PHOSPHATASE ACTIVITY IN ANIMAL MANURE-AMENDED SOILS AND MOLECULAR PROFILING OF MICROBIAL COMMUNITY IN TNT-SPIKED SOILS

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FORMAT OF THESIS

This thesis is presented in a combination of formats as outlined by Environmental Microbiology, Applied and Environmental Microbiology journals and Oklahoma State University graduate college style manual. The use of this format allows the independent chapters to be suitable for submission to scientific journals. Each paper is complete in itself with an abstract, introduction, materials and methods, results, discussion and reference sections.

Chapter I

INTRODUCTION

Microorganisms mediate nutrient cycling and degradation of organic materials in soil. Microbial responses to anthropogenic alterations of soil fertility and quality are often reflected by changes in microbial activity and community structure. Therefore, microbial activity and community structure are sensitive indicators of environmental impact (Kandeler et al. 1999; Zak et al. 1994) and soil health (Doran et al., 1994).

Agricultural production increasingly depends on input of large quantity of chemical fertilizers to maintain productivity. However, nutrient input exceeding crop requirements could become environmental contaminants. Ample evidence suggests that phosphorus (P) and nitrogen (N) from agro-ecosystems may contaminate nearby water bodies through leaching and runoff, resulting in eutrophication (Whalen and Chang, 2001). Environmental concerns are intensified with increasing development of confined animal feeding operations. Large quantity of animal manure is often applied to a limited land area nearby the facility, exceeding nutrient requirement for crop production. It is reported that Mehlich-3 extractable P in animal manure amended soil reached over 1100 kg P ha^{-1} soil (Reed et al., 1998). Some of the accumulated P may find its way to water bodies nearby.

Environmental impact by human activities is not limited to agricultural production. Manufacturing and processing of chemicals generated noticeable

quantity of environmental contaminants and left the nation with abundance of hazardous sites to clean and reclaim. Many of the contaminants are toxic to microorganisms and suppress microbial activity to limited degradation activities, resulting in persistence of these contaminants. For instance, over 50 years of soil contamination with explosives, cultural microbial population was reported to be as low as 5-colony-forming unit g^{-1} soil (Meyer, 2002).

Seeking solutions for these environmental problems demands understanding of microbial responses to these environmental impacts. Microorganisms and their released enzymes dictate transformations of nutrients to forms that are more mobile, subject to leaching, or less mobile and retained in soil. Although toxic wastes often significantly reduce microbial activity in a contaminated environment, extensive studies indicated that many microbes persist in toxic waste contaminated sites. These persisting microbes may not only be tolerant to the toxic compound, but may also possess the ability to break down the toxic waste and detoxify the environment. Identification of such microorganisms is desirable for potential application in bioremediation of contaminated sites.

Therefore, the objectives of this study were (1) to determine the effects of TNT contaminations on soil bacterial community, aiming at identification of unique bacterial strains induced by TNT contamination; and (2) to evaluate the impact of manure application on soil P levels and phosphatase activities. Understanding biological and biochemical properties to prevailing soil conditions can benefit soil management strategies.

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Chapter II

LITERATURE REVIEW

A. Animal Manures in Agriculture

Animal manures are not only nutrient sources for plant growth, but can also contribute to enhanced aggregation, and improved soil structure for better aeration (Lal, 2004). As a leading producer of animal products (i.e. 86 billion pounds of meat and poultry products in 2002), the United States generated more than 350 million tons of animal manure from livestock and poultry farms in 2002 (Ribaudo, 2003). In Oklahoma, Concentrated animal feeding operations (CAFO) generate nearly 9 million tons of manure from cattle, poultry, horse, and swine in a year (Johnson et al., 2000).

Environmental concerns of land applied manure

Due to the confinement, large quantity of animal manure is applied repeatedly to limited land area near CAFO, exceeding the needs for crop production. As are a result, environment concerns of nutrient loading in nearby water bodies are intensified (Beegle and Lanyon, 1994).

One nutrient of the concern is phosphorus (P). Soils usually contain between 100 to 3000 mg P kg^{-1} soil as orthophosphate compounds and organic forms of P can range from 30-65% (Harrison, 1987). Both inorganic and organic P (Po) forms are found in animal manure (Peperzak et al., 1959). P loading in

animal manure is in part due to a compound known as phytate. Phytate (inositol hexaphosphate) is a P-storage compound in plant seeds. Approximately 75% of P in plant seeds is in the form of phytate (Nelson et al., 1968; Morse et al., 1992). Unfortunately, monogastric animals, including humans, chicken, and pigs, do not sustain microbes in their intestine to digest phytate. As a result, 70% of the total P ingested by domestic farm animals is excreted (Church, 1979). The concern is that application of animal manure exceeding crop requirements would lead to P accumulation in soil, which may ultimately result in potential P contamination in waters. It has been reported that soil test P (Olsen P) increased from 15 to 187 mg kg^{-1} following over 35 years of manure applications at 37–270 kg P ha^{-1} yr^{-1} (Sharpley and Smith, 1995). The accumulated soil P could reach water bodies through leaching or run off (Edwards and Daniel, 1993). Run off concentrations of total phosphorus ranged between 47 to 300 mg/L (Novotny and Harvey, 1994).

Other nutrients such as nitrogen (N) resulting from animal manure application are also of environmental concern. Ammonia emission from animal manure is a prime concern for air quality because approximately 50% of the total U.S. anthropogenic ammonia emissions are contributed from animal production activities (Van Aardenne et al., 2001). Ammonia emission not only degrades air quality, but also could lead to acid deposition (Roelofs and Houdijk, 1991). Ammonia deposited on land can also damage vegetation (Holtan-Hartwig and Bøckman, 1994; Van der Eerden et al., 1998) and reduce plant biodiversity in natural ecosystems (Heil and Bruggink, 1987; Sutton et al.,

1993). In addition to ammonia emission, animal manure degradation could result in the release of other gasses such as nitrous oxide (N_2O) and methane (CH_4) that contribute to global warming and stratospheric ozone depletion (Crutzen, 1981). These concerns accompany increased animal production in the U.S and the average amounts of N and P in manure generated per livestock operation of 1000 animal units (AU) nearly doubled between 1982 and 1997 (from 4,910 to 10,180 kg N $farm^{-1}$; from 2,110 to 4,740 kg P $farm^{-1}$) (Kellogg et al., 2000).

Regulations for land application of manure

The U.S. federal legislation has set several standards and legislations to regulate the environmental issues arising out of land-applied manure. The recent ones include the promulgation of 40 CFR Part 503 regulations for land-applied biosolids (USEPA, 1993), the USDA-NRCS Conservation Practice Standards for Nutrient Management Code 590 (USDA, 1999), the US Environmental Protection Agency (USEPA, 2003) concentrated animal feeding operation (CAFO) regulations, and the USDA National Organic Standards (USDA, 2000). The phosphorus issues were considered in Code 590 nutrient management standards which required the application of animal wastes based on nitrogen when phosphorus losses are low (USDA, 1999) and it also required that in case of any negative effects on the environment from a CAFO, the operation must obtain a nutrient management plan at any point of time. Meeting nutrient application standards will require CAFOs to spread their manure over a

much larger land base than they are currently using, and most will need to move their manure off the farm (USEPA, 2003). The USEPA has proposed new regulations requiring operations with the large number of animals to manage their manure according to a nutrient management plan. More recently, a revised regulation has been released by the agency about the concentrated animal feeding operations to improve management practices for livestock waste and it established performance expectances for manure storage, wastewater management, and land use (USEPA, 2003).

Methods for quantification of soil phosphorus

Phosphorus in soil is often quantified by extracting P with a chemical extractant, followed by determination of P in solution using a colorimetric method or by Inductively Coupled Plasma (ICP). These procedures are based on the assumption that chemical extractants selectively dissolve discrete groups of P compounds; therefore, such operationally defined P fractions are subject to broad interpretation (Sui, et al., 1999).

For plant available P, extraction methods vary depending upon soil types and mechanisms controlling the chemistry of soil P. Various soil extractant s are available for acid soils where Al and Fe dominate P chemistry and for basic or calcareous soils where Ca dominates soil P reactions (Pierzynski, 2000). For multi-element extractions, Mehlich-3 soil test is widely accepted and used for P, K, Ca, Mn, Cu, Fe, Mg, and Zn estimations (Mehlich, 1984). The Mehlich-3 extractant is an acidic solution prepared using ammonium fluoride and acetic

acid contributing to the release of available P in most soils and the determination of extracted orthophosphate P is based on a colorimetric method (Mehlich, 1984). Mehlich-3 test is suitable for wide range of soils, both acidic and basic in reaction and hence it has been proposed to be used as a standard reference procedure for soil test P determination (Tucker, 1992). A Mehlich-3 value of 45-50 mg P/kg soil is generally considered optimum for plant growth and crop yields (Pierzynski, 2000) and in manure applied soils, Mehlich 3 extractable P can reach up to 1,121 kg P ha^{-1} $year^{-1}$ (Reed et al., 1998).

For total soil phosphorus extractions, most commonly used methods are sodium carbonate (Na_2CO_3) fusion and acid digestion methods. Dick and Tabatabai, (1977) proposed an alkaline oxidation method using sodium hypobromite (NaOBr) and it is known to give better results than original method wherein $HClO_4$ Digestion was used (Pierzynski, 2000).

Phosphorus in solutions is often quantified by colorimetric methods because of their high sensitivityand reproducibility. These methods are based on reaction of P with molybdenum that forms molybdenum blue, which is proportional to P concentration in the solution (Olsen and Sommers, 1982). The most commonly used method was the one developed by Murphy and Riley (1962). Inductively Coupled Plasma (ICP) spectrophotometry for solution P quantification is based on characteristic optical emission of atoms excited in a high-temperature (5000–8000 K) Ar (Argon) plasma wherein the molecules injected into the plasma undergo instantaneous vaporization, dissociation, and ionization. ICP measures total P in the solution (Soltanpour, et al., 1996). This

method is gaining preference over colorimetric methods because it provides simultaneous analysis of many elements in addition to P (Khiari et al., 2000; Masson et al., 2001). It is reported that the P estimations from colorimetric analyses are not always directly comparable to those from ICP because ICP determines total P in solution, while colorimetric procedures measure only the color-reactive P (Pierzynski, 2000). Measurement of P with ICP is reported to be giving 50% higher P estimations than those using colorimetric methods and the additional P could be mainly organic P (Hylander et al., 1995; Eckert and Watson, 1996; Masson et al., 2001).

Phosphorus transformation in soil

Transformation of nutrients in soil is mediated mainly by microbiological activities. Microorganisms are known to be involved in regulating the size and dynamics of different pools of soil P (Stevenson, 1986; Richardson, 1994). Many transformations in soil are governed by enzyme activities where most enzymes are of microbial and plant origin (Tabatabai, 1994). Widely studied enzyme systems in soil include oxidoreductases, transferases, and hydrolases (Tabatabai, 1994).

There are several phosphatases that have been detected in soil, including alkaline and acid phosphomonoesterases (EC 3.1.3.1 and EC 3.1.3.2), phosphodiesterase (EC 3.1.4), and inorganic pyrophosphatase (EC 3.6.1.1). Methods for quantification of these enzymes are described in detail in Tabatabai (1994), which are well established and widely used.

B. Explosives and Nitroaromatic Compounds

Nitroaromatic compounds, including explosives, pesticides, and herbicides, are common organic contaminants found in soil. Explosives are one group of nitroaromatic compounds that are widespread in military site, resulting from manufacture and handling of explosives (ATSDR, 1995). There are three classes of explosives, including nitroaromatics, nitroamine and nitrate esters (Kaplan, 1998). Explosive nitroaromatics include 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotolene (2,6-DNT), *N*-2, 4, 6-tetranitro-*N*-methylamine (tetryl), and picric acid (2, 4,6-trinitrophenol). The nitramines include hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 1-acetylhexahydro-1,3-dinitro-1,3,5-triazine (AcRDX), 1-acetyloctahydro-3,5,7-trinitro-1,3,5,7-tetrazocine (AcHMX), and nitroguanidine. The nitrate esters comprise of nitroglycerin (glycerol trinitrate), nitrocellulose, propylene glycerol dinitrate, triethylene glycol dinitrate, trimethylolethane trinitrate, hydroxylammonium nitrate (HAN), isopropyl ammonium nitrate (IPAN), trimethyl ammonium nitrate (TMAN), triethanol ammonium nitrate (TEAN), triaminoguanidine nitrate, and pentaerythritol tetranitrate (PETN) (Kaplan, 1998). The principal contaminants polluting munitions sites include 2,4,6-trinitrotoluene (TNT), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) which is often used in combination with octahydrol-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Pennington, 1998).

Historical perspective

Explosives entered human civilization thousands of years ago. It was during 220 B.C that blackpowder or gunpowder was accidentally prepared by Chinese alchemists and later during 13th Century and an English monk, Roger Bacon, prepared blackpowder using potassium nitrate and used it for mining and tunneling operations (Akhavan, 2004). During the mid 19th century, a Swedish chemist, Alfred Nobel, invented dynamite by using mixture of nitric and sulfuric acids and nitroglycerine. He developed a 'blasting cap' also patented the dynamite mixture known as ghur dynamite in 1867 (Russell, 2000). Alfred Noble also used ammonium nitrate in his later experiments as ammonium nitrate powder was known to explode upon mixing with charcoal and for his experiments, he is known as father of explosives. During 1888, blackpowder was replaced by Picric acid or trinitrophenol ($C_6H_3N_3O_7$) in British munitions and first military use of explosives was reported (Akhavan, 2004).

Trinitrotoluene (TNT) was first prepared by Wilbrand in 1863 and produced by DuPont company in Wilmington, DE in 1880 (World Book Online Americas Edition, 2002). Subsequently, the German Army used TNT instead of picric acid starting 1902. In 1912, the US Army started the use of TNT. By 1914, TNT was the standard and most widely used explosive for all armies involved in World War I. The use of nitramine and other explosives including RDX, HMX and PETN, did not start until World War I was over (Gilbert, 1980; Boileau et al.,

1987). During World War II, there were nearly ten different types of explosives or explosive compositions in use (Akhavan, 2004).

The industrial production of explosive ordnance and decommissioning of old ordnance have been carried out on a vast scale, resulting widespread contamination in soils near manufacturing sites (Spain et al., 2000). Although TNT is no longer been produced in the US, years of past manufacturing activities have resulted in many contaminated sites. Compounds like 2,4-DNT and 2,6-DNT are still being produced in the US by DuPont Company, Air products and Chemical Inc.

In addition to explosives, nitroaromatics contaminations may also come from productions and application of agricultural chemicals and manufacturing process. These contaminants include dyes, polyurethane foams, plastics, pesticides, herbicides, insecticides, solvents and pharmaceuticals (Davis et al., 1997; Snellinx et al., 2002). Agricultural use of pesticides like Dinoseb, Dinitrocresol, Parathion, Methylparathion (Spain et al., 2000), and use of RDX as rodenticide (Wildman and Alvarez, 2001) often lead to intentional release of nitroaromatic compounds in soil.

It was estimated that there were more than 1000 sites contaminated with explosives, of which >95% were contaminated with TNT (Walsh et al., 1993). There were about 50 million acres of land contaminated by bombing and training in the USA alone (Armstrong, 1999b, 1999a).

Explosives not only cause physical injury and property damage by detonation, they also have detrimental impact on human health. TNT has high

intrinsic toxic potential on mammalian including humans, aquatic life (algae, fish, and bacteria) and terrestrial organisms (plants, soil invertebrates) (Simini et al., 1995; Robidoux et al., 1999). Depending on the type of explosive compound, health concerns associated with exposure vary and the Environmental Protection Agency (EPA) has set the lifetime exposure drinking water advisory limits for TNT, RDX, and HMX as 2, 2, and 400 $\mu g\ L^{-1}$, respectively (Crockett A. et al., 1999). During the handling of nitroaromatic compounds, dinitrotoluenes (mixture of 71-77% 2,4-DNT and 18-20% 2,6-DNT) can enter into human body via the skin, the respiratory tract, and the gastrointestinal tract leading to formation of methemoglobin (MetHb) in the blood that can be detrimental to health. Furthermore, general symptoms such as headache, irritation of the mucous membranes, nausea, and vomiting can also be seen in people exposed to these chemicals (National Institute for Occupational Safety and Health, 1985). The exposure to TNT through inhalation or skin can cause anemia, cataracts, headache, skin irritation, and liver injury (Morton et al., 1976; Hathaway, 1977). More over, TNT and some other nitroaromatic compounds including 2-amino-4-6-DNT and 2,6-diamino-4-nitrotoluene can act as mutagens (George et al., 2001). Thus, the U.S. EPA has designated TNT as a hazardous waste (U.S. EPA, 1990).

The exposure to RDX occurring through skin and inhalation was reported as early as 1965 (Kaplan et al., 1965) and being termed as a class C carcinogen, it is known to cause epileptiform seizures and unconsciousness in humans (Harvey et al., 1991). The minimal risk level (MRL) (an estimated dose

not likely to cause adverse systemic effects) for RDX is set to 0.03 mg kg^{-1} day^{-1} (Mclellan et al., 1998).

Explosives once entered into soil, can also contribute to groundwater contamination because solubilized RDX and HMX could leach into groundwater (U.S. Army, 1979, 1986). Among the 1000 sites contaminated with TNT in the U.S., nearly 87% of them had ground waters that exceeded the permissible contaminant levels (Walsh et al., 1993).

Biodegradation of 2,4,6-trinitrotoluene

Microorganisms in the environment have the ability to break nitroaromatic compounds, which may result in detoxification of these organic contaminants. Biodegradation of nitroaromatic compounds involves the activities of aerobic, facultative and anaerobic bacteria as well as fungi (Esteve-Nunez et al., 2001). Nitroaromatic compounds can be degraded through anaerobic and aerobic pathways by bacteria or fungi (Spain, 1995). Anaerobic bacteria break down nitroaromatic compounds by reducing the nitro group via nitroso and hydroxylamio intermediates to the corresponding amines. Subsequently these amines can be degraded aerobically or anaerobically to small aliphatic acids to enter metabolic pathways for complete degradation. Under aerobic degradation, bacteria and fungi use nitroaromatic compounds as growth substrates. The initial step in the degradation often involves removal or metabolism of nitro groups, followed by ring cleavage, and then degradation to small aliphatic acids for subsequent degradation (Spain, 1995). Mechanism and

degradation pathways of TNT are further discussed below as examples to illustrate the concepts.

Aerobic bacterial degradation

There are three known aerobic TNT degradation pathways (UMBBD, 2005). Pathway A involves two bacteria or actinomycetes using nonspecific NAD(P)H nitroreductases during the entire process with a final product 4-acetamido-2-amino-6-nitrotoluene. Pathway B involves only bacterial species, but uses some specialized enzymes such as nitrobenzene reductase and 4-amino-2-nitroso-6-nitrotoluene reductase (degradation is less complete, with the final product 4-amino-2-nitroso-6-toluene), and pathway C involves bacteria as well, but uses nonspecific NAD(P)H reductases like pathway A. Pathway C differs in the metabolites produced during degradation, ending with the same final metabolite as pathway A. In addition, an aerobic degradation pathway for TNT via 2,4-dihydroxylamino-6-nitrotoluene (2,4-DHANT) and 2-hydroxylamino-4-amino-6-nitrotoluene (2HA4ANT) by *Pseudomonas pseudoalcaligenes* JS52 was reported (Fiorella and Spain, 1997).

Although both gram-positive and gram-negative bacteria were reported to be involved in TNT degradation, research data suggested that culturability of gram-positive bacteria were severely reduced by TNT in media (Fuller and Manning, 1997). On the other hand, gram-positive bacteria were shown complete degradation of several nitroaromatic compounds at concentrations

exceeding 100 μg mL^{-1} (Gundersen and Jensen, 1956; Lenke and Knackmuss, 1992; Lenke et al., 1992).

Anaerobic bacterial degradation

The original studies on biodegradability of nitroaromatics indicated that anaerobic biodegradation was not possible (Kaplan, 1992). However, pure culture studies demonstrated that strictly anaerobic organisms could completely mineralize recalcitrant aromatic compounds such as toluene and naphthalene (Rabus et al., 1993; Galushko et al., 1999). Although no strict anaerobe has yet been isolated that can completely mineralize TNT, there are a few reports of microbial consortia that can oxidize TNT partially or completely in the absence of molecular oxygen (Funk et al., 1993; Boopathy and Manning, 1996). Preliminary investigations indicated that Fe (III) reducing bacteria (FeRB) might play a significant role in the transformation of nitroaromatics in the natural environments (Hofstetter et al., 1999).

There are four possible microbial pathways in anaerobic degradation of TNT (UMBBD, 2005). Pathway A involves a bacterial species or consortia that use specific enzymes such as nitrobenzene reductase during the reduction of TNT. The metabolites produced include the dead end intermediate 2,4-dihydroxyl-amino-6-nitrotoluene and 4-amino-2,6-dinitrotoluene, which joins into the main pathway. Pathway B has many possible bacterial species which also produces the metabolite 4-amino-2,6-dinitrotoluene, but with a nonspecific NAD(P)H nitroreductase that merges into the center pathway. Pathway C

involves three possible bacterial species using a nonspecific NAD(P)H reductase producing 2-amino-4,6-dinitrotoluene that merges into the main pathway. Pathway D uses an anaerobic consortia and a nonspecific NAD(P)H nitroreductases producing the intermediate 2-amino-4,6-dinitrotoluene that merges into the center pathway. After the four pathways merge the final products of degradation include 4-hydroxytoluene, which can be used in the toluene pathway or uncharacterized organic acids.

The sequential reduction of the nitro groups of TNT by a *Desulfovibrio spp.* growing on pyruvate and sulfate with TNT as the sole nitrogen source (Preuss et al., 1993). *D. sulfidogen* reduced TNT completely to triaminotoluene, which was further transformed to unknown compounds. In this study, 2,4-diamino-6-nitrotoluene was reduced to triaminotoluene by *Clostridium pasteurianum* and *Clostridium thermoaceticum*, in which 2,4-diamino-6-hydroxy laminotoluene was an intermediate. A hydrogenase from *C. pasteurianum*, and a carbon monoxide dehydrogenase from *C. thermoaceticum* catalyzed the reduction, with methyl viologen and/or ferrodoxin as electron carriers. Thus, ferrodoxin-reducing enzyme systems, such as sulfite reductase (Livingston, 1993), play an important role in the complete reduction of nitroaromatic molecules by these anaerobes. Research data suggested that *Clostridium bifermentans* degrade TNT and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in the presence of co-substrates (Crawford, 1995).

Fungal degradation

Of the biological systems investigated, fungi have proven to be the most effective in the degradation of nitroaromatic compounds (Spain, 1995). However, hydroxylamino intermediates produced during the process are toxic to degraders (Spain, 1995). As a result, the rates of degradation are limited. In general, fungal degradation of nitroaromatic compounds has received little attention other than a modest amount of work on white-rot fungi. The wood-decay white-rot basidiomycete fungus *Phanerochete chrysosporium* has been shown to degrade nitroaromatic compounds, such as TNT, through lignolytic enzymes (Field et al., 1993). Although lignolytic enzymes are not involved in the initial degradation (Stahl and Aust, 1993; Michels and Gottschalk, 1994), these are essential to further degrade amino-dinitrotoluenes through oxidative attack, which eventually lead to complete mineralization (Stahl and Aust, 1993; Michels and Gottschalk, 1994).

However, much of the recent studies indicated concerns about the feasibility of using *P. chrysosporium* for treatment of TNT-contaminated soils because TNT suppress growth of this fungus (Spiker et al., 1992). White rot fungi have a major limitation as bioremediation agents. Establishing mycelium in soil for bioremediation purposes requires the soil be inoculated with the fungus growing on an organic substrate (usually wood chips) (Lamar et al., 1993). Once the carbon source has been depleted, they will become ineffective in bioremediation unless additional woody substrates are provided (Meharg et al., 1997).

Achromobacter spp. in chemical degradation

Achromobacter sp. is a group of gram-negative, nonfermenting bacteria. Originally described by Yabuuchi and Ohyama (1971), these organisms are known as causal agents of diseases like meningitis (Shigeta et al., 1978; Namnyak et al., 1985), pneumonia (Dworzack et al., 1978), surgical wound infections (Pien and Higa, 1978), septicemia (Holmes et al., 1977), urinary tract infections, peritonitis and pharyngitis (Igra-Siegman et al., 1980). It has also been isolated from aqueous environmental sources, some of which have been associated with nosocomial outbreaks of infections (Shigeta et al., 1978; McGuckin et al., 1980; Reverdy et al., 1984).

Achromobacter spp. were shown to be involved in degradation of some chemical compounds, including nicotine (Hylin, 1958), and pesticides Carbofuran (Karns et al., 1986). These organisms are capable of producing N-methylcarbamate hydrolase (carbofuran hydrolase) enzyme to hydrolyze a range of N-methylcarbamate insecticides, including Carbaryl and Aldicarb. (Derbyshire et al., 1987; Tomasek and Karns, 1989). The bacterial strain *Achromobacter piechaudii* TBPZ-N61 was used in degrading 2,4,6-tribromophenol (TBP) that displayed kanamycin resistance as a selective marker (Nejidat et al., 2004).

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Chapter III

IMPACT OF ANIMAL WASTE AMENDMENTS ON PHOSPHORUS LEVELS AND ACTIVITY OF PHOSPHATASES IN SOIL

Abstract

Soil phosphorus (P) accumulation following long-term manure applications exceeding crop needs leads to concerns of soil health and subsequent potential threat to nearby water bodies. Studies were conducted to investigate effects of swine effluent and beef manure on soil P levels in a continuous corn experiment established in the southern Great Plains. Beef manure and swine effluent were each applied at 56, 168, and 504 kg N ha^{-1} yr^{-1} (corresponding to P rate ranging from 4 to 91 kg P ha^{-1} yr^{-1}) for consecutive six years. Total soil P levels ranged from 400 to 770 mg P kg^{-1} soil with the highest detected in the high rate beef manure-treated soil. Phosphorus accumulation in comparison to the controls was detected mostly in the surface soils of 0-10 cm depth following beef manure application. Annual addition of swine effluents at the two lower levels did not result in detectable increase of total soil P. Similar trends were observed for Mehlich-3 extractable P. Among the phosphatase activities evaluated, acid and alkaline phosphomonoesterase activities dominated at 0-10 cm soils, and decreased with increasing soil depth; while activities of phosphodiesterase and inorganic pyrophosphatase increased with increasing soil depth up to 30 cm. These two distinctly different trends among

the four enzymes evaluated imply potential differences in origin and activity of phosphatases in agro-ecosystems.

Introduction

Soils sustain immense diversity of microorganisms essential for nutrient cycling in terrestrial ecosystems. Due to close association between microbial diversity, soil and plant quality, and ecosystem stability, the microbial characteristics are often considered as sensitive indicators of soil health (Doran et al., 1994). Soil microbial community is composed of physiologically heterogeneous (Korsaeth et al., 2001) and diverse microbial groups (Curtis et al., 2002; Zhou et al., 2002), and the nature of soil microbial community depends largely on land use practices (Bossio et al., 1998; NRCS, 2004). Characterization of a community composition is important in assessing the impact of soil management practices on soil health.

In addition to meeting crop nutrient demands for the disposal of animal manure agricultural producers and animal feeding operations increasingly rely on land applications of large quantities animal manure that often exceed agronomic demands (NRC, 1993; Gollehon, et al., 2001). The long term and repeated applications of manure may lead to accumulation of soil phosphorus (Whalen and Chang, 2001) and could contribute to potential contamination of adjacent water bodies (James et al., 1996).

It has long been recognized that the cycling and transformation of nutrients in soil are closely associated with microbial activities and studies conducted by Parham et al., (2002, 2003) suggested that long-term application of cattle manure may result in increased soil P mobility and a shift of soil microbial community structure. Therefore, this study was initiated to assess the impact of animal manures and anhydrous ammonia on soil P levels and activities of phosphatases at soil depths of 0-30 cm.

Materials and Methods

Soil sampling and preparation

Soil samples were taken in March, 2000 from a continuous corn (*Zea mays* L.) experiment initiated during 1995 in western Oklahoma on a Richfield (fine, smectic, mesic Aridic Argiustolls) clay loam soil. The mean particle-size distribution of soils was 30% sand, 42.5% silt, and 27.5% clay. Treatments included beef manure, swine effluent, and anhydrous ammonia each applied at 56, 168, and 504 kg N ha^{-1} yr^{-1} (referred as L, M, and H, respectively). Controls received no fertilizer amendments. Experimental design and soil sampling procedure can be found in Deng et al (unpublished). Field-moist soil samples were sieved to pass through a 2-mm screen, mixed and divided into two parts. One part was air-dried for chemical analysis and the other was stored at 4^{o}C and kept field-moist for use in biochemical and microbiological analyses.

Soil chemical properties are reported in Deng et al. (unpublished). Briefly, pH values in control and manure treated soils ranged from 7.1 to 7.5, organic carbon contents ranged from 7.0 to 15.6 g kg^{-1} soil, and total N contents ranged from 0.76 to 1.72 mg N kg^{-1} soil. Soils with high beef manure treatments showed the highest organic carbon and total nitrogen contents when compared with control and other treatments. In anhydrous ammonia treated soils, soil pH valued ranged from 4.3 to 6.9, which decreased with increasing application rates. When compared with the control soils, anhydrous ammonia treatments did not affect soil organic carbon and total nitrogen contents significantly in majority of anhydrous ammonia added soils matched closely with control. The detailed soil properties table is included in Deng et al (unpublished).

Total and Mehlich-3 extractable phosphorus

Total soil phosphorus analysis involved acid digestion of organic phosphorus and then subjecting directly to inductively coupled argon plasma atomic emission spectrometry (ICP-AES) for P analysis as described by Jones and Case (1990). Briefly, 1 g air-dried soil samples were wet digested for 48 h at room temperature and for 4 h at 120°C with 5 mL of concentrated nitric acid (HNO_3) and followed by 180°C for 2 h with 10 mL of Perchloric acid ($HClO_4$). The samples were then cooled, filtered and diluted to 50 mL with deionized water and P content in the solution was quantified by ICP-AES. The available

soil P was extracted using Mehlich-3 extractant (Mehlich, 1984), filtered through 0.45 μm, and analyzed by ICP analysis (Jones and Case 1990).

Phosphatase assay

Four phosphatases, including phosphomonoesterases (acid and alkaline phosphatases; EC 3.1.3.2 and EC 3.1.3.1 respectively), phosphodiesterase (EC 3.1.4) and inorganic pyrophosphatase (EC. 3.6.1.1), were determined. Activities of phosphomonoesterases were quantified by incubating soils in a buffer solution and with sodium ρ-nitrophenyl phosphate for one hour at 37°C followed by spectrophotometer measurement of ρ-Nitrophenol released at 405 nm (Tabatabai and Bremner, 1969; Eivazi and Tabatabai, 1977). For the phosphodiesterase activity, a similar procedure was used with bis-ρ-nitrophenol phosphate (BPNP) as the substrate (Browman and Tabatabai, 1978). Inorganic pyrophosphatase activity was determined by quantifying orthophosphate released during incubation of a soil sample in a buffer with pyrophosphate as the substrate (Dick and Tabatabai, 1978).

Statistical analyses were performed to test the significant difference among the treatments using Statistical Analysis Systems (SAS) and means were compared using Least Significant Difference (LSD) test at $P \leq 0.05$. All reported results are averages of duplicated assays and analyses.

Results

Soil phosphorus

When compared with the control soil there was significant increase in total P (720 mg P kg^{-1} soil) of surface soils applied with high rates beef manure (Fig. 1A). There was a trend that application of high rate swine effluents increased total soil P levels in surface soils, though this increase was not statistically significant. Overall, soil total P levels decreased with increasing soil depth. Similar trend was shown with Mehlich-3 P levels detected (Fig. 1B). High rates of beef manure treatments resulted in Mehlich-3 extractable P up to 156 mg P kg^{-1} soil. This was 2.6-9.8 fold increase in Mehlich-3 extractable P when compared with other treatments (Fig. 1B). Mehlich-3 extractable P increased with increasing swine effluent application rates. However, most of these increases were not statistically significant, with exception of comparing high and low rates for the surface soils. Mehlich-3 extractable P contents were 2.3 to 24.6 % of soil total P, with highest in high rates beef manure amended surface soils (Fig. 1C).

Phosphatases

Activity of acid phosphatase in beef manure and swine effluent treated surface soils of 0-10 cm depth did not differ significantly when compared with control soils (Fig. 2A). Medium and high rates anhydrous ammonia treatments

increased acid phosphatase activity significantly ($P \leq 0.05$) in surface 0-10 cm soils, but decreased in soils of 10-20 and 20-30 cm depths (Fig. 2B).

With the exception of anhydrous ammonia treated soils, alkaline phosphatase activities were significantly ($P \leq 0.05$) higher in the surface soils when compared with the lower soil profiles. Highest activity of alkaline phosphatase was shown in high rate beef manure treated surface soils, which was significantly ($P \leq 0.05$) higher than the control. Although low rates of anhydrous ammonia application did not affect alkaline phosphatase activity significantly when compared with control, increasing rates of anhydrous ammonia application led to significant decrease in alkaline phosphatase activity (Fig. 2B).

Phosphodiesterase and inorganic pyrophosphatase activities increased with increasing soil depth (Fig. 2C, D), a very different trend from those of phosphomonoesterase. Highest activity of phosphodiesterase detected was in 20-30 cm depth soils treated with high rate swine effluent. Among surface soils tested, high rate beef manure significantly increased phosphodiesterase activity when compared with the control. Though there was decreased activity of this enzyme in swine effluent treated surface soils, this decrease was not statistically significant. Phosphodiesterase activity decreased with increasing anhydrous ammonia application rates. The decrease was statistically significant for the lower soil profiles (10-20 and 20-30 cm depth) when compared within the

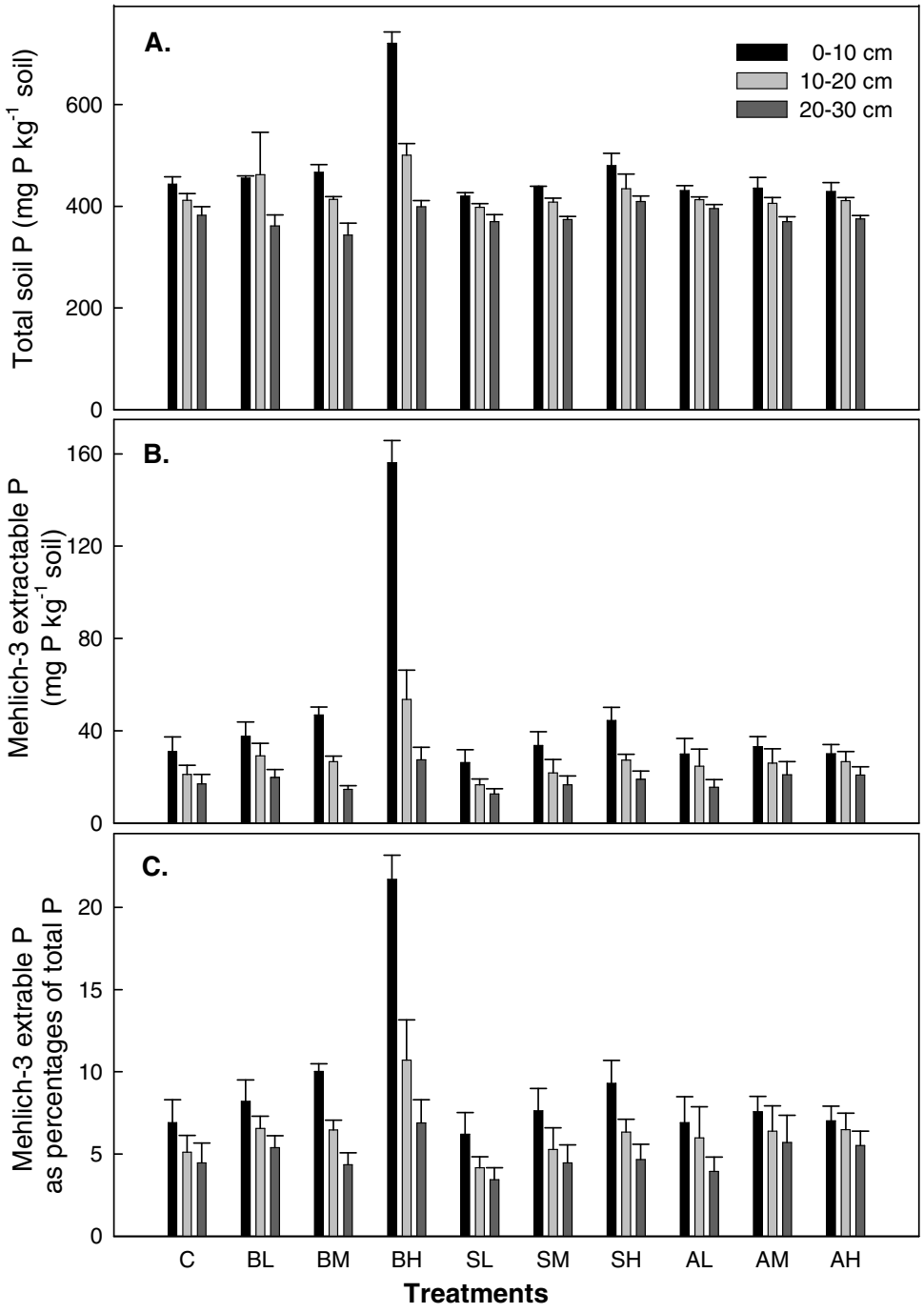

Fig. 1. Effect of different levels of animal manure and anhydrous ammonia applications on total phosphorus (A), Mehlich-3 extractable phosphorus (B) and percentages of Mehlich-3 extractable P in total P (C) in soils from 0-30 cm depth. Bars indicate standard error. C=Control, B=Beef manure, S=Swine effluent, and A=Anhydrous ammonia. Application rates were L=Low (56 kg N ha^{-1} yr^{-1}), M=Medium (168 kg N ha^{-1} yr^{-1}), and H=High (504 kg N ha^{-1} yr^{-1})

swine effluent application. When compared with the controls, the increase was statistically significant ($P \leq 0.05$) for the high rates swine effluent treated soils. Mixed responses were shown in the anhydrous ammonia treated soils. Although activity of this enzyme decreased with increase application rates of beef manure, none of the decrease was statistically significant when compared with the control.

In summary, high rates of beef manure and swine effluent treatments resulted in significantly higher activities of alkaline phosphatase and phosphodiesterase in surface 0-10 cm soils, while the activities of acid and inorganic pyrophosphatase were considerably enhanced by medium to high rates of anhydrous ammonia treatments.

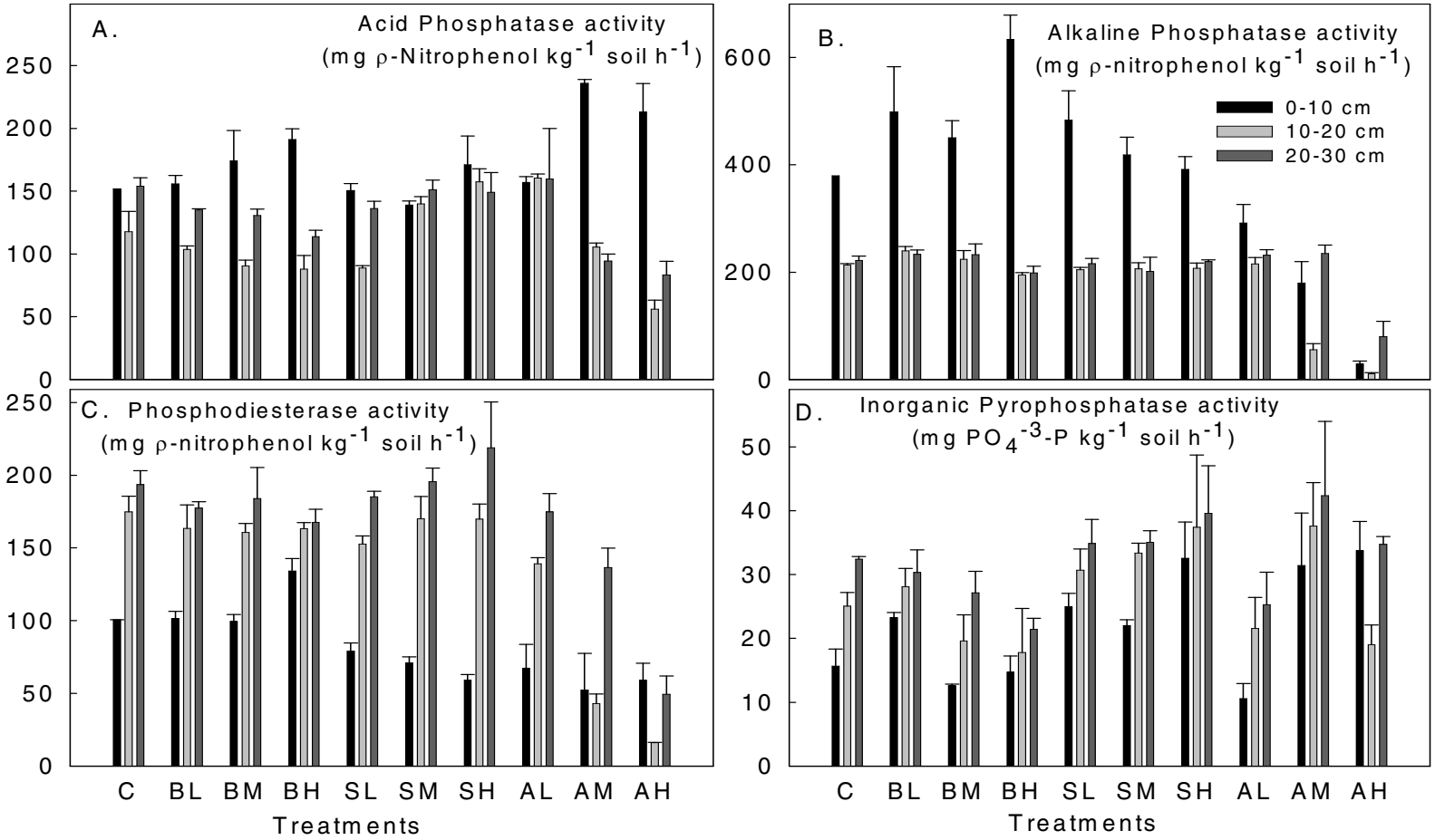

Fig. 2. Effect of different levels of animal manure and anhydrous ammonia on activities of acid phosphatase (A), alkaline phosphatase (B), phosphodiesterase (C), and inorganic pyrophosphatase (D) in soils at depths from 0-30 cm. Bars indicate standard error. C=Control, B=Beef manure, S=Swine effluent, and A= Anhydrous ammonia. Application rates were L=Low (56 kg N ha^{-1} yr^{-1}), M=Medium (168 kg N ha^{-1} yr^{-1}), and H=High (504 kg N ha^{-1} yr^{-1}).

Discussion

As soil microorganisms mediate organic matter decomposition and nutrient cycling depending on several factors including nutrient additions, results of this study indicated that soil P levels and biochemical soil characteristics varied depending on soil depth, manure type and rates of application. Significantly higher levels of P accumulation and Mehlich-3 extractable P in surface soils following addition of beef manure at high rates (nearly 1.5 fold for total P and about 4 fold for Mehlich-3 extractable P when compared with the control) depict impact of repeated and high rate manure applications on soil P. It was reported that the upper limit for crop response to P fertilizer was about 120 lb $acre^{-1}$ based on Mehlich-3 extractable P (Johnson et al., 2000). Repeated application of high rate beef manure led to soil Mehlich-3 extractable P reaching 156 mg P kg^{-1} soil, (equivalent to 312 lb $acre^{-1}$). Thus, soil test P in these soils exceeded crop response range, which could potentially contaminate adjacent water bodies through runoff water and subsurface drainage (Sharpley, et al., 1994; McDowell and Sharpley, 2001). However, swine effluent treatments may be of less concern, as they did not result in significant increase in total or Mehlich-3 extractable P levels. The percentages of Mehlich-3 extractable P in total P levels did not exceed 10% for most of the treatments, with exception of high rate beef treatments.

High P accumulation in beef manure applied soils could be in part due to greater P input from beef manure when compared with other treatment

evaluated. Cattle excrete around 80-85% of their P intake (Kornegay, 1997), resulting in high P content in beef manure. Nitrogen and P ratios in beef manure were reported to be around 1, while those in swine effluent were around 4 (Zhang et al., 1998). Manure application rates were N-based. Therefore, about 4-fold of P was applied in the beef manure treated soils comparing with swine effluent treated soils. Moreover, limited P removal (60 lb $acre^{-1}$ yr^{-1}) by corn (Bundy et al., 2001) grown in these soils could further contribute to elevated soil P levels.

Phosphorus in soil exists in both organic and inorganic forms and the conversion of organic P to orthophosphate (Pi) requires activities of phosphatases. Soilenzymes could be intracellularor extracellular, that originate from microbes, plant and animal cells (Skujins, 1976); the associated biochemical activities may change depending on environmental conditions and presence of enzymes and substrates. The activity of soil phosphatases reported to be dependent on factors such as soil pH and organic carbon content (Eivazi and Tabatabai, 1977, Juma and Tabatabai, 1978). In this study, mixed responses of acid phosphatase activity resulted from manure treatment. The relatively high acid phosphatase activity in surface soils added with anhydrous ammonia can be attributed in part, to reduced soil pH from 7.5 (control soils) to 6.9 (low rates application) to 4.3 (high rates application) (Deng et al., unpublished). Alkaline phosphatase activity in control, beef manured and swine effluent treated surface soils on the other hand was favored by their alkaline pH, which is consistent with data reported by Eivazi and Tabatabai (1977). It is,

therefore, not surprising that activities of this enzyme was reduced significantly following anhydrous ammonia treatment. Among the soils at 10-20 and 20-30 cm depths, there were no significant differences in activity of alkaline phosphatase, except in soils that were treated with anhydrous ammonia. Although acidic pH ranges generally favor acid phosphatase activity and alkaline phosphatase is dominant in alkaline pH ranges (Eivazi and Tabatabai, 1977), the pH optima of these enzymes may also be related to the origin of these enzymes in soils. Acid phosphatase in soil mainly comes from microbes and plants, whereas, alkaline phosphatase in soil is reported to be mostly from microbial and animal sources but not from plant roots (Dick et al., 1983; Juma and Tabatabai, 1988).

Activity of phosphodiesterase and inorganic pyrophosphatases showed a different trend than phosphomonoesterases with their increased activity in deeper soils. However, since the control soils also showed this trend, the higher activities with increasing soil depth may not be directly related to treatments. The activity of phosphodiesterase could be related to availability organic P and soil pH. It has been reported that solubility and availability of organic P increased with increasing soil pH (Gerke, 1992). In this study, soil pH was relatively high in manured and control soils, ranging from 7.1 to 7.5, which resulted in relatively higher phosphodiesterase activity when compared with other soils tested. Organic P is more mobile in soil than Pi and has showed downward movement in soil profiles (Chardon et al., 1997; Parham et al., 2002). Diester phosphates are especially mobile in soil profiles because they do

not adsorb to soil particles (Cosgrove, 1967). Thus, increased availability of organic P in deeper soil profiles could have lead to enhanced synthesis of phosphodiesterase. The significant effects of high rates of swine effluent and anhydrous ammonia on inorganic pyrophosphatase activity in the surface soils could in part be due to their effect on soil pH, which resulted in altered solubility of Ca^{+2} and Mg^{+2} in soils. In general, Ca^{+2} availability is significantly reduced in pH less than 5, while Mg^{+2} availability is significantly higher in pH ranges of 4.5 to 6.5 (Lucas and Davis, 1961). It has been suggested that inorganic pyrophosphatase is Mg^{+2} dependent and high input of Ca^{+2} (via beef manure) may displace Mg^{+2} (Avaeva et al., 2000) which could have lead to its reduced activity in beef manured soils.

Thus, it is imperative that high rate and repeated manure applications affect activity of phosphatases responsible for organic phosphorus mineralization reflecting altered soil microbial activity and community structure. Manure additions could result in P accumulation in soils and greater concentrations of runoff P can occur mainly by amending soils with high rates beef manure.

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Chapter IV

DOMINANT BACTERIAL DIVERISTY AND COMMUNITY STRUCTURE IN TNT-SPIKED SOILS

Abstract

Microbiological processes significantly influence degradation of nitroaromatics in the environment, while contamination of nitroaromatics could also affect microbial community inhabiting the environment. The effect of 2, 4, 6-trinitrotoluene (TNT) on soil bacterial community was evaluated using denaturing gradient gel electrophoresis (DGGE) analyses of 16S rRNA genes. Following incubation of soils that were treated with TNT at 250 to 5000 mg TNT kg^{-1} soil for a minimum of 10 days, distinct changes in soil bacterial communities were detected as evidenced by changes in DGGE fingerprints. Out of the 11 DGGE bands sequenced, four bands showed sequence similarity with bacteria belonging to β-Proteobacteria. There are two intense DGGE bands shown in the soils spiked with 2500 to 5000 mg TNT kg^{-1}, suggesting increasing dominance of these two bacterial ribotype in the community following TNT addition. Sequence of these two bands showed that one of them had 99% similarity to the 16S rRNA gene of *Achromobacter xylosoxidans* belonging to β-Proteobacteria, while the other was an unknown bacterial ribotype.

Introduction

Contamination of toxic chemicals is a wide spread problem that occurs in air, soil, sediments, and water, threatening the global community of inhabiting organisms. Environmental contaminants are contributed by agricultural, industrial and military activities. Explosive contamination, for example, could be contributed by the handling of explosive materials during their manufacture, processing and packaging at military ammunition plants (Widrig et al., 1997). The principal nitroaromatic contaminants found in defense sites are 2, 4, 6-trinitrotoluene (TNT), and hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX). RDX is often used in combination with octahydrol-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) (Pennington, 1998). Human health effects associated with exposure to high explosive compounds vary by explosive type and the USEPA has set lifetime exposure of drinking water health advisory limits for TNT, RDX, and HMX as 2, 2, and 400 $\mu g\ L^{-1}$, respectively (Crockett et al., 1999).

Soil contamination with TNT can be detrimental to soil microorganisms by inhibiting the growth and survival of bacteria, fungi, actinomycetes (Klausmeier et al., 1973; Fuller and Manning Jr., 1997, 1998) and can adversely affect microbial biomass and microbial processes (Gong et al., 1999). Contaminant concentrations often lead to reduced microbial activities, biomass and biodiversity due to the stress of contamination and as low as 1-2 mg acetonitrile extractable TNT kg^{-1} can significantly inhibit soil microbial activity (Gong et al., 1999).

Although nitroaromatic compounds inhibit growth of many microorganisms, degradation of this class of organic chemicals is also undertaken by microorganisms. Therefore, it is anticipated that microbial community would change towards a community that is tolerant or even capable of using nitroaromatic compounds as nutrient and energy sources in a nitroaromatics-contaminated soil environment (Gong et al., 2000). Numerous studies have demonstrated microbial degradation of nitroaromatics (Melius, 1990; Fuller and Manning 1998). Revealing microbial communities that dominate in a nitroaromatics-contaminated soil is of interest in developing strategies for bioremediation of these contaminated soils

Limited information is available about specific microbial species that are involved in explosive degradation. Bacteria belonging to the genus *Achromobacter* are found to present in gunshot wounds (D'Amato et al., 1988), indicating potential role in breakdown of explosive. *Achromobacter* sp. is a gram-negative, nonfermenting bacteria originally isolated from clinical specimens (Namnyak et al., 1985), and are shown to be involved in degradation of chemicals such as nicotine (Hylin, 1958), pesticides (Karns et al., 1986) and 2,4,6-tribromophenol (TBP) (Nejidat et al., 2004). Therefore, this study was initiated to reveal changes in dominant bacterial ribotypes following amendment of TNT to soil.

Materials and Methods

Soil samples and analysis

An agricultural soil was taken from a continuous winter wheat (*Triticum aestivum*) experiment site located in central Oklahoma. The soil is a silt loam with mean particle size distribution of 37.5% sand and 22.5% clay containing no detectable level of TNT or other explosives. Soil pH was 5.6 with total nitrogen of 0.9 g N kg^{-1} soil, and total organic carbon of 10.2 g C kg^{-1} soil (Meyer, 2002). A 50 g (<2 mm) of soil was taken in six different glass beakers and spiked with 0, 250, 500, 1000, 2500, and 5000 mg TNT kg^{-1} soil respectively. The spiked soils were incubated at 23^{o}C and maintained at 60% field moisture content.

Extraction of soil DNA and PCR amplification of 16S rRNA genes

Following a minimum of 10 days incubation, soil DNA was extracted using an UltraClean^{TM} soil DNA kit (Mo Bio Laboratories Inc., Solana Beach, CA, U.S.A.). 16S rRNA genes of bacterial community were obtained by polymerase chain reaction (PCR) amplification using soil DNA as templates and universal primers that are specific for the domain bacteria. PCR primers used were BF1092 (5'-AAGTCCCGTAACGAGCGCAA-3') (Woese, 1987) and U1392GC with 40 bases of GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCACGGGCG GTGTGTAC-3') (Meyers et al., 1985; Olsen et al., 1986; Ferris et al., 1996).

These two primers are reported to maximize the recovery of soil bacterial community, and correspond to *E. coli* positions 1092-1111 and 1392-1406, respectively. Therefore the PCR products should be around 354 bp with about 314 bp from the 16S rRNA genes of the members from bacteria domain.

The PCR amplification was performed using Promega Chemicals (Madison, WI, USA) supplied 10x Magnesium free buffer, 1.25 mM $MgCl_2$, 1.5 units of *Taq* DNA polymerase stored in PCR buffer A, and Fisher scientific Inc., (Fisher Bioreagents, Pittsburgh, PA, USA) supplied PCR nucleotide mix (300 μM) along with 1.5 μg bovine serum albumin and 0.6 μM of each primer mentioned above. All reagents were mixed with PCR grade distilled water along with 50 ng of soil DNA, and the final volume was 100 μL. The PCR was performed using a modified procedure as described by Ferris et al. (1996) that involved an initial denaturation of 2 min at 94°C, followed by 9 cycles with 1 min denaturation at 94°C, 1 min annealing at 56°C with a touchdown of 1°C per cycle, and 2 min of extension at 72°C. Additional 22 cycles were also performed with cycle composed of 40 sec at 94°C, 40 sec at 47°C, and 2 min at 72°C. A final extension at 72°C for 8 min was included. The PCR products were checked for the expected size and quantified on 2.5% agarose gels using molecular weight markers as a reference.

DGGE analysis of rRNA genes

The PCR amplicons were separated using a DCode™ Universal Mutation Detection System (Bio-Rad, Inc., Hercules, CA, USA). A 6.5% acrylamide gel with a 30-55% parallel denaturing gradient was prepared using a Hoefer SG100 gradient maker (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The denaturing solution of 100% contained 7 M urea and 40% deionized formamide. The gel was polymerized for at least 4 hours and about 2 μg PCR products along with loading dye were added to each well. The DGGE gel was run for 5 h at 200 mV constant voltage in 1x TRIS-acid EDTA (TAE) buffer at 60°C. The gel image was taken using a Kodak 1D Scientific Imaging System attached with a Kodak DC 290 zoom digital camera (New Haven, CT, USA).

Isolation and Sequencing of dominant DGGE bands

Prominent bands from DGGE gels were excised, suspended with 50 μL sterile water in eppendorf tubes and kept overnight at 4°C for the DNA to diffuse into water. A 10 μL of the suspended DNA was used as a template to reamplify the band of interest using the same primers and PCR conditions described above. The reamplified PCR products were then purified using UltraClean™ PCR clean-up DNA purification kit (Mo BIO laboratories, Inc., Solana Beach, California, USA) to remove the unused primers and short oligonucleotides. Purified DNA was sequenced using primers used in the PCR reaction as

described above on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Similarity searches for all the sequences were done using the BLAST program (Altschul et al., 1997) in the GenBank database to identify the nearest relatives of the excised bands. Phylogenetic analysis were conducted with Biology WorkBench, version 3.2 (San Diego Supercomuter Center; http://workbench.sdsc.edu).

Nutrient agar-culturable bacterial population and its DGGE fingerprints

Results from PCR-DGGE analysis indicated that there were two ribotypes enriched by TNT addition, an uncultured unknown and a ribotype that was closely related to *Achromobacter* spp. Therefore, *Achromobacter xylosoxidans* (ATCC 31040) was evaluated for its ability in degrading TNT. Since this bacterial species is usually cultured on nutrient agar plates, nutrient agar-culturable bacterial community and DGGE fingerprints in TNT-spiked soils were evaluated for involvement of *Achromobacter* spp in TNT degradation.

A 2.0 g of TNT-spiked soil was suspended in 18.0 mL of 25% sterile ringer solution and 36 μl of 10% Sodium pyrophosphate solution (0.18%). Following serial dilutions, 100 μl of 10^{-3} and 10^{-6} were plated on nutrient agar plates in five replicates and incubated at 37°C. Bacterial cells appeared at day 1, day 2 and day 10 were scraped off the plates for genomic DNA extraction (Richter et al., 1991). 16S rRNA genes of the recovered and spiked soil

bacterial community and that of *Achromobacter xylosoxidans* (ATCC 31040) were obtained by PCR amplification and analyzed by DGGE as described above. Extraction of *Achromobacter xylosoxidans* genomic DNA was conducted as described by Richter et al. (1991).

Achromobacter xylosoxidans in TNT degradation

A 50 mL of basal broth supplemented with 5 ppm of TNT in 250 mL of Erlenmeyer flasks was inoculated with 100 μL of overnight *Achromobacter xylosoxidans* (ATCC 31040) culture. The flasks were placed on a rotary shaker at 37^{o}C. TNT concentrations in the culture medium were monitored on daily basis for 5 days. TNT degradation was evaluated by comparing TNT concentrations in different days of incubation and with the uninoculated controls.

TNT in solution was quantified by High Performance Liquid Chromatography (HPLC) using acetonitrile and water (80:20) as a mobile phase and a UV detected at 254 nm, and calibrated with 0-10 ppm TNT standard curve developed under the same analysis conditions.

Results

Microbial Community analysis using DGGE

DGGE banding pattern obtained from the contaminated soils showed only a few bands, indicating effect of contamination. The control soil showed no discrete or dominant bands and the soils treated with TNT showed prevailing bands indicating dominance of several bacterial ribotype induced by the presence of TNT (Fig. 1).

The sequence analyses of excised DGGE bands and subsequent comparisons to sequences in GenBank database showed presence of both uncultured and known bacteria (Table 2). Of the 11 bands sequenced, four of the sequences matched with β-proteobacteria and identified to be *Achromobacter sp.* and *Alcaligens sp.* Three of the sequences showed over 98% sequence similarity with *Achromobacter xylosoxidans* that was dominated to be present in soils treated with increased levels of TNT. The rest 7 bands belonged to uncultured soil bacteria.

Phylogenetic analysis of 16S rRNA sequences of the 11 excised bands excised showed presence several clusters of soil bacterial community (Fig. 2) differing with TNT treatments. An uncultured bacterial ribotype (L11) was shown closely related to *Achromobacter xylosoxidans*.

In general, fewer bacterial ribotypes were detected on nutrient agar plates in comparison with PCR-DGGE fingerprints obtained using soil DNA as a PCR template (Fig. 3). High concentration of TNT also led to reduction of

bacterial ribotypes in the culturable population (comparing A and B groups, Fig. 3). There is evidence that bacterial ribotypes closely related to *Achromobacter* spp. were enhanced by TNT contamination (indicated by arrows a, and b in Fig. 3). There were three bands shown in the PCR-DGGE fingerprint for *Achromobacter xylosoxidans* (ATCC 31040), suggesting presence of multiple operon copy number heterogeneity of rRNA in this bacterium (Crosby and Criddle, 2003). However, *Achromobacter xylosoxidans* did not break down TNT under conditions evaluated (Table 2).

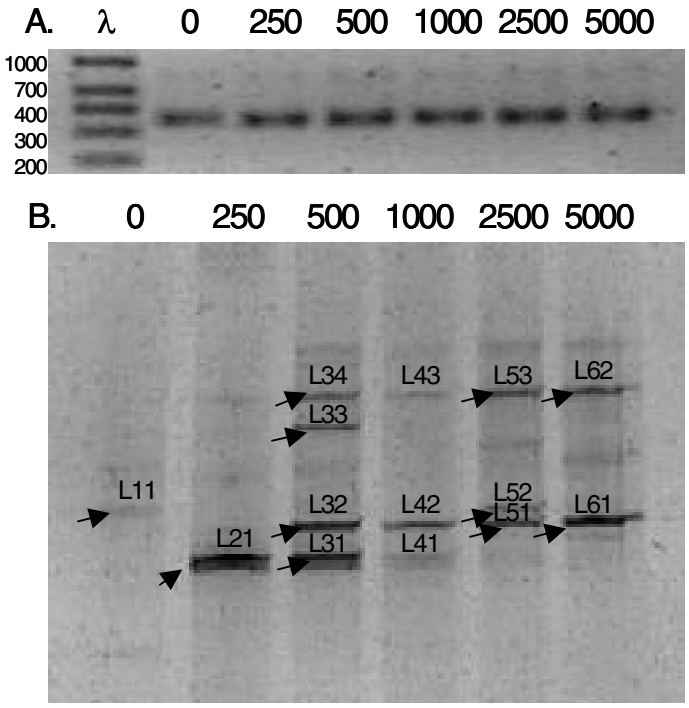

Fig. 1. (A) A 2.0% agarose gel electrophoresis of PCR-amplified 16S rRNA gene fragments. The template DNAs were extracted from soil amended with different concentrations of TNT, ranging from 250 to 5000 mg TNT kg^{-1} soil. (B) Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes. Bands marked are excised and sequenced (see Table 1).

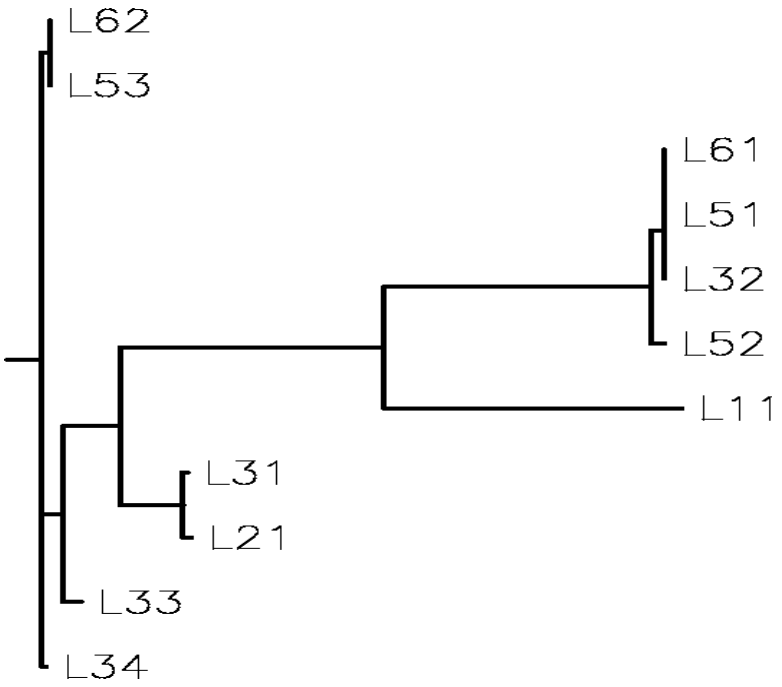

Fig. 2. Phylogenetic analysis of 16S rRNA sequences of the 11 excised bands from DGGE gel (as indicated in Fig. 1). The dendrogram was constructed using Biology WorkBench, version 3.2 (San Diego Supercomputer Center; http://workbench.sdsc.edu).

Table 1. Tentative Identification of dominant DGGE bands by sequencing the excised bands and BLAST analysis*

Band Name	Sequence size (bp)	Closest relative	Alignment & % similarity	Score	Taxonomic affiliation
L11	314	Uncultured soil bacterium	314/315 99	609	Unknown
L21	312	Uncultured soil bacterium	311/314 99	575	Unknown
L31	313	Uncultured soil bacterium	313/313 100	620	Unknown
L32	298	*Achromobacter xylosoxidans*	278/281 98	527	β-Proteobacteria
L33	311	Uncultured soil bacterium	308/314 98	553	Unknown
L34	311	Uncultured soil bacterium	306/314 97	537	Unknown
L51	291	*Achromobacter xylosoxidans*	273/278 98	488	β-Proteobacteria
L52	289	*Alcaligens sp.*	274/276 99	523	β-Proteobacteria
L53	319	Uncultured soil bacterium	308/320 96	476	Unknown
L61	301	*Achromobacter xylosoxidans*	281/282 99	543	β-Proteobacteria
L62	316	Uncultured soil bacterium	309/316 97	547	Unknown

*Sequences were aligned with the closest relatives (highest score) in the GenBank database by using BLAST. Bands correspond to those excised from DGGE gel as L11 for band 1 in lane 1 (Fig. 1). The percentage of similarity was calculated without taking gaps into account. The part of the total sequence used for alignment is indicated by the alignment data.

Table 2. TNT (mg L^{-1}) detected in basal broth (BB) following different treatments and up to five days of incubation at 37°C

Days of incubation	BB+TNT	BB+TNT+ *A. xylosoxidans*
1	8.41	8.51
2	9.02	10.36
3	8.99	9.44
4	9.40	9.51
5	8.89	8.54

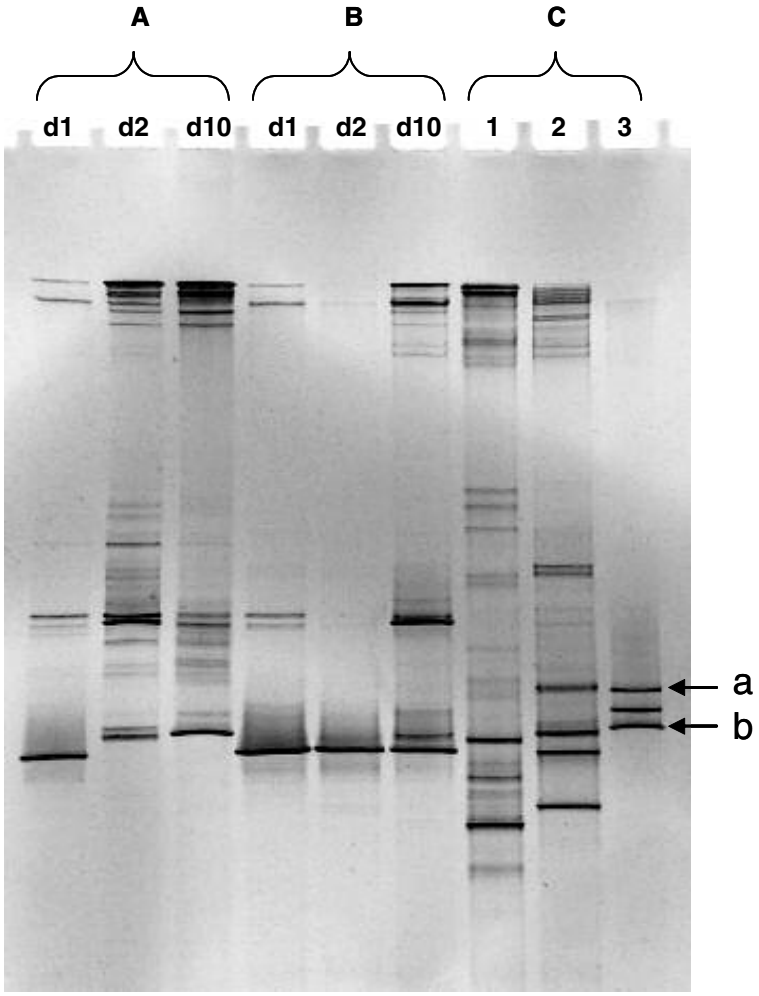

Fig. 3. PCR-DGGE banding patterns of 16S rRNA genes obtained by PCR amplification of 16rRNR genes using template DNA extracted from soil bacteria cultured on nutrient agar plates, soil, and *Achromobacter xylosoxidans* (ATCC 31040). A, bacteria recovered on nutrient agar plates from soils spiked 250 mg TNT kg^{-1} soil; and B, bacteria recovered from the soil spiked with 5000 mg TNT kg^{-1} soil. Lanes d1, d2 and d10 indicate recovery at day 1, day 2 and day 10 incubation times. PCR template DNA for lanes 1 and 2 in the C group was extracted directly from soils spiked with 250 and 5000 mg TNT kg^{-1} soil; respectively; that of lanes 3 was genomic DNA of *Achromobacter xylosoxidans*. Arrows a and b indicate possible presence of some 16S rRNA bands of *Achromobacter xylosoxidans* in TNT-spiked soils.

Discussion

The PCR-DGGE fingerprints of 16S rRNA genes from soils spiked with different levels of TNT showed increased band intensity and dominance of few bands reflecting a shift in bacterial community towards TNT tolerant and potentially TNT degrading bacterial community.

The biodegradation of soil contaminants usually limited by factors such as bioavailability, chemical toxicity, diffusion and transportation of contaminants to microbial cell, and associated low populations of microorganisms capable of degrading contaminants (Pieper and Reineke, 2000) in soil. Thus, the fewer dominant bands in TNT contaminated soils depicted potentially reduced diversity of bacterial community, due to persistence and toxic effects of TNT contamination in soil.

Contamination of soils with TNT limits proliferation of microbial population, microbial biomass, and activity of enzymes, such as dehydrogenases (Gong et al., 1999; Meyer,2002). Thereby, TNT contamination of soils affects survivability of soil microorganisms and lead to lesser microbial diversity. This is evidenced by the presence of fewer bands in the PCR-DGGE fingerprints of 16S rRNA genes in the bacterial community following spiking the soils with TNT. A few bacterial ribotypes were enhanced by TNT contamination, which could be due to reduced competition and preferential growth of TNT-tolerant bacteria.

Upon sequencing the dominant PCR-DGGE bands in TNT contaminated soils, the sequence similarity search indicated one of the bands was closely related to *Achromobacter* spp. The cluster analysis indicated that these bacteria formed a distinct group and dominated in soils spiked with elevated levels of TNT. The increasing band intensity with increasing TNT concentrations up to 5000 mg kg^{-1} pointed out that growth of these bacteria might be stimulated by the presence of TNT in soil.

The apparent presence of ribotypes that are closely related to *Achromobacter* spp in soils spiked with TNT indicated their high tolerance to TNT contaminations, especially when TNT concentrations as low as 1-2 mg kg^{-1} soil were shown to inhibit the native soil bacterial activity (Gong et al., 1999). The indirect evidences of involvement of *Achromobacter* spp. in degrading nitroaromatic compounds like pesticides and Nicotine (Hylin, 1958; Karns et al., 1986), and their isolation of *Achromobacter xylosoxidans* in gunshot wounds (D'Amato et al., 1988) indicates their potential involvement in degradation of TNT.

However, preliminary laboratory incubation studies indicated that *Achromobacter xylosoxidans* (ATCC 31040) did not break down TNT. The increased growth of this bacterium in basal broth was observed when 2% N solution using NH_4Cl was supplied instead of TNT indicating its requirement for nitrogen (data not shown). When TNT was used as sole nitrogen source, the growth of *Achromobacter xylosoxidans* was observed at slower rate but there was no detectable reduction in TNT concentrations (up to five days incubation).

Thus, it can be inferred that either this bacterium degrades TNT at very slow rates or obtained N nutrition by fixing atmospheric N (Goerz and Pengra, 1961). On the other hand, the ribotypes that were induced by the presence of high concentrations of TNT are not identical to *Achromobacter xylosoxidans* (ATCC 31040), evidenced by 99% sequence similarity and DGGE banding patterns. Further studies are needed to confirm the obtained results.

In addition, the uncultured soil bacteria in TNT spiked soils could also be breaking down TNT. In fact, TNT degradation may require consortium of bacteria and not by *Achromobacter xylosoxidans* alone. Complete breakdown of TNT by a single bacterium in soil could be hindered because of the possible release of nitrite via nucleophilic attack (Vorbeck et al., 1994), and since nitrite is known to be detrimental for bacterial growth (Stein and Arp 1998), there could be possible occurrence of incomplete TNT degradation. TNT detection methods also need to be improved due to co-elution of more than one peak shown in basal medium (data not shown).

However, when comparing PCR-DGGE fingerprints developed using soil DNA or nutrient agar-cultured bacterial genomic DNA as a PCR template, it was clear that high concentrations of TNT led to fewer number of bacterial ribotypes. One of the dominant bands in the PCR-DGGE fingerprint developed by using DNA extracted from the soil spiked with 5000 mg TNT kg^{-1} soil demonstrated similar mobility with one of the three bands for *Achromobacter xylosoxidans* on a DGGE gel, suggesting potential involvement of *Achromobacter* spp. in TNT

degradation and/or tolerance. With increasing interest in developing effective bioremediation technologies, the obtained results warrant further studies.

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Chapter V

Summary and Conclusion

Results from this study suggested that P transformation were enhanced, as evidenced by enhanced phosphatase activities following manure applications at high rates. Accumulation of P would be of environmental concern only if animal manures were applied repeatedly at excessive amounts. Beef manure is more of a concern than swine effluent when application rates are N-based.

Nitroaromatic compounds, such as 2,4,6-trinitrotoluened (TNT), altered microbial community structure. Spiking soil with TNT induced dominance of an unknown soil bacterium and a potential human pathogen *Achromobacter xylosoxidans* (ATCC 31040). The potential role of *Achromobacter xylosoxidans* in degradation of TNT deserves special attention in future studies.

APPENDIX

L11 **AAGTCCCGTA** **ACGAGCGCAA** CCTTATCAAT AGTTGCCAGC GGTTCGGCCG
GGCACTCTAT TGAGACTGCC GTTGACAAAA CGGAGGAAGG TGGGGATGAC
GTCAAGTCCT CATGGCCTTT ATGTCCAGGG CTACACACGT GCTACAATGG
CGAGTACAAA GCGCTGCAAA CCTGCAAGGG GGAGCCAATC GCAAAAAGCT
CGTCTCAGTT CGGATTGGAG TCTGCAACTC GACTCCATGA AGCTGGAATC
GCTAGTAATC GCAGATCAGC ATGCTGCGGT GAATACGTTC CCGGGTCTT**G**
TACACACCGC **CCGT**

L21 **AAGTCCCGTA** **ACGAGCGCAA** CCTCGTCTCT AGTTGCTACC ATTTAGTTGG
GCACTCTAGA GAAACTGCCG GTGATAAGCC GGAGGAAGGT GGGGATGACG
TCAAGTCCTC ATGGCCCTTA CGCGCTGGGC TACACACGTG CTACAATGGC
GGTGACAGTG AGCAGCGACC CCGCGAGGGT GAGCTAATCT CCAAAAGCCC
GTCTCAGTTC GGATTGTTCT CTGCAACTCG AGAGCATGAA GGCGGAATCG
CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC CAGGCCT**GTA**
CACACCGCCC **GTT**

L31 **AAGTCCCGTA** **ACGAGCGCAA** CCCTCGTCTC TAGTTGCTAC CATTTAGTTG
GGCACTCTAG AGAAACTGCC GGTGATAAGC CGGAGGAAGG TGGGGATGAC
GTCAAGTCCT CATGGCCCTT ACGCGCTGGG CTACACACGT GCTACAATGG
CGGTGACAGT GAGCAGCGAC CCCGCGAGGG TGAGCTAATC TCCAAAAGCC
GTCTCAGTTC GGATTGTTCT CTGCAACTCG AGAGCATGAA GGCGGAATCG
CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC CAGGCCTT**GT**
ACACACCGCC **CGT**

L32 **AAGTCCCGTA** **ACGAGCGCAA** TTAGTTGCTA CGAAAGGGCA CTCTAATGAG
ACTGCCGGTG ACAAACCGGA GGAAGGTGGG GATGACGTCA AGTCCTCATG
GCCCTTATGG GTAGGGCTTC ACACGTCATA CAATGGTCGG GACAGAGGGT
CGCCAACCCG CGAGGGGGAG CCAATCCCAG AAACCCGATC GTAGTCCGGA
TCGCAGTCTG CAACTCGACT GCGTGAAGTC GGAATCGCTA GTAATCGCGG
ATCAGCATGT CCCGGTGAAT ACGTTCCCGG GTC**GTACACA** **CCGCCCGT**

Fig. 1. Partial gnces of L11, L21, L31 and L32 DGGE bands from the TNT-treated soils.

L33 **AAGTCCCGTA** **ACGAGCGCAA** CCTCGTCTTT AGTTGCCATC CATTTAGTTG
GGCACTCTAA AGAAACTGCC GGTGATAAGC CGGAGGAAGG TGGGGATGAC
GTCAAGTCCT CATGGCCCTT ACGCGCTGGG CTACACACGT GCTACAATGG
CGGTGACAGT GGGCTGCAAA CTCGCGAGAG TGAGCAAATC CCCAAAAACC
GTCTCAGTTC GGATTGTTCT CTGCAACTCG AGAGCATGAA GGCGGAATCG
CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC CAGGCC**GTAC**
ACACCGCCCG **T**

L34 **AAGTCCCGTA** **ACGAGCGCAA** CCTCGTCTCT AGTTGCCATC ATTTAGTTGG
GCACTCTAAA GAAACTGCCG GTGATAAGCC GGAGGAAGGT GGGGATGACG
TCAAGTCCTC ATGGCCCTTA CACGCTGGGC TACACACGTG CTACAATGGC
GGTGACAGTG GGCAGCAAAC TCGCGAGAGT GAGCAAATCC CCAAAAACCG
TCTCAGTTCG GATTGTTCTC TGCAACATCG AGAGCATGAA GGCGGAATCG
CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC CAGGCC**GTAC**
ACACCGCCCG **T**

L51 **AAGTCCCGTA** **ACGAGCGCAA** CCTTGTCATT AGTTGCTACG AAAGGGCACT
CTAATGAGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA TGACGTCAAG
TCCTCATGGC CCTTATGGGT AGGGCTTCAC ACGGTCATAC AATGGTCGGG
ACAGAGGGTC GCCAACCCGC GAGGGGGAGC CAATCCCAGA AACCCGATCC
GTAGTCCGGA TCGCAGTCTG CAACTCGACT GCGTGAAGTC GGAATCGCTA
GTAATCGCGG ATCAGCATGT CTCGGT**GTAC** **ACACCGCCCG** **T**

L52 **AAGTCCCGTA** **ACGAGCGCAA** CCTTGTCATT AGTTGCTACG AAAGGGCACT
CTAATGAGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA TGACGTCAAG
TCCTCATGGC CCTTATGGGT AGGGCTTCAC ACGTCATACA ATGGTCGGGA
CAGAGGGTCG CCAACCCGCG AGGGGGAGCC AATCCCAGAA ACCCGATCGT
AGTCCGGATC GTAGTCTGCA ACTCGACTAC GTGAAGTCGG AATCGCTAGT
AATCGCGGAT CAGCATGTCG CGGT**GTACAC** **ACCGCCCGT**

Fig. 2. Partial 16S rRNA gene sequences of L33, L34, L51 and L52 DGGE bands from the TNT-treated soils.

L53 **AAGTCCCGTA** **ACGAGCGCAA** CCTCGTCTCT AGTTGCCATC ATTTAGTTGG
GCACTCTAAA GAAACTGCCG GTGATAAGCC GGAGGAAGGT GGGGATGACG
TCAAGGTCCT CATGGCCCTT ACACGGCTGG GCTACACACT GTGCTACAAT
GGCCGGTGAC AAGTGGGCAC GCAAACTCGC GAGAGTGAGC AAATCCCCAA
AAACCGTCTC AGTTCGGATT GTTCTCTGCA ACTCGAGAGC ATGAAGGCGG
AATCGCTAGT AATCGCGGAT CAGCATGCCG CGGTGAATAC GGTTCCCAGG
CCTT**GTACAC** **ACCGCCCGT**

L61 **AAGTCCCGTA** **ACGAGCGCAA** TTAGTTGCTA CGAAAGGGCA CTCTAATGAG
CACTGCCGGT GACAAACCGG AGGAAGGTGG GGATGACGTC AAGTCCTCAT
GGCCCTTATG GGTAGGGCTT CACACGTCAT ACAATGGTCG GGACAGAGGG
TCGCCAACCC GCGAGGGGGA GCCAATCCCA GAAACCCGAT CGTAGTCCGG
ATCGCAGTCT GCAACTCGAC TGCGTGAAGT CGGAATCGCT AGTAATCGCG
GATCAGCATG TCGCGGTGAA TACGTTCCCG GGTCTT**GTAC** **ACACCGCCCG**
T

L62 **AAGTCCCGTA** **ACGAGCGCAA** CCCTCGTCTC TAGTTGCCAT ACATTTAGTT
GGGCACTCTA AAGAAACTGC CGGTGATAAG CCGGAGGAAG GTGGGGATGA
CGTCAAGTCC TCATGGCCCT TACACGGCTG GGCTACACAC TGTGCTACAA
TGGCGGTGAC AGTGGGCAGC AAACTCGCGA GAGTGAGCAA ATCCCCAAAA
ACCGTCTCAG TTCGGATTGT TCTCTGCAAC TCGAGAGCAT GAAGGCGGAA
TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT TCCCAGGCCT
T**GTACACACC** **GCCCGT**

Fig. 3. Partial 16S rRNA gene sequences of L53, L61 and L62 DGGE bands from the TNT-treated soils.

VITA

Dinesh Babu

Candidate for the Degree of

Master of Science

Thesis: PHOSPHATASE ACTIVITY IN ANIMAL MANURE-AMENDED SOILS AND MOLECULAR PROFILING OF MICROBIAL COMMUNITY IN TNT-SPIKED SOILS

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Scope and Method of Study: This study was conducted to examine microbial communities in soil agro-ecosystems amended with organic and inorganic fertilizers and in soils contaminated with nitroaromatic 2,4,6-trinitrotoluene (TNT). Effects of fertilizer treatment on soil phosphorus concentrations and phosphatases were determined. Another soil treated with TNT at 250-5000 mg kg^{-1} soil, an untreated control was used. Bacterial community changes induced by treatments were tested using fingerprints of bacterial 16S rRNA genes.

Findings and Conclusions: Beef manure application increased soil total and Mehlich-3 extractable phosphorus. Acid and alkaline phosphatase activities were significantly high at 0-10 cm soil and decreased with increasing soil depth. Phosphodiesterase and inorganic pyrophosphatase activities increased with increasing soil depths from 0-30 cm. Distinct trends among four phosphatases implied their activity in different agro-ecosystems. Soils contaminated with TNT showed presence of TNT tolerant bacteria and their potential involvement in TNT degradation needs further attention.

ADVISER'S APPROVAL: Dr. Shiping Deng