NITROREDUCTASES AND THE ANAEROBIC DEGRADATION OF NITRO ORGANICS IN SOIL

By

RUSSEL RAHI

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Oklahoma State University

Stillwater, Oklahoma

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Thesis Approved:

Dr. Mark Krzmarzick

Thesis Adviser

Dr. Gregory Wilber

Dr. Gilbert John

Name: RUSSEL RAHI

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Abstract: The anaerobic degradation of 2,4-dinitrophenol and number of nitro organic compounds with soil microbial communities was investigated in two experiments. The goal of this study is to develop a method for the isolation for nitroreductase enzymes responsible for the anaerobic transformation of nitro organic compounds. The degradation of 2,4-dintrophenol was studied with the coamendment of 2-nitroethyl benzene under anaerobic conditions and the change in concentration was measured with GC-FID. This experiment revealed that the coamendment had no effects on the degradation. For the second experiment anaerobic microcosms were set up using anaerobic mineral media and soil collected from Ray Harrell regional park (Broken Arrow, OK). Nitro organic compounds were used in this study include, 2,4-dinitrophenol, 2-amino-4-dinitrophenol, 4-amino-2-dinitrophenol, nitrobenzene, 2,4,6-trinitrotoluene and 4-amino-2,6-dinitrotoluene. After 50 days only 2,4-dinitrophenol microcosm showed a decline in concentration but reduction to amino compounds was not confirmed. The isolated DNA from this microcosm along with sludge DNA from WWTP was used to test 8 different primers targeting nitroreductases designed from 98 unique amino acid sequences of nitroreductase enzymes. Primers were used with different concentrations of $MgCl_2$ and a gradient of temperature from 42 ºC to 56 ºC. The clone library analysis on the PCR amplification products did not succeed in recovering nitroreductases in this study.

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CHAPTER I

INTRODUCTION

Nitro organic compounds are widely used and highly contaminating. These compounds consist of an organic molecule with at least one nitro group attached to it, and they can occur naturally or be chemically synthesized (Ju & Parales, 2010). Nitro organics are used in munitions, pesticides, dyes and pharmaceuticals (Singh et al., 2012; Kimura et al., 2014). Because of nitro organics' broad use, they are present as contaminants in soil, water and ground water (Ye et al., 2004; Singh et al., 2012; Kimura et al., 2014). Nitro organics possess the potential to be toxic, mutagenic and carcinogenic to all living cells (Rieger & Knackmuss 1995; Purohit & Basu, 2000; Padda et al., 2003; Sunahara., et al 2009). Many nitro organic compounds are listed on the U.S. Environmental Protection Agency (US EPA) list of priority pollutants (U.S. EPA. 2006).

The unique chemistry of these compounds make them resistant to degradation by microbial communities. Efforts to understand the biodegradation and biotransformation of these nitro organic contaminants have been made over the years (Ye et al., 2004; Kulkarni & Chaudhari, 2007). Most former research was focused on aerobic degradation, but these contaminants are present in deep layers of soil and ground water. It has been proven that some aerobic microorganism adapted to metabolic pathways that gave the

ability to utilize nitro organics as their sole nitrogen, carbon and energy source (Peres & Agathos, 2000; Esteve-Núñez et al., 2001; Heiss & Knackmuss, 2002; Ramos et al., 2005). The reduction of the nitro-group occurs in three steps, all reduction reactions: nitro- to nitroso, nitroso- to hydroxylamino-, and hydroxylamino- to amino groups (Roldan et al., 2008). Another issue with the previous research is the absolute elimination of other carbon, nitrogen and energy source to drive the degradation process, which made the conditions environmentally irrelevant (Spain et al., 1995). The need for understanding the anaerobic degradation pathways is important to the development of remediation processes. Also the need to develop a method to isolate nitroreductases and study their unique chemistry is crucial.

In an effort to develop a method for the isolation of nitroreductases, the anaerobic degradation of 2,4-dinitrophenol along with a number of other nitro organic compounds was attempted. 2,4-Dinitrophenol is a yellowish crystalline solid and fairly soluble in water (5.6 g/L at 18 ^{o}C) (Shukla et al., 2009). It is commonly used in the manufacturing of explosives, pharmaceuticals, pesticides and dyes (She et al., 2005). As a result, there are several sites contaminated with this compound, which is potentially carcinogenic to humans and is one of the priority pollutants under the EPA, which recommends it be kept below 10 ng/L in the environment (U.S. EPA 1976). The goal of this study is to achieve the degradation of 2,4-dinitrophenol in an anaerobic environment and develop a method for the isolation of nitroreductase enzymes.

In this research the microcosms were constructed using anaerobic mineral media with yeast extract, which contains all the needed macro and micro nutrients essential for microbial growth. Also, potassium acetate was used as carbon source and bicarbonate as pH buffering agent. The inoculating soil was collected from Ray Harrell regional park (Broken Arrow, OK). The change in concentration of compounds was measured until degradation of the nitro compounds is achieved. DNA was extracted from the microcosms with degradation to optimize molecular methods (DNA) for nitroreductases.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Introduction to Nitro Organics

Nitro organic compounds are among the most important groups that display industrial and biological diversity. They consist of an organic molecule with at least one nitro group attached. Naturally, a number of compounds containing nitro groups have been isolated from microorganisms, plants and animals (Ju & Parales, 2010). These compounds display a wide range of biological activities such as antibacterial, antitumor and cell signal properties (Parry et al., 2011). A variety of bacteria, fungi and plants have been discovered to produce nitroaromatic compounds (Parry et al., 2011). For example, chloramphenicol, which exhibits anti-microbial activities against both gram positive and gram negative bacteria (Parry et al., 2011), is produced by *Streptomyces venezuelae* (Ehrlich et at., 1948). *Streptomyces thioluteus* produces other nitro groups containing antibiotics including aureothin and polyketide (Cardillo et al., 1972; Hirata et al., 1961; Madeda, 1953).

Nitroaromatics are also produced chemically for numerous uses, predominately for explosives. These compounds can be persistent pollutants. The degradation of nitroaromatics is

poorly understood. Importantly, molecular tools targeting nitroreductases are totally absent making the study of these processes limited in mixed cultures and real-world conditions.

2.2 Naturally Occurring Nitro organics

A huge variety of natural nitro organics are known to exist (Parry et al., 2011), such as chloramphenicol (Ahmed & Vining, 1983), nitropyoluteorn (Ohmori et al., 1978), oxypyrrolnitrin (Hashimoto & Hattori, 1966; Kirner et al., 1998) and phidolopin (Tischler et al., 1986). Winkler and Hertweck reviewed three mechanisms in order to understand the biosynthesis of nitro compounds that occurs naturally (Parry et al., 2011). Some compounds are produced by the oxidation of an amino group. The second mechanism involves the action of a nitric oxide synthase during the biosynthesis of the thaxtomins, and the third mechanism involves the introduction of nitro groups by an electrophilic nitration reaction (Parry et al., 2011). Other unknown formation mechanisms might exist due to the structural variety for naturally occurring nitro compounds.

Nitroaromatics can also abiotically form in nature in both atmospheric and aqueous environments. The incomplete combustion of fossil fuels and other natural combustion processes release hydrocarbons which serve as substrates for nitration with atmospheric nitrogen dioxide. Through this hydroxyl radical-initiated mechanism, several nitro-polyaromatic hydrocarbons are produced, such as nitrobenzene, 3-nitrobiphenyl and 3-nitrotoluene (Atkinson et al., 1987; Yamaguchi, 1998; Purohit & Basu 2000; Nishino & Atkinson, 2010). In an aqueous environment, sunlight catalyzes the nitration reaction of

naturally occurring nitroaromatic compounds. Solar irradiation of nitrate or nitrite generates the hydroxyl radical; which serves as a catalyst for a direct nitration reaction of organic compounds. For example, nitrophenols, bromophenols and chlorophenols can be produced by irradiation of seawater containing phenol (Calza, et al., 2008). Despite the widespread diversity and presence of these compounds, little is known regarding the microbiology of their degradation in natural systems which may elucidate better degradation for the anthropogenic nitro organics which are problematic environmental pollutants.

2.3 Chemical Synthesis of Nitroaromatic Compounds

The main reaction used for the synthesis of nitro aromatics is direct nitration. In a lab setting, nitronium ion could be generated in a mixed acid reaction of sulfuric and nitric acid and then added to an aromatic substrate by electrophilic substitution (Booth, 2007). Using this method, aromatics such benzene, toluene, and phenol are converted to nitrobenzene, nitrotoluene and nitrophenol, respectively. Conditions could be modified to direct nitration on the ortho, meta or para position. Nitration can also be tailored to multiple substitutions on one molecule, for example, benzene nitration with nitrous acid can produce 1,3,5-trinitrobenzene (Raiford, 1922; Raiford, & LeRosen, 1944; Raiford & Miller, 1933).

2.4 Nitroaromatic Compounds for Industrial Uses

Nitroaromatic compounds have been used in high energy explosives due to the unique chemistry of the nitro group (Singh et al., 2012). Nitrogen atoms readily accept electrons in the (+III) oxidation state and that allows nitroaromatics to act as self-oxidants, leading to energy release from the compound when detonated (Schwarzenbach et al., 2005). An example nitroaromatic that has been used as explosive since 1902 is 2,4,6-trinitrotolune (TNT) (Higson et al., 1992). TNT derivatives are also used as explosives such as 1,3,5-trinitrobenzene, a TNT without the methyl group, which is a higher energy explosive with decreased shock sensitivity. Hexanitrostilben is also produced by fusing two TNT molecules to increase thermal stability (Zukas & Walters, 1998)

Other commonly used industrial products derived from nitroaromatics include dyes, pesticides and pharmaceuticals (Singh et al., 2012; Kimura et al., 2014). Nitrobenzene, nitrophenols, and nitrotoluenes are used in the production stages of a wide variety of pesticides. Nitrophenols are used to synthesize chemicals including carbofuran (Singerman, 1977), parathion (Friemann et al., 2005) and bifenox (Ware, 1994). Nitroaromatics are also used to produce pharmaceuticals such as indoles which are bioactive and used in the production of drugs and agrochemicals (Dalpozzo & Bartoli, 2005). Derivatives of phenothiazines which have antipsychotic properties, include acetaminophen, which is an over-the-counter pain reliever, and the synthesis of lidocaine,

a local anesthetic (Bhattacharya et al., 2006). Foam and rubber are also produced on an industrial scale using halogenated nitrobenzene (Travis, 2009).

2.5 Nitroaromatic Contamination

The unique chemistry for nitroaromatics that led them to be broadly used also gives them toxicity to humans and wildlife. Nitroaromatic compounds are usually toxic and mutagenic, and are often suspected to be carcinogenic (Rieger & Knackmuss, 1995; Purohit & Basu, 2000; Padda et al., 2003; Sunahara et al., 2009). Many nitro organic compounds are listed on the U.S. Environmental Protection Agency (US EPA) list of priority pollutants (U.S. EPA, 2006). An unfortunate consequence of the extensive use of these compounds is the widespread contamination of ground water and soil (Ye et al., 2004; Singh et al., 2012; Kimura et al., 2014). Munitions from World War II caused soil contamination in the United States and Europe in the locations where they were stored or manufactured (Spain et al., 1995; Singh et al., 2012). Nitro containing pesticides and water soluble nitroaromatic solvents are also responsible for widespread water and soil contamination. TNT is one of the most widely used nitroaromatics and there are several reports of contamination in soil and groundwater (Mulla et al., 2014). It has been identified in at least 20 of the 1,397 hazardous wastes sites in the United States (U.S. EPA, 2011).

A number of monocyclic, polycyclic, and heterocyclic nitroaromatic compounds have been shown to cause DNA mutations, including transition, transversion and frame shift mutations (Purohit & Basu, 2000) The toxicity of nitro compounds is also well-associated with the products formed during the reduction of the nitro group (Roldan et al., 2008). The hydroxyamino derivatives are highly reactive with organic matter and can interact with biomolecules including DNA, causing toxic and mutagenic effects (Roldan et al., 2008).

2.6 Nitroaromatic Compounds Degradation

Many natural organic compounds degrade easily and can serve as a great source of carbon and energy for many microorganisms. Chemically synthesized nitro organic compounds are much harder to degrade and be utilized by microorganisms due to the nitro group repelling oxygen and hydroxyls which are normally used in degradative pathways, the unusual chemical bonds, steric hindrance of the large nitro group, and further complications from additional substitutions with halogens and other functional groups (Spain et al., 1995; Roldan et al., 2008).

In order to develop better remediation processes for these contaminants from the environment, a better understanding of their biodegradation is required. Biodegradation of nitro organics is done by some aerobic bacteria, anaerobic bacteria and by fungi (Ye et al.,

2004; Kulkarni & Chaudhari, 2007; Krzmarzick et al., 2015). Some microorganisms, especially aerobes are able to adopt metabolic pathways that allow them to utilize nitroaromatics as their source for carbon, nitrogen and energy (Peres & Agathos, 2000; Esteve-Núñez et al., 2001; Heiss & Knackmuss, 2002; Ramos et al., 2005). Most research has focused on this degradation mechanism which is often accomplished only by absolute elimination of other carbon, nitrogen and energy sources and is thus potentially environmentally irrelevant. Degradation by microorganisms requires overcoming several obstacles such as the toxicity of the nitroaromatics, complications caused by mixtures of nitroaromatics, and the lack of catabolic systems capable of biodegradation of these compounds (Spain et al., 1995).

It is common for microbial communities to contain enzymes with the ability to transform the aromatic nitro group with nitroreductases, but it is less common for them to have a catabolic pathway allowing them to use nitroaromatics as their nitrogen or carbon source (Spain et al., 1995). Microorganisms able to degrade nitroaromatic compounds grow very slowly because of the lower solubility and high toxicity of these compounds (Ramos et al., 2005). Biodegradation ability decreases when the number of nitro substituents increase, because nitro groups withdraw electrons from the aromatic ring leaving it electron deficient and impairing the electrophilic attack mediated by oxygenases (Rodan et al., 2008). This is why the microbial degradation of nitroaromatic compounds, especially explosives, have been the center of attention for much research.

Bioremediation is the biotechnological use of organisms to remove pollutants from the environment due to the ability of their enzymes for biodegradation. Many microbial strains have been isolated from contaminated soil or water for the purpose of studying their ability to biodegrade nitroaromatic contaminants. TNT, widely studied for bioremediation, and its metabolites exhibit toxic and mutagenic potential on both prokaryotes and eukaryotes (Spanggord et al., 1995; Honeycutt et al., 1996; Lachance et al., 1999). TNT biodegradation by several microorganism including methanogens, *Clostridia*, denitrifyers and sulfate reducing bacteria has been investigated. For example, TNT can be reduced to its nitro substituent under anaerobic conditions by *Methanococcus* sp. and *Desulfovibrio* sp. (Roldan et al., 2008). Several strains of *Desulfovibrio* have also been shown to utilize TNT as their nitrogen source and possibly an electron acceptor (Boopathy & Kulpa, 1993). These studies proposed nitro compounds are reduced to their corresponding amine compounds which are then removed by reductive deamination mechanisms (Schnell & Schnik, 1991). In other research, *Clostridium* strains were the microorganisms of interest because of their ability to reduce nitroaromatic compounds (McConnick et al., 1976; Angermaier & Simon, 1983; Preuss et al., 1993; Marvin-Sikkema & De Bont, 1994). These papers reveal evidence of the presence of enzymes in *Clostridium kluvyeri* that are responsible for a one electron reduction of nitroaromatic compounds such as hydrogenases and ferredoxins. These enzymes were able to reduce 4-nitrobenzoate and several nitropyrenes to their corresponding amines (Rafil et al., 1991).

A fungus known as *Phanerochaete chrysosporium* has the ability to produce a nonspecific system known as the ligninolytic system by producing extracellular peroxidases, small organic molecules, and hydrogen peroxide to degrade lignin (Barr & Aust, 1994). This system, as many studies proposed, is found to biodegrade a variety of synthetic chemicals including nitro aromatics (Barr & Aust, 1994).

Aerobic bacteria are able to degrade a wide range of nitroaromatics by several mechanisms that remove or transform nitro groups. These mechanisms include elimination of the nitro group as nitrite, catalyzed by monooxygenase, or insertion of two hydroxyl groups catalyzed by dioxygenase which coincides with subsequent elimination of the nitro group as nitrite (Spain et al., 1995).

As discussed above, biodegradation pathways do exist in many organisms and efforts to understand these pathways will allow development of a system for contaminant removal. Therefore, in the current study, 2,4-dinitrophenol is used as an example of an anthropogenic nitroaromatic compound for discussion in more detail.

2.7 2,4-Dinitrophenol Chemical Properties and Toxicity

2,4-Dinitrophenol is among the most widely used organic compounds. It exists as a yellowish crystalline solid, is fairly soluble in water (5.6 g/L at 18 ^{o}C) and is volatilized with steam. It has a weakly acidic property, with a pKa value of 4.03 (Shukla et al., 2009).

It is a common building block in the manufacturing of explosives, pharmaceuticals, pesticides, dyes, wood preservatives and rubber chemicals (She et al., 2005). Therefore, 2,4-dinitrophenol occurs as a contaminant in rivers, groundwater, wastewater, the atmosphere, and pesticide and herbicide treated soil.

This nitroaromatic compound is very toxic to living organisms. Among the produced nitrophenols, 2,4-dinitrophenol is the most toxic, followed by 4-nitrophenol and 2-nitrophenol (Parry et al., 2011). 2,4-Dinitrophenol can cause "dinitrophenol poisoning" (Leftwich et al., 1982) due to its ability to uncouple oxidative phosphorylation in the mitochondria and photophosphorylation in chloroplast in the cells (Ilvicky & Casida, 1969; Simpson, 1953; Hirooka et al., 2006). Symptoms for dinitrophenol poisoning in humans include headache, thirst, and increased respiration, weight loss and respiratory failure (Leftwich et al., 1982: Yoon et al., 2000). 2,4-Dinitrophenol also suppresses immunity, disrupts energy metabolism and can cause DNA mutations (Kitova et al., 2004). 2,4-Dinitrophenol is also potentially carcinogenic to humans and one of the priority pollutants under the EPA and it is recommended to keep its concentration in the environment below 10 ng/L (U.S. EPA, 1976).

2.8 Degradation of 2,4-Dinitrophenol

Nitrophenols are readily degradable in sunlight due to photochemical oxidation (Uberoi & Bhattacharya, 1997). However, degradation is difficult without solar irradiance such as in soil and in groundwater.

Despite the toxicity of this compound, a number of microorganisms are able to degrade it. Many useful strains have been isolated due to their ability to utilize 2,4-dinitrophenol as their sole source for nitrogen, carbon and energy. For example, two *Rhodococcus erthropolis* strains, HL 24-1 and HL 24-2, both isolated from river and soil, are gram positive aerobic bacteria found to reduce an aromatic nuclei and utilize the compound as a nitrogen source (Lenke et al., 1992; Hirooka et al., 2006). *Corynebacterium* sp. PNK-26 is able to completely degrade concentrations up to 750 mg/L of 2,4-dinitrophenol and utilize it as carbon and nitrogen sources (Hirooka et al., 2006). Some biodegradation studies under anaerobic conditions showed that 2,4-dinitrophenol is not easily degraded and inhibits methanogens at high concentrations (Battersby & Wilson, 1989; Oren et al., 1991). It is also more inhibitory to acetate utilizer methanogens then propionate utilizers (Uberoi & Bhattacharya, 1979). Research was conducted to study biodegradation of 2,4-dinitrophenol by methanogens and the toxic effects on them using both a glucose fed system and volatile fatty acid fed system (She et al., 2005). This study revealed that 2,4-dinitrophenol was degraded without inhibiting methanogens when concentrations less than 25 mg/L were used in both systems (She et al., 2005). Additionally, it was found that halophilic, anaerobic eubacteria *Halanaerobium praevalens*

and *Haloarcula marismortui* have the ability to degrade 2,4-dinitrophenol if the concentration is less than 10 mg/L without inhibiting them (Oren et al., 1991).

Another study showed that *Sphinomonas* sp. UG30 can oxidatively degrade some nitrophenolic compounds containing a nitro group in the para position and eventually assimilate the majority of the nitrite released from the compound into biomass (Zablotowicz et al., 1999). This study showed that, with additional carbon and readily available nitrogen sources for initial growth, UG30 can degrade 2,4-dinitrophenol. The degradation could be enhanced by adding an amino acid like glutamate, which serves as both nitrogen source and the Krebs cycle intermediate upon deamination (Zablotowicz et al., 1999).

It is possible to design an industrial wastewater treatment system for the removal of 2,4-dinitrophenol in low concentrations using microalgae and cyanobacteria (Hirooka el al., 2006). Nitrophenols are known to reduce to nitrosamines and amines. It has been verified that *Anabaena variabilis* reduces 2,4-dinitrophenol to 2-amino-4-nitrophenol, which is a potent mutagen and must be removed from wastewater (Hirooka el al., 2006), but the removal of the reduction product by this strain is low. It is been shown that *Chlamydomonas reinhardtii* and *Anabaena cylindrical* has high removal ability for 2-amino-4-nitrophenol (Hirooka el al., 2006). Based on these studies, the authors proposed that a mixed culture using these strains would be very useful to remove 2,4-DNP from industrial wastewater. Another aerobic biodegradation product of 2,4-DNP is nitrite

(Gemini et al., 2007). This biodegradation has been done by a gram positive, pleomorphic, aerobic and non-motile bacterial strain (Gemini et al., 2007). This strain was identified on the basis of its 16S rRNA gene sequence closely related to *Rhodococcus opacus* with 99% identity. This strain is capable of degrading 2,4-DNP by initial reduction of the aromatic ring (Rieger et al., 1999; Heiss et al., 2002). It was reported the nitrite released from this aerobic degradation could be removed by adding a denitrification step in an anoxic process performed by a mixed inoculum from a pollutant-free fresh water stream (Gemini et al., 2007).

Meanwhile, studies have shown the ability for some microbial communities to biodegrade 2,4-DNP. Efforts toward enhancing the mineralization and biodegradation were undertaken to optimize these biological treatment systems. A study was conducted to research the effect of supplemental substrate enhancement on mineralization of 2,4-DNP by a consortium containing two strains of bacteria. In this study two microbes were used, first an *Actinomycete* from fresh water and *Janthinobacterium* isolated from forest soil (Hess et al., 1990). In order to identify possible supplement substrates for enhancement, degradation studies on toxic chemicals in bioreactors that contain heterogeneous microbial communities were done (Hess et al., 1990). This research revealed that using glucose in concentration of 100 µg/L would enhanced the growth rate of 2,4-dinitrophenol mineralizing bacteria, which will enhanced the degradation of this compound.

2.9 Nitroreductases

Bacterial nitroreductase is a flavoenzyme that catalyzes the NAD(P)H-dependent reduction of the nitro groups on nitroaromatic and nitrohetrocyclic compounds (Roldan et al., 2008). Nitroreductase coding genes are widely spread and present in the bacterial genome, as well as many archaea, and some eukaryotic species (Roldan et al., 2008). Bacterial nitroreductase is a very important enzyme due to it its potential in environmental and clinical biotechnological applications (Roldan et al., 2008).

Nitroreductases may reduce a wide range of nitroaromatic compounds due to their ability to catalyze the sequential transfer of an electron pair to the nitro group of the aromatic compound to the nitroso, hydroxylamino, and amino derivatives (Roldan et al., 2008). It is difficult to isolate the nitroso intermediates because they react rapidly with nitroreductases, and the resulting hydroxyamino reacts with their nitroso precursors to yield stable azoxy compounds (Pitsawong et al., 2014). This enzyme contains a flavin mononucleotide as a prosthetic group (Roldan et al., 2008). It is common to distinguish between two types of bacterial nitroreductases: “oxygen insensitive” or type I and “oxygen sensitive” or type II (Roldan et al., 2008). The former catalyzes the sequential reduction of the nitro group by adding electron pairs from NAD(P)H to produce nitroso, hydroxylamine and other amine derivatives, while the latter one catalyzes the single electron reduction of the nitro group to produce a nitro anion radical, which will be reoxidized aerobically to the original structure with the production of a superoxide anion (Roldan et al., 2008).

2.10 Conclusion

Nitroreductases are not well understood as far as their specific function, diversity, and possible terminal electron acceptor for bacteria. Surprisingly, molecular DNA or RNA based tools to study nitroreductases have not been developed, limiting the ability to study these enzymes in mixed cultures. A set of experiments were operated to better understand nitroreduction processes and novel molecular methods (PCR) targeting nitroreductases were developed and tested.

CHAPTER III

METHODOLOGY

3.1 Soil and Sediment Collection

For microbial seed material, a 500 mL grab sediment sample was collected from a slow running stream at Ray Harrell Nature Park (Broken Arrow, OK) in March 2014. The sample was collected 1 foot from the stream edge in 6 inches of running water. The sediment was shoveled to about 4 inches of depth and funneled into a 500 mL bottle until it was filled completely. Approximately 2 kg of surface soil was collected from a forest with oak and pecan tree cover in Payne County, Oklahoma. The material is rich with decaying detritus and collected only from the top 4 inches of the soil horizon.

3.2 Anaerobic Mineral Media Preparation

Anaerobic mineral media was prepared for many experiments and consisted of (per L) 1000 mg NaCl, 500 mg $MgCl_2*6H_2O$, 200 mg KH_2PO_4, 300 mg NH_4Cl, 300 mg KCl, 15 mg $CaCl_2*2H_2O$, 1 mL of trace element A and 1 mL of trace element B (Shelton and Tiedje, 1984; Wolin et al., 1963). The mixture was placed on a hot plate and stirred with a magnetic stir bar until all the salts were completely dissolved. The media was autoclaved for 15 min, cooled under a stream of N_2 gas and moved to the anaerobic chamber.

3.3 Nitro Organic Compounds

2,4-Dinitophenol, nitroethyl benzene, 2-amino-4-nitrophenol, 4-amino-2-nitrophenol, nitrobenzene, 2,4,6-trinitrotolune and 4-amino-2,6-dinitrotoluene were purchased from (Sigma-Aldrich; St. Louis, MO).

3.4 Experiment One – Nitroreduction of Dinitrophenol with and without Co-Amendment of Nitroethylbenzene

In experiment 1, the effect of another nitro-compound (nitro-ethyl benzene) on DNP reduction was tested. Anaerobic batch microcosms were used in this research and Table 3.1 shows a summary of all microcosms used in this experiment. Each microcosm treatment was operated in duplicate.

Table 3.1 Summary of Microcosms Used in Experiment One

Microcosm	**Description**
A (DNP and Nitroethyl benzene)	To study the degradation of DNP in the presence of nitroethyl benzene
B (DNP only)	To determine the rate of DNP degradation independent of nitroethyl benzene
C (Nitroethyl benzene only)	To determine the rate of nitroethyl benzene degradation
D (soil only)	Control without nitro compounds
E (Autoclaved) containing DNP and Nitroethyl benzene	Control on abiotic degradation

Microcosms were constructed in silanized 160 mL serum bottles capped with Teflon

stoppers and aluminum crimps similar to previously published research (Krzmarzick et al., 2012; Krzmarzick et al., 2014). Each microcosm contained the respective nitro organics or control amendment, 5 g of soil and sediment collected above, 100 mL of anaerobic mineral media reduced with sodium sulfide and cysteine, 10 mM acetate, and 1 mL of vitamin solution to provide cobalamin, an essential cofactor (Shelton et al., 1984; Wolin et al., 1963; He et al., 2007; Krzmarzick et al., 2012). Microcosms were constructed in an anaerobic glove box with a 3% H_2/ 97% N_2 headspace. Autoclaved controls were prepared and then autoclaved three times on three subsequent days.

3.4.1 Sample Collection

Samples were collected at Days 0, 1, 4, 9, 16, 23, 32, 48, 65, 73 and 78 for DNA analysis. Bottles were vigorously hand-shaken for 30 seconds before they were opened in the anaerobic glovebag and approximately 1.6 mL of sediment slurry was transferred to microcentrifuge tubes with sawed-off Pasteur pipettes (Yan et al., 2006, Krzmarzick et al., 2012). For DNA extraction, 1.6 mL of slurry was centrifuged at 1000 × g for 1 min, the supernatant was transferred to another microcentrifuge tube and the pellet was frozen at -20 ^{o}C until further analysis. Another sample was collected by pipetting 2 mL of liquid into small weighted salinized serum vials for gas chromatography (GC) analysis.

3.4.2 Extraction of Nitro Organics

Silanized serum vials (12 mL) were weighed with a Teflon cap before and after the addition

of the 2 mL samples collected from each microcosm. The vials were transferred to a fume hood where 8 mL of dichloromethane were added for extraction. Each vial was shaken for 1 min and allowed to sit undisturbed for 1 min to separate into two layers. The dichloromethane was transferred to a second vial where it was reduced to about 4 mL and the exact amount of dichloromethane extract was determined gravitationally.

3.4.3 Gas Chromatography with Flame Ionization Detector (GC-FID) Analysis

The extracted dichloromethane solution was analyzed using an Agilent Technologies 7890B gas chromatograph system equipped with a split/splitless injector, and flame ionizing detector (FID). Capillary column used was a Supelco Equilty-5 (5% methyl silica, 30 m × 250 µm × 0.25 µm). The analysis was performed at an initial temperature of 35 ^{o}C for 0.5 min and ramped at 10.167 ^{o}C/min to 325 ^{o}C Samples of dichloromethane extract (2 µL) were introduced by glass syringe to a splitless inlet. The carrier gas was helium with a flow rate of 1 ml/min. For data acquisition Agilent Open LAB CDS software was used. Concentrations between 0 and 100 mg/L in dichloromethane of 2,4-dinitrophenol and nitroethyl benzene were prepared by serial dilution using dichloromethane for external standards. For 2,4-dinitrophenol a peak appeared at approximately 7.6 min and nitroethyl benzene appeared at 6.7 minutes. Concentration vs peak area were used to create standard curves for both compounds (see Appendix A).

3.5 Experiment 2: Enrichment Culture of DNP reducing bacteria

An enrichment culture of DNP degrading community was then developed. A 1 mL sample each of microcosm A (DNP and nitroethyl benzene) and B (DNP) were transferred into a silanized 160 mL serum bottle containing 100 mL of anaerobic mineral media and 50 mg of DNP. A control microcosms were also prepared to be treated identically with the exception of no DNP amendment. Microcosms were kept in the anaerobic chamber. Samples (1.5 mL) were collected in a microcentrifuge tubes in the anaerobic glovebag on Days 0, 3, 8, 12, and 15 for DNA analysis. Samples were frozen at -20 ^{o}C for possible future analysis on the nitro organic degrading bacteria. On Day 17, The 1 mL samples from previous enrichment culture microcosms A and B were transferred to a silanized 160 mL tube with 100 mL of anaerobic mineral media and 50 mg of DNP. Samples were collected as stated above for spectrophotometer analysis on Days, 0, 3, 7 and 26 thereafter.

3.6 Experiment Three – The Nitroreduction of Different Nitro-Organics to Study Nitroreductases.

A second set of duplicate microcosms were prepared to study DNP and other nitro organic compounds degradation. Table (3.2) shows a summary of the microcosms used in this experiment. These microcosms were prepared as mentioned above and kept in the anaerobic chamber glove bag.

Table 3.2 Summary of the microcosms used in experiment 3

Microcosm	Description
A	DNP
B	2-Amino-4-Nitrophenol
C	4-Amino-2-Nitrophenol
D	Nitrobenzene
E	2,4,6-Trinitrotolune
F	4-Amino-2,6-Dinitrotoluene
G	Control

3.6.1 Sample Collection

For each microcosm, 1.5 mL samples were collected in microcentrifuge tubes on Days 0, 2, 6, 9, 11, 13, 15, 18, 20, 22, 25 and 27. Samples were centrifuged for 10 min at 10,000 × g and the pellet was frozen at -20 ^{o}C for DNA analysis and the supernatant was used for spectrophotometric analysis.

3.6.2 Spectrophotometer

Samples were analyzed in triplicate using Thermo Scientific Mulitiscan Go Spectrophotometer. A 100 µL volume from each sample was loaded into a 96 well plate. The machine was set to capture wavelength between 250 and 425 nm. A triplicate of 100 µL water samples were used to subtract water absorbance from each sample reading.

For each compound from the third experiment a standard curve was created using the spectrophotometer with the specific wavelength. For 2,4-dinitrophenol; 2-amino-4-nitrophenol; 4-amino-2-nitrophenol and nitrobenzene, serial dilution with DI water were used to make

concentration between 0-100 mg/L. For 2,4,6-trinitrotoluene and 4-amino-2,4-dintrotoulne the curves were made for concentrations between 0-10 mg/L. Absorbance vs concentration data were used to graph the standard curves.

3.6.3 DNA Extraction

DNA was extracted from the 2,4-dinitrophenol sample that showed the lowest concentration. The pellet was transferred to bead-beating tubes for DNA extraction with the PowerSoil DNA isolation kit (MoBio Laboratories). Then DNA was extracted according to the manufacturer's recommendations and frozen at 20 ºC for PCR analysis.

3.7 Development, Optimization, and Verification of PCR Method Targeting Nitroreductases

Nitroreductase protein sequences were collected from the NIH NCBI data base (NCBI). The 100 gene sequences collected were aligned using MEGA 6.1 software with MUSCLE alignment and a phylogenetic analysis using the Neighbor Joining Method with 500 bootstraps was performed. Unique phylogenetic groups of nitroreductases from the bacteria were then aligned and analyzed for conserved amino acid sequences for the development of degenerate primer pairs for the amplification of nitroreductase genes for PCR. For each group, the least degenerate primers were selected that could give an amplification product with at least 150 bp (corresponding to at least 50 amino acids). See results for primers designed.

DNA extracts from the duplicate microcosm with 2,4-dinitrophenol and sludge, with concentration of 0.0178 ng/µL, 0.0121 ng/µL and 0.156 ng/µL respectively, were used for PCR

analysis. PCR was performed for community analysis similarly as described in (McNamara & Krzmarzick, 2013). Briefly, the interspacer region between the 16S and 23S sections of the rRNA gene were amplified with polymerase chain reaction. Each PCR reaction (25 µL) contained 1 µL of DNA extract 1.0-3.0 mM of magnesium chloride, 1 × DNA GoTaq buffer (Promega), 1 µg of bovine serum albumin (BSA), 1.6 mM of dNTPs, 0.25 µM of each forward and reverse primer (Cardinale et al., 2004), and 0.625 U of GoTaq DNA polymerase (Promega). PCR was performed in a T100 Thermal Cycler (BioRad) with a thermocycling protocol of 34 cycles with an annealing temperature gradient of 56-42 ^{o}C. To test PCR methods, eight primers were designed and for each primer set three different magnesium chloride concentrations were tested with 1.0, 2.0 or 3.0 mM of magnesium chloride. Each unique PCR condition (24 total for each primer pair with combinations of three different $MgCl_2$ concentrations and eight different annealing temperatures) contain the same three DNA templates (two from DNP reducing microcosms and a DNA extract from anaerobic digester sludge) and a negative control. Nested PCR was also attempted in which the product from a PCR amplification was cleaned, and used as template for a second amplification. PCR product was analyzed using gel electrophoresis and the samples with amplification at the expected sizes (200-500 bp) were used for clone library analysis. Cloning on successful amplifications were performed using pGEMT-Easy Vector kit II (Promega) according to manufacturer's directions and sequencing was performed at the DNA/Protein Core Facility at Oklahoma State University using an ABI 3730xl sequencer (Applied Biosystems).

CHAPTER IV

FINDINGS

4.1 Nitroreduction of DNP with and without Co-Amendment of Nitroethylbenzene

In the anaerobic microcosms (Table 3.1) the degradation of 2,4-dinitrophenol was measured with and without coammendment with nitroethyl benzene. The concentration was directly measured with gas chromatography quipped with a flame ionization detector (GC-FID) to determine the degradation in the microcosms over time versus the control. The concentration of 2,4-dinitrophenol varied significantly making quantitative analysis difficult. There was a decrease in the concentration from day 0 to day 50. On Day 63, an additional 50 mg/L of 2,4-dinitrophenol was added and the concentration of DNP decreased from day 63 to day 76 showing degradation in the microcosms (Figure 4.1). No significant differences between the microcosms with and without co-amendment was measured, and thus there was no measurable impact on DNP degradation from the addition of a second nitro organic compound. The autoclaved control also showed degradation, indicating that it was either not fully killed or was contaminated during the experiment. Additionally, nitroethyl benzene was not observed to decrease over time. Nonetheless the results suggest that in this particular case, at least, nitroreduction of the main

contaminant, DNP, was not stimulated from a nitro organic coamendment, so the experiment was not repeated.

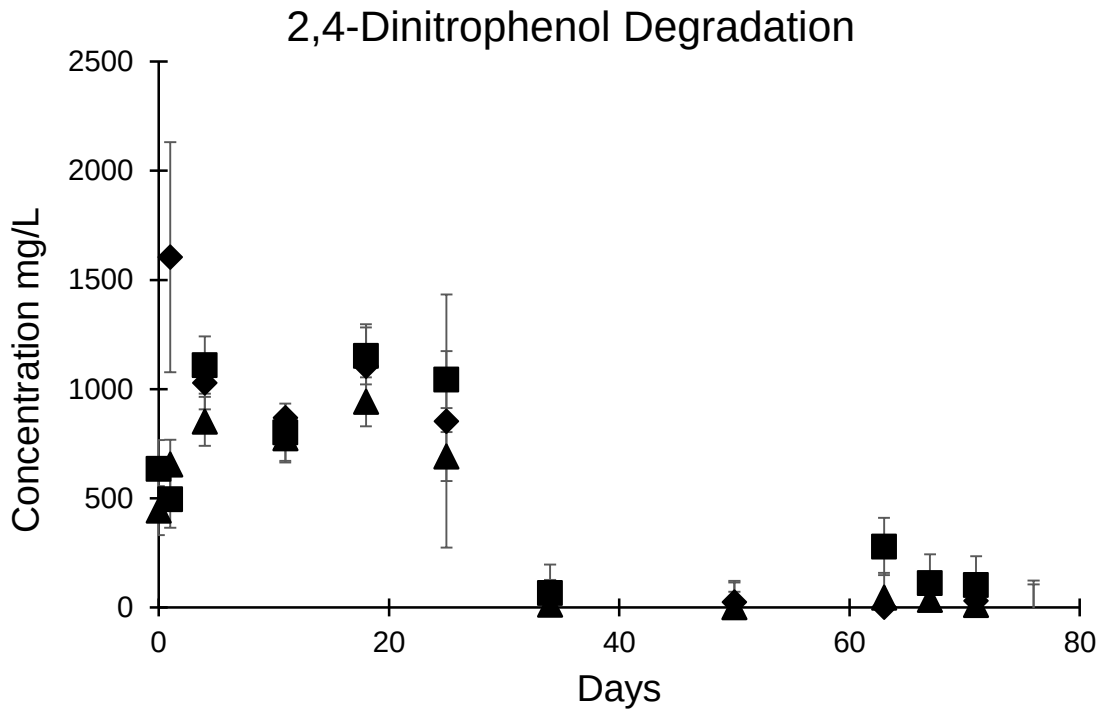


Figure 4.1. The change in 2,4-dinitrophenol concentration over period of 76 days with coamendment with nitroethyl benzene (▲), without coamandment of nitroethyl benzene (■) and the autoclaved control (♦). Error bars indicate the standard deviations of duplicate microcosms.

4.2 Enrichment Culture of 2,4-Dinitrophenol Reducing Bacteria

Enrichment cultures from microcosms (containing DNP and a control without any nitroorganic) were made for the study of 2,4-dinitrophenol reducing bacteria. The measured concentrations of 2,4-dinitrophenol with the spectrophotometer from duplicate enrichment culture microcosm did not show definitive degradation (Figure 4.2). Unexpectedly, there was no statistically significant differences in the concentration of 2,4-dinitrophenol from day 0-13. Another 50 mg/L of 2,4-dinitrophenol was added and the concentration was monitored. In the duplicate microcosm the concentration still did not show a decrease but completely disappeared on Day 62. The reduction of 2,4-dinitrophenol to amino derivatives in this experiment was not conclusive using gas chromatography and significant dark rust coloring in the microcosms indicated the likely presence of compounds other than the amines, such as azoxy compounds. Therefore, this experiment was not continued.

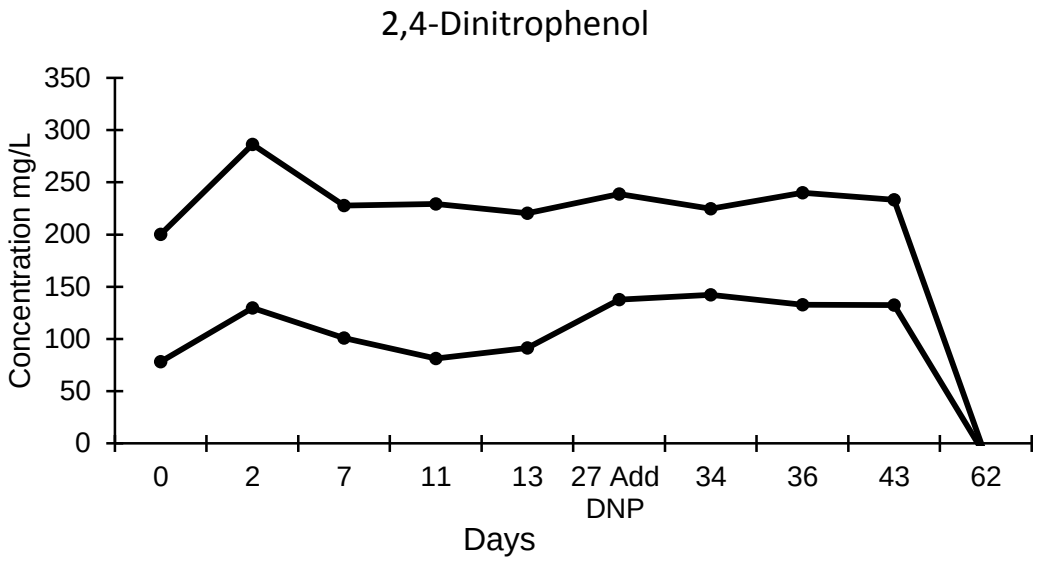


Figure 4.2. The change in 2,4-dinitrophenol concentration in 62 days in the duplicate enrichment culture microcosms.

4.3. The Nitroreduction of Different Nitro Organics to Study Nitroreductases

In this experiment the degradation of several nitro compounds (Table 3.2) by soil bacteria under anaerobic conditions was measured using a Multiscan spectrophotometer that was set to capture each compound at a specific wave length. The concentration then was calculated using external standard curves prepared for each nitro compound. The concentration of these nitro compounds varied significantly over time which made it hard to detect degradation (Figures 4.4-4.8). As for 2,4-dinitrophenol, the concentration was going down with time but not consistently. This could have been the results of 2,4-dinitrophenol transformation to nitroso, hydroxylamine or amino product which could interfere with the spectrophotometer reading.

For the nitro compounds (Figures 4.4-4.8) the final concentration is equal to or higher than the initial concentration that was added to the microcosms. With these results it is hard to see any degradation or biotransformation patterns. Since these microcosms were made with anaerobic mineral media which contain all the necessary requirements for microbial growth, microbial activities may have been occurring. But with no chromatography and no other separation of the compounds it cannot be concluded whether there was biotransformation resulting in the high absorptions and high readings or not complete dissolution

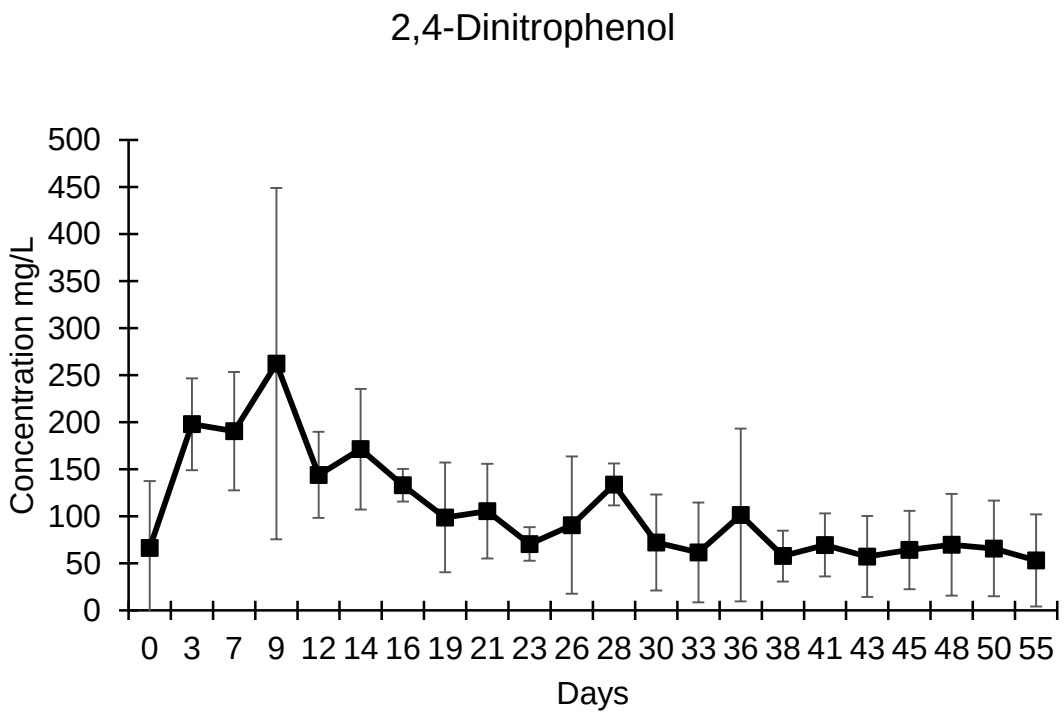


Figure 4.3. The change of 2,4-dinitrophenol concentration in 50 days. Error bars are the standard deviations of duplicate microcosms.

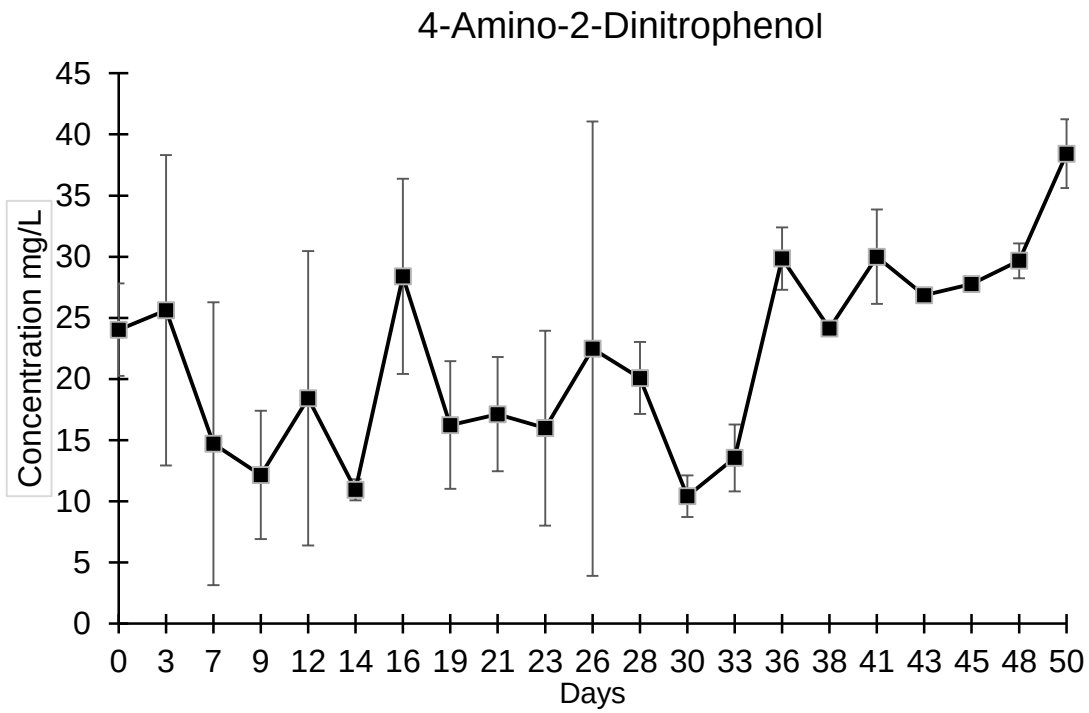


Figure 4.4. The change of 2-amino-4-nitrophenol in 50 days. Error bars are the standard deviations of duplicate microcosms.

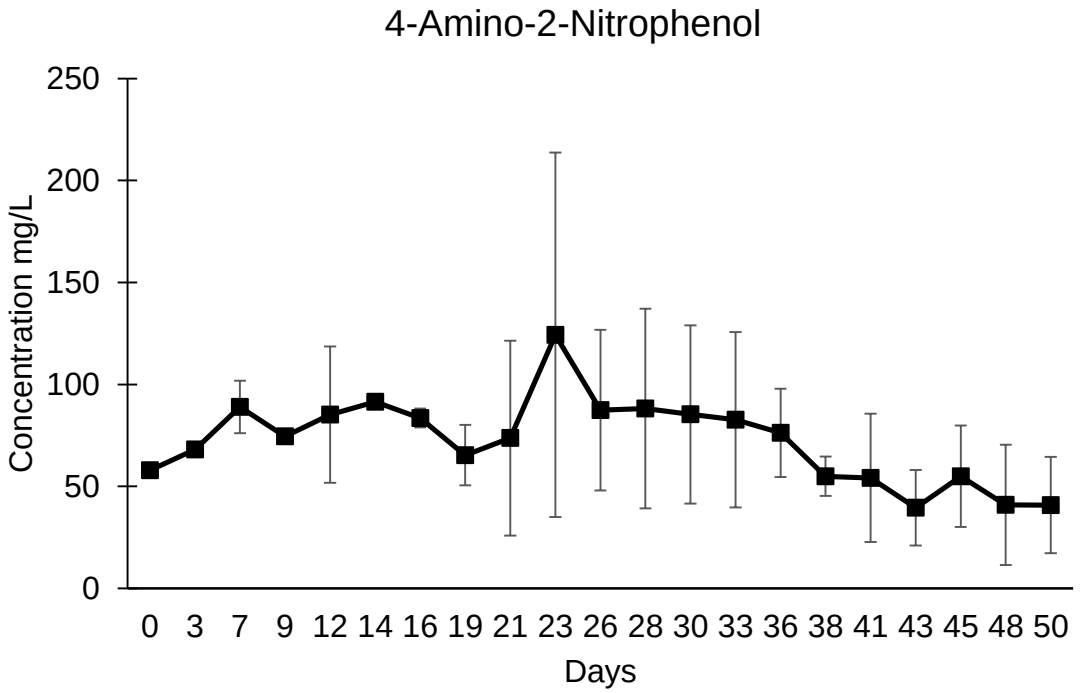


Figure 4.5. The change in 4-amino-2-nitrophenol in 50 days. Error bars are the standard deviations of duplicate microcosms.

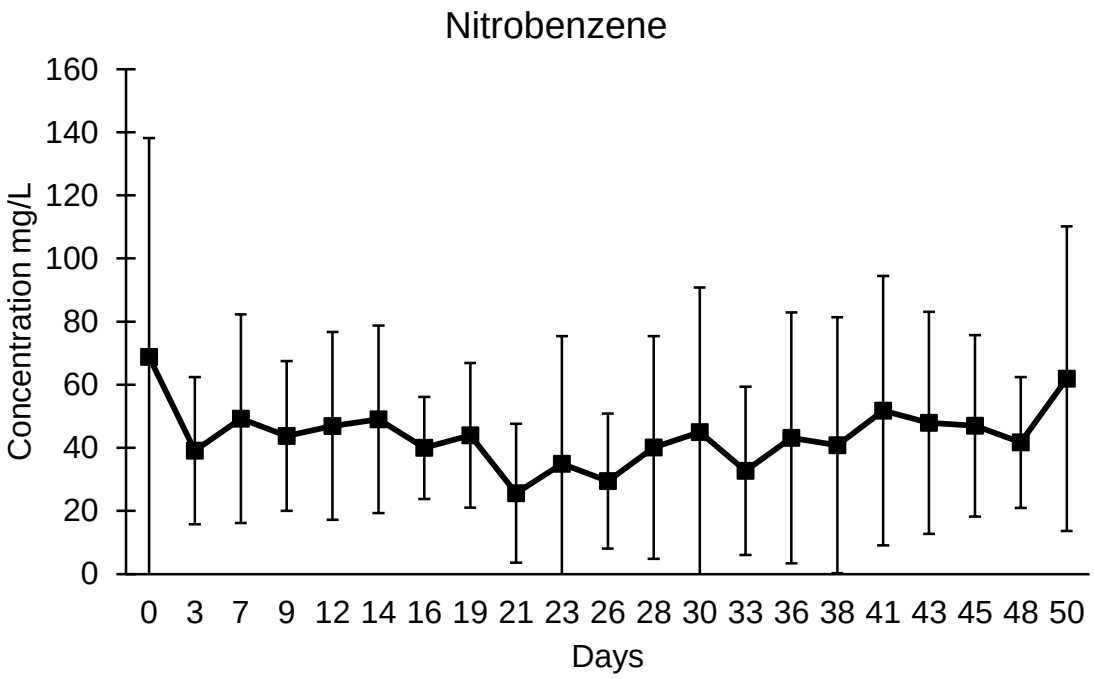


Figure 4.6. The change of nitrobenzene concentration in 50 days. Error bars are the standard deviations of duplicate microcosms.

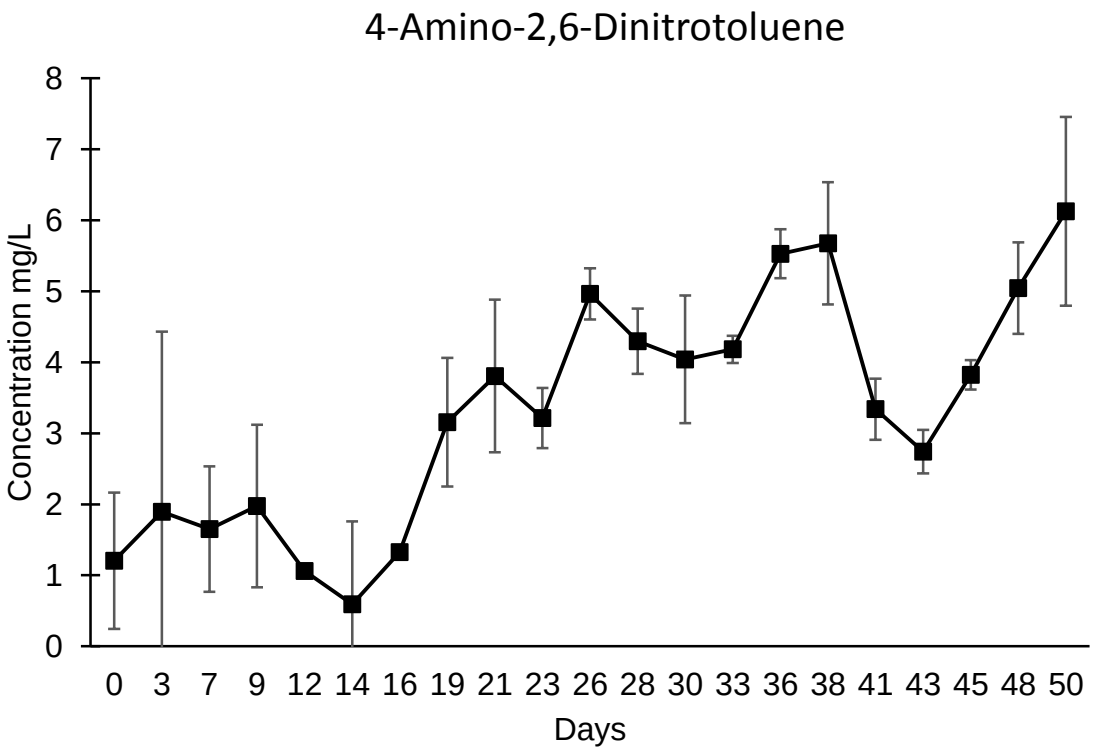


Figure 4.7. The change of 4-amino-2,6-dinitrotoluene concentration in 50 days. Error bars are the standard deviations of duplicate microcosms.

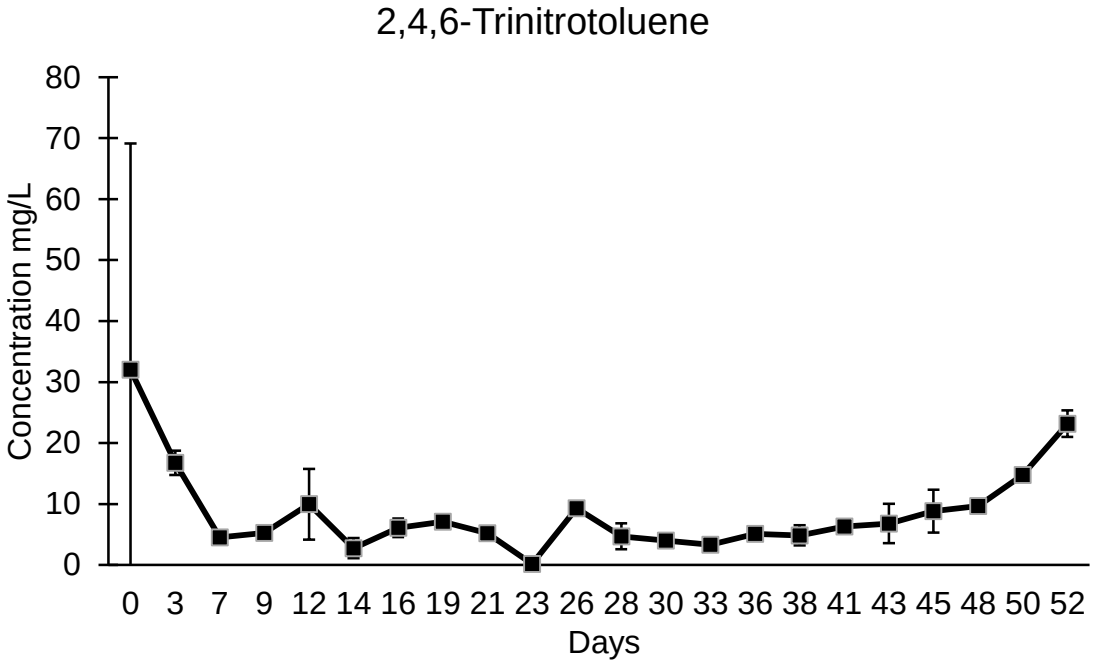


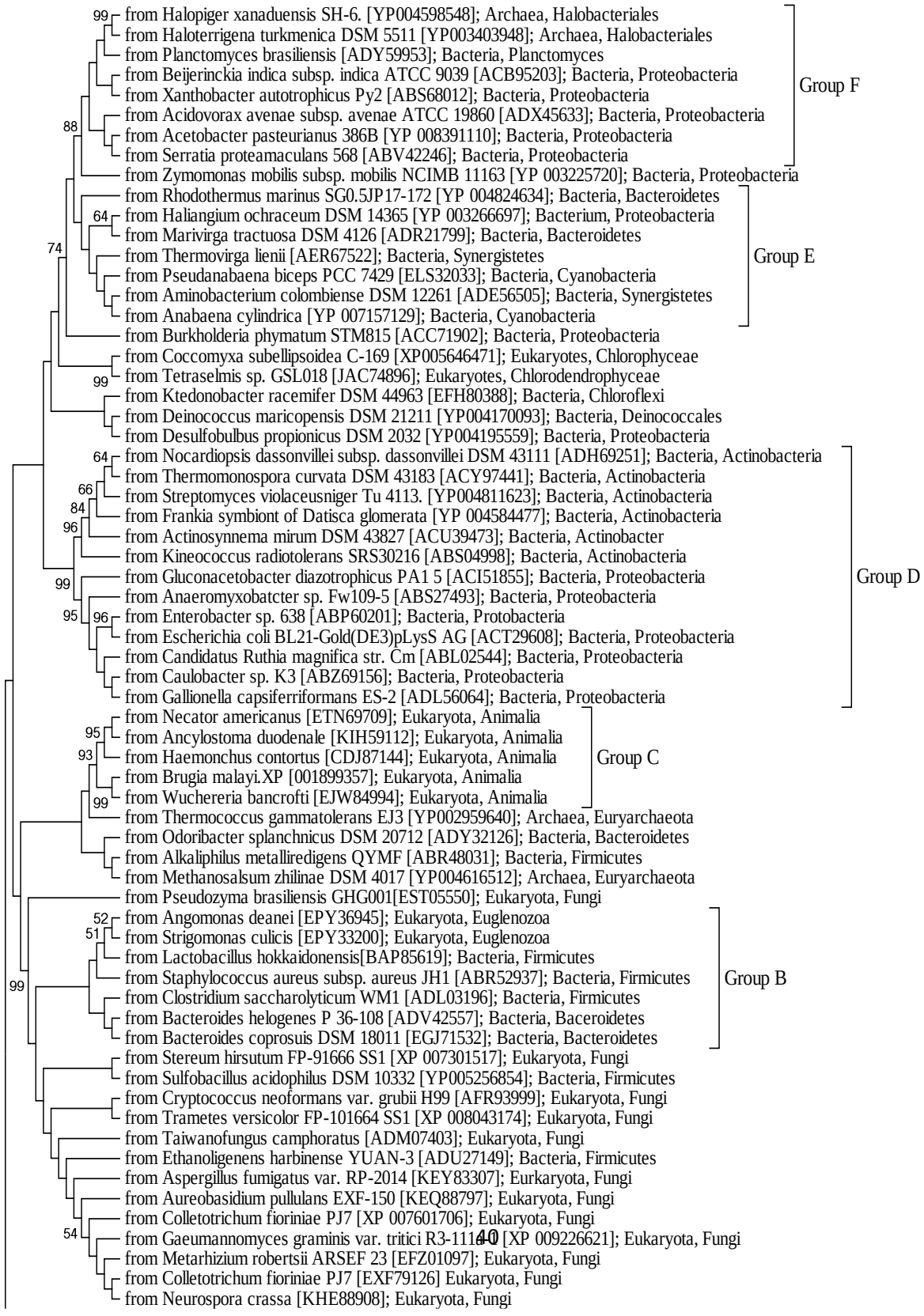
Figure 4.8. The change of 2,4,6-trinitrotoluene concentration in 52 days. Error bars are the standard deviations of duplicate microcosms.

4.4 Phylogeny of Nitroreductases, Development of PCR Primers and Results of PCR.

The phylogenetic analysis of 98 unique nitroreductase genes is shown in Figure 4.9. These nitroreductases came from *Bacteria, Fungi, Archaea, Eukaryota* and the kingdom *Animalia*. These genes are very divergent with a likely long evolutionary history. Most *Fungi* are relatively more homologous to each other than other genes, while *Bacteria* and *Archaea* are mixed throughout the major homologous groups.

Homology was not significant enough to design just one primer pair for all nitroreductases, so homologous groups indicated in the figure were analyzed. Seven groups were analyzed for primer design, which contained the majority of nitroreductases from the *Bacteria* domain. Adjacent sequences to each group could not be included without addition of degeneracy to the amino acid sequences where the primers were designed to target. Except for Group C which consisted of genes from *Eukaryota* and the kingdom *Animalia*, the focus of the design was on the groups dominated by *Bacteria*. To reduce degeneracy, group D, which consists of genes from *Actinobacteria* and *Proteobacteria* was split into two different forward primers to cover the group. Primers with melting temperature ranges extending less than 42 ^{o}C had a GC-clamp attached to increase the annealing temperature during PCR. Inosine was used in place of 'N' for the 3' end of the primers to increase effective primer concentration and reduce the DNA degeneracy of the primers. Table 4.1 lists the primers designed in this study.

Seven clone libraries were produced from four primer sets that were successful in producing an expectedly sized amplicon (Table 4.2). The plasmid sequence was deleted prior to analysis and the amplicons was translated in all six possible reading shifts and the pBLAST of NCBI's BLAST database function was used to identify the encoded proteins. Table 4.3 lists ten of the unique identified genes based on amino acid sequence for those amplicons in which a function was identified. Other sequences (30 additional unique) had poor protein homology with amino acid sequences of the database but none were putatively identified as a likely nitroreductase enzyme. Though in this study, these primers were not able to identify unique nitroreductases in weakly enriched DNP-degrading cultures or in digester sludge, later studies on nitro organic reducing cultures, possibly combined with next generation sequencing, may benefit from using these primers to identify and study these genes.


from Halopiger xanaduensis SH-6. [YP004598548]; Archaea, Halobacteriales
from Haloterrigena turkmenica DSM 5511 [YP003403948]; Archaea, Halobacteriales
from Planctomyces brasiliensis [ADY59953]; Bacteria, Planctomyces
from Beijerinckia indica subsp. indica ATCC 9039 [ACB95203]; Bacteria, Proteobacteria
from Xanthobacter autotrophicus Py2 [ABS68012]; Bacteria, Proteobacteria
from Acidovorax avenae subsp. avenae ATCC 19860 [ADX45633]; Bacteria, Proteobacteria
from Acetobacter pasteurianus 386B [YP 008391110]; Bacteria, Proteobacteria
from Serratia proteamaculans 568 [ABV42246]; Bacteria, Proteobacteria
Group F
from Zymomonas mobilis subsp. mobilis NCIMB 11163 [YP 003225720]; Bacteria, Proteobacteria
from Rhodothermus marinus SG0.5JP17-172 [YP 004824634]; Bacteria, Bacteroidetes
from Haliangium ochraceum DSM 14365 [YP 003266697]; Bacterium, Proteobacteria
from Marivirga tractuosa DSM 4126 [ADR21799]; Bacteria, Bacteroidetes
from Thermovirga lienii [AER67522]; Bacteria, Synergistetes
from Pseudanabaena biceps PCC 7429 [ELS32033]; Bacteria, Cyanobacteria
from Aminobacterium colombiense DSM 12261 [ADE56505]; Bacteria, Synergistetes
from Anabaena cylindrica [YP 007157129]; Bacteria, Cyanobacteria
Group E
from Burkholderia phymatum STM815 [ACC71902]; Bacteria, Proteobacteria
from Coccomyxa subellipsoidea C-169 [XP005646471]; Eukaryotes, Chlorophyceae
from Tetraselmis sp. GSL018 [JAC74896]; Eukaryotes, Chlorodendrophyceae
from Ktedonobacter racemifer DSM 44963 [EFH80388]; Bacteria, Chloroflexi
from Deinococcus maricopensis DSM 21211 [YP004170093]; Bacteria, Deinococcales
from Desulfobulbus propionicus DSM 2032 [YP004195559]; Bacteria, Proteobacteria
from Nocardiopsis dassonvillei subsp. dassonvillei DSM 43111 [ADH69251]; Bacteria, Actinobacteria
from Thermomonospora curvata DSM 43183 [ACY97441]; Bacteria, Actinobacteria
from Streptomyces violaceusniger Tu 4113. [YP004811623]; Bacteria, Actinobacteria
from Frankia symbiont of Datisca glomerata [YP 004584477]; Bacteria, Actinobacteria
from Actinosynnema mirum DSM 43827 [ACU39473]; Bacteria, Actinobacter
from Kineococcus radiotolerans SRS30216 [ABS04998]; Bacteria, Actinobacteria
from Gluconacetobacter diazotrophicus PA1 5 [ACI51855]; Bacteria, Proteobacteria
from Anaeromyxobatcter sp. Fw109-5 [ABS27493]; Bacteria, Proteobacteria
from Enterobacter sp. 638 [ABP60201]; Bacteria, Protobacteria
from Escherichia coli BL21-Gold(DE3)pLysS AG [ACT29608]; Bacteria, Proteobacteria
from Candidatus Ruthia magnifica str. Cm [ABL02544]; Bacteria, Proteobacteria
from Caulobacter sp. K3 [ABZ69156]; Bacteria, Proteobacteria
from Gallionella capsiferriformans ES-2 [ADL56064]; Bacteria, Proteobacteria
Group D
from Necator americanus [ETN69709]; Eukaryota, Animalia
from Ancylostoma duodenale [KIH59112]; Eukaryota, Animalia
from Haemonchus contortus [CDJ87144]; Eukaryota, Animalia
from Brugia malayi.XP [001899357]; Eukaryota, Animalia
from Wuchereria bancrofti [EJW84994]; Eukaryota, Animalia
Group C
from Thermococcus gammatolerans EJ3 [YP002959640]; Archaea, Euryarchaeota
from Odoribacter splanchnicus DSM 20712 [ADY32126]; Bacteria, Bacteroidetes
from Alkaliphilus metalliredigens QYMF [ABR48031]; Bacteria, Firmicutes
from Methanosalsum zhilinae DSM 4017 [YP004616512]; Archaea, Euryarchaeota
from Pseudozyma brasiliensis GHG001[EST05550]; Eukaryota, Fungi
from Angomonas deanei [EPY36945]; Eukaryota, Euglenozoa
from Strigomonas culicis [EPY33200]; Eukaryota, Euglenozoa
from Lactobacillus hokkaidonensis[BAP85619]; Bacteria, Firmicutes
from Staphylococcus aureus subsp. aureus JH1 [ABR52937]; Bacteria, Firmicutes
from Clostridium saccharolyticum WM1 [ADL03196]; Bacteria, Firmicutes
from Bacteroides helogenes P 36-108 [ADV42557]; Bacteria, Baceroidetes
from Bacteroides coprosuis DSM 18011 [EGJ71532]; Bacteria, Bacteroidetes
Group B
from Stereum hirsutum FP-91666 SS1 [XP 007301517]; Eukaryota, Fungi
from Sulfobacillus acidophilus DSM 10332 [YP005256854]; Bacteria, Firmicutes
from Cryptococcus neoformans var. grubii H99 [AFR93999]; Eukaryota, Fungi
from Trametes versicolor FP-101664 SS1 [XP 008043174]; Eukaryota, Fungi
from Taiwanofungus camphoratus [ADM07403]; Eukaryota, Fungi
from Ethanoligenens harbinense YUAN-3 [ADU27149]; Bacteria, Firmicutes
from Aspergillus fumigatus var. RP-2014 [KEY83307]; Eurkaryota, Fungi
from Aureobasidium pullulans EXF-150 [KEQ88797]; Eukaryota, Fungi
from Colletotrichum fioriniae PJ7 [XP 007601706]; Eukaryota, Fungi
from Gaeumannomyces graminis var. tritici R3-111a-1 [XP 009226621]; Eukaryota, Fungi
from Metarhizium robertsii ARSEF 23 [EFZ01097]; Eukaryota, Fungi
from Colletotrichum fioriniae PJ7 [EXF79126] Eukaryota, Fungi
from Neurospora crassa [KHE88908]; Eukaryota, Fungi

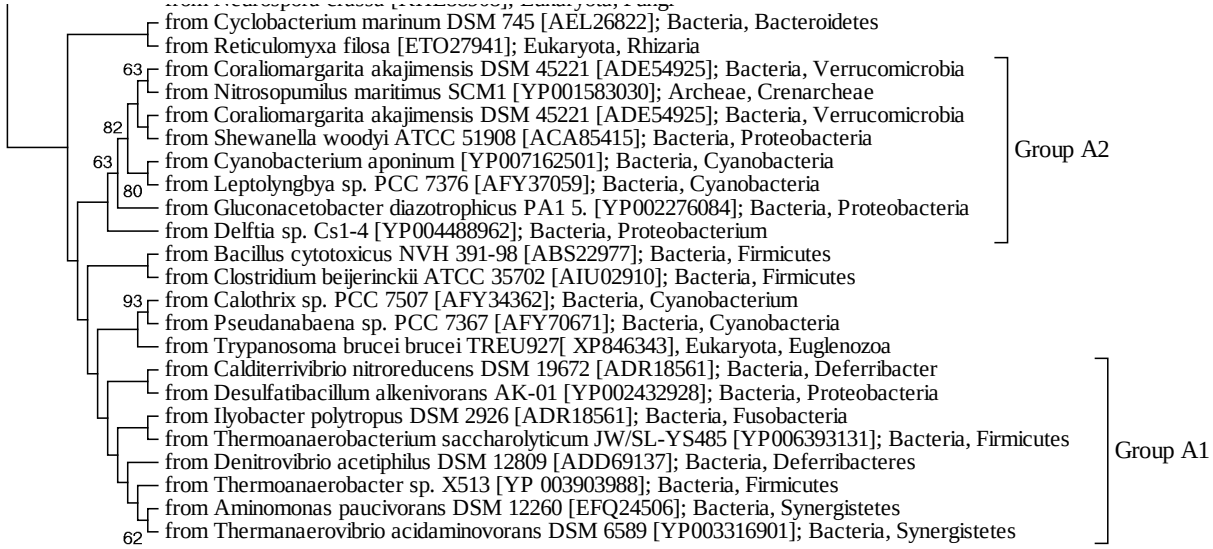


Figure 4.9. Phylogeny of Nitroreductases in Bacteria, Archaea, and Eukaryotes. Bootstrap values over 50% are shown. The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 100 replicates is shown and bootstrap values over 50% consensus are shown. The tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 88 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 116 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 4.1 Primers designed and used in this study

Target Group	Forward Primer	Reverse Primer
A1	[A]5'-AAYYTICARCCITGGRA-3'	[B]5'-AANCCIHYCATIGSRTG-3'
A2	[A]5'-AAYHTICARMAYTGG-3'	[B]5'-TCRAANCCIWYCATICC-3'
B	[A]5'-TAYDMIGAYAAYTTY-3'	[B]5'-CCRAAIGGCATYASIGC-3'
C	5'-TTYTAYGARMAIATGAARATGMG-3'	[B]5'-TRCTIADYTGRTTRTAITG-3'
D1	[B]5'-GSNCCNCANWSIRYIAA-3'	5'-TCRAANCCNSWCATIGGICC-3'
D2	[B]5'-GSNCCNCANGCIRYIAA-3'	5'-TCRAANCCNSWCATIGGICC-3'
E	5'-GARGCNGCIMGITGG-3'	5'-AWNCCNSCCATIDRTGIAC-3'
F	5'-GARGCNGCNMGITGGGCICC-3'	5'-CCANGCNGCICCIGYRTCRAA-3'

[A] A 5'-GCGCGGAATTC-3' clamp was added to these primers
[B] A 5'-GCGCGCAAGCTT-3' clamp was added to these primers

Table 4.2. Optimized PCR conditions for clone libraries

Target Group	Temperature ºC	$MgCl_2$ mM
A1	46	2
D	46	2
E	56	1
F	46	3

Table 4.3. Results from PCR methods targeting nitroreductase genes.

Primer Pair	Length (amino acids)	Closest Match from Protein Blast
E	94	Hemolysin D from *Syntrophus aciditrophus*
A1	154	DNA polymerase I from *Sphaerochaeta globosa*
E	183	Dehydratase from *Variovorax paradoxus*
F	34	NAD-dependent epimerase from *Verminephrobacter eiseniae*
F	80	Heme utilization protein from *Pseudomonas* sp. 45MFCol3.1
E	55	Putative membrane protein from unculture bacterium
E	103	Acetyl-CoA carboxylase alpha subunit from *Brachyspira* sp. CAG:700
E	95	Pyruvate Kinase superfamily. 95 aa. pyruvate kinase from *Tolumonas auensis*
E	58	Lactamase B 2 Superfamily. Hypothetical protein from *Thermomicrobium roseum*
E	102	5-oxoprolinase from *Rhizobiales* bacterium YIM 77505

CHAPTER V

CONCLUSION

The goal of this thesis is to set up an experiment for the degradation of 2,4-dinitrophenol and other nitro organic compounds with environmentally relevant conditions. The soil microbes in the microcosms had all the essential nutrients for growth. The anaerobic mineral media included the addition of acetate as a carbon source and ammonia as a nitrogen source. The source of bacteria was soil collected at four inches of depth to provide anaerobic bacteria. The microcosms were kept in the anaerobic glove chamber for the duration of the experiment to maintain anaerobic conditions to favor the reduction of the nitro organics. The use of a natural nitro organic compound (nitroethyl benzene) as coamendment to the degradation of 2,4-dinitrophenol was also tested. Also a molecular method for the isolation of nitroreductases was attempted.

The anaerobic degradation of nitro organic compounds was not successful. The soil microbial communities used in the microcosms were mostly unable to degrade or transform the nitro compounds. Nitroethyl benzene coamandment had no impact on the degradation of DNP. This could have been the result of the high toxicity of these compounds or the bacterial enzymatic limitations. Another factor could have contributed to the high absorption reading is

incomplete dissolution of the compound when added to the microcosms. The novel PCR techniques for nitroreductases failed to amplify nitroreductases in our sludge or the DNP degrading sample.

- In this research, I found that coamendment of nitroethyl benzene did not have an impact on the degradation of DNP under anaerobic conditions.
- Gas chromatography and the spectrophotometry were not the ideal method to monitoring the change in concentration in the microcosms. With gas chromatography, the amino derivatives and the nitroethylbenzene were difficult to reproducibly extract and analyze. With spectrophotometry, there was no good separation of compounds which likely allowed other products interfere with the measurement. A liquid chromatography method would likely be more ideal for analytical measurement.
- The enrichment culture did not produce degradation patterns to confirm 2,4-dinitrophenol degradation.
- The degradation of many nitro organics with the soil bacterial communities under anaerobic conditions in this study was not successful, indicating the difficulty of degrading nitro-organics in contaminated environments.
- A novel phylogenetic analysis of nitroreductases was performed in this study which identified that these enzymes are deeply divergent and in general the *Eukaryotic* reductive dehalogenases share a unique homology than *Bacteria* and *Archaea* nitroreductases.

- Novel PCR methods with degenerate primers targeting unique nitroreductase groups were unsuccessful at detecting nitroreductases in anaerobic digestion sludge from WWTP and in the microcosms degrading 2,4-dinitrophenol. Nonetheless, these primers and methods provide a starting point for better design and optimization of culture-independent methods to study these enzymes.

In order to develop a bioremediation technique for the removal of nitro organic contaminants, more research should be done on the anaerobic degradation of these compounds in environmentally relevant conditions and with no limiting nutrients. Acclimation of microbial communities should be considered to overcome toxicity effects. In addition, a more specific GC method to measure the formation of degradation products of nitro compounds such as nitroso, hydroxyamine and amino compounds is needed. Finally, we need more understanding of nitroreductases and that starts with developing a successful method for isolation of the enzyme.

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APPENDIX A. Standard Curves for Analytical Methodologies

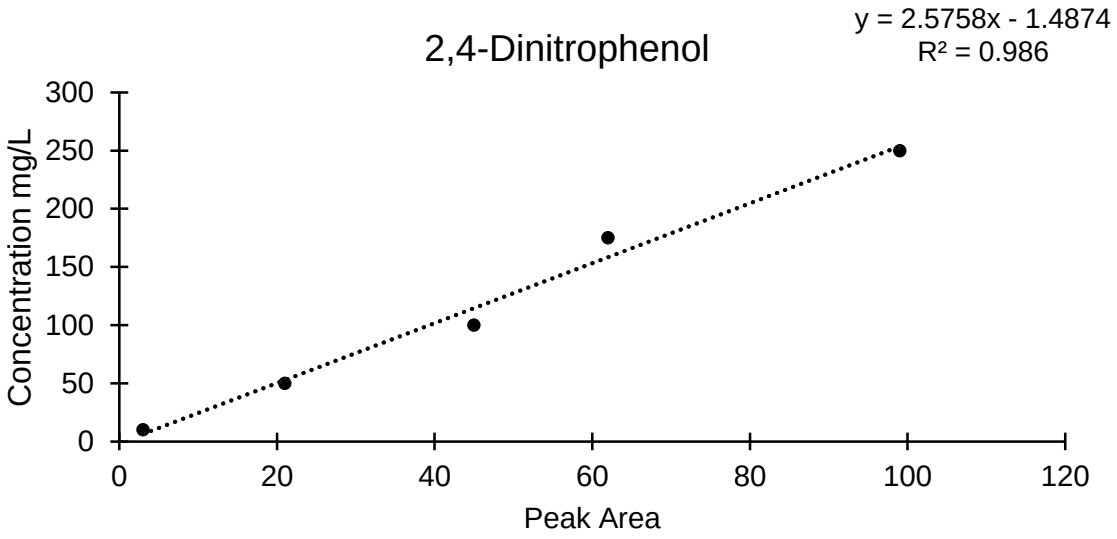

Figure A1. Calibration curve of 2,4-dinitrophenol plotted with peak area appeared at approximately 7.5 min against different concentration in µg/mL with gas chromatography method with an flame ionization detector.

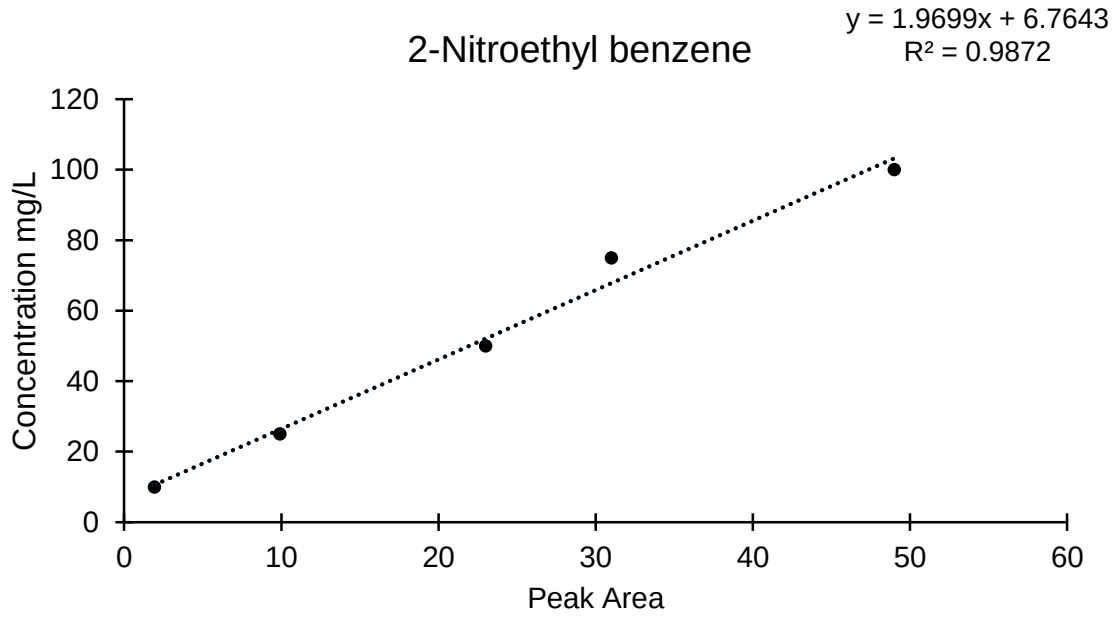

Figure A2. Calibration curve of 2-nitroethyl benzene. with gas chromatography method with an flame ionization detector plotted with peak area appeared at approximately 6.6 min against different concentration in µg/mL.

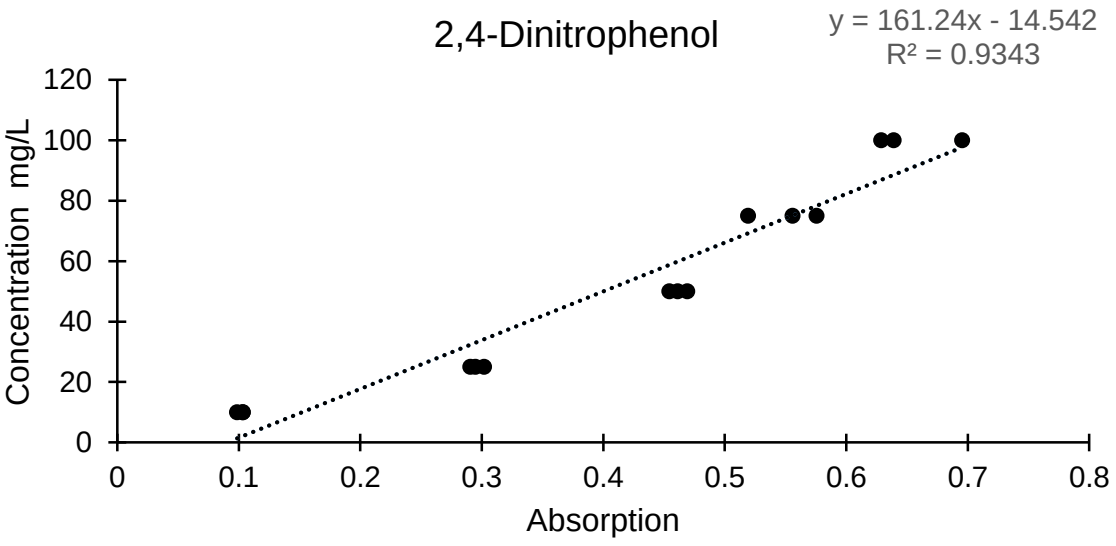

Figure A3. Calibration curve of 2,4-dinitrophenol with the spectrophometer. Absorption at 350 nm is plotted with different concentrations in mg/L.

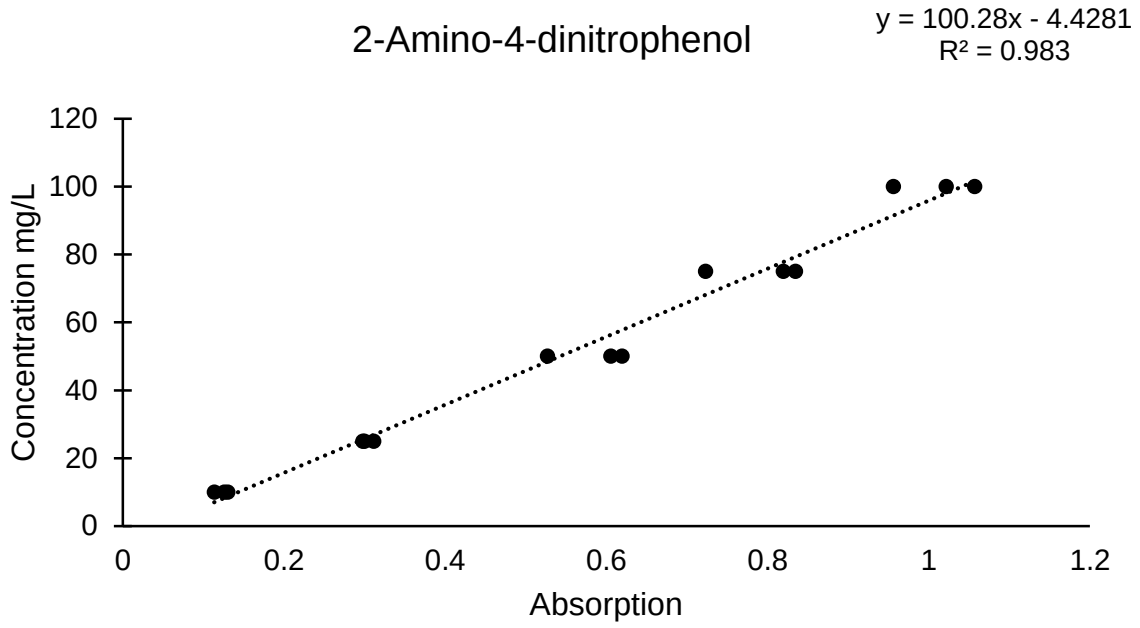

Figure A4. Calibration curve of 2-amino-4-dinitrophenol plotted with absorption appeared at 372 nm against different concentration in mg/L.

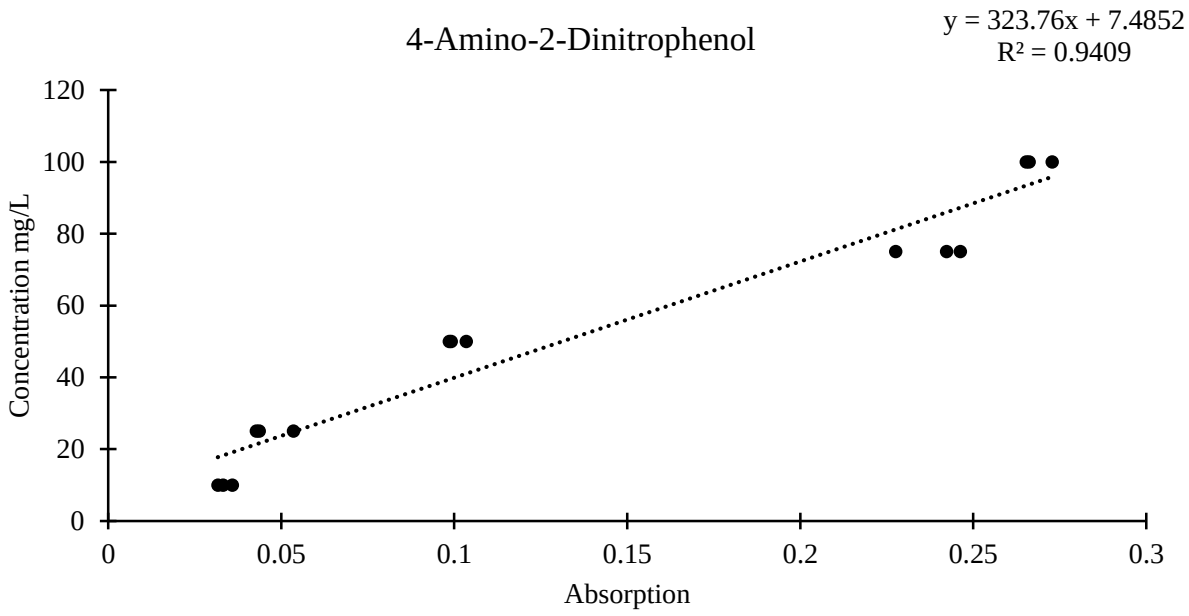

Figure A5. Calibration curve of 4-amino-2-dinitrophenol plotted with absorption appeared at 424 nm against different concentration in mg/L. R^2 value is close to one which means concentration of 4-amino-2-dinitrophenol closely related to absorption.

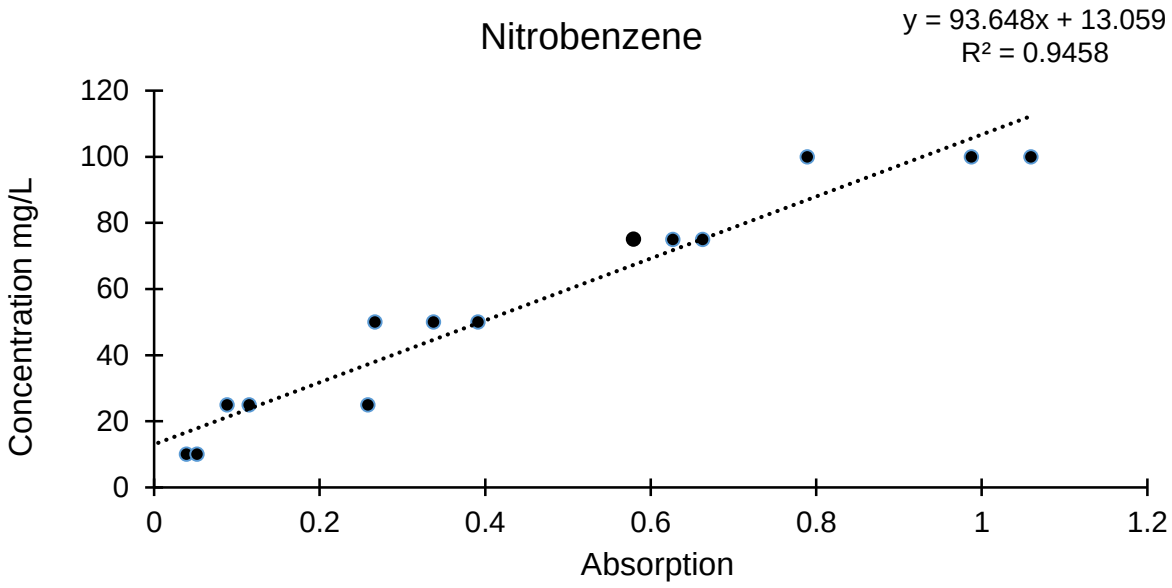

Figure A6. Calibration curve of nitrobenzene plotted with absorption appeared at 284 nm against different concentration in mg/L. R^2 value is close to one which means concentration of nitrobenzene closely related to absorption.

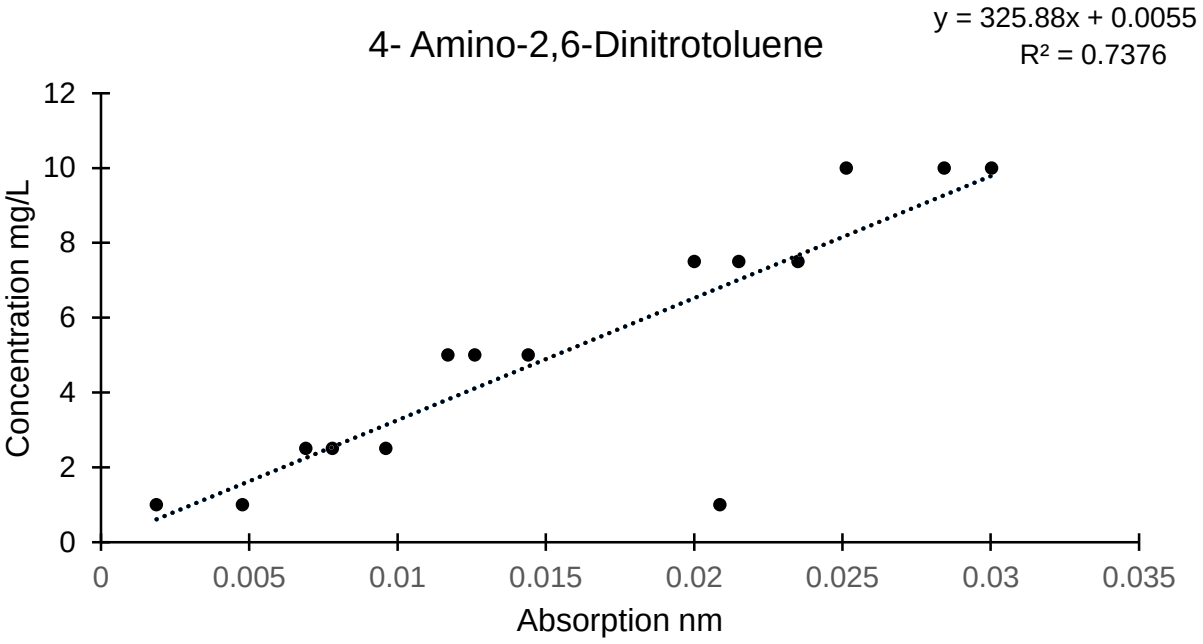

Figure A7. Calibration curve of 4-Amino-2,6-Dinitrotoluene plotted with absorption appeared at 360 nm against different concentration in mg/L. R^2 value is close to one which means concentration of 4-Amino-2,6-Dinitrotoluene closely related to absorption.

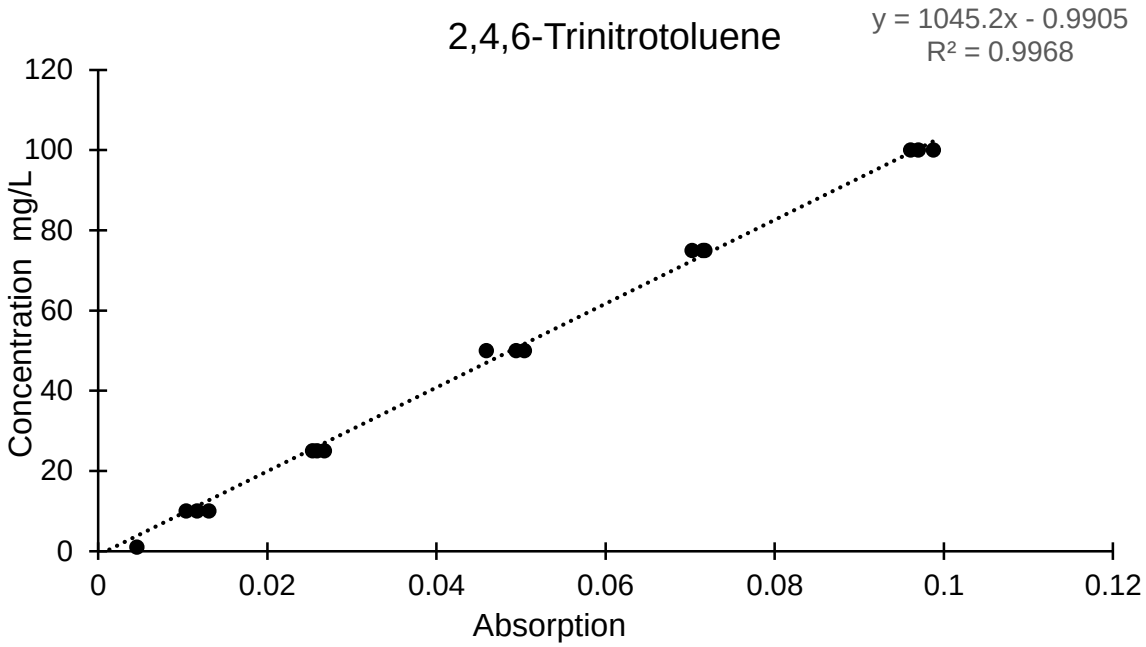

Figure A8. Calibration curve of 2,4,6-Trinitrotoluene plotted with absorption appeared at 340 nm against different concentration in mg/L. R^2 value is close to one which means concentration of 2,4,6-Trinitrotoluene closely related to absorption.

VITA

RUSSEL RAHI

Candidate for the Degree of

Master of Science

Thesis: RUSSEL RAHI

Major Field: ENVIRONMENTAL ENGINEERING

Biographical:

Education:

Completed the requirements for the Master of Science in environmental engineering at Oklahoma State University, Stillwater, Oklahoma in May, 2015.

Completed the requirements for the Bachelor of Science in microbiology at Oklahoma State University, Stillwater, Oklahoma 2012

Experience:

Environmental Lab Technician at Stover's Group.

Graduate Research Assistant, dissertation focused on nitroreductases and the anaerobic degradation of nitro organic in soil advised by Dr. Mark J. Krzmarzick,

Graduate Teaching Assistant for Arabic I and Arabic II at Oklahoma State University.