BACTERIAL COMMUNITIES CAPABLE OF ENHANCED EPTC AND ATRAZINE DEGRADATION IN OHIO SILT LOAM AND LURAY SILTY CLAY LOAM SOILS

DISSERTATION

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ABSTRACT

Approximately 7.5 – 8.0 million kg of EPTC (S-ethyldipropylthiocarbamate) and 32 million kg of atrazine (2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine) are used each year for crop production in the United States. Recent reports have raised concern about continued use of these herbicides because of their negative impacts on aquatic life and potential endangerment of animal/human health. Therefore rapid degradation of these herbicides after intended action is important to prevent non-target pollution associated with their presence in the environment.

In this study, a culture independent metagenome approach was used to identify bacteria capable of degrading EPTC and atrazine. Two different soils (Wooster silt loam and Luray silty clay loam) were evaluated for development of enhanced degradation by successive application of these herbicides. A polymerase chain reaction (PCR) technique was used to monitor the appearance of the specific genes *thc*A, *thc*B, *thc*C, *thc*D and *thc*R for EPTC degradation and *atz*B, *atz*D, *trz*D and *trz*N for atrazine degradation. Whereas no EPTC or atrazine degrading genes were detected in control soils, presence of these genes in the enhanced soils confirmed the higher degradation potential observed.

A PCR-DGGE (Polymerase Chain reaction – Denaturing Gradient Gel Electrophoresis) method was used to amplify and characterize the V-3 (338 to 518) region of the 16s rRNA gene from soil DNA extracted from the enhanced soils to identify the dominant bacteria involved in the degradation of the herbicides. Unique DGGE band

profiles obtained for each soil sample were used to calculate the bacterial richness index and Dice similarity index. The values for these indices showed that microbial community abundance and distribution was greatly impacted due to successive exposure of these two herbicides.

Selected DGGE bands were excised, cloned and sequenced to identify the dominant bacterial species in the EPTC and atrazine enhanced soils. From the 16S rRNA clone libraries, a total of 64 clones were sequenced. DNA sequence data confirmed the presence of known EPTC and atrazine degrading bacterial species such as *Sphingomonas* sp., *Rhodococcus* sp., and *Actinobacterium* sp. Several uncultured bacterial species, earlier detected in aromatic chemicals contaminated sites, were also identified. Two bacterial species, *Kaistobacter* sp. and *Gemmatomons* sp., were identified that have not been reported yet as degraders of these two chemicals.

Identification of novel bacterial species capable of degrading these herbicides provides evidence for the vast diversity in microbial communities that still remains to be explored. Knowledge gained about these bacterial degraders will be useful in elucidating novel pesticide degradation pathways and in developing methods for bioremediation to reclaim contaminated soils.

Key Words: EPTC, atrazine, enhanced degradation, PCR, DGGE, bacterial community, bioremediation.

Dedicated to the individuals
who shared the sacrifices during my endeavor,
my parents, wife and daughter.

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CHAPTER 1

INTRODUCTION

Over the last few decades, there has been a steady increase in the development of xenobiotic compounds considered harmful to humans, plants, and animals. Synthetic chemicals include medicines, pesticides, fertilizers, industrial products and processing chemicals. Although these chemicals are directly related to the quality of our daily lives, widespread contamination resulting from their injudicious use is rampant. Serious negative impacts on plant and animal life have been reported because of accumulation of these chemicals in the environment.

Microbes, especially bacteria, have the ability to degrade, transform and detoxify a huge range of compounds such as hydrocarbons, polychlorinated biphenyls (PCBs), poly aromatic hydrocarbons (PAHs), pharmaceuticals, pesticides, metals, and radionuclides. Bacteria either use the substrates to derive energy or transform the compounds through a process called cometabolism. Cometabolism is probably the most widespread mechanism for biodegradation, in which bacteria partially degrade a substrate that does not influence their growth directly.

EPTC (S-ethyldipropylthiocarbamate) and atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) are two of the most widely used

herbicides in agriculture around the world. Among the million tons of pesticides that are applied in agricultural cultivation around the world, it is estimated that only about 1% actually works against the target pests, while the rest is either adsorbed in soil or dispersed in the environment. Although the extent of negative impact depends on the half-life, some herbicides have been reported to cause environmental pollution (Allran and Karasov, 2000). Both EPTC and atrazine can be toxic at higher concentrations and prolonged exposure. Rapid biodegradation is the critical step in reducing nonpoint source pollution of such agricultural pesticides in the environment. Pesticide degradation can occur in plants, animals and in soil and water through chemical or biological processes. However, microbial degradation is the primary route of agricultural pesticide degradation in the environment.

Initial research on pesticide degradation focused on the fate of pesticides mostly from chemical and physical attributes of the decomposition process. Tam et.al. (1987) and Mueller et al. (1988) were pioneers in isolation and characterization of EPTC-degrading bacterial strains that were able to thrive on EPTC as the sole carbon source. Since then several other studies have identified bacterial species capable of degrading EPTC in soil (Dick et al., 1990; Behki, 1991; Nagy et al., 1995). Microbial regulation has also been established as the primary route of atrazine degradation in soils. Several bacterial cultures and mixed cultures have been identified which are capable of degrading atrazine (Assaf and Turco, 1994; Mandelbaum et al., 1993; Topp et al., 1995).

Although several EPTC and atrazine degradation microorganisms have been identified and isolated in laboratory conditions, few studies have attempted to explore the bacterial diversity involved in the pesticide degradation in soil. Soil bacteria are very

diverse and each gram of soil can contain up to 10,000 species of bacteria (Torsvik, 1990). Overall it is estimated that 2 to 3 million bacterial species thrive in the earth's crust (Truper, 1992). Since only about 0.1 to 10% of the bacterial population in soil can be actively cultivated in the laboratory conditions, there is a possibility of existence of several other bacterial species, which are not cultivable but capable of degrading EPTC and atrazine.

This study investigated the soil metagenome using culture independent techniques in an effort to identify novel bacterial species capable of degrading EPTC and atrazine. I hypothesized that when exposed to EPTC and atrazine, soils develop an enhanced degradation potential due to the enrichment of components of bacterial populations able to degrade these herbicides.

The specific objectives developed to test this hyothesis are to:

(1) Obtain enhanced degradation potential for the herbicides EPTC and atrazine in soils,

(2) Relate expression of specific herbicide degrading genes to enhanced degradation,

(3) Observe any change in bacterial diversity due to exposure of these herbicides, and

(4) Identify specific bacterial communities involved in the degradation of EPTC and atrazine.

CHAPTER 2

LITERATURE REVIEW

Soils and Biodegradation

Soil is the most important natural resource that sustains life on the planet. Soil provides nutrition for plant growth, foundation for buildings and infrastructure, serves as habitat for microbes and animals, recycles water and nutrients, decomposes residues and biodegrades and detoxifies natural and artificial chemicals. Most important of the soil processes takes place out of our sight, below the soil surface.

Innumerable life forms dwell in the soil and carry out many of the aforementioned soil processes. Soil macroflora, microflora and fauna include burrowing animals, earthworms, mites, springtails, small insects, bacteria, algae, fungi and protozoa. All soil organisms have specific roles in the soil food web. Microorganisms such as bacteria, actinomycetes, algae and fungi influence the major components of nutrient cycling, decomposition of organic matter (living, nonliving, or xenobiotic), and maintaining soil productivity. Even though primary producers drive most food webs, decomposers (heterotrophic bacteria, fungi and micro and macro fauna) initiate the breakdown of detritus and residues and controls availability of nutrients for higher plants or primary producers.

Among all soil processes, biodegradation and detoxification of chemical compounds is critical to proper functioning of the ecosystem. Toxicity resulting from the presence of a chemical compound can lead to alteration of life forms or loss of biodiversity and in extreme cases can make the soil unproductive. In general, it is widely accepted that biodiversity, or presence of more species in an ecosystem, increases the "resilience" in the system (Tilman et al., 2006). Resilience is the capacity of a particular ecosystem to reduce the effects of a change in an environmental condition. A resilient system has the capacity to revert to its original steady-state condition and can rebuild itself after a perturbation. However, it is extremely difficult to quantify the factors that provide resilience to a particular ecosystem.

Manufactured chemicals in the environment

The number and amount of xenobiotic compounds considered harmful to human, plants and animals have been increasing steadily in our society. Our well-being depends on these chemicals, so much that it is impossible to sustain the world population without the use of these chemicals. There are several types of synthetic chemicals that are being used around the world such as medicines, pesticides, fertilizers, industrial products and processing chemicals. These chemicals are often directly related to our food production systems, helping to maintain our health and other critical functions of our daily lives. Although synthetic chemicals are an integral part of our lives, serious negative impacts on plant and animal life have been reported because of accumulation of these chemicals in the environment (Allran and Karasov, 2000).

Chemicals are released into the environment during their manufacture, processing or use. Since many of these chemicals are synthetic with unknown environmental impacts, the excess release of such chemicals in our natural environments may lead to contamination. The extent and ability of a particular chemical to contaminate a system depends on its form, mode of release, contaminating potential, half-life and mobility. For example, a recent study reported that in 1998, an estimated 450 million kg of developmental and neurological toxins were released in the United States by different companies (Third World Network report, 2000). They also reported that due to the release of these toxic chemicals in the environment, children who are exposed are facing developmental and learning disabilities.

Until recently, because of scientific and technological challenges to conduct long-term animal bioassays, the negative effect of such processes was not clearly understood. Because of the use of sophisticated technology and recent advances in experimentation and modeling, we now have a greater understanding about the potential impacts of these chemicals.

EPTC and atrazine: Agricultural pesticides and environmental pollutants

The regular development of many new chemicals for use as herbicides in agriculture has increased since WWII. The undesired exposure of these new generation herbicides to natural environments has serious implications. Entry of herbicides into natural environments can be varied and may include manufacturing sites, loading sites, and application sites (Allard and Neilson, 1997). Although the extent of negative impact depends on the half-life, some herbicides have been reported to cause environmental

pollution (Allran and Karasov, 2000). Among the million tons of pesticides that are applied in agricultural cultivation around the world, it is estimated that only about 1% actually works against the target pests, while the rest of it is either absorbed in soil or dispersed in the environment. To prevent the negative impacts of pesticides and other chemicals, over the years several regulations have been imposed by USEPA such as the Clean Water Act (CDA) of 1977, the Safe Drinking Water Act (SDWA) of 1974 and the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) of 1980. The goal of this legislative activity was to ensure proper application and disposal of pollutants and remediation of contaminated environments.

EPTC (S-ethyldipropylthiocarbamate) and atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) are two of the most widely used herbicides in agriculture around the world. EPTC belongs to a class of pesticides called thiocarbamates, which are synthetic organic pesticides that have been used in agriculture for the past several decades. Thiocarbamates consist of several pesticides including insecticides, herbicides, and fungicides. The general formula of thiocarbamates includes a sulfur atom bonded with three R-groups along with an oxygen and nitrogen atom. Thiocarbamates are volatile and can exist either in solid or liquid form with a low melting point. Because of their pesticidal properties, thiocarbamates are widely used around the world in agriculture and other operations. First registered in the United States in 1958, EPTC is primarily a pre-emergence and early post-emergence thiocarbamate herbicide used to control broadleaf weeds, grasses and sedges. EPTC is also used in urban and home gardens where vegetable and ornamental plants are grown. Annually around 20 million pounds (7.5 – 8.0 million kg) active ingredients of EPTC are used in the United

States. EPTC inhibits the cuticle formation during the early stages of seedling growth. EPTC is available as emulsifiable concentrate and granular formulations. The half-life of EPTC has been reported to range from 6 to 35 days in soil.

Atrazine, a selective systemic herbicide, belongs to the class of triazine herbicides and is used to control broadleaf and grassy weeds. Atrazine is a chlorotriazine and consists of a ring structure, called the triazine ring, along with five nitrogen atoms and a chlorine atom. Atrazine was also introduced in the United States in 1958 and currently is the most widely used herbicide with an estimated annual application of more than 75 million pounds (~32 million kg) (EPA, 2009). Approximately 75% of corn field acreage in the United States is treated with atrazine. The mode of action for atrazine is through inhibition of photosynthesis in the target plants. Atrazine formulations are available in emulsifiable concentrate, wettable powder, granular and ready to use formulations. Persistence of atrazine in the soil is critical and has been shown to vary from 8 days (Mathess, 1994) to up to 60 days (Tomlin, 1994).

With implementation and adoption of no-till farming practices, use of herbicides has gained added importance to maintain crop productivity. At the same time, leaching of chemicals through well-developed pores to the ground water system, in such no-till management systems, has become a concern. Atrazine is water-soluble and can be transported in dissolved form (Humburg et al., 1989) and has been detected consistently both in surface and ground waters in the United States (Hallberg, 1989; Thurman et al., 1992). As a result, atrazine is quite susceptible to leaching and/or runoff, especially if a subsequent precipitation event occurs shortly after application of the herbicide. Atrazine is mostly applied after rainfall, as the chemical is most effective in wet soils which can

promote vertical movement of the chemical in the soil profile. Atrazine has also been reported in precipitation so that it can lead to contamination of pristine water resources (Cromwell and Thurman, 1993).

Like most chemicals, the persistence of EPTC and atrazine varies depending on their respective chemical properties and physical and environmental conditions. Since large quantities of these herbicides are used in agriculture, non-point source pollution of these two chemicals has been a longtime concern for ecologists studying streams and rivers. They have potential to pollute both surface and groundwater. Atrazine has been dubbed as the most controversial “crop protector” and has been detected in surface and drinking water more frequently than any other chemical in the United States (USEPA Fact Sheet, 2008). The use of atrazine has been banned in the European Union since 2003 because of it's role in water contamination. The Safe Drinking Water Act of 1974 regulates drinking water standards and in 1992 the maximum contaminant level (MCL) in drinking water for atrazine was set not to exceed 3 μg/L (3 ppb). In Canada, the threshold concentration for atrazine in aquatic systems is limited to 1.8 μg/L.

Exposure to atrazine can be harmful and in extreme cases, can cause animal health problems (Allran and Karasov, 2000; Koprivnikar et al., 2006) while results from human health studies are still being debated. Atrazine has been classified as moderately toxic and according to USEPA (2003), lifetime exposure of atrazine above the MCL (maximum contaminant level) of 3 ppb can cause certain types of cancer. Atrazine has been reported as an endocrine disruptor in animals. Even 0.1 ppb atrazine exposure in frogs has been shown to severely impact the reproductive system and eventually resulted

in chemical castration. It was also found that prolonged exposure to these chemicals can have a negative impact on human tissues such as nerve, muscle, heart and liver.

Scientific investigations have found that EPTC can also cause negative health impacts when humans and animals are exposed for a prolonged period of time (Extoxnet Fact Sheet, 1996). EPTC has been classified as slightly toxic compound and maximum negative effect from EPTC can result from ingestion or inhalation. Exposure of EPTC has resulted in increased occurrence of neuronal degeneration and cardiomyopathy in the central and peripheral nervous system of rats and dogs. Therefore, rapid degradation of both atrazine and EPTC in soil and the environment is highly desirable.

Microbial biodegradation of pesticides

According to the Institute of Chemistry and Dynamics of the Geosphere, the soil microflora has an essential role in agriculture as well as degradation of xenobiotic compounds (Fig 1) and acts as a pivot for proper balance in the ecosystem. Mineralization of natural organic matter controls the fertility of agricultural soils and highly influences the production of biomass. Biomass production in turn provides substrate as well as habitat for microbial population in the soil. Soil microflora is also responsible for the degradation of xenobiotic compounds that might be harmful if left unaltered. As a result of degradation, the organisms utilize the compounds as substrate and derive energy. There are, however, certain effects of these xenobiotic compounds on the microbial populations that can either be negative or positive.

Microorganisms are integral to nutrient cycling and maintaining fertility of agricultural soils. Both natural organic matter and xenobiotic compounds are present in

an agricultural ecosystem and are readily utilized by microorganisms, primarily bacteria, resulting in degradation and mineralization of these compounds. Although all soil life has a role in the process, soil bacteria are most suited and highly specialized for the job.

Bacteria are the most primitive life form on the planet and through billions of years of evolution have developed the capacity to utilize almost any substrate to derive energy. Soil bacteria are very diverse and each gram of soil can contain up to 10,000 species of bacteria (Torsvik, 1990). Overall it is estimated that 2 to 3 million bacterial species thrive in the earth's crust (Truper, 1992). The presence of a high diversity in the soil microbial population would imply a higher efficiency in the ability to control soil functions responsible for proper ecosystem functioning. Because of this high diversity, soil bacteria can degrade almost any synthetic or natural chemical compound whether it is plant residues, oil or toxic materials.

Most bacteria use oxygen as the electron acceptor while under certain circumstances bacteria can use other electron acceptors such as nitrate, sulfate, Fe (III), CO_2, or other acceptors. Agricultural and forest soils generally remain aerobic and under proper conditions can easily degrade most chemical compounds. However, the microbial aspect of pesticide degradation was not investigated until a few years ago.

Pesticide degradation has been studied for a long time as part of agronomic, fate and transport investigations. However, since the microbial population is the primary facilitator in the degradation mechanism of these pesticides, it is imperative to investigate the microbial basis of degradation of EPTC and atrazine. Usually a group of bacteria work in consortium to completely mineralize a particular chemical. However, some

members of that consortium are very specific to certain parts of a pathway and are essential to degrade a specific chemical.

Soil bacteria have a significant influence in the fate and persistence of pesticides. Bacteria either uses the substrates in the pesticide to derive energy or transforms the pesticide compound through a process called cometabolism. Cometabolism is probably the most widespread mechanism for pesticide degradation in which bacteria partially degrade a substrate that does not influence their growth directly.

Apart from proximity and exposure of the chemicals to the microbial population, other major factors that influence microbial biodegradation in soil are usually optimal microbial growth conditions that include resources for growth, pH and soil chemical properties, soil physical properties and presence of microbial predators. Alexander (1999) has postulated a set of conditions that leads to biodegradation of a chemical in an environment. The conditions are: (1) specific organism or a consortium of organisms with proper metabolic mechanisms that can lead to degradation, (2) bioavailability of the chemical to microorganisms, (3) exposed functional groups in the compound to be degraded, especially in the case of extracellular degradation, (4) cellular transport of chemical in case of internal degradation, and (5) optimal physical, chemical, and environmental condition for microbial growth.

In absence of any of these requirements, the chemicals could persist in the environment for long periods of time. Most of these factors can be easily changed in the presence of artificial chemicals that are applied in soil. Thus their application can change the native bacterial composition and, in some cases, alter the functions of the soil bacterial communities.

Enhanced Microbial Degradation of EPTC and Atrazine

Rapid biodegradation is the critical step in reducing nonpoint source pollution of agricultural pesticides in the environment. Decomposition of these compounds often is carried out by microbial species that may or may not utilize the chemical as substrate. Because of the gradual increase of a specific microbial population over the years with ability to rapidly degrade the pesticides, several studies have reported a decline in efficacy of many pesticides.

Repeated application of a particular pesticide or structurally similar chemicals in the same soil leads to accelerated biodegradation and the subsequent loss in efficacy have been documented in several studies (Roeth, 1986; Katan and Aharonson, 1989; Tal et al., 1990). Mahia and Diaz-Ravina (2007) reported that in two different soils with history of atrazine application for 10 years and 40 years, respectively, the initial rate of atrazine degradation was much higher in the 40-year history soil compared to the 10-year history soil. The results from this study indicated that the activity and abundance of the atrazine degrading microbial species were most likely dependent on dose. This corresponds well with the high initial rate of atrazine degradation in the 40-year history soil implying that enrichment and adaptation of specific atrazine degraders took place for 40 years with repeated application of the same chemical. Enrichment of specific bacterial population occurs through substrate selection and through horizontal gene transfer between degraders and non-degraders. It is important to mention that horizontal gene transfer is an important component of bacterial evolution (Jain and Lake, 1999). McClung et al. (1994) reported that more than 25 commercial pesticides that included insecticides, herbicides, and fungicides have shown accelerated degradation, also sometimes called enhanced

degradation, in “problem soils”. Aharonson et al. (1990) found that during the enhanced degradation of carbendazim (Methyl *1H*-benzimidazol-2-ylcarbamate) or diphenamid (N,N-Dimethyl-2,2-diphenylacetamide) the fungal communities were not different between the degradation in previously treated or non-treated soils. However differences were found between the mixed bacterial cultures. The authors thus concluded that bacterial population had the most influence on enhanced degradation even though fungi were also involved in the process.

Even though microbial populations are the primary source of herbicide degradation, there have been few studies that attempted to isolate and identify herbicide-degrading bacterial species. Tam et al. (1987) and Mueller et al. (1988) are the pioneers in isolation and characterization of EPTC-degrading bacterial strains that were able to thrive on EPTC as the sole carbon source. Since then several other studies have identified bacterial species that are capable of degrading EPTC in soil (Behki and Khan, 1990; Dick et al., 1990; Behki, 1991; McClung et al., 1994; Ankumah et al., 1995; Nagy et al., 1995; Tal and Rubin, 1993). EPTC degradation can follow several catalytic pathways. The initial reaction for degradation may involve i) sulfoxidation of the parent molecule to a sulfoxide followed by further cleavage at the carbamoyl bond (Casida et al., 1974), ii) hydroxylation of an ethyl C or a propyl C atom (Chen and Casida, 1978), or iii) direct hydrolysis without an initial hydroxylation that results in formation of mercaptan, CO_2, and an amine (Fang1969).

Ankumah et al. (1995) proposed bacteria-mediated EPTC degradation pathways that included i) oxidation of the S atom to form sulfoxide, or initial hydroxylation of the

ii) ethyl C or the iii) propyl C (Figure 2.2). The authors concluded that EPTC can be rapidly mineralized by *Rhodococcus* species.

Atrazine degradation pathways involve several possible hydrolytic cleavages of the halogen, amino, and alkylamino groups to produce cyanuric acid. Cyanuric acid is then mineralized by hydrolytic ring cleavage to CO_2 and NH_4^+ (Cook and Hutter, 1981; Cook, 1987). After application of atrazine to soils with enhanced degradation potential, the reaction can proceed either by formation of hydroxyatrazine or deisopropylatrazine (DIA) (Mahia and Diaz-Ravina, 2007) with DIA being the dominant pathway. Deethylatrazine (DEA) was not detected as a secondary metabolite in the same soils. Based on their findings, Mahia and Diaz-Ravina (2007) proposed a schematic diagram of the first steps of the microbial-mediated atrazine degradation pathway in agricultural soils (Figure 2.3).

Several bacterial pure cultures have been identified capable of degrading atrazine. However, these microorganisms were unable to completely metabolize atrazine. The microorganisms derive energy from the side chains in the atrazine molecule. Recently microorganisms capable of complete degradation of atrazine (i.e. complete breakdown of atrazine ring structure) have been isolated (Mandelbaum et al., 1995). Evidence for complete breakdown of the atrazine ring structure was verified because the bacteria could grow with atrazine has the only source of nitrogen, and nitrogen can only be obtained after ring breakage. At present *Pseudomonas* sp. strain ADP, containing the genes for *atzABC* enzymes, is used for the bioremediation of atrazine polluted sites (Shapir et al., 2000). For complete mineralization of atrazine, mixed microbial populations are more efficient and ubiquitous. There are now additional studies that have identified atrazine-

degrading microorganisms and microbial mixed cultures capable of atrazine mineralization (Assaf and Turco, 1994; Barriuso and Houot, 1996; Mandelbaum et al., 1993; Mirgain et al., 1993; Stolpe and Shea, 1995; Topp et al., 1995).

Genetic basis of EPTC and atrazine degradation

For several pesticides that are used extensively, genes involved in the degradation mechanism have already been identified. Degradation of EPTC and atrazine are carried out in soil mainly by microbial species from the genera *Rhodococcus, Streptomyces, Pesudomonas, Nocardia* and *Clavibacter*. EPTC and other thiocarbamates are efficiently degraded and the carbon and nitrogen is utilized by the members of the *Rhodococcus* genus. Bacterial degradation of EPTC has been investigated to better understand the degradation pathway and the enzymes involved in the degradation of EPTC (Nagy et al., 1995a; Nagy et al., 1995b; Shao and Behki, 1995; Cunningham et al., 1996). During the initial step of EPTC degradation a P-450 system becomes active in the *Rhodococcus* species strain NI86/21(Nagy et al., 1995a). The genes involved in the degradation of EPTC include a cytochrome P-450 system (*Thc*B), and two genes downstream from *ThcB* producing a rhodocoxin (*Thc*C), and a rhodocoxin reductase (*Thc*D). The authors reported that the *ThcB* gene is the first member of a new gene family, CYP116. A regulatory protein (*Thc*R) is divergently transcribed from the cytochrome P-450 system that is a member of the AraC-XyIS family of transcription regulators. It has been suggested that *Thc*R probably regulates the expression of cytochrome P-450 system during EPTC degradation (Shao and Behki, 1996). Also identified during EPTC degradation was a NAD^+ dependent aldehyde dehydrogenase (*Thc*A). Although the

cytochrome P-450 appeared to be unique to the *Rhodococcus* species strain NI86/21, the dehydrogenase was found in other *Rhodococcus* species and most likely metabolizes the aldehyde that is generated after dealkylation.

Shao and Behki (1995) reported that *Rhodococcus* species strain TE1 could degrade EPTC because of the presence of an indigenous plasmid. A gene responsible for EPTC degradation, *eptA* was found to be located on a 6.2 kb KpnI fragment. Further cloning and characterization of the KpnI fragment suggested that the genes in the plasmid of *Rhodococcus* species strain TE1 where similar to the genes in the cytochrome P-450 system of *Rhodococcus* species strain NI86/21. The findings are important because the same gene sequences were found in two different bacterial strains from two widely divergent geographical regions of the world. Also in one strain the genes were located on the chromosome and the other on a plasmid. This information is clear indication of gene mobilization and transfer into a new bacterial strain with EPTC degradation potential. Several EPTC-degrading bacterial species have also been reported to degrade atrazine. Behki (1995) reported there was a threefold increase in degradation of atrazine by *Rhodococcus* species strain TE1 when EPTC was added. However addition of atrazine did not produce a similar result on the degradation of EPTC. Mulbry (1994) reported that a dechlorinating enzyme, s-triazine hydrolase catalyzes the dechlorination of deisopropylatrazine and deethylatrazine.

de Souza et al. (1995) reported that a pMD1, 22-kb EcoRI DNA fragment from the *Pseudomonas* species strain ADP, encoded the genes for atrazine degradation. A 1.9-kb *Ava*I fragment was cloned into pACYC184 and the new plasmid, named pMD4, was

expressed in *E. coli*. A 0.6-kb *Apa*I-*Pst*I fragment from the pMD4 was later identified as the putative atrazine chlorohydrolase gene (*atz*A) responsible for the removal of chlorine. The *atz*B gene, which is responsible for the dealkylation reaction, could only metabolize hydroxyatrazine, a product of *atz*A metabolism, but could not dealkylate atrazine (Boundy-Mills et al., 1997).

Shao et al. (1995) elucidated the structure and sequence of the 2450 bp long *trz*A gene which was not expressed in *E. coli* or other gram-negative bacteria. The gene, however, was expressed in the bacteria *Rhodococcus*. It was also found that *Rhodococcus* sp. that carried the *trz*A gene was able to dechlorinate the dealkylated atrazine products. The *trz*A gene in *Rhodococcus* has only a 41% sequence identity with the *atz*A gene in the *Pseudomonas* species strain ADP. However, both genes have similar function and are responsible for a chlorohydrolase enzyme. The atrazine degrading genes in Pseudomonas species strain ADP (*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, and *atz*F) have been reported to be widespread and highly conserved (de Souza et al., 1998). In *Nocardia* species, the dechlorination of atrazine was mostly carried out through another dechlorination gene (*trz*N) (Smith et al., 2005). Several other atrazine degradation gene combinations have been reported (Piutti et al., 2003; Rousseaux et al., 2001). Mineralization of atrazine in gram-negative bacteria was carried out by the *atz*ABC-*trz*D gene combination in pure cultures. In contrast, gram-positive bacteria could transform atrazine into cyanuric acid and only possessed the *atz*B and *atz*C genes. A list of the different atrazine metabolites, their common names, genes responsible for degradation and corresponding enzymes that catalyze the reaction has been provided (Table 2.1).

16S rRNA gene as a molecular tool

In the last few decades, identification and monitoring of bacterial communities in different complex environments has involved the use of the 16S rRNA gene. Since culture-based methods can only explore about 0.1 to 10% of the total microbial diversity (Torsvik and Ovreas, 2002), analysis of the 16S rRNA gene to identify individual species and communities provides a definite advantage over culture-based methods. Moreover it is also not known whether the cultural fraction of soil microbial population is a representative community or not.

The 16S rRNA molecule is an integral part of the ribosomes found inside both eukaryotic and prokaryotic cells. The ribosome is one of the most important cell components responsible for synthesis of protein by translating mRNA molecules. Structure of ribosomes differs significantly between bacteria, archea, and eukaryotes. Ribosomes are composed of ribosomal RNA (rRNA) and many protein molecules. Ribosomes are divided into two parts, one larger than the other. Prokaryotic ribosomes consist two subunits—a 50S large subunit and a 30S small subunit. The 30S small subunit is composed of a single 16S rRNA and 21 proteins ranging in molecular weight from 9 kD to 61 kD. This subunit is the site of translation initiation and is important for understanding protein synthesis and drug discovery.

The 16S rRNA molecule is the major component of the 30S subunit. The gene that codes for 16S rRNA has 1542 bases and contains three substrate binding sites (A-, P-, and E-). It has both variable and conserved regions in it’s sequence. Using sequence analysis of the 16S rRNA gene, it is possible to identify bacterial species and phylogenetic relationships among different species. The 16S rRNA can also be compared

with the 16S rRNA gene of archea as well as the 18S rRNA gene for eukaryotes. The 16S rRNA gene sequence for a very large number of strains has been determined and is accessible in the GenBank database. The most important characteristic of the 16S rRNA gene is that it is universal in all bacteria which allows for analysis of the 16S rRNA gene for the measurements of relationships between bacteria. As a result the 16S rRNA gene has been often used by researchers as a phylogenetic marker to determine microbial diversity and structure (Hill et al., 2003).

The 16S rRNA gene of bacteria contains nine highly variable regions named V1 through V9 interspersed between stretches of conserved regions. The nine hyper variable regions can be found between the nucleotides 69 – 99, 137 – 242, 433 – 497, 576 – 682, 822 – 879, 986 – 1043, 1117 – 1173, 1243 – 1294, and 1435 – 1465, respectively, for V1 through V9. As evident from the nucleotide positions, the sizes of these hypervariable regions are different. Because these variable regions are spaced in between conserved regions, it is possible to amplify these variable fragments by polymerase chain reaction (PCR) and subsequently compare and identify the bacterial species.

Although there are nine variable regions, their importance in identifying species and establishing relationships is different. The degree of variation within each hypervariable region is also not uniform, rendering some regions more useful from a phylogenetic standpoint than others. For example, V1 can be used to differentiate among different *Staphylococcus* species. Likewise, V6 is capable of differentiating most bacterial species with specialty to differentiate between bacterial species from the genus *Bacillus*. The V4, V5, V7 and V8 regions are not as useful as the other regions. On the

other hand, V2 and V3 are highly efficient in differentiating almost all bacterial species to the genus level except for a few Enterobacteriaceae.

Using the 16S rRNA gene for microbial ecology research in the last couple of decades has altered the foundation of taxonomy. With the new sequence-based taxonomy, the classification of five kingdoms has become obsolete. Instead current knowledge has developed that indicates all life can be grouped into three domains of life—the archaea, eukarya, or bacteria (Hugenholtz et al., 1998).

Molecular microbial diversity techniques

Until recently, soil bacterial populations were very difficult to monitor and only culturable bacteria were studied in the laboratory. Such investigations were limited by the fact that only a maximum of about 10% of the soil bacterial population can be cultured in the laboratory. Thus we had no knowledge of the remaining 90% of the soil bacteria that was present in the soil. With the advent of modern molecular tools in the last couple of decades, we now have resources to gain information about soil bacterial communities and functions that was not possible earlier. Studies have since investigated bacterial functions *in situ* and efforts have been made to link different bacterial species to specific soil functions.

Traditionally, microbial diversity studies were based on cultivating the organisms in a laboratory and efforts to cultivate diverse organisms centered on the use of different kinds of nutrient rich or oligotrophic medium to grow the organism. The identification of physiologically distinct groups of microorganisms was restricted to isolation of bacteria from pure culture. However, since we know that we have only cultured about 1 – 10% of

the total microbial population (Torsvik, 1990), there is a great possibility that we have overlooked previously unknown and unique microorganisms with separate enzyme-mediated pathways for EPTC and atrazine degradation.

Molecular techniques such as denaturing gradient gel electrophoresis (DGGE) community profiling (Muyzer et al., 1993; Øvreas et al., 1997), DNA cloning, probing and sequencing (Borneman et al., 1996), amplified rDNA restriction analysis (ARDRA) (Vaneechoutte et al., 1992) and DNA melting and reassociation profiles (Torsvik et al., 1990, 1996) have been used by scientists to better identify the microbial community structure in different ecosystems. Use of such techniques has transformed microbial ecology research. For example, it is now possible to focus more attention on the molecular mechanisms involved for most ecosystem functions by going directly to the genes expressed and studying the products of these genes.

Denaturing gradient gel electrophoresis

One of the most widely used techniques in microbial ecology is denaturing gradient gel electrophoresis (DGGE). PCR-DGGE has been applied for the exploration of microbial communities in different ecosystems and in response to change. DGGE was invented for the purpose of detecting single base. However over the years, DGGE has evolved and Muyzer et al. (1993) first used 16S rDNA fragments to fingerprint microbial communities in complex environmental samples. The technique relies on the separation of desired PCR fragments based on their sequences in a gel matrix, usually a polyacrylamide gel, as they encounter increasing concentration of a denaturant. Since then, the PCR-DGGE technique has been used to monitor prokaryotic communities after

16S rRNA gene amplification, eukaryotic communities such as fungi after 18S rRNA gene amplification, or by use of other specialized PCR products to evaluate different ecosystem functions and/or microbial communities.

In a PCR-DGGE method, same-sized DNA fragments are denatured while moving through the gel matrix and their movement is impeded during the process. The denaturing or melting of DNA fragments depends on each individual sequence and more precisely on the extent of guanidine and cytosine (GC) bases present in the DNA fragments. As a result DNA fragments with different sequences denature at different gradients in the gel and appear as separate electrophoretic bands. PCR amplified DNA fragments of sizes ranging between 300 to 1000 base pairs long can be efficiently separated in a DGGE gel.

The denaturants used in PCR-DGGE polyacrylamide gel are urea and formamide. A 100 percent denaturing solution contains 7M urea and 40% formamide. The concentration of urea and formamide is changed in proportion to obtain lower denaturing gradients and thus more discriminating power to separate DNA fragments. Depending on the specific gradient that is desired, gels are poured in a gel casting equipment to form a linear gradient. The electrophoresis is carried out at a constant temperature of 60°C. PCR products loaded in a DGGE gel are amplified using primers sets that have a GC clamp (30-40 bp GC bases) added to one of the primers in the PCR reaction to prevent complete separation of the two DNA strands that can result in smeared bands (Muyzer and Smalla, 1998). Another method to achieve the same purpose is by using chemiclamps, psoralen-derivatized PCR primers, in place of the regular GC clamps (Fuhr, 1996). The problem with using the chemiclamps is that it prevents downstream sequencing without adding a

nested PCR step as the chemiclamps are covalently linked to the DNA strands (Ercolini, 2004).

DGGE has been validated as an excellent tool to investigate microbial community dynamics and species diversity. The advantage of using a PCR-DGGE method is that many samples can be analyzed at the same time to obtain a fingerprint profile for each set of samples. Monitoring a shift in microbial community diversity due to change in environmental conditions or other external stimuli has become more routine as a result of applying the PCR-DGGE method.

PCR-DGGE was introduced in food microbiology when Ampe et al. (1999) reported the microbial community interactions during the spontaneous fermentation of maize for the production of Mexicans pozol dough. The authors compared the PCR-DGGE method with traditional food microbiology studies and concluded that the culture-independent PCR-DGGE method is better in comparison. Use of this technique to study the diversity and composition of marine picoeukaryotic assemblages also revealed that the PCR-DGGE method provided a detailed composition of marine picoeukaryotic assemblages and was robust enough to allow phylogenetic identification of the dominant members of the ecosystem (Diez et al., 2001).

Researchers have used the PCR-DGGE approach to study the composition and shift in bacterial, archeal, nematodal and fungal communities in lake waters (Crump et al., 2003), in an estuarine salinity gradient (Crump et al., 2004), waste water treatment plants (Gary et al., 2002; Stamper et al., 2003), fungal infections (Kowalchuk et al., 1997), arbascular mycorhhizal fungi (Liang et al., 2008). Other studies involving the PCR-DGGE method investigated microbial diversity in the rumen epithelium (Sadet et

al., 2007).The PCR-DGGE method might also be useful for detecting changes in the dominant microbial species in an ecosystem, but that rare species are not detected efficiently (Cook et al., 2005). However, it should be emphasized that the success or failure of this method also depends on the success of the PCR reaction and efficiency of the primers used to amplify the representative population in that particular ecosystem.

Limitations of molecular methods

Cell lysis, DNA extraction and inhibitors

Even though molecular methods to study microbial diversity are more efficient than traditional culture based methods, there is also a certain amount of limitation with the molecular techniques. Cell lysis can be the first major limitation, as lysis efficiency varies between different groups of microorganisms (Prosser, 2002). Gentle lysis methods will preferentially work for gram-negative bacteria and leave gram-positive bacteria intact in the soil. Harsh lysis protocols, involving use of beads, may be successful in breaking open both gram-negative and gram-positive bacteria, but in the process might shear or degrade the extracted DNA (Wintzingerode et al., 1997). Feinstein et al. (2009) found that extraction bias caused a significant shift in community composition in the terminal restriction fragment length polymorphism profiles. They also reported that the relative abundance of sequences from rarely cultivated groups of bacteria were higher in the first extraction than in the sixth. To circumvent the problem, they recommended three successive extractions and the use of the pooled sample for community analysis. If downstream PCR reaction is required, presence of humic acids and other inhibitors can cause problems. PCR inhibitors usually inhibit the amplification by directly interacting

with the DNA or altering the function of the thermostable DNA polymerase. Presence of inhibitors that reduce Mg^{2+} availability for DNA polymerase can also inhibit PCR.

Universal primers

Bias can be introduced during the PCR reaction for any of the following reasons : differential primer affinity for templates, varying copy number of target genes, hybridization efficiency, and primer specificity (Wintzingerode et al., 1997). In many PCR-based microbial ecology methods, the 16S rRNA gene is amplified using universal primers. Farris et al. (2007) reported that for the detection of actinobacteria cultivated from environmental samples, different sets of universal primers used in the study had different amplification efficiency. Even when there was a hundred percent sequence similarity, in some cases, the primers could not amplify the genes. Luo et al. (2006) reported that while testing different universal primers, they found that by using the primer sets 63f/518r, 341f/926r, and 933f/1387r there was a significantly lesser number of discernible DGGE bands in comparison with primer set 968f/1401r.

Bacterial Species Classification and Phylogenetic trees

Classification of bacterial species into genera and species has remained a complicated topic since the late 19th century. Since the classification used for microorganisms was really developed and based on the system used for highly evolved organisms, there was significant anomaly that existed in the classification of bacterial species (Stackebrandt and Goebel, 1994). Researchers like Ravin (1963) argued that the zoological definition of species/group/organisms based on “potentially interbreeding natural populations that are reproductively isolated from other such groups” cannot be applied to prokaryotes.

However with the advent of molecular microbiological techniques, attempts have been made to define a constant bacterial taxonomy. Even then a disparity exists between the use of techniques like DNA similarity and 16S rRNA gene homology. Organisms have been identified with almost identical 16S rRNA gene sequences but with less than 70% DNA hybridization, implying the existence of two different species (Stackebrandt and Goebel, 1994). Liu et al. (2008) reported that using different portions of the 16S rRNA gene resulted in different taxonomic classifications for the same species or the same sequence set. They also recommended a fragment size of a minimum of 250 bases and a few specific primers.

Amann et al. (1992) reported that even with 99.8% sequence similarity, the DNA hybridization value may only be 25%. Such statistics mean that DNA hybridization has a greater degree of resolution capacity between closely related individuals. Fox et al. (1992) also argued that 16S rRNA gene sequence identity is a rather inaccurate way to classify bacterial species and that DNA hybridization is by far the most reliable tool available at present. At the same time it should be noted that the DNA hybridization method is unable to differentiate results where there are differences in genome size or plasmid content. Even the hybridization conditions and methods used can influence the results (Williams et al., 2001). It is thus apparent that the classification of bacterial species varies and may be dependent on the different techniques used by individual researchers. A consensus bacterial taxonomic classification system would be needed to generate reproducible phylogenetic trees of bacterial species.

However, investigations based on the 16S rRNA gene sequence have been extensively used in microbial ecology research. Numerous bacterial phylogeny analyses

conducted using 16S rRNA gene sequence (Woese et al., 1990) has resulted in establishment of public domain databases (Maidack et al., 1996) for large number of uncultured microorganisms from complex environmental samples. The additional fact that 16S rRNA gene sequence is universally present in prokaryotes along with species-specific hypervariable regions qualifies provides a unique edge for use of this molecular method in microbial ecology research. Drancourt et al. (2000) compared the effectiveness of the 16S rRNA gene sequence to identify a collection of 177 bacterial isolates from environmental, veterinary and clinical sources. At the same time they emphasized that development in the accuracy of the gene sequence databases will further improve the effectiveness of the method. They reported that 16S rRNA gene sequence analysis yielded more than 90% efficiency and is an excellent tool for molecular identification and phylogenetic analysis.

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Common name	Abbreviation	Gene	Corresponding enzyme
Atrazine	CIET	-	-
Deisopropylatrazine	CAET	*atrA*,	P450 enzyme system
Deethylatrazine	CIAT	*atrA*,	P450 enzyme system
Hydroxyatrazine	OIET	*atzA*,	Atrazine chlorohydrolase
N-	OIOT	*atzB*	Hydroxyatrazine
Cyanuric acid	OOOT	*atzC*	N-sopropylammelide
Biuret	-	*atzD*,	Cyanuric acid amidohydrolase

Table 2.1: Atrazine degradation genes and corresponding enzymes (Adinda and De Mot 1999)

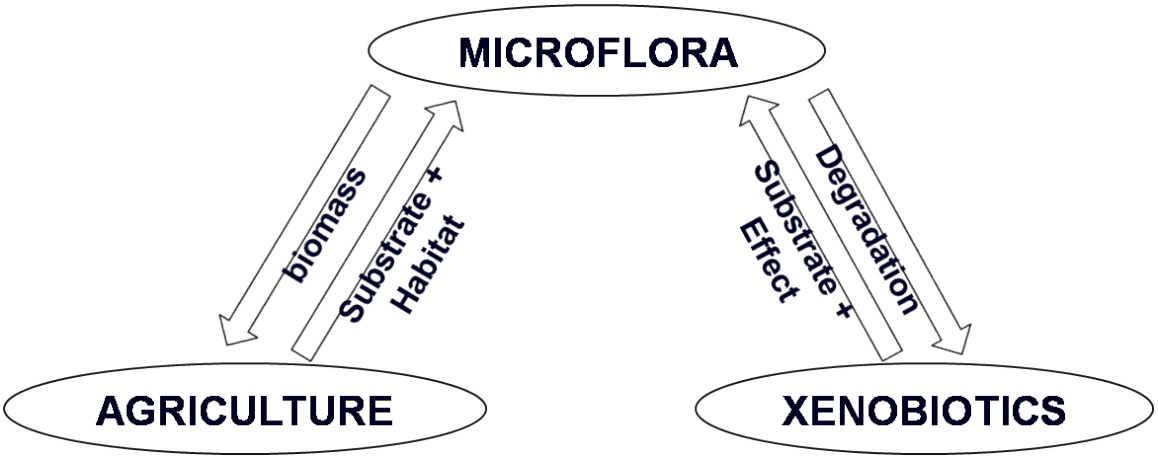

Fig 2.1: Microbial interaction in soil systems (ICDG, 2010**)**

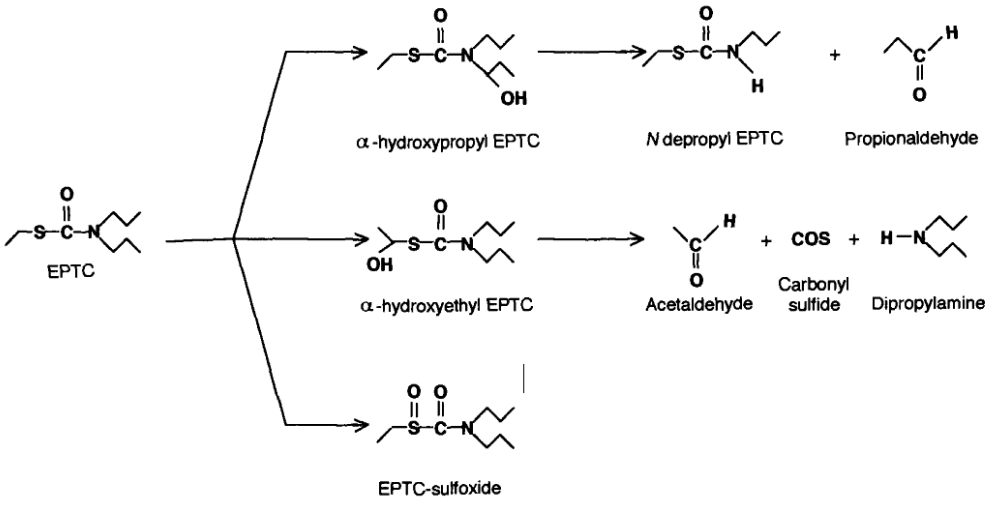

Fig 2.2: Microbial mediated EPTC degradation pathway (Ankumah et al. 1995)

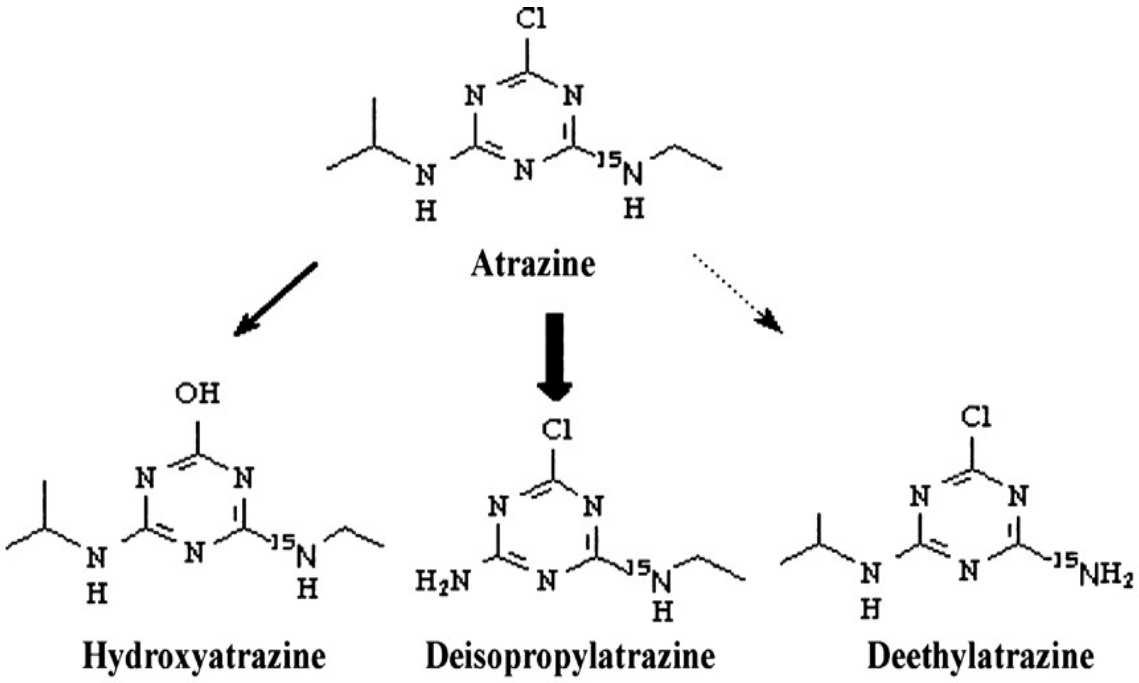

Figure 2.3: First metabolites of atrazine degradation pathway. Bold arrow represents dominant mechanism and dotted arrow represents least dominant mechanism with thin arrow being intermediate. (Mahia and Diaz-Ravina 2007)

CHAPTER 3

SELF AND CROSS ENHANCED DEGRADATION OF HERBICIDES IN WOOSTER SILT LOAM AND LURAY SILTY CLAY LOAM SOILS

Abstract

Use of pesticides is a common component of crop production in agriculture, horticulture, lawn care and urban/agroforestry practices. EPTC and atrazine are two of the most widely used herbicides in the world. Due to repeated exposure of a particular herbicide, soils develop enhanced degradation potential for such herbicides. The objective of this study was to develop enhanced degradation potential for the herbicides EPTC and atrazine in two Ohio soils. The two soils used in this study are Wooster silt loam and (WSiL) and Luray silty clay loam (LSiCL) soils. Four experiments were conducted to evaluate the degradation potential of EPTC and herbicide in these two soils. In the first experiment, we observed enhanced EPTC degradation in both soils after three repeated incubations. The first two incubations were continued for two weeks and the third incubation was continued for four days. In the third incubation experiment, more than 85 to 90% of the EPTC added was dissipated within four days. Similar result was obtained for degradation of atrazine in these two soils. For the atrazine degradation study, the natural soils were inoculated with EPTC enhanced soils in order to monitor the shift

in microbial population. Evidence of cross enhancement was also found for EPTC in atrazine enhanced field soils. Lastly, effects of different antibiotics on the enhanced degradation potential of EPTC were investigated. The effect of three antibiotics-kanamycin, chloramphenicol, and cycloheximide was monitored. Chloramphenicol was the most effective antibiotic in reducing the degradation of EPTC and atrazine from the soils.

Introduction

Large quantities of pesticides are used in agriculture, horticulture, landscape and other industries to control insects, microbial pathogens, and rodents and other small animals. Though active against the target pest population, significant quantities of many pesticides persist in the environment and may be harmful and toxic to other nontarget plants and animals and even effect human health. A recent study reported that less than 1% of the total amount of pesticide active ingredients applied worldwide actually reaches the target pests while the rest accumulates in the soil or is dissipated in the environment.

Both EPTC (S-ethyldipropylthiocarbamate) and atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) are widely used for weed control in the United States and around the world. EPTC, a sulfur containing carbamothioate, is used for the pre-emergent control of annual grasses, broadleaf and perennial weeds. EPTC is classified as moderately to slightly toxic and long-term exposure can impact the nervous system and the heart. The primary routes of EPTC dissipation are microbial degradation and volatilization. Degradation, especially complete degradation, will convert the applied pesticide into inactive and less toxic forms.

Atrazine belongs to the class of s-triazine compounds and is used for the pre and post-emergence control of broadleaf and grassy weeds. Atrazine is heavily used in corn with more than 75% of US corn acreage receiving some application of atrazine. Atrazine has a much higher relative persistence in the environment in comparison to EPTC. Because of this fact, atrazine is routinely detected in both ground water and surface waters around the world. Atrazine is also more potent than EPTC in terms of it's toxic impacts. Several studies have reported negative impacts of atrazine on the reproductive system of amphibians. Long-term exposure impacts on human health are still not conclusive, but early indications suggest atrazine can be carcinogenic after prolonged exposure.

The half-lives of EPTC and atrazine have been determined empirically by various researchers, and range from 6 to 112 days, depending on soil and environmental conditions. Singh et al. (1990) measured the half-lives of both EPTC and atrazine in California soils for various moisture regimes and found that the half-life of EPTC ranged between 25 to 44 days while the half-life of atrazine ranged between 26 to 34 days.

To achieve better pesticide use and management, slight persistence of the applied chemical is desired by agricultural managers for the purpose of control of the pest population. But environmentalists are also concerned by the residual toxicity of agricultural chemicals and their negative impact on the nontarget species and nonpoint source pollution. Repeated application of the same pesticide or structurally similar compounds can cause the efficacy of the pesticides to kill the target pests to be greatly reduced. This is because too rapid degradation removes the pesticide before it has a chance to control its target. Therefore degradation of both EPTC and atrazine at a rate

that maintains concentrations high enough to control the target pest, but then dissipates afterwards is desired. In the 1980s, an estimated 80% of the herbicides and 67% of the insecticides applied in the North central region of the United States exhibited enhanced degradation (Ankumah, 1988). McClung et al. (1994) reported that more than 25 commercial pesticides have been reported to show "enhanced degradation" in soils. Enhanced degradation is mostly influenced by the microbial population present in soil, specifically the bacterial population. Exploring the pesticide degradation mechanism is important to develop better pesticide management regimes.

The capacity of soil microorganisms to degrade similar or closely related group of compounds has been investigated by several researchers. Cross enhancement degradation is when microbial species adapt to one compound but is then able to metabolize another compound that is structurally similar. In soil, cross enhancement occurs when one pesticide is degraded at an enhanced rate when the soil has previously been treated with a different, but structurally similar pesticide.

Initially there is a degradation lag phase as the microbial population capable of degrading the new compound adjusts to the presence of the new compound. The lag phase has been attributed to several mechanisms including time needed to enrich a specific portion of the microflora or time needed for the substrate to induce enzyme synthesis (Rao, 2000). Evidence of cross enhancements has been documented in several studies (Suett and Jukes, 1988; Bean et al., 1998; Morel-Chevillet et al., 1996; Warton et al., 2003).

Because evaluation of enhanced degradation potential for a particular soil can often be difficult to monitor, the objectives of this study were to: i) obtain enhanced

degradation potential for EPTC with repeated applications, ii) evaluate cross-enhancement for degradation of atrazine in EPTC-enhanced soils and vice versa, and iii) determine the effect of antibiotics in suppressing the degradation of EPTC in EPTC-enhanced soils.

Materials and Methods

Wooster silt loam soil was collected from The Ohio State University – The Ohio Agricultural Research and Development Center farms located in Wooster, Ohio. Luray silty clay loam soil was collected from a private firm in Wooster, Ohio. Soil samples were collected near agricultural fields that have not been exposed to EPTC or atrazine to the best of our knowledge. Collection of soil from a site that has never been exposed to these herbicides will ensure an unbiased response as a result of repeated applications of the herbicides. Development of accelerated degradation of the herbicide, if observed, will thus be a direct result of the repeated exposure of the soil to the herbicide of interest. The two soils sampled were Wooster silt loam and Luray silty clay loam) that had contrasting physical and chemical properties. Luray silty clay loam soil has higher clay and organic matter content, and a slightly more alkaline pH in comparison to the Wooster silt loam soil (Table 3.1).

Replicated soil samples were collected from the 0 - 15 cm surface layer using a shovel. Samples were placed into Ziploc bags and transported in coolers to the laboratory where they were maintained at 4° C until analysis. Similarly two field soil samples were also collected from the King Range B and the OARDC Old Weather Shed Lot B sites located on the campus of The Ohio State University-Ohio Agricultural Research and

Development Center, Wooster, Ohio. King Range B last had atrazine applied in 1999, with a herbicide called Guardsman at 5 kg ha^{-1}. Old Weather Shed Lot B had atrazine applications starting in 1999, with a herbicide called Harness Extra applied at a rate of 5.4 kg ha^{-1}. Atrazine was applied three more times in 2002 (8.4 kg ha^{-1}Degree Extra plus 0.75 kg ha^{-1}Aatrex 4L), 2005 (9.6 kg ha^{-1}Bullet), and 2008 (8.4 kg ha^{-1}Degree Extra plus 0.75 kg ha^{-1}Aatrex 4L).

Chemicals used in this study were purchased from commercial vendors within the USA. Certified grade EPTC (S-ethyl N,N-dipropyl carbamothioate) and atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) were purchased from Chem Service Inc., West Chester, Pennsylvania, USA. EPTC was a clear liquid with 99.5% purity while atrazine was a white powder with 99.8% purity. Pesticide grade toluene, used in the extraction of herbicide from soil, was purchased from Sigma-Aldrich Company, St. Louis, Missouri, USA. Cycloheximide, kanamycin, and chloramphenicol were purchased from Thermo Fisher Scientific, Waltham, Massachusetts, USA.

Soil analysis. Before the experiment, soils stored in 4°C were air-dried at room temperature, ground, and sieved through a 2 mm mesh. The different soil properties measured were pH, organic matter content, and soil particle size distribution. Soil pH was measured using a glass electrode (1:1 soil:water ratio prepared with 25 g of soil and 25 mL of deionized water). Soil organic matter content was measured by the loss on ignition (LOI) method (Combs and Nathan, 1998) where dried and ground soil is incubated for two hours at 105°C (W1) followed by two hours at 360°C (W2). Organic matter content is measured based on the difference in weight between W2 and W1. Soil particle size

distribution was measured using the hydrometer method (Bouyoucos, 1927). Salient characteristics for the two soils are reported in Table 3.1.

Soil spiking, herbicide extraction and detection. Air-dried soil samples and EPTC or atrazine were mixed with sufficient amount of water corresponding to the gravimetric water content (Black, 1965) for each soil. A spatula was used to obtain a thoroughly mixed sample with uniform distribution of herbicide and the soil. EPTC and atrazine were added at the rate of 10 mg kg^{-1} of soil.

Residual herbicide in the soil after incubation was extracted with toluene as a solvent using the following procedure. Five g soil sample was removed from the incubation container and placed into a 50 ml Falcon tube. Three mL of deionized water and 5 mL of pesticide grade toluene were added to the soil in the Falcon tube. The tube was capped and vortexed for 30 seconds in a vortex mixer. The tubes were then placed in a horizontal shaker for an hour of shaking at a rate of 75 oscillations per minute. The tubes were then centrifuged at 200 g for 30 seconds to allow for the separation of the toluene fraction at the top. A 200 µL sample from the top layer was carefully aspirated and transferred to a small glass vial. Anhydrous sodium sulfate was added to the glass vial to trap any moisture present in the sample.

The amount of EPTC and atrazine present in the sample was measured using a Varian CP 3800 gas chromatograph fitted with a thermoionic specific detector (TSD). A TSD detector is similar to a flame-ionization detector but uses a ceramic bead for the detection of nitrogen or phosphorus. A fused silica column, CP 0.32 mm X 7 µM X 30m, was used for the detection of both herbicides. A splitless injection was performed with

1.0-µL injection volume. An initial oven temperature of 90 °C was maintained for 0.5 min. A temperature gradient of 15 °C/min was initiated until a temperature of 160 °C was reached, followed by a 25 °C/min gradient to a final temperature of 200 °C. The oven was maintained at 200° C for an additional 0.5 min. The entire analysis required a run time of 7.2 min. Injector and detector temperatures were maintained at 280 and 300 °C, respectively. Helium was used as the carrier gas and maintained at a flow rate of 1.0 mL/ minute. Helium was also used as the makeup gas and was set at a flow rate of 28 mL/minute. Hydrogen and medical grade air where introduced to activate the element in the TSD at the flow rates of 4.2 mL/minute and 175 mL/ minute respectively.

The program for detection of atrazine was slightly different with an initial oven temperature set at 90° C. A temperature gradient of 15 °C/min up was used up to a temperature of 160 °C, followed by a 25 °C/min gradient until a final temperature of 270 °C. The entire analysis required a run time of 10 min. Injector and detector temperatures were maintained at 280 and 300 °C, respectively. The gas flow through the column and at the detector was kept same as the EPTC program described above. A 1/100 split injection was done for atrazine samples.

At these operating conditions, the retention time for EPTC was 6.47 minutes while the retention time for atrazine was 8.49 minutes in the 30 m fused silica column. For both herbicides, the detection limit was 0.05 ppm and average recovery was about 70%.

Self enhancement of EPTC enhanced degradation. Wooster silt loam and Luray silty clay loam soils were used for the first incubation experiment to develop enhanced EPTC

degradation potential. The soils were collected from outside the crop production areas that were least likely to be exposed to the herbicides. In the laboratory, gravimetric water content for Wooster silt loam and Luray silty clay loam was determined at 27% and 36% respectively. Air-dried samples from these two soils were ground and sieved through a 2 mm mesh before spiking with the herbicide. For each incubation chamber, 300 g soil sample was placed in a 500 ml plastic container. Three mg EPTC was uniformly mixed with sufficient amount of deionized water to obtain field moisture capacity for the respective soil types. The water and EPTC mixture was slowly poured into the soil in the container while continuously mixing with a spatula until the entire solution was mixed with the soil. Similar protocol was followed for each soil type and all the replicated containers. Five g soil sample from each container was collected in 50 ml Falcon tubes for initial extraction to determine the recoverability of the herbicide from soil. This sample was also used as the time zero sample. The containers were then capped and incubated at 25°C in the dark for two weeks. Subsequent extraction following the same procedure mentioned earlier was carried out after 4, 7, 11, and 14 days of incubation. The herbicides in the extracts were quantified using gas chromatography.

Each container was uncapped after 14 days of incubation and the soil was air-dried. The soil chunks were broken and ground and then re-treated with the same rate of EPTC as before. Residual EPTC in the soil was measured after the same intervals of 0, 4, 7, 11, and 14 days as in the first incubation experiment. However for the third round of incubation, residual EPTC samples was extracted every 24 hours starting at time 0 and continuing after 1, 2, 3 and 4 days.

Enhancement of atrazine degradation in EPTC enhanced soils. Enhanced degradation of atrazine was developed in the same two soils as used for the EPTC degradation experiment (i.e. Wooster silt loam and Luray silty clay loam soils) were used for the atrazine cross enhancement experiment. Initially 75 g of EPTC enhanced soil (after the third treatment) was mixed with 225 g of natural Wooster silt loam and Luray silty clay loam soil. The soils were thoroughly mixed to obtain a uniform sample. Atrazine was added to the soil following the same rate and procedure as in the EPTC self enhancement experiment. For each incubation chamber a 300 g soil sample was placed in a 500 ml plastic container and atrazine was added at the rate of 10 mg kg^{-1} soil following the same procedure used to add EPTC to the soil. The moisture contents in the incubation containers were maintained at 27% and 36% for Wooster silt loam and Luray silty clay loam, respectively. The containers were then capped and incubated at 25°C in the dark for two weeks. Five g soil sample from each container were then removed for the extraction of atrazine following the same protocol as for EPTC extraction. All extractions were carried out following the same procedure mentioned earlier for EPTC. A total of three cycles was repeated to develop the enhanced atrazine degradation potential in the two soils, with the same frequency of sampling and sampling interval as the EPTC self-enhancement experiment.

EPTC degradation in enhanced atrazine degradation soil: Two field soils with different histories of atrazine application were collected. These soils were not treated in the laboratory in any way to increase either atrazine or EPTC degradation. Atrazine was last applied in the King Range B 10 years ago in 1999. Soil from the OWS Lot B site

received atrazine application every three years with an application occurring in the most recent growing season. None of the soils received any EPTC application during this entire period. Replicated soil samples were collected from corn fields at these two sites in 2009. Soils were also collected from an adjoining forested area near the King farm as a control soil.

EPTC was mixed with the soil following the same procedure as the described previously. For extraction of herbicide from soil, a 5 g soil sample was placed in a 50 mL Falcon tube. Residual EPTC was extracted and analyzed at the onset of experiment (time zero) and after 4 and 7 days following the extraction procedures described previously.

Effect of Antibiotics on EPTC degradation: Three antibiotics were used for this experiment—kanamycin, cycloheximide and chloramphenicol. In general, kanamycin and chloramphenicol are effective against bacteria while cycloheximide works mostly against fungi. Three doses for each antibiotic were investigated and were 50, 75 and 100 mg kg^{-1} soil. The soil used was that obtained after the third consecutive treatment of EPTC and that exhibited enhanced EPTC degradation abilities. A total of 75 g of the EPTC-enhanced soil was mixed with 225 g of Wooster silt loam or 225 g of Luray silty clay loam soil. The soils were thoroughly mixed to obtain a uniform sample. EPTC was then added to the soil following the same procedure as in the enhanced EPTC degradation experiment. Five g replicated soil samples were individually incubated in 50 mL Falcon tubes for each soil. EPTC enhanced soil without any antibiotic was used as a control. Residual EPTC was extracted at the onset of experiment (time zero) and after 4 and 7

days. Similar extraction and analysis procedures for EPTC were used as previously described.

Experimental design and data analysis. A randomized design with two replicates was used for the soil incubation experiments. The residual EPTC and atrazine in the soil were analyzed statistically using JMP (SAS Institute) and Minitab statistical software while the graphs were generated using Microsoft Excel 2007. Comparison of treatments, at a predetermined level of significance ($p < 0.05$), was done using a HSD-Tukey method (JMP, SAS Institute). In all figures and table, mean values denoted by different letters are significantly different ($p < 0.05$).

Results and Discussion

Self enhancement of EPTC degradaton. Successive application of EPTC to Wooster silt loam and Luray silty clay loam soils resulted in a gradual increase in the degradation potential in each cycle for both soils (Figure 3.1). Although many soils exhibit conditions required for enhanced pesticide degradation, it is often difficult to detect. The first indication of enhanced herbicide degradation is often the reduction in the efficacy of the herbicide to kill its target weeds. This reduction in efficacy would be difficult to monitor if recommendations for pesticide application have been calculated that unintentionally consider the effects of enhanced degradation, i.e. the recommendations are increased due to enhanced degradation. When enough pesticide is applied in soil, most undesired plants and weeds will be killed before the chemical is mineralized resulting in optimum weed control. The process can become more complicated because

even a 10% inoculation by an enhanced soil onto an unenhanced soil can result in accelerated pesticide degradation (Engvild and Jensen, 1969) in the receiving site. Runoff and wind erosion transport soils between landscapes on a regular basis and this can result in the mixing of enhanced and unenhanced soils.

In the first incubation cycle (Figure 3.1), both Wooster silt loam soil and Luray silty clay loam soil exhibited similar rates of degradation. For each soil, after four days of incubation during the first cycle only 15% to 20% of the EPTC in the soil was degraded. After 14 days of incubation, approximately 70% of the EPTC was degraded but 30% still remained in both soils. By the third cycle (Figure 3.1), most of the EPTC was degraded by the fourth day of incubation. During this third cycle, EPTC degradation in the Wooster silt loam soil as compared to the Luray silty clay loam soil, was much faster for days 1 and 2. By day 4, 85% and 95% of the EPTC was degraded in Luray silty clay loam soil and Wooster silt loam soil, respectively. During the second cycle the degradation curve for both soils proceeded at a higher rate in comparison to the first cycle but was much slower than the third cycle.

Many researchers have reported the existence of enhanced pesticide degradation mechanisms in soil (Engvild and Jensen, 1969; Tal et al., 1990; Bean et al., 1988; Roeth et al., 1989; McClung et al., 1994; Rouchaud et al., 1997; Cotterill et al., 1989; Felsot 1989; Moorman et al., 1992; Karpouzas et al., 1999; Ankumah, 1988). Felsot (1989) reported that chemically similar herbicides and insecticides can all exhibit enhanced degradation. The result of rapid or enhanced degradation of insecticides, such as carbofuran and diazinon, can result in economic losses in crops such as corn, cabbage and rice.

Bean et al. (1988) found that enhanced pesticide degradation is influenced by types of pesticide in rotation, time and site of application. When a particular pesticide is not used for a period of time, the soil that once showed enhanced degradation potential can revert back to natural condition without the capability to rapidly degrade an added pesticide. The authors also reported that through cross enhancement, a chemical may be rapidly degraded as a result of an earlier application of another, but chemically similar pesticide. Investigations of such mechanisms are important because the knowledge of enhanced degradation of pesticides will help in the formulation of bioremediation techniques for contaminated areas.

The gradual increase in the rate of degradation confirms the conditioning effect of soil in order to develop enhanced degradation. The conditioning of soil is attributed to a proliferation of inducible biological agents against a particular substrate (Kaufman and Edwards, 1983). The results from this experiment clearly show that enhanced EPTC degradation develops in both Wooster silt loam and Luray silty clay loam soils.

Repeated applications have also resulted in enhanced degradation of several other herbicides such as glyphosate (Forlani et al., 1999), carbetamide (Hole et al., 2001), propyzamide, linuron, and alachlor (Walker and Welch, 1991).

Enhancement of atrazine degradation in EPTC enhanced soils.

Like EPTC, s-triazene herbicides can also be susceptible to enhanced microbial degradation in soil. Zablotowicz et al. (2007) found that after a single application microbial population develops adaptation for rapid degradation of atrazine. Both atrazine

and simazine suffered reduction in the efficiency of weed control in s-triazine adapted soils (Krutz et al., 2007; 2008). In the first incubation cycle (Figure 3.2), both soils exhibited a similar trend for degradation of atrazine. After 14 days of incubation, approximately 85 to 90% of the atrazine present in the soil was degraded. During the second cycle degradation of atrazine in the Wooster silt loam soil was more rapid in comparison with the Luray silty clay loam soil. By day four of the incubation experiment, almost 88% of the atrazine in the Wooster silt loam soils was degraded. The degradation of atrazine in the Luray silty clay loam soil was more gradual and at the end of 14 days, 90% of the atrazine was degraded. In the third cycle (Figure 3.2) most of the atrazine was degraded by the fourth day of incubation and both soils followed an almost identical degradation curve.

Development of enhancement for atrazine degradation potential in both soils is evident by comparing the degradation curves between the first on the third cycle. The degradation proceeded faster in the first cycle in comparison to the EPTC experiment most likely because some EPTC degrading microbial population are also capable of degrading atrazine. Behki (1995) showed EPTC induced atrazine degradation in the *Rhodococcus* species. The soils were thus "conditioned" for rapid degradation of atrazine after going through three repeat applications of EPTC.

EPTC degradation in enhanced atrazine degradation soil. The two soils collected for this experiment had different atrazine application history. King Farm received the last atrazine application in 1999 (10 years ago) while the old weather shed site received atrazine application every three years starting in 1999 with the latest application

occurring in the most recent growing season. The control soil for each site was collected from an adjacent area with no exposure to EPTC or atrazine.

The results from the incubation experiment with King Farm soils shows that ETPC degradation in both the control and the treatment soils were quite similar (Figure 3.3A). Since King Farm received an atrazine application 10 years previously, any enhanced degradation potential in the soil seems to have dissipated and the soil has returned to its original unenhanced state. Enhanced degradation potential is not permanent and can decrease over a period of time without exposure to the chemical. Smelt et al. (1996) found that enhanced degradation potential aldicarb or oxamyl remained stable for up to five years following which it began to diminish. Other researchers have found that enhanced degradation of 1,3-Dichloropropene can diminish in a much shorter time period of two years or less (Chung et al., 1999). When microorganisms are not regularly exposed to a chemical, the herbicide-degrading microbial species decrease in number and the soil loses its ability to degrade the chemical at an enhanced degradation.

Pesticide rotations have often been recommended to prevent the development of enhanced pesticide degradation. However increased microbial degradation potential for a pesticide due to cross enhancement can be a major cause of concern for farmers and agricultural managers. Suett (1987) first observed that the efficacy of benfurcarb, carbosulfan and furathicarb were reduced in soils that had prior application of carbofuran. The authors also reported that aldicarb lost it’s efficacy quicker in a soil that had been previously treated with carbofuran. This enhancement was even more rapid in carbofuran treated soils than in soils previously exposed to aldicarb (Suett, 1989), thus implying that

cross enhancement can sometimes be more advanced than self-enhancement. Cross enhancement of one compound against another has been documented in several studies (Suett and Jukes, 1988; Bean et al., 1998; Morel-Chevillet et al, 1996; Warton et al., 2003).

Through cross enhancement even in the absence of a particular pesticide, the enhanced degradation potential can persist for several years (Smelt et al., 1996). Behki (1995) reported that enhanced atrazine degradation by *Rhodococcus* strain TE1 can be induced by the addition of EPTC. This study was conducted to evaluate the cross enhancement of EPTC degradation in atrazine history soil.

The Old Weather Shed (OWS) site, in contrast to the King Farm soil, received atrazine application every three years starting in 1999, with the last application occurring in the most recent cropping season. The microbial population in this soil was conditioned to degrade atrazine. When EPTC was applied in a laboratory incubation study (Figure 3.3B), the degradation rate was much faster in the Old Weather Shed corn soil in comparison with the control soil that was not conditioned. The difference in degradation between these two soils was more pronounced after four days of incubation. Even after seven days of incubation, EPTC degradation was higher in the OWS soil than the control soil. However it was apparent from the degradation curve that the microbial population in the control soil had started to adapt to the presence of EPTC. The result also indicates that in the OWS corn site, some of the atrazine-degrading microbial species were also able to degrade EPTC through the mechanism of cross enhancement.

Warton et al. (2003) found cross-enhanced degradation for 2-propenyl isothiocyanate, benzyl isothiocyanate, and 2-phenylethyl isothiocyanate in soils capable

of accelerated degradation of methylisothiocyanate. None of the other three isothiocyanates were applied in the soil, and this implied that application of methylisothiocyante to soil caused increased levels of degradation of the other three compounds due to cross enhancement between. Other researchers have also observed cross enhancement of pesticide degradation (Hole et al., 2001). According to Smelt et al. (1996), enhanced degradation potential, or cross enhancement, for a pesticide can persist for several years. The objective of this experiment was to evaluate the existence of any cross enhancements for degradation of EPTC in soils with a history of atrazine application.

The rate of degradation in the unenhanced King Farm soil was similar to the rate of enhanced degradation in the OWS corn site. Other factors such as soil physical and chemical properties and difference in the community profiles of microbial species affect degradation of organic compounds in soil and so this result is not surprising. It is certainly possible that a similar rate of degradation between the King Farm site (last atrazine application 10 years ago) with OWS corn site (4 atrazine applications in the last 10 years) could occur. Since the degradation rate in the King Farm soil was almost as high as in OWS enhanced corn soil, one would expect that repeat applications of EPTC to the King Farm soil will ultimately result in a higher final rate of enhancement than what would occur in the OWS corn site soil.

These results are similar to the findings of Mahia and Diaz-Ravina (2007) who reported that in two different soils with history of atrazine application for 10 years and 40 years, respectively, the initial rate of atrazine degradation was much higher in the 40-year

history soil compared to the 10-year history soil. The difference between their study and this study is that the result here was also seen in cross enhancement against EPTC.

Effect of antibiotics on EPTC degradation. Degradation of organic compounds in soil is primarily mediated primarily by bacteria, which is the most active and diverse component of the soil microbial population. Application of antibiotics in soil reduces microbially-mediated degradation of organic compounds (Karpouzas et al., 2004). Three different antibiotics were used (a fungicide, a narrow spectrum bactericide and a broad spectrum bactericide) to determine the specific group of microorganisms involved in the degradation of EPTC. The results obtained from the Luray silty clay loam and Wooster silt loam soils are reported in Tables 3.3 and 3.4. In both soils, only chloramphenicol seemed to impact the degradation rates of EPTC in enhanced soil. Ankumah (1988) used three different antibiotics at the rate of 100 mg kg^{-1} to monitor suppression of EPTC degradation and found significant reduction in the rate of EPTC degradation due to the effect of chloramphenicol.

To determine the most effective antibiotic dose to reduce EPTC degradation, three different rates for each antibiotic was used in this experiment. The effect of antibiotics on EPTC degradation was investigated to develop an understanding of the microbial components in the soil responsible for degradation of EPTC. Previous studies have used a 50 mg kg-1 dose and reported effective inhibition of specific microbial groups such as Gram− bacteria, Gram+ bacteria and fungi (Roberts et al., 1998). With a 100 mg kg^{-1} dose of chloramphenicol, there was a significant reduction in the degradation of EPTC during the seven days of incubation compared to the control and the other antibiotics.

The effect of all antibiotics in reducing EPTC degradation was slightly greater in the Wooster silt loam soil compared to the Luray silty clay loam soil. The Luray silty clay loam soil has higher organic matter and clay content that probably resulted in adsorption of some of the antibiotics. Clay-bound antibiotics are generally considered less effective against the microbial populations, although antibiotics absorbed onto clay particles can still be biologically active (Chander et al., 2005). In contrast, lesser amount of antibiotic was probably bound to organic matter and clay particles in the Wooster silt loam soil resulting in a more effective reduction in the degradation of EPTC.

Cycloheximide had the least effect among the different antibiotics in reducing EPTC degradation. Lee (1984) reported that an investigation of the EPTC-degrading microorganisms in soil identified several EPTC degrading fungal species. However, the lack of effect of cycloheximide to reduce EPTC degradation suggests that in these two soils, fungi contributed little to the degradation. Tal et al. (1990) also did not find a major fungal contribution in the degradation of EPTC.

Conclusion

The results from this study confirmed that enhanced degradation of herbicides in soil can be obtained with repeat applications. Enhanced soils are also conditioned for cross-enhanced degradation for herbicides that are structurally or chemically similar. Enhanced soil can lose the capacity for the accelerated degradation if left unexposed to the chemical for a prolonged period of time. Enhanced degradation of EPTC is mainly influenced by the soil bacterial population as evident from the reduction in degradation when soil was treated with the bactericide, chloramphenicol. Enhanced degradation is the

direct result of microbial adaptation to the exposure of a particular chemical. Since this is a natural process, proper management strategies for pesticide application should consider the existence of this phenomenon.

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	Wooster Silt Loam	Luray Silty Clay Loam
Suborder	Typic Fragiudalf	Typic Argiaquoll
pH	5.5	6.5
Organic matter	4.3	7.7
Sand %	18	16
Silt %	70	52
Clay %	12	32

Table 3.1. Major soil characteristics for Wooster silt loam and Luray silty clay loam soil

Experiment	**Soil**	**Herbicide Rate**
Self Enhancement of EPTC	WSiL[a] and LSiCL[b] Soil	10 mg kg^{-1}
Atrazine Degradation in EPTC Enhanced Soil	EPTC enhanced WSiL and LSiCL Soil	10 mg kg^{-1}
Effect of Antibiotics on EPTC Degradation	WSiL and LSiCL Soil	10 mg kg^{-1}
EPTC Degradation on Atrazine Enhanced Soils	OWS[c] and King Farm Soil	10 mg kg^{-1}

[a]Wooster silt loam, [b]Luray silty clay loam, [c]Old Weather Shed

Table 3.2. **Different experimental treatments and soils for each experiment**

Dose		0 Day	4 Days	7 Days
		EPTC Remaining		
		------ μg kg^{-1} soil-----		
None	Control	5.6	1.8^{c}	0.7^{c}
50 mg kg^{-1}	Kanamycin	4.55	2.13^{ab}	1.44^{b}
	Chloramphenicol	4.57	3.74^{a}	3.33^{a}
	Cycloheximide	3.69	0.56^{b}	0.95^{b}
75 mg kg^{-1}	Kanamycin	4.93	2.82^{ab}	1.23^{b}
	Chloramphenicol	4.88	3.77^{a}	4.32^{a}
	Cycloheximide	5.80	1.69^{b}	1.87^{b}
100 mg kg^{-1}	Kanamycin	4.76	3.34	2.66^{b}
	Chloramphenicol	6.17	5.23	5.25^{a}
	Cycloheximide	6.48	3.20	2.27^{b}

Table 3.3: **EPTC degradation in Wooster silt loam soil effected by the addition of the antibiotics kanamycin, chloramphenicol and cycloheximide.**

Dose		0 Day	4 Days	7 Days
		EPTC Remaining		
		------ μg kg^{-1} soil-----		
None	Control	4.90	0.90^{c}	0.20^{c}
50 mg kg^{-1}	Kanamycin	4.70	1.10^{b}	0.90^{b}
	Chloramphenicol	5.25	4.90^{a}	2.20^{a}
	Cycloheximide	4.00	0.90^{b}	0.50^{b}
75 mg kg^{-1}	Kanamycin	5.25	1.15^{b}	0.10
	Chloramphenicol	5.00	4.80^{a}	2.80
	Cycloheximide	5.30	1.40^{b}	0.80
100 mg kg^{-1}	Kanamycin	5.15	1.20	1.20^{b}
	Chloramphenicol	6.15	5.60	4.60^{a}
	Cycloheximide	6.80	2.70	0.65^{b}

Table 3.4: **EPTC degradation in Luray silty clay loam soil effected by the addition of the antibiotics kanamycin, chloramphenicol and cycloheximide.**

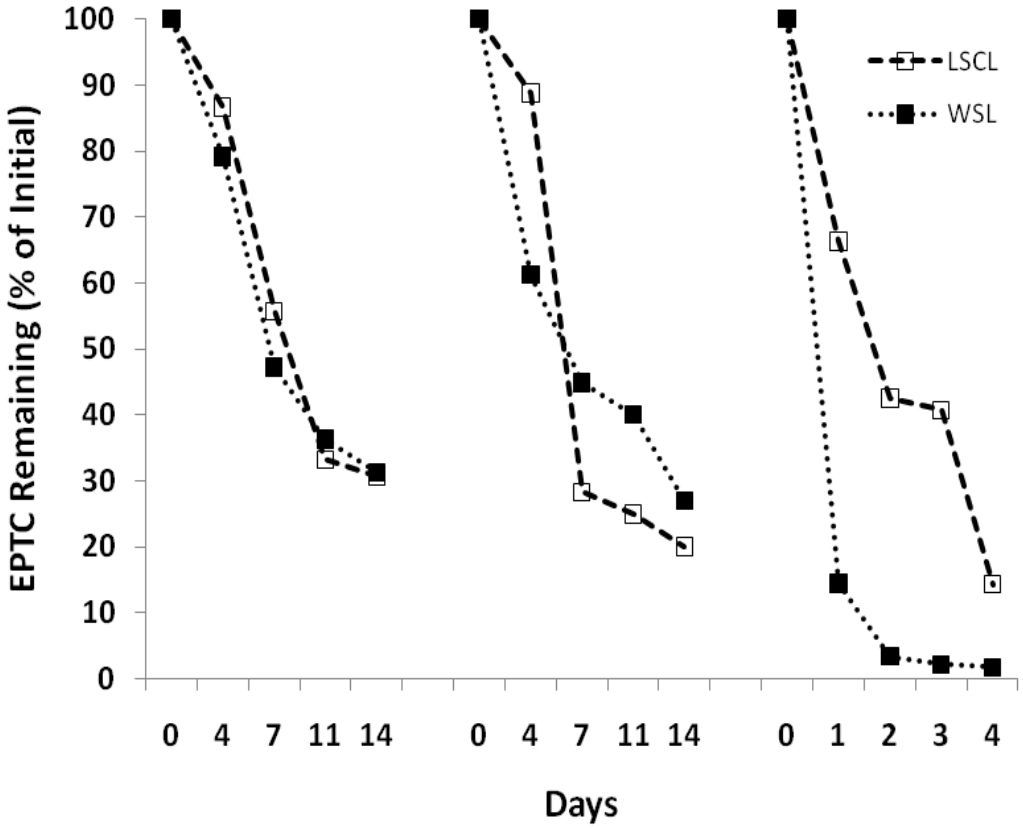

Figure 3.1: **Degradation curve of 10 mg kg^{-1} EPTC in Luray silty clay loam (LSiCL) and Wooster silt loam (WSiL) soil. Three set of graphs represent three repeated incubation cycles. Incubations one and two were continued for two weeks while incubation three was continued for four days.**

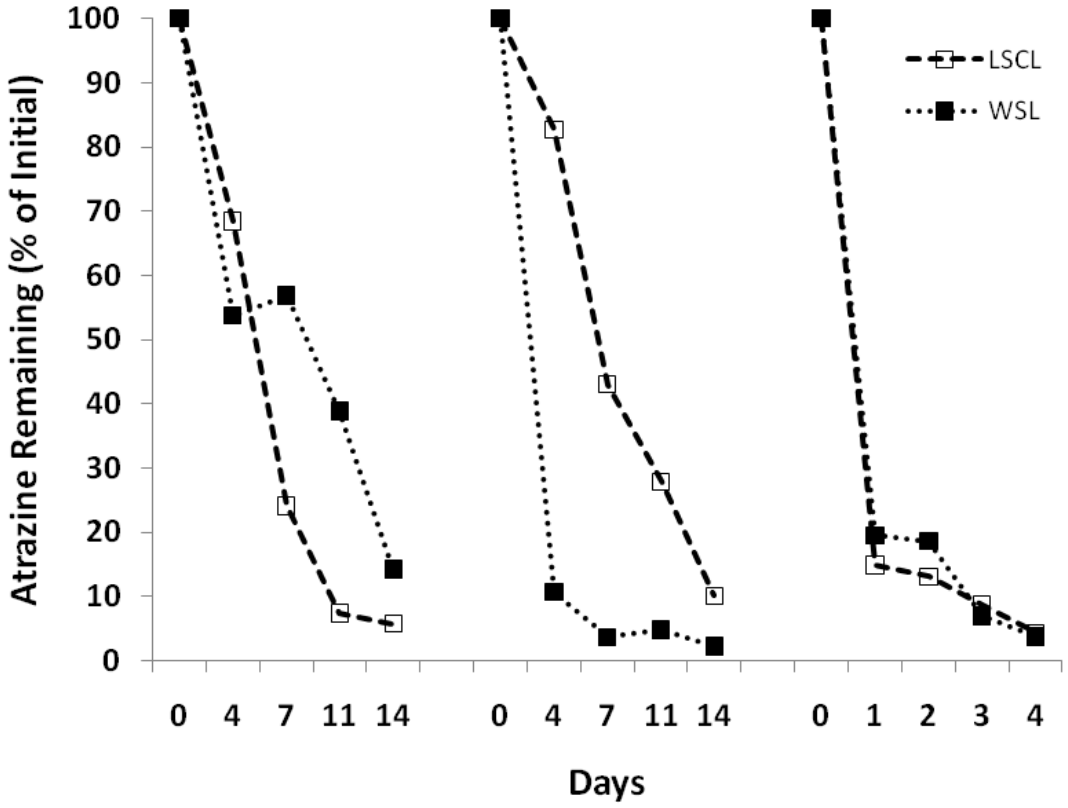

Figure 3.2: **Degradation curve of 10 mg kg^{-1} atrazine in Luray silty clay loam (LSiCL) and Wooster silt loam (WSiL) soil. Three set of graphs represent three repeated incubation cycles. Incubations one and two were continued for two weeks while incubation three was continued for four days.**

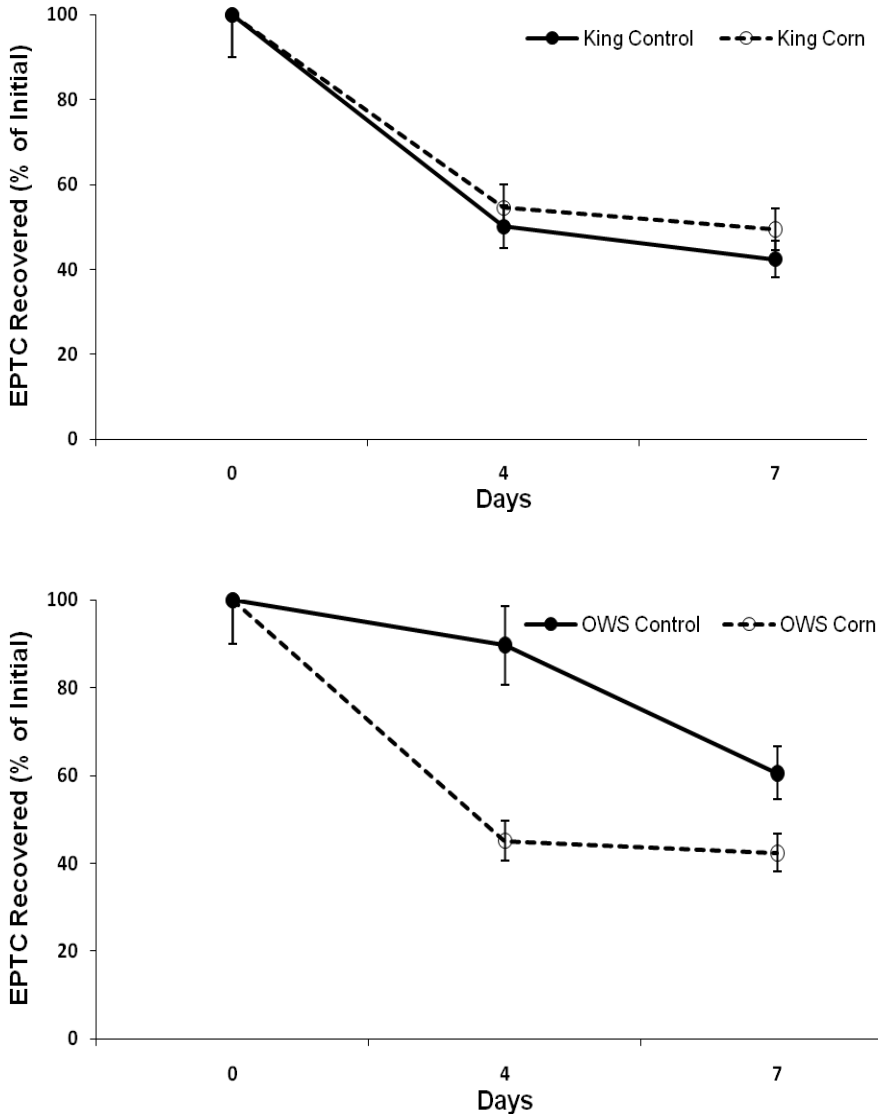

Figure 3.3: **Degradation curve of 10 mg kg^{-1} EPTC in atrazine history soil in Old Weather Shed (OWS) and adjacent control (top) and King Farm and adjacent control (bottom).**

CHAPTER 4

EXPRESSION OF EPTC AND ATRAZINE DEGRADING GENES AND SHIFT IN BACTERIAL DIVERSITY IN SOILS CAPABLE OF ENHANCED DEGRADATION

Abstract

Rapid biodegradation is the critical step in reducing nonpoint source pollution of agricultural pesticides in the environment. Presence of a pesticide in the environment will lead to the gradual increase of a specific microbial population that can degrade the pesticide over a period of time. As a result, expected changes in the soil biochemical functions would be observed as well as a shift in bacterial diversity. In this study, specific EPTC- and atrazine-degrading genes were detected only in soils that were capable of enhanced degradation of these two chemicals. The EPTC genes that were found to be related to the presence of enhanced EPTC degradation in soils were *thc*A, *thc*B, *thc*C, *thc*D, and *thc*R.. Similarly atrazine degrading genes such as *atz*B, *trz*N, and *trz*D were detected in soils enhanced for atrazine degradation. A dilution PCR experiment was conducted to estimate the increasing number of specific herbicide-degrading genes. At thousand-fold dilution of DNA extracted from soil still showed that the herbicide-degrading genes were easily detected in the enhanced soils. In contrast, the control soils did not show the presence of these genes, even in the non-diluted DNA samples. This provides ample evidence of the sharp increase in either the numbers of the specific

components of bacterial communities that are able to degrade these herbicides or the number of genes within a stable degrading population. A shift in bacterial community diversity, characterized by the difference in the banding pattern of DGGE profiles, was also evident by analyzing the DGGE banding profile of the different control and enhanced degradation soils.

Introduction

EPTC (S-ethyldipropylthiocarbamate) and atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) are used extensively to control broadleaf weeds, grasses and other perennial weeds. Both EPTC (a thiocarbamate) and atrazine (a triazine) can have negative environmental and ecosystem impacts.

In modern no-till farming systems, herbicides are applied more frequently for weed control and to maintain crop productivity. This increases the likelihood of nontarget toxicity and nonpoint source pollution. According to the EPA, EPTC has the potential to be redistributed off-site through vapor phase movement. Dissolved atrazine in water can be easily transported (Humburg et al., 1989) and is the most widely detected herbicide in ground and surface waters in the United States (Hallberg, 1989; Thurman et al., 1992). EPTC and atrazine have been detected in the rain water samples implying that these two herbicides can become part of the precipitation cycle (Cromwell and Thurman, 1993).

Microbial degradation is the primary route of xenobiotic compounds degradation, including agricultural pesticide degradation, in the environment. Initial research on pesticide degradation focused on the fate of pesticides mostly from chemical and physical attributes of the decomposition process. Alexander and Aleem (1961) were

some of the first researchers to explore microbial degradation of aromatic herbicides. Tam et.al. (1987) and Mueller et al. (1988) were pioneers in isolation and characterization of EPTC-degrading bacterial strains that were able to thrive on EPTC as the sole carbon source. Since then several other studies have identified bacterial species that are capable of degrading EPTC in soil (Behki and Khan, 1990; Dick et al., 1990; Behki, 1991; McClung et al., 1994; Ankumah et al., 1995; Nagy et al., 1995; Tal and Rubin, 1993). Microbial regulation has also been established as the primary route of atrazine degradation in soils. Several bacterial cultures and mixed cultures have been identified which are capable of degrading atrazine (Assaf and Turco, 1994; Barriuso and Houot, 1996; Mandelbaum et al., 1993; Stolpe and Shea, 1995; Topp et al., 1995).

Although several EPTC and atrazine degradation microorganisms have been identified and isolated in laboratory conditions, few studies have attempted to relate enhanced degradation activity with the expression of the specific herbicide degrading genes *in situ*. The genes involved in the degradation of EPTC include a cytochrome P-450 system (*Thc*B), and two genes downstream from *ThcB* producing a rhodocoxin (*Thc*C), and a rhodocoxin reductase (*Thc*D). The *ThcB* gene is the first member of a new gene family, CYP116. A regulatory protein (*Thc*R) is divergently transcribed from the cytochrome P-450 system that is a member of the AraC-XyIS family of transcription regulators. It has been suggested that *Thc*R probably regulates the expression of cytochrome P-450 system during EPTC degradation (Shao and Behki, 1996). Also identified during EPTC degradation was a NAD^+ dependent aldehyde dehydrogenase (*Thc*A). Although the cytochrome P-450 appeared to be unique to the *Rhodococcus*

species strain NI86/21, the dehydrogenase was found in other *Rhodococcus* species and most likely metabolizes the aldehyde that is generated after dealkylation.

The atrazine degrading genes (*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, and *atz*F) in *Pseudomonas* species strain ADP have been reported to be widespread and highly conserved (de Souza et al., 1998). In *Nocardia* species, the dechlorination of atrazine was mostly carried out through another dechlorination gene (*trz*N) (Smith et al., 2005). Several other atrazine degradation gene combinations have been reported (Piutti et al., 2003; Rousseaux et al., 2001). Mineralization of atrazine in gram-negative bacteria was carried out by the *atz*ABC-*trz*D gene combination in pure cultures. In contrast, gram-positive bacteria could transform atrazine into cyanuric acid and only possessed the *atz*B and *atz*C genes.

Microbial populations control gene expression in response to an environmental stimulus by “turning on” and “turning off” specific genes required for specific functions. The detection of specific herbicide-degrading genes in soil at a highly increased concentration will establish the that widespread use of that herbicide had occurred in the past. It signifies the significant role of the microbial population in the degradation mechanism of these two herbicides. The objective of this study was to compare the expression of specific herbicide degrading genes in two Ohio soils that showed enhanced degradation potential for EPTC and atrazine. An attempt was made to enumerate the increase in the detection level of specific herbicide-degrading genes in the enhanced soils compared to the control soils. Comparison was also made between the different soils for bacterial diversity and richness.

Materials and Methods

Development of enhanced ETPC and atrazine degradation in soil: Wooster silt loam and Luray silty clay loam soils were used to develop enhanced EPTC degradation potential (as described in chapter 2). EPTC or atrazine were added at the rate of 10 mg kg^{-1} of soil with sufficient amount of water corresponding to the field capacity measured gravimetrically in the laboratory (Black, 1965) for each soil. Wooster silt loam soils were maintained at 27% moisture while Luray silty clay loam soils were maintained at 36% moisture content. Residual herbicide in the soil after incubation was extracted with toluene as a solvent and measured in a gas chromatograph following the protocol described in chapter 2. The atrazine degradation experiment was conducted with a mixture of 1 part EPTC enhanced soil to 3 part natural soil. Three successive incubation experiments were conducted with repeated application of each herbicide. Control samples were incubated with deionized water following the same conditions for the EPTC or atrazine incubations. Enhanced degradation was verified in the sample by periodically extracting the residual herbicide and measuring the samples with a gas chromatograph.

Soil DNA extraction: Soil samples were collected after the third incubation experiment with EPTC and atrazine in soils. Soil was also collected from the control, which was incubated only with deionized water. DNA was extracted and purified from 250 mg of fresh soil from each incubation sample by using the Power Soil DNA Kit (MoBio Laoratories, CA) following manufacturer's recommendations. DNA integrity was checked by electrophoresis on a 1% agarose gel. Purity of extracted DNA was further

measured and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA yields ranged between 7.5 - 12.5 µg g^{-1} dried soils.

Polymerase Chain Reaction for gene detection: GoTaq Green Master Mix (Promega, Milwaukee, WI) was used to amplify the different herbicide-degrading genes. The master mix reagent is a pre-mixed solution of bacterial *Taq* DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers. Two dyes (blue and yellow) mixed for easy monitoring during electrophoresis allows for direct loading of samples onto agarose gels. A final reaction volume of 50 µl was used for the amplification reactions containing 25 µl GoTaq Green Master Mix, 2X, 0.5 µM of each primers, 1 µl DNA (10 – 20 ng) template. The PCR reactions were performed using an automated thermal cycler (PTC-100, MJ Research, Waltham, MA). The temperature program for the PCR reaction started with a 94ºC denaturation step for 9 min. Then 30 cycles were conducted with each cycle having a denaturing step of 94ºC for 30 s, an annealing step of 55ºC (for the atrazine degrading genes, *atz*B, *atz*D and *trz*D, the annealing temperature was set at 60°C) for 30 s and an extension step of 72ºC for 30 s. The last step in the PCR program was a final extension at 72ºC for 7 min. The samples were then held at 4ºC before being stored in a freezer at -20ºC before being visualized in 1% agarose gel. The primers used for the amplification of the EPTC and atrazine degrading genes are shown in Tables 4.1 and 4.2, respectively.

Dilution PCR: A dilution PCR experiment was conducted whereby serial dilutions were made for the DNA template extracted from enhanced soils. The starting DNA concentrations in sample extracts were 12 ng $µl^{-1}$ and 18 ng $µl^{-1}$ for the Wooster silt loam

soil and the Luray silty clay loam soils, respectively. A total of 25 µl of each DNA sample was added with 75 µl of nuclease free water to obtain a four-fold dilution. Similar four-fold serial dilutions were made from this sample to yield the highest dilution of 1:1096. Polymerase chain reactions using the specific herbicide-degrading gene primers were conducted following the same procedure as described above. PCR products were resolved and visualized in a 1% agarose gel.

Polymerase Chain Reaction and DGGE: A polymerase chain reaction (PCR) was conducted using a set of universal bacterial primers, PRBA 338 and PRUN518R primers, that amplify the 338 to 518 region of the 16s rRNA gene of bacteria. For PCR reactions, 100 µl of final mixture volume were used containing 1 µM of each primer, 50 µl of GoTaq Green Master Mix, 2X (Promega, Madison, WI) and 1 µl DNA (10 – 20 ng) template. The PCR reactions were performed using an automated thermal cycler (PTC-100, MJ Research, Waltham, MA). The temperature program for the PCR reaction started with a 94ºC denaturation step for 9 min. Then 30 cycles were conducted in which each cycle included a denaturing step of 94ºC for 30 s, an annealing step of 55ºC for 30 s and an extension step of 72ºC for 30 s. The last step in the PCR program was a final extension at 72ºC for 7 min. The samples were then held at 4ºC before being stored in a freezer at -20ºC.

A BioRad DCode apparatus (BioRad, Hercules, CA) was used to conduct the denaturing gradient gel electrophoresis (DGGE) analysis. An 8% (w/v) polyacrylamide gel, with denaturing gradients ranging from 35 – 65 % was used for separation of PCR products obtained as described above. Urea and formamide were used as denaturants to

facilitate the separation of DNA fragments. DGGE was performed using the Dcode Universal Mutation Detection System (BioRad Laboratories) and a 16 cm/16 cm gel apparatus. The gel was loaded and run in 1 X TAE (20 m*M* tris-Cl, 10 m*M* acetate, 0.5 m*M* Na_2EDTA) buffer at 60° C for a total of 780 V hours (constant voltage of 130 V for six hours). Gels were then stained with ethidium bromide and visualized on a UV transilluminator and photographed (Gel Logic Unit, Kodak, California, USA).

Calculation of Bacterial Richness and Dice Similarity index: A diversity richness index calculated using the DGGE banding pattern was used to quantify the different soils numerically. The mean band number for each soil was used to calculate the richness index. For our analyses, bands that could be clearly discerned as being distinct and separated from other bands, even if faint, were marked. The existence of the bands was further confirmed by comparing the normal gel pictures with an inverted image.

A maximum value of 1.00 was assigned to the Luray silty clay loam soil due to the maximum number of bands for this soil. By using the richness index it was possible to differentiate the soils based on the bacterial diversity observed in each soil. The phylotype richness (S, number of bands) was calculated for each soil and was normalized in comparison to the Luray silty clay loam soil that was assigned a index value of 1.00. In this evaluation of richness, the higher the value, the more diverse in terms of the number of dominant species that were in the soil sample.

The Dice similarity index was also calculated based on the DGGE profiles obtained for the different soils (Sigler et al., 2004), S_D

$$S_D = \frac{2N_C}{N_Q + N_T}$$

where N_Q is the number of bands in the query soil, N_T is the number of bands in the test soil, and N_C is the number of bands common to both soils.

Experimental design and data analysis. A randomized design with two replicates was used for the soil incubation experiments. The residual EPTC and atrazine in the soil were analyzed statistically using JMP (SAS Institute) and Minitab statistical software while the graphs were generated using Microsoft Excel 2007. Comparison of treatments, at a predetermined level of significance ($p < 0.05$), was done using a HSD-Tukey method (JMP, SAS Institute). Soil DNA extraction, PCR amplification, and DGGE were conducted in duplicate. In all figures and tables, mean values denoted by different letters are significantly different ($p < 0.05$).

Results and Discussion

The amount of genomic DNA extracted from the two soils varied with a considerably higher DNA yield for the Luray silty clay loam soil in comparison with the Wooster silt loam soil. This trend was observed for all the different treatments and control, i.e. in EPTC-enhanced soils and in atrazine-enhanced soils (Figure 4.1).

Significant differences in the expression of different herbicide degrading genes were observed between the control and enhanced soils. These results are consistent with the herbicide degradation curves obtained from the self enhancement experiment. In

Figure 4.2, none of the five EPTC degrading genes (*thc*A, *thc*B, *thc*C, *thc*D, and *thc*R) were detected in control soils. However in the EPTC enhanced soils, *thc*B, *thc*C, *thc*D, and *thc*R were easily detected. De Schrijver and De Mot (1999) reported that bacterial degradation of thiocarbamates was mainly confined to *Rhodococcus* species that were able to use the carbon and nitrogen in these herbicides to derive energy. *Rhodococcus erythropolis* NI86/21 produces N-dealkylated and hydroxylated metabolites during the biodegradation of EPTC and atrazine.

The signal for the *thc*A gene was very weak and was present in both the control and enhanced soils. Whyte et al. (1998) found the *thc*A gene to be widespread in the *Rhodococcus* genus, along with another actinomycetes, with a high degree of homology. There was a 95% similarity in the nucleotide sequence and a 98% similarity in the amino acid sequence between two *thc*A fragments extracted from different species. Moreover this gene fragment was found to be part of the chromosome rather than the large plasmid that harbors the EPTC degrading genes. Further, the *thc*A gene is not part of the P-450 cytochrome system which codes for the genes that are responsible for the degradation of EPTC (Nagy et al., 1995).

The detection of the *thc*B, *thc*C, *thc*D genes confirmed the involvement of the cytochrome P-450 system that was identified in *Rhodococcus* species NI86/21. This system is thus considered responsible for the degradation of the herbicide EPTC in our soils. The *thc*B gene, is classified as the first member of a new gene family (CYP116). To achieve the degradation of EPTC, the cytochrome CYP116 (*thc*B gene) needs a iron-sulfur protein, rhodocoxin (*thc*C gene) and a flavoprotein, rhodocoxin reductase (*thc*D gene) for the transfer of electrons from NADH to P450 for oxygen activation (Nagy et

al., 1995). Another gene, *thc*R, is divergently transcribed from *thc*B and is believed to control the regulation of the P-450 system for EPTC degradation.

The detection of these genes in the soils with enhanced ability to degrade EPTC is evidence that microbial degradation is a major route of dissipation in soil. The microorganisms that are capable of degrading EPTC "turn on" their genetic mechanism when exposed to the specific substrate, in this case the herbicide EPTC. Along with some bacterial species that are traditionally able to degrade these herbicides, other species, unable to degrade the herbicide may also develop the new trait through relaxation of substrate specificity or inducer specificity of existing enzymes or by acquisition of specific enzymes by genetic exchange (van der Meer, 1994). Such species which are able to adapt their pre-existing genetic mechanism will have an advantage that may allow these species to predominate within the community (Aislabie and Lloyd-Jones, 1995).

The EPTC degrading genes were also detected in the soils that showed enhanced ability to degrade atrazine (Figure 4.3), although with a lesser intensity. The soil for the atrazine degradation experiment was inoculated with 25% EPTC-enhanced soils. The primary intention of using the EPTC inoculated soils was to monitor the shift in EPTC degrading bacterial community diversity due to the exposure of atrazine. Several researchers have documented that many EPTC degrading bacterial species from the genera *Rhodococcus,* are also capable of degrading atrazine, at least partially (Vancov et al., 2005; Behki and Khan, 1994; Fazlurrahaman et al., 2009). Therefore it is reasonable to expect a change in microbial community diversity in the EPTC-enhanced soil because of the subsequent application of atrazine. Since the DNA extraction and the PCR

amplification was carried out after the third treatment of atrazine it is likely that some of the EPTC degrading bacterial population were still actively degrading atrazine in the soil.

Bacterial populations, specialized in the degradation of atrazine, responded positively to three repeat applications of atrazine. The slope of the degradation curve for atrazine was relatively higher in comparison with EPTC degradation curves. Soils for the atrazine degradation experiment were inoculated with soils with enhanced EPTC degradation capacity. The higher initial rate of degradation could be attributed to the components of EPTC degrading bacterial communities that are capable of at least partial degradation of atrazine, such as *Rhodococcus erythropolis* (Vancov, 2005) and *Rhodococcus* species strain TE1 (Behki, 1993). The detection of the genes *atz*B, *trz*D and *trz*N (Figure 4.4) in the atrazine-enhanced soils provides indication that the atrazine degrading bacterial species became metabolically enriched after being exposed to atrazine.

The atrazine degrading genes in *Pseudomonas* species strain ADP (*atz*A, *atz*B, *atz*C, *atz*D, *atz*E, and *atz*F) have been reported to be widespread and highly conserved (deSouza et al., 1998). In *Nocardia* species, the dechlorination of atrazine was mostly carried out through another dechlorination gene (*trz*N) (Smith et al., 2005). Several other atrazine degradation gene combinations have been reported (Piutti et al., 2003; Rousseaux et al., 2001). Mineralization of atrazine in gram-negative bacteria was carried out by the *atz*ABC-*trz*D gene combination in pure cultures. In contrast, gram-positive bacteria could transform atrazine into cyanuric acid and only possessed the *atz*B and *atz*C genes. The *trz*D gene has a similar function as the *atz*D gene in the ring cleavage of the s-triazine ring.

Both *atz*B and *trz*N were detected in the atrazine enhanced Wooster silt loam and Luray silty clay loam soils. However, the *atz*D gene was not detected in either soil. The *trz*D gene, which has a similar function as the *atz*D gene, was detected in the atrazine enhanced Luray silty clay loam soil. The detection of *trz*D gene in the Wooster silt loam soil was very faint. This suggests that none of the ring cleavage genes were readily detected in the Wooster silt loam soil which may imply that there might have been only partial degradation of atrazine in the Wooster silt loam soil. Although the degradation curves for atrazine in both soils were almost identical in the third incubation experiment, this only measured disappearance of the parent atrazine molecule and does not provide evidence of the extent of degradation.

Overall the detection of the herbicide degrading genes in the enhanced soils verified the accelerated degradation of EPTC and atrazine obtained in the Wooster silt loam and Luray silty clay loam soils (chapter 2). Detection of metabolically active Herbicide-degrading genes can be used to monitor the degradative potential of contaminated sites. Introduction of microbial "hotspots" to an area of contamination has been effective in developing natural bioremediation and bioaugmentation of herbicide degradation potential (Grundmann et al., 2007).

Dilution PCR

EPTC Degrading Genes: The detection of the herbicide degrading genes in the dilution PCR experiment at the highest dilution of 1:1096 is evidence that the genes responsible for EPTC and atrazine degradation were highly enriched in the soils with ability to degrade these two pesticides at enhanced rates. This may be due to either increased

populations of degrading microorganisms or to an increase in gene and gene products within a numerically stable degrading population. A preliminary PCR test reaction showed that the EPTC degrading genes were not detected in the atrazine enhanced soils at the highest dilution sample. All future PCR reactions were done with five dilutions for the EPTC-enhanced soils and four for the atrazine-enhanced soils.

*Thc*B gene was detected at the highest dilution (1:1096) in the EPTC enhanced Wooster silt loam soil and at 1:256 dilution in the EPTC enhanced Luray silty clay loam soil (Figure 4.5). In the atrazine enhanced soils, *thc*B gene was consistently detected at 1:256 dilution in both Wooster silt loam and Luray silty clay loam soils. The detection of *thc*R gene followed similar fashion as the *thc*B gene in Wooster silt loam soils but amplification was not successful in a higher dilution in the Luray silty clay loam soils. Similar results, as the *thc*B gene, were obtained for the other two EPTC degrading genes, *thc*C and *thc*D. It is important to note that in the control soils, a PCR reaction, even with the non-diluted DNA template, failed to amplify any of the specific herbicide degrading genes. The *thc*A gene was not included in the diluted PCR experiment because the sensitivity of detection of this particular gene, even with the non-diluted sample, was very low.

Compared to the Luray silty clay loam soil, the genes were detected at a higher dilution in the Wooster silt loam soils. This could result from either greater enhancement of the gene numbers in the Wooster silt loam soil compared to the Luray silty clay loam soil or because of PCR inhibitions in the Luray silty clay loam soil. Luray silty clay loam soils had relatively higher organic matter content then the Wooster silt loam soils. There is a possibility that at very low concentration of the template DNA, the humic substances

present in the solution may have interfered with the primer annealing. Wilson (1997) reported that along with many other substances, humic materials can inhibit PCR reactions. Tebbe and Rubin (1993) found negative interference of humic acids on PCR amplification with DNA extracted directly from soil.

Atrazine Degrading Genes: For the atrazine degrading genes, the dilution PCR experiment was only conducted for atrazine-enhanced soils. The reason being, the atrazine degrading genes were not detected either in the control or the EPTC-enhanced soils. PCR amplification was carried out with all five dilutions (1:4 to 1:1096) for *atz*B, *trz*N, and *trz*D genes. Both *atz*B and *trz*N genes were easily detected up to a 1:256 dilution in Wooster silt loam and Luray silty clay loam soils. Detection of these genes at the highest dilution was, however, more sensitive in the Wooster silt loam soils. A similar effect was also observed with the dilution PCR detection of the EPTC degrading genes. On the contrary, detection of *trz*D gene was very faint in the Wooster silt loam soil. Microbial species harboring this gene most likely were naturally present in the Luray silty clay loam soil at a much higher number than the Wooster silt loam soil.

Bacterial richness and diversity. Electrophoresis of 16S rRNA gene PCR fragments in the DGGE gels (Muyzer et al., 1993) resulted in many bands, some of which were often only faintly visible (Figure 4.7). For our analyses, bands that could be clearly discerned by visual comparison as being distinct and separated from other bands, even if faint, were marked (in green). The presence of bands at each location was further confirmed by comparing the true image with an inverted image generated in Photoshop. The results

indicate that among all the different treatments in the study, the Luray silty clay loam control soil had the highest number of distinguishable bands in the gel. While the two LSiCL control soils had 23 and 28 discernible bands for the two replicate samples, the two Wooster silt loam field soil samples had only 10 bands each. The Luray silty clay loam field soil had a higher number of bands than the Wooster silt loam field soil. Since species diversity and richness has been linked to better ecosystem functioning (Tilman et al., 2006), a higher diversity of bacteria for the Luray silty clay loam control soil may be interpreted as an indication of better potential for pesticide degradation compared to Wooster silt loam soils.

The phylotype richness (S, number of bands) was calculated (Table 4.3) for each soil combination and was normalized with the respective soil which was assigned an index value of 1.00 (Table 4.3). In this evaluation of richness, the higher the value, the more diverse in terms on number of dominant species that were in the soil sample. Thus, the Luray silty clay loam control soil had the greatest average richness index value (1.41) of bacterial species and the Wooster silt loam field soils, the least (0.54). The band richness index for the Luray silty clay loam soils in both the EPTC and atrazine enhanced profiles were highly similar with the control profile, with each average value being 1.35.

The Dice index results (Table 4.4) were also calculated based on the number of dominant bands in the DGGE gel. Dice similarity index values were also calculated using each soil as test soil, for all eight soil profiles. The average values for most soil profiles had a Dice index value between 0.47 – 0.58. A Dice similarity index value of 1.00 means that all bands in the DGGE profile for two different treatments were at identical positions. The low value of the Dice similarity index provides evidence for a shift in

bacterial diversity.

The results from the Dice index also shows that even for the Luray silty clay loam soils that had very similar band richness index (0.96, 0.96, and 1.00), there was a shift in bacterial community diversity compared to the control sample. The corresponding dice index values for these three soils are 0.63, 0.67 and 1.00. The use of the band richness index and the Dice similarity index provides a snap shot of the relative bacterial diversity in the soils with different treatments.

The results from this study support the notion that a huge range of metabolic capabilities associated with a highly diverse microbial population exists in natural environments. It is interesting to mention that until a few decades ago due to the scientific challenges soil systems were considered lifeless. In spite of the fact that atrazine is more persistent than EPTC and the ring structure is less susceptible to degradation, a relatively higher rate of atrazine degradation in the first incubation cycle than the first incubation cycle for the EPTC degradation was observed. This indicates either the involvement of EPTC degrading bacterial communities in the degradation of atrazine or existence of other metabolic pathways that can catalyze atrazine. This prediction is further confirmed as none of the atrazine degrading genes targeted in this experiment were detected in the EPTC enhanced soils or in the control soils.

According to Voget et al. (2005), NCBI database consists of 215 completely sequenced prokaryotic genome comprising approximately 4.5×10^8 bps with an average genome size of 6.1×10^6 bps. Based on the average gene size of 10^3 bps, the authors calculated that 6.1×10^{10} bps can potentially code 6.1×10^7 genes in one gram of soil. The total genome sequencing data collected from 1995 up to 2005 amounted to two

orders of magnitude lower than the possible number of genes in 1 g of soil implying that there is, in every likelihood, immense diversity of genes and metabolic pathways in soil than has been estimated or discovered until today.

Conclusion

A clear-cut relationship was observed for enhanced EPTC and atrazine degradation and the detection of the herbicide degrading genes in soil. None of the EPTC or atrazine degrading genes were detected in control soils. Atrazine degrading genes were not detected before the soil was treated with atrazine. This evidence suggests that the microbial communities acclimatize with the environment and the substrate available in the environment for degradation. Detection of genes after more than a thousand-fold dilution of the DNA templates indicated that the population of the specific microbial communities able to degrade these two herbicides was greatly increased. This is even more significant because none of these genes were detected in the non-diluted DNA control samples.

Detection of functional genes can be beneficial in determining potential soil functions and ecosystem condition. For example, a contaminated site with no detectable genes for the enhanced degradation of the contaminant would imply a lack of microbial population capable of degrading that particular contaminant. Management efforts by introducing microbial “hotspots” can be developed to aid in the rapid dissipation of the contaminant. The bacterial richness and Dice similarity index verified a shift in bacterial community diversity among the control, EPTC enhanced, and atrazine enhanced soils.

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Gene	Primer	Nucleotide sequence (5' →	Reference
thcA	thcA-f	TTCgCTTTCggAgATCAACT	Wang, Y., 2002
	thcA-r	CgAgAACgTCggAgAAgAAg	
thcB	thcB-f	AgATCACCCCgATCAgTgAC	Wang, Y., 2002
	thcB-r	AAACACACCACCgAACATgA	
thcC	thcC-f	ACCTACgTTCACCCTgATgg	Wang, Y., 2002
	thcC-r	CgAgCATTTCgTCTTCTTCC	
thcD	thcD-f	CgAAgCAgAgTCCCTCACAT	Wang, Y., 2002
	thcD-r	CgAggAAgCAgAAgATggAg	
thcR	thcR-f	CTTgggTTCgTACAgCCTTG	Wang, Y., 2002
	thcR-r	ggAAACggCACAAAgACCTA	

Table 4.1: Primer sets used for the amplification of the five EPTC-degrading genes.

Gene	Primer	Nucleotide sequence (5' →	Reference
atzB	atzB-f	TCACCggggATgTCgCgggC	de Souza et al., 1995
	atzB-r	CTCTCCCgCATggCATCgg	
atzD	atzD-f	gggTCTCgAggA TTT gAT	Devers et al., 2004
	atzD-r	TCCCAC CTg ACA	
trzN	trzN-f	CACCAgCACCTgTACgAA	Mulbry et al., 2002
	trzN-r	gATTCgAACCATTCCAAA	
trzD	trzD-f	CCTCgCgTTCAAggTCTA	Rousseaux et al., 2001
	trzD-r	TCgAAgCgATAACTgCAT	

Table 4.2: Primers sets used for the amplification of the four atrazine-degrading genes.

	Soil Profiles Corresponding to Treatments							
Treatments	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
WSiL Field (1)	1.00							
LSiCL Field (2)	1.60	1.00						
WSiL Cont. (3)	1.90	1.19	1.00					
LSiCL Cont. (4)	2.60	1.63	1.37	1.00				
WSiL EPTC (5)	2.10	1.31	1.11	0.81	1.00			
LSiCL EPTC (6)	2.50	1.56	1.32	0.96	1.19	1.00		
WSiL Atz. (7)	1.90	1.19	1.00	0.73	0.90	0.76	1.00	
LSiCL Atz. (8)	2.50	1.56	1.32	0.96	1.19	1.00	1.32	1.00

Table 4.3. Band richness index values of species calculated using data from the DGGE profiles. Columns represent band richness index values normalized with individual soil profile for comparison.

	Soil Profiles Corresponding to Treatments							
Treatments	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
WSiL Field (1)	1.00							
LSiCL Field (2)	0.54	1.00						
WSiL Cont. (3)	0.48	0.63	1.00					
LSiCL Cont. (4)	0.39	0.57	0.40	1.00				
WSiL EPTC (5)	0.32	0.43	0.40	0.34	1.00			
LSiCL EPTC (6)	0.34	0.54	0.45	0.55	0.43	1.00		
WSiL Atz. (7)	0.41	0.40	0.32	0.49	0.65	0.41	1.00	
LSiCL Atz. (8)	0.34	0.54	0.45	0.63	0.35	0.68	0.36	1.00

Table 4.4. Dice Similarity index of species calculated using data from the DGGE profiles. Columns represent Dice similarity index values normalized with individual soil profile for comparison.

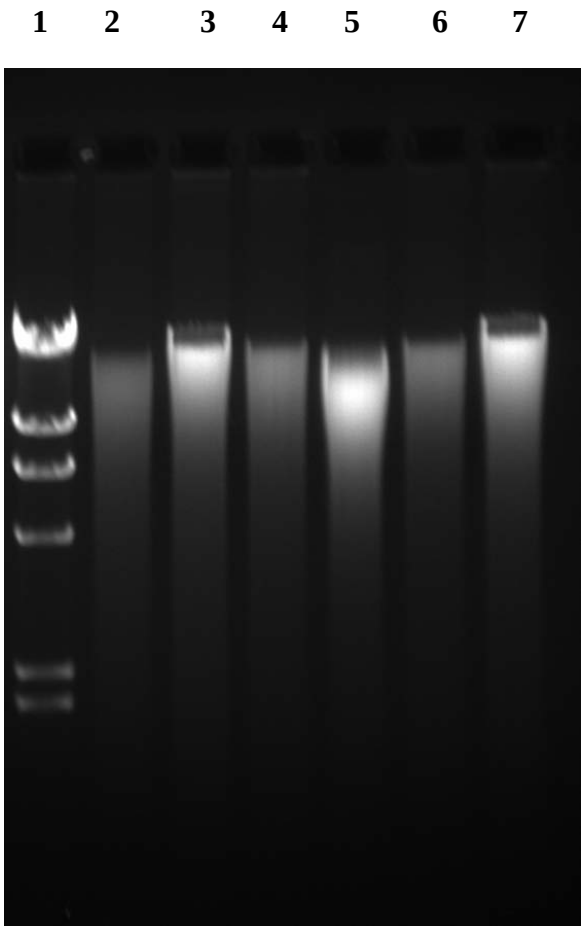

Figure 4.1: Genomic DNA extracted from the different treatment soils. Lane 1 = HindIII Lambda DNA marker, 2 & 3 = control WSiL and LSiCL soils, 4 & 5 = EPTC enhanced WSiL and LSiCL soils, 6 & 7 = atrazine enhanced WSiL and LSiCL soils. Equal volume of DNA sample was loaded in each lane extracted from 250 mg soil sample for each treatment.

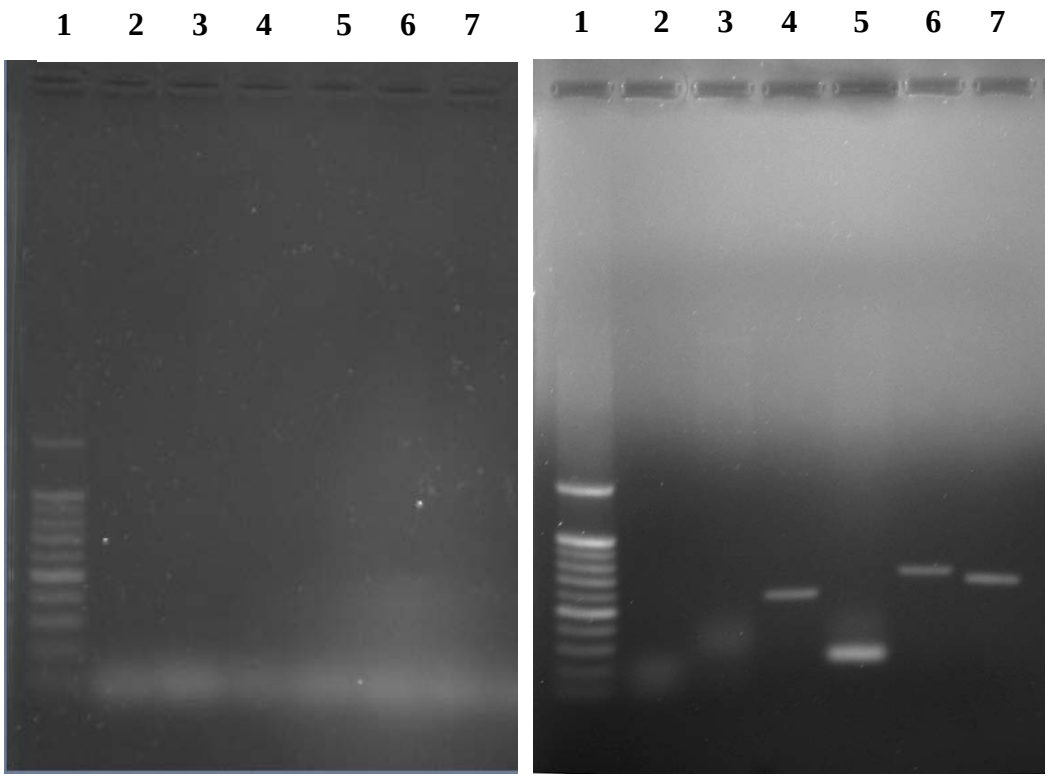

Figure 4.2: Detection of the EPTC degrading genes *thc*B, *thc*C, *thc*D, and *thc*R in control soils (left) and EPTC enhanced soils (right). Lane 1 = 100 bp marker, 2 = -ve control, 3 = *thc*A, 4 = *thc*B, 5 = *thc*C, 6 = *thc*D, and 7 = *thc*R

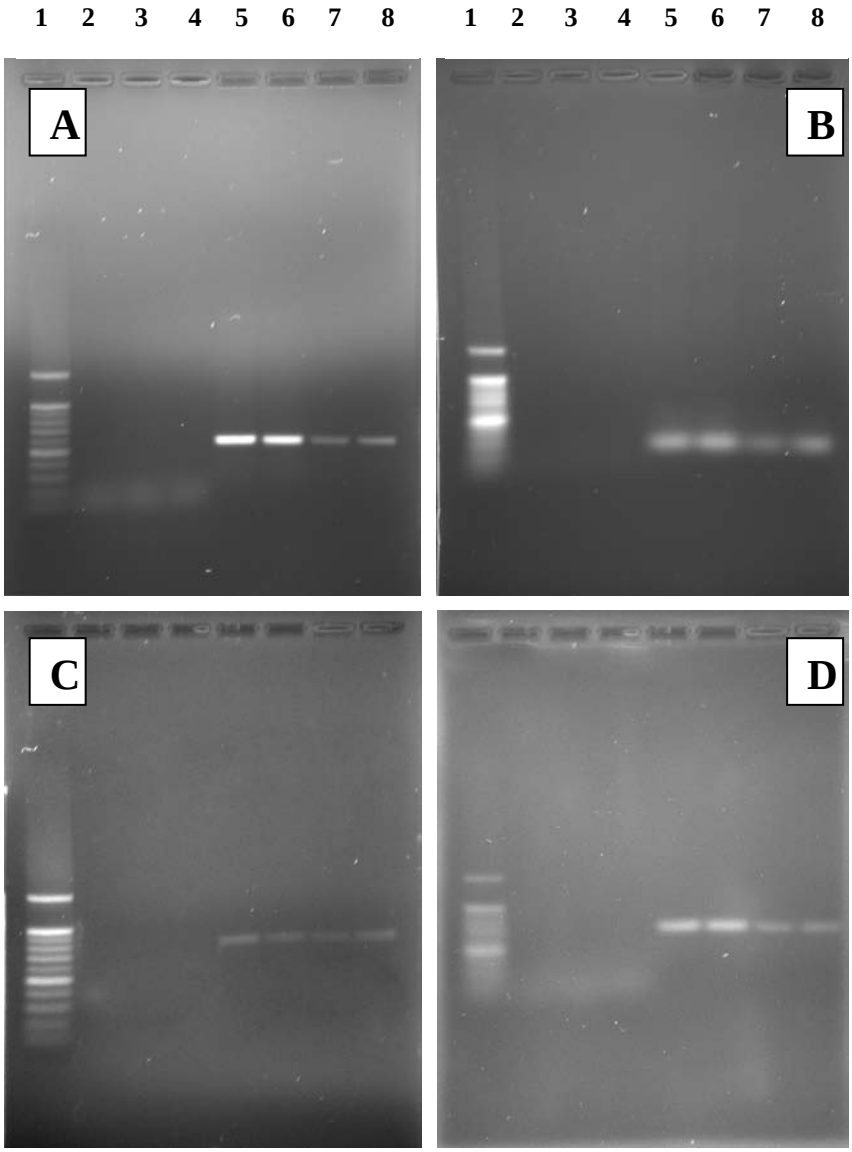

Figure 4.3: Detection of the EPTC degrading genes *thc*B (A), *thc*C(B), *thc*D (C), and *thc*R (D). Lane 1=100 bp marker, 2=-ve control, 3 & 4 = control (WSiL and LSiCL) soils, 5 & 6 = EPTC enhanced (WSiL and LSiCL) soils, and 7 & 8 = atrazine enhanced (WSiL and LSiCL) soils.

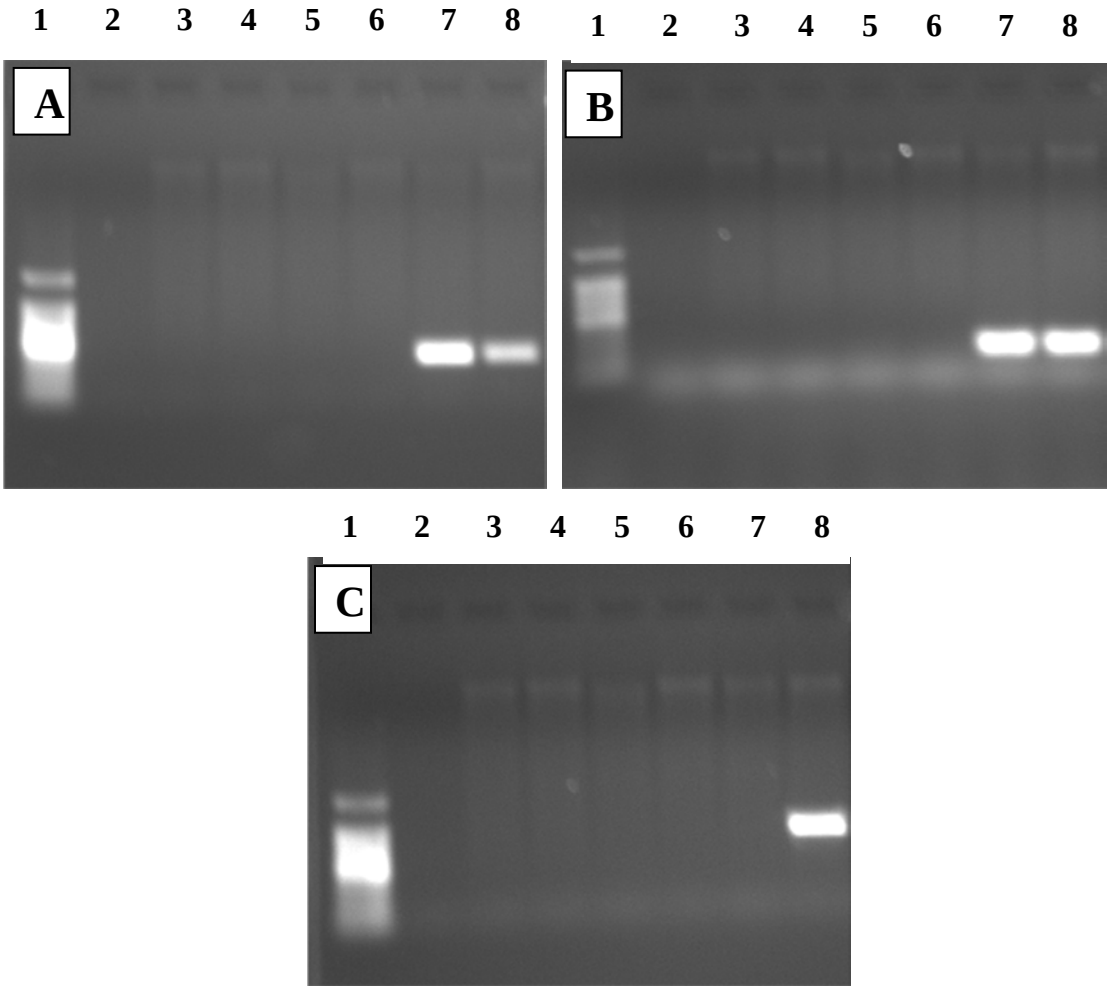

Figure 4.4: Detection of the atrazine degrading genes *atz*B (A), *trz*N(B), and *trz*D (C). Lane 1=100 bp marker, 2=-ve control, 3 & 4 = control (WSiL and LSiCL) soils, 5 & 6 = EPTC enhanced (WSiL and LSiCL) soils, and 7 & 8 = atrazine enhanced (WSiL and LSiCL) soils.

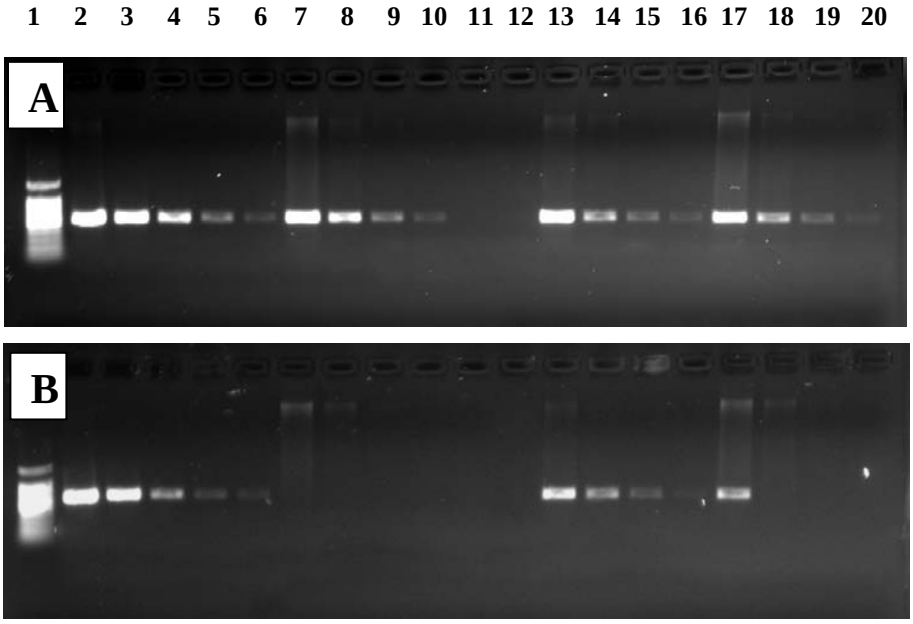

Figure 4.5: Dilution PCR reaction for *thc*B (A), and *thc*R(B) genes in WSiL and LSiCL soils. Lane 1=100 bp marker, lanes 2, 3, 4, 5, 6 = dilution 1:4, 1:16, 1:64, 1:256, 1:1096 in EPTC enhanced WSiL soil, lanes 7, 8, 9, 10, 11 = dilution 1:4, 1:16, 1:64, 1:256, 1:1096 in EPTC enhanced LSiCL soil, lane 12= -ve control, lanes 13, 14, 15, 16 = dilution 1:4, 1:16, 1:64, 1:256 in atrazine enhanced WSiL, lanes 17, 18, 19, 20 = dilution 1:4, 1:16, 1:64, 1:256 in atrazine enhanced LSiCL soil.

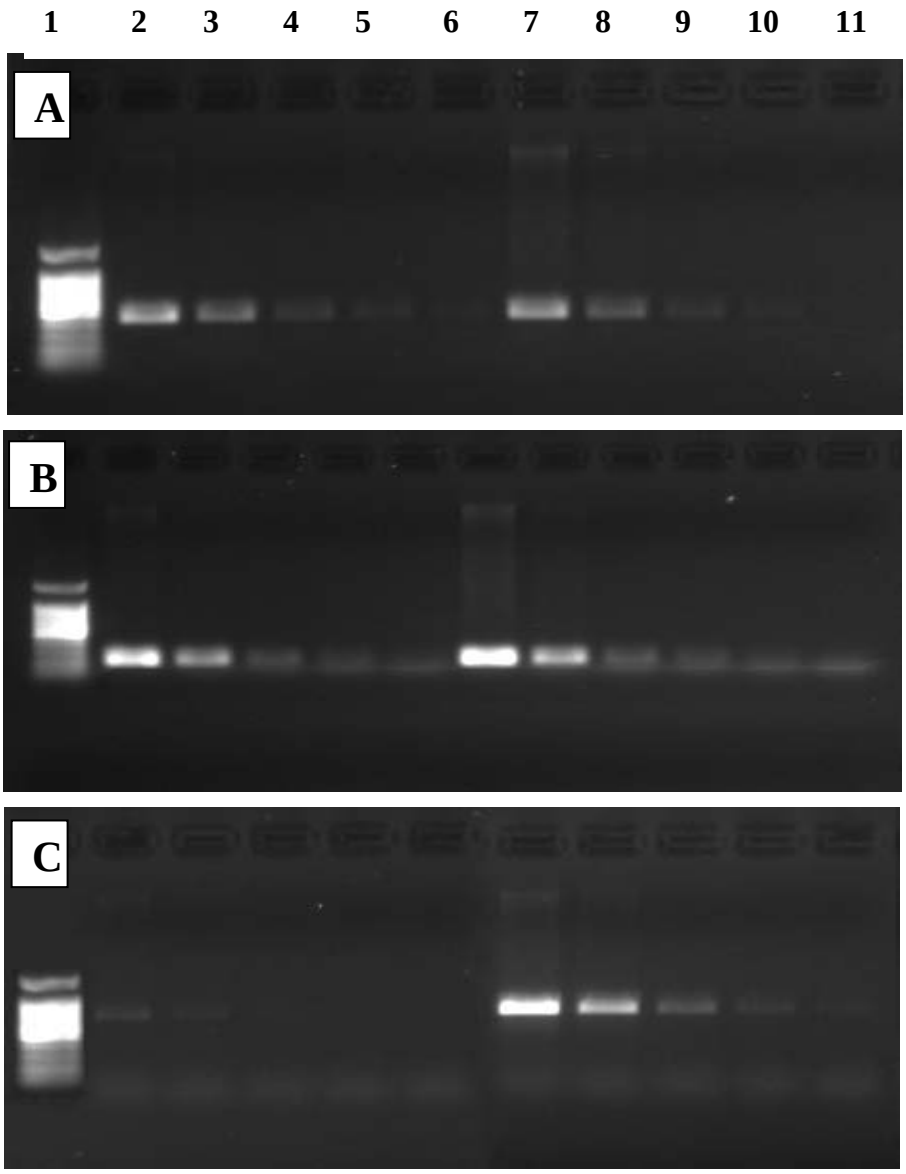

Figure 4.6: Dilution PCR reaction for *atz*B (A), and *trz*N(B) and *trz*D (C) genes in WSiL and LSiCL soils. Lane 1=100 bp marker, lanes 2, 3, 4, 5, 6 = dilution 1:4, 1:16, 1:64, 1:256, 1:1096 in atrazine enhanced WSiL soil, lanes 7, 8, 9, 10, 11 = dilution 1:4, 1:16, 1:64, 1:256, 1:1096 in atrazine enhanced LSiCL soil.

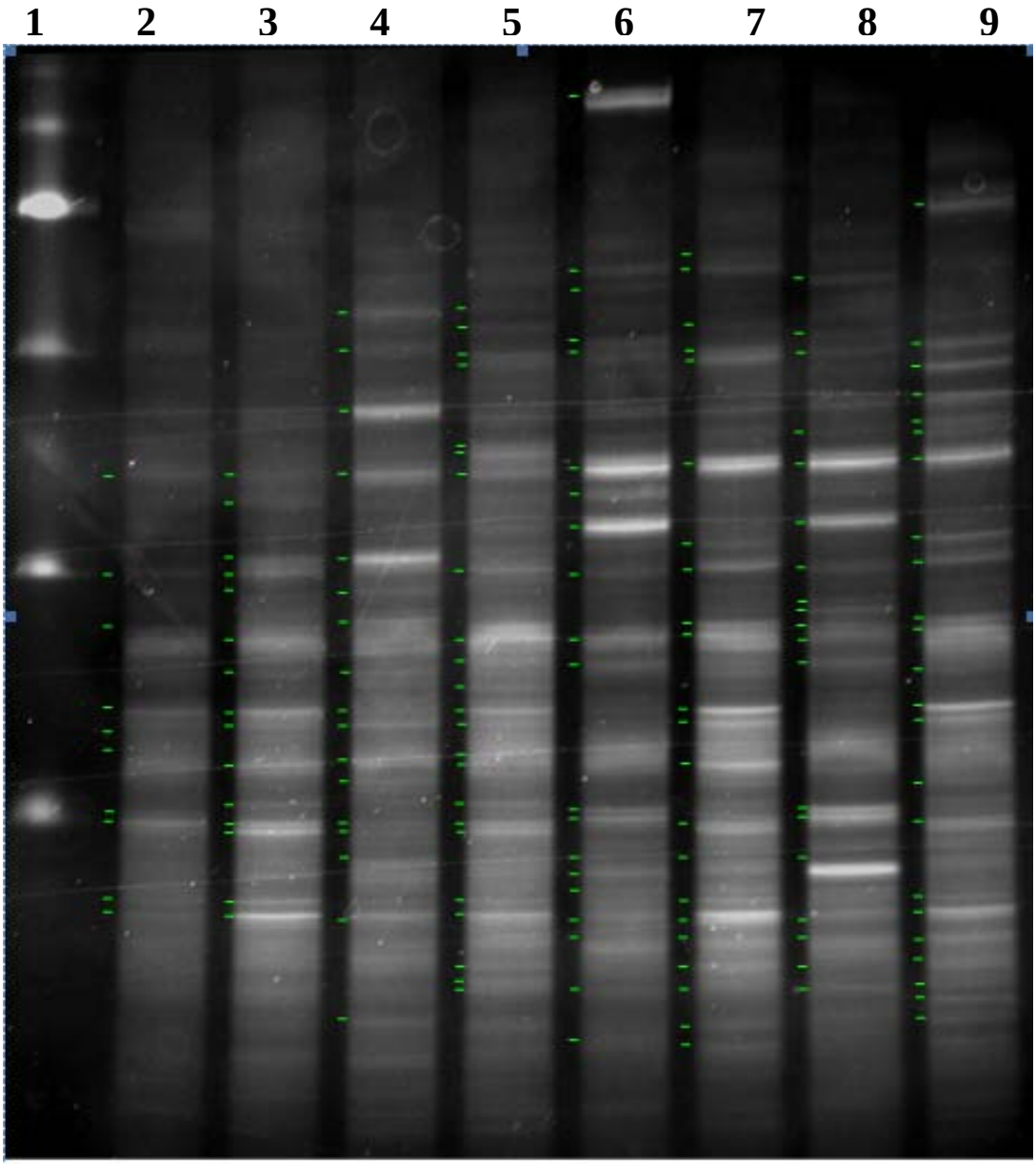

Figure 4.7: DGGE profiles (35 – 65 % gradient) of PCR products of different soil samples. Lane 1 = 100 bp DNA ladder. Lanes 2 and 3 = field (WSiL and LSiCL) soil, Lanes 4 and 5 = control (WSiL and LSiCL) soil, Lanes 6 and 7 = EPTC enhanced (WSiL and LSiCL) soil, Lanes 8 and 9 = atrazine enhanced(WSiL and LSiCL) soil. Green dots represent presence of discernible bands.

CHAPTER 5

DOMINANT EPTC AND ATRAZINE DEGRADING BACTERIAL COMMUNITY IN WOOSTER SILT LOAM AND LURAY SILTY CLAY LOAM SOILS

Abstract

Pesticide application in crop production and other agricultural landscapes can lead to pollution of nontarget areas. EPTC and atrazine are two of the most widely used herbicides in the world and have been detected in ground and surface waters. Potential toxicity and negative environmental impacts have caused concern for continued use of these chemicals. Rapid degradation of EPTC and atrazine by microbial communities can prevent the undesired effects of these herbicides. The objective of this study was to identify bacterial species capable of degrading EPTC and atrazine in two Ohio soils. Two new dominant bacterial species in the EPTC and atrazine degrading soils, *Kaistobacter* sp. and *Gemmatimonas* sp., were identified. Along with these species, other known EPTC and atrazine degraders were also detected by sequence analysis of 16S rRNA genes and included *Rhodococcus, Actinobacterium, Rhizobiales, Xanthomonadaceae, Oxalobacteraceae,* and *Rhizobium*. Several of these bacteria have been previously identified as pesticide or pesticide metabolite degraders. Identification of novel bacterial species capable of degrading these herbicides will be useful in elucidating novel pesticide

degradation pathways and in developing methods for bioremediation to reclaim contaminated soils.

Introduction

Though insecticides may be the most prevalent image of an agricultural pesticide, since 1970s, the use of herbicides has been increasing while that of insecticides has been decreasing. EPTC (S-ethyldipropylthiocarbamate) and atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) have been used to control weeds for more than 50 years in the United States and around the world. Large quantities of herbicides applied to crop lands in the midwestern United States are transported with surface runoff (Thurman, 1991) and deposited in lakes and rivers. Approximately 1 – 6 % of the applied herbicides can be released to the aquatic environment, with atrazine being one of the most commonly detected herbicides (Rice et al., 1997). At very high concentrations, herbicides can be persistent as microbial population may not be able to degrade the pesticides quickly. Aged and persistent herbicides can become recalcitrant due to increased sorption and decreased bioavailability over time (Felsot and Dzantor, 1997).

Both EPTC and atrazine can be toxic at high concentrations or after prolonged exposure. Negative impacts of atrazine on the reproductive system of amphibians have been reported (Allran and Karasov, 2000; Koprivnikar et al., 2006). Long-term exposure impacts of both herbicides on human health are still not conclusive, but early indications suggest that prolonged exposure can cause some types of cancer (Biradar & Rayburn, 1995).

Because of the human health hazards and negative ecological impacts, rapid degradation of these herbicides in contaminated environments is essential. Bioremediation is arguably the most popular method of reclamation of contaminated sites with tangible technical and financial benefits (Day et al., 1997). It is a natural process, simpler than excavation or incineration, and can be highly specific. However, to obtain efficient bioremediation there is a need to develop a system based on empirical identification of indigenous microbial organisms capable of efficiently degrading a chemical compound.

Until recently, due to technical limitations, identification of microbial species capable of degrading xenobiotic compounds in soil was limited to culturable microorganisms. With the availability and advancement of newer techniques such as phospholipid fatty acids analysis (PLFA) (Green & Scow, 2000), denaturing and temperature gradient gel electrophoresis (DGGE/TGGE) (Muyzer et al., 1993; Øvreas et al., 1997), fluorescent in situ hybridization (FISH) (Amann et al., 1995), amplified rDNA restriction analysis (ARDRA) (Vaneechoutte et al., 1992) and DNA melting and reassociation profiles (Torsvik et al., 1990, 1996), it is now possible to conduct in-depth analysis of microbial species, both culturalable and unculturable, in complex environmental samples.

The objective of this study was to explore the microbial community structure in soils with enhanced EPTC degradation potential. Since several EPTC degrading bacterial species from the genera *Rhodococcus* are capable of at least partially degrading atrazine (Vancov et al., 2005; Behki and Khan, 1994; Fazlurrahaman et al., 2009), an effort was

also made to identify the shift in microbial community structure in the EPTC enhanced soils because of subsequent exposure of atrazine.

Materials and Methods

Development of EPTC and atrazine enhanced soils: Wooster silt loam and Luray silty clay loam soils were used to develop enhanced EPTC degradation potential (as described in Chapter 2). EPTC or atrazine were added at the rate of 10 mg kg^{-1} of soil with sufficient amount of water corresponding to the water content (Black, 1965) for each soil. Wooster silt loam soils were maintained at 27% moisture while Luray silty clay loam soils were maintained at 36% moisture content. Residual herbicide in the soil after incubation was extracted with toluene as a solvent and measured in a gas chromatograph following the protocol described in Chapter 2. Three incubation experiments were conducted with repeated application of EPTC. The atrazine degradation experiment was conducted with a mixture of 1 part EPTC-enhanced soil to 3 part natural soil. Soil samples were collected after the third incubation cycle for DNA extraction. Control samples were incubated with deionized water following the same conditions as for the EPTC or atrazine incubations. Enhanced degradation was verified in the sample by periodically extracting the residual herbicide and measuring the concentrations remaining in the samples with a gas chromatograph.

Soil DNA extraction: DNA was extracted and purified from 250 mg of fresh soil from each incubation sample by using the Power Soil DNA Kit (MoBio Laoratories, CA)

following manufacturer's recommendations. DNA integrity was checked by electrophoresis on a 1% agarose gel. Purity of extracted DNA was further measured and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). DNA yields ranged between 7.5 - 12.5 µg g^{-1} dried soils.

Polymerase Chain Reaction and DGGE: A polymerase chain reaction (PCR) was conducted using a set of universal bacterial primers - PRBA 338 (5′-AC TCC TAC GGG AGG CAG CAG-3′) and PRUN518R (5′-ATT ACC GCG GCT GCT GG-3′) primers that amplify the 338 to 518 region of the 16s rDNA of bacteria. A 40 base GC clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) was added to the 5' end of the forward primer For PCR reactions 100 µl of final mixture volume was used containing 1 µM of each primer, 50 µl of GoTaq Green Master Mix, 2X (Promega, Madison, WI) and 1 µl DNA (10 – 20 ng) template. The PCR reactions were performed using an automated thermal cycler (PTC-100, MJ Research, Waltham, MA). The temperature program for the PCR reaction started with a 94ºC denaturation step for 9 min. Then 30 cycles were conducted in which each cycle included a denaturing step of 94ºC for 30 s, an annealing step of 55ºC for 30 s and an extension step of 72ºC for 30 s. The last step in the PCR program was a final extension at 72ºC for 7 min. The samples were then held at 4ºC before being stored in a freezer at -20ºC.

A BioRad DCode apparatus (BioRad, Hercules, CA) was used to conduct the denaturing gradient gel electrophoresis (DGGE) analysis. An 8% (w/v) polyacrylamide gel, with denaturing gradients ranging from 35 – 65 % was used for separation of PCR products obtained as described above. Urea and formamide were used as denaturants to

facilitate the separation of DNA fragments. DGGE was performed using the Dcode Universal Mutation Detection System (BioRad Laboratories) and a 16 cm/16 cm gel apparatus. The gel was loaded and run in 1 X TAE (20 m*M* tris-Cl, 10 m*M* acetate, 0.5 m*M* Na_2EDTA) buffer at 60° C for a total of 780 V hours (constant voltage of 130 V for six hours). Gels were stained with ethidium bromide and visualized on a UV transilluminator and photographed (Gel Logic Unit, Kodak, California, USA).

DGGE Band excision and DNA purification: **U**nique bands were cut from the DGGE gels using a sterile razor blade and placed in clean Eppendorf tubes. Bands were selected based on visual comparison between the control and the EPTC and atrazine enhanced soils. Dominant bands in the EPTC and atrazine enhanced soil profiles that were absent in the control samples were selected for excision. Few bands were also selected that were very faint in the control profile but became very intense in the enhanced soils. Six bands were excised from the EPTC-enhanced Wooster silt loam soil profile, two from the EPTC-enhanced Luray silty clay loam soil, three from atrazine-enhanced Wooster silt loam soils, and five from the atrazine-enhanced Luray silty clay loam soils. In total, 16 bands were excised from one gel (Figure 5.1) and a total of 32 bands were excised from two replicated gels. The gel fragments were purified with the Qiaex II Gel Extraction Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The purified DNA was resuspended in 20 µL of nuclease free water.

Cloning of 16S rRNA gene fragments: The purified DNA from the DGGE gel bands was cloned into plasmid vector pCR 2.1 and the ligation product transformed into

chemically competent E. coli TOP10 cells using the TA Cloning Kit, Catalog # K204040 (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocols. The transformed cells were plated in LB (Luria-Bertani) agar plates (1.0% Bacto-Tryptone, 0.5% Bacto-yeast extract, 1.0% NaCl, 1.5% Bacto agar, pH 7.0) containing 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-*β*-d-galgacto-pyranoside). X-Gal was added in the plates in order to identify white-colored transformed colonies.

Clone libraries, screening and sequencing: Two random white colonies were selected from each plate representing an individual band from the DGGE gel. Thus in total 64 individual colonies were selected and screened to confirm the presence of inserts. A colony PCR with the GoTaq Green Master Mix (Promega, Madison, WI, USA) was performed for each clone with a final volume of 25 µl and 0.5 µM of each vector specific primer M13 forward (5′-GTAAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′). The PCR reactions were performed using an automated thermal cycler (PTC-100, MJ Research, Waltham, MA). The temperature program for the PCR reaction started with a 94ºC denaturation step for 9 min. Then 30 cycles were conducted with each cycle having a denaturing step of 94ºC for 30 s, an annealing step of 55ºC for 30 s and an extension step of 72ºC for 30 s. The last step in the PCR program was a final extension at 72ºC for 7 min. The PCR products were analyzed by agarose gel electrophoresis and positive clones were identified based on the size of the fragments (approximately 430 bp).

PCR products from the above agarose gel were purified using the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI, USA). Purified products were sent to

the Molecular Cloning and Imaging Center (MCIC, http://www.oardc.ohio-state.edu/mcic/) located at the Ohio State University, Ohio Agricultural Research and Development Center (OARDC) in Wooster, Ohio. Sequencing was performed on a single strand.

Phylogenetic analysis: The partial 16S rRNA gene sequences were subjected to the NCBI BLASTN (http://www.ncbi.nlm.gov/blast/) in order to identify sequences with maximum similarity. The sequences were aligned and visually compared using the MacVector program before creation of the phylogenetic tree.

The phylogenetic tree for the EPTC and atrazine degrading bacterial population were prepared using the Phylogeny.fr platform (http://www.phylogeny.fr/). The 'One Click' method was used to run the default programs: MUSCLE for multiple alignments, Gblocks for automatic alignment curation, PhyML for tree building, and TreeDyn for tree drawing.

Results

The total genomic DNA isolated from the different soil samples was of high molecular weight (> 20 kb) and sufficiently pure to allow PCR amplification for downstream analysis. Successful PCR amplification with universal bacterial primer sets - PRBA 338 (5′-AC TCC TAC GGG AGG CAG CAG-3′) and PRUN518R (5′-ATT ACC GCG GCT GCT GG-3′) produced 16S rDNA fragments of size 220 bp confirmed by agarose gel electrophoresis. Since there was no inhibition of PCR reaction in any of the samples, the genomic DNA was not further purified through the gel extraction procedure.

The PCR-DGGE profiles for each soil contained several unique, distinct, and intense bands. The DGGE banding pattern for the two replicates were highly similar and reproducible. Overall, between ten (WSiL field soil) and 28 (LsiCL control) discernible bands were observed in the different DGGE profiles. The soil profiles (figure 5.2) were coded as WE (EPTC-enhanced Wooster silt loam soil), WA (atrazine-enhanced Wooster silt loam soil), LE (EPTC-enhanced Luray silty clay loam soil), and LA (atrazine-enhanced Luray silty clay loam soil). The bands in each soil profile were coded to indicate their relative position in the DGGE profile. The top most band was numbered 1 and increasing for the bands below.

All bands were not of same intensity in the different profiles. The air-dried field soil samples expectedly had a lower number of DGGE bands compared to the soils that were incubated with EPTC, atrazine or deionized water. Microbial activity in the field soils were limited by less available moisture and thus resulted in fewer bands in the DGGE profiles. Mean number of dominant bands in the two different soils for the EPTC and atrazine enhanced soils were similar (Table 5.1).

The Dice similarity index calculated (as described in Chapter 3) for EPTC and atrazine enhanced Wooster silt loam soils was 0.65 while for the Luray silty clay loam soil it was 0.72. The Dice similarity values thus indicate that along with some overlap of bacterial species that were active in both herbicide treatments, there was considerable shift in the dominant population in each system (Figure 5.2).

Duplicate analysis of the DGGE profiles revealed well-separated intense and faint bands for each treatment. Profiles for each treatment in the two replicates were very similar in times of the locations of bands in the DGGE profile. Soil samples from the

EPTC enhanced Wooster silt loam soil (WE) yielded two very bright bands and 20 bands of lesser intensity. In contrast, the atrazine enhanced Wooster silt loam soil (WA) had four bright bands along with 15 other bands in different locations in the profile. The Luray silty clay loam soils yielded relatively more bands in comparison with the Wooster silt loam soils and several highly intensity bands. The EPTC enhanced Luray silty clay loam soil (LE) had four very bright bands while the atrazine enhanced Luray silty clay loam soil had three very bright bands. The Luray silty clay loam soils on average had more than 26 total bands in each profile.

A very prominent band (WE 1) at the top of the DGGE profile in EPTC enhanced Wooster silt loam soil was not detected in any other soil profile. Some very intense bands were detected across the soil profiles for both EPTC and atrazine enhanced soils. Bands WE 9, WA 8, LE 8, and LA 8 all appear to have very similar sequence configuration as they were located in the same position in the DGGE profile for the respective soils. Band WE 11 and WA 9 were not detected in the Luray silty clay loam soils implying that these bacteria were present only in Wooster silt loam soil and not in the Luray silty clay loam soil. The sequences obtained in this study were compared to those available in the NCBI GenBank database and the sequence similarities for the dominant organisms in both EPTC and atrazine-enhanced soils are presented in Table 5.2 and 5.3.

Discussion

The unique band, WE1, was detected in the EPTC enhanced Wooster silt loam soil and was not detected in the DGGE profile of the EPTC-enhanced Luray silty clay loam soil. It appeared that the members of these taxa existed only in the Wooster silt

loam soils and are probably highly specialized in the degradation of EPTC or in utilizing the metabolites of EPTC degradation. Sequence analysis from this band revealed the identities of two bacterial species– *Nitrosospora* sp. and an uncultured soil bacterium.

Nitrosospora sp. is a nitrifying ammonia oxidizer found in most soils that have also been identified as the dominant population in nitrifying fluidized bed reactors (Schramm et al., 1998). Nitrifying bacteria are ubiquitous and are responsible for microbial nitrogen cycling in soil. Identification of *Nitrosospora* sp. associated with such a dominant band in the EPTC-enhanced soil DGGE profile may be the result of co-migration of DNA fragments with similar sequence. The sequence similarity with *Nitrosospora* sp. was 91% while the sequence similarity with the uncultured soil bacteria was 96%. GenBank information revealed that the uncultured soil bacterium was identified in contaminated soils with exposure to a mixture of chlorinated hydrocarbons (Low et al., 2007). Bacteria capable of degrading chlorinated hydrocarbons often share similar degradative enzyme systems that are capable of degrading EPTC, such as the dealkylation mechanism in *Rhodococcus* sp. which is able to dealkylate both EPTC and atrazine.

The sequences obtained from the bands WE 9, WA 8, LE 8, and LA 8 showed 100% similarity to the both these species belonging to the family Sphingomonadaceae. *Shingomonas* sp. possess biodegradative and biosynthetic capabilities and have been documented to have a role in degradation of several pesticides such as isoproturon (3-*p*-cumenyl-1,1-dimethylurea), 2, 4-D (2,4-Dichlorophenoxyacetic acid) and EPTC (Barreiros et al., 2008; Kitagawa, 2002; Wang, 2002). However, we are not aware that *Kaistobacter* sp. has ever been previously identified as either an EPTC- or atrazine-

degrader. Further investigation is therefore required to confirm the role of *Kaistobacter* sp. in EPTC and atrazine degradation. Since both *Shingomonas* sp. and *Kaistobacter* sp. belong to the same family, there is a high likelihood of transfer of genetic materials and development of novel catabolic functions. Moreover, existence of many pesticide degrading genes in plasmids increases the likelihood of transfer of genetic material between bacterial species, especially within the same family.

Identification of unique common bands that are present in both in the EPTC and atrazine enhanced soils may indicate existence of bacterial species that are capable of degrading both herbicides. Presence of *Shingomonas* sp. and *Kaistobacter* sp. in all soil profiles, i.e. in EPTC- and atrazine-enhanced soils, indicates that these species might have the required enzymatic mechanism to degrade both EPTC and atrazine. Capacity to degrade both these chemicals is not uncommon to bacteria. Several EPTC-degrading bacterial species, from the genera *Rhodococcus,* have been shown to be capable of degrading atrazine, at least partially (Vancov et al., 2005; Behki et al., 1993; Behki and Khan, 1994; Fazlurrahaman et al., 2009). Behki et al. (1993) observed that *Rhodococcus* species that are capable of degrading EPTC can also partially degrade atrazine. The authors also reported that the dealkylation of the s-triazine herbicides was associated with a 77-kb plasmid known to be an essential component for EPTC degradation. Fazlurrahaman et al. (2009) identified *Rhodococcus* sp. strain MB-P1 as being able to degrade very high concentrations of atrazine as well as utilizing atrazine has the sole source of carbon and energy. They found that the atrazine degrading genes were located on a 10 kb plasmid.

Some bands were specific to EPTC- and atrazine-degrading Wooster silt loam soils but not in the Luray sility clay loam soils. Band WE 11 and WA 9 were not detected in the Luray silty clay loam. Blast search of the sequence generated from these two brands revealed that the dominant bacterial population are *Shingomonas* sp. and *Kaistobacter* sp. DNA fragments with identical or similar sequences have been reported to migrate to different vertical positions in DGGE gel profile. Opik et al. (2003) also found that the DNA fragments with highly similar sequence analysis can migrate to different vertical positions in a DGGE profile. Therefore careful interpretation should be made during analysis of DGGE profiles based on mobility of different bands (Liang et al., 2008).

Another bacteria consistently found in all the soil profiles belonged to the bacterial phyla *Gemmatimonadetes*. Sequence identities for these bacteria were obtained from the different bands WE 18, WA 15, LE 13, and LA 14 in the DGGE profile. Members of these species were identified recently (Zhang et al., 2003) as belonging to candidate division BD (also called KS-B), a phylum-level lineage in the bacterial domain. The authors classified these bacteria as gram-negative, rod shaped aerobic organisms capable of enhanced phosphorous removal from wastewater treatment plants. Recently, Takaichi et al. (2010) reported the identification of biosynthetic pathway for carotenoids and the corresponding genes and enzymes in *Gemmatimonas aurantiaca* strain T-27.

Because *Gemmatimonadetes* bacteria are newly identified in soil, their role and function in soil are not yet known. However, identification of the bacterium in both soil types and herbicide treatments provides circumstantial evidence that these bacteria where either actively involved in the degradation of EPTC or atrazine or the metabolites of

EPTC and atrazine degradation pathways. Consistent recovery of *Gemmatimonadetes* bacteria from the different soil profiles warrants detailed investigation for their capacity to degrade EPTC and atrazine.

A phylogenetic tree revealed other bacterial species belonging to several bacterial genera, such as *Rhodococcus, Actinobacterium, Rhizobiales, Xanthomonadaceae, Oxalobacteraceae, Rhizobium* in the EPTC- and atrazine-enhanced Wooster silt loam and Luray silty clay loam soil profiles (Figure 5.3 & 5.4). These bacteria have been previously isolated and/or enriched from EPTC and atrazine degrading bacterial communities. Several *Rhodococcus* sp. have been identified that are capable of degrading EPTC and at least partial degradation of atrazine in soil (Behki and Khan, 1990; Dick et al., 1990; Behki, 1991; McClung, 1994; Ankumah et al., 1995; Nagy et al., 1995; Tal and Rubin, 1993).

Macias-Flores (2009) isolated *Xanthomonas* sp., along with other bacteria, from selective atrazine enrichment cultures. Although they later found that *Xanthomonas* sp. is not able to use atrazine as the sole source for carbon or nitrogen. However, detection of this bacteria in the enhanced atrazine degradation soil in this study as well provides indication that *Xanthomonas* sp. might have the capacity to utilize some of the atrazine degradation metabolites more efficiently than other bacteria present in the environment. Detailed investigation of the genes expressed by *Xanthomonas* sp. will help in better understanding the role of these bacteria in the atrazine degradation mechanism.

Atrazine degrading *Rhizobium* sp. have also been isolated and reported by Bouquard et al. (1997). Mehmannavaz et al. (2001) achieved biofiltration of residual atrazine in bioaugmented columns inoculated with *Rhizobium meliloti* A-025. There was

significant reduction in residual atrazine through bioaugmentation by *Rhizobium meliloti* A-025 verifying the role of *Rhizobium* sp. in atrazine degradation.

Several soil bacterial species have been identified in this study (accession numbers EF173332, FJ479554, DQ278835, GQ467811, DQ189897, AB486360) that have been isolated from contaminated environments exposed with chlorinated hydrocarbons, PAHs and other organic compounds. As these were the dominant bacteria with unique bands in the DGGE profiles of enhanced soils it is assumed that these organisms had a role in the degradation of EPTC and atrazine. Efforts to isolate and further identify these organisms and elucidate the enzymatic systems for these organisms will help in developing a thorough knowledge base for degradation of these two herbicides in soils and natural environments.

Theoretically each DGGE band represents a dominant microbial community or an operational taxonomic unit (OTU) and the higher the number of bands in a profile, the more diverse is the bacterial population. Separation of bands in the DGGE profile is not based on the size of the fragments but on the sequence variation in each fragment (Muyzer and Smalla, 1998). Excision and direct sequencing of DGGE bands has been used by many researchers to identify the bacterial taxa and elucidated the microbial community diversity (Ampe et al., 1999; Ovreas et al., 1997). According to recent studies, direct excision and sequencing can often lead to ambiguous and biased identification of bacterial taxa because of co-migration of DNA fragments from different taxa in the same position in a DGGE profile (Ecorlini et al., 2003; Ecorlini, 2004). Therefore in this study, after excision and purification of DGGE bands, the 16S rDNA fragments were cloned into *E. coli* and multiple clones were selected and sequenced.

Variation in banding patterns between the EPTC enhanced and atrazine enhanced soils were also observed for the Luray silty clay loam soils. In comparison to the EPTC-enhanced Luray silty clay loam soil, most of the unique bands in the atrazine-enhanced soils appear in the top half of the DGGE profiles. Bands that are formed in the upper portion of the DGGE profiles are most likely bacteria with a low G+C content in their sequences. In contrast, the sequences of bands forming lower in the DGGE profiles have higher G+C content and are less susceptible to denaturation, allowing them to move further through the denaturing environment.

Although most of the EPTC degrading bacterial species identified so far are from the genera *Rhodococcus*, atrazine degrading bacterial species are widespread and have been identified in every continent (Kurtz et al., 2009). Bacterial species have been identified from four different bacterial phyla, including numerous bacterial species that are capable of degrading atrazine. Moreover, the genes that are responsible for the degradation of atrazine are generally located on plasmids and are susceptible to horizontal gene transfer. Therefore possibility exists that more bacterial species, hitherto unidentified, exist and are capable of degrading EPTC and atrazine in natural environments. Continuous exploration of the immense bacterial diversity in soils and natural environments for enhanced EPTC and atrazine degradation potential may lead to (1) the discovery of “super degraders” that can be effectively used for the bioremediation of contaminated sites and (2) new insights of how microbial communities interact and evolve in relation to the presence of an introduced xenobiotic like either atrazine or EPTC.

Functional diversification of gene products is part of the evolution of gene families that allows for adaptation to changing environments. Prokaryotes often develop such function through horizontal or lateral gene transfers. The phenomena of horizontal gene transfer and functional recruitment of genes has been traced back to millions of years. It is considered the principal cause of increased drug resistance (Barlow, 2009).

Although horizontal gene transfers are less frequent in eukaryotes, in comparison with prokaryotes, the mechanism has been detected in eukaryote chloroplasts, mitochondria and even in the nuclear genome. Under laboratory conditions, plant genetic materials have been successfully introduced into target bacteria capable of accepting the DNA fragments. For example, evidence exists that gene transfer from sugar beet plant into bacteria is possible, although the success and rate of transfer is very low (GMO Safety Report, 2008). Since bacteria are capable of direct exchange of genetic materials, there is the possibility that unidentified bacterial enzyme systems capable of EPTC and atrazine degradation may exist in plants and can be transferred from plants to bacteria living in soil. Because we can cultivate only about 1 – 10% of the total microbial population in the laboratory (Torsvik, 1990), continuous exploration of unknown and unique microorganisms with separate enzyme-mediated degradation pathways for EPTC, atrazine or other xenobiotic chemical is desirable.

Future exploration of soil bacterial biodiversity for specific functions, including pesticide degradation, can develop progressively with the implementation of techniques such as functional cloning. By using functional cloning technology it is possible to explore pathways or enzyme systems similar to an already known function. Functional cloning has been used to characterize novel drug resistance and heavy metal

detoxification efflux carriers in *Arabidopsis* (Li et al., 2002). Functional cloning techniques have been used extensively in biological research to understand cellular functions of vast numbers of genes identified in the post sequence era. Functional cloning techniques have also been used to investigate prokaryotic functions such as nitrogen cycling in natural environments (Braker et al., 2000).

Certain plants and grasses such as switchgrass have the capacity to transform and detoxify atrazine (Lin et al., 2008). Wenger et al. (2005) showed that corn root exudates can effectively degrade atrazine in order to reduce its toxicity and mobility. They suggested the possible use of certain plants that exude high amounts of benzoxazinones into the rhizosphere to degrade atrazine as a means of environmental protection and sustainable management of agricultural land. Functional cloning strategies using probes for plant genes for degrading enzymes that can then be transferred into microorganisms may reveal novel unexplored degradation diversity.

Another technique to monitor microbial gene expression is the use of pyrosequencing of total RNA extracted from complex microbial assemblages. Such techniques, also called metatranscriptomics, can overcome inherent limitations of other techniques such as qPCR and microarrays and at the same time explore all the genes expressed in a complex environment (Cardenas and Tiedje, 2008; Warnecke and Hess, 2009) in order to discover and characterize microbial diversity and function. By controlling environmental variation such as substrate, contaminant, or physical parameters it is possible to explore changes in gene expression in the entire system. Comparing the complementary DNA sequences and known peptide sequences from a soil with a known set of conditions with another set of conditions can led to the identified of

uncharacterized DNA and peptide sequences. Metatranscriptomic data have been used successfully to explore natural ecosystems to reveal the diversity, abundance, and identification of functional proteins in ocean water column (Shi et al., 2009), comparative day/night microbial communities (Poretsky et al., 2009), and soil eukaryotic diversity (Bailly et al., 2007).

This study revealed the dominant bacterial population present in EPTC and atrazine degrading soil systems using PCR-DGGE profiles and sequencing of 16S rRNA gene bands from the DGGE gels. Several bacterial species such as *Kaistobacter* sp., G*emmatimonas* sp. were dominant in the EPTC- and atrazine-degrading soils in this study. Identification of bacterial species capable of EPTC and atrazine degradation, not reported to date in the literature, provides valuable information on the microbial ecology of EPTC- and atrazine-degrading bacterial communities in soil. However, further exploration of EPTC- and atrazine-degrading environments, using techniques such as functional cloning and metatranscriptomics, will shed new light on microbial diversity and function of EPTC- and atrazine-degradation in soil.

Conclusion

This study identified two new dominant bacterial species in the EPTC- and atrazine-degrading soils. *Kaistobacter* sp. is a member of the bacterial family Sphingomonadaceae and was one of the dominant bacterial populations in both EPTC- and atrazine-degrading soils. Along with this, *Gemmatimonas* sp. was also identified which was consistently present in the EPTC- and atrazine-degrading soils. *Sphingomonas* sp. was dominant in both EPTC- and atrazine-degrading soils and confirmed findings by

other researchers about the contribution of *Sphingomonas* sp. in EPTC degradation. Other bacterial species belonging to several bacterial genera, such as *Rhodococcus, Actinobacterium, Rhizobiales, Xanthomonadaceae, Oxalobacteraceae, and Rhizobium* were identified in the EPTC- and atrazine-enhanced Wooster silt loam and Luray silty clay loam soil profiles. Several of these bacteria have been previously identified degraders of pesticides or pesticide degradation metabolites.

Use of culture independent metagenomic techniques can be used to identify bacterial species and biodegradation pathways in natural environments. Such studies will not only solve the problem of contaminant degradation and dissipation but also will open doors for identifying novel gene products. Identification of bacterial species capable of enhanced degradation of EPTC and atrazine will be useful in development of bioremediation mechanisms for contaminated sites.

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Treatments	*Mean number of bands*	*Common bands between two herbicide treatments*
WSiL EPTC Enhanced Soil	21	13
WSiL Atrazine Enhanced Soil	19	13
LSiCL EPTC Enhanced Soil	25	18
LSiCL Atrazine Enhanced Soil	25	18

Table 5.1: **Mean number of unique DGGE bands detected in the EPTC and atrazine enhanced Wooster silt loam and Luray silty clay loam soils and number of common bands for each soil type.**

Clone Number	*Accession Number*	*Closest Match in GeneBank database*	*Similarity*	*Division*
Seq 1	GU097360	Uncultured Nitrosospira sp.	91%	Betaproteobacteria
Seq 2	EF173332	Uncultured Soil Bacterium	96%	Betaproteobacteria
Seq 4	FN794227	Kaistobacter sp.	100%	Alphaproteobacteria
Seq 5	FN794222	Sphingomonas sp.	100%	Alphaproteobacteria
Seq 6	FJ479554	Uncultured Soil Bacterium	94%	Uncultured bacteria
Seq 11	FM176841	Uncultured Rubrobacterineae	95%	Actinobacteria
Seq 12	GU257581	Uncultured Gemmatimonadetes sp.	90%	Gemmatimonadetes
Seq 14	DQ278835	Uncultured Soil Bacterium	99%	Uncultured bacteria
Seq 15	GU784866	Nocardioides sp. Cr7-14	98%	Actinobacteria
Seq 16	GQ467811	Uncultured Soil Bacterium	100%	Uncultured bacteria

Table 5.2: **Identity of selected 16S rRNA bands excised from the DGGE profiles of EPTC enhanced Wooster silt loam and Luray silty clay loam soils in NCBI database.**

Clone Numbe	*Accession Number*	*Closest Match in GeneBank database*	*Similarity*	*Division*
Seq 17	DQ189897	Uncultured gamma proteobacterium	95%	Gammaproteobacteria
Seq 18	FN794227	Kaistobacter sp.	100%	Alphaproteobacteria
Seq 20	AM934841	Uncultured Gemmatimonadetes sp.	100%	Gemmatimonadetes
Seq 21	FN794222	Sphingomonas sp.	100%	Alphaproteobacteria
Seq 23	GQ366518	Uncultured Acidimicrobiales	100%	Actinobacteria
Seq 24	GQ338810	Uncultured Bacteroidetes bacterium	97%	Bacteroidetes
Seq 26	FJ391493	Oxalobacteraceae bacterium	98%	Betaproteobacteria
Seq 28	FJ568975	Uncultured Acidobacteria bacterium	99%	Acidobacteria
Seq 30	AB486360	Uncultured Soil Bacterium	95%	Uncultured bacteria
Seq 31	AY360613	Uncultured Xanthomonadaceae sp.	100%	Gammaproteobacteria
Seq 32	EF019894	Uncultured actinobacterium	98%	Actinobacteria

Table 5.3: **Identity of selected 16S rRNA bands excised from the DGGE profiles of atrazine enhanced Wooster silt loam and Luray silty clay loam soils in NCBI database.**

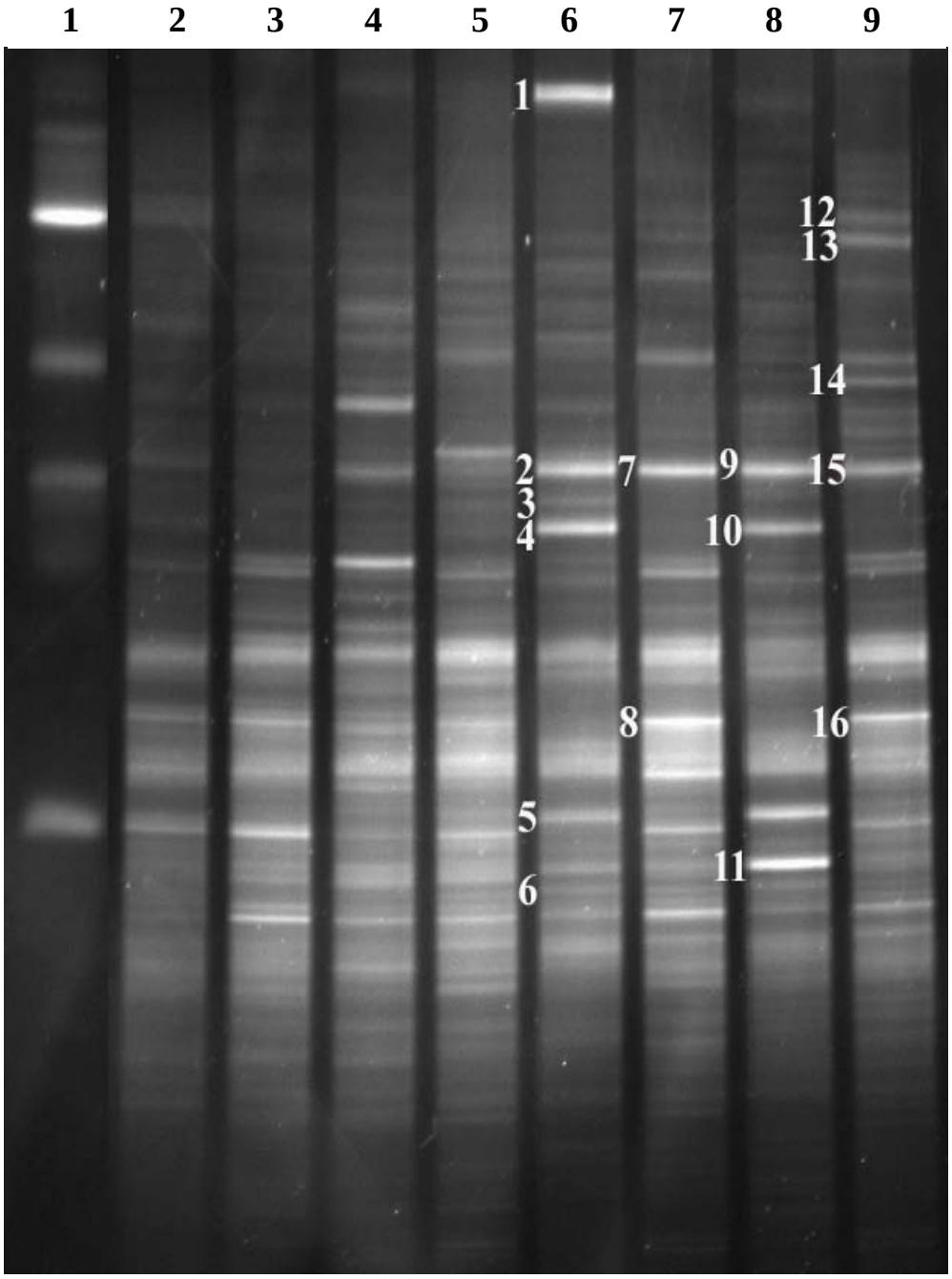

Figure 5.1. Unique DGGE bands excised from different soil profiles. Lane 1 = 100 bp DNA ladder. Lanes 2 and 3 = field (WSiL and LSiCL) soil, Lanes 4 and 5 = control (WSiL and LSiCL) soil, Lanes 6 and 7 = EPTC enhanced (WSiL and LSiCL) soil, Lanes 8 and 9 = atrazine enhanced(WSiL and LSiCL) soil.

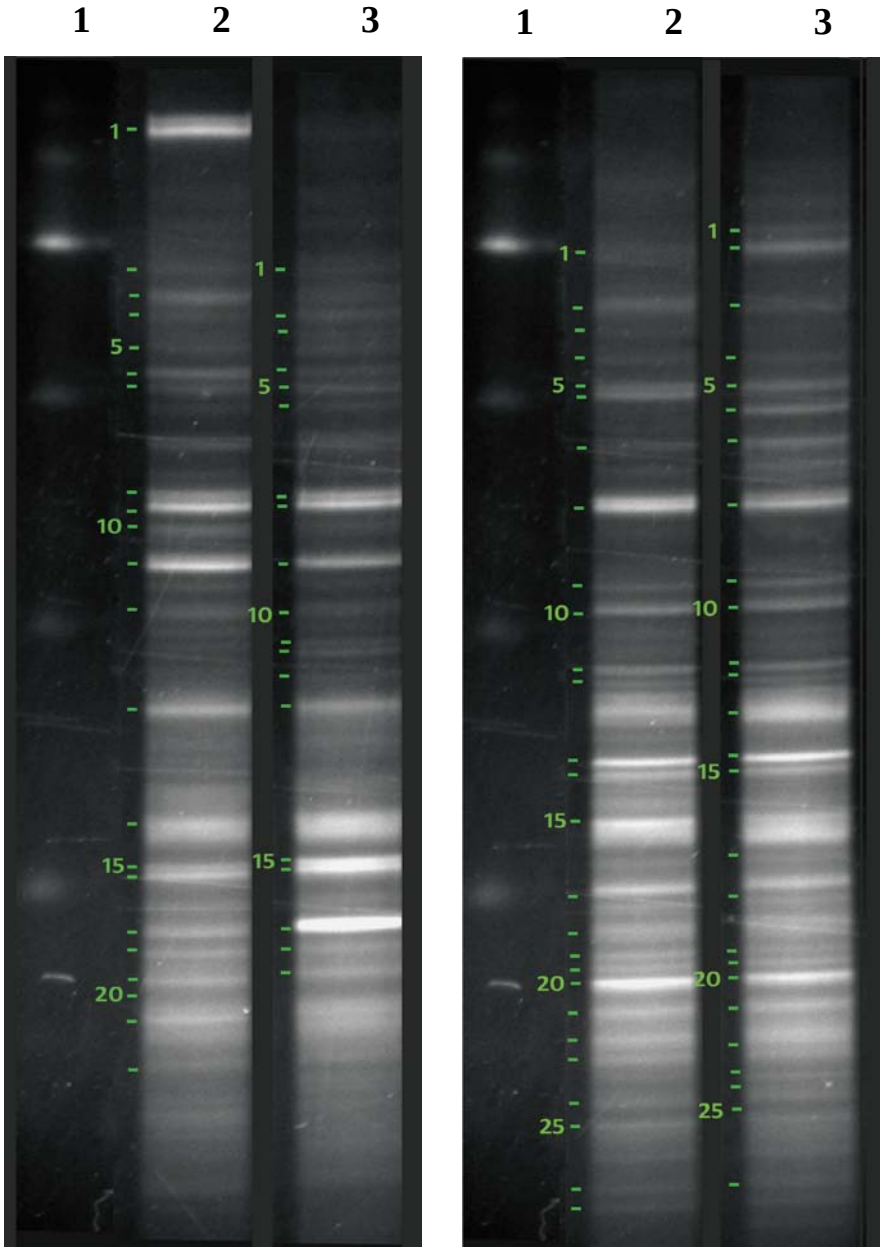

Figure 5.2: **DGGE profiles for Wooster silt loam soil (left) following EPTC (WE) (lane 2) and atrazine (WA) (lane 3) enhancement and Luray silty clay loam soil following EPTC (LE) (lane 2) and atrazine (LA) (lane 3) enhancement. Lane 1 is 100 bp DNA ladder.**

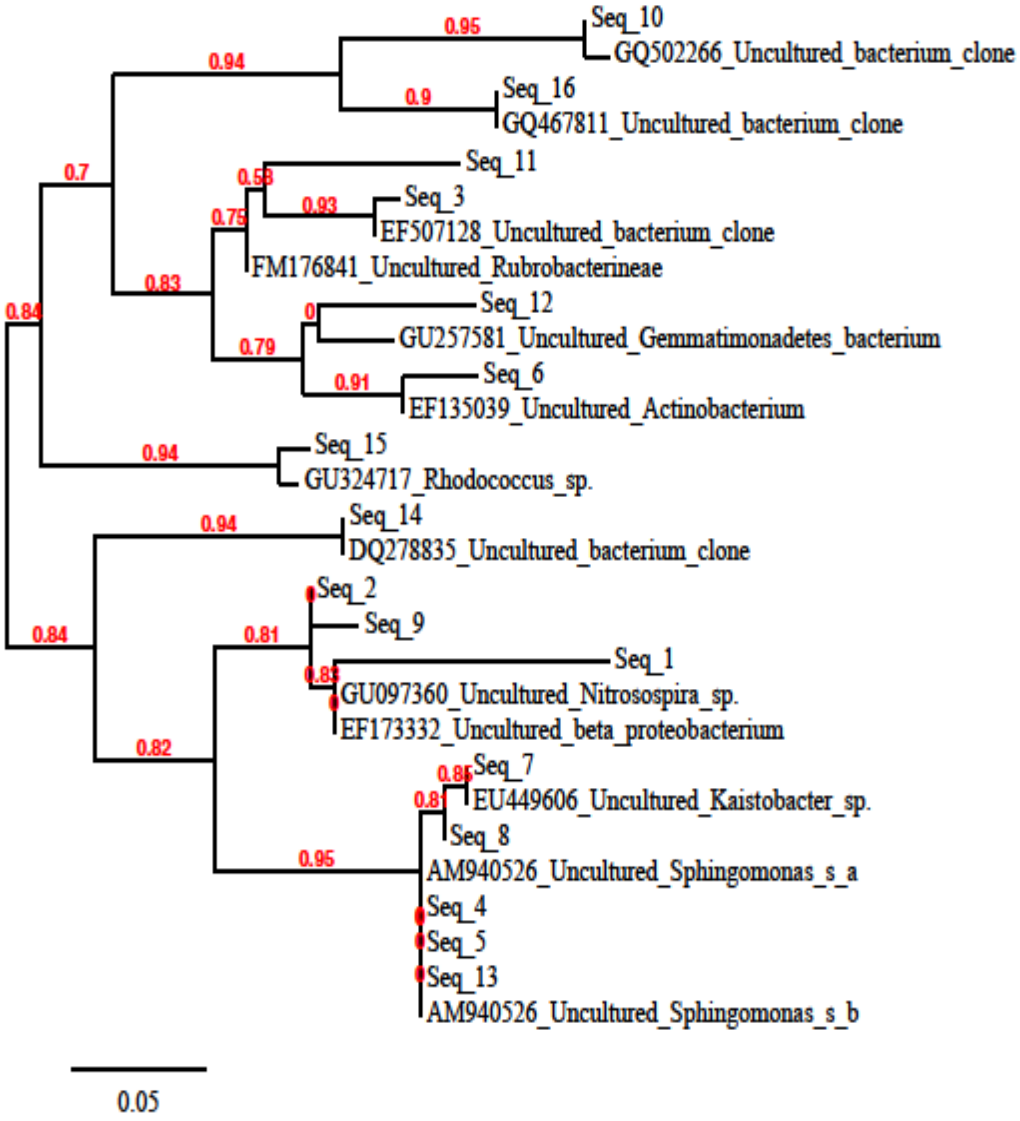

Figure 5.3: **Phylogenetic tree of partial 16S rRNA gene (V3 region) sequences amplified from excised DGGE fingerprint bands from EPTC enhanced Wooster silt loam and Luray silty Clay loam soils. The space bar indicates 10% sequence variation.**

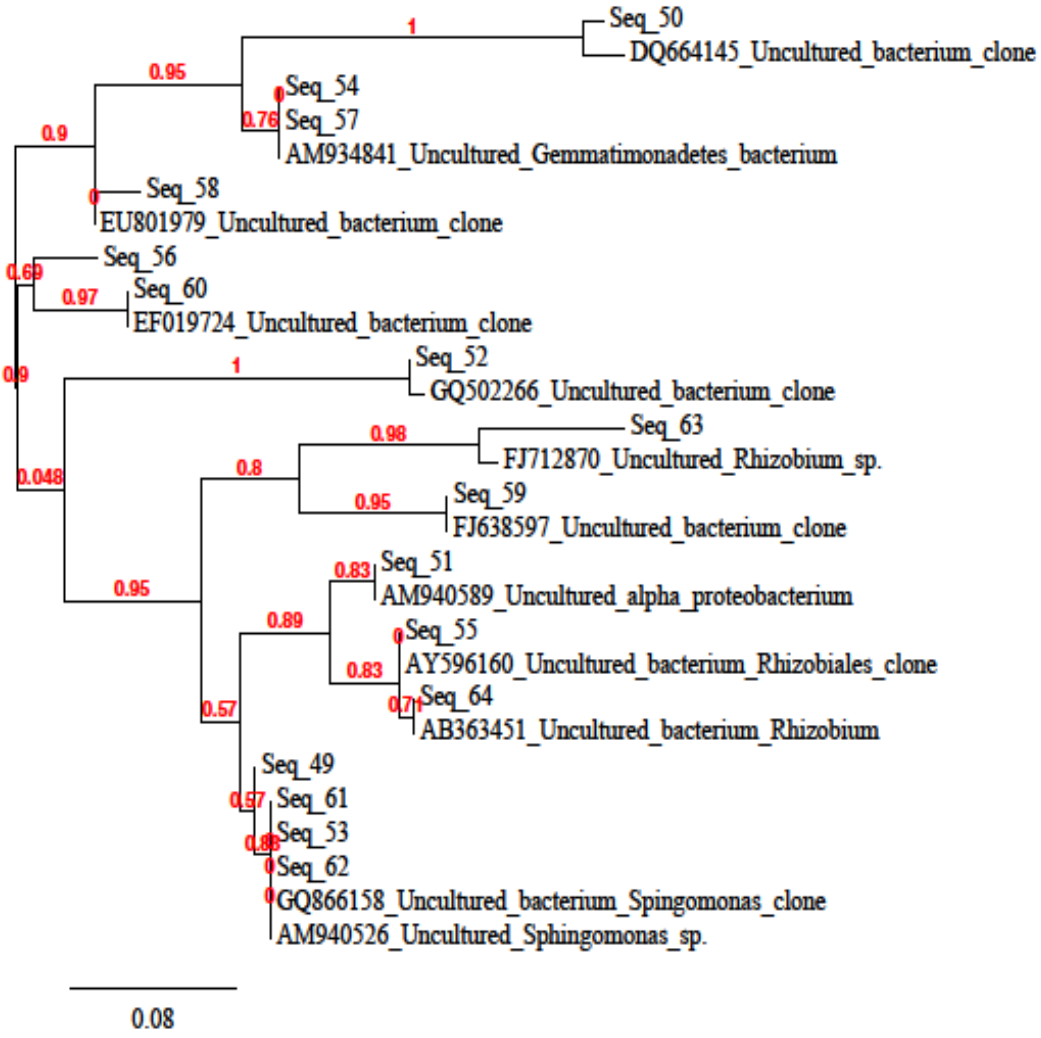

Figure 5.4: **Phylogenetic tree of partial 16S rRNA gene (V3 region) sequences amplified from excised DGGE fingerprint bands from atrazine enhanced Wooster silt loam and Luray silty Clay loam soils. The space bar indicates 10% sequence variation.**

TERMS AND DEFINITIONS

ANOVA	Analysis of Variance
ARDRA	Amplified rDNA restriction analysis
CDA	Clean water act
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act
CFU	Colony forming unit
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxy ribonucleic acid
EPTC	s-Ethyl-N,N.-dipropylthiocarbamate
GC	Gas chromatograph
LSiCL	Luray silty clay loam soil
MCL	Maximum contaminant level
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
SDWA	Safe drinking water act
U.S. EPA	United States Environmental Protection Agency
WSiL	Wooster silt loam soil

APPENDIX

Sequences of selected clones

>Seq_1 Uncultured Soil Bacterium clone C1Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGG
TCCAGCTATGCCGCGTGTGTGAAGAAGGCCTTCTGGTTGTAAAGATCATTTTC
ACGGAAAGAAAGCTTACCTACTAATACTANGTGAGGTGGCGGTACCTTGATA
ACATTGACCAATATAACCCCTGCTGCCTCCCGTAGGAGTA

>Seq_2 Uncultured Soil Bacterium clone C2Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTC
GCAAGGAAAGAAAACTTACCTACTAATACTGGGTGAGGTTGACGGTACCTGA
TAGGAGGCACCGGCTAACTACGTGCCGGCGGCCGCGGTAAT

>Seq_3 Uncultured Soil Bacterium clone C3Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTG
ACGCAGCGACGCCGCGTGAGGGAAGACGGCCTTCGGGTTGTAAACCTCTTTT
AAGAGGGAAGAAGCCACTCGGGTGAATAGCCCAGAGGGTGACGGTACCTCT
AGAATAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAAT

>Seq_4 Uncultured Soil Bacterium clone C4Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATA

>Seq_5 Uncultured Soil Bacterium clone C5Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_6 Uncultured Soil Bacterium clone C6Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGACGAAAGTCTGA
CGCAGCGACGCCGCGTGGGGGATGAAGGCCCTCGGGTTGTAAACCCCTTTCG
GCAGGGACGAAGCGCATGTGACTGTACCTGCAAAAAAAACACCGGCTAACT
ACGTGCCAGCAGCGAGTA

>Seq_7 Uncultured Soil Bacterium clone C7Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCGGCCGCGGTAAT

>Seq_8 Uncultured Soil Bacterium clone C8Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATA

>Seq_9 Uncultured Soil Bacterium clone C9Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTG
ATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTC
GCAAGGAAAGAAAACTTACCTACTAATACTAGGTGAGGTTGACGGTACCTTG
ATAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT

>Seq_10 Uncultured Soil Bacterium clone C10Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAAGGGATATTGCACAATGGGCGCAAGCCTGA
TGCAGCAACGCCGCGTGCAGGATGAAGGGCTTCGGCTCGTAAACTGCTTTTC
TGGGGGACGAGGATGACGGTACCCCAGGAAGAAGTCTCGGCTAACTACGTGC
CAGCAGCCGCGGTAATA

>Seq_11 Uncultured Soil Bacterium clone C11Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCAACGCCGCGTGCGGGAGGAAGGCCTTCGGGTTGTAAACTGCTTTTA
GGAGGGAAGAATATGCTCGGGTGAATAGCCCAGAGGGTGACTAACCCTCCAC
CAGAAGCCGCGGTTATCAACCTGCCTCTGCCCATGGTCATA

>Seq_12 Uncultured Soil Bacterium clone C12Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGGCAATGGGCGAAAGCCTGA
CCCAGCAACGCCGCGTGTGGGATGAAGGCCTTCGGGTTGTAAACCACTTTCG
AGAGGGACGAAGATCTGACGGTTCCTCGAAAGGGAGCTGCGGGTACCTGTGG
GCCAACCGCCGCGGTAATA

>Seq_13 Uncultured Soil Bacterium clone C13Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_14 Uncultured Soil Bacterium clone C14Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCAACGCCGCGTGGGTGAAGAAGGCTCTTGGGTCGTAAAGCCCTTTCG
ACAGGGAAGAAGGGTCTCGTGGTGAACAATTACGAGATTTGACGGTACCTGA
TGAAGAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT

>Seq_15 Uncultured Soil Bacterium clone C15Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TCCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA
GTACCGACGAAGCGCAAGTGACGGTAGGTACAGAAGAAGCACCGTCCAACT
ACGTGCCAGCAGCCGCGGTAATAA

>Seq_16 Uncultured Soil Bacterium clone C16Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAAGGAATCTTCCACAATGGGCGAAAGCCTGA
TGGAGCAACGCCGCGTGCAGGATGAAGGCCCTCGGGTCGTAAACTGCTTTTA
TGAGTGAAGAATATGACGGTAACTCATGAATAAGCACCGGCTAACTACGTGC
CAGCAGCCGCGGTAAT

>Seq_17 Uncultured Soil Bacterium clone C17Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTA
AGTTGGGAGGAAGGCCTTGGCGCTAATATCGCTGAGGATTGACGTTACCAAC
AGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT

>Seq_18 Uncultured Soil Bacterium clone C18Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_19 Uncultured Soil Bacterium clone C19Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCGACGCCGCGTGTGGGATGACGGCCTTCGGGTTGTAAACCACTGTCG
GGAGGGACGAAGATCTGACGGTACCTCCAAAGGAAGCACCGGCTAACTCTGT
GCCAGCAGCCGCGGTAATAA

>Seq_20 Uncultured Soil Bacterium clone C20Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCGACGCCGCGTGTGGGATGACGGCCTTCGGGTTGTAAACCACTGTCG
GGAGGGACGAAGATCTGACGGTACCTCCAAAGGAAGCACCGGCTAACTCTGT
GCCAGCAGCCGCGGTAATAA

>Seq_21 Uncultured Soil Bacterium clone C21Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq_22 Uncultured Soil Bacterium clone C22Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGCAAGCCTGA
TCCAGCAATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTA
CCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTGC
CAGCAGCCGCGGTAATAA

>Seq_23 Uncultured Soil Bacterium clone C23Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCAACGCCGCGTGCGGGATGAAGGCCTTCGGGTTGTAAACCGCTTTCA
GTGGGGACGAACCAAGACGGTACCCACAGAAGAAGCCCCGGCCAACTACGT
GCCAGCAGCCGCGGTAATAA

>Seq_24 Uncultured Soil Bacterium clone C24Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAAGGAATATTGGTCAATGGACGCAAGTCTGA
ACCAGCCATGCCGCGTGAAGGATGAAGGCCCTCTGGGTTGTAAACTTCTTTTA
CAGGGGAAGAAATCTTTTGATTCTTCGAGAGTTGACGGTACCCTGGGAATAA
GCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT

>Seq_25RC Uncultured Soil Bacterium clone C25Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_26 Uncultured Soil Bacterium clone C26Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGA
TCCAGCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGT
CCGGGAAGAAACGGTTGTGGCTAATATCCATGGCTAATGACGGTACCGGAAG
AATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAA

>Seq_27 Uncultured Soil Bacterium clone C27Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_28 Uncultured Soil Bacterium clone C28Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGGCAATGGGCGCAAGCCTGA
CCCAGCAACGCCGCGTGAAGGAGGAAATCCTTCGGGATGTAAACTTCACAAG
CAAGGGAAGAATGCCTCAGGGTGAATACCCCTGAGGAGAGACGGTACCTTGC
GTAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA

>Seq_29 Uncultured Soil Bacterium clone C29Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq_30 Uncultured Soil Bacterium clone C30Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTGTG
GGGAGAGACGAATAAGTGCAGCCTAATACGCTGCATGATGACGGTATCTCCT
TAGCAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATAA

>Seq_31 Uncultured Soil Bacterium clone C31Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTT
GTCCGGAAAGAAATCCTGTCGGCTAACACCCGGCGGGGATGACGGTACCGG
AAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAA

>Seq_32 Uncultured Soil Bacterium clone C32Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGCGCGAAAGCGTGA
CGCAGCAACGCCGCGTGGGGGATGAAGGCCCTCGGGTTGTAAACCCCTTTCG
GCAGGGACGAAGCGATCGTGACGGTACCTGCAGAAGAAGCCTCGGCTAACT
ACGTGCCAGCAGCCGCGGTAAT

>Seq_33 Uncultured Soil Bacterium clone C33Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGNGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGA
TGCAGCAACGCCGCGTGCGGGAAGAAGGCCTTAGGGTTGTAAACCGCTTTCA
GCAGGGAAGAAACTGACGGTACCTGCAGAAGAAGGTGCGGCCAACTACGTG
CCAGCAGCCGCGGTAATAA

>Seq_34 Uncultured Soil Bacterium clone C34Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTC
GCAAGGAAAGAAAACTTACCTACTAATACTAGGTGAGGTTGACGGTACCTTG
ATAAGAAGCACCGGCTAACTACGTGCCAGCGGCCGCGGTAAT

>Seq_35 Uncultured Soil Bacterium clone C35Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTC
AACGGTGAAGATAATGACGGTAGCCGTAGAAGAAGCCCCGGCTAACTTCGTG
CCAGCAGCCGCGGTAATAA

>Seq_36 Uncultured Soil Bacterium clone C36Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq37 Uncultured Soil Bacterium clone C37Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq_38 Uncultured Soil Bacterium clone C38Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAACGAATCTTCCGCAATGCGCGAAAGCGTGA
CGGAGCAATGCCGCGTGCAGGATGAAGCCCTTCGGGGTGTAAACTGCTGTCA
GGGTTTAGGAAGCACGTGACCAAACCCAAAGGAAGAGACGACTAACTCTGT
GCCAGCAGCCGCGGTAATA

>Seq_40 Uncultured Soil Bacterium clone C40Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCAATACCGCGTGTGTGAAGAAGGCCTGAGGGTTGTAAAGCACTTTC
AGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAAC
TACGTGCCAGCAGCCGCGGTAAT

>Seq_41 Uncultured Soil Bacterium clone C41Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGGGTGATGAAGGCCCTAGGGTTGTAAAGCCCTTTC
GGCGGGGAAGATAATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATAA

>Seq_42 Uncultured Soil Bacterium clone C42Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_43 Uncultured Soil Bacterium clone C43Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAAGGAATCTTCCACAATGGGCGAAAGCCTGA
TGGAGCAACGCCGCGTGCAGGATGAAGGCCCTCGGGTCGTAAACTGCTTTTA
TGAGTGAAGAATATGACGGTAACTCATGAATAAGCACCGGCTAACTACGTGC
CAGCAGCCGCGGTAATAA

>Seq_44 Uncultured Soil Bacterium clone C44Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCAACGCCGCGTGGAGGATGAAGGCCCTTGGGTTGTAAACTCCTGTCG
GCTGGGAAGAAATCTTCCGGGGCTAATACCTCCGGGAGTTGACTGTACCAGC
AAAGGAAGCCCCGGCTAACTCTGTGCCAGCAGCCGCGGTAAT

>Seq_45 Uncultured Soil Bacterium clone C45Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGGGTGATGAAGGCCCTAGGGTTGTAAAGCCCTTTC
AGCGGGGAAGATAATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATAA

>Seq_46 Uncultured Soil Bacterium clone C46Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq_47 Uncultured Soil Bacterium clone C47Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGA
TCCAGCCATTCCGCGTGCAGGAAGAAGGCCCTCGGGTTGTAAACTGCTTTTG
GACGGAACGAAAAGCGCTGAGTTAATACCTCGGCGTCATGACGGTACCGTCA
GAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT

>Seq_48 Uncultured Soil Bacterium clone C48Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGA
TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA
GTAGGGACGAAGCGTAAGTGACGGTACGTGCAGAAGAAGCACCGGCTAACT
ACGTGCCAGCAGCCGCGGTAAT

>Seq_49 Uncultured Soil Bacterium clone C49Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq_50 Uncultured Soil Bacterium clone C50Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGCGACGAATCTTCCGCAATGGGCGAAAGCCTGA
CGGAACGACGCCGCGTGTGGGATGAAGCGTCTACGACGTGTAAACCACTGTC
AGAGACCAGTAACACTTATCGTTGTCAGAGGAAGGGGCGACTAACTCTGTGC
CAGCAGCCGCGGTAAT

>Seq_51 Uncultured Soil Bacterium clone C51Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGCGCAAGCCTGA
TCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTAGGGTTGTAAAACTCTTTCG
CCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGC
CAGCAGCCGCGGTAATAA

>Seq_52 Uncultured Soil Bacterium clone C52Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAAGGGATATTGCACAATGGGCGCAAGCCTGA
TGCAGCAACGCCGCGTGCAGGATGAAGGGCTTCGGCTCGTAAACTGCTTTTC
TGGGGGACGAGAATGACGGTACCCCAGGAAGAAGTCTCGGCTAACTACGTGC
CAGCAGCCGCGGTAAT

>Seq_53 Uncultured Soil Bacterium clone C53Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGAAGATAATGACTGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_54 Uncultured Soil Bacterium clone C54Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCGACGCCGCGTGTGGGATGACGGCCTTCGGGTTGTAAACCACTGTCG
GGAGGGACGAAGATCTGACGGTACCTCCAAAGGAAGCACCGGCTAACTCTGT
GCCAGCAGCCGCGGTAAT

>Seq_55 Uncultured Soil Bacterium clone C55Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGGGTGATGAAGGCCCTAGGGTTGTAAAGCCCTTTC
GGCGGGGAAGATAATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGT
GCCAGCAGCCGCGGTAAT

>Seq_56 Uncultured Soil Bacterium clone C56Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGCGCGAAAGCGTGA
CGGCAGCAACGCCGCGTGAGGGAAGAAGGCCTTCGGGTTGTAAACCTCTTTTA
TCAGGGAAGATAATGACGGTACCTGATGAATGAGCCCCGGCTAACTACGTGC
CAGCAGCCGCGGTAATAA

>Seq_57 Uncultured Soil Bacterium clone C57Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGGGCGAAAGCCTGA
CGCAGCGACGCCGCGTGTGGGATGACGGCCTTCGGGTTGTAAACCACTGTCG
GGAGGGACGAAGATCTGACGGTACCTCCAAAGGAAGCACCGGCTAACTCTGT
GCCAGCAGCCGCGGTAAT

>Seq_58 Uncultured Soil Bacterium clone C58Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTGCTCAATGGGCGAAAGCCTGA
CGCAGCAACGCCGCGTGCGGGATGAAGGCCTTCGGGTTGTAAACCTCTTTCA
GCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACCGGCCAACT
ACGTGCCAGCAGCCGCGGTAAT

>Seq_59 Uncultured Soil Bacterium clone C59Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGGGCAACCCTGA
TCCAGCGATGCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTAG
GTTGGGAAGAAGTTGCTAGGGGGATAATCCCTAGCAGTTGACGGTACCAACA
GAATAAGCACCGGCAAACTCTGTGCCAGCAGCCGCGGTAAT

>Seq_60 Uncultured Soil Bacterium clone C60Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTCGGGAATTTTGCGCAATGGACGAAAGTCTGA
CGCAGCAACGCCGCGTGAGGGATGAAGGCCTTCGGGTTGTAAACCTCTTTTA
TCAGGGAAGATAATGACGGTACCTGATGAATAAGTCACGGCTAACTACGTGC
CAGCAGCCGCGGTAAT

>Seq_61 Uncultured Soil Bacterium clone C61Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAT

>Seq_62 Uncultured Soil Bacterium clone C62Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCAATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTT
ACCCGGGATGATAATGACAGTACCGGGAGAATAAGCCCCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATAA

>Seq_63 Uncultured Soil Bacterium clone C63Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATCTTGGTCAATGGGCGCAAGCCTGA
ACCAGCGACGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAAAGCTCTGTGG
GGAGGGACGAAAGACTGAGGTCTAATAGGCTTCAGGTTGACTGTCCCCCCTC
AGCAAGCACCGGGTAACTCTGTGCCTGTAGCCGCCTCCATCTTCTTCTCACAC
GCGGAATTGCTTGATCAGGGTTGCCCCCATTGTTGATTCAGCCTCGCGGCTAT
TTCCCAAGATCCCCCCCCGGTTGCCTCC

>Seq_64 Uncultured Soil Bacterium clone C64Y2010 16S ribosomal RNA gene, partial sequence
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG
ATCCAGCCATGCCGCGTGGGTGATGAAGGCCCTAGGGTTGTAAAGCCCTTTC
AGCGGGGAAGATAATGACGGTACCCGCAGAAGAAGCCCCGGCTAACTTCGT
GCCAGCAGCCGCGGTAATAA

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