarding gene expression and global genetic responses for poplars exposed to TNT.

1.2 Objectives

1) Determine if a specific plant tissue cultures is representative of the metabolic biochemistry of the whole plant in aqueous systems.
2) Design a method using the Microtox® toxicity testing system platform to determine toxicity reduction in aqueous systems.
3) Use poplar plant tissue cultures as a model plant system that will remove and transform explosive compounds resulting in the reduction of residual toxicity from hydroponic solutions.
4) Develop a consistent laboratory method for extracting RNA from poplar tree root samples.
5) Perform an initial study of gene expression of poplar trees to TNT over a 48 hour time course experiment.
6) Identify global genetic response patterns in the *Populus* genome after exposure to TNT over a 48 hours time course experiment using microarray technology.

CHAPTER II

LITERATURE REVIEW

2.1 History and Use of Nitro-Substituted Explosives

Nitro-substituted explosives, 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX, Royal Demolition eXplosive) and1,3,5,7-tetranitro-1,3,5,7-tetrazacyclooctane (HMX, High Melting eXplosive) are produced in large quantities every year. Extensive contamination from manufacturing, testing and using these explosives has become a serious problem for the military and regulatory agencies. Produced in tens of thousands of kilograms each year, TNT has been heavily manufactured for over 100 years (Tadros *et al*, 2000). More stable RDX and HMX have greater detonation power than TNT. These compounds have replaced TNT as the most widespread, conventional explosives used in military applications today (McCormick *et al.*, 1981; Faust, 1994; Roberts *et al.*, 1998).

Soil and water contaminated with these energetic compounds are primarily located on military properties including bases, ranges, and munitions manufacturing centers, both in the U.S. and throughout the world (Van Aken et al., 1999; Hawari et al., 2000b; Van Aken and Schnoor, 2002; Jenkins, 2006; Ryu, 2007). These properties have been systematically contaminated over a period of more than one hundred years. Due to environmental health concerns and toxicity issues, clean-up of these military properties has recently become a priority for the U.S. military and other agencies. The added importance of sustaining the bases and land available to the military for use into the next 150 years is also a priority consideration (Van Aken and Schnoor, 2002; Jenkins, 2006; Ryu, 2007).

2.2 Physical and Chemical Properties of TNT, RDX, and HMX

TNT is a nitro-aromatic compound with three nitro functional groups attached to an aromatic ring structure (Figure 2-1). It is also known as trinitrotoluene, tolite, and 1-methyl-2,4,6-trinitrobenzene. TNT is considered reactive and energetic because the three nitro groups can be reduced/reacted under both aerobic and anaerobic conditions (Hawari et al., 2000b). It is a colorless to pale yellow, odorless solid that is considered insoluble (0.1 g per 100) (Chemfinder). TNT binds readily (and irreversibly) to soils and is not very mobile in the environment. Several transformation products have been identified for TNT including 2, 6-dinitrotolune (2,6-DNT), 2-amino-6-nitrotoluene (2A-6NT), 2,6-diaminotoluene (2,6-DAT), 2,4-dinitrotoluene (2,4-DNT), 2-amino-4-nitrotoluene (2A-4NT), 4-amino-2-nitrotoluene (4A-2NT), and 2,4-diaminotoluene (2,4-DAT) (Dodard et al., 1999).

RDX is a nitramine with a heterocyclic ring structure (Figure 2-1). It is also known as hexagen, hexolite, trinitrohexahydrotriazine and cyclotrimethylenetrinitramine. RDX is even less soluble than the insoluble TNT. RDX is more mobile in the environment than TNT, as it is not easily sorbed to soil particles. RDX is more easily degraded under anaerobic conditions than aerobic (Pennington and Brannon, 2002). Transformation products of RDX are difficult to locate in field conditions and are rarely even seen in laboratory conditions.

HMX is a heterocyclic compound with an eight-membered ring in a stable crown conformation (Figure 2-1). HMX is also known as octogen, cyclotetramethylene tetranitramine and HW4. HMX is by far the most stable of these three explosives under a variety of conditions both in the laboratory and in field samples (Pennington and Brannon, 2002). HMX is the least soluble and is therefore the most difficult to accurately test. Little is known about the transformation products of HMX. Fournier et al (2004)

proposed these ring-cleavage products: 4-nitro-2,4-diazabutanal (NDAB), nitrite (NO_2^-), nitrous oxide (N_2O), and formaldehyde (HCHO).

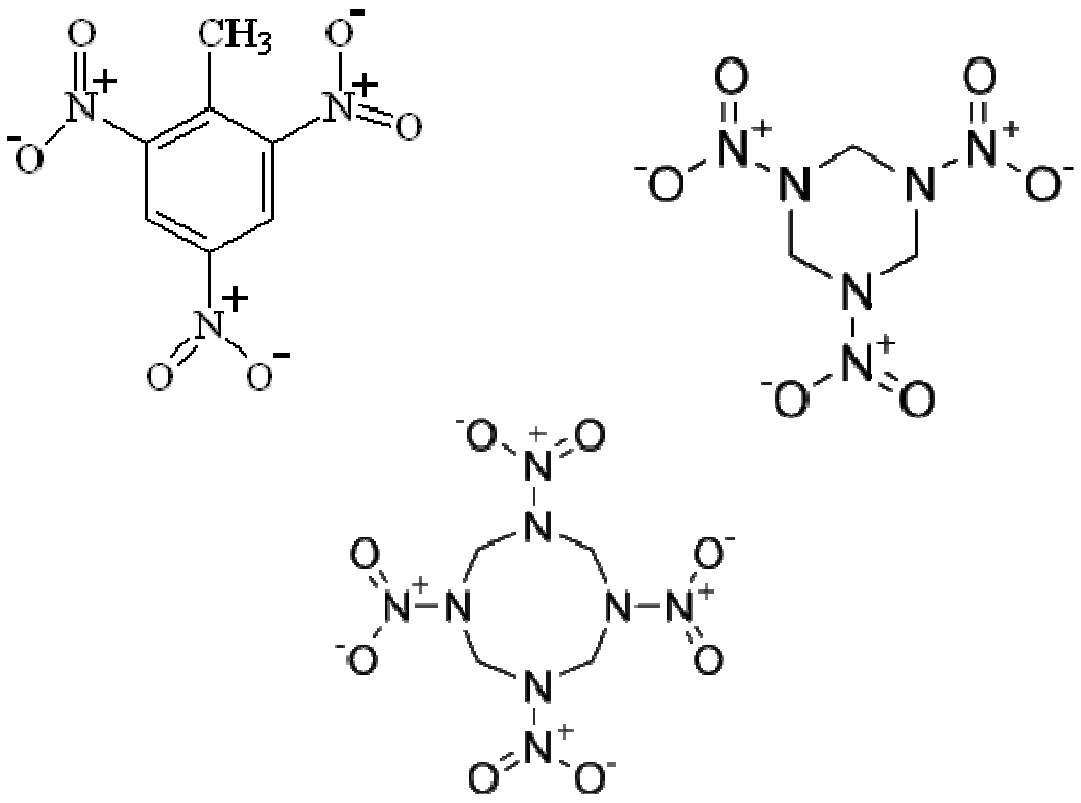


Figure 2-1 Chemical Ring Structure Representations of TNT (top left), RDX (top right), and HMX (bottom)

2.3 Toxicity of TNT, RDX, and HMX

Trinitrotoluene is highly toxic, RDX is toxic, and HMX is non-toxic at soluble levels (Won et al., 1976; Faust, 1994; Honeycutt et al., 1996; Lynch, 2002; Brunjes,

2007; Rosen and Lotufo, 2007; Smith, 2007). There is some disagreement as to whether TNT is mutagenic, with both positive and negative *in vitro* results reported (Honeycutt et al., 1996). Research also indicates that the typical derivatives of TNT are all less toxic than the parent compound, though the exact order of their toxicity depends on the testing procedure used (Simini et al, 1995; Lachance et al, 2004). There are, however, a few uncommon azoxy derivatives that have been found to be more toxic than TNT (Won et al., 1976; Honeycutt et al., 1996; Dodard et al., 1999; Fournier et al., 2002).

Toxicity testing for explosives on various organisms have shown TNT to be toxic to bioluminescent bacteria (*Vibrio fischeri*), unicellular algae (*Selenastrum capricornutum*), oyster larvae (*Crassostrea gigas*), and copepods (*Tigriopus californicus*), midge (Chironomous tentans) and amphipod (Hyalella azteca) (Won et al., 1976; Burton et al., 1994; Sunahara et al., 1998; Dodard et al., 1999; Tadros et al., 2000; Gong et al., 2001; Frische, 2002; Steevens et al., 2002). In addition some benthic invertebrates (marine polychaete *Neanthes arenaceodentata* and estuarine amphipod *Leptocheirus plumulosus*) have had adverse reactions (Green et al., 1999; Lotufo et al., 2001). Soil microbial communities have also been studied and only TNT has any clear negative effect on soil microbial populations (Fuller and Manning, 1998; Gong et al., 1999b; Gong et al., 2002; Juck et al., 2003; Kuperman, 2006; Neuwoehner, 2007).

Earthworm (*Eisenia foetida* and *Eisenia andrei*) growth inhibition has been observed for TNT, RDX and HMX , though the last only slightly (Robidoux et al., 2001; Gong et al., 2002). Tiger salamanders (*Ambystoma tigrinum*) were found to accumulate TNT and metabolites in skin, lung, liver and kidneys (Johnson et al., 2000b; Johnson et al., 2000a). TNT has been found to be toxic in mammals such as rats, dogs, birds and other wildlife (Dilley et al., 1982; Johnson et al., 2000b; Gogal et al., 2002; Gong et al., 2002).

2.3.1 Toxicity to Plants (Phytotoxicity)

TNT is toxic to hybrid poplar trees at 5 mg L^{-1} (Thompson, 1997) and at 50 mg kg^{-1} of soil there were negative effects on seed germination and seedling growth of turnips and cress (Gong et al., 1999a). Lettuce and barley both had significant emergence reduction when exposed to 1,040 mg kg^{-1} of soil (Robidoux et al., 2003). RDX was not toxic to hybrid poplars up to 20 mg L^{-1} (Thompson et al., 1998a) and HMX was not toxic up to 4500 mg kg^{-1} dry soil using barley and lettuce (Robidoux et al., 2003).

2.3.2 Toxicity to humans

The Environmental Protection Agency (EPA) lists TNT and RDX as priority pollutants. TNT can enter the human system through eating, drinking, touching or inhaling contaminated water, food or air. Adverse human health effects from exposure to TNT can include anemia, liver function abnormalities (toxic hepatitis is the most common), and respiratory complications (Agency for Toxic Substances and Disease Registry-ASTDR). In addition, TNT is also a suspected carcinogen. RDX enters the human body primarily through inhalation or touching contaminated soil. Some can be ingested through water or food, but these incidents are considered rare. If exposed, RDX can preferentially target the neurological system and cause seizures or convulsions (ATSDR). HMX is not considered to be a primary pollutant by the EPA, nor does it exhibit the same toxic properties as TNT and RDX. No serious health problems are given for HMX exposure. TNT, RDX and HMX have been given a lifetime health advisory of 2, 2 and 400 µg L^{-1} in drinking water, respectively, by the EPA (Ross and Hartley, 1990; Lynch et al., 2002).

2.4 Removal and Remediation of TNT, RDX, and HMX

2.4.1 Traditional Removal Methods

Traditional treatments of explosive wastes have included detonation and incineration, which can cause as many problems as they supposedly remove (Van Aken and Schnoor, 2002; Lynch et al, 2002). These traditional treatments are high in cost to both the companies in charge of remediation and to the environment. Problems with incineration include hazardous particulate emissions, high cost of equipment, and the relatively few places that such a treatment can be carried out. Traditional treatments have also become increasingly difficult to implement effectively due to the sheer magnitude of the contamination problem (Van Aken and Schnoor, 2002). Cost effective and environmentally friendly alternatives are needed to treat the many explosive contaminated sites. To date, these cleaner treatment choices have primarily involved abiotic remediation, microbial bioremediation and phytoremediation efforts.

2.4.2 Bioremediation

Bioremediation consists of the biodegradation of toxic substances by microbial activity. Bioremediation is effective, but can sometimes take a very long time to remove toxicity from contaminated soils and water. Explosive bioremediation efforts have included field site in-situ addition of microbial species, composting, bioslurries and soil-flooded biopiles (Jarvis et al., 1998) (Boopathy et al., 1997). In addition to field research, laboratory studies have shown there to be several species that can transform nitro-substituted explosives.

TNT is microbially transformed under both anaerobic and aerobic conditions and has been extensively studied in simple soil systems (Hawari et al., 2000a). Anaerobic degradation has been shown by *Desulfovibrio* (Spain, 1995; Esteve-Nunez et al., 2001) and aerobic degradation occurred by *Actinomycetes* (PastiGrigsby et al., 1996).

RDX has been shown to be degraded anaerobically in a sludge by methanogens and acetogens (McCormick et al., 1981) and aerobically by Rhodococcus (Jones et al., 1995; Coleman et al., 1997).

Van Aken *et al* report biodegradation of TNT, RDX and HMX by a phytosymbiotic *Methylobacterium* species in association with poplar trees (Van Aken et al., 2004). It was reported that a pure culture of the *Methylobacterium* species "fully transformed" (not mineralized) 20 mg TNT L^{-1}. Van Aken *et al* also report that 58% of the initial 20 mg RDX L^{-1} and 61.4% of the initial 2.5 mg HMX L^{-1} were mineralized.

2.4.3 Phytoremediation

Phytoremediation is the use of plants to remove contaminants from the environment or to biotransform them into less toxic constituents. It is based on the "green liver" model which describes the fate of organic contaminants within plant tissues (Sandermann, 1994; Burken and Schnoor, 1998; Salt, 1998; Hannink, 2002). Both cost effective and environmentally friendly, phytoremediation is sometimes faster than most microbial bioremediation schemes.

Phytoremediation includes phytoextraction (uptake through roots), phytodegradation, phytovolatilization through leaves and rhizodegradation (roots and associated microbes) (Burken and Schnoor, 1997; Salt et al., 1998; Burken, 2000).

Depending on the location of the contamination, the soil type and the study purposes, several plant species have been used in the phytoremediation of explosives.

Those plants that have been shown to take up and biotransform explosives in laboratory settings include Arabidopsis, aquatic plants (*Myriophyllum aquaticum* and *Catharanthus roseus*), Stonewort, poplar trees, oat, wheat, cress, turnip and bush beans (Thompson, 1997; Gong et al., 1999a; Burken, 2000; Ekman et al., 2003; Hitchcock et al., 2003; Yoon, 2004; Ekman et al., 2005; Kurumata et al., 2005)

Some studies on the fate of explosives in edible plants have also been done with tomatoes, corn, radishes, and lettuce (Hughes et al., 1997; Bhadra et al., 2001; Price et al., 2002). A few reports of complete plant-assisted fungal mineralization of TNT have been documented (Scheibner and Hofrichter, 1998; Van Aken et al., 1999).

2.5 Phytoremediation Model Plant Systems

2.5.1 Poplar Trees

Poplar trees from the genus *Populus* (family Salicaeae) is an extensively studied woody plant. Poplars are members of the Salix family and are closely related to willows, cottonwoods, and aspens (Tuskan et al., 2004). Poplars are a C3 plant (uses the Calvin cycle for synthesis of cellular components), deciduous (loses leaves yearly), dicotyledon (have net-veined leaves) and dioecious (having both male and female individual trees) (Thompson, 1997). Poplar trees have been used for bioenergy, fiber. They can be grown throughout the contiguous United States, excepting only the most arid regions (Tuskan et al., 2004).

Hybrid poplars, such as *Populus deltoides x nigra*, DN34, are considered ideal plants for phytoremediation of large quantities of explosives in soil and water for many reasons. Primarily, hybrid poplar trees:

- grow very quickly, becoming 65-100m within a few years,
- take up large quantities of water, exerting hydraulic control at the site,
- are hardy and survive limited periods of soil anoxia,

- can be grown from a small cutting of another tree, or clonal propagation and,
- live longer (25-50 years) and are perennial (Thompson, 1997; Dietz, 2000; Snellix, 2002; Taylor, 2002; Moore, 2006; Gunderson, 2007).

Poplar trees have been shown to take up explosives in both laboratory and field studies (Figure 2-5) (Thompson et al., 1998b; Thompson et al., 1998a, 1999; Yoon et al., 2002; Mezzari et al., 2004; Van Aken et al., 2004). Laboratory phytoremediation of explosives have shown that TNT tends to accumulate in the roots, RDX in the shoots and leaves and HMX accumulates more slowly in the shoots and leaves. (Pennington and Brannon, 2002; Price et al., 2002; Yoon, 2006).

Figure 2-2 Hybrid poplar cuttings with increasing concentrations of TNT from left to right (in units of mg L^{-1}): 0, 4, 6, 8, 10, and 20.

2.5.2 Plant Tissue Cultures

In addition to traditional plant species, plant tissue cultures (PTCs), which are micropropagated plant material from a whole plant species, can be grown from hybrid poplars. Based on the type of growth medium used to propagate the cells, these tissue cultures can take the form of whole plant clones, root or shoot growths, and undifferentiated callus tissue (Donnelly and Vidaver, 1998; Van Aken *et al*., 2004). For our purposes, PTCs are grown in a sterile growth medium (Figure 2-6) that induces undifferentiated callus tissue that contain the same DNA found in the whole plant. PTCs are a unique model plant system because large quantities of genetically identical plant material can be rapidly reproduced in a small amount of space and time. These micropropagated cultures are ready for experimentation in a matter of weeks and can be refreshed with new media monthly to keep them healthy and growing. Once a suitable stock amount is obtained, keeping any number of PTCs ready for experimentation needs little effort, time or expense.

Plant tissue cultures of *Soanum aviculare* and *Rheum palmatum* have been used for *in-vitro* degradation of TNT with success (Nepovim et al., 2004). Hairy root cultures have been used to verify transformation and toxicity studies (Hughes et al., 1997). PTCs of poplar trees have recently been used to create a mathematical model of the transformation pathways of RDX and HMX in poplar trees (Mezzari et al., 2004; Yoon, 2004).

2.5.3 Arabidopsis thaliana

Arabidopsis thaliana is a small flowering plant that has been widely used as a model plant species in biology, toxicology and phytoremediation. It is a member of the mustard (Brassicacea) family. *A. thaliana* has a rapid life cycle, from germination to

mature seed in about six weeks. At that time, a plethora of seeds are produced and can be easily cultivated. There is a large quantity of information available concerning research and this plant, which makes it ideal for comparison and confirmation studies.

Figure 2-3 Poplar Tree Plant Tissue Cultures in 250 mL flask with growth solution.

A. thaliana's genomic sequence has been fully mapped and available since the year 2000 (Rhee et al., 2003; Mentewab, 2005).

A. thaliana has been used as a model plant system for xenobiotic experiments and genetic studies for decades. Its genetic sequence is comparable to that of poplar tree genetic codes, which allow for a comparison between the smaller *A. thaliana's* responses

to toxins and that of the poplar trees. Up to this point, *A. thaliana* has been used in genetic studies because its genome is sequenced and annotated.

2.6 Ecotoxicogenomics

Ecotoxicogenomics is described as "the integration of genomics (transcriptomics, proteomics and metabolomics) into ecotoxicology" or also "the study of gene and protein expression in non-target organisms that is important in response to environmental toxicant exposures" (Snape et al., 2004).

2.6.1 Background

In the last ten years technological advances in genomic research have changed the face of toxicity testing and genomic research. Prior to 1995, DNA, RNA, proteins and enzymes were isolated and studied, but entire genomic sequences were unavailable. The first complete sequencing of a free-living organism, *Haemophilus influenzae*, was reported by Fleishmann *et al* in 1995. Soon after, the first complete sequence of a eukaryote, *Saccharomyces cervisiae,* was introduced by Goffeau et al. Human genetic sequencing began to take priority and since the mid-nineties hundreds of plants, mammals, microbes, insects, fish, nematodes and amphibians, as well as the entire human genome have been sequenced (Goffeau et al., 1996b; Goffeau et al., 1996a; Nuwaysir et al., 1999; Snape et al., 2004).

Many of these known genetic sequences have become available on small microchips known as microarrays. Microarrays can contain an entire species genome on one chip. This can mean a range of 3,000 to 90,000 genes on one array that can be used for toxicity testing or other purposes.

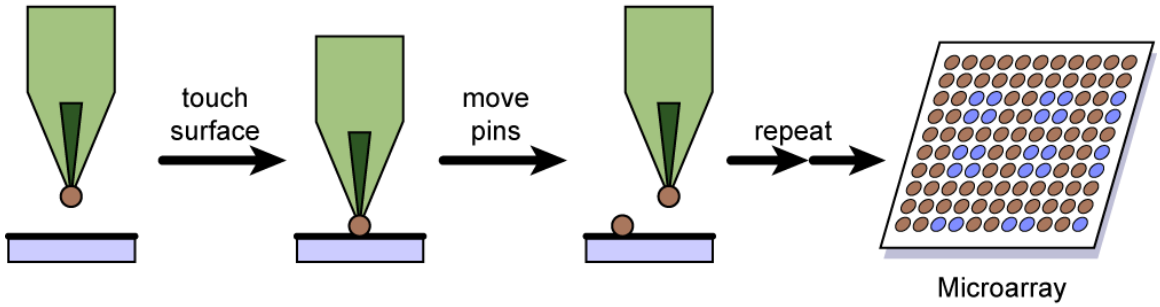
2.6.2 Microarray Platforms

There are two main types of microarrays: spotted and Affymetrix® GeneChips (or Affymetrix® DNA chips). Spotted arrays are created by mechanically placing the DNA on a glass slide (Figure 2-7a). The volume of DNA spotted to the slide is in the nano or picoliter range and therefore is placed there using a robotic-arm instrument. The DNA is available in a microtiter plate, in which the pins are placed and then the arm "spots" a slide by touching all the tiny pins to the slide at once.

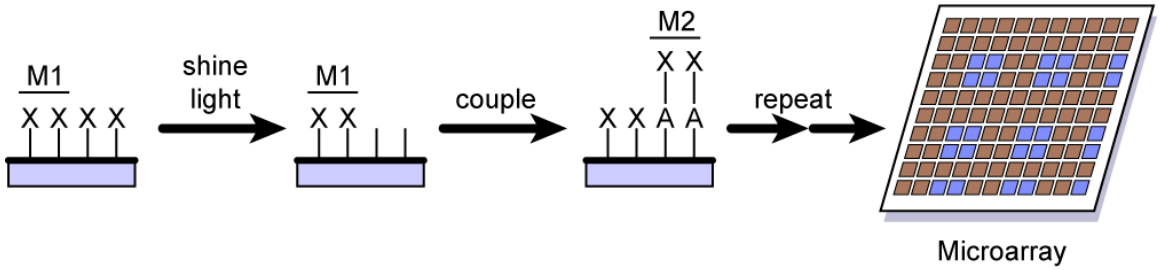
The disadvantages to this type of array arise from technical difficulties with the robotic arms or pins. If one pin is bent, the whole array can be off. This limits reproducibility. Spotted arrays cannot be compared with one another due to this limitation in reproducibility. The advantages of spotted arrays are the cost and flexibility. Spotted arrays are lower in overall price than GeneChips because so many can be made in a short period of time. The flexibility advantage comes from being able to spot whatever DNA the researcher wishes to the given microarrays. Many genomes are not available on chips yet and if the researchers have isolated whole or parts of genomes that they wish to study, they can provide the DNA themselves. There is additional error introduced when the DNA is isolated by the researchers, but this can be minimized. (Nuwaysir et al., 1999; Vrana et al., 2003; Snape et al., 2004).

GeneChips (or Affymetrix® DNA chips) are the second main type of microarray. GeneChips are created by synthesizing oligonucleotides on a silicon chip one base at a time. This process uses photolithography, a light activated chemical process, to attach to oligonucleotides to the array (Figure 2-7b). Between 20 and 25 base pairs are added to the array with 10-20 oligonucleotides for each gene.

These arrays are more costly than spotted arrays because of their reproducibility and minimal variance. GeneChips studied in different labs can be compared because there is no significant variability between slides of the same genome (Lee et al, 2009).



a



b

Figure 2-4 Simple Diagrams of a) A Spotted Microarray and b) An Oligonucleotide array (Mount, 2004)

The University of Iowa's Microarray facility can process the Affymetrix® oligonucleotide microarrays once the cDNA has been isolated. This process involves standardized Affymetrix® microarrays purchased for the genomic sequences that will be studied. The information provided will then be able to be compared to other oligonucleotide microarrays and toxicity testing.

2.6.3 Basic Overview of Microarray Process:

RNA Isolation

RNA isolation is achieved through the specialized extraction kits for plants and eukaryotes. In both cases, DNA is denatured and the proteins and are removed leaving only RNA. The RNA is then converted into cDNA by reverse transcription, a process that synthesizes complementary DNA using RNA as a template and reverse transcriptase as a catalyst. Reverse transcriptase is a DNA polymerase that can use RNA as a template, thus reversing the usual order of genetic information (DNA to RNA) to the opposite (RNA to DNA).

Degradation of the remaining RNA is achieved using Ribonuclease H (RNase H), an enzyme that specifically targets the phosphodiester bonds of RNA hybridized to DNA and then hydrolyzes the bonds, removing excess RNA from the solution and leaving only the cDNA. DNA polymerase is added to synthesize copies of the cDNA. DNA polymerase is an enzyme that creates exact copies of the DNA and also double-checks the strands for any errors in the bases and eliminates those errors so that the remaining DNA copies are all identical (Figure 2-8).

Amplification

The cDNA for each RNA species is transcribed into cRNA incorporating Biotinylated dUTP. Biotin has a very high affinity for streptavidin, which is essential during the signal amplification phase. Biotin can be incorporated within an oligonucleotide and has good binding kinetics and capacity.

Fragmentation

The biotin-labeled cRNAs are fragmented to prevent any interference with probe proximity or secondary structure interferences. Fragmenting also helps hybridization

kinetics, which can, in turn, enhance the signal output. Fragmentation is achieved through adding a reagent and heating the cRNA.

Hybridization

Hybridization is the process of binding the labeled cRNA to the Microarray and is usually done by the laboratory technicians who have a Microarray reader. During the hybridization step only cRNAs with complimentary sequences to the oligonuclueotides in a particular probe cell will bind. A single oligonucleotide microarray contains tens of thousands individual probes cells, which in turn contain thousands of oligonucleotide probes.

Signal Amplification

Signal amplification detects target sequences that are found when the streptavidin attaches to the biotinylated cRNA fragments and then a conjugate is formed.

Analysis

The microarray chip is scanned and the intensity of each spot is proportional to the amount of cRNA which hybridized to that spot. The expression level of a single gene is calculated from the sum of the perfect match oligos minus the mismatch oligos, divided by the beginning amount of oligos (20-25).

2.6.4 Microarray Toxicology and Phytotoxicity Studies

Whole genome genetic testing can now be done on this very small platform with unlimited results. Genetic responses are being studied in a variety of research areas, including pharmaceuticals, xenobiotics, toxic metals, and endocrine disruptors.

(Nuwaysir et al., 1999; Snell et al., 2003; Vrana et al., 2003; Newton et al., 2004). Genetic response to toxic compounds can be analyzed for any testable toxic compound, such as xenobiotic explosives.

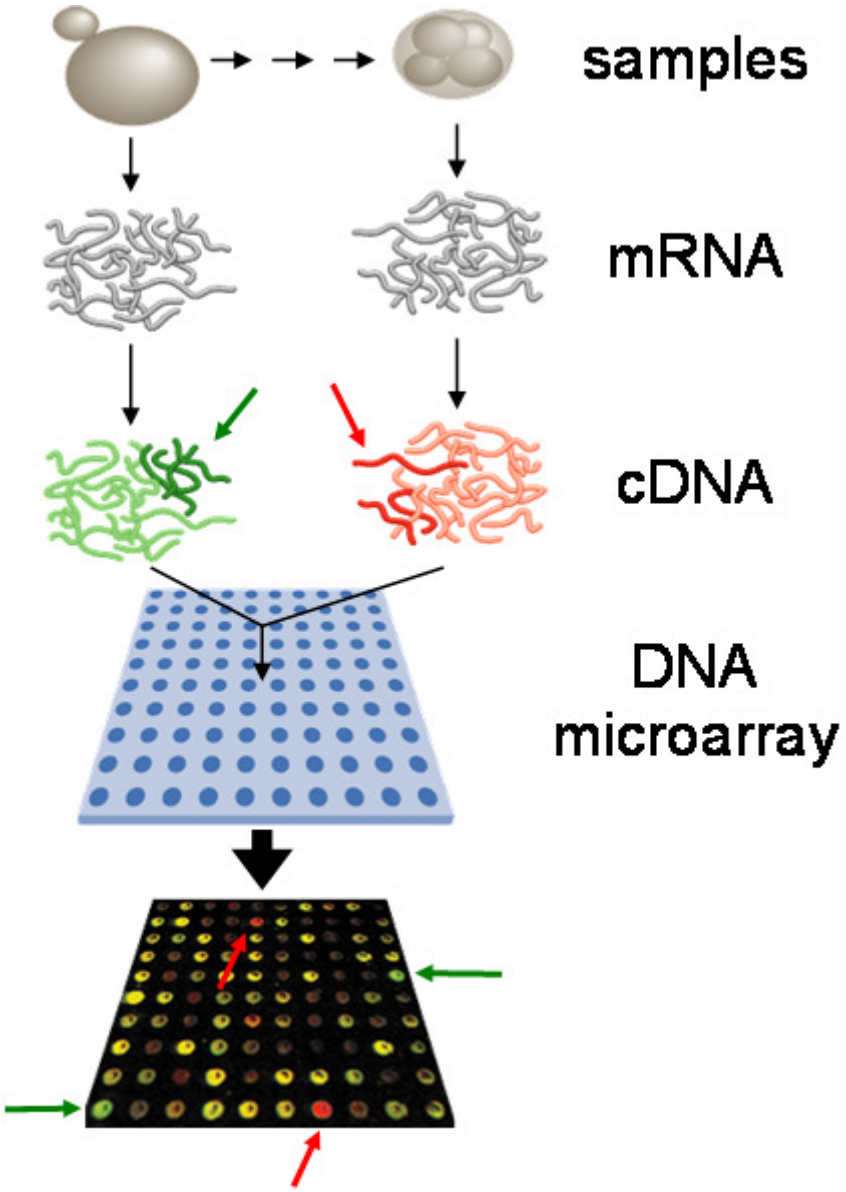


Figure 2-5 Schematic of Affymetrix® GeneChip application of samples (Mount, 2004)

Microarray technology was initially used in the health and medical fields for determining responses in mammals and humans with respect to various chemicals or drugs or new pharmaceuticals. Currently there are many GeneChips (Affymetrix®) available commercially. These include the entire human genome, mouse, rat, *Arabidopsis*, the bacteria *B. subtilis*, barley, bovine, *C. elegans*, canine, chicken, citrus, *Drosophila*, *E. coli*, maize, poplar, porcine, Rheus, rice, soybean, sugar cane, tomato, wheat, yeast and Zebra fish. Poplar is the only woody plant with a GeneChip available.

Toxicity testing using Microarrays has been reported in literature and is proving to be a very important procedure for toxicity testing (Morgan et al., 2002; Snell et al., 2003; Vrana et al., 2003; Kim et al., 2004; Sirisattha et al., 2004; Snape et al., 2004).

2.6.5 Model Species and Microarrays

Some SAGE (Serial Analysis of Gene Expression) studies have been conducted using *A. thaliana* and explosives (TNT & RDX) (Ekman et al., 2003; Ekman et al., 2005). These studies focused primarily on mechanisms and metabolism of TNT and RDX. Since the advent of the Affymetrix® chip for *A. thaliana*, many more studies have been conducted for that model species.

In late December 2005, Affymetrix® announced the availability of a poplar genome microarray. No studies concerning TNT phytoremediation have been published using this new chip since its release. Combining information from *A. thaliana* studies and previous poplar genomic studies, a simple phytotoxicity and biotransformation study was performed using this GeneChip.

The Populus GeneChip contains "more than 61,000 probe sets representing over 56,000 transcripts and gene predictions" (Affymetrix®). It is designed to detect specific expressed sequence tags (EST) and m-RNA based poplar UniGene clusters. The EST, m-RNA and UniGene cluster information is available through the library files of the

Affymetrix® website and are available for downloading into statistical software. The GeneChip includes built-in poplar probes, controls, and rRNA probes.

CHAPTER III

PLANT TISSUE CULTURES AS MODEL SYSTEMS FOR DETERMINING TOXICITY REDUCTION IN A PHYTOREMEDIATION SCHEME[1]

3.1 Introduction

The use of poplar trees in a phytoremediation scheme to remove nitro-substituted explosives has proven a viable option in both laboratory and field experiments. As stated, the fast growth and ability to take up large quantities of contaminated water, as well as providing a large number of roots for rhizosphere degradation, make this an ideal phytoremediation species. Once a phytoremediation scheme has been decided upon it is important to be able to evaluate the overall effectiveness of this process.

In the environment explosives are found primarily in soil. Through rain filtration and typical soil processes, other avenues, such as groundwater, may become contaminated. Groundwater can transport the contaminants into civilian drinking systems and local crop soil systems thereby becoming a vector for human consumption. One purpose of phytoremediation is to help prevent and contain the spread of toxic contamination into human systems by the taking up of large quantities of contaminated water and filtering it through the "green liver" of the poplar tree.

In order ascertain if a phytoremediation scheme is effective, groundwater monitoring must be considered. Measurements of aqueous toxicity should be taken prior to phytoremediation efforts and then regularly during the phytoremediation process. In determining the success of a phytoremediation project one must consider whether there is a reduction of toxicity in groundwater, or soil-water, where the trees are planted.

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[1]This chapter appeared in 2008 in the journal *Chemosphere* (Environmental Toxicology and Risk Assessment) V. 71 (10) pp. 1970-1976.

Plant tissue cultures (PTCs) of poplar trees present an opportunity to test the question of toxicity reduction in aqueous solutions during phytoremediation. Since the liquid medium only needs refreshing once a month, experiments taking less than one month can be accomplished without the addition of more media. Whole plant cuttings need to have new media up to two times per week and are open to the external contamination sources. It would be more difficult to maintain an experimentally controlled environment with poplar tree cuttings. However, the environment for plant tissue cultures is closed to external microbial intervention and is therefore a good indicator of a phytoremediation scheme that doesn't rely on external microbial bioremediation.

A simple, standardized test to determine whether or not a reduction in toxicity of the explosive-contaminated aqueous system occurs after exposure to plant tissues would be beneficial to researchers and military agencies alike. Rojickova,1998, examined the Microtox® standardized toxicity test for use with poplar plant tissue cultures for the remediation of TNT, RDX and HMX. The Microtox® toxicity test is based on the reduction in light emission from the bioluminescent bacteria *Vibrio fischeri* to indicate acute toxic stress, a protocol that is accepted as standard methods in several countries including France, Germany and the USA (ASTM method D5660-1995).

Renoux *et al*, used Microtox® to study the detoxification of fluorine, phenanthrene, carbazole and *P*-Cresol by aerobic bacteria in aquifer sand (Renoux et al., 1999). Pandey (2003) used Microtox® to confirm reduction of chromium in tannery effluent (Pandey et al., 2003). The effluent of several other industries, including textiles and paper wastewater has also been evaluated using the Microtox® system. (Araujo, 2005; Frijters, 2006; Wang, 2007) Sewage plants have recently begun using Microtox® as part of a series of toxicity tests (Alonso-2005; Muller-2007) Explosive monitoring using Microtox® has been performed by Frische (2002) to confirm microbial detoxification in soils contaminated with TNT. However, the Microtox® system has not

been previously used to study the detoxification of explosives in aqueous (groundwater) phytoremediation systems involving plant transformations of parent compounds.

This study used poplar tissue cultures, mimicking whole plant transformation of explosives, in a controlled aqueous environment. The aqueous solution was analyzed for reduction in residual aqueous toxicity using the Microtox® system. The study establishes that poplar tissue cultures can be used as a model plant system that will remove and transform explosive compounds resulting in the reduction of residual toxicity from hydroponic solutions, and it suggests that phytoremediation may be a viable technology for treating contaminated sites.

3.2 Materials and Methods

3.2.1 Chemicals and Supplies

All chemicals were of analytical grade or higher and were purchased from Fischer Scientific or Sigma. The TNT and radiolabeled [U-^{14}C]TNT were purchased from Dupont NEN. Radiolabeled [U-^{14}C]RDX was purchased from PerkinElmer Life Sciences and RDX was synthesized in the W.M. Keck Phytotechnologies laboratory. Microtox® supplies, including cuvettes, freeze-dried bioluminescent bacteria, diluent, osmotic adjusting solution, and reconstitution solution, were purchased directly from Strategics Diagnostics, Inc. (formerly Azur Environmental.)

3.2.2 Cultivation and Growth

Poplar plant tissue cultures (PTCs) were cultivated from explants (pieces of young leaves and stems) and grown on a solid, sterile Murashige & Skoog (MS) solution that included the addition of sucrose, kinetin, 2, 4-dichlorophenoxyacetaic acid (2,4-D) and benomyl as growth regulators, according to the method of Van Aken *et al* (2004)

(Appendix A.1). After approximately one month of growth under a 16 hour light and 8 hour dark diurnal cycle, healthy callus pieces were placed in a sterile MS liquid solution that included sucrose as a carbon source. The flasks containing the callus pieces and the MS medium were placed on a shaker table at about 125 rpm. In a matter of weeks, they had become green and spherical cellular material. Gradually, the spherical plant tissue cultures (a.k.a. nodules) grew and became differentiated with green cells on the outside and tan/grey cells on the inside of the approximately 1" spheres. They were heterotrophic, but also green and able to use light energy.

3.2.3 Experimental Procedure and Sampling

For both TNT and RDX explosive experiments, poplar tissue cultures were placed in sterile 250 mL flasks containing the MS solution and initial concentrations of 20 mg ^{14}C TNT L^{-1} or 20 mg ^{14}C RDX L^{-1}. Positive controls contained both the MS solution and the explosive. Negative controls contained only the MS solution and the poplar tissue cultures.

Transformation experiments and positive and negative controls were all done in triplicate. Sampling, addition of explosives, and addition of plant materials were performed in a sterile laminar hood, with additional flame sterilization.

Initial samples were taken immediately after the explosives were added to solution, but before the poplar tissue cultures were added. The second sample was taken 24 hours after plant tissue cultures were added. Additional samples were taken every two days. Sampling continued through day 15 for TNT and day 23 for RDX. Previous experiments had shown that the plant material takes up RDX slower than TNT.

3.2.4 Analysis

Samples were held at 4°C and analyzed within three days of sampling. Analysis included Microtox®, high performance liquid chromatography (HPLC) with radiochromatography (RC) and liquid scintillation counter (LSC).

The Microtox® Basic Test Protocol was performed on a Microtox® Model 500 Analyzer using reagents and bacteria from Azur Environmental. Instrument readings were entered into the MicrotoxOmni® data reduction software for analysis. Further statistical analyses, including sample replicate standard deviation and linear regression analysis were done in Excel and SigmaPlot. Results are given in EC_{50} concentrations, the effective concentration where 50% of the originally emitted light was reduced. EC_{50} concentrations in Microtox® are similar to standard lethal doses to 50% of orgaisms (LD_{50}) reported in toxicity literature.

Samples were analyzed on a Hewlett-Packard Series 1100 high-performance liquid chromatography with variable wavelength detector (HPLC/UV) and a Supelcosil LC-18 column. Samples were filtered through 0.2-µm microfilters and diluted 1:1 with acetonitrile before analysis. Mobile phase through the HPLC column was 50:50 deionized water and acetonitrile with 1% NH_4ACO and a flow rate of 1 mL per minute. The injection volume was 100 µL.

The HPLC instrument was connected with a 525TR Packard flow scintillation analyzer radiochromatogram, which received sample flow-through after analysis by HPLC. The radiochromatogram measured the radioactive peaks of samples run at 3 mL per minute with a chemiluminescent detector. Peaks were analysed by the FLO-ONE computer program provided with the instrument. Internal controls and integration were performed for each group of samples run.

Additional Liquid Scintillation Counter tests were performed on all radioactive samples on a Beckman LS 6000IC scintillation counter (Fullerton, CA). Samples were

placed in a glass vial containing 10 mL Optima Gold® scintillation cocktail, shaken and inserted into the counter for a 5 minute analysis. Readings were given in disintegrations per minute (DPM) and compared to a standard Optima Gold control.

3.3 Results and Discussion

Microtox® results are shown on an effective concentration scale based on a 50% response (EC_{50} concentration), where 1% is the most toxic and $\geq$ 100% is the least toxic (Bennet & Cubbage, 1992). A decrease in the toxicity of the explosive solution is shown by an increase in the EC50. Values for EC50 are qualitative values that can be expressed in varying toxicity levels from extremely toxic to non-toxic (Table 3-1). Readings for EC50 that are $\geq$ 100% indicate an extrapolated concentration greater than the concentration tested in the sample and are non-toxic.

3.3.1 TNT

Microtox® readings indicated that the toxicity of the solution rapidly decreased in the flasks containing TNT and plant tissue cultures over a period of five days, when the toxicity became equivalent to the negative control (Figure 3-1). The Microtox® test is a very sensitive test for general toxic stress. The negative control flasks began at time zero with 54% EC_{50} concentration readings, indicating some toxicity of the control solution of MS medium plus plant tissue cultures. The positive control (MS solution and TNT only) EC_{50} percent concentration of around 20% for all 15 days of the test indicates TNT is very toxic (Table 3-1). Further calculation of the concentration of TNT that the positive control represented was approximately 4 mg L^{-1}. This was comparable to poplar tree cuttings for which the toxicity level was about 5 mg TNT L^{-1} (Thompson et al., 1998a).

Figure 3-2a shows the dynamic response of the concentration of TNT using results from the Liquid Scintillation Counter (LSC) analysis of the radiolabeled TNT.

Plant tissue cultures removed approximately 80% of the ^{14}C TNT by day 15. The steady drop of radiolabeled TNT for the first few days indicates as much as half of the initial TNT was taken up into the plant by day three. After day three the concentration in the solution seemed to level off for the remainder of this test. As expected, ^{14}C TNT in plant-free positive control solutions remained at or near 100% concentration for the entire experiment (Figure 3-2a). The results from the control solutions show that the plant tissue cultures, and not light or some other factor, transform TNT in hydroponic solutions, as poplar cuttings have been shown to do (Hughes et al., 1997; Thompson et al., 1998a; Van Aken et al., 2004).

Analysis by High Performance Liquid Chromatography (HPLC) allowed identification of the parent compound and one metabolite. HPLC results support the LSC findings showing a rapid decrease in TNT over two days, followed by removal from the system by day 5 (Figure 3-2b). Day 5 corresponds to the Microtox® analysis where the toxicity of the experimental solution became equivalent to the negative control at that same time. After day 5, the solution became moderately toxic and finally only slightly toxic (Figure 3-1 and Table 3-1). In addition to the decrease in TNT, one metabolite was detected by HPLC, peaking on days two and three and steadily declining to almost zero by day 15. This metabolite was most likely 2-ADNT, as the elapsed column detection time of around 5.5 to 6 minutes was consistent with detailed analyses and metabolite identification in a similar plant tissue culture experiment (Figure 3-3). These results and mass balance confirmed the removal of TNT and daughter products by day 10. The final unknown metabolite was investigated by Yoon and hypothesized to be a conjugated, polar product of low or negligible toxicity (Yoon et al., 2006).

3.3.2 RDX

The experimental design to investigate RDX toxicity was similar to the TNT plant tissue culture experiment, but extended to 23 days, which were required for toxicity reduction. Samples were analyzed on the same instruments used to analyze TNT. Microtox® results showed that toxicity of the RDX solution decreased by about 15 % over the entire experiment, but never reached the level of the non-toxic negative control (Figure 3-4). Positive control RDX solutions remained toxic, but not as much as TNT, confirming previous findings of relative toxicity TNT > RDX > HMX (Won et al., 1976; Honeycutt et al., 1996; Dodard et al., 1999; Fournier et al., 2002). There was a large decrease in the toxicity of the negative control solution after day 15, as shown by increasing EC_{50} concentrations in Figure 3-4. Both the TNT and RDX experiments showed that the MS solutions without explosives were somewhat toxic alone. After a period of 10 to 15 days this toxicity became less apparent, indicating that there might be some constituent in the growth solution that was toxic to the bioluminescent bacteria and was later taken up by the plant tissue cultures and a loss of toxic response occurs.

Additional tests were performed on the MS medium in order to find a solution that would not be even slightly toxic to the bioluminescent bacteria. Results of these tests are provided in Figure 3-5 and clearly show that only DI water was entirely non-toxic. However, even though some plants can survive for short time periods in only DI water, plant tissue cultures require certain nutrients and inhibitors to maintain the callus stage and prevent cell differentiation.

Liquid scintillation counting of the radiolabeled RDX showed that 20% of the initial concentration of ^{14}C RDX had been removed by the plant tissue cultures by day 21 (Figure 3-6). This was a much slower rate of removal than that of the ^{14}C TNT (Figure 3-2a). Control RDX solutions remained constant at or near 100% of the initial concentration of ^{14}C RDX for the entire experiment, confirming the hypothesis that the

plant tissue cultures are the reason the RDX is being removed (Yoon et al., 2002; Van Aken et al., 2004)

3.3.3 Comparison of TNT and RDX

Trinitrotoluene (TNT) was removed from solution when plant tissue cultures were present, indicating a clear phytoremediation uptake effect. Uptake of RDX was less clear, Liquid Scintillation Counter (LSC) results indicated that about 20 % was removed by the plant tissue cultures. Both TNT and RDX controls remained toxic to the Microtox® luminescent bacteria from starting concentrations and throughout the experiments. This steady toxicity in positive controls indicates that no form of toxicity reduction (bioremediation or photolysis) was removing RDX or TNT from the positive control solutions. This confirmed the Liquid Scintillation Counter findings. In the experimental flasks, the uptake of RDX was much slower than in those containing TNT. Similar results have been found in whole plant uptake of TNT and RDX (Yoon et al., 2002; Yoon, 2004)

3.3.4 HMX

Experiments were conducted with HMX, but toxicity levels of both the controls and the HMX-spiked medium were all, essentially, non-toxic. The Microtox®Omni software, which mathematically interprets the Microtox® instrument readings, recommended “testing at a higher concentration” indicating that the instrument was unable to accurately measure toxicity at the concentration tested. This confirms previous tests that HMX is non-toxic, even at saturated concentrations (solubility limit) (Yoon, 2004).

3.4 Conclusions

The use of the Microtox® system to confirm the removal of residual aqueous toxicity in plant-assisted remediation efforts is potentially quite useful. Microtox® analysis showed the toxicity of the TNT solution decreased to match the control in 5 days and the RDX solution toxicity slowly declined over the entire time of the experiment. Poplar plant tissue cultures took up TNT and RDX from solution, whereas the plant-free controls remained consistent with respect to initial concentration and toxicity.

Microtox EC50%	Toxicity Level
0-19	Extremely toxic
20-39	Very toxic
40-59	Toxic
60-79	Moderately toxic
80-99	Slightly toxic
>100	Non-toxic

Table 3-1 Microtox® effective concentration levels of toxicity (Bennett and Cubbage, 1992)

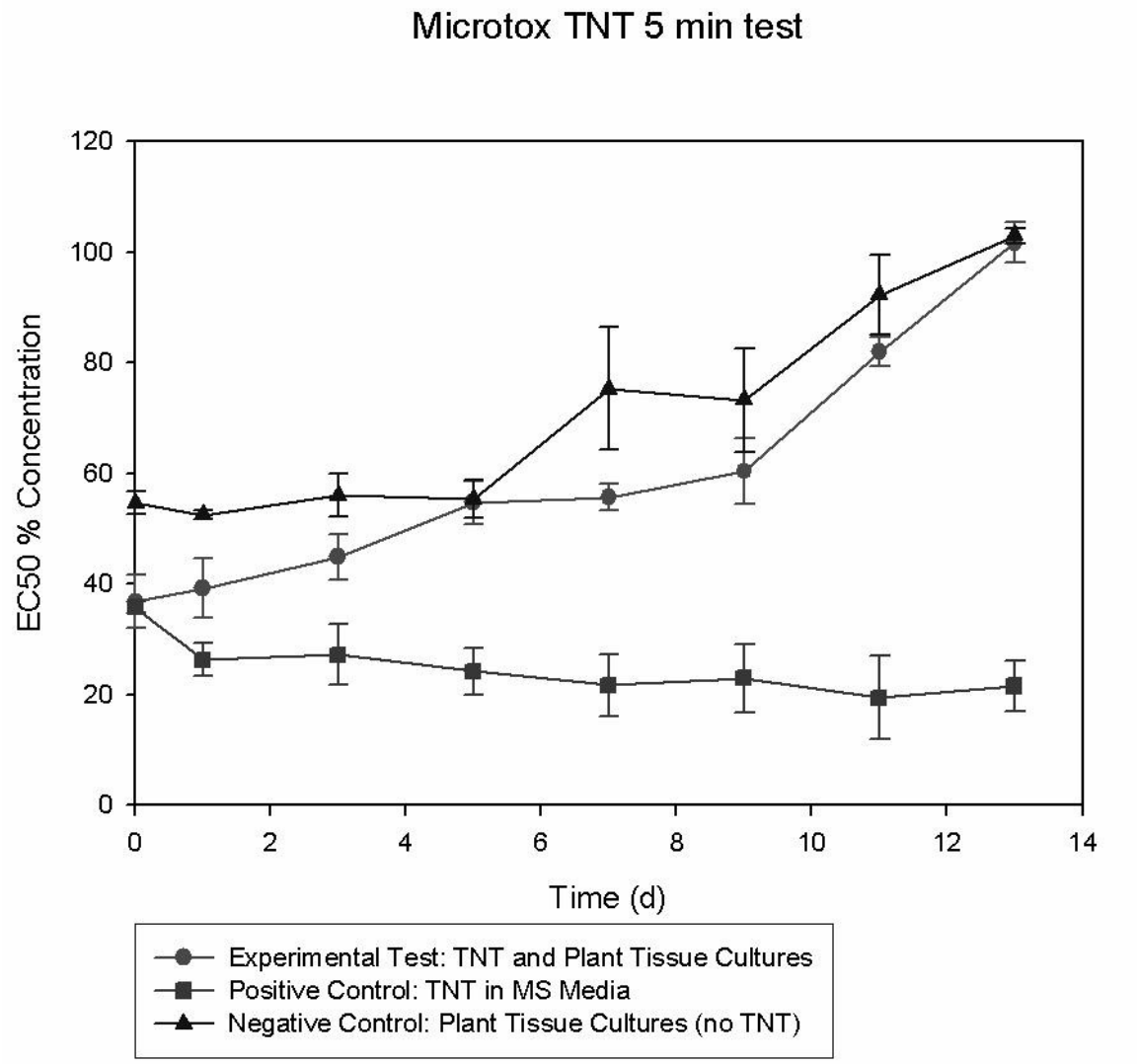

Figure 3-1Microtox® 5-min test EC_{50} percent concentrations over the duration of the experiment beginning with 20 mg TNT L^{-1} in solution with poplar tree plant tissue cultures in sterile 250 mL flasks.

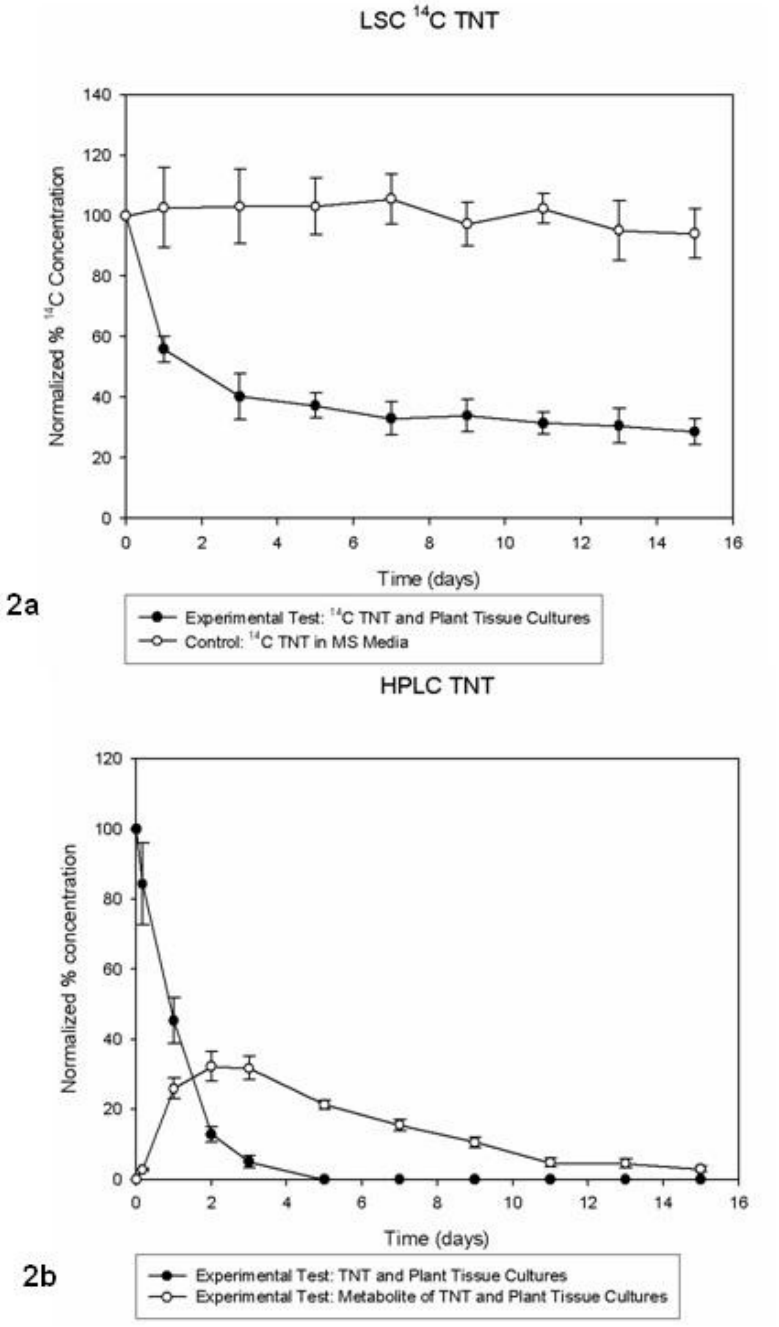

Figure 3-2 LSC results from experimental and control samples for the duration of the experiment. Concentrations of TNT are expressed as a percentage of the initial TNT radioactivity. HPLC results from experimental flask samples for the duration of the experiment. Concentrations of TNT are expressed as a normalized percentage of the initial TNT concentration of 20 mg TNT L^{-1}.

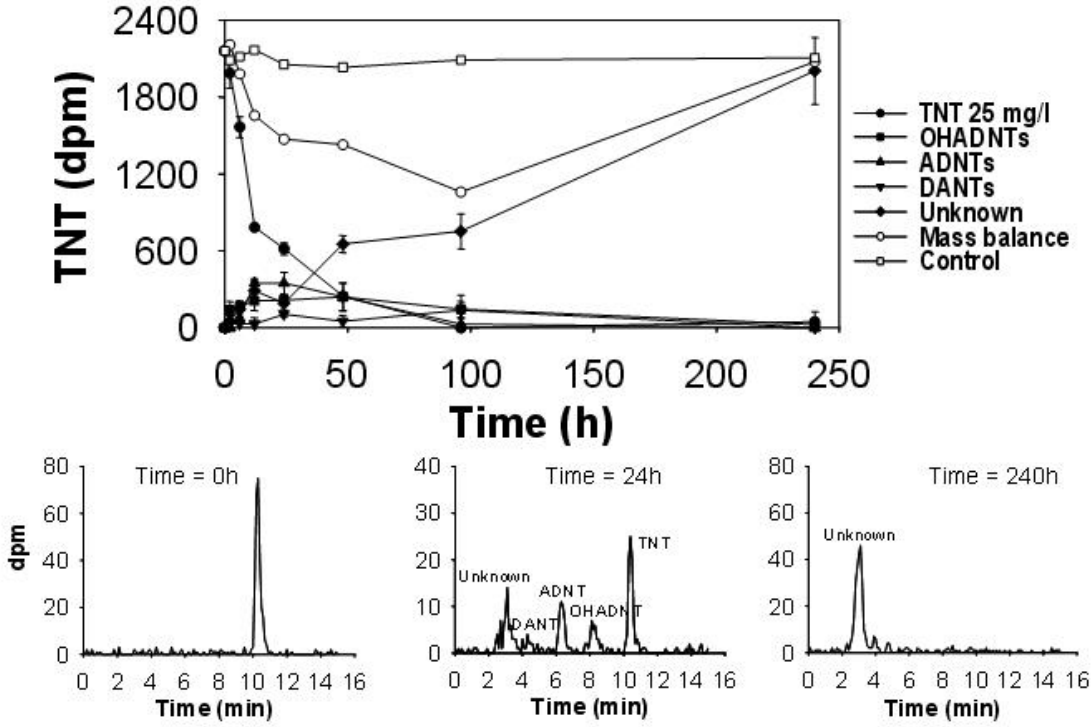

Figure 3-3 HPLC radiochemical results from ^{14}C-TNT standard degradation by poplar plant tissue cultures including metabolite and mass balance studies. Unknown was recovered as $^{14}CO_2$ by biovidizing radiolabeld TNT metabolites in plant tissue cultures. (Courtesy of B. VanAken, previously unpublished data.)

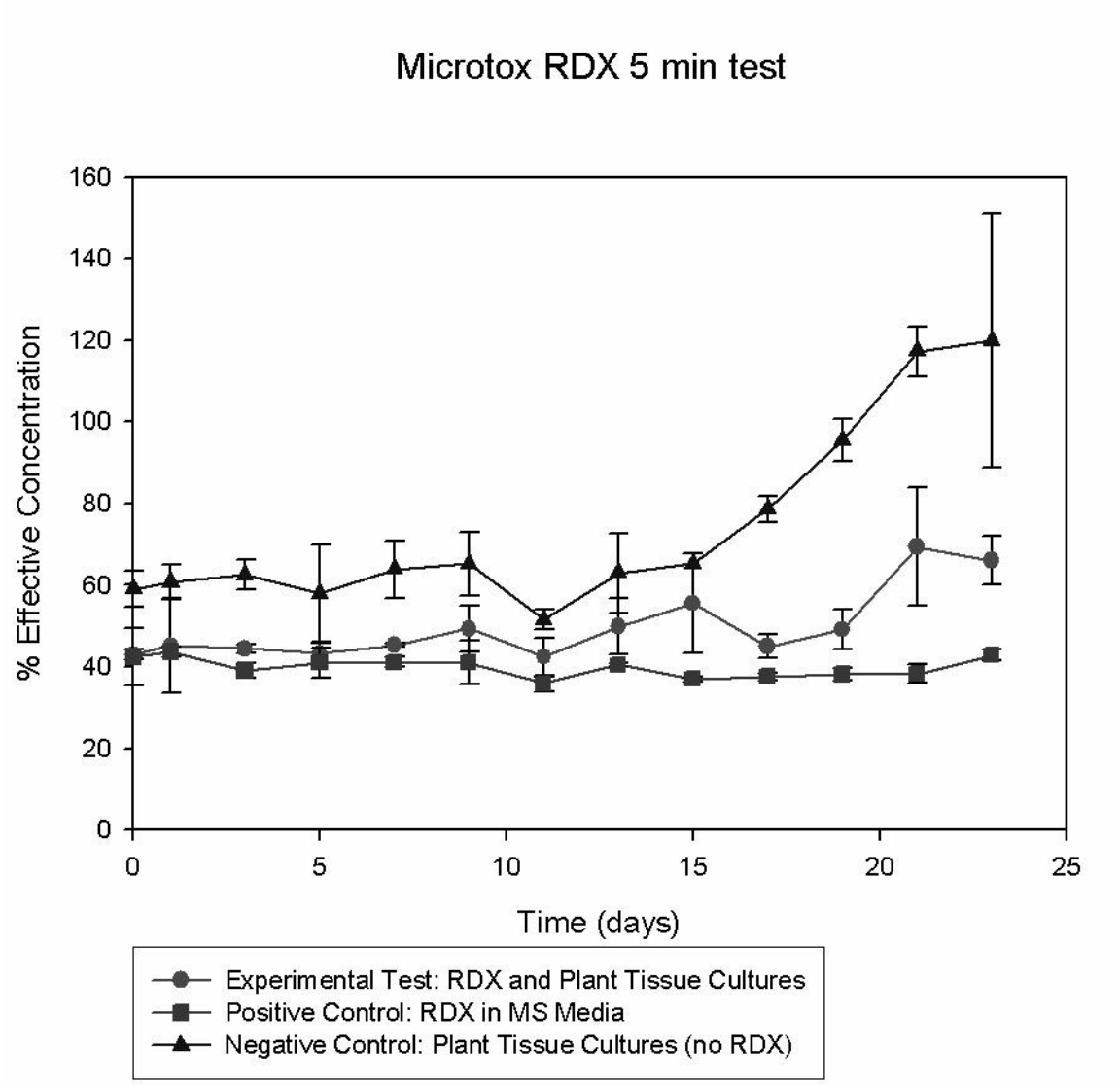

Figure 3-4 Microtox® 5-min test EC_{50} percent concentrations over the duration of the experiment beginning with 20 mg RDX L^{-1} in solution with poplar tree plant tissue cultures in sterile 250 mL flasks.

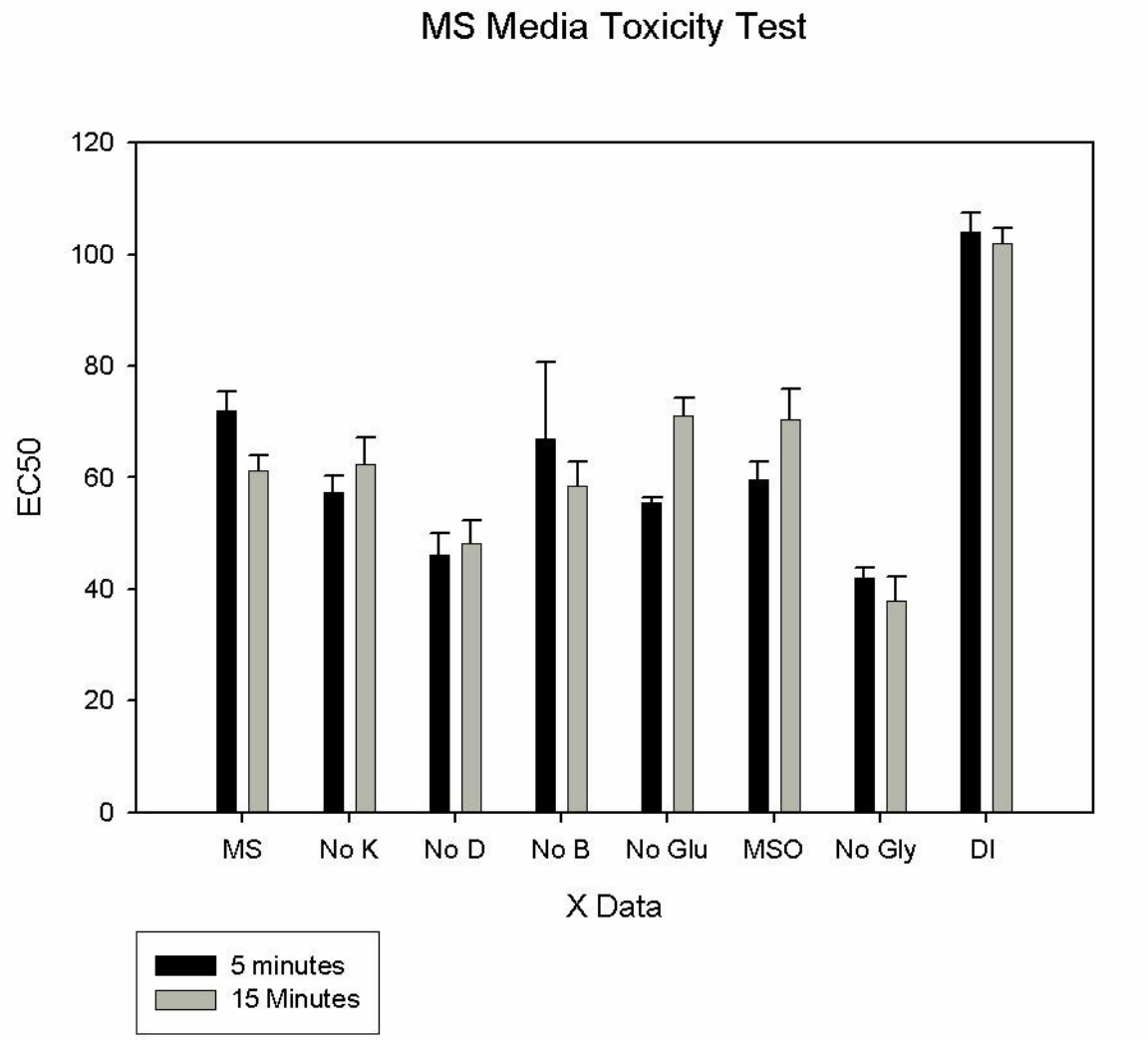

Figure 3-5 Microtox® EC_{50} of various growth solutions constituents at both the 5- and 15-min acute toxicity test times. MS, Murashige and Skoog medium with everything added; K, kinetin; D, 2-4, D; B, benomyl; Glu, glucose; MSO, MS medium only (no additions); Gly, glycine; and DI, de-ionized water.

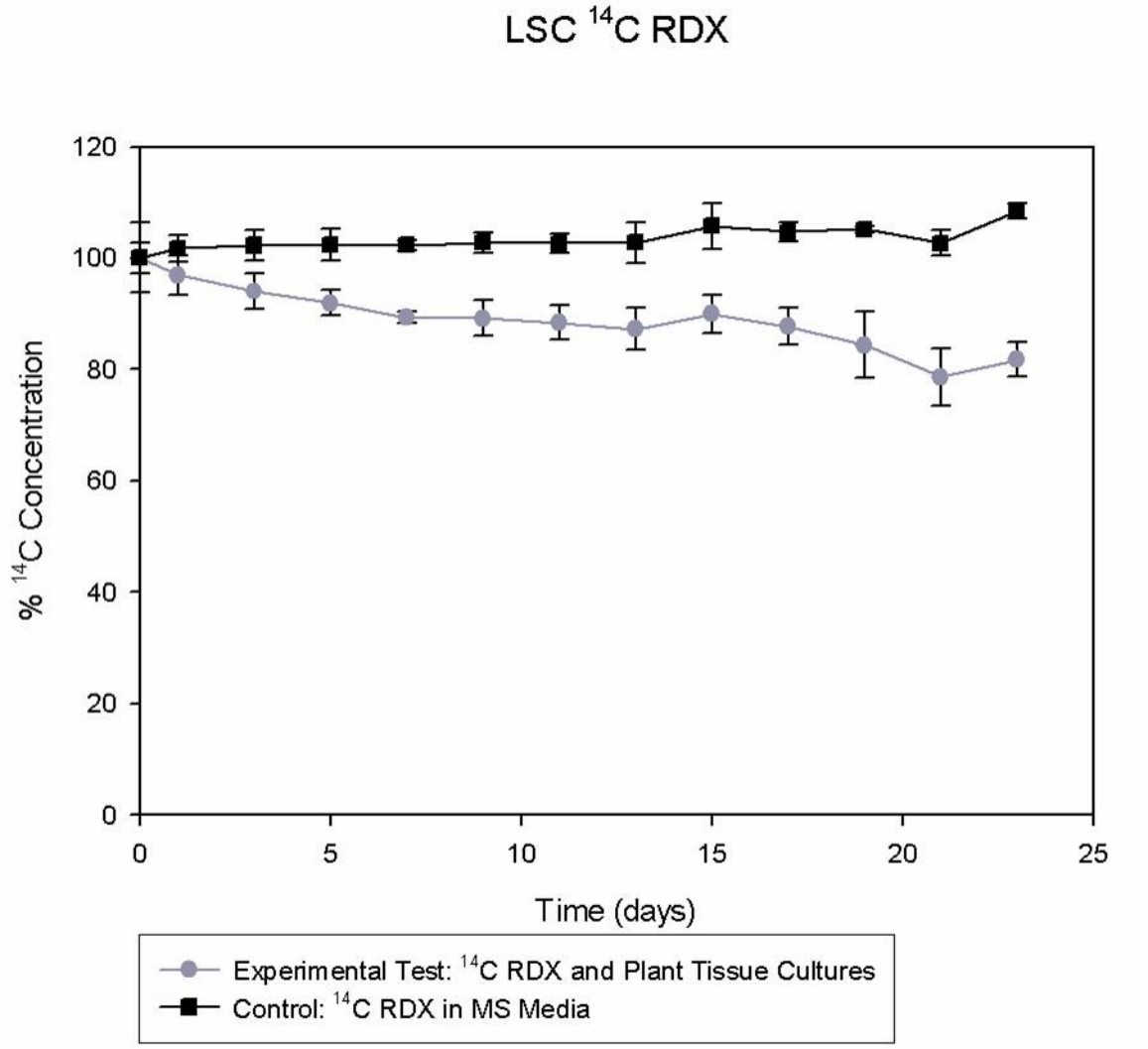

Figure 3-6 LSC results from experimental and control samples for the duration of the experiment. Concentrations of RDX are expressed as a percentage of the initial RDX radioactivity.

CHAPTER IV

POPLAR TREE PHYTOTOXICITY AND GENETIC RESPONSES[2]

4.1 Introduction and Background

The nitro-substituted explosive 2,4,6-trinitrotoluene (TNT) poses a significant contamination problem on military bases and testing and training ranges world-wide. TNT has been produced and used in large quantities for over 100 years. Many sites are in need of extensive treatment and remediation due to contamination of soils and groundwater which threatens terrestrial and aquatic biota. The US Environmental Protection Agency (EPA) estimates at least 30 munitions sites across the United States are currently contaminated with explosives (EPA530-F-97-045). TNT is also considered a priority pollutant by the EPA.

Trinitrotoluene is toxic to bacteria, unicellular algae, midge, amphipods, benthic invertebrates, earthworms, salamanders, rats, dogs, birds, and other wildlife (Burton *et al.*, 1994; Dodard *et al.*, 1999; Frische, 2002; Gong *et al.*, 2001; Steevens *et al.*, 2002; Sunahara *et al.*, 1998; Tadros *et al.*, 2000; Won *et al.*, 1976). Phytotoxicity of lettuce, barley, *Arabidopsis* and *Populus* species have been reported, as well as the adverse effects on seed germination and sprouting of both turnips and cress (Thompson, 1997; Gong *et al.*, 1999; Robidoux *et al.*, 2003).

Phytoremediation research involving the hybrid poplar tree *Populus deltoides x nigra* DN34, and *Arabidopsis thaliana* has been conducted and indicate that both these species can take up and transform nitro-substituted explosives (Ekman *et al.*, 2003; Mentewab *et al.*, 2005; Ekman *et al.*, 2005; Tanaka *et al.*, 2007; Gandia-Herrero *et al.*, 2008).

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[2] This chapter is currently under review by the journal *Environmental Pollution*.

Gene expression studies concerning phytotoxicity and plant responses to TNT have primarily been focused on *Arabidopsis*, since this genome is well documented and annotated (Mentewab *et al.*, 2005). Due to the numerous similarities between the *Populus* genome and that of *Arabidopsis*, comparisons and extrapolations between *Arabidopsis* genetic responses and those of poplar trees have been predicted (Tanaka *et al.*, 2007; Brentner *et al*., 2008). In 2006, Affymetrix® released the *Populus* genome on a GeneChip® which allows direct genetic experimentation on a standardized platform. Though the annotation process of the *Populus* genome is still far behind that of *Arabidopsis*, an initial study to determine genetic responses of poplars to TNT is possible. Using the Joint Genome Institute (JGI) annotation website and the Affymetrix® online genetic libraries, this research endeavors to understand global genetic responses of the *Populus* genome when exposed to TNT and compare these results with previously reported gene expression in *Arabidopsis*.

4.2. Methods:

4.2.1 MIAME for Affymetrix® Experiments

Minimum Information About a Microarray Experiment (MIAME) guidelines were consulted with respect to Affymetrix® designed experiments. MIAME reporting is primarily geared towards spotted arrays and not the manufactured arrays used in this experiment. However, we have used the MIAME guidelines as applicable.

4.2.2 Plant Growth and Treatment

Poplar tree cuttings (*Populus deltoides x nigra* DN-34) were obtained from Segal Ranch Greenhouse (Grandview, WA) and kept in a 4°C cooler to maintain dormancy until needed. Cuttings were grown in a modified Hoagland's solution under a 16 hour

light and 8 hour dark diurnal cycle. The growth solution was replaced twice a week for approximately eight weeks when plant shoots and roots reached sufficient size for experimentation.

Similar sized cuttings with extensive roots were transferred to foil wrapped 500 mL flasks with modified Hoagland's Solution. Treatment flasks contained Hoagland's with a concentration of 5 mg TNT L^{-1} ($2.2x10^{-5}$ M) dissolved in acetonitrile (Sigma-St. Louis), and negative control flasks contained the Hoagland's solution and acetonitrile. Three control plants were sacrificed at time zero with triplicate plants sacrificed at 8, 24, and 48 hours after exposure to TNT. These sampling times were pre-determined in the lab by several experiments specifically conducted by myself to find the optimum sampling times for the course of exposure in this system. The optimal sampling times were found to be at 8 hours, when TNT had just begun to be removed from the aqueous solution by plant uptake, at 24 hours, when approximately half of the initial concentration remained in the aqueous solution and half was metabolized, and at 48 hours, when all of the original TNT concentration had been taken-up and metabolized from the aqueous solution. These sampling times have since been confirmed by a similar time-course experiment duplicated in the same lab setting (Brenter *et al*., 2008).

4.2.3 RNA Extraction and Microarray Analysis

RNA extraction took place under a sterile laminar-flow hood specifically used for this purpose. All instruments, tubes, surfaces and containers and gloves were sprayed or wiped down with RNase *AWAY*™ (Invitrogen). All plant cuttings chosen for extraction were taken from solution and the roots were immediately and fully immersed in liquid nitrogen. The frozen roots were then broken-off into a mortar containing liquid nitrogen and ground with a pestle. There were no pooled root samples, each plant's roots were individually sampled and RNA extracted. Approximately 150 mg of crushed and frozen

root material from each cutting was then placed in a tube containing 450 μL of RNAlater®(Ambion) and vortexed to allow the stabilizer to permeate the entire sample. Small (1.0 mm) zirconia/silicon beads were added and the tubes were shaken on a bead beater for maximum cellular disruption. Total RNA extraction was completed using the RNeasy® Plant Mini Kits from Qiagen and included the DNase treatment (Appendix B).

The *Populus* genome microarrays were purchased from Affymetrix® through the University of Iowa DNA Facility. The array design is a synthesized oligonucleotide array on a glass plate. Extracted RNA concentration and quality was determined using the Agilent Bioanalyzer® at the DNA Facility. Samples met the facility guidelines of an absorbance $A_{260/A280}$ ratio between 1.9 and 2.1 and a minimum of 10 μg of total RNA mass. Gel analysis showed distinctive 28S rDNA and 18S rDNA peaks with little to no degradation. Hybridization of the samples to the GeneChips® was done at the DNA facility using a model 250 GeneChip® Fluidics Station by the DNA facility staff.

4.2.4 Data Analysis:

Affymetrix® microarray files were uploaded and analyzed using ArrayAssist® and a time course package "expression and analysis of differential gene expression (EDGE)" designed to run on the R statistical program platform (Leek *et al.*, 2006; Storey *et al.*, 2005). Standard Affymetrix® probe level analyses and quality control checks for the GeneChips® were performed as part of the GeneChip® Fluidics Station protocols and no abnormalities were noted. Data was made available through the a specialized ArrayAssist® program available through the University of Iowa DNA facility. Files were also transferred to the Affymetrix® website for identification and additional information. Using ArrayAssist® microarray protocols, the data was transformed using Probe Logarithmic Intensity Error (PLIER) method and standard log transformations. Data was subjected to the Student's t-test and normalized over the four time points and

among the triplicate samples to the probe control responses. Triplicate columns were combined and means and standard deviations were calculated.

Statistically significant differences between the controls and three treatments at different sampling times were reported when a greater than (or less than) 2-fold change and a P-value of less than 0.05 was observed. Approximately 2305 genes met these criteria using the ArrayAssist® program. The R "EDGE" program (specifically designed for time-course experiments) analysis resulted in 9327 statistically significant genes. An overlap analysis was performed that resulted in 1443 genes over the entire time-course experiment, which were identified as significant in both statistical programs. An individual analysis of these genes was subsequently undertaken.

4.3. Results and Discussion:

Plant uptake and metabolic transformation of xenobiotic compounds involves three main phases (Sandermann, 1994; Coleman, 1997; Madhou *et al.*, 2006). Phase one involves the transformation of the parent compound into a more soluble and typically less toxic daughter product. In phase two, conjugation begins with an increase in metabolic activity that is catalyzed by glycosyl-, malonyl-, and glutathione *S*-transferases. In the final phase, the conjugated compound is transported into the vacuole or cell wall from the cytosol via ATP-binding, ABC transporters and multi-drug resistant proteins for sequestration or compartmentation . This study incorporates these phases as an outline to understand the genetic responses of hybrid poplar to trinitrotoluene (TNT).

4.3.1 Phase 1: Transformation

In phase one, TNT can be degraded by various enzymes including nitroreductases, monooxygenases, and, more specifically, cytochrome P450's. Previous *Arabidopsis* research has focused primarily on the cytochrome P450's, but it has also

included several unknown nitroreductases and other monooxygenases implicated in this phase of transformation (Madhou *et al.*, 2006; Rylott *et al.*, 2006). Over the 48 hours of this study, cytochrome P450's were the primary gene superfamily upregulated with respect to phase one. As indicated in Table 4-1 and Figure 4-1, the expression trend of these genes was noticeable beginning at 8 hours, and it peaked around 24 hours, typically declining in expression levels by 48 hours. The range of expression was between 2-fold and 4-fold greater than the unexposed control cuttings for those same times. This result is consistent with previous findings that poplar tree cuttings take up TNT quickly, usually within 10 hours, and immediately begin transformation (Brentner *et al.*, 2008).

During the transformation of the nitro groups, cupin was also significantly upregulated, ranging between 2-fold and 5-fold upregulation in gene expression (Table 4-2). Cupin is a nutrient reservoir for nitrogen, which would increase in concentration as transformation continued (JGI). Not much has been discussed in *Arabidopsis* studies with respect to cupin, but we found it to be one of our most significantly upregulated genes. Cupin and nitrogen reservoir upregulation accounted for around 11% of the identifiable significantly upregulated genes. This response could be important in determining new pathways for the transformation and nutrient uptake of nitro-substituted xenobiotics.

4.3.2 Phase 2: Conjugation

In phase two, metabolic activity tends to increase as metabolites are deactivated by conjugation (Madhou, *et al.*, 2005). This deactivation can be catalyzed by glycosyl-, malonyl-, and glutathione *S*-transferases. The most common of these transferases, glutathione *S*-transferases (GSTs), has been reported in several TNT related studies (Tanaka, *et al.*, 2007; Yoon, *et al.*, 2007; Brentner, *et al.*, 2008; Gandia-Herrero, *et al.*, 2008) Some recent studies have also reported glycosyl- and glucosyl-transferase activity

as a part of phase two conjugation (Weis *et al.*, 2006). This study also found upregualtion of GST, glycosyl- and glucosyl-transfereases, but no significant malonyl-transferase upregulation. These results are reported in Figures 4-2, 4-3, 4-4 and Table 4-3.

GST and glycosyltransferase both followed similar patterns to the cytochrome P450's over the course of this experiment. Results indicated upregulation in the range of 2-5 fold from the poplar controls, and they reached their peak expression at approximately 24 hours. Glucosyltransferases, however, did not follow the same trend (Figure 4-4). Their average expression indicated a steady increase with a possible tapering-off just past 24 hours. Some of the genes began to return to a lower level of expression during the time course, while others maintained a high expression. This would be consistent with phase two conjugation following a slight time lag after phase one transformation by P450s.

4.3.3 Phase 3: Transport and Sequestration

Phase three involved the transport of the conjugated TNT products from the cytosol into the vacuole or cell wall by transport proteins. This transport is typically marked by an increase in ATP-binding/ABC transporter activity and multi-drug resistance (MDR) proteins (Madhou, *et al.*, 2005). For our study, we found all of these genes were significantly expressed in the 24-48 hour time periods (Table 4-4). No significant expression was observed at the 8-hour time period, and very little upregulation was noted at the 24 hour sampling time. Most of the significant upregulation occurred between 24 and 48 hours and corresponded to the gene superfamily of protein transporters. In addition to the ATP, ABC and MDRs, many other transporter proteins were also upregulated at the 48 hour sampling time.

4.3.4 Other Upregulated Genes

Additional genes groups that were induced at greater than 2-fold expression values are shown in Figure 4-5. Metabolic processes accounted for 30% of the significantly upregulated genes in this experiment. Previously discussed genes involved in the three main phases are located in the "other" expression group that makes up about 24% of the significantly expressed genes. Standard categories of biosynthesis, transport, and binding account for the rest of the genes significantly expressed compared to the control plants.

4.3.5 Downregulated Genes

In order to develop a more complete, global understanding of the poplar tree's response to TNT, we also looked at those genes that were significantly downregulated during the 48 hours of this study. Some of these genes are of interest, as they play contrary roles in the processes previously discussed for phases 1-3. Other downregulated genes from this study have previously been reported to be significant in the areas relating to energy regulation, respiration, transcription, translation, and growth. Of the 286 significantly downregulated genes, only 14% were downregulated at the 8-hour sampling time. This doubled to approximately 30% at the 24-hour sampling time. At the 48-hour sampling time, we saw the largest combination of gene repression comprising 82% of the significantly downregulated genes. Whereas gene expression of up-regulated genes peaked primarily in the 24 to 48 hour sampling range, the downregulation of genes did not exhibit an overall peak time. As downregulation is often a response to other factors, these genes may point to lasting inhibition or toxic effects of TNT to *Populus*.

One pattern of significant downregulation can be correlated to the process of respiration. As the metabolism of the plant increased to compensate for the phase two

conjugation of the TNT metabolites, downregulation of respiration-related genes became obvious. Of the 286 downregulated genes, 73 were respiration process related. Initially, we noticed that several phosphofructokinases (PFKs) had been downregulated from the controls ranging between -2.9 and -5.7-fold changes. The 8 and 24-hours sampling times revealed about a -3.2 fold decrease in PFK expression. At 48 hours this had changed to an average -5.5-fold change in response from the controls. This reduction of PFKs during an increase in metabolism has been shown to occur in previous sugarcane plant studies, but has not been reported in either *Arabidopis* or *Populus* studies (Groenewald & Botha, 2007). PFKs are considered to be an important part of the rate-limiting step of glycolytic flux during periods of increased metabolic activity (Winkler *et al.*, 2007). PFKs and other pyrophosphates relate directly to glycolysis, which is an integral part of cellular respiration.

This pattern of significant downregulation in respiration related genes continued with key genes involved in pathways leading to the citric acid cycle. These include pyruvate kinases, hexokinases, phyrophosphorylases, fatty acid desaturases, acetyl co-A enzymes and aspartate kinase. The most commonly downregulated respiration related activity in this study was the protein-tyrosine kinase activity and protein serine/threonine kinase activity. Protein-tyrosine is an intermediate on the shikimate pathway of phosphorylation and plays a key role in plant metabolism. Peroxidases were also downregulated, Table 4-5. These were previously reported to be important with respect to lignin content and composition in hybrid aspen trees as well as a response to oxidative stress (Li *et al.*, 2003).

Significant downregulation may imply that the cell was over stimulated by this gene family for a period of time and the expression of that receptor protein was decreased to protect the cell. This downregulation pattern is likely indicative of an increase in metabolic activity due to the plant's phytotoxic response to stress. This is supported by the overall percentage of downregulation relating to both respiration and the more

specific pathways of shikimatic acid and glycolysis in response to the conjugation of the xenobiotic in the cell.

During phase three, transport proteins carry large conjugated organic molecules into the vacuole for sequestration. As this transportation occurs, pumps extrude calcium ions into the cytosol as a balancing mechanism (Taiz & Zeiger, 2002). When there is an abundance of these ions in the cell, downregulation begins in order to maintain homeostasis (Nagata et al, 2004; Taiz & Zeiger, 2002). This research found eleven genes encoding for calcium ion binding that were significantly downregulated at the 48 hour sampling time. This information supports the hypothesis that TNT is being sequestered in the cell wall during phase three.

Another pattern of downregulation in the area of transcription, RNA processing, translation and posttranslation was also identified. Thirty-seven total genes encoding for methylation, targeting endoplasmic reticulum (ER) and DNA binding, including helix-turn-helix, zinc fingers, helix-loop-helix, leucine zippers and basic zippers, were significantly downregulated (Figure 4-6). These are commonly downregulated genes and support the consistency of this research to previous findings in this area.

In addition to these observable patterns, other genes were significantly repressed. This study found a large number of heat shock proteins were downregulated in our samples. This is common for toxic responses in plants and serves only to confirm that our results are consistent with previously observed phytotoxic response mechanisms. Heat shock proteins reflect the general toxicity of TNT to Populus, but they are likely not involved in the actual transformation and detoxification of TNT.

4.4 Conclusions

In this report, we have identified several new genes that were expressed in the detoxification and metabolism of TNT by *Populus*. In particular, our results support the

"green liver" model of different gene families being expressed during the time course experiments demonstrating successive Phase I transformation, Phase II conjugation, and Phase III compartmentation processes. Many of the genes identified in this study were related to those significantly expressed in previous *Arabidopsis* studies, indicating the similarity between these plants. However, genes potentially involved in the uptake and transformation of TNT in hybrid poplar trees were identified here. These genes included the significantly upregulated cupin, glucosyltransferases, and protein transport families. Also we observed several interesting downregulation patterns in the areas of respiration, citric acid cycle, shikimatic pathway, and toxic responses. This is the first report of genes cupin, phosphofructokinases, and glucosyltransferases, being implicated in the detoxification and metabolism of TNT by *Populus*.

Affymetrix Probe ID	8 hours	24 hours	48 hours	Molecular & Biological Functions	Gene type
PtpAffx.132618.1.S1_at		3.163	2.550	monooxygenase activity; electron transport	Cytochrome P450
PtpAffx.138857.1.A1_at	3.311	3.750	3.134		
PtpAffx.151850.1.A1_at		2.452	2.130		
PtpAffx.222544.1.S1_at		2.426	3.284		
PtpAffx.225096.1.S1_s_at			2.051		
PtpAffx.33300.1.A1_at	2.251	3.361	3.270		
PtpAffx.35496.1.A1_at		2.565	2.372		
PtpAffx.90126.1.S1_at	2.188	3.105	3.109		

Table 4-1 List of Phase 1 Cytochrome P450 genes (by Affymetrix ID) upregulated at 8, 24, and 48 hours after exposure to TNT.

Affymetrix Probe ID	8 hours	24 hours	48 hours	Molecular & Biological Functions	Gene type
PtpAffx.211043.1.S1_at	2.544	2.926	2.618	nutrient reservoir activity	Cupin
PtpAffx.211091.1.S1_s_at	4.051	5.126	5.628		
PtpAffx.217785.1.S1_at			2.014		
PtpAffx.3487.1.S1_at			2.595		

Table 4-2 Upregulation of Cupin during Phase 1

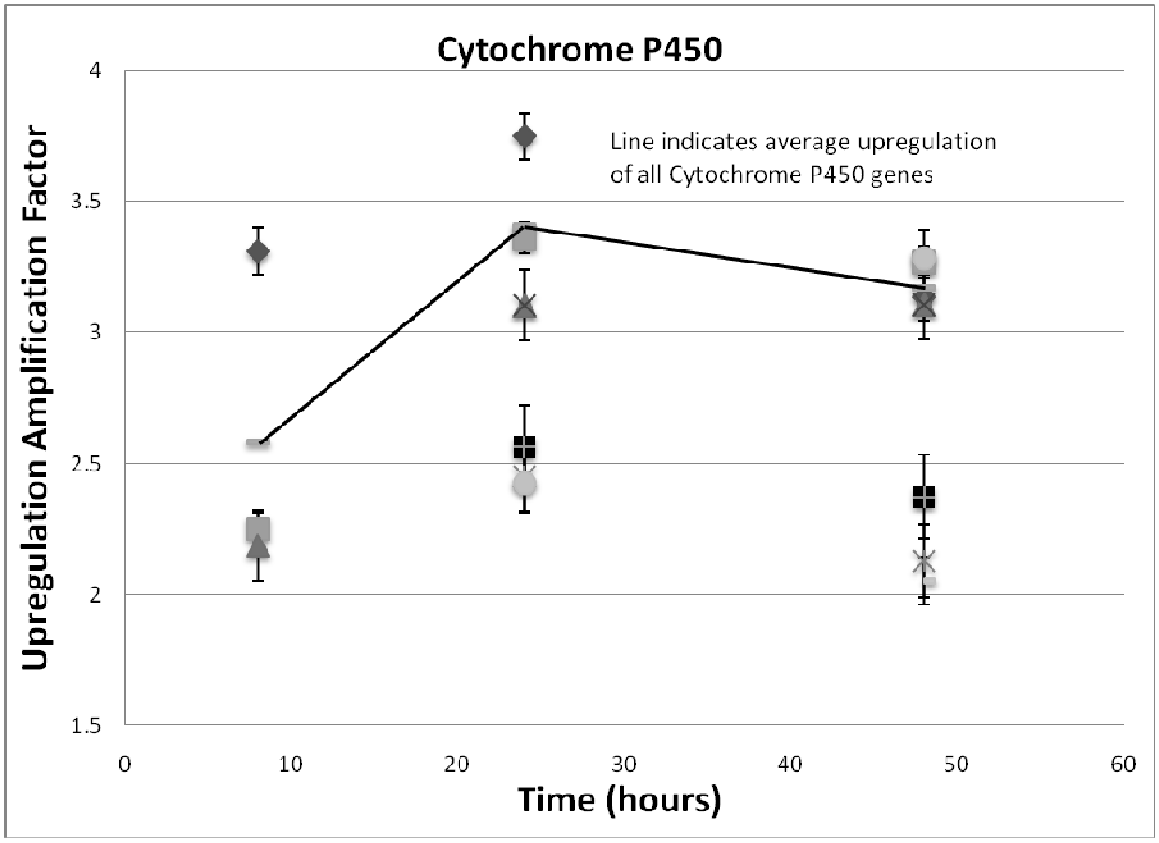

Figure 4-1 Upregulation of Cytochrome P450s at 8, 24, and 48 hours. The average of all CytP450 genes is plotted as a line and standard deviation error bars are shown. Cytochrome P450 genese are identified by Affymetrix® ID in Table 4-1

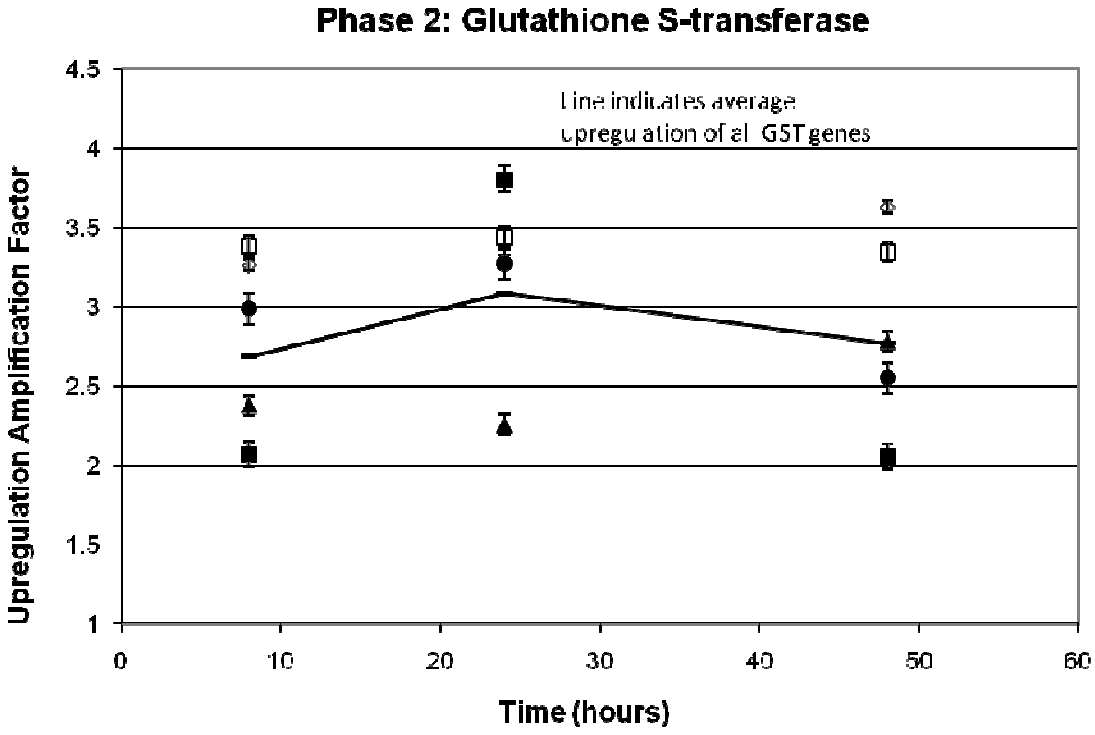

Figure 4-2 Phase 2 glutathione S-transferase (GST) upregulation over time, with standard deviation error bars.

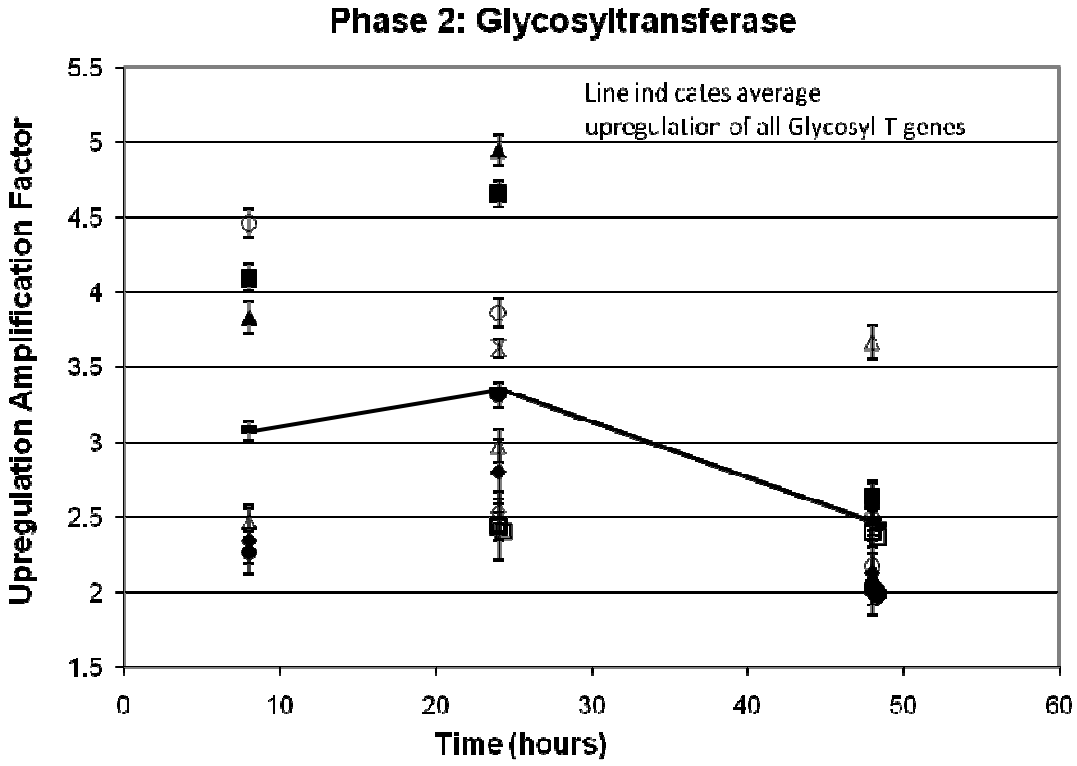

Figure 4-3 Phase 2 glycosyltransferase upregulation over time, with standard deviation error bars.

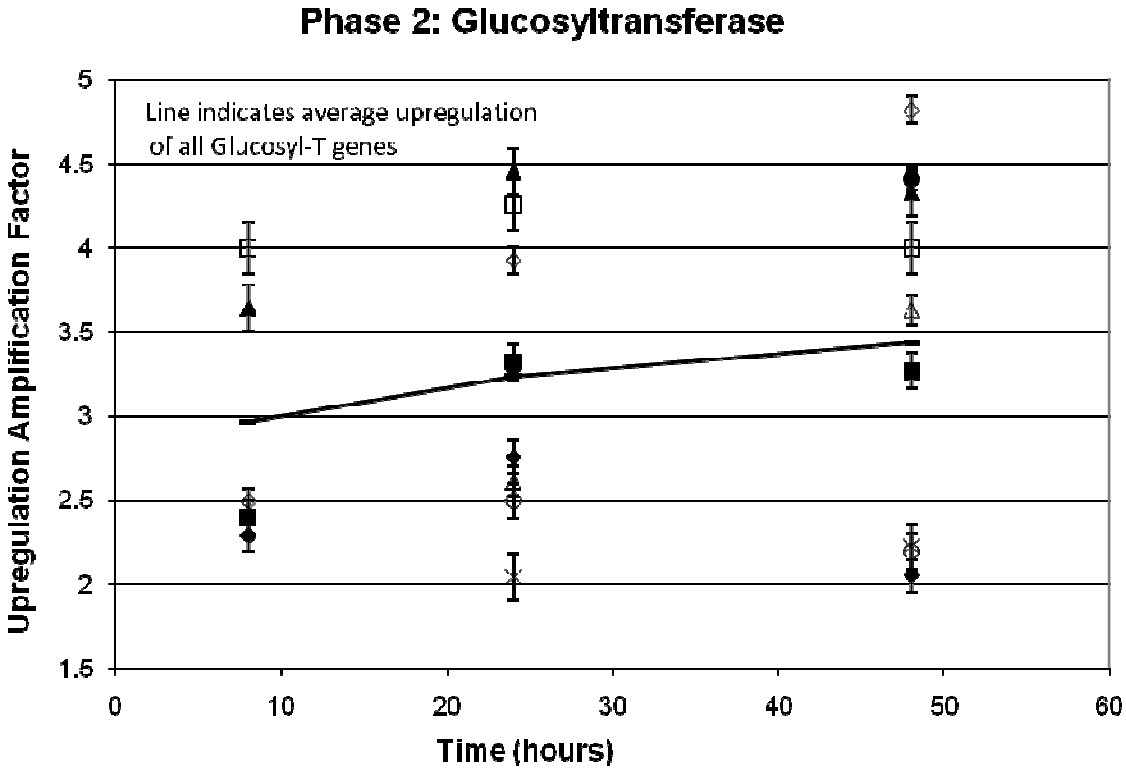

Figure 4-4 Phase 2 glucosyltransferase upregulation over time, with standard deviation error bars.

Affymetrix Probe ID	8 hours	24 hours	48 hours	Molecular & Biological Functions	Gene type
Ptp.5289.1.S1_s_at	2.03	2.43	2.28	metabolism	glutathione S-transferase (GST)
PtpAffx.224201.1.S1_at	2.07	3.8	2.06		
PtpAffx.224631.1.S1_s_at	2.38	2.26	2.78		
PtpAffx.4246.2.S1_a_at	3.38	3.44	3.34		
PtpAffx.43231.1.A1_at	3.26	3.29	3.63		
Ptp.3709.1.S1_at	2.99	3.27	2.55		
PtpAffx.203138.1.S1_at			2.49	hydrolase activity, hydrolyzing O-glycosyl compounds	glycosyl transferase
PtpAffx.223848.1.S1_at			2.03		
Ptp.3522.1.S1_s_at			2		
Ptp.6729.1.S1_at		2.44	2.4		
PtpAffx.127059.1.A1_at	2.47	2.97	3.66		
PtpAffx.200185.1.S1_s_at	2.27	3.31	2.58		
PtpAffx.209038.1.S1_s_at	2.26	2.54	2.52		
PtpAffx.217237.1.S1_at	4.46	3.86	2.17		
PtpAffx.221654.1.S1_s_at		2.42	2.05		
PtpAffx.224410.1.S1_at	2.34	2.8	2.13		
Ptp.4506.1.S1_s_at	4.09	4.66	2.64		
PtpAffx.32356.3.S1_at	3.83	4.95	2.64		
PtpAffx.83041.1.A1_s_at	3.07	3.62	2.56		
Ptp.6958.1.S1_s_at	2.29	2.76	2.05	metabolism	UDP-glucuronosyl/UDP-glucosyltransferase
PtpAffx.139063.1.S1_at	2.4	3.32	3.27		
PtpAffx.215169.1.S1_s_at	3.64	4.45	4.32		
PtpAffx.216568.1.S1_s_at	2.49	3.93	4.82		
PtpAffx.224189.1.S1_at	4	4.26	4		
PtpAffx.22666.1.A1_s_at		3.3	4.41		
PtpAffx.2665.1.S1_at		2.5	2.19		
PtpAffx.31211.1.A1_at		2.04	2.22		
PtpAffx.23657.1.S1_s_at	2.05	2.47	3.59		
PtpAffx.23657.2.S1_s_at	2.02	2.56	3.74		
PtpAffx.158231.1.A1_at	2.26	2.78	2.55		

Table 4-3 List of Phase 2 gene upregulation of glutathione S-transferases, glycosyl- and glucosyltransferases.

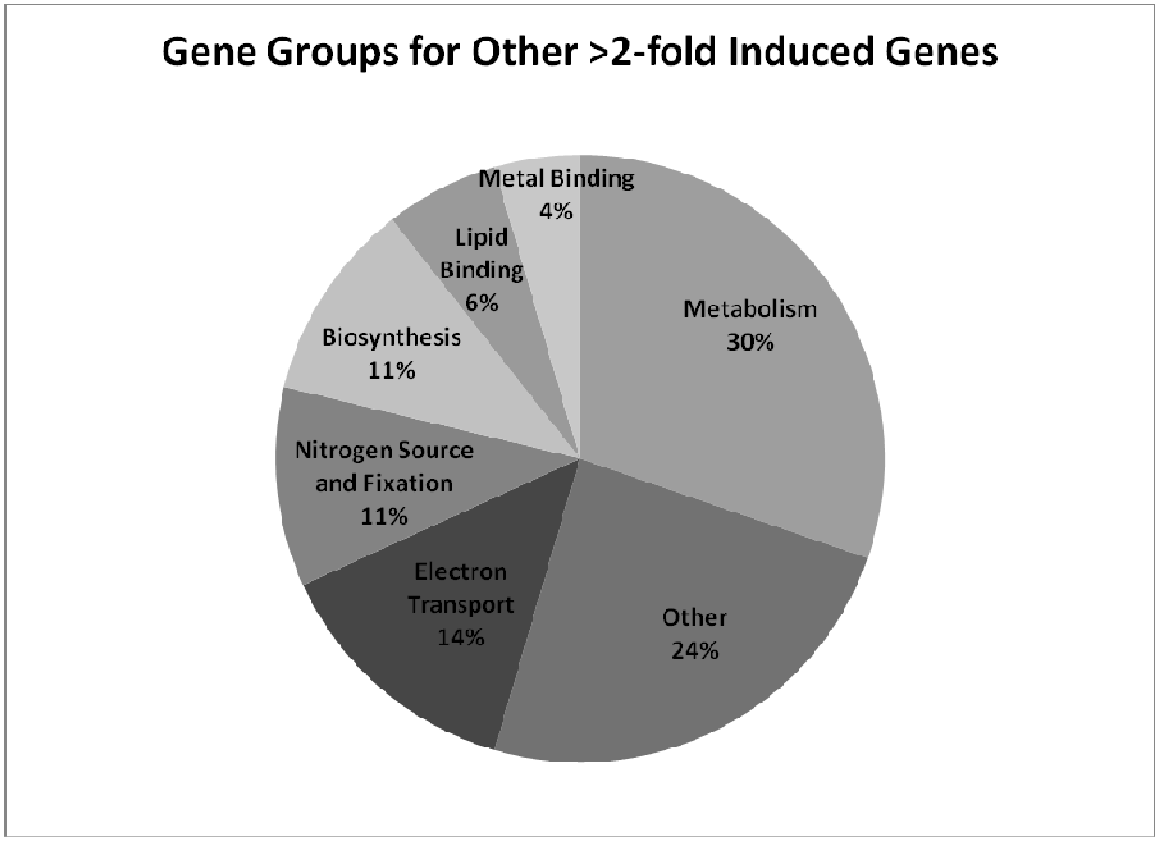

Figure 4-5 Chart of gene categories for other significantly upregulated genes over the course of 48 hours when exposed to TNT.

Affymetrix Probe ID	8 hours	24 hours	48 hours	Molecular & Biological Functions	Gene type
PtpAffx.208904.1.S1_at		2.24	4.07	antiporter activity; membrane	multidrug transporters
PtpAffx.208905.1.S1_at			3.88		
PtpAffx.213048.1.S1_at			2.58		
PtpAffx.212897.1.S1_s_at			2.28	ATP-binding cassette; ABC transporter activity	membrane transport; ABC transporter
PtpAffx.214961.1.S1_s_at		2.4	2.39		
PtpAffx.225122.1.S1_s_at			3.37		
PtpAffx.225531.1.S1_s_at			2.01		
PtpAffx.225717.1.S1_s_at			2.27		
Ptp.2887.1.S1_at			4.1		
PtpAffx.114367.1.A1_at			2.56		
PtpAffx.141628.1.S1_at			3.81		
PtpAffx.202418.1.S1_at		2.22	2.69		
PtpAffx.218920.1.S1_s_at			3.39		
PtpAffx.22198.1.S1_at			3.35		
PtpAffx.223705.1.S1_x_at			4.21		
PtpAffx.61049.1.S1_at			3.09		
PtpAffx.87677.1.S1_at			2.94		
Ptp.7558.1.A1_at		2.01	2.32	transporter activity; membrane	Adenine nucleotide translocator 1
PtpAffx.14146.1.A1_s_at		2.01	2.45		
PtpAffx.159072.1.S1_at			2.76		
PtpAffx.83667.1.A1_at			2.95		
PtpAffx.204923.1.S1_at			2.2	oligopeptide transport; membrane	TGF-beta receptor, type I/II extracellular region
PtpAffx.204923.1.S1_x_at			2.03		
PtpAffx.216706.1.S1_at	2.22	3.21	3.98		
PtpAffx.216706.1.S1_x_at	2.2	3.16	3.92		
PtpAffx.75314.1.A1_at			2.31		
PtpAffx.221953.1.S1_at			2.17	transporter activity; membrane	plasma membrane intrinsic protein
PtpAffx.221953.1.S1_s_at			2.43		
Ptp.4477.1.S1_at			2.21		
Ptp.1224.1.S1_s_at			2.27		Lipocalin-related protein

Table 4-4 List of Phase 3 upregulated genes.

Affymetrix Probe ID	8 hours	24 hours	48 hours	Molecular & Biological Functions	Gene type
Ptp.719.1.S1_at			-2.180	peroxidase activity	response to oxidative stress
PtpAffx.10355.2.S1_at	-4.430	-4.097	-5.207		
PtpAffx.16117.1.A1_a_at		-2.311			
PtpAffx.18226.1.A1_a_at			-2.335		
PtpAffx.211356.1.S1_at	-2.562	-3.294	-3.301		
PtpAffx.213709.1.S1_at		-2.862	-2.845		
PtpAffx.216844.1.S1_at			-2.417		
PtpAffx.224549.1.S1_s_at			-2.034		
PtpAffx.225157.1.S1_at			-2.533		
PtpAffx.5.2.S1_a_at	-2.567	-3.320			

Table 4-5 List of downregulated genes in response to oxidative stress.

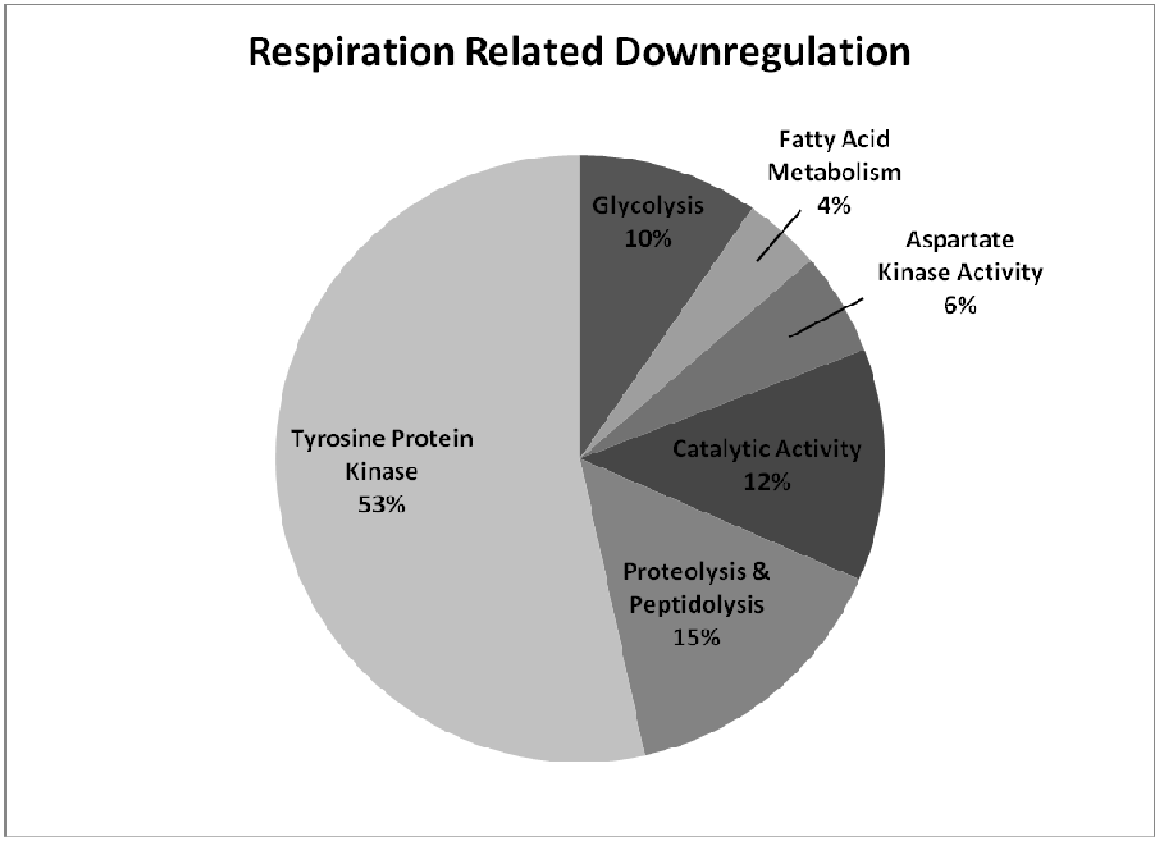

Figure 4-6 Chart of gene functions of 73 respiration related downregulated genes.

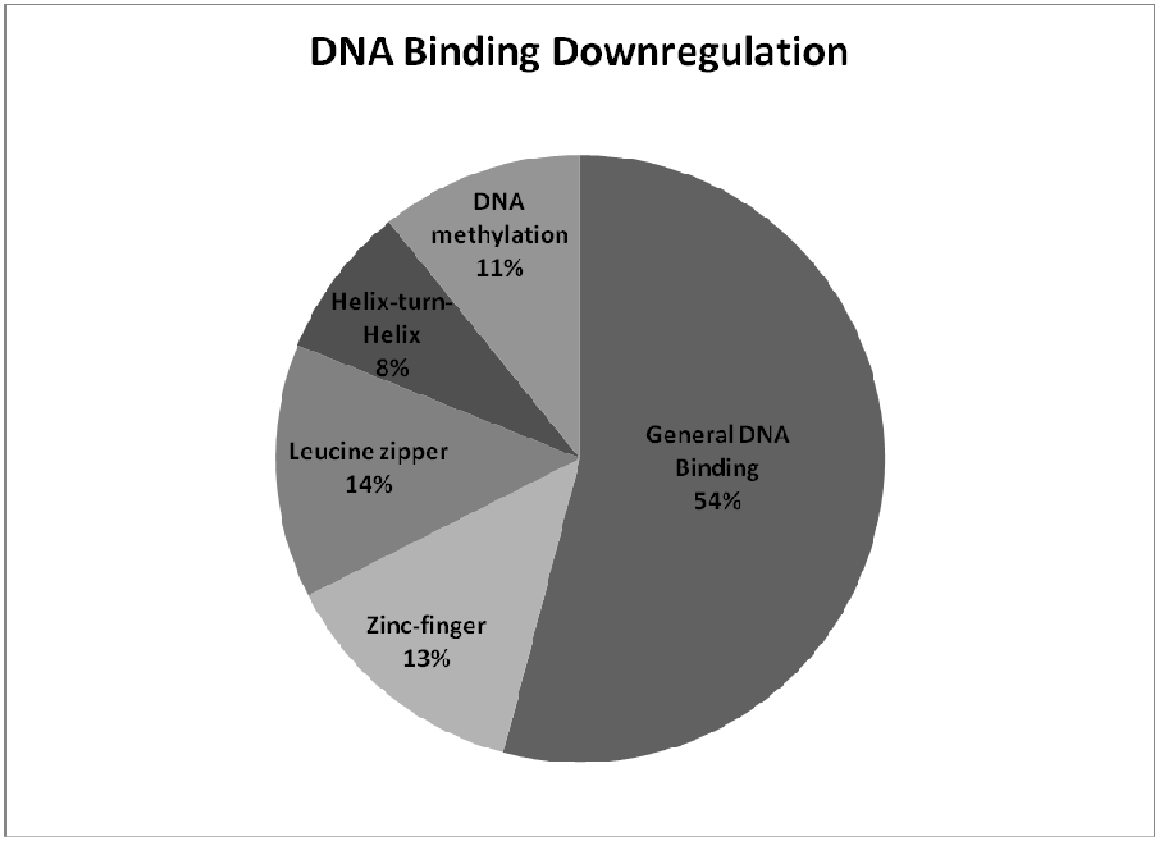

Figure 4-7 Chart of gene functions of 37 DNA Binding related downregulated genes.

CHAPTER V

CONCLUSIONS

Phytoremediation of nitro-substituted explosives at military and other sites around the world has been of great interest. Regulatory and government agencies need simple, repeatable methods to determine if the phytoremediation scheme is removing residual toxicity from the application site. Laboratory researchers also need access to phytoremediation results in a timely manner. This research studied the viability of using plant tissue cultures as models for the whole plant system and, in conjunction, determined if the Microtox® toxicity platform could be used to determine toxicity reduction in aqueous environments. Specific conclusions for the poplar tree plant tissue cultures that can be drawn from this research are as follows:

1) Poplar (*Populus deltoides x nigra* DN34) tissue cultures removed 2,4,6-trinitrotoluene (TNT) from an aqueous solution in five days. High Performance Liquid Chromatography (HPLC) measurements confirmed removal of TNT from the solutions containing poplar plant tissue cultures and showed no change in the positive, plant-free, controls.

2) The toxicity of the aqueous solutions containing TNT was reduced from a highly toxic Microtox® EC value to that of the negative control by day 15.

3) Uniformly ring-labeled ^{14}C-TNT was used to confirm the removal of TNT from the hydroponic solutions containing plant tissue cultures and to verify that toxicity did not change in solutions where no plant cultures were present (positive controls). Liquid Scintillation (LSC) measurements confirmed 80% removal of TNT, by day 15, from the solutions containing poplar plant tissue cultures and showed at or near 100% of the initial concentration in the positive, plant-free, controls.

4)Poplar tissue cultures removed 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) more slowly than the TNT removal.

5) Toxicity reduction of the aqueous solution containing RDX was evident using the Microtox® method. There was a steady decrease in toxicity over 23 days, but the solutions never reached the same Microtox® EC value as the controls.

6) Radiolabeled RDX was used to confirm the removal of RDX from the hydroponic solutions containing plant tissue cultures. Liquid Scintillation (LSC) measurements confirmed around 20% removal of initial RDX from the solutions containing poplar plant tissue cultures and showed no change in the positive, plant-free, controls.

In summary, the measurement for the reduction of toxicity of both TNT and RDX was performed using a novel method developed specifically for use with the Microtox® testing system. This methodology, described in Chapter 3, is potentially very useful in confirming the removal of residual aqueous toxicity in plant-assisted remediation efforts.

This research also examined the global genetic responses of the Poplar tree when exposed to TNT. Understanding the phytotoxic responses of the plant to the chemicals may give us additional information on degradation pathways and what genes are significantly up- or down-regulated when exposed to TNT. Specific conclusions involving the genetic responses of hybrid poplars to TNT that can be drawn from this research are as follows:

1) Phase 1 (Transformation) gene upregulation was consistent with previous *Arabidopsis* studies and included significantly expressed cytochrome P450 genes.

2) In addition, this research determined that Cupin, a nutrient reservoir for nitrogen, was also significantly upregulated in Phase 1.

3) Phase 2 (Conjugation) gene upregulation was consistent with previous *Arabidopsis* studies and included significantly expressed glutathione S-transferase and glycosyl transferase

4) In addition, this research found significant upregulation of glucosyl transferase during Phase 2.

5) Phase 3 (Transport and Sequestration) global gene patterns were identified. These included ATP-binding/ABC transporter upregulation and other transporter proteins involved in Phase 3 transport.

6) Other global patterns of upregulation were identified in the areas of metabolic processes, electron transport, lipid binding and metal ion binding.

7) Downregulated global gene patterns were determined in response to Phase 2 upregulated genes. The most significant downregulated gene category related to respiration, the citric acid cycle and shikimate pathway of phosphorylation.

8) This research identified phosphofructokinases (PFKs) as a significantly downregulated gene possibly responding to Phase 2 conjugation of TNT metabolites.

9) Global downregulated gene patterns in response to Phase 3 upregulated genes included significant downregulation in the areas of transcription, RNA processing, translation and posttranslation.

10) This research identified Phase 3 downregulated calcium ion binding genes that may be a response to the transport proteins carrying the conjugated organic molecules into the vacuole, or cell wall, for sequestration.

In summary, this research has confirmed several genes that match previous Arabidopsis findings for both Phase 1 and Phase 2. These genes include cytochrome P450, glutathione s-transferase, and glycosyl-transferase. In addition to these previously reported genes, this research identified Cupin as a significantly upregulated Phase 1 gene and glucosyl-transferase as a significantly upregulated Phase 2 gene. It also identified global upregulation patterns in the areas of protein transport (Phase 3) and metabolic processes. This research explored the global downregulation of genes and found patterns in the areas of respiration, citric acid cycle, shikimatic pathway and toxic responses.

APPENDIX A: PLANT MEDIA PREPARATION

A-1. Plant Tissue Culture MS Media Information from Caisson Labs

MSP009 1 Liter packets contains:

mg/L	compound	catalog #
1650	Ammonium Nitrate	CN-A015
332.20	Calcium Chloride Anhydrous	CN-C021
37.26	Na2 EDTA	CN-E004
27.80	Ferrous Sulfate	CN-F006
180.70	Magnesium Sulfate	CN-M022
1900	Potassium Nitrate	CN-P012
170	Potassium Phosphate Monobasic	CN-P031
32.83	Trace Mineral Stock	CTM01
2.0	Glycine	CN-G008
100	myo-Inositol	CN-M014
0.50	Nicotinic Acid	CN-N004
0.50	Pyridoxine HCl	CN-P018
0.10	Thiamine HCl	CN-T009

Nodule Culture Medium (NCM)

(Benoit Van Aken, 2004)

For 1 L:

-Fill a 2-L conical flask with about 850 - 900 mL DI water

-Under stirring, add

-30 g sucrose

-1 MSP009 Packet

-0.5 mL kinetin (1 mg/mL in 0.1 N NaOH)

-2.5 mL 2,4-D (1 mg/mL in 0.1 N NaOH)

-2.5 mL benomyl (1 mg/mL in MeOH)

-Mix

-Adjust pH 5.7 – 5.8

-DI water to 1 L

-Place 400 mL in 500 mL flasks

-Plug flasks with foam plug and cover foam with aluminum (Al) foil

-Autoclave 30 min, 121 C

-Cool down under the laminar hood, remove the Al foil to allow the plug to dry

Inoculation

-Under laminar Hood (clean, ethanol sterilized, exposed to UV 15 – 20 min)

-Using ethanol-dipped, flame-sterilized tweezers, add 5-10 pieces of small nodules/flask

-Incubate under a 16 h/8 h light/dark photoperiod, under stirring 130 rpm

Remarks

1. Kinetin and 2,4-D are dissolved in 0.1 N NaOH at a concentration of 1 mg/mL.

2. Nodules are transferred in fresh medium every 3-4 weeks or when needed (cloudy solution).

3. The best "bioreactors" are 500-mL Erlenmeyer flasks with large neck and stoppered with foam plugs or equivalent

4. The addition of 0.05 % PPM (500 µl L^{-1}) or 2.5 mg/L benomyl (2.5 mL of 1 mg/mL stock in methanol) has been experienced with success, i.e. does not seem to affect nodule growth and help to control contaminations.

A-2. Poplar Tree Growth Solution (modified Hoagland's)

Modified 0.5X Hoagland Nutrient Solution (Hoagland, 1950; Epstein, 1972)

Stock Solution	**Vol. Stock per 4 L**	**Vol. Stock per 12 L**
1M $Ca(NO_3)_2 \cdot 4H_2O$	8.0 mL	**24.0 mL**
2M KNO_3	6.0 mL	**18.0 mL**
2M $NH_4H_2PO_4$	4.0 mL	**12.0 mL**
Micronutrients	4.0 mL	**12.0 mL**
20mM Fe-EDTA *Use Sterile Pipet!!!!	4.0 mL	**12.0 mL**
1M $MgSO_4 \cdot 7H_2O$	2.0 mL	**6.0 mL**
1M NaOH	≈ 6.0 mL	**(until pH = 6.8)**

Stock Solution Preparation:

2M KNO_3	202.20 g	1L
1M $Ca(NO_3)_2 \cdot 4H_2O$	236.16 g	1L
2M $NH_4H_2PO_4$	230.06 g	1L
1M $MgSO_4 \cdot 7H_2O$ *Store in brown bottle	123.24 g	500 mL

20mM Fe-EDTA (store in brown bottle)

heat 800 mL water

add 7.44 g Na_2EDTA, stir

add 5.56 g $FeSO_4 \cdot H_2O$

bring to 1 L; cover with parafilm and let stir overnight (turn heat off when gone)

This solution should NOT be cloudy. If it is, heat it to nearly boiling and stir more.

Micronutrient Solution- combine in 1 L brown bottle or bottle wrapped in foil

KCl	3.728 g
H_3BO_3	1.546 g
$MnSO_4 \cdot H_2O$	0.338 g
$ZnSO_4 \cdot 7H_2O$	0.575 g
$CuSO_4 \cdot 5H_2O$	0.125 g
H_2MoO_4 (85% MoO_3)	0.081 g

APPENDIX B: RNA PROTOCOLS

B-1 RNA Extraction using RNeasy Qiagen extraction kit

(adapted from the Qiagen plant extraction instructions)

1. Weigh plant material (*Start with about 50 mg of plant material, do not overload column.)
2. Grind in liquid Nitrogen, do not allow material to thaw
3. Add 450 µL Buffer RLT to ground powder (***β-ME must be added to Buffer RLT before use)

-Vortex vigorously

-Incubate at 56°C for 1-3 min.

4. Pipet lysate directly onto a QIAshredder spin column (lilac) in 2 mL collection tube

-Centrifuge for 2 min at max speed

-Transfer supernatant of flow-thru fraction to a new microcentrifuge tube (not supplied) WITHOUT disturbing the pellet in tube (Only supernatant is used henceforth)

5. Add 0.5 volume (~225 µL) ethanol (96-100%) to lysate and mix by pipetting
6. Apply sample (~650 µL), including precipitate, to Rneasy mini column (pink) in 2 mL tube

-Close tube gently and Centrifuge for 15 sec at >8000 x g

-Discard flow-thru, keep tube for next step

7. **Intermediate steps using RNase-Free DNase Set:

D1. Pipet 350 µL Buffer RW1 into Rneasy mini column

-Centrifuge for 15 sec at >8000 x g

-Discard flow-thru, keep tube for next step

D2. In a separate 2mL tube, add 10 µL DNase 1 stock soln. to 70 µL Buffer RDD for every sample.

-Mix by gently inverting the tube

D3. Pipet the DNase 1 incubation mix (80 µL) directly onto the Rneasy silica-gel membrane

-Incubate at room temp for 15 min.

D4. Pipet 350 µL Buffer RW1 into the Rneasy mini column

-Centrifuge for 15 sec at >8000 x g

-Discard flow-thru

8. Transfer the RNeasy column to a new 2 mL collection tube (supplied)

-Pipet 500 µL Buffer RPE onto column

-Close tube gently, Centrifuge for 15 sec at >8000 x g

-Discard flow-thru, keep tube for next step

9. Add 500 µL Buffer RPE to column

-Close tube gently Centrifuge for 2 min at >8000 x g

9a. Place the RNeasy column in a new 2 mL tube (not supplied)

-Discard old tube with flow-thru

-Centrifuge 1 min at max speed

10. Transfer column to new 1.5mL tube (supplied)

-Pipet 50 µL RNase-free water directly onto the membrane

-Close tube gently Centrifuge for 1 min >8000 x g

11. Repeat previous step (#10) into same collection tube

APPENDIX C: ATTEMPTED EXPERIMENTS

C-1 *Eisenia fetida* Studies

We performed laboratory toxicity experiments on the earthworm species *Eisenia fetida* following ASTM standard method protocols #E1676-97. Worm stock was purchased from New York Worms and maintained at constant light and temperature in plastic, lidded containers. Fed the worms aged alfalfa pellets soaked in water and left in a one quart sealed glass jar for two weeks.

Experiments included subjecting worms to varying amounts of TNT-exposed alfalfa pellets as the only source of food. Concentrations ranged from 0 to 300 gL^{-1} of TNT soaked alfalfa pellets. The worms refused to eat the pellets, therefore accurate results could not be determined.

TNT-contaminated soil was then used and the worms exhibited no signs of toxicity at lower levels of TNT, but died on contact with soils containing high concentrations of TNT. However, all samples concentrations were lower than reported toxic results for earthworms. In addition, earthworms from control samples without TNT, but containing the acetonitrile solution used to dissolve TNT in the soil slurries were showing identical results as the TNT-contaminated soils. We hypothesized that the acetonitrile needed to dissolve TNT in the soil slurries was more toxic to the earthworms than the TNT and therefore the experimental results were nullified.

Further work to fix these problems was futile and the earthworm experiments were cancelled.

C-2 *Caenorhabditis elegans* Studies

A brood stock solution of dauerlarval stage *Caenorhabditis elegans*, wild type strain N2, was obtained from the *Caenorhabditis* Genetics Center (*C*GC) in Minneapolis,

Minnesota. *Escherichia coli strain* OP50, for feeding *C. elegans*, was purchased from the *C*GC in Minneapolis, MN.

Caenorhabditis elegans (*C. elegans*) were cultured according to Annex A.1 of ASTM method E 2172-01. The brood stock solution, from *C*GC in Minneapolis, was kept at 20°C in no light conditions in an M9 buffer and was renewed monthly. Age-synchronized

Age-synchronized adults were used for toxicity testing. E.coli food was contaminated with TNT, but there were no obvious effects or results to report. Additional testing trials proved futile.

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