BIOAUGMENTATION FOR THE REMEDIATION OF PESTICIDE-
CONTAMINATED SOIL WITH MICROORGANISMS DIRECTLY ENRICHED
IN SOIL OR COMPOST

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

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2003

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EPTC (s-ethyl-N,N’-dipropylthiocarbamate) and atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) are commonly used agricultural herbicides. Atrazine, especially, can be found in soils and waters at concentrations that exceed the 3 µg/L health advisory level set by U.S. EPA. Developing an effective inoculum with high activity to bioaugment degradation of EPTC or atrazine in contaminated environments is a goal related to bioremediation of such sites.

Direct enrichment of microorganisms in soil capable of EPTC or atrazine degradation was accomplished. The effectiveness of adding these soils (hereafter called ACTIVATED soils) for enhancing the biodegradation of EPTC or atrazine in contaminated soil was evaluated. Effectiveness of compost as an inoculum carrier and stability of ACTIVATED soils and compost under different storage conditions were also tested.

EPTC-degrading microorganisms were readily enriched in a Brookston clay loam soil that was sampled from a field previously treated with EPTC, and then air-dried and stored at room temperature for 14 years. The most probable number (MPN) method revealed that the number of EPTC-degraders increased by about 3 logs of magnitude after a 7 day water pretreatment followed by a single treatment with EPTC at a rate of 20 mg/kg
soil. Additional applications at the same rate did not result in any further increase in the number of EPTC-degrading microbes. EPTC degradation was also enhanced considerably after the first EPTC treatment. Degrading activity in ACTIVATED soil was not inhibited at initial EPTC concentrations up to 2,000 mg/kg soil. Inoculation of four EPTC-contaminated soils with ACTIVATED soil, at rates ranging from 0.05% to 5% (w/w), resulted in significantly ($p < 0.05$) increased degradation of EPTC with little variation among soils tested. At the 0.5% inoculation rate, the concentration of EPTC in soil after 5 days of incubation was about 20% of that initially measured in all soils tested. This was in contrast to EPTC concentration remaining at 60% or higher in non-inoculated soils.

Atrazine-degrading and mineralizing microbial populations, based on MPN, were also readily enriched in a Wooster silt loam soil. Microorganisms utilizing atrazine as their sole carbon or nitrogen source increased by 3 logs and 1 log of magnitude, respectively, after three treatments of atrazine at a rate of 4 mg/kg soil. Atrazine degradation was greatly accelerated after the first treatment. Atrazine degrading activity was not inhibited at initial concentrations up to 400 mg/kg soil. Inoculation of atrazine-contaminated soils with ACTIVATED soil significantly ($p < 0.05$) increased atrazine degradation in all four soils tested. In soils with near neutral pH and organic content less than 10%, inoculation at a rate of 0.5% reduced atrazine concentration to less than 5% of the atrazine added (initial concentration of 4 mg/kg soil) after 5 days of incubation, compared to about 70% atrazine remaining in non-inoculated contaminated soil.
Neither EPTC nor atrazine degradation was further accelerated by nutrient (glycine, sucrose, urea, or yeast extract) amendment at a rate of 1% or 0.1%.

Both atrazine-degrading and mineralizing microbial populations were successfully transferred to and enriched in compost. The degradation activity in ACTIVATED compost was much greater than in an ACTIVATED soil. The concentration of atrazine (initially 400 mg/kg) decreased about 90% after 9 hours of incubation in ACTIVATED compost, compared to 3 days in ACTIVATED soil. This suggests that compost can be used as an effective carrier of EPTC and atrazine degraders and thus an effective inoculant for bioaugmentation.

EPTC and atrazine degradation activity in ACTIVATED soil and compost remained stable when they were stored at low temperature (below 10 °C) and in a wet state (water content above 15%, w/w). ACTIVATED soil and compost, containing increased numbers of EPTC- or atrazine-degrading microorganisms, are to be useful inoculants that will bioaugment the remediation of EPTC- or atrazine contaminated soil.
Dedicated to my parents and wife
ACKNOWLEDGMENTS

I would like to express my deep appreciation to Dr. Warren A. Dick, my academic advisor, for encouraging me and for his advice and support in every aspect of my graduate school experience. I would like to thank Drs. Edward McCoy, Fredrick C. Michel, Jr., and Lynn B. Willett for their assistance and advice as members of the committee for my dissertation.

I also would like to thank Ahmad Al-Turki and Guilherme Holtz for sharing pleasure and experience in classes and laboratories, We all had a great time working together. It was also a pleasure for me to work with Ying Wang, and Drs. Liming Chen and Ping Wang. Special thanks also go to Jeanne Durkalski for her technical assistance. The assistance of all members of the OARDC is gratefully acknowledged.

I also would like to express deep gratitude to Mrs. Mary Misner who provided my family with many chances to learn and enjoy various activities while I was preoccupied with my research.
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BMN</td>
<td>Basal minimal salts-nitrogen</td>
</tr>
<tr>
<td>BMNA</td>
<td>Basal minimal salts-nitrogen amended with atrazine</td>
</tr>
<tr>
<td>BMNE</td>
<td>Basal minimal salts-nitrogen amended with EPTC</td>
</tr>
<tr>
<td>BTEX</td>
<td>Benzene, toluene, ethylbenzene, and xylene</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegration pr minute</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>EPTC</td>
<td>s-Ethyl-(N,N)'-dipropylthiocarbamate</td>
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<tr>
<td>GEMs</td>
<td>Genetically engineered microorganisms</td>
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<td>HDPE</td>
<td>High density polyethylene</td>
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<td>GC</td>
<td>Gas chromatograph</td>
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<tr>
<td>LSD</td>
<td>Least Significant Difference</td>
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<td>MPN</td>
<td>Most probable number</td>
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<tr>
<td>OPH</td>
<td>Organophosphorus hydrolase</td>
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<td>PAHs</td>
<td>Polycyclic aromatic hydrocarbons</td>
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<tr>
<td>PCBs</td>
<td>Polychlorinated biphenyls</td>
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<tr>
<td>PCE</td>
<td>Perchloroethylene</td>
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<tr>
<td>PCP</td>
<td>Pentachlorophenol</td>
</tr>
<tr>
<td>TCE</td>
<td>Trichloroethylene</td>
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<tr>
<td>TGYe</td>
<td>Trypton-glucose-yeast extract</td>
</tr>
<tr>
<td>U.S. EPA</td>
<td>The United State Environmental Protection Agency</td>
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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

A variety of synthetic chemicals are used throughout the world, and environmental pollution by some of these compounds is a worldwide problem. Regulations have been imposed to ensure appropriate disposal of chemicals, to protect the environment, and to encourage the remediation of polluted environments. Among the most commonly encountered compounds in soils and waters are hydrocarbons, pesticides, heavy metals, and essential growth nutrients. These chemicals enter natural environments from various sources. Sites of interest, with respect to environmental pollution, include agricultural areas and their vicinity, industrial areas, dumpsites, coal-distillation areas, oil refinery areas, wood preservation sites, and groundwater (Allard and Neilson 1997).

EPTC (s-ethyl-$N,N'$-dipropylthiocarbamate) and atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine commonly used herbicides, and it was reported that EPTC is slightly toxic (Class III) and atrazine are moderately toxic to human and animals. Atrazine, especially, is often found in soils and waters at levels that exceed 3 µg/L health advisory level set by U.S. EPA, and is a compound that brings about public concern.
Once released to the environment, chemicals undergo various dissipation pathways, and the persistence of chemicals in the environment varies widely. Among factors affecting the local concentration of a compound are the amount of compound released, the rate of compound released, its persistence in the environment under various conditions, the extent of its dilution, its mobility, and the rate of biological or non-biological degradation (Ellis 2000, Harayama 1997, Janssen et al. 2001).

Both organic and inorganic contaminants in soil and groundwater can be degraded or immobilized by naturally occurring processes. These naturally occurring processes include sorption, dilution, dispersion, volatilization, precipitation, ion exchange, and abiotic transformation and biological degradation by intrinsic organisms (Christensen et al. 2001). The toxicity, mass and/or mobility of the contaminants can thus be reduced, even in the absence of human intervention. Depending on their properties and environmental conditions, some chemicals may disappear in few days or weeks, but others may persist for years (Gibson and Parales 2000).

Microorganisms are ubiquitous, and it has been estimated that 1000 or more species may exist in each gram of soil. A total of 2-3 million bacterial species are thought to exist in the biosphere (Torsvik et al. 1990, Truper 1992). Microorganisms are thought to be the principal mediators of the natural attenuation of many pollutants, such as organic molecules, inorganic compounds, and metals (Christensen et al. 2001, Lovley 2001). Microorganisms with the ability to degrade a specific compound in a specific place where the compound exists are required for active degradation of chemicals in the environment. The degradation of some compounds in some environments is, however, not great enough
to remove it from the environment. Therefore, enhancement of degradation using bioaugmentation, either by stimulation of indigenous microbes or addition of exogenous microorganisms, has been extensively studied (Chen et al. 1999, Johan et al. 2001, Mishra et al. 2001, Watanabe 2001).

Inoculation, that is the introduction of microorganisms to a new environment, is conducted to bioaugment degradation of chemicals in the environments. These microorganisms are normally species that can be isolated and cultured. Other species in consortium, genetically engineered microorganisms (GEMs) or directly isolated microorganisms from such as soils or sludges, may also be used in bioaugmentation. However, recent studies have shown that the majority of species present in an ecosystem are non-culturable (Torsvik et al. 1990), which means that microorganisms having a major role in degradation of certain compounds may not be isolated. In addition, it has been found that inoculation is effective in some cases, but not at all in other cases. Among the main reasons for unsuccessful results are low survival rate of the introduced microorganisms, loss of activity, or both. Therefore, developing inoculant with high survival rate and activity for degradation of target compounds is a goal that holds promise to enhance the effectiveness of bioaugmentation.

Previous studies have shown accelerated degradation of chemicals in fields having a history of treatment with the same chemical or its analogs. EPTC (s-ethyl dipropylthiocarbamate), one of the most widely used herbicide in the North America, is an example, and there have been a number of reports on enhanced degradation of EPTC in

To date, EPTC-degrading soil isolates have mostly belonged to the genus *Rhodococcus* (Table 1.1), and single species could degrade EPTC to inorganic forms. Atrazine (2-chloro-4-ethylamino-6- isopropylamino-1,3,5-triazine) is another herbicide that has been widely used for more than 50 years. A number of atrazine-degrading microorganisms and some microbial mixed cultures capable of atrazine mineralization have been reported (Assaf and Turco 1994, Barriuso and Houot 1996, Mandelbaum et al. 1993a, Mirgain et al. 1993, Stolpe and Shea 1995, Topp et al. 1995)). However, only recently have a few microorganisms capable of complete degradation of atrazine (breaking down s-triazine ring to carbon dioxide) been isolated (Table 1.1). Enhanced degradation of atrazine in soils has rarely been reported (Barriuso and Houot 1996).

1.2 OBJECTIVES

In this work, ACTIVATED soil and compost were prepared by treating them with EPTC or atrazine to develop enriched microbial populations capable of degrading or mineralizing EPTC or atrazine in soils. Degradation rates and microorganism number were monitored during treatments. In addition, various soils, artificially contaminated with EPTC or atrazine, were inoculated with ACTIVATED soil or compost. The effect of this inoculation on degradation of EPTC or atrazine in soils was evaluated at various inoculation rates. The effect of nutrient addition on EPTC or atrazine degradation and the effect of storage conditions on the stability of ACTIVATED soil or compost were also investigated.
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<td></td>
</tr>
<tr>
<td>Unspecified</td>
<td>Lee et al. 1984</td>
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<tr>
<td><em>Rhodococcus</em> sp. strain TE1 (Formerly known as <em>Arthrobacter</em> sp. strain TE1)</td>
<td>Tam et al. 1987</td>
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<tr>
<td><em>Rhodococcus</em> sp. strain JE1</td>
<td>McClung and Dick 1987</td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp. N186/21</td>
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<td><em>Rhodococcus</em> spp. strains J10, B30, B40, and D20</td>
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<td><strong>Atrazine-mineralizing isolates</strong></td>
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<td><em>Pseudomonas</em> strain YAYA6</td>
<td>Yanze-Kontchou and Gschwind 1994</td>
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<tr>
<td>M91-3</td>
<td>Radosevich et al. 1995</td>
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<tr>
<td><em>Pseudomonas</em> sp. strain ADP</td>
<td>Mandelbaum et al. 1995</td>
</tr>
<tr>
<td><em>Pseudaminobacter</em> sp. strains C147 and C195</td>
<td>Topp 2001</td>
</tr>
<tr>
<td><em>Chelatobacter heintzii</em> Cit1</td>
<td>Rousseaux et al. 2001</td>
</tr>
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Table 1.1. Some of the EPTC-degrading or atrazine-mineralizing bacterial isolates reported.
Specific objectives of this research were:

(1) to determine whether the number of microorganisms capable of degrading and/or mineralizing EPTC or atrazine in soil could be increased, thus resulting in an ACTIVATED soil or compost,

(2) to evaluate the effectiveness of the ACTIVATED soil and compost as inoculants to bioaugment degradation of these same target compounds in contaminated soils,

(3) to investigate whether nutrient amendment, with or without inoculation with ACTIVATED soil, could further accelerate the degradation of EPTC or atrazine

(4) to evaluate compost as an inoculum carrier, and

(5) to find conditions for storage of inoculum (ACTIVATED soil or compost) in a readily usable state.
CHAPTER 2

LITERATURE REVIEW

2.1 ENVIRONMENTAL CONTAMINATION

2.1.1 Environmental contamination: a worldwide problem

Modern society depends on a variety of synthetic chemicals, and the amount of these chemicals used is huge. Many chemicals, produced on a large scale as parts of the normal activities of industrialized societies, are considered hazardous to humans, plants and animals. The application of highly sensitive analytical techniques to environmental samples has provided society with disturbing information: the air, the water, and the soil are contaminated with a variety of synthetic chemicals. Thus, contamination of soils, sediments, waters, and air with hazardous and toxic chemicals is one of the major problems facing the industrialized world today.

Among the most commonly encountered contaminants in soils and waters are hydrocarbons (both aliphatics and aromatics), pesticides, heavy metals and nutrients. These chemicals enter natural environments from various sources. Aromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, and xylene) result form oil refinery waste, leakage from oil pipelines and underground storage tanks or basins, and
spills of crude oil in marine environments after accidents at sea. Chlorinated aliphatics such as trichloroethylene (TCE), tetrachloroethylene, perchloroethylene (PCE), and 1,1,1-trichloroethane, are used for degreasing and released to the environments. Pesticides and/or products of their degradation are frequently found in agricultural areas and adjacent ground and surface waters (McCarty 1991). Industrial chemicals are discharged deliberately or advertently into waters or onto soils following their intended use. By-products of manufacturing operation and pollutants contained in wastes that were inadequately treated are also released to the environments (Alexander 1999).

Unacceptable pollution concentrations in water, for example, range from less than a milligram per liter for a single compound (Ralebitso et al. 2002), to grams per liter of highly complex mixtures (Christensen et al. 2001). Sites that can become contaminated include agricultural areas and vicinity with pesticides, industrial areas with chemical production waste, dumpsites with municipal waste, coal-distillation areas with coal tar, oil refinery areas and groundwater with petroleum hydrocarbons, and wood preservation sites with chlorophenolic compounds (Allard and Neilson 1997).

Although regulations are strictly enforced in developed countries like the United States and most of the western European countries, these regulations often remain unenforced in many of the developing countries.

2.1.2 Fate of contaminants in the environments

Chemicals released to the environment undergo various dissipation pathways, and the persistence of chemicals in the environment varies widely. Depending on their behavior in the environment, contaminants are often classified as biodegradable,
persistent, or recalcitrant. Factors affecting the local concentration of a contaminant include the amount of compound released, the rate at which the compound is released, its stability in the environment under various conditions, the extent of its dilution, its mobility in a particular environment, and its rate of biological or non-biological degradation (Ellis 2000, Harayama 1997, Janssen et al. 2001).

Both organic and inorganic contaminants in soil and groundwater can be degraded or immobilized by naturally occurring processes, and the toxicity, mass and/or mobility of the contaminants can be reduced without human intervention when suitable conditions prevail. Naturally occurring processes involved in the attenuation of pollutants in the environment include sorption, dilution, dispersion, volatilization, precipitation, ion exchange, abiotic transformation, and biological degradation by intrinsic organisms (Christensen et al. 2001).

Microorganisms are ubiquitous and natural environments contain a variety of microorganisms with various catalytic functions. Consequently, microorganisms are considered the principal mediators of the natural attenuation of many pollutants, such as organic molecules, inorganic compounds, and metals (Christensen et al. 2001, Lovley 2001). Therefore, degradation of pollutants by microorganisms has been considered as a major pathway, among natural processes, by which various industrial compounds in the environment are attenuated.

Microorganisms transform or mineralize pollutants, thereby decreasing their masses and toxicities in contrast to most other methods of natural attenuation. Chemicals that can be degraded by organisms or decompose spontaneously under natural conditions
may disappear in days or weeks, but others may persist for years (Gibson and Parales 2000).

2.2 REMEDIATION OF CONTAMINATED SITES AND TREATMENT OF WASTES

2.2.1 Natural attenuation

Natural attenuation refers to the decrease in the mass and/or concentration of a contaminant due to sorption, dilution, volatilization, non-biological degradation and naturally occurring biodegradation. The use of natural attenuation as a cleanup method for underground storage tank sites with petroleum-contaminated soil and groundwater has increased dramatically over the last few years. According to Tulis (1997), natural attenuation, as of 1995, has been the most common treatment for contaminated groundwater and the second most common treatment for contaminated soil at these sites. Natural attenuation has been being applied to about 47% of contaminated groundwater sites, and 28% of polluted soil sites as a remediation option.

Among natural attenuation, intrinsic bioremediation, i.e. natural bioremediation by indigenous microorganisms, is becoming a favored treatment technology for contaminated sites. This is partly because natural processes have been found to be satisfactory for removal of many pollutants (Rügge et al. 1995, Semprini et al. 1995), and other more aggressive treatments still do not totally eliminate contaminants or do not result in expected removal or destruction rates (Bredehoeft 1992, Valkenburg 1994, Uhlman 1995). So far, intrinsic bioremediation has been most successfully applied to fuel hydrocarbon plumes (Davis et al. 1994, Rifai et al. 1995, Rügge et al. 1995, Wiedemeier et
al. 1994, Wilson et al. 1995). Intrinsic bioremediation used for other compounds such as chlorinated aliphatics (Cox et al. 1995, Lee et al. 1995, Major et al. 1995, Semprini et al. 1995), polychlorinated biphenyls (U.S. EPA 1990), and explosives have also been reported.

In 1999, U.S. Environmental Protection Agency provided guidance on the use of monitored natural attenuation (U.S. EPA 1999). The guidelines offered by the U.S. EPA include: (1) the site must be closely monitored, (2) natural attenuation should not necessarily be considered a stand-alone technology, (3) contingency option(s) must be in place in the event natural attenuation is ineffective or receptors are threatened, and (4) natural attenuation must be accomplished within a reasonable time frame. The U.S. EPA views natural attenuation as an acceptable means of treatment if there is a high degree of certainty it will happen before contaminants migrate to a sensitive receptor. Consequently, it is required that, in order to get approval from a regulatory agency for the use of natural attenuation as a cleanup technology of contaminated sites, various lines of evidence on the occurrence of natural attenuation must be provided.

2.2.2 Land farming

One technology, which retains benefits of simplicity and low cost, is land farming (Morgan et al. 1989). The term, land farming was originally used to describe the method used by farmers for centuries to decompose organic nonhazardous waste. This process simply spreads the waste and tills the soil to incorporate the waste into the soil matrix, thus providing the aeration necessary to optimize microbial degradation. Land farming dates
back to the early 1900s when it was used more as a disposal technique than a remediation process. At that time, it was common for petroleum refineries to dump the sludge generated in processing onto nearby land. This option, as a remediation method, has been applied mainly for the remediation of such petroleum-contaminated sites (Hinchee 1998).

2.2.3 Excavation

*Ex situ* remediation is the excavation of contaminated media and then treating it elsewhere. This option is applicable when the soil must be excavated to restrict the spread of contaminant or when the soil must be used for different purposes and cannot be held for treatment. It is also necessary if the contamination level is so high that biodegradation is inhibited. Some technologies involve partial or total removal of contaminated material, so it can be cleaned elsewhere. It takes a relatively short period for restoration, but it is a very expensive option.

2.2.4 Percolation, extraction, treatment, and injection

Typical equilibrium oxygen concentrations in groundwaters are below 8 mg/L and most shallow groundwaters are found to have 4 to 7 mg/L of dissolved oxygen. Therefore, because of the oxygen concentration and transport limitations associated with aquifers, the rate of aerobic biodegradation is severely constrained in aquifers, and *in situ* remediation of contaminated aquifers is conducted differently than for the contaminated vadose zone. Methods for the removal of contaminant from groundwater include (1) percolation and (2) extraction, treatment, and injection. In percolation, materials such as oxygen, nutrients or sometimes microorganisms are added to increase the rate of biodegradation. However,
percolation is limited to shallow aquifers because of the time required to move materials into the contaminated zone. Extraction, treatment, and then injection is a combination of \textit{ex situ} (pump and treat) and \textit{in situ} (oxygen and nutrient injection) remediation. In this process, groundwater is removed along with contaminants from the contaminated sites, treated and then discharged to a receiving water or land. Water with oxygen, nutrients, and in some cases bacteria, are added to stimulate \textit{in situ} bioremediation (Eweis 1998).

\textbf{2.2.5 \textit{In situ} Bioremediation}

Another way to remediate a contaminated site or to treat waste is via \textit{in situ} bioremediation, which is enhanced biodegradation using added indigenous or exogenous microorganisms. This approach is considered as being different from intrinsic bioremediation (i.e. natural bioremediation by indigenous microorganisms). \textit{In situ} bioremediation works best if an impermeable layer exists under the contaminated soil, which would eliminate the threat of groundwater contamination. The concentration level of the contaminant should not be inhibitory to microbial activity for successful results (Eweis 1998). Biodegradation and \textit{in situ} bioremediation are discussed further in the following sections.

\textbf{2.3 BIODEGRADATION}

Biodegradation can be defined as the biologically catalyzed reduction in complexity of chemicals. Biodegradation of organic contaminants in the natural environment has been extensively studied to evaluate its potential in bioremediation and to understand microbial ecology and physiology (Bouwer and Zehnder 1993, Chen et al.)

In case of organic compounds, biodegradation frequently leads to the conversion of much of the C, N, P, S, and other elements in the original compound to inorganic products. Such a conversion of organic substrates to inorganic forms, such as CO₂, H₂O and other inorganic compounds is known as mineralization. Consequently, mineralization is sometimes used as a synonym for \textit{ultimate biodegradation}. In the mineralization of organic compounds by microorganisms, inorganic forms of elements are released to the surrounding environment. Because mineralization results in the total destruction of the parent compound, it is viewed as a beneficial process.

Although plant and animal respiration and non-biological processes also play a considerable role in the mineralization process, mineralization of synthetic chemicals appears to result largely or entirely in most environments from microbial activity. Microorganisms are thought to be ubiquitous, and it has been assumed that there might exist 1000 or more species per gram soil, with accumulative total of 2-3 million bacterial species in the biosphere (Torsvik et al. 1990, Truper 1992). Natural microbial populations in various habitats contain microorganisms with a diverse array of catabolic activities and an amazing physical versatility (Greer et al. 1992, Watanabe et al. 1996). A number of microorganisms with ability to degrade a wide variety of compounds have been isolated and characterized (Sangodkar et al. 1989, Dickel et al. 1993). Most synthetic products,
regardless of their complexity, are degraded by one or more species in some particular environment. Indeed, microorganisms are frequently the sole means of converting synthetic chemicals to inorganic products (Alexander 1999).

2.3.1 Factors affecting biodegradation rates of chemicals

There has been extensive research to improve biodegradation ability of microorganisms under laboratory conditions, and the rate of microbial biodegradation of a chemical in the environments is known to be affected by a number of physicochemical, biological, and environmental factors. These parameters have been discussed in many recent publications (Alexander 1999, Eweis 1998, Hinchee et al. 1995, Vogel 1996). Among the parameters that appear to be important include the properties of the chemicals to be degraded, the presence/absence of predators or interspecies competition, the conditions for microbial degradation activity (e.g. presence of nutrients, oxygen, pH, and temperature), and the physicochemical characteristics of the environments.

2.3.1.1 Intrinsic ability of the microflora at the site

Since the major pathway by which chemicals are dissipated in the environments is degradation by microorganisms, the presence or absence of microbe(s) or microbial communities with relevant activity affect their rate of degradation. It is well known that many chemicals are not degraded by a single microbial species; but require the cooperation of two or more species. In such cases, transformation that one species alone cannot perform, results in the amount of chemical degraded by the community that is greater than the sum of individual species degradation.
2.3.1.2 Properties of microbial association

Microorganisms capable of degrading chemicals have central roles in biodegradation, but other organisms also affect the process through increased predation and parasitism of the degrading bacteria and/or a fungal biomass. Although all of these can affect the rate of biodegradation, protozoan grazing is thought to most effective in controlling the rate of biodegradation. According to Alexander (1999), protozoan grazing is substantial when a prey density is greater than $10^6$ to $10^7$ bacterial cells per milliliter or per cubic centimeter for nonaqueous environments.

2.3.1.3 Characteristics of chemicals

Biodegradability of compounds is sometimes related to compound structure and its related physico-chemical characteristics such as solubility and bioavailability. One of the main reasons for the prolonged persistence of hydrophobic organic compounds in the environment is their solubilization-limited bioavailability, which itself is not intrinsic to the compound but related to the interaction between the compound, the microbes, and soil (Vogel 1996). It is known that toxicity of chemicals at high concentration affect microbes and their efficiency as degraders, although indigenous microbial population can target constituents over a wide range of concentrations in the environments (Barbeau et al. 1997).

2.3.1.4 Availability of nutrients

The nutrient requirements for microbes are approximately the same as the composition of their cells, and microbial nutrients are categorized into three groups based
on the quantity and essential need for them by microorganisms: macro-, micro, and trace nutrients (Baker and Herson 1994, Sutherson 1997). The absence of any of these nutrients, in suitable forms and states, in a particular environment will prevent the growth and/or activity of microorganisms. In contrast, enhanced and accelerated biodegradation in fertilized soils have been reported (Atlas and Bartha 1992, Lindstrom et al. 1991, Maresin and Schinner 1997, Mills and Frankenberger, Jr. 1994, Morgan and Watkinson 1989, Prichard et al. 1992, Rosenberg et al. 1992). Bioremediation of hydrocarbon-contaminated soils, in particular, is known to be limited by nitrogen (N) and phosphorus (P) since the contaminant, itself, usually functions as a carbon source. Microbial growth may be limited by several elements at the same time and additions of combinations of nutrients can enhance biodegradation (Swindoll et al. 1988). Polycyclic aromatic hydrocarbons (PAHs) degradation was found to be optimal when a material containing approximately 75% S, 3% N, and 11% P, was applied to soil (Liebeg and Cutright 1999).

Although the optimal C:N:P mole ratio for bioremediation applications is thought to be approximately 100:10:1 (Cookson 1995), there are no specific methods for determining the exact nutrient sources available at a site. In addition, the successful implementation of a nutrient combination at one site for decomposition of one contaminant does not guarantee similar success at a different environment and for a different contaminant.

2.3.1.5 Presence of other substrates

Natural or contaminated environments characteristically contain more than one organic compound, either natural or synthetic, that can be utilized by microorganisms. The
contaminant concentrations may range from quite high to extremely low. Simultaneous metabolism or utilization of several contaminants at the same time, is affected by the interaction of the contaminants (Schut et al. 1995, Wang et al. 1996). Many cases in which one substrate enhances the rate of biodegradation of a second compound have been reported (Alvarez and Vogel 1991, Hendriksen et al. 1992, Hess et al. 1990, Millette et al. 1995, Ogunseitan and Olsen 1993). In contrast, the presence of one contaminant can inhibit the degradation of a second contaminant (Steffensen and Alexander 1995). The stimulating effect of one contaminant to biodegrade another might result from the greater population size or biomass arising due to additional C source. The inhibiting effect might be due to: (1) toxicity of the second contaminant or its degradation product, (2) competition for limiting nutrients or electron acceptors among microbes, or (3) grazing of degraders by enriched protozoa (Alexander 1999).

2.3.1.6 Environmental characteristics

Environmental conditions play a pivotal role in determining biological activity, whether of indigenous microorganisms, cultured indigenous microbes returned to the soil, or exogenous microorganisms introduced to soil. These conditions are classified into two categories: those that reduce the microbial activity such as temperature, humidity, and ionic strength; and those that restrict the mass transfer of the compound to the microorganisms such as clay and organic matter content (Ralebitso et al. 2002).

The temperature of the environment is an important factor governing microbial activity and some physical of compounds. The optimum temperature for biodegradation of contaminants in temperate climates is generally in the range of 20 to 30°C (Atlas 1992).
However, the metabolism of microorganisms can be adapted to work and function optimally at low temperatures (Gounot 1991, Margesin and Schinner 1994, Russell 1990, Whyte et al. 1996), and there are reports of microbial degradation at low temperatures. Those reports include mineral oil degradation at temperatures below 0°C (ZoBell 1973), biodegradation activities of indigenous soil microorganisms at 10 to 16°C from an oil spill in Alaska (Prichard et al. 1992), and the elevated biodegradation of diesel oil by inoculation of cold-adapted indigenous microorganisms in alpine soils under laboratory conditions (Margesin and Schinner 1997).

According to Norris et al. (1994), the redox potential must be greater than 50 millivolts for optimal aerobic condition. Christensen et al. (2000) demonstrated that the redox environment forms the boundaries for attenuation of many compounds. The activities of degrading microorganisms are often restricted to certain redox environments, and degradation processes occur at different rates in different redox environments.

2.3.2 Conditions for successful biodegradation by microorganisms

Because microorganisms are frequently the major cause of contaminant degradation, the absence of a microorganism from a particular environment, or its inability to function, often means that the compound disappears very slowly. However, other factors also are involved and Alexander (1999) has summarized conditions that must be satisfied for successful biodegradation to take place in an environment. The conditions are: (1) organism(s) with proper metabolic activity for the biodegradation of a compound must exist in the environment containing the chemicals, (2) the chemical to be degraded by microorganisms must be exposed to the organism having the requisite enzymes, (3) the
functional groups of the compound to be degraded must be exposed if the biodegradation
is extracellular, (4) molecules must be transported, either actively or passively, to the
internal sites of the cells where the enzyme acts if degradation occurs by intracellular
enzymes, and (5) since biodegradation, is a result of microbial activity, environmental
conditions must be favorable for microbial growth and activity.

If any of the conditions mentioned are not satisfied, it is likely the chemical
contaminant will remain undegraded in the soil. It is not certain, at present, how many
compounds persist in the environment because of the complete absence of species having
the capacity to bring about degradation or the conditions not favorable for microbial
biodegradation (Alexander 1999).

2.3.3 Methods used for enhanced biodegradation

2.3.3.1 Enhanced degradation by increasing bioavailability of pollutants

A possible way to enhance bioavailability and, hence, biodegradation of organic
contaminants is by increasing the surface area of hydrophobic, water-insoluble substrates.
In hydrocarbon degradation, it essential for bacteria to come in direct contact with the
hydrocarbon substrates, which are usually hydrophobic. Therefore, it is not surprising that
bacteria growing on hydrocarbons such as petroleum usually produce potent emulsifiers.
A remediation strategy to enhance contact between bacteria and water-insoluble
hydrocarbon is the addition of emulsifier to soil. These molecules are consist of both a
hydrophilic and hydrophobic part, and are often called biosurfactants.
Bacterial biosurfactants can be classified into two types - low molecular and high molecular weight biosurfactants. There have been numerous reports on biosurfactants produced by microorganisms (Abraham 1998, Navon et al. 1995, Rosenberg and Ron 1998, Lang and Wullbrandt 1999), and their role in enhancing bioremediation (Golyshin et al. 1999, Lang and Wullbrandt 1999, Rosenberg et al. 1999, Zhang and Miller 1994).

The net effect of a surfactant on biodegradation depends on the benefits that result from enhanced solubility of target compounds versus the problems caused by a reduction in the adhesion of bacteria to those compounds. There is certainly a need to design an optimal surfactant/biodegrader/target environment combination and to further unravel the underlying complex interactions.

2.3.3.2. Improved biodegradation by augmenting species richness

There are different strategies for increasing the catabolic activity of soil including adding specific microbial strains, introduction of specific mobile genetic elements into microbial strains to enhance biodegradation, or encouraging the activity of highly diverse microbial communities directly in natural environments. Several tests have shown a positive effect of seeding strains on the degradation of certain compounds in soil. The addition of any species capable of living under the given environmental conditions will give rise to increased biodiversity of the soil for days to months. According to the concept of the carrying capacity of microbial communities, the added populations will generally stabilize at $10^3$ cfu/g soil (Postma et al. 1990, Vandenhove et al. 1991, Vandepitte et al. 1995). Hence, inoculation is a valuable approach to broadening the biodegradation potential of soil.
Genetic information can also be transferred from an introduced donor strain to well-established and competitive indigenous bacterial populations of soil. An advantage of this approach is its independence of the long-term survival of the introduced donor strain, which is often the major bottleneck in bioaugmentation processes (Akkermans 1994).

The third strategy is the introduction of an unspecified group of bacteria such as those present in soil, sludge, manure or compost (Barbeau et al. 1997, Mikesell and Boyd 1988). These materials normally contain a high diversity of microorganisms in which the species necessary to destroy the pollutant might be present. Addition of these materials to bioaugment degradation may occur without adaptation to the pollutant because an interaction between different microorganisms may result in improved removal of the contaminant. Moreover, in such samples, the cooperating species might be optimized relative to one another. Using this approach eliminates the difficult task of isolating and characterizing a specific bacterium able to degrade a specific compound. The key question is how to add these strains or microbial associations in a way that achieves the most positive effect.

2.4 **IN SITU BIOREMEDIATION**

The term “bioremediation” has been used to describe the process of “purposefully” using microorganisms to degrade or remove from the environment hazardous components or wastes (Glazer and Nikaido 1995). Bioremediation is a resilient and adaptable technology that can be used with a surprising range of treatment approaches to improve removal efficiency and reduce the life cycle cost of a treatment project (Brown et al. 1999).
Although the use of bioremediation in the treatment of hazardous waste is a relatively new concept, it is a rapidly growing technology in environmental management, and there have been numerous reports on the application of bioremediation of contaminated sites (Middeldrop et al. 1990). Examples of bioremediation include land farming, composting, bioreactors, bioventing, biofilters, bioaugmentation, biostimulation, intrinsic bioremediation, and pump and treat (Boopathy, 2000).

Treatment of contaminated sites rather than disposal is increasingly being emphasized in most industrialized nations since 1970. One factor in the development of bioremediation has been the enhancement of environmental laws and regulations that favor waste treatment rather than waste disposal (Caplan, 1993). Bioremediation has numerous applications including cleanup of soils, waters, lagoons, sludges, and process-waste streams. The shore-line cleanup efforts in Prince William Sound, Alaska, after the Exxon-Valdes oil spill is a good example of large-scale application of bioremediation. At this site, the U.S. Environmental Protection Agency (EPA) and Exxon Company demonstrated effectiveness of bioremediation technology on oil-contaminated beaches (ADEC et al. 1990).

A number of bioremediation strategies have been developed to treat contaminated wastes and sites. Selecting the most appropriate strategy to treat a specific site can be guided by considering three basic principles: (a) the amenability of the pollutant to biological transformation to less toxic products (biochemistry), (b) the accessibility of the contaminant to microorganisms (bioavailability), and (c) the opportunity for optimization of biological activity (bioactivity).
2.4.1 Processes and systems used in bioremediation (types of bioremediation)

Based on the place where the contaminated materials are treated, bioremediation technologies can be broadly classified as ex situ or in situ. Ex situ technologies refer to treatments that remove contaminants at a separate treatment facility, while in situ bioremediation technologies is the term used for treatments of contaminants in the place itself.

In situ bioremediation, especially by indigenous microbial population, is one of the most attractive features of bioremediation of sites containing readily degradable contaminants. For more recalcitrant compounds, bioaugmentation with adapted or specially designed microbial inoculants is a useful alternative (Vogel 1996). In situ bioremediation processes currently being utilized in the field are classified into three categories: bioattenuation, biostimulation, and bioaugmentation.

Biostimulation, the artificial creation of an environment that promotes the growth of naturally occurring microorganisms capable of degrading the target contaminants, is the method in which biodegradation by indigenous microorganisms is stimulated and the reaction rates are increased. This option is adopted when there are indigenous microbes with degradation capacity but natural degradation does not occur or the degradation is too slow. Biostimulation includes supplying the environment with nutrients such as carbon, nitrogen, phosphorus, or other substrates.

Bioaugmentation is a way to enhance the biodegradative capacities of contaminated sites by inoculation of microorganisms with the desired catalytic capabilities. Bioaugmentation is discussed later in detail in section 2.5.
There is another type of bioremediation, which is called bioattenuation (i.e., intrinsic bioremediation by indigenous microorganisms). This is a natural process of degradation, without stimulating indigenous microbial population or inoculation of exogenous microorganisms. Bioattenuation is widely used in the United States as a clean up method for petroleum-contaminated soil and groundwater at underground storage tank sites (Dojika et al. 1998). However, this option is not generally included in the category of \textit{in situ} bioremediation.

2.4.2 Advantages and disadvantages of bioremediation

Bioremediation, which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention as effective biotechnological approach to clean up a polluted environment. Bioremediation offers several advantages over the conventional chemical or physical technologies, especially for diluted or widely spread contaminants. These treatment methods have generally been found to be advantageous compared to other treatment methods in the following aspects (Brown et al. 1999, Iwamoto and Nasu 2001): (1) The intensity of the process can be adjusted from highly aggressive to passive, (2) the incremental costs for adding a bioremediation component to most types of treatments is lower than other options, (3) the process can be applied to a wide range of purposes, from mass removal to formation of a migration barrier to final polishing, (4) it can be done on site with minimal site disruption, and (5) it can be applied to diluted and widely diffused contaminants.

Although bioremediation is a generally accepted technology for removal of contaminants from the environments, bioremediation has its limitations and it is still an
immature technology. Some chemicals, (i.e., heavy metals, radionuclides, and some chlorinated compounds) are not amenable to biodegradation. It also is not always possible to obtain complete contaminant removal because there might be a threshold concentration below which rates of biodegradation are slow or negligible (Allard and Neilson 1997). Bioavailability of pollutants may decrease as biodegradation proceeds, and thus recalcitrant compounds may persist. Secondary effects that develop during bioremediation is also must be considered. Biodegradation sometimes generates products with higher toxicities than the parent molecules (Bradley 2000).

Bioremediation frequently must address multiphasic, heterogeneous environments such as soils, in which the contaminant is present in association with the soil particles, dissolved in soil liquids, and in the soil atmosphere. Because of these complexities, successful bioremediation is dependent on an interdisciplinary approach involving disciplines such as microbiology, engineering, ecology, and chemistry (Boopathy 2000).

Our current knowledge of changes in the microbial communities during bioremediation is limited and the microbial community in natural environments is still treated as a “black box. The reason for this is that many environmental microorganisms cannot yet be cultured by conventional laboratory techniques. Therefore, attention has to be paid to the application of bioremediation, especially bioaugmentation, because of its unknown effects on the ecosystem. This has led to two essential questions related to bioremediation in the field: (1) how to clarify the biological contribution to the effectiveness of bioremediation, and (2) how to access the environmental impact of bioremediation (Iwamoto and Nasu 2001).
2.5 BIOAUGMENTATION

Bioaugmentation, in general, is defined as the application of indigenous and exogenous wild-type, or genetically modified organisms to polluted sites or bioreactors in order to accelerate the removal of undesired compounds. There have been numerous reports on feasibility and field application of bioaugmentation as a remediation technology (Vogel 1996). Microorganisms are thought to be ubiquitous and bioremediation often uses naturally occurring indigenous microorganisms. The addition of supplemental inoculum consisting of either indigenous or non-indigenous microbes can enhance the diversity of the indigenous population and thus increase the degradation rate of target compounds. This is necessary when no microorganism capable of degradation of target compounds exists in the natural community, or when the activity of the natural community is inhibited.

2.5.1 Types of bioaugmentation based on the origin of microorganisms

2.5.1.1 Use of isolated and cultured microorganisms

Successful efforts have been reported to isolate microorganisms from natural environments with degradation activity against various chemicals (Ashok et al. 1995, Haugland et al. 1990, Horvath et al. 1990, Kharoune et al. 2001, Parekh et al. 1994, Qureshi and Purohit 2002, Rousseaux et al. 2001, Saber and Crawford 2003, Smith-Grenier and Adkins 1996). Application of isolate(s) to bioaugment degradation of contaminants, in either laboratory or field-scale, has also been reported (Baud-Graset and Vogel, 1995, Colores et al. 1995, Newcombe and Crowley, 1999, Rousseaux et al. 2003, Struthers et al. 1998, Widada et al. 2002).
A high cell density was maintained up to 21 days after inoculation when *Pseudomonas resinovorans* strain CA10, at a $10^7$ cells/g dry soil, was introduced into soil treated with carbazole at an initial rate of 100 µg/g soil (Widada et al. 2002). In addition, almost all of the 2,3-dichlorodibenzo-\(p\)-dioxin (1 µg/g soil) was degraded within 14 days after inoculation of *P. resinovorans* strain CA10 at an even higher density of $10^9$ cfu/g soil. Mohn et al. (2001) documented that inoculation with cold-adapted, mixed microbial cultures stimulated hydrocarbon removal during the summer immediately following inoculation in Arctic tundra soils. Fast removal of 2,4,6-trichlorophenol (2,4,6-TCP) was observed in soils contaminated with 2,4,6-TCP and inoculated with *Alcaligenes eutrophus* TCP (Andreoni et al. 1998).

Topp (2001) demonstrated differences in the efficacy of atrazine-degrading bacteria for bioremediation of soil contaminated with atrazine. Although all three strains, *Pseudaminobacter*, *Norcardioides* and *Pseudomonas* were able to mineralize atrazine, only *Pseudaminobacter* and *Norcardioides*, at inoculum density of $10^5$ cells/g soil, accelerated atrazine dissipation. The author suggested that the ability to utilize atrazine as a carbon source is important to establish “enhanced degradation” at ecologically meaningful inoculum densities.

2.5.1.2 Use of directly enriched microorganisms

Direct enrichment of microorganisms can be accomplished by stimulating indigenous microorganisms to degrade a specific compound(s) in the natural media in which the microbes exist. Soil activation is an example of direct enrichment in which an acclimated consortium is produced that can degrade a specific compound (Otte et al. 1994).
Soil particles act as microniche for microorganisms protecting them from predators. Soil also provides nutrients as well as physical support for microbial growth. Direct enrichment has several advantages over isolating and culturing microorganisms. During direct enrichment, many species, which are not culturable but may be of importance in degradation of chemicals in natural environment, can be enriched. While the use of pure culture of isolated strains can be associated with the accumulation of partial degradation products which might be more toxic than parent materials, mixed consortia are more likely to completely degrade the target compounds. Mixed consortia have also been known to have the advantage of being more resistant to natural environmental conditions and predation (Fewson 1988) compared to pure cultures which often fail to generate the desired activities when released to the environments.

2.5.1.3 Use of genetically engineered microorganisms

Genetic engineering has been used to confer new functions to microorganisms and to enhance their catabolic activities. The microorganisms constructed are called genetically engineered microorganisms (GEMs). It also provides microorganisms with multiple metabolic activities in single strain, which is often required for degradation of specific pollutants. Bioaugmentation of contaminated sites with microbes that are genetically engineered for degradation of specific compounds is an area that is currently being explored as a cleanup option.

There have been a number of on GEMs designed for enhanced biodegradation and their application for bioremediation (Brierley 1985, Chen and Mulcandani 1998, Erb et al.)
Shao et al. (1995) reported that the recombinant *Rhodococcus* TE1, carrying the gene encoding *s*-triazine hydrolase from *Rhodococcus corallinus*, was capable of degrading atrazine to cyanuric acid. Trichloroethylene could efficiently be degraded by either a recombinant *E. coli* with the phenol hydroxylase gene transferred from *Pseudomonas putida* BH (Fujita et al. 1995) or a *P. fluorescens* strain with the toluene o-monooxygenase genes transferred from *Burkholderia* PR1 (Yee et al. 1998). Lange et al. (1998) reported that *Deinococcus radiodurans* with a *tod* gene encoding toluene dioxygenase was developed and successfully applied to the bioremediation of mixed wastes containing both radionucleotides and organic solvents. A genetically modified strain of *Pseudomonas fluorescens* HK44 was designed for the purpose of sensing environmental contaminants and signaling with bioluminescence. Use of this GEM as an online tool for *in situ* monitoring of bioremediation has been proved effective by field tests (Ripp et al. 2000, Sayler et al. 1999, Sayler and Ripp 2000).

Application of GEMs to environmental biotechnology has been very limited (Ripp et al. 2000, Sayler et al. 1999, Sayler and Ripp 2000, Van Veen et al. 1997) even though several laboratory studies have shown that GEMs could improve the efficiency of pollutant removal. Recently, a number of opportunities for improving degradation performance using GEMs for bioremediation have been described including their use in pure-culture systems (Erb et al. 1997; Timmis and Piper 1999). It has been suggested that field-scale application of GEMs may be adopted for *in situ* reduction of recalcitrant
organics in the subsurface, for use in bioreactors for remediation or waste treatment, for greenhouse gas control, for carbon sequestration, and for conversion of wastes to value-added products (Sayler and Ripp 2000).

However, there are two basic limitations with the use of GEMs in remediation. One limitation is ecological. A laboratory-developed strain must be designed to fit into the natural community. The ability of GEMs to survive in the environment is quite controversial with some studies reporting that GEM survival is inherently unpredictable (Giddings 1998). The other limitation comes from regulatory controls. The release of organisms developed by genetic engineering is subjected to worldwide regulation. Getting governmental approval is often a difficult and lengthy endeavor, and this regulation has made some researchers concentrate on the optimization and commercial development of naturally occurring microorganisms (Cha et al. 1999).

According to Drobnik (1999), gene transfer, one of the expected problems in the use of GEMs for remediation, may not be a serious limitation to their use because transgene formation is usually a result of intrinsic microorganisms and thus no new gene is added to the environment.

2.5.2 Factors affecting success of bioaugmentation

Although several releases of microorganisms into soil have been found to be successful, many failures or inconsistencies in achieving the objective have been reported as well (Akkermans 1994, van Elsas and Heijnen 1990). Factors that determine the efficiency, efficacy and cost of bioaugmentation include the nature of the microorganisms,
the nature of the xenobiotics, the physicochemical conditions and the metabolic potential of the microflora.

2.5.2.1 Competition and survival of the introduced microorganisms


The introduced strain may face intense competition, predation or parasitism in sewage, natural water or soil. Laboratory strains, especially genetically engineered microorganisms, have difficulty competing with the established microbial community (Goldstein et al. 1985). The grazing of protozoa is the main biological process that controls allochthonous bacterial density (Barcina et al. 1997). According to Blasco et al. (1997), accumulation of toxic intermediate or end-products of pollutants by members of indigenous bacteria also has negative effects on the survival of introduced microorganisms. The competition can be controlled by adding specific nutrients that the inoculant can utilize (Ogunseitan et al. 1991, Van Veen et al. 1997) or by changing operation parameters (Blumenroth and Wagner-Döbber 1998, Fujita et al. 1994). In addition, it has been reported that survival of bacteria added to soil was improved by pre-adaptation of the strains on a minimal medium with soil extract (Timmis 1997) or by starvation in an
inorganic medium (Van Elsas et al. 1994, Watanabe et al. 2000). However, good survival and growing capabilities of the incorporated strains do not always insure the breakdown of the xenobiotic (Lewis et al. 1986, McClure et al. 1991).

2.5.2.2 Ability of introduced microorganisms to move or distribute throughout the sites

Either direct or indirect contact between microbes and contaminant is a prerequisite for biodegradation by microorganisms. One of the serious limitations in the widespread application of bioaugmentation for remediation of contaminated sites is the inability of microorganisms to transport or move throughout the contaminated area. According to Harvey et al. (1989), more than 99% of bacteria injected into a sand gravel aquifer in Cape Cod, Mass. did not move to a sampling well located 1.7 m from the injection site. Improving microbial transport has been considered as an option for more effective bioaugmentation strategy, and various techniques have been studied as methods to improve microbial transport and distribution. Such techniques include the application of surfactants (Brown and Jaffe 2001, Gross and Logan 1995, Jackson et al. 1994, Kane et al. 2001, Lee and Logan 1999, Streger et al. 2002), the reduction of cell size by starvation (Lappin-Scott and Costerton 1992), and the development of adhesion- deficient variants (Deflaun et al. 1990, Deflaun et al. 1999, Streger et al. 2002). Gannon et al. (1991) demonstrated the relationship between cell surface properties and transport of bacteria through soil. Retention of the 19 strains tested in Kendaia loam was statistically related to cell size, whereas either transport or retention of these strains was not correlated with hydrophobicities, net surface charges of cells, or the presence of capsule. The presence of flagella was not correlated with transport, either. The authors suggest that more than one
characteristic of bacterial cells determines whether the organisms are transported through soil with moving water. According to Streger et al. (2002), the transport of the adhesion-deficient strain *Hydrogenophaga flava* ENV735:24 was dramatically improved in both Ottawa sand and in the Oyster sediment columns.

### 2.5.3 Examples of bioaugmentation applied for remediation of contaminated sites

#### 2.5.3.1 Remediation of hydrocarbon-contaminated soil

One of the major problems faced by oil refineries is the safe disposal of oily sludge generated during the processing of crude oil. Improper disposal of oily sludge leads to soil pollution and poses a serious threat to ground water. According to Mishra et al. (2001), chemical and physical properties of soil were significantly improved by inoculum addition to stimulate *in situ* bioremediation of oily-sludge-contaminated soil.

Chlorinated ethanes and ethenes are commonly used as cleaning solvents and in dry cleaning operations. Among chemicals belonging to these categories, trichloroethylene (TCE) has received the most attention because of its toxicity and the magnitude of its pollution. So far, microbes capable of utilizing TCE as energy source have not been isolated. However, it is well known that some microbes can degrade TCE via a special type of metabolism, called cometabolism. In cometabolism, microbes gratuitously metabolize TCE using the enzymes that are synthesized to degrade the primary substrate (Eweis et al. 1998). After Wilson and Wilson (1985) demonstrated cometabolism of TCE by methanotrophs, many other researchers reported similar results. Some representative microbes capable of cometabolizing TCE are methanotrophs.
(Oldenhuis et al. 1989), phenol oxidizers (Nelson et al. 1988), toluene oxidizers (Shields and Reagan 1992), ammonia oxidizers (Rasche et al. 1991), and propane utilizers (Wackett et al. 1989). Methanotrophs (i.e. the methane oxidizing bacteria) are a unique group of bacteria that utilize methane as their sole source of carbon and energy. Methanotrophs play an important role in the oxidation of methane in natural environments, such as wetlands and freshwater systems. Methanotrophs are considered important in trichloroethylene (TCE) bioremediation, and TCE bioremediation often employs methane injection to stimulate the TCE-degrading activity of indigenous methanotrophs.

Polychlorinated biphenyls (PCBs) are a group of manmade compounds composed of biphenyl molecules containing from one to ten chlorines. They are oily fluids with high boiling point, high chemical resistance, low electrical conductivity, and high refractive index. Because of these properties, they have been used mainly as insulators in electrical transformers and capacitors, as heat exchange fluids, and as plasticizers. Their toxicity, bioconcentration, and persistence have been well documented. PCB use and discharge of in the United States has been under government regulation since 1978. However, PCBs are still serious environmental pollutants globally since previously contaminated sediments, landfills, and older electric transformers still exist as sources of PCBs. Although PCBs are relatively resistant to biodegradation, it has been shown that a number of bacteria can cometabolize various PCB components (Khan and Walia 1989, Kimbara et al. 1989). Genes related to PCB catabolism have also been cloned and sequenced (Ahmad et al. 1990, Erickson and Mondello 1992, Furukawa et al. 1987, Furukawa and Miyazaki 1986, Hayase et al. 1990, Mondello 1989).
2.5.3.2 Waste and wastewater treatment

Sewage sludge has been used in agriculture as a valuable source of plant nutrients and organic matter in many countries. Inoculation of activated sludge systems with specialized bacterial strains has been explored as a possible tool to improve several aspects in wastewater treatment processes, such as improved flocculation and degradation of recalcitrant compounds. In some studies, degradative bacteria and mobile genes were introduced into activated sludge to enhance biodegradation of xenobiotics (McClure et al. 1991, Nußlein et al. 1992, Selvaratman et al. 1997). However, in spite of several successes of small-scale tests involving inoculation of activated sludge and other waste-treating bioreactors, this technique is not yet widely applied, despite its low cost. This is partly because the inoculation of activated sludge to enhance pollutant removal is less predictable and controllable than direct physical or chemical destruction of the pollutants (Boon et al. 2000).

2.5.3.3 Pesticide detoxification

Pesticides are indispensable tools for the control of weeds, insect, and fungi in agriculture, and there are approximately 1290 registered active ingredients for pesticides in the U.S.A. (40 Code of Federal Regulations (CFR) 152.25 (g)(1), US EPA). In 1995, approximately 500 million kilograms of pesticides were applied in the U.S.A. One of the most important features pesticides should exhibit is that they persist only long enough to control the target weeds or pest(s). In reality, many pesticides are known to be either recalcitrant or biodegrade very slowly, and consequently pesticide contamination in soil, surface water and ground water has long been a concern. According to the data developed
by the U.S. Environmental Protection Agency (US EPA), 74 pesticides were found in ground water in 38 states (Ritter, 1990).

Pesticide contamination results from normal agricultural use and/or point source misuse. High pesticide concentration in the soil environment occurs from spillage during pesticide mixing, loading, and rinsing at dealerships and sprayer fill sites, and from direct dumping of pesticides into disposal sites. Pesticides in soils can further contaminate surface and ground water depending on their properties of persistence, mobility, and interaction with soils.

The pesticides that have been of concern include pentachlorophenols (PCP), \(s\)-triazines (primarily atrazine), carbamates and organophosphates (Kumar et al. 1996). These compounds are widely used, toxic, and highly persistent in the environment. Conventional landfills and incineration have been used for detoxification of pesticide-contaminated sites. However, because of the problems associated with the conventional methods, e.g. leaching and emission of toxic gases, there has been increasing interest in the use of microorganisms with pesticide degradative capacity.

Chen and Mulchandani (1998) reviewed detoxification of pesticides using microorganisms, either natural isolates or genetically engineered strains. Natural isolates of \textit{Arthobacter}, \textit{Mycobacterium}, \textit{Flavobacterium}, \textit{Pseudomonas} and \textit{Rhodococcus} have been used for PCP degradation in bioreactors (McAllister et al. 1996). Microorganisms used for \(s\)-triazine degradation include \textit{Klebsiella terragena} strain DRS-I (Hapeman et al. 1995), the mixed culture of \textit{Rhodococcus corallinus} and \textit{Pseudomonas} sp. D,
Rhodococcus rhodocorous SL1 (Arnold et al. 1996), and Acinetobacter junii WT1 (Feakin et al. 1995).

There are four classes of carbamates used as pesticides – methyl carbamates, thiocarbamates, phenyl carbamate, and dithiocarbamates. Pure bacterial cultures capable of degrading the first three classes of carbamates have been reported (Mulbry and Kearney 1991). The enzymes involved in degradation of carbamates have been purified from a soil isolate of Pseudomonas alcaligenes (Marty and Vouges 1987) and Achromobacter sp. (Karns et al. 1986). However, there is no report of the use of either these enzymes or bacteria for carbamate degradation in bioreactors. Organophosphates are known to be susceptible to hydrolysis by bacterial enzymes, thus resulting in fewer toxic products. The use of Pseudomonas putida and Pseudomonas sp. A3 as bioaugments for degradation of organophosphates in bioreactors has been reported (Lakshmi and Lalithakumari 1994, Ramanathan and Lalithakumari 1996).

Several studies reported pesticide detoxification using genetically engineered strains. Genes coding for the enzymes responsible for the initial steps of PCP degradation have been transferred form a Flavobacterium strain to Escherichia coli, and its expression and PCP degradation by the genetically engineered E. coli have been demonstrated (Orser et al. 1993). With respect to s-triazine degradation, the atrA gene of Rhodococcus strain TE1 that encodes the enzymes for N-dealkylation was introduced to Rhodococcus corallinus. The recombinant, however, was not found effective in enhancing biodegradation because of poor expression of the cloned gene (Shao and Behki 1994). On the other hand, it has been shown that the recombinant Rhodococcus, bearing the trzA
gene encoding the s-triazine hydrolase from *Rhodococcus corallinus*, efficiently dechlorinated the corresponding metabolites (Shao et al. 1995). In addition, a plasmid carrying both *atr* A and *trz* A genes were successfully transferred from *Rhodococcus* strains to *E. coli*, but expression of these genes at high levels was not successful (Shao et al. 1995).

In terms of carbamate degradation, the structural *mcd* gene, encoding carbofuran hydrolase, has been isolated from a strain of *Achromobacter* and its expression in *Pseudomonas* and *E. coli* was found to be very low (Hauschild et al. 1990). Genetically engineered *Pseudomonas diminuta* and *Flavobacterium* ATCC 27551 has been shown to express plasmid-encoded organophosphorus hydrolase (OPH) (Mulbry and Karns 1989, Serdar et al. 1989, Serdar and Gibson 1985). High-level expression of OPH in the soil fungus has also been reported (Dave et al. 1994, Xu et al. 1996).

### 2.6 CURRENT RESEARCH INTEREST ON BIOAUGMENTATION

#### 2.6.1 Isolating or developing useful microorganisms

Conventional isolation methods have resulted in the isolation of only a fraction of the diverse population in the environment (Torsvik et al. 1990). Thus, natural microbial populations are much more diverse than those expected from the catalog of isolated microorganisms are. This is the same for pollutant-degrading microorganisms which implies that, in the natural environment, there may be a wide range of unidentified pollutant-degrading microorganisms that have high potential for bioremediation. Furthermore, most of the pollutant-degrading microorganisms isolated and characterized in the laboratory make only a minor contribution to bioremediation.
2.6.2 Preparation, preservation, and application of inoculum

To successfully apply bioaugmentation, a supplement must be prepared such that a sufficient number of microorganisms will become or remain viable in the environment and degrade the target contaminants. Preservation of inoculum in a readily usable form is required because it is generally not convenient or practical to cultivate the necessary quantity of microorganisms for application to the contaminated area or media prior to use. The requirements for successful inoculum include: (1) the ability to degrade the target contaminant, (2) competence to compete with the indigenous population for the necessary nutrients, (3) simplicity to use or distribute, (4) high stability during storage, and (5) low costs associated with its production, storage, and transportation (Romich et al. 1995).

Most widely used methods for inoculum preparation for bioaugmentation involve culturing or enriching microorganisms in the presence of target compound to acclimate microorganisms. However, it has also been found that cultivation of microbial supplement on the target substrate is not necessary. Instead other substrates can be used to stimulate the desired activity. For example, there was no difference in growth and degrading activity of a polychlorinated biphenyl degrader, *Alcaligenes eutrophus* on biphenyl whether it was grown on biphenyl or succinate (Romich et al. 1995).

Spray-drying, freeze-drying, and freezing have been evaluated as methods for preservation of inoculum of a pure culture of a biphenyl degrader (Romich et al. 1995). Among the three methods tested, freezing was found more effective in terms of microbial survivals and ability to grow on biphenyl compared with spray-drying and freeze-drying.
The ideal carrier material transfers the microorganisms without affecting their population or capacity to degrade chemicals. Therefore, inoculum carrier is one of the primary factors that affects bioaugmentation, and several different types of inocula carriers for biological control and bioaugmentation have been developed. Carriers include agricultural by-products, peat, wood chips, wheat straw, corncobs, commercial mushroom spawn, compost, granular vermiculite mixtures, grains, and alginate pallets (Cho et al. 1997, Wolter et al. 1997).

2.6.3 Monitoring changes in microbial communities

Microbial communities play an essential role in biogeochemical cycles and contribute to the maintenance of the ecosystem. Therefore, it is important to evaluate the impact of the introduced microorganisms in natural ecosystems. Investigating the effect of bioaugmentation on the microbial community is indispensable to prove the safety of this in situ bioremediation technology (Iwamoto and Nasu 2001, Iwamoto et al. 2000, Nakamura et al. 2000). Therefore, several new molecular biological techniques for the rapid and specific identification of microorganisms within their natural environment have been used to identify, enumerate and isolate introduced and indigenous microorganisms with degradative potential (Erb and Wagner-Döbber 1993, Fode-Vaughan et al. 2001, Matheson et al. 1997).
CHAPTER 3

USE OF ACTIVATED SOIL TO BIOAUGMENT EPTC DEGRADATION IN SOILS

3.1 ABSTRACT

Bioaugmentation, an option to bioremediate contaminated sites by introduction of exogenous microorganisms, is becoming more common. Success of bioaugmentation largely depends on the introduced microorganisms’ survival and activity as well as the properties of chemicals to be degraded and the ability of microorganisms to degrade that specific compound. EPTC (s-ethyl-\(N,N’\)-dipropylthiocarbamate) is one of the world’s most widely used herbicides. The effectiveness of ACTIVATED soil as an inoculant to bioaugment the remediation of EPTC-contaminated soils was investigated. EPTC-degrading microorganisms were first enriched in a Brookston clay loam soil (Typic Argiaquoll), and the soil was then stored in an air-dry state at room temperature for 14 years. After the soil was rewetted and incubated for 7 days, a single treatment of 20 mg EPTC/ kg soil caused the number of EPTC-degraders to increase by about 3 logs of magnitude. Inoculation of EPTC-contaminated soils with this ACTIVATED soil at a rate as low as 0.05% resulted in acceleration of the EPTC decontamination process similar to the effect when soils were inoculated with EPTC degrading microbial strain \textit{Rhodococcus}
sp. TE1 at $10^8$ cfu/g soil. The ACTIVATED soil was stable, with respect to its ability to degrade EPTC, up to at least 6 months when stored at temperatures below $10\,^\circ\text{C}$. This study confirms that EPTC-degrading microorganisms are readily enriched in soil by treatment of EPTC, and that use of this ACTIVATED soil to bioaugment remediation of EPTC-contaminated soils is effective.

3.2 INTRODUCTION

Pesticides are indispensable tools for the control of weeds, insects, and fungi in agriculture. It is known that aerobic microbial degradation is a major pathway of dissipation of organics including various pesticides. Unfortunately, some pesticides are quite persistent in the environment. For example, once pesticides reach the deep soil or groundwater, their potential to be degraded is much less due to low oxygen concentration and transportation limitations. Therefore, there has been considerable interest and need to develop low-cost remediation practices that reduce or remove pesticides from soils before they reach such environments.

Currently, methods for detoxifying pesticides depend largely on incineration and landfills. Incineration, a method approved by the U.S. EPA, has experienced serious public opposition because of emissions of potentially toxic volatile and particulate materials. It is also very costly because it requires large amounts of energy to reach the high temperatures needed to destroy the pollutants. Landfills are effective in the short term, but leaching of pesticides into surrounding soil and groundwater is a concern. Bioremediation of contaminated sites, based on use of either indigenous or exogenous
microorganisms capable of degrading specific pollutants including pesticides, is becoming more common.

EPTC (s-ethyl-\(N,N'\)-dipropylthiocarbamate) is one of the most widely used carbamothioate herbicides in North America for the control of weeds in corn (\textit{Zea mays} L.) and soybean (\textit{Glycine max} L.) fields. Although carbamothioate herbicides can be dissipated via metabolism by mammals, plants, and microbes (Fang, 1969), photolysis (Konstantinou et al. 2001) and volatilization and sorption (Elker 1988, Singh et al. 1990a, 1990b), microbial degradation is thought to be the major route of their dissipation in the environment. There have been numerous reports of accelerated degradation of EPTC in natural environments (Ankumah 1988, Dick et al. 1990, Roeth et al. 1989, Skipper 1990) and several species capable of degrading EPTC, largely confined to the genus \textit{Rhodococcus}, have been isolated from soils either with or without history of carbamothioate application (Anthony et al. 1987, Assaf 1991, Ankumah et al. 1995, Behki and Khan 1990, Behki et al. 1993, Dick et al. 1990, McClung et al. 1994, Nagy et al. 1995, Tal and Rubin 1993). Unidentified microorganisms with EPTC degradation activity have also been reported (Moorman 1988, Mulbry and Kearney 1991, Wang 2002).

Microbial degradation pathways of EPTC include sulfoxidation (Casida et al. 1974), hydroxylation of either the \(\alpha\)-carbon of the ethyl group or one of the \(\alpha\)-carbons of the \(N,N'\)-dipropyl moiety (Chen and Casida 1978), and direct hydrolysis without hydroxylation (Fang 1969). McRae and Alexander (1965) reported that EPTC detoxification occurs without appreciable mineralization of the ethyl-moiety group. Kaufman (1967) demonstrated that the \(^{14}\text{CO}_2\) evolution from ethyl-moiety-\(^{14}\text{C}\)-EPTC
applied to soil occurred relatively slowly. According to Dick et al. (1990), hydroxylation and sulfoxidation are possible pathways of EPTC degradation by *Rhodococcus* sp. strain JE1, and the α-propyl hydroxylation was thought to be the dominant pathway of EPTC degradation. Proposed microbial degradation pathways of EPTC by soil isolate JE1 are summarized in Figure 3.1.

Genetic information related to EPTC degradation by microbial isolates has partly been elucidated. In some bacteria, the EPTC degradation is associated with an indigenous plasmid (Assaf 1991, Anthony et al. 1987, Behki et al. 1993). Plasmids are mobile genetic elements, and this fact opens up the possibility of rapid genetic transfer of EPTC-degrading genes among soil organisms, thus increasing degradation potential. A 6.2-kb *KpnI* fragment from a plasmid of EPTC-degrading *Rhodococcus* sp. strain TE1 contains both the *eptA* gene, encoding the enzyme required for EPTC degradation, and the *atrA* gene involved in atrazine *N*-dealkylation. In addition, the enzymes involved in degradation of carbamates have been purified from soil isolates of *Pseudomonas alcaligenes* (Marty and Vouges 1987) and *Achromobacter* sp. (Karns et al. 1986). A single cytochrome P-450 system has been found to be involved in degradation of the herbicide EPTC and atrazine by *Rhodococcus* sp. strain NI86/21 (Nagy et al. 1995).

Enhanced degradation of carbamate herbicides in soils with a history of application of the same or similar compounds implies that the activity of carbamate degraders or their biomass has been increased. This also implies that the microorganisms involved in degradation of carbamothioates can be enriched even in natural media such as soil. It is highly likely, however, that not all species in natural environments, involved in
Figure 3.1. Proposed pathways for the metabolism of EPTC by microbial isolate JE1. (Redrawn from Dick et al. 1990)
carbamothioate degradation, can be cultured. This is because only a small percentage (≈ 0.1 – 5%) of the species in a typical soil can be isolated using current culture-based methods (Alexander 1977, Klung and Tiedje 1994, Torsvik et al. 1990).

Isolation of microorganisms from soils may require excessive time and effort, or not be successful at all. Thus, using soil, in which indigenous degraders of a pesticide have been enriched, as an inoculant has advantages in time and economics. In addition, soil provides nutrients and supporting materials for microorganisms as they are introduced into new environments. Microorganisms carried in soil are protected from predation by other organisms in the environment.

The specific objectives of this study were: (1) to investigate the possibility of direct enrichment of EPTC-degrading microorganisms in soil, (2) to evaluate the effect of inoculation of soils with directly enriched (i.e. ACTIVATED) soil to bioaugment the degradation of EPTC, (3) to test the effect of nutrient amendment on EPTC-degradation in soils, and (4) to find storage conditions for ACTIVATED soil containing enhanced number of EPTC-degrading microorganisms.
3.3 MATERIALS AND METHODS

3.3.1 Chemicals and soils

Standard-grade EPTC (s-ethyl \(N,N\)-dipropylcarbamothioate) was purchased from Chem Service, Inc. (West Chester, PA). Pesticide-grade toluene used for the extraction of residual EPTC was obtained from Sigma (St. Louis, MO).

A Brookston clay loam (Soil I of Table 3.1) was sampled from an experimental field near Canal Winchester, Ohio. This soil had been previously treated with Eradicane (EPTC + antidote, 2,2-dichloro-\(N,N\)-di-2-prophenylacetamide) for four consecutive years at field application rate (approximately 3.5 kg active ingredient/ha). The Brookston soil was collected in 1986 and then stored in an air-dried state at room temperature for 14 years without additional treatment for storage (for more information on this soil, refer to Ankumah 1988). Other surface (0-20 cm depth) soils were obtained with different textures, pH values, organic matter content and land use. A Wooster silt loam (Soil II) and a Fremont sandy loam (Soil III) were sampled from agricultural areas near Wooster and Fremont, Ohio, respectively. These soils may have received occasional pesticide applications, but their pesticide application history is not known. A forest soil (Soil IV) was collected from Mohican State Park, Ohio, and had no known history of pesticide application. Soil V (a Muck soil, Typic Histosol) was obtained in 2000 from the Muck branch of OARDC at Celeryville, Ohio, and stored at greenhouse without further treatment.

Each sample (approximately 30 kg total) except for the Brookston soil (Soil I) was collected in 2000 using a spade to a depth of 20 cm and combining soil from closely
This was a Brookston clay loam soil used to prepare the ACTIVATED soil that was subsequently used as inoculum to bioaugment remediation of EPTC-contaminated soil.

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<tr>
<th>Properties</th>
<th>Soils</th>
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<td>Soil I</td>
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<td>Sand (%)</td>
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<td>Particle Size Composition (%)</td>
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<td>Stilt (%)</td>
<td>36.0</td>
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<td>Clay (%)</td>
<td>33.0</td>
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<td>pH</td>
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<tr>
<td>Organic Matter (%)</td>
<td>7.63</td>
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<tr>
<td>Total Nitrogen (%)</td>
<td>0.345</td>
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* This was a Brookston clay loam soil used to prepare the ACTIVATED soil that was subsequently used as inoculum to bioaugment remediation of EPTC-contaminated soil.

Table 3.1. Selected properties of soils used to evaluate EPTC degradation in soils.
spaced sampling points. The soils were partially air-dried (to a moisture content of approximately 10%, w/w), passed through a 2-mm sieve, and then stored in a cold room (10 °C). Soil texture was analyzed by the hydrometer method (Buoyoucos 1962). Total organic matter was analyzed using methods described by Combs and Nathan (1998), and total nitrogen was determined using a Carbon/Nitrogen Analyzer, Model MAX-CN from Elementar Americas, Inc. (Mt. Laurel, NJ). Soil pH was measured by the glass electrode method (McKeague 1978) in a 1/1 (w/v) soil/water suspension. Selected properties of soils used for EPTC degradation are summarized in Table 3.1.

3.3.2 Application, extraction and determination of EPTC

For preparation of artificially EPTC-contaminated soils, EPTC (water solubility equal to 375 mg /L at 25 ± 2 °C) was applied to soil as follows. Aqueous standard-grade EPTC solution (200 mg /L) was sprayed onto soil at a ratio of 1:10 (v/w) while thoroughly mixing with a spatula. Final concentration target was 20 mg EPTC/kg soil. For the experiments in which higher concentrations of EPTC were desired, a commercial formulation of EPTC (i.e. Eptam) was purchased from a local farmer coop and diluted to give approximately 100 mg EPTC/mL, and serial dilution was then made prior to application soil as described above. The concentration of active EPTC in the commercial formulation was checked using a gas chromatographic method as described below.

The EPTC concentrations in soil were determined as follows. Eight mL of distilled water was added to 10 g soil contained in a 125-mL high-density polyethylene (HDPE) bottle, and the contents in the bottle were vortexed for one min to disperse soil particles. Ten mL of pesticide-grade toluene was added and the bottle was shaken on a horizontal shaker at
120 strokes per min for one hour. The mixture was transferred to a Teflon tube and centrifuged at 8,000 x g for 10 min. After centrifugation, a 2-mL portion of the toluene layer was transferred to a 5-mL glass vial and stored at 4 °C until analyzed. Tests indicated that samples could be stored for several months in the glass vials without change in EPTC concentration.

To extract EPTC from microbial culture used for MPN determination, 0.8 mL aliquots of the culture were transferred to Eppendorf tubes, and an equal volume of pesticide-grade toluene was added. After mixing on a vortex for one min, the mixture was centrifuged at 15,000 rpm (≈ 10,000 x g) for 10 min, and 0.5 mL of the toluene layer was transferred to a 5-mL glass vial and stored at 4 °C until analyzed.

The amount of EPTC in the extracts was determined with a Varian 3700 gas chromatograph equipped with a 3% OV-1 on 100/200 Supelcoport packed column (3 m in length) and a nitrogen-specific thermionic detector. Helium was used as carrier gas at a flow rate of 28 mL/min. Temperatures were set at 210 °C for injector, 160 °C for column, and 250 °C for detector. EPTC standards were made by dissolving standard-grade EPTC (Chem Services Inc., West Chester, PA) in pesticide-grade toluene. EPTC concentrations in toluene were calibrated by regression equation between EPTC concentration and peak area of the chromatogram provided by the instrument integrator.

The retention time for EPTC at the above given condition was 1.60 min, and the detection limit for EPTC was approximately 0.1 mg/kg soil. The average recovery rate of EPTC, measured for sterile soils spiked with EPTC at various rates (20, 200, and 2000 mg/kg soil) and placed at room temperature for 24 hours, was between 91 and 96%. The
average recovery rate of EPTC from liquid culture containing dead cells of TE1 (cell density was 1.0 as OD_{600}) was 97%.

3.3.3 Degradation rates of EPTC in soils

In this chapter, the term “degradation of EPTC” is defined as disappearance of EPTC, based on extractable EPTC remaining in the soil. The amount of EPTC biodegraded was calculated by subtracting the amount of EPTC remaining in non-sterilized soil from that of sterilized soils.

Degradation of EPTC in soils was measured as follows. A batch of each soil was treated with EPTC at different rates (20, 200, or 2,000 mg/kg soil) by the method described in the previous section, and then the moisture content of the EPTC-treated soil was adjusted to 25% (w/w) with sterilized water. Each soil was divided into 10 g (as dry weight) subsamples and put into 125-mL high-density polyethylene (HDPE) bottles. The bottles containing the treated soil were incubated in a temperature-controlled (25 ± 2 °C) incubator. Triplicate samples were randomly selected at various times of incubation, and EPTC remaining in soil was extracted and analyzed as previously described (section 3.3.2).

The rates of EPTC degradation in soils were compared by plotting the percentage of EPTC remaining in soils vs. incubation time. The averaged amount of EPTC extracted from triplicate samples at the very beginning of the incubation (i.e. at time zero) was regarded as 100%. For test of significant statistical difference in EPTC degradations (as measured by percent recovery of EPTC) in soils, Analysis of Variance (ANOVA) was conducted for each individual incubation time.
3.3.4 Creation and evaluation of ACTIVATED soil

ACTIVATED soil was prepared by direct enrichment, or acclimation, of EPTC-degrading microorganisms in soil. The Brookston clay loam soil (Soil I of Table 3.1) was used as the source of EPTC-degrading microorganisms. As described previously, this soil was collected from an experimental field with history of four successive annual applications of Eradicane (EPTC + antidote, 2,2-dichloro-\(N,N\)-di-2-prophenylacetamide) at normal field application rates. The soil was stored at room temperature for 14 years. The soil was initially treated by adjusting moisture content to 25% (w/w) with distilled water and placing it at room temperature (20 – 25 °C) for 7 days. This soil was then additionally treated three times with EPTC at an initial concentration of 20 mg/kg soil, and the soil was incubated at 25 ± 2 °C. EPTC was allowed to dissipate until no further decrease was observed before the next EPTC treatment was applied. The concentration of EPTC in soil was monitored as previously described, and the number of total or EPTC-degrading bacteria were enumerated by the most probable number (MPN) method described in the following section. At the end of the third treatment, the moisture content was adjusted again to obtain a water content of 25% (w/w) and the ACTIVATED soil was stored in a refrigerator (4 °C).

The ACTIVATED soil containing enriched numbers of EPTC degrading microorganisms was treated with EPTC at different initial concentrations (20, 200, or 2000 mg/kg), and the degradation of EPTC in this ACTIVATED soil was monitored by the method previously described (section 3.3.3) for evaluation of ACIVATED soil. Statistical comparison were made for EPTC degradation (as measured by EPTC
recovered) in the ACTIVATED soil. Comparisons were made to test for differences in EPTC degradations at the 20, 200, and 2000 mg/kg EPTC application rates for each individual incubation time.

3.3.5 Most probable number (MPN)

The number of total or EPTC-degrading microorganisms in soils was determined based on the most probable number (MPN) method modified from Alexander (1982). A 10 g soil sample was suspended in 100-mL of sterilized water in a 250-mL Erlenmeyer flask and gently shaken for 20 min. Ten-fold dilutions were made by sequential transfer of 1 mL subsamples into 9 mL of each medium: TGYe (0.5% tryptone, 0.1% glucose, and 0.25% yeast extract, w/v) for total bacteria and BMNE (a basal minimal-salts-nitrogen medium amended with EPTC at 50 mg/L concentration) for EPTC-degrading bacteria. MPN tubes (5 replicates) were prepared for each of the 10-fold serial dilutions, and tubes were incubated at 25 ± 2°C for 5 days. Positive tubes for total bacteria were identified by visual turbidity and those for EPTC-degrading bacteria were determined by measuring EPTC remaining. The tubes containing less than 20% of the initial EPTC recovered were considered positive.

3.3.6 Inoculation of contaminated soil with ACTIVATED soil

Artificially EPTC-contaminated soils were prepared by applying EPTC to target soils (Soil II, III, IV, and V) at a rate of 20 mg/kg soil. Aqueous EPTC solution (200 mg/L) was applied as described in section 3.3.2. Then the EPTC-contaminated soil was inoculated with ACTIVATED soil at different rates (0.05, 0.5, and 5%, w/w), and the soil
was thoroughly mixed by stirring with a spatula. The mixture was divided into 10 g (as dry weight) subsamples and placed into 125-mL HDPE bottles and loosely-capped. These bottles were incubated in a temperature-controlled incubator (25 ± 2 °C). Triplicates samples were selected at various times of incubation, and the EPTC remaining in soil was extracted and analyzed as described in section 3.3.2.

The amount of EPTC recovered (determined as a percentage of EPTC initially added and recovered at time zero) vs. incubation time was plotted. For test of significant statistical difference among EPTC degradations (as measured by percent recovery of EPTC) in soils non-inoculated and inoculated with ACTIVATED soil at different rates, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each contaminated soil.

3.3.7 Bacterial culture and inoculation

The strain *Rhodococcus* sp. TE1 (formerly known as *Arthrobacter* sp. strain TE1, Tam et al. 1987), an isolate from a soil exposed to EPTC, was used in order to evaluate the effects of inoculation with pure cultured microorganisms on EPTC degradation in soils. This strain is known to degrade EPTC effectively and to grow on EPTC as its sole carbon source (Behki and Khan 1990).

*Rhodococcus* sp. TE1 was grown in a basal mineral salts-nitrogen medium (BMN, Behki and Khan 1986) amended with 50 mg EPTC per liter as a sole carbon source (herewith called BMNE). The composition of BMN is as follows. Each liter of medium contains 0.1 g NaCl, 0.2 g MgSO₄·7H₂O, 0.025 g CaCl₂·2H₂O, 0.0025 g FeCl₃·6H₂O, 0.5 g NH₄NO₃, and 0.0125M phosphate buffer (pH 7.0). Cultures were continued by
transferring a 100 µl of culture to 10 mL of fresh BMNE medium every two weeks and incubating at 28 °C on a shaker (120 strokes/min). A growth curve was used to determine the growth state of *Rhodococcus* sp. TE1 so that harvest of cells could occur during the log phase of growth.

Plate counting was used for enumeration of the cultured *Rhodococcus* TE1 prepared for inoculum. *Rhodococcus* TE1 culture was grown in BMNE and harvested at mid log phase by centrifugation. The cells were resuspended and serially diluted in sterilized 0.0125M phosphate buffer (pH 7.2), and then plated on BMNE-agar (1%) medium. The colony-forming units (cfu) were counted at appropriate dilutions after 5-day incubation at 28 °C. The relationship between colony forming units of TE1 and optical density (OD$_{600}$) was established during this time.

*Rhodococcus* sp. TE1, cultured and harvested at its mid log phase by the method described above, was diluted in fresh BMN medium to yield a specified colony forming unit (cfu) concentration (based on the relationship between OD$_{600}$ and cfu previously established). The diluted *Rhodococcus* sp. TE1 culture was introduced into artificially EPTC-contaminated soil at a 1:10 ratio of culture:soil (v/w). Degradation of EPTC, initially added at a rate of 20 mg/kg soil, was monitored by determining the EPTC remaining in soil after certain times of incubation at 25 ± 2 °C. The rates of EPTC degradation were compared by plotting the percentage of EPTC remaining in soil with EPTC recovery at time zero arbitrarily set at 100% vs. incubation time, followed by statistical analysis at each given incubation time.
For test of significant statistical difference among EPTC degradations (as measured by percent recovery of EPTC) in soils non-inoculated and inoculated with cultured *Rhodococcus* sp. strain TE1 at different rates, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each soil.

### 3.3.8 Nutrient amendment

Nutrients tested for their effect on EPTC degradation in soil were glycerol, sucrose, urea, and yeast extract. All nutrients were reagent grade obtained from certified suppliers. Each nutrient solution was prepared as 10% aqueous solution, and added to soil at a 1:10 or 1:100 ratio (nutrient solution:soil, v/w), which gives 1% or 0.1% (w/w), respectively, of nutrient added. The soil and nutrient solution were thoroughly mixed using a spatula, and the samples were incubated at 25 ± 2 °C. The effect of nutrient amendment on EPTC degradation in Soils II, III, IV, and V was evaluated without and with 0.2% inoculation with ACTIVATED soil.

For test of significant statistical difference among EPTC degradations (as measured by percent recovery of EPTC) in soils with and without nutrient amendment, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each nutrient, using means of results from four soils tested.

### 3.3.9 Evaluation of stability of inoculant after inoculated to soil

Stability of degradation activity was investigated under different laboratory conditions imitating conditions that might be encountered in the environment. ACTIVATED soil was mixed with Soil II at a 1:50 ratio (2% inoculation), and the
inoculated soil was stored at 4 different conditions: (1) at room temperature without soil moisture adjustment, (2) at room temperature and with soil moisture content between 15 ~ 25 %, (3) at low temperature (10 °C), and (4) at frozen state (-20 °C). Stabilities at 10 and 4 °C were found to be almost the same, and so these storage conditions were regarded as one condition.

After various times, the rates of degradation were determined by treating the soil with EPTC at a rate of 20 mg/kg. The inoculated soils that had been stored at low temperature and in a frozen state were allowed to adjust to room temperature (20 – 25 °C) for 1 and 2 days, before EPTC was applied.

The degradation activity of the stored soils after a specific time of storage were compared with that of the beginning of the storage by plotting the percentage of EPTC remaining in soil vs. time of incubation. For test of significant statistical difference among EPTC-degrading activities in soils stored at different conditions, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each condition.

3.3.10 Evaluation of storage conditions on the stability of ACTIVATED soil

The ACTIVATED soil had its water content initially adjusted to 25% (w/w), and stability of the ACTIVATED soil, in terms of EPTC-degrading activity, was investigated under 4 different storage conditions as previously described.

For the storage at room temperature (20 – 25 °C) with and without moisture adjustment, two 200 g subsamples of ACTIVATED soil were each placed in a plastic container with lid. One of the subsamples had water content maintained at 25% (w/w) by adding sterilized water every other week. The other did not have water content maintained,
but this soil was allowed to naturally dry down over time. If no additional water added, soil water content in this soil dropped below 15% (w/w) after 1 month of storage at room temperature. Ten g of the stored ACTIVATED soil was taken from each container at specific times of storage and inoculated into 500 g of artificially EPTC-contaminated (20 mg/kg) Soil II. The rates of EPTC degradation were monitored by the method previously described (section 3.3.3).

For the storage at low temperatures or frozen state (10, 4 and -20 °C), the ACTIVATED soil was subdivided into ten g subsamples and placed in small (16-mL) vials and capped. The vials were placed in a cold room (10 °C), a refrigerator (4 °C), and a freezer (-20 °C), respectively. Subsamples stored at different conditions were randomly taken out at specific times of storage. The subsamples stored at low temperature (4 °C) and at frozen state (-20 °C) were allowed to adjust to room temperature (20 – 25 °C) for 1 and 2 days, respectively. Ten g of each of these ACTIVATED soils were inoculated into 500 g of artificially EPTC-contaminated (20 mg/kg) Soil II. The rates of EPTC degradation were monitored by the method previously described (section 3.3.3).

The rates of EPTC degradation and the remaining activity were determined by the method previously described (sections 3.3.3 and 3.3.9). For test of significant statistical difference among EPTC-degrading activities of ACTIVATED soils stored at different conditions, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each storage condition.
3.3.11 Statistical analysis

All statistical analyses were done using MINITAB (ver. 13.1) from MINITAB Inc., State College, PA. Analysis of Variance (AVOVA) was done using the General Linear Model menu in MINITAB, and the Least Significant Difference (LSD) was calculated at the 5% level.
3.4 RESULTS AND DISCUSSION

3.4.1 EPTC degradation in non-activated soils

EPTC degradation in non-activated soils were measured over a 7 day period. The rate of EPTC degradation in non-activated Soil I, which was a Brookston clay loam soil, was measured after pretreating the soil with water to achieve a content of 25% (w/w) and incubating at room temperature (20 – 25 °C) for 7 days. EPTC was then added and degradation was tested. The rates of EPTC degradation in other soils (Soils II – V) were tested right before the soils were placed in a cold room (10 °C) for storage as described in section 3.3.1.

Figure 3.2 shows the rates of EPTC degradation in all five non-activated soils. The rates and extents of EPTC degradation varied among soils tested. The percentage of EPTC recovered after 7 days in soils treated with EPTC at a rate of 20 mg/kg soil ranged from approximately 50 to 70% of EPTC initially recovered. It was also observed that there was considerable decrease in EPTC recovered from sterile soils, based on extractable EPTC, which means that there was degradation and/or dissipation and sorption of EPTC by processes other than by biological activity. However, the percentage of EPTC dissipated by non-biological processes in a typical soil (such as Soil I) treated with EPTC at much higher rates (200 or 2000 mg/kg soil) was generally much less. For example, after 7 days of incubation, it was determined that the percentage of EPTC lost by non biological means was less than 5% in Soil I treated with 200 or 2000 mg/kg soil (see Figure 3.4A).
Figure 3.2. Degradation of EPTC in non-activated soils. Soils were applied with EPTC at a rate of 20 mg/kg soil. Soil I, which was stored in an air-dried state for 14 years, was pretreated with water (water content of 25%, w/w) and incubated at room temperature (20 – 25 °C) for 7 days. Error bars represent Least Significant Difference (LSD) between treatment means at given incubation time.
The amount of EPTC biodegraded, which was defined as the difference in the amounts of EPTC recovered from non-sterile and sterile soils, was also different among soils tested. After 7 days of incubation, Soil III seemed to have highest EPTC biodegradation activity. However, Soil I was used to prepare the ACTIVATED soil because some EPTC-degrading microorganisms were isolated from this soil (Assaf 1991) and because previous study (Behki 1991) had shown that EPTC-degrading microorganisms had survived for several years when stored at 4 °C.

It has been widely reported that the rate of EPTC degradation is accelerated in soils with history of previous application of the same or similar compounds (Lee et al. 1984, Obrigawitch et al. 1983, Wilson 1984). As described previously, Soil I was a Brookston clay loam soil sampled from a field with high EPTC-degrading activity attributed to four successive annual EPTC treatments at field application rates, The soil was brought to the laboratory and stored at room temperature (20 – 25 °C) in an air-dry state for 14 years. However, the high EPTC-degrading activity originally observed in this soil at the time of sampling (Ankumah 1988) was not recovered by the initial water pretreatment only.

### 3.4.2 Creation of EPTC-ACTIVATED soil

Although high activity for EPTC degradation in Soil I, previously observed at the time of sampling (Ankumah 1988), was not recovered by water pretreatment only (see Figure 3.2), both EPTC-degrading activity and microorganisms were readily recovered and enriched during subsequent EPTC treatments at a rate of 20 mg/kg soil (Figure 3.3). After a single treatment with EPTC, a second treatment caused the extractable residual EPTC in soil to drop below 10% of that initially recovered after only 2 days of incubation.
Figure 3.3. EPTC degradation and enrichment of EPTC-degrading microorganisms in soil. The rate of EPTC at the beginning of each treatment cycle was 20 mg/kg soil.
The amount of EPTC recovered 2 days after the third treatment was similar to that recovered 2 days after the second treatment. This contrasts to a time of 20 days to achieve similar level of degradation during the first EPTC treatment. Thus a single treatment of EPTC at a rate of 20 mg/kg soil was sufficient to achieve a high level of EPTC degradation activity. This result is similar to the observations reported by Obrigawitch et al. (1983) and Schman and Harvey (1980). According to these authors, a single application of EPTC resulted in maximum increase in the rate of degradation of EPTC in the soils studied. Similarly, according to Ankumah (1988), self-enhancement of EPTC by repeated application of EPTC at a rate of 4 mg active ingredient/kg soil was observed in all soils tested. Ankumah (1988) also reported additional enhancement of EPTC degradation in soil occurred after a second treatment of EPTC, but further enhancement of EPTC degradation by additional treatments of EPTC was not significant.

The number of EPTC-degrading microbes, based on MPN, increased significantly (> 3 log units) during the first cycle of treatment, whereas total number of microorganisms remained relatively constant during treatments (Figure 3.3). The number of EPTC-degrading microorganisms almost reached its maximum during the first treatment, and there was little further increase in the number of EPTC-degraders with two additional treatments. However, it might be possible to increase the number of EPTC degrading microorganisms in soil more by application of EPTC at a higher rate.

This Brookston clay loam soil (i.e. Soil I) which had been treated with EPTC three times at a rate of 20 mg/kg soil, and that exhibited increased number of EPTC degraders and enhanced activity for EPTC degradation, is hereafter defined as ACTIVATED soil.
Moorman (1988) reported that populations of EPTC-degrading microorganisms, based on $^{14}$C-MPN technique, in soils with accelerated rates of EPTC degradation were not larger than those of other soils. Degrader populations increased in soil previously treated with EPTC for 6 years when treated with EPTC at a rate of 60 mg/kg soil, but not when treated with EPTC at a rate of 6 mg/kg soil. Either treatment at a rate of 60 or 6 mg/kg soil did not result in a population increase in soil that was not previously exposed to EPTC. The author suggested that increased rates of metabolism of EPTC, rather than increased populations of degraders, was apparently responsible for the increased rates of EPTC degradation.

Some microbial species are known to be very stable even under extreme conditions, and Behki (1991) has reported that EPTC-degrading microorganisms survived at 4 °C for 3 years in soil. This study showed that EPTC-degraders were readily enriched by one treatment of EPTC at a rate of 20 mg/kg soil even in a soil stored in an air-dried state for 14 years without any treatment for preservation of the microbial population. This supports the widely reported enhancement of EPTC degradation in soils with history of previous treatment of EPTC or its analogs (Ankumah 1988, Dick et al. 1990, Roeth et al. 1989, Skipper 1990).

Although the use of indigenous microbial consortium has been proposed as a bioaugmentation method for enhanced biodegradation (Otte et al. 1994), direct enrichment of microorganisms capable of degrading specific compounds in natural media and its application for bioremediation have rarely been reported. Laine and Jorgensen (1996) reported induced biotransformation of pentachlorophenol (PCP) and
mineralization of [U-14C]-PCP in either PCP-adapted straw compost (prepared by circulating compost in a percolator containing mineral salts medium amended with 5 to 10 mg PCP/L for 3 months) or remediated soil (obtained from successfully remediated, full-scale 3-year composting of chlorophenol-contaminated soil in biopiles to which bark chips and nutrients but no inoculum had been added). Recently a report was published (Barbeau et al., 1997) in which activated soil was used to bioaugment the bioremediation of soil contaminated with PCP. According to the authors, in a mixed soil slurry bioreactor (10% soil) fed with PCP at increasing rates ranging from 50 to 300 mg/L, PCP-degrading bacteria increased from 10^5 to 10^8 cfu/g soil within 30 days.

3.4.3 Effect of initial EPTC concentration on degradation of EPTC in ACTIVATED soil

Chemicals become toxic to microorganisms at concentrations exceeding a certain threshold, although they are not harmful at lower concentrations. Bioaugmentation may not be effective when the contamination exceeds a threshold concentration above which inhibitory effects occur on microbial growth or degradation activity.

Toxicological effects on human and animals and ecological effects of EPTC have been known (EXTONET 1996). However, there is no previous work on the effect of EPTC concentration on microbial growth or degradation of EPTC by microorganisms in soil. In this work, degradation of EPTC in the ACTIVATED soil was evaluated at three different initial EPTC concentrations (20, 200, and 2000 mg/kg soil). At all levels tested, i.e., even at an initial EPTC concentration of 2000 mg/kg soil, degradation of EPTC in the ACTIVATED soil proceeded very rapidly. The amount of extractable EPTC remaining in
soil after 8 days of incubation was about 5 to 15% of the EPTC initially recovered (Figure 3.4A). The amount (Figure 3.4B) but not the percentage (Figure 3.4A) of EPTC biodegraded in ACTIVATED soil increased with an increase in the initial concentration of EPTC added to soil from the beginning of incubation (Figure 3.4B). The total amount of EPTC biodegraded in ACTIVATED soil during 8 days of incubation was almost directly proportional \( (r^2 = 0.999) \) to the amount of EPTC initially added to ACTIVATED soil.

The effect of initial concentration of EPTC on degradation of EPTC, however, may be different in soil with EPTC at much higher initial concentrations \( (>> 2000 \text{ mg/kg soil}) \) or in soils with lower numbers of EPTC-degrading organisms.

### 3.4.4 Effect of inoculation on EPTC degradation in soils

There have numerous reports on the effect of introducing microorganisms, either cultured isolate(s), consortium of unknown members, enriched microbe(s), or commercial bioaugmentation product (bioadditive), to bioaugment degradation of pollutants under laboratory or field conditions (Barbeau et al. 1997, Boon et al. 2000, Mendoza-Espinosa and Stephenson 1996, Pritchard 1992, Rojas-Avelizapa et al. 2003, Schwartz and Scow 2001, Watanabe et al. 1996, Wilderer et al. 1991). The results have not always been promising because many environmental factors also affect the success of bioaugmentation, and several reasons for failure in bioaugmentation have been proposed (Fujita et al. 1994, Goldstein et al. 1985, McClure et al. 1989, McClure et al. 1991, Schmidt and Alexander 1985). The use of ACTIVATED soil is proposed because it offers several advantages over that of cultured microorganisms. Soil serves as a carrier material that retains microorganisms introduced in required places, and it also provides inoculated
Figure 3.4. EPTC degradation as a function of EPTC concentration in ACTIVATED soil. Degradation of EPTC in ACTIVATED soil expressed as (A) percentage of EPTC remaining and (B) amount of EPTC biodegraded. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time. Means of ‘Non-activated’ soils were not included in LSD calculation.
microorganisms with shelter for survival from predation and/or nutrients, which may not exist in the environment introduced, essential for survival and/or activity.

The effectiveness of ACTIVATED soil as an inoculum to bioaugment the degradation of EPTC in soils was studied by adding the ACTIVATED soil at different rates (0.05, 0.5 and 5%, w/w) to a soil artificially contaminated with 20 mg EPTC/kg soil. Based on MPN method, a 5% inoculation corresponds to introduction of $10^3$ EPTC-degrading microorganisms per g soil.

Inoculating contaminated soils with ACTIVATED soil significantly ($p < 0.05$) enhanced EPTC degradation at almost all incubation time with a little variation among soils tested (Figure 3.5). As expected, the effect of inoculation on EPTC degradation in soils was proportional to the rate of inoculation. The EPTC-degrading activity was not significantly inhibited even in a soil with relatively low pH (pH of 4.5, Figure 3.5 Soil IV). However, the effect of inoculation was slightly less in soil with high organic matter (43.3% organic matter, Figure 3.5 Soil V), especially at the low rate of inoculation (0.05%).

The effect of inoculating soil with the cultured *Rhodococcus* strain TE1 on EPTC degradation in soils treated with EPTC was evaluated at two different inoculation rates: $10^4$ and $10^8$ cfu/g soil (Figure 3.6). At low inoculation rate ($10^4$ cfu/g soil), there was almost no effect ($p < 0.05$) on EPTC degradation in soils, regardless of types and properties of soils tested. Inoculation at a rate of $10^8$ cfu/g soil was similar to the effect of inoculation with the ACTIVATED soil at a rate of 0.05% (refer to Figure 3.5). The equivalent number of EPTC-degrading microorganisms at this level of ACTIVATED soil
Figure 3.5. Effect of inoculation rate of ACTIVATED soil on degradation of EPTC in soils treated with EPTC. Soil was applied with EPTC at a rate of 20 mg/kg soil. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
Figure 3.6. Effect of inoculation rate of cultured strain TE1 on EPTC degradation in soils treated with EPTC at a rate of 20 mg/kg soil. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
inoculum, based on MPN method, was 10 cfu/g soil. It does not seem likely that only 10 microorganisms per gram of ACTIVATED soil could enhance degradation greatly. There could be other microorganisms enriched but not enumerated by the MPN method which is based on culture technique.

The introduction of cultured TE1, compared to ACTIVATED soil inoculation, seemed to be less effective for EPTC degradation in Soil IV (Figure 3.6, compare results in Figure 3.5 with results in Figure 3.6). This might be attributed to decreased activity of the introduced microorganisms into this new environment. Adding ACTIVATED soil for bioaugmentation provides EPTC-degrading microorganisms a more favorable microenvironment where activity can be maintained.

3.4.5 Effect of nutrient addition on EPTC degradation

There have been numerous reports of acceleration or deceleration in degradation of specific compounds due to addition of nutrients or presence/absence of other substrates (Alvarez and Vogel 1991, DuPont 1994, Gallert and Winter 2002, Liebeg and Cutright 1999, Pfiffner et al. 1997, refer also to the Chapter 2 of this dissertation for more information). However, the effect of nutrients or other substrates on EPTC degradation in soils or other media has not been studied widely, although several isolates capable of EPTC degradation have been reported and microbial pathways of EPTC degradation and cross-enhancement of degradation among EPTC and its analogs have been extensively studied (Ankumah et al. 1995, Bean et al. 1988, Dick et al. 1990. Harvey 1990, Lawrence et al. 1990).
Most of the isolated microorganisms capable of degrading EPTC belong to the genus *Rhodococcus*, and among those isolates, the strain *Rhodococcus* TE1 is the most studied. According to Tam et al. (1987), *Rhodococcus* sp. TE1 can utilize various compounds as sole carbon sources: i.e. citrate, malonate, ketoglutarate, D-ribose, D-xylose, *m*-,inositol, uracil, ethanol, acetate, glucose, aspartate and succinate. However, TE1 could not grow on maltose, rhamnose, *m*-hydroxybenzoate, or ketogluconate as sole carbon source. After 4 hours of incubation, the amounts of EPTC (initially added at a rate of 1 mg/kg) degraded by *Rhodococcus* TE1 growing on BMN supplemented with EPTC (60 µg/mL), glycerol (1 mg/mL), or glucose (1 mg/mL) were 30, 9.2, and 8.3 µg/mL, respectively. The authors suggested the enzyme(s) involved in EPTC degradation is regulated, but catabolic repression by glucose does not seem to occur since there was no significant difference in degradation of EPTC between cells growing on glycerol and those growing on glucose.

In this study, effect of nutrient addition on EPTC degradation in soils was evaluated by adding glycerol, sucrose, urea, or yeast extract at rates of 1 and 10 g/kg soil (0.1 and 1%, w/w). However, there was no significant effect on degradation at the 0.1% level of nutrient addition, and degradation at the 1% nutrient application rate is presented. Furthermore, since degradation rates in all soils tested (Soils II – V) responded similarly to nutrient addition, only mean values for the four soils are reported. First, the effect of nutrient addition on EPTC degradation for the soils without inoculation by ACTIVATED soil (Figure 3.7A) showed there was no significant effect, acceleration or deceleration on EPTC degradation. It seemed that EPTC degradation in natural soils was not subject to
nutritional limitations of the environment. In soils inoculated with 0.2% ACTIVATED soil, addition of glycerol actually slightly decreased the rate of EPTC degradation (Figure 3.7B). Addition of glycerol or yeast extract decreased the rate of EPTC degradation by 20 and 10%, respectively, during 5 days of incubation. The reasons for this result may include: (1) the introduced microorganisms were outcompeted by other microorganisms in the environment which might be stimulated by the added nutrient, and (2) the added nutrients were favored over EPTC by the introduced EPTC-degrading microorganisms.

### 3.4.6 Stability of inoculant after introduction to soil

EPTC is a herbicide that exhibits enhanced degradation in the environment. Enhanced degradation of a compound is generally attributed to increased number or adaptation of microorganisms capable of degrading this compound. This may imply that there always exist relatively high activity or number of microorganisms for EPTC degradation.

In order to investigate how long the inoculant is active in soils, the Soil II was inoculated with ACTIVATED soil at a rate of 2% (w/w) and EPTC degrading activity was monitored at various times and under various conditions. Environmental conditions tested were (1) room temperature (20 – 25 °C) without soil moisture adjustment, (2) room temperature with soil moisture adjustment between 15 and 25% (w/w), (3) low temperatures (10 and 4 °C), and (4) in a frozen state (-20 °C). All inoculated soils had their moisture contents adjusted to 25% (w/w) before they were placed at given conditions. At room temperature, the inoculated soil lost EPTC-degrading activity after 1 or 2 months.
Figure 3.7. Effect of nutrient addition on EPTC degradation (A) without and (B) with inoculation of ACTIVATED soil at 0.2% rate. Results shown are averaged data from four soils tested. Each nutrient was added at a rate of 10 g/kg soil. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
depending on moisture content (Figure 3.8). In contrast, little loss of EPTC-degrading activity occurred even after 6 months when the inoculated soil was stored at low temperature.

These results imply that at temperatures above 10 °C, EPTC-degrading microorganisms in soils become inactive, presumably outcompeted by other microbes, especially if there is no EPTC available in soil. However, it seems that sufficient EPTC-degraders survive such conditions, and they can be rapidly enriched again in the presence of EPTC as confirmed by my study and as reported by Behki (1991). According to author, a single application of EPTC at a rate of 5 mg/kg moist soil was sufficient to increase the rate of EPTC degradation by 37%, in soils pretreated with EPTC for 4 years, compared to 11% in a similar soil not previously exposed to EPTC.

3.4.7 Effect of storage condition on stability of ACTIVATED soil

Since it is generally not convenient or practical to prepare the necessary quantities of inoculum immediately prior to use, inoculum is often prepared and then preserved so that it is immediately available when need. Therefore, inoculum must be stable during storage and remain effective after it is added to soil for bioaugmentation.

The stability of ACTIVATED soil, in terms of EPTC-degrading activity, was evaluated under various storage conditions. The ACTIVATED soils had their water content initially adjusted to 25% (w/w) and then the following four storage treatments were imposed: (1) at room temperature (20 – 25 °C) without moisturizing, (2) at room temperature with additional water added to maintain moisture level, i.e., water content between 15 – 25%, (3) at low temperatures (10 and 4 °C), and (4) frozen. The results
Figure 3.8. Stability, in terms of EPTC-degrading activity, of inoculant in various conditions after inoculated into Soil II at a rate of 2%. Imitated environmental conditions are: (A) room temperature without water content adjustment, (B) room temperature with water content adjustment between 15 to 25%, (C) low temperature (10 and 4 °C, water content remained above 15% during storage), and (D) frozen. Error bars represent Least Significant Difference (LSD) among means at given incubation time.
Figure 3.9 show that when the ACTIVATED soil was stored at room temperature (20 – 25 °C), the ACTIVATED soil, regardless of its moisture content, lost its EPTC-degrading activity in a relatively short period. Degradation activity after 1 month of storage dropped to that of non-activated soil. However, when stored at lower temperatures (10 or –20 °C), degrading activity of the ACTIVATED soil was preserved for at least 6 months. The stability of ACTIVATED soil at these lower temperatures was not monitored more than 6 months.

Previously, Behki (1991) observed EPTC-degrading microorganisms survived for three years in a soil exposed to EPTC for four consecutive years, when the soil was stored at 4 °C. Furthermore, it was evident in this study that EPTC-degraders could survive for a much longer period (14 years) even under harsh conditions (i.e. at room temperature and low moisture content) although degradation activity was lost. Furthermore, this study found that the ACTIVATED soil carrying directly enriched EPTC-degrading microorganisms, when stored at low temperature, was stable even in terms of EPTC-degrading activity. However, EPTC degradation activity was not maintained very well at high temperature or low moisture content of soil. Under these conditions, EPTC-degraders are either not capable of surviving or they are outcompeted by other microorganisms under these conditions. Similarly, there was a loss of activity for atrazine degradation caused by low moisture content of soil (Barriuso and Houot 1996). According to the authors, however, keeping moisture content of the ACTIVATED soil between 15 and 25% (w/w) did not ensure preservation of atrazine-degrading activity when the soil was stored at room temperature (20 – 25 °C).
Figure 3.9. Effect of storage conditions on the stability, in terms of EPTC degrading activity, of ACTIVATED soil. (A) room temperature without water content adjustment; (B) room temperature with water content adjustment between 15% and 25%; (C) low temperature (10 and 4 °C); (D) frozen. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
Therefore, the ACTIVATED soil with EPTC-degraders can be stored in a readily usable state if both the temperature is kept below 10 °C and moisture content is maintained above 15% (w/w). For longer term storage of soil, room temperature and air-dry conditions are probably more suitable as it is easier to preserve soil in this way. Even though the air-dried soil loses degradation activity, it can respond, in terms of degrader number, upon introduction to an environment which contain EPTC. Storing at room temperature and occasional treatment with EPTC can be another effective strategy for the storage of ACTIVATED soil in a readily usable state.
3.5 SUMMARY AND CONCLUSION

The rates of EPTC degradation in natural soils was relatively slow. However, EPTC-degraders survived well for 14 years in a Brookston clay loam soil (Soil I), that was sampled, air-dried, and placed at room temperature without any additional treatments for storage. After the soil was pretreated with addition of water and incubated at room temperature for 7 days, EPTC-degrading microorganisms in this soil were readily enriched by repeated treatments of EPTC at a rate of 20 mg/kg soil. Most probable number method revealed that the number of EPTC-degraders increased by about 3 logs of magnitude after a single treatment with EPTC at a rate of 20 mg/kg soil. Treatments of EPTC, beyond the first treatment, did not significantly increase the number of EPTC-degraders. This Brookston clay loam soil, which contains directly enriched EPTC-degrader and had enhanced activity for EPTC degradation as a result of three repetitive treatments of EPTC at a rate of 20 mg/kg soil, was defined as ACTIVATED soil.

Degradation of EPTC in ACTIVATED soil was not inhibited in soils treated with EPTC at rates up to 2,000 mg/kg soil, and total amount of EPTC degraded after 8 days of incubation was directly proportional to the amount of EPTC initially added to soil. Inoculation of EPTC-contaminated soil with the ACTIVATED soil (Brookston clay loam) carrying enriched EPTC-degrading microorganisms resulted in significant (p < 0.05) increase in rates of EPTC degradation in soil. Inoculation at a rate as low as 0.05% could result in considerable acceleration of EPTC degradation in soils. When soils were inoculated at a rate of 5%, the extractable EPTC remaining in soil (Based on recovered EPTC) after about 4 days in all four soils tested dropped to below 10% of EPTC initially.
added and recovered at time zero. As expected, rates of inoculation ranging from 0.05 to 5% were directly related to rates of EPTC degradation in soils. When compared with the effect of inoculation of *Rhodococcus* sp. TE1, inoculation of ACTIVATED soil at a rate of 0.05% was similar to the effect generated by inoculation of *Rhodococcus* sp. TE1 at $10^8$ cfu/g soil. There was no significant acceleration on EPTC degradation observed when *Rhodococcus* sp. TE1 was introduced at a rate of $10^4$ cfu/g soil.

Addition of glycerol, sucrose, urea, yeast extract, at 10 g/kg soil, did not accelerate or decelerate EPTC degradation in any of the four soils tested when they were not inoculated with ACTIVATED soil. In contrast, addition, at the same rate of glycerol into the soil inoculated with ACTIVATED soil slightly decreased ($p < 0.05$) degradation of EPTC in soils. This may be a result of outcompetition by other microorganisms stimulated by the nutrient added, or preference of inoculated microorganisms for nutrient over EPTC. Nutrient amendment seemed not to be an effective option for enhancing the remediation of EPTC-contaminated soils. The ACTIVATED soil was found to be stable, in terms of EPTC-degrading activity, up to 6 months when it was stored in a moist state at temperature below 10 °C. This stability, along with the effectiveness of the ACTIVATED soil inoculum, suggest that use of this ACTIVATED soil to bioaugment EPTC degradation in contaminated soils is effective.
CHAPTER 4

USE OF ACTIVATED SOIL AND COMPOST TO BIOAUGMENT DEGRADATION OF ATRAZINE IN SOILS

4.1 ABSTRACT

Atrazine has been found in soils and waters at concentrations that exceed the 3 µg/L health advisory level set by the U.S. Environmental Protection Agency. Technologies that can rapidly and inexpensively remediate such atrazine-contaminated sites are needed. The purpose of this study was to investigate effectiveness of ACTIVATED soil or compost carrying directly enriched atrazine-degrading soil microorganisms as inoculant to bioaugment degradation of atrazine in soils. To prepare ACTIVATED soil, Soil I (a Wooster silt loam, Typic Fragiuudalf) was treated three successive times with atrazine at a rate of 4 mg/kg soil. After the second treatment, there was an increase in the number, based on MPN, of microorganisms utilizing atrazine as a C and N source by 3 logs and 1 log of magnitude, respectively. Atrazine degradation was also significantly enhanced ($p < 0.05$) after the first treatment. The atrazine degrading and mineralizing microbial populations, developed in soil during activation, were successfully transferred with inoculated ACTIVATED soil and enriched in compost. Inoculation of atrazine-contaminated soils with ACTIVATED soil or compost effectively accelerated
atrazine degradation in four soils tested, although the effects were different among soils and with inoculation rate. Inoculation of typical agricultural soils from Ohio with ACTIVATED soil or compost at a rate as low as 0.5% reduced extractable atrazine remaining in soils below 2% of that initially recovered (initially added at 4 mg/kg soil rate) after 4 days. Inoculation at a higher rate was required to achieve the same result in soils with non-typical properties (pH of 4.5 or organic matter of 43%). Addition of glycerol, sucrose, urea, or yeast extract at 1 g/kg level seemed to have little effect on atrazine degradation in Soil II inoculated with ACTIVATED soil at a rate of 0.5%. ACTIVATED soil and compost were stable, in terms of atrazine degradation activity, at least up to 6 months when kept at low temperature (below 10°C) and moistened (water content above 15%). The results of this study indicate that microorganisms capable of degrading and mineralizing atrazine are relatively easily enriched in soil or compost to create an ACTIVATED soil. Use of these ACTIVATED materials to bioaugment degradation and/or mineralization in atrazine-contaminated soil represents a practical option for bioremediation of such soils.

4.2 INTRODUCTION

The herbicide, atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), is used in many parts of the world to control broadleaf and grassy weeds primarily in the production of corn. Atrazine is frequently detected at trace levels in surface and groundwater samples (Goodrich et al. 1991, Thurman et al. 1992) and has been found in water samples at levels exceeding the 3 µg/L health advisory level set by the U.S. EPA
Atrazine is thought to be persistent in the environment, especially in aquifers or anaerobic sediments (Agertved et al. 1992, Widmer and Spalding 1995). Half-life of atrazine in natural soils ranges from a few days to a few months. The frequency of the occurrence of atrazine in the environment is related to extensive usage, atrazine’s moderate persistence, and its mobility through the soil (Burkart and Kolpin 1993). Therefore, there has been considerable interest in developing management practices that minimize the potential for atrazine pollution of surface water and groundwater resources.

Although atrazine has been known to be recalcitrant, both aerobic and anaerobic microbial degradation (Chung et al. 1996), as well as sorption, are key processes governing the persistence of atrazine in the environment. Microbial atrazine degradation pathways include four major processes: dehalogenation (dechlorination), N-dealkylation, deamination, and s-triazine ring cleavage (Cook et al. 1985, Erickson and Lee 1989). A variety of bacteria (Behki and Khan 1986, Behki et al. 1993, Bouquard et al. 1997, Nagy et al. 1995b) and fungi (Donnelly et al. 1993, Kaufman and Blake 1970, Mougin et al. 1997), that dealkylate or dechlorinate atrazine but do not mineralize its s-triazine ring, have been isolated.

biodegrade atrazine but none of the purified organisms alone or any possible combination of those five bacteria as 2- or 3-members were able to degrade atrazine. More recently, several microorganisms capable of atrazine mineralization have been isolated from sediments or soils that have frequently contact with atrazine (Mandelbaum et al. 1993b, 1995, Mirkaim et al. 1993, Moscinski et al. 1996, Radosevich et al. 1995, Rousseaux et al. 2001, Struthers et al. 1998, Topp 2001, Vanderheyden et al. 1997, Yanze-Kontchou and Gschwind 1994). However, it seems likely that atrazine mineralization in natural environments is more a result of microbial consortia than a single species.

Use of atrazine-metabolizing microorganisms for bioremediation of atrazine-contaminated soils, wastes and waters has been suggested (Cook 1987, Karns et al. 1987). A number of microbial strains and microbial consortia have been tested for their ability to bioaugment the remediation of atrazine-contaminated soils and waters. Either specific strains or microbial consortia capable of atrazine degradation resulted in improved atrazine degradation in both laboratory and natural environments (Barriuso and Houot 1996, Feakin et al. 1995, Habecker 1989, Newcombe and Crowley 1999, Risatti and Zagula 1993, Runes et al. 2001, Struthers et al. 1998).

Microorganisms with ability to degrade atrazine can be directly enriched in natural media, but only a limited portion of this naturally degrading microbial can be isolated and cultured. Zeng et al. (2002) has summarized what is known about the metabolic steps and microorganisms involved in atrazine degradation and the low diversity of microbial strains capable of atrazine mineralization (Figure 4.1).
Figure 4.1. Atrazine mineralization pathway map. Genes and corresponding enzymes involved in each step and bacterial strains that initiate each pathway are indicated (modified from Zeng et al. 2002).
In most previous studies on bioremediation of atrazine-contaminated soils or waters using bioaugmentation with atrazine degraders, pure cultures of microorganisms have been used as inoculum (Grigg et al. 1997, Newcombe and Crowley 1999). In this study, soil and/or compost enriched in numbers of atrazine-degrading microorganisms were tested for their ability to bioaugment the degradation of atrazine-contaminated soil. Compost, for several reasons, has been proposed as an effective carrier for microorganisms to be introduced to the environment to bioaugment remediation of contaminated sites. Compost is easy to produce with low cost. It can provide microniches and nutrients required for the survival of desired microorganisms, thereby making the introduced microorganisms more competitive. It can also provide supplementary nutrients needed for the introduced microorganisms to degrade pollutants in the environments or necessary for effective preservation of the introduced microorganisms.

In the previous Chapter (i.e. Chapter 3 of this dissertation), it was found that EPTC-degrading microorganisms were readily enriched in soils. EPTC is one of the well-known herbicides that can be mineralized by single species of microorganisms (mostly belonging to the genus *Rhodococcus*), and is often rapidly degraded in soil. This fact may imply that EPTC-degraders are relatively easily enriched. However, since enhanced degradation of atrazine and atrazine-mineralizing microorganisms have rarely been reported (Barriuso and Houot 1996), atrazine-mineralizing microorganisms may not be readily enriched in natural media. In this study, the possibility of direct enrichment of atrazine-degrading and mineralizing populations in soil and/or compost and their effectiveness as inocula to bioaugment the degradation of atrazine-contaminated soil were
tested. It was hypothesized that the microorganisms capable of complete degradation of atrazine are enriched in soils or compost, and the ACTIVATED soil and compost are effective inoculants to bioaugment degradation of atrazine in soils contaminated with atrazine. The specific objectives of this study were: (1) to determine if atrazine-degrading or mineralizing soil microorganisms are readily stimulated or enriched in soils when treated with atrazine, (2) to evaluate effect of inoculation of atrazine-contaminated soils with ACTIVATED soil containing directly enriched atrazine-degrading microorganisms, (3) to evaluate effectiveness of compost as carrier for microorganisms to be introduced to the environment for bioaugmentation, and (4) to investigate proper storage condition for ACTIVATED soil or compost in a readily usable state.
4.3 MATERIALS AND METHODS

4.3.1 Chemicals, soils, and compost

Standard-grade atrazine was purchased from Chem Service, Inc. (West Chester, PA), and uniformly ring-labeled $^{14}$C-atrazine (specific activity of 8.7 mCi per mmole) was purchased from Stauffer Chemical Co. (Richmond, CA). Pesticide grade toluene was purchased from Sigma (St. Louis, MO). Liquid scintillation cocktail, EcoLite, was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA).

Soils used were collected from various areas including agricultural areas and forest. Surface (0-20 cm depth) soils were obtained with different textures, pH values, organic matter content and land use. Soils I and II are Wooster silt loam soils sampled from an agricultural field near Wooster, Ohio, and Soil III is a Fremont sandy loam collected from an agricultural area near Fremont, Ohio. These soils may have received occasional pesticide applications, but their pesticide application history is not known. Soil IV is a forest soil collected from Mohican State Park, Ohio, and had no known history of pesticide application. Soil V (a Muck soil) was obtained in 2000 from the MUCK branch of OARDC at Celeryville, Ohio, and stored at greenhouse without further treatment.

Each sample (approximately 30 kg total) was collected in 2000 using a spade and combining soil from closely spaced sampling points. The soils were partially air-dried (to a moisture content of approximately 10%, w/w), passed through a 2-mm sieve, and then stored in a cold room ($10^\circ$C). Soil texture was analyzed by the hydrometer method (Buoyoucos 1962). Total organic matter was analyzed using methods described by Combs and Nathan (1998), and total nitrogen was determined using a Carbon/Nitrogen Analyzer,
Model MAX-CN from Elementar Americas, Inc. (Mt. Laurel, NJ). Soil pH was measured by the glass electrode method (McKeague 1978) in a 1/1 (w/v) soil/water suspension. Some of the properties of the soils used are summarized in Table 4.1.

The soil designated as Soil VI and used in some experiments, was a “mixed soil” prepared by combining equal weights of Soil I, II, III, and IV. This soil was prepared to generate a soil with more (or less) complexity in its microbial population. One soil (designated as Soil VII) had been previously spiked with atrazine and artificially aged as follows. The soil used for preparation of atrazine-spiked and aged soil, was a Wooster silt loam collected from a field that was routinely sprayed with various herbicides. It was sieved through a 2-mm sieve and stored in a greenhouse in an air-dried state with nothing planted for two years. The artificially atrazine-contaminated soil was created by spraying commercial formulation of atrazine (i.e. AATREX, 40.8 % active ingredient, w/v) diluted in water (100 x dilution, 4.8 mg active ingredient/mL). Atrazine was applied at a rate of 500 mg/kg soil, and the atrazine treated soil was placed in a plastic bag, air-dried under a hood for 18 days, and stored in a cold room (10 °C) for 6 years. The concentration of atrazine measured in the soil at the start of this experiment was approximately 400 mg/kg soil.

The compost used to test its effectiveness as an inoculum carrier was created from manure obtained from the Northwest branch of Ohio Agricultural Research and Development Center located in Hoytville, Ohio in 1999. The raw material of the compost was a mixture of beef steer (Bos Taurus) manure and wheat (Triticum aestivum, L) straw bedding. This mixture was transported to Wooster and placed into long piles (windrows)
<table>
<thead>
<tr>
<th>Properties</th>
<th>Soil I</th>
<th>Soil II</th>
<th>Soil III</th>
<th>Soil IV</th>
<th>Soil V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>16.1</td>
<td>12.1</td>
<td>53.7</td>
<td>36.1</td>
<td></td>
</tr>
<tr>
<td>Silt</td>
<td>62.9</td>
<td>72.7</td>
<td>32.9</td>
<td>53.1</td>
<td>Ash Content 33.2 %</td>
</tr>
<tr>
<td>Clay</td>
<td>21.0</td>
<td>15.2</td>
<td>13.4</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.7</td>
<td>6.8</td>
<td>6.6</td>
<td>4.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Organic Matter (%)</td>
<td>3.10</td>
<td>3.23</td>
<td>2.74</td>
<td>4.13</td>
<td>43.31</td>
</tr>
<tr>
<td>Total Nitrogen (%)</td>
<td>0.17</td>
<td>0.16</td>
<td>0.52</td>
<td>0.15</td>
<td>2.37</td>
</tr>
</tbody>
</table>

Table 4.1. Selected properties of soils used for atrazine degradation study.
on a cement/concrete pad. These rows were turned by machine about once a week for 2 to 6 months. Water content was maintained above 55% during composting. Mature compost was stored in a vinyl bag in a cold room (10 °C). Among some properties of the compost were: pH of 7.28, total carbon 38.7%, inorganic carbon 1.25%, total nitrogen 3.44%, NO₃-N 2,280 µg/g; and NH₃-N 132 µg/g.

4.3.2 Application, extraction, and determination of atrazine

Atrazine stock solution was prepared by dissolving 20 mg of atrazine in 1 mL of methanol. All experiments in which soils and compost were treated with atrazine (i.e. artificially atrazine-contaminated soil and compost) at various rates (4, 40 and 400 mg/kg soil) were prepared by applying this atrazine stock solution correspondently diluted in sterilized water. Soils and compost were thoroughly mixed with added atrazine using a spatula to achieve uniform distribution of atrazine.

Atrazine was extracted from soil or compost as follows. Eight mL of distilled water was added to 10 g soil or compost in a 125-mL high-density polyethylene (HDPE) bottle, and was vortexed to disperse soil particles. Ten mL of pesticide-grade toluene was added, and the soil-water-toluene mixture was shaken on a horizontal shaker at 120 strokes per min for 1 hr. The mixture was then centrifuged at 8,000 x g for 10 min, and a 2-mL aliquot of the toluene layer was transferred to a 5-mL glass vial and stored at 4 °C until analyzed. Tests indicated that samples could be stored for several months in the glass vials without change in atrazine concentration.

To extract atrazine from microbial cultures used for MPN determination, 0.8 mL aliquots of the culture were transferred to Eppendorf tubes, and equal volume of
pesticide-grade toluene was added. After mixing on a vortex shaker for 1 min, the mixture was centrifuged at 15,000 rpm (≈ 10,000 x g) in a microcentrifuge for 10 min, and 0.5 mL of the toluene layer was transferred to a 5-mL glass vial and stored at 4°C until analyzed. As for atrazine extracted from soil, these samples could be stored several months without change in atrazine concentration.

Atrazine concentration in the extracts was determined with a Varian 3700 gas chromatograph equipped with a 3% OV-1 on 100/200 Supelcoport packed column (3 m in length) and a nitrogen-specific thermionic detector. Helium was used as carrier gas at a flow rate of 28 mL/min. Temperatures were set at 210°C for injector, 250°C for column, and 310°C for detector. The retention time for atrazine was 2.10 min. The average recovery rate of atrazine from soils, when atrazine-treated sterile soils were left at room temperature for 24 hours, was between 82 to 95% at low atrazine treatment rate (4 mg/kg), and above 95% at higher treatment rates (40 mg/kg or above). The average recovery rate of atrazine under the condition used for MPN was 95%.

4.3.3 Monitoring the rate of atrazine degradation

Atrazine degradation in this chapter is defined as disappearance of parent material during incubation. Disappearance of atrazine was measured based on the amount of extractable atrazine remaining in soils. The amount of atrazine degraded by microorganisms was calculated by subtracting the amount of extractable atrazine remaining in non-sterile soil sterile soil from that of sterile soil at certain time of incubation. The rate of degradation was primarily expressed by plotting percentage of
atrazine remaining after various incubation times compared to the amount of atrazine extracted at time zero immediately after treatment.

The rate of atrazine degradation in (1) atrazine-treated natural soils and compost, (2) ACTIVATED soil and compost, and (3) soils inoculated with ACTIVATED soil or compost at various rates were evaluated as follow. Since uniformity of reaction mixture, in terms of atrazine concentration and inoculant density is essential for reliable data with small standard deviations, soils and compost were partially dried to achieve water content of about 15%, to ensure easier mixing, before application of atrazine or inoculation of ACTIVATED soil or compost. Care was given to ensure each soil and compost was thoroughly mixed. The water content of soil and compost reaction was then adjusted to 25% (w/w) with sterilized water. The reaction mixture was divided into 10 g (as dry weight) subsamples and the subsamples were placed into 125-mL high density polyethylene (HDPE) bottles. Bottles were loosely capped and incubated at 25 ± 2°C.

The rates of atrazine degradation in the experiments for evaluation of the effect of storage condition on the stability of ACTIVATED soil and compost were measured similarly to that described in Chapter 3 of this dissertation. Triplicates sample were randomly selected, and atrazine remaining in soil was extracted and analyzed by the procedure previously mentioned.

Atrazine degradations in soils were monitored by plotting the percentage of atrazine recovered from soils vs. incubation time. The averaged amount of atrazine initially recovered from triplicate samples at the very beginning of the incubation (i.e. at time zero) was regarded as 100%. For test of significant statistical difference in atrazine
degradations (as measured by percent recovery of atrazine) in soils or compost, Analysis of Variance (ANOVA) was conducted for each individual incubation time.

4.3.4 Creation, evaluation, and preservation of ACTIVATED soil and compost

Soil activations were achieved by direct enrichment, or acclimation, of atrazine-degrading microorganisms. Four different methods were used for preparation of activated soils and compost, depending on the purpose of activation.

First, Soil I was treated three times with atrazine at a rate of 4 mg/kg soil. Atrazine dissipation rate was monitored by the method previously described in section 4.3.3, and additional atrazine was applied when there was no apparent change for 6 days. No further degradation of atrazine was found after 18, 4, and 2 days of incubation for the first, second, and third treatment, respectively. During activation, total bacteria, microbes utilizing atrazine as carbon source, and those utilizing atrazine as nitrogen source were counted (see next section). The term “ACTIVATED soil” in this chapter refers to this soil, unless otherwise specified, and this is the soil used as inoculant for subsequent experiments. This soil was used in all experiments unless noted otherwise.

Second, to check the ease of enhancement in a range of soils, a separate experiment was conducted using Soils I - IV, and IV (Table 4.1). These soils were treated with atrazine at a rate of 4 mg/kg. However, additional treatment of atrazine was applied up to two more times only when the extractable atrazine remaining in soil dropped to less than 20% of initially recovered. Therefore, there was only one treatment of atrazine at a rate of 4 mg/kg for Soil IV, because the atrazine concentration in this soil from the first treatment remained above 40% of initially recovered atrazine during one month of incubation and no
further decrease was observed. The number of bacteria was not counted during this activation process.

Third, the atrazine-spiked and aged Wooster soil was activated by addition of water to obtain a water content of 25% (w/w), followed by incubation at 25 ± 2 °C for 7 days. Activation as a result of degradation of preexisting atrazine at a concentration of approximately 400 mg/kg soil is expected in this atrazine-spiked and aged soil.

Fourth, for cross enhancement investigation of atrazine and EPTC-degradation, Soil I, without activation with atrazine, was activated with EPTC at a rate of 20 mg/kg soil by the same method used in preparation of atrazine activated soil.

Compost was activated as follows. The ground compost stored in a cold room was rewetted to obtain a water content of around 30%. Then, the compost was inoculated with activated soil at a 1 % (w/w) rate, and the inoculated compost was activated further by three successive applications of atrazine (4 mg/kg compost). Each treatment was applied when the extractable atrazine remaining in compost was reduced to the level below 20% of initially recovered atrazine. The initial moisture content of compost during each treatment for activation was adjusted to 40% (w/w), so that the water content would remain above 30% during the activation treatments. The compost containing atrazine-degrading microorganisms transferred from ACTIVATED soil is herewith called ACTIVATED compost.

Atrazine-degrading activity of the ACTIVATED soil or compost containing enriched numbers of EPTC degrading microorganisms was evaluated at different rates of initial atrazine concentrations (4, 40, or 400 mg/kg soil or compost), and the degradation
of atrazine ACTIVATED soil or compost was monitored by the method previously described (section 4.3.3) for evaluation of ACTIVATED soil. Statistical comparison were made for atrazine degradations (as measured by EPTC recovered) in the ACTIVATED soil or compost. Comparisons were made to test for differences in atrazine degradations at the 4, 40, and 400 mg/kg atrazine application rates for each individual incubation time.

The ACTIVATED soil and compost were moisturized to obtain a water content of 25% (w/w) and 40% (w/w), respectively, and were placed in a plastic container with lid in a cold room (10 °C) for storage.

4.3.5 Most probable number (MPN)

Total bacteria and microorganisms capable of utilizing atrazine as a carbon or nitrogen source in soil or compost were counted by the MPN method described for the enumeration of total and EPTC-degrading bacteria, with a slight modification, in Chapter 3 of this dissertation. TGYe (0.5% tryptone, 0.1% glucose, and 0.25% yeast extract, w/v) was used as medium for culturing total bacteria. For microorganisms utilizing atrazine as carbon source, BMNA (a basal minimal salts nitrogen (BMN) medium amended with atrazine at 4 mg atrazine/L concentration to serve as the sole carbon source for microbial growth) was used. The medium, BMN, was described by Behki and Khan (1986) and its composition is given in Chapter 3 of this dissertation. A modified ‘atrazine medium’ described by Mandelbaum et al. (1993a), containing 4 mg atrazine/L, was used for MPN counting bacteria that could utilize atrazine as sole nitrogen source. The composition and preparation of the modified atrazine medium is given in Table 4.2.
### Table 4.2 Composition and preparation of modified atrazine medium used in MPN for bacteria utilizing atrazine as nitrogen source.

<table>
<thead>
<tr>
<th>Medium and stock solution</th>
<th>Composition</th>
</tr>
</thead>
</table>
| **Modified atrazine medium**<sup>a)</sup> | Per liter of deionized water,  
 1.6 g K$_2$HPO$_4$  
 0.4 g KH$_2$PO$_4$  
 0.2 g MgSO$_4$·7H$_2$O  
 0.1 g NaCl  
 0.02 g CaCl$_2$ (or 0.0265 g CaCl$_2$·2H$_2$O)  
 1 g Sucrose  
 1 g Sodium citrate (Na$_3$C$_6$H$_5$O$_7$·2H$_2$O)  
 20 mL Salt stock solution  
 20 mL Vitamin stock solution  
 0.2 mL Atrazine stock solution |
| **Salt stock solution**<sup>b)</sup> | Per liter of deionized water,  
 2.5 g EDTA (disodium salt)  
 11.1 g ZnSO$_4$ (or 19.76 g ZnSO$_4$·7H$_2$O)  
 5.0 g FeSO$_4$ (9.14 g FeSO$_4$·7H$_2$O)  
 1.54 g MnSO$_4$·H$_2$O  
 0.4 g CuSO$_4$·5H$_2$O  
 0.25 g Co(NO$_3$)$_2$·6H$_2$O  
 0.18 g Na$_3$B$_4$O$_7$·10H$_2$O |
| **Vitamin stock solution**<sup>c)</sup> | Per liter of deionized water,  
 2 mg d-Biotin (Vitamin H)  
 2 mg Folic acid  
 10 mg Nicotinamide (Niacinamide)  
 10 mg Pyridoxine-HCl  
 5 mg Thyamine-HCl (Vitamin B$_1$) |
| **Atrazine stock solution**<sup>d)</sup> | 20 mg atrazine/mL methanol |

<sup>a)</sup> pH was adjusted to 7.3, and cyclohexamide was added at 50 mg/L rate.  
<sup>b)</sup> 5.0 mL of c-H$_2$SO$_4$ was added to retard precipitation of salts  
<sup>c)</sup> Filter-sterilized and stored at 4°C  
<sup>d)</sup> Shaken vigorously for several hours prior to incorporate into the medium
Five replicates of MPN tubes were incubated at 25 ± 2 °C for 7 days. Positive tubes for total bacteria were identified by visual turbidity. Those for atrazine utilizing bacteria were determined based on the amount of atrazine remaining in tubes and extracted by the method described in previous section, after 7 days of incubation. Tubes containing atrazine less than 20% of the initial concentration were considered positive.

4.3.6 Inoculation of contaminated soil with ACTIVATED soil or compost

Inoculation of contaminated soils with ACTIVATED soil and evaluation of its effect on degradation of atrazine were performed as follows. The artificially atrazine-contaminated soils were prepared by applying atrazine to target soils (Soil II, III, IV, and V) at a rate of 4 mg/kg soil. Then the atrazine-contaminated soil was inoculated with ACTIVATED soil at different rates (0.05, 0.5, and 5%, w/w), and the soil was thoroughly mixed by stirring with a spatula. The mixture was divided into 10 g (as dry weight) subsamples and placed into 125-mL HDPE bottles and loosely capped. These bottles were incubated in a temperature-controlled incubator (25 ± 2 °C). Triplicate samples were selected at certain times of incubation, and the atrazine remaining in soil was extracted and analyzed as described in section 4.3.2. Atrazine remaining (calculated as a percentage of atrazine initially added at time zero and recovered) vs. incubation time was plotted. The same procedures were followed in the investigation of the effect of inoculation of ACTIVATED compost on degradation of atrazine in soils.

For test of significant statistical difference among atrazine degradations (as measured by percent recovery of EPTC) in soils non-inoculated and inoculated with
ACTIVATED soil or compost at different rates, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each contaminated soil.

4.3.7 Nutrient amendment and its effect on atrazine degradation

The effect of nutrient amendment on atrazine degradation was tested for glycerol, sucrose, urea, and yeast extract in Soil II inoculated with ACTIVATED soil at a rate of 0.5% to see if further acceleration is possible. Each nutrient solution was prepared as a 1% aqueous solution, and added to soil at a 1:10 ratio (nutrient solution:soil, v/w), which gives a final concentration of 0.1% (w/w) nutrient added. The reaction contents were thoroughly mixed, and the mixture was incubated at 25 ± 2°C.

For test of significant statistical difference among atrazine degradations (as measured by percent recovery of EPTC) in soils with and without nutrient amendment, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each nutrient.

4.3.8 Determination of atrazine mineralization

Atrazine mineralization was determined by measuring $^{14}$CO$_2$ evolved and captured in NaOH solution due to s-triazine ring cleavage of uniformly ring-labeled $^{14}$C-atrazine. Soil (20 g) was weighed into a 300-mL canning jar, and one set of soils was treated with unlabeled atrazine to achieve a concentration of 20 mg/kg for degradation measurement. The other set of soils was treated with both unlabeled and $^{14}$C-labeled atrazine to achieve a concentration of 20 mg/kg as unlabeled and 44,400 dpm/g as radioactive atrazine, respectively. A 20-mL glass vial containing 5-mL of 1N NaOH was placed in each
canning jar to capture CO₂ evolved from degradation of s-triazine ring of atrazine.

Canning jars containing soil were capped tightly and incubated in a vented hood located within a temperature (20 - 25 °C) controlled room. A 0.1-mL aliquot of the NaOH solution from the glass vials inside the canning jars was taken out and mixed with 10-mL of liquid scintillation cocktail for radioactivity measurement. Radioactivity was determined with a Beckman LS-1801 Liquid Scintillation System (Fullerton, CA) following instructions given in the operation manual.

For test of significant statistical difference in atrazine mineralizations (as measured by percent recovery of atrazine) in non-activated and activated soils or compost, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each soil or compost.

4.3.9 Cross enhancement

Cross enhancement between EPTC and atrazine degradation was investigated by activating Soil I either with EPTC at a rate of 20 mg/kg soil or with atrazine at a rate of 4 mg/kg soil, and by comparing degradation rates of EPTC or atrazine in soils activated by counter compound. Cross activated soil is defined as the soil activated with EPTC but used for test of atrazine degradation, or vice versa. Soils were activated as described in section 4.3.4.

Cross enhancement between EPTC and atrazine was evaluated by comparing the amounts of EPTC or atrazine recovered in cross activated soils at specific incubation time. For test of significant statistical difference in EPTC or atrazine degradation (as measured
by percent recovery of EPTC or atrazine) in cross activated soil, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each soil or compost.

### 4.3.10 Evaluation of conditions for storage of ACTIVATED soil and compost

Stability of the ACTIVATED soil and compost, in terms of atrazine-degrading activity, was investigated by first adjusting water content of the ACTIVATED soil and compost to 25 (w/w) and 40% (w/w), respectively, and then storing them at different conditions. Four different storage conditions were imposed as follows: (1) at room temperature without soil water content adjustment, (2) at room temperature and with soil water content maintained between 15 ~ 25%, (3) at low temperature (10 or 4 °C), and (4) at frozen state (-20 °C). Results observed at 10 and 4 °C were found to be almost identical, and so these two conditions were combined and regarded as one condition.

For storage at room temperature (20 – 25 °C) with and without water content adjustment, two 200 g lots of ACTIVATED soil were placed in separate plastic containers with a lid. The subsamples stored at room temperature with water content adjustment were treated with sterilized water. Ten g of soil was taken from each container at specific times of storage and inoculated into 500 g of artificially atrazine-contaminated (4 mg/kg) Soil II. The rates of atrazine degradation were monitored by the method previously described (section 4.3.2).

For storage at low temperatures or frozen state (10, 4 and -20 °C), the ACTIVATED soil was subdivided into 10 g subsamples and placed in small (16-mL) vials and capped. The vials were placed in a cold room (10 °C), in a refrigerator (4 °C), or in a freezer (-20 °C). Subsamples stored at different conditions were randomly taken out at
specific times of storage. The subsamples stored at low temperature (4 °C) and at frozen state (-20 °C) were allowed to adjust to room temperature (20 – 25 °C) for 1 and 2 days, respectively. Ten g of each of these ACTIVATED soils were inoculated into 500 g of artificially atrazine-contaminated (4 mg/kg) Soil II. The rates of atrazine degradation were monitored by the method previously described (section 4.3.2).

The ability of ACTIVATED soil to serve as an inoculum to increase atrazine degradation after a specific time of storage was compared with ability of the ACTIVATED soil at the beginning of the storage (i.e. at time zero) by plotting the percentage of atrazine remaining in soil vs. time of incubation. For test of significant statistical difference among atrazine-degrading activities of ACTIVATED soils stored at different conditions, Analysis of Variance (ANOVA) was conducted for each individual incubation time for each storage condition.

4.3.11 Statistical analysis

All statistical analyses were done using MINITAB (ver. 13.1) from MINITAB Inc., State College, PA. Analysis of Variance (AVOVA) was done using the General Linear Model menu in MINITAB, and the Least Significant Difference (LSD) was calculated at the 5% level.
4.4 RESULTS AND DISCUSSION

4.4.1 Rates of atrazine degradation in various soils

The amount of atrazine degraded, presumably disappearance due to degradation by microorganisms and sorption by soil particles and soil organic matter in various soils, is shown in Figure 4.2. With exception of Soil VII (the atrazine-spiked and aged soil), dissipation of atrazine in these non-activated soils was relatively slow. After 10 days of incubation, about 50 to 70% of the atrazine initially recovered (added initially at 4 mg/kg soil rate) still remained in the soils tested. It was also observed that there was considerable decrease in atrazine recovered from sterile soils, based on extractable atrazine, which means that there was degradation and/or dissipation of atrazine by processes other than biological degradation. However, the percentage of atrazine dissipated by non-biological processes in a typical soil (such as Soil I and II) was relatively low.

Soil VII (i.e. atrazine-spiked and aged soil), that initially contained a high concentration of atrazine (about 500 mg/kg) and stored at low temperature (10°C) without moisture adjustment for 6 years, yielded high degradation rate. The atrazine concentration in this soil rapidly dropped to below 10 mg/kg (about 2% of initial concentration) in only 4 days of incubation at room temperature after the time water was added to reactivate the soil (Figure 4.2). It seems as if enrichment of atrazine degrading microorganisms occurred during the artificial aging process. However, because change in atrazine concentration during incubation at 25 ± 2°C was very slow when no water was added, the degradation potential seemed to have been governed by low moisture content of the soil.
Figure 4.2. Degradation and disappearance of atrazine in soils. Soils, except for Soil VII (atrazine-spiked and aged soil), were treated with atrazine at a rate of 4 mg/kg soil, and incubated at 25 ± 2 °C. For Soil VII, no additional atrazine was added. Initial level of atrazine was about 400 mg/kg soil, and degradation was followed in soil kept dry or water-added to achieve water content of 25%. Error bars represent Least Significant Difference (LSD) between treatment means at given incubation time.
Atrazine biodegradation seemed to be relatively high in typical agricultural soils that may have had previous application of s-triazine herbicide, but was lower in non-agricultural soil (Soil IV, a forest soil). Barriuso and Houot (1996) reported similar results. Factors affecting atrazine degradation rate include the soil pH (a low pH of 4.5 in Soil IV) and organic content (as high as 43% in Soil V).

4.4.2 Creation of atrazine-ACTIVATED soil and compost

Atrazine-degrading microorganisms were enriched in soil by applying atrazine (4 mg/kg soil) for three consecutive treatment cycles. The soil used was Soil I (a Wooster silt loam) found to have a higher atrazine degrading activity than the other soils tested. The changes during enrichment process are shown in Figure 4.3. The extractable atrazine concentration was reduced much faster during the second atrazine treatment cycle, compared to the first atrazine treatment at a rate of 4 mg/kg soil. The extent of atrazine degradation also increased with each treatment cycle; i.e. the equilibrium amount of extractable atrazine remaining in soil was less with each successive treatment.

Based on most probable number (MPN), the number of total bacteria remained almost constant during all three treatments. However, the number of microorganisms utilizing atrazine as a carbon source and nitrogen source increased by about 3 logs and 1 log of magnitude, respectively, after three treatments. In this study, the population changes during enrichment were not monitored after the third treatment cycle of atrazine. However, further enrichment of atrazine-degrading microorganisms might be possible with additional cycles of atrazine application at the same rate, or by a single treatment at a higher atrazine application rate. The rate of atrazine degradation seemed to be relatively
Figure 4.3. Atrazine degradation and enrichment of atrazine-degrading microorganisms in soil. Application rate of atrazine at the beginning of each treatment cycle was 4 mg/kg soil.
easily enhanced, which is in contrast to the established understanding that atrazine is not
generally susceptible to enhanced biodegradation by repetitive application.

The soil prepared by the process described above, which contains enriched
numbers of atrazine-degraders, is hereafter defined as ACTIVATED soil. The
ACTIVATED compost was prepared by inoculation compost with this ACTIVATED soil
at a 1% rate and by treating with atrazine three times at a rate of 4 mg/kg.

4.4.3 Atrazine degradation in ACTIVATED soil

Many chemicals, not toxic at low concentrations and easily biodegraded, are
known to become toxic when concentrations exceed a threshold. For example, among
various factors investigated (soil type, pH, moisture content, organic matter content,
microbial activity, and pesticide concentration), pesticide concentration was found to be
the single most important factor affecting degradation rate of pesticide mixture of atrazine,
captan, carbaryl, 2,4-D, diazinon, fenitrothion, and triflurarin when treatments of 100 and
1,000 mg pesticide/kg soil were compared (Schoen and Winterlin 1987). Degradation
rates in soils treated with pesticide at a rate of 1,000 mg/kg soil were much lower than in
soil treated at a rate of 100 mg/kg soil.

In this study, the degradation rates were compared when atrazine was applied at
different concentrations (4, 40, and 400 mg/kg soil). A short acclimation or lag period
seemed to exist for the 40 and 400 mg/kg treatments, whereas degradation began
immediately in the ACTIVATED soil treated with atrazine at a rate of 4 mg/kg soil
(Figure 4.4A). As shown in Figure 4.4B, the total amount of atrazine degraded in
ACTIVATED soil after 5 days of incubation was positively proportional ($r^2 = 0.999$) to
Figure 4.4. Degradation of atrazine as a function of atrazine concentration in ACTIVATED soil. Degradation of atrazine in ACTIVATED soil expressed as (A) percentage of atrazine remaining and (B) amount of atrazine biodegraded. Graph C is an enlargement of initial degradation (shown in Graph B) up to day 1. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
the concentration of atrazine initially applied to soil. However, initially (i.e. for the first 12 hours of incubation), the amount of atrazine degraded in ACTIVATED soil treated with 400 mg atrazine/kg soil appeared to be less than when soil was treated with 40 mg atrazine/kg, although the differences were not statistically significant (Figure 4.4C).

The overall degradation pattern observed was similar to the results reported by Gan et al. (1996). They found that the amount of atrazine degraded in soils under laboratory conditions also increased proportionally with the increase of concentration of atrazine over the range of 5, 50, 500, and 5000 mg/kg. Their experiments were performed in non-activated soils with relatively low atrazine degrading activity over a long time (40 weeks). A delayed degradation or lag phase was not mentioned. Grigg et al. (1997) reported incomplete and lower extent of atrazine degradation at a very high initial concentration of atrazine (0.046 mole/kg ≈ 10,000 mg/kg, and 0.23 mole/kg ≈ 50,000 mg/kg) by a mixed microbial population. The amount of atrazine degraded when 10,000 mg/kg was applied was less than that for the 50,000 mg/kg rate in both soil or liquid culture. The authors postulated that decreased degradation resulted from atrazine decreasing bioavailability rather than due to inhibitory effect of high concentration. Also, incomplete degradation was attributed to phosphorus depletion.

4.4.4 Effect of inoculation of ACTIVATED soil on atrazine degradation

Inoculation of natural soils with ACTIVATED soil significantly increased degradation of atrazine, and the effects of inoculation rates on atrazine degradation in various soils are shown in Figure 4.5. Atrazine degradation rates and extents increased with increased inoculation rates ranging from 0.05, 0.5 and 5% (w/w) on dry weight basis.
Figure 4.5. Effect of inoculation rate of ACTIVATED soil on degradation of atrazine in soils treated with atrazine. The rate of atrazine applied to soil was 4 mg/kg soil. Error bars represent Least Significant Difference (LSD) among treatment means, excluding that of sterile soil, at given incubation time.
In all soils inoculated with ACTIVATED soil at 5% rate, atrazine concentrations were reduced very rapidly from the beginning of incubation. Several previous studies have shown similar accelerated biodegradation of atrazine in soil augmented with pure culture of isolates, soil with higher atrazine degrading activity, or non-sterile poultry litter.

At low inoculation rates, especially at the 0.05% level of inoculation, enhancement in atrazine degradation was much less as compared to when higher inoculation rates were used (Figure 4.6). This is thought to be due to addition of lower numbers of degrading microorganisms, lower amount of total degradation activity, or to lower bioavailability due to poor contact between inoculant and atrazine. Similar to degradation patterns observed in the non-activated soils (refer to Figure 4.2), inoculation effects were greater in soils with neutral pH and moderate organic matter content. A pH of 4.5 in Soil IV may have inhibited microbial growth or inactivated enzyme function. Lower degradation rate in the high organic Soil V is attributed to either lower bioavailability of atrazine because of sorption to organic matter or presence of other substrates that are preferred over atrazine as a carbon source for the microbial population.

According to Assaf and Turco (1994a), when 200 g of soil was augmented with cells harvested at mid-log phase from 50 mL of medium, 80% and 100% of applied atrazine was degraded after 10 days and 30 days, respectively. Barriuso and Houot (1996) reported enhanced degradation in soil after repeated field application. They also demonstrated that, when a soil (40 g) with low mineralization activity was treated with soil (5 g) with a higher activity, the mineralization, based on $^{14}$CO$_2$ evolved from [U-ring-$^{14}$C] atrazine, of atrazine reached 15% of initially applied (0.42 mg/kg soil) after 82 days of
Figure 4.6. Effect of (A) inoculation rate on atrazine degradation when atrazine was applied at different concentrations and (B) atrazine concentration on atrazine degradation when artificially contaminated soil (Soil II) was inoculated with different rates of ACTIVATED soil. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
incubation, whereas that in non-inoculated soil was 5% of applied. Gupta and Baummer (1996) have also shown that augmenting soils with non-sterile poultry litter resulted in almost a two-fold faster atrazine degradation rate than without addition of litter or addition of sterilized litter. Inoculation of soil, to increase atrazine degradation rate, with a Pseudomonas strain grown in liquid media was less efficient at reduced water contents, limited oxygen supply, and a low pH, and high organic content of the soil (Yanze Kontchou and Gschwind 1995).

The relationship between inoculation rate and atrazine concentration on atrazine degradation in Soil II (Fig. 4.6A) revealed that degradation of atrazine started with a short lag when atrazine was applied at a higher rate (40 or 400 mg/kg). Once started, however, degradation was rapid, even in the non-inoculated soils. It seemed that when atrazine was applied at a higher rate (40 or 400 mg/kg soil), it quickly stimulated atrazine degrading activity. This lag in the development of degradation was not evident at application rate of 4 mg/kg soil, and stimulation of atrazine degradation was not observed during 15 days of incubation. The extent of degradation in non-inoculated soil treated with atrazine at a rate of 4 mg/kg soil was low and almost completely stopped after only 50% of the initially applied atrazine was degraded. The reason for this might be low concentration of atrazine to support increase in atrazine-degrading microbial population. Figure 4.6B shows that a higher rate of inoculation reduces the lag period even at high atrazine concentrations. The microbial population required for rapid degradation was delayed at the lowest inoculation rate (0.05%) but developed rapidly over just a few days of time to achieve rapid atrazine degradation.
Grigg et al. (1997) suggested that a mixed culture could be used for bioremediation of atrazine at concentrations up to and exceeding those reported for agrochemical mixing-loading facilities; e.g., atrazine at agrochemical mixing-loading sites in Illinois occurred at levels up to 1.9 mmole/kg soil (≈ 413 mg/kg soil). This study found that ACTIVATED soil, carrying enriched atrazine degrading microbial consortia, is also an effective bioaugmentation option. Recently, Rousseaux et al. (2003) reported the effect of inoculation of *Chelatobacter heintzii* Cit1 on degradation of atrazine in four different soils. This organisms carries atrazine-degrading *atzABC* and *trzD* genes and is able to mineralize up to 80% of the initially added $^{14}$C-ring labeled atrazine in 14 days in liquid culture. Inoculation of soil with *C. heintzii* Cit1 at $10^4$ cfu/g resulted in a 3-fold increase of atrazine mineralization in soils with low atrazine mineralization activity, while it did not accelerate the rate of atrazine mineralization in soil with high activity for atrazine mineralization. In contrast, Topp (2001) documented that inoculation of the *Pseudomonas* sp. strain ADP, which was able to rapidly mineralize atrazine in pure culture, had no positive effect on atrazine dissipation in soils at inoculum density of $10^5$ cells/g.

**4.4.5 Effect of nutrient and other substrate addition on atrazine degradation**

There have been numerous papers reporting effects of nutrients or other substrate on degradation rates of pesticides in both laboratory and field conditions (Alvey and Crowley 1995, Duquenne et al. 1996, Dzantor et al. 1993, Felsot and Dzantor 1995, Moorman et al. 2001, Schoen and Winterlin, 1987, Thom et al. 1997). Organic amendments often stimulate pesticide biodegradation, but some organic amendments and inorganic N addition can reduce biodegradation. This is also true for atrazine degradation.
According to Struthers et al. (1998), *Agrobacterium radiobacter* J14a can utilize $^{14}$C-U-ring]-atrazine as sole C and N source, and the presence of sucrose affected the mineralization and biomass incorporation of [ethyl-$^{14}$C]-atrazine by the strain J14a. In N-limited medium amended with 50 mg atrazine/L, the mineralization and biomass incorporation was about 10-times and two-times higher, respectively, in the presence of sucrose than in the absence of sucrose after 5 days (Mandelbaum et al. 1993a). Although the inoculation of J14a to soil with low indigenous population of atrazine degraders resulted in significantly higher mineralization of atrazine, the addition of sucrose with J14a showed no additional effect on the mineralization of atrazine in soils either with low or high population of atrazine degraders.

Assaf and Turco (1994b) documented the effect of soil carbon and nitrogen on the mineralization of atrazine in soil, as well as the effect of an external carbon source added to soil. There was no significant change in atrazine mineralization by addition of carbon as mannitol, nitrogen as urea, or both mannitol and urea, at levels of 10, 30, 50, 80 mg/kg. However, a second mannitol application at a 1 g/kg rate resulted in a 17% increase in mineralization of the atrazine metabolites. Similarly, biodegradation rate of s-triazine by a stable mixed bacterial community increased in the presence of other carbon sources (Yanze-Kontchou and Gschwind 1999). In contrast, it has been documented that degradation of atrazine by bacterial isolate M91-3 was inhibited in the presence of exogenous nitrogen (Gebendinger and Radosevich 1999). Gan et al. (1996) have also shown that addition of corn meal and (NH$_4$)HPO$_4$ decreased atrazine mineralization. Ostrofsky et al. (2001) reported that amendment of cyanuric acid, one of the central
metabolites in the degradation pathway of s-triazines prior to ring-cleavage (Karns 1999), had a variable effect on the subsequent mineralization of atrazine in soils tested. Cyanuric acid amendment had a negligible effect on the subsequent mineralization of atrazine in some soils; it enhanced atrazine mineralization in other soils. According to Alvey and Crowley (1995), when the soil was spiked with atrazine at a rate of 100 mg/kg, 73% was mineralized in the absence of organic amendment and 59 to 88 % in soil amended with other organic matter (such as rice hills, starch, and compost). In contrast, only 10%, based on captured $^{14}$CO$_2$, of atrazine was mineralized in soils amended with glucose. They also demonstrated that atrazine mineralization is suppressed under high N conditions in soil.

According to previous works, it seems that the effect of nutrient supplementation or the effect of presence/absence of other substrates on atrazine degradation is dependent on the microbial community or properties of soils, or both. In general, however, it might be expected that nutrient addition does not greatly affect the degradation of atrazine in soils with moderate nutrient contents. In this work, the effect of nutrient addition at a 0.1% level on atrazine degradation (initially added at a rate of 4 mg/kg soil) was investigated for Soil II inoculated with ACTIVATED soil at 0.5% rate, in order to see if further acceleration of atrazine degradation is possible. As shown in Figure 4.7, nutrient addition effect on atrazine degradation was moderate although some treatments effects were significant at certain incubation times. This might indicate that sufficient nutrients required for degradation of atrazine (initially added at a rate of 4 mg/kg soil) were present in this soil.
Figure 4.7. Effect of nutrient addition on atrazine degradation in soil treated with atrazine at a rate of 4 mg/kg soil. The effect of each nutrient addition was tested in Soil II inoculated with ACTIVATED soil at rate of 0.5%. Nutrients were added at 0.1% level. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
4.4.6 Atrazine mineralization

Many metabolites, sometimes more harmful than the parent material, can be generated during microbial degradation. Therefore, breakdown pathways, intermediates, and products are of particular importance in biodegradation of pesticide during remediation. Complete mineralization is, therefore, a desirable characteristic in microbial degradation of pesticides. In atrazine degradation, the s-triazine ring from atrazine is considered toxic and quite recalcitrant to further breakdown.

There have been numerous reports of pathways of atrazine degradation and mineralization by microorganisms, and various pathways, including dechlorination, N-dealkylation, deamination, and s-triazine ring cleavage, have been elucidated (Cook et al. 1985, Erickson and Lee 1989). Bacterial strains initiating each pathway, and genes and enzymes involved in atrazine mineralization have been intensively studied. A summary of our current understanding of how atrazine is biodegraded was provided previously (see to Figure 4.1).

The degradation pathway of atrazine is quite complicated, and this might be one of the reasons why there have been only a few microbial isolates capable of complete atrazine mineralization. It seems plausible that atrazine mineralization in natural environments is facilitated more by microbial consortia than by microorganisms of single species with atrazine mineralization capability.

To determine the relative ease of atrazine activation and to investigate the relationship between atrazine degradation and mineralization in different soils, a separate activation study (described in section 4.3.4) was conducted. In this study, uniformly
ring-labeled $^{14}$C-atrazine was added to both activated and non-activated soils to determine complete mineralization (i.e. CO$_2$) occurred. Atrazine degradation increased greatly in the activated Soils I, II, III, and VI (Figure 4.8). However, no acceleration of atrazine degradation was observed in Soil IV (a forest soil with pH of 4.5). Disappearance of atrazine in this soil seems to be attributed to non-biological process(es), and little $^{14}$CO$_2$ was evolved during 10 days of incubation. The patterns of atrazine mineralization were different from those of degradation. In activated Soils I, II, III, and VI, total amount of atrazine mineralized during 10 days of incubation directly reflected the amount of degraded atrazine. The % of $^{14}$CO$_2$ evolved from triazine ring of atrazine and captured after 7 days of incubation was almost the same in these soils (Soils I, III, and VI), although the rates of $^{14}$CO$_2$ evolution were slightly different during the early stage of incubation. Atrazine degradation and mineralization seemed to occur at the same time in Soils I and IV, but mineralization of atrazine in Soil III was slightly delayed. Similar to degradation, there was no effect of activation on atrazine mineralization for Soil IV. The low pH of this soil might inhibit growth and/or activity microbial populations that could degrade the parent atrazine molecule and then subsequently breakdown of the triazine ring of atrazine. Rapid mineralization in Soil VI seems to be attributed to microorganisms transferred and activated from Soil I or II rather than form Soil III. In contrast, there was no $^{14}$CO$_2$ evolution corresponding to atrazine degraded in non-activated Soil II, but the amount of $^{14}$CO$_2$ evolved in activated Soil II reflected the amount of atrazine degraded.

Soil VII (i.e. the atrazine-spiked and aged soil) was an interesting case. This soil exhibited accelerated degradation, as measured by disappearance of atrazine, but almost
Figure 4.8. Rates of atrazine degradation measured by disappearance of parent compound and atrazine mineralization measured by appearance of $^{14}$CO$_2$ in non-activated and activated soils. Note that the Soil VII (atrazine-spiked and aged soil) was activated by addition of water only and incubation at 25 ± 2 °C for 7 days. Initial level of atrazine before water addition was about 400 mg/kg soil. There was no change in atrazine concentration when water was not added. Error bars represent Least Significant Difference (LSD) between treatment means at certain time of incubation. Means of degradation and mineralization were compared separately.
no mineralization was observed. This may be due to (1) microorganisms capable of breaking the s-triazine ring of atrazine not being present in this soil, (2) the high concentration of atrazine originally present inhibited the enzymes involved in ring breakage, (3) the alkyl side chains may have been removed and supplied (at least initially) sufficient nutrients so that ring breakage was not required for microbial growth of atrazine degraders, or (4) the atrazine mineralization population may have been inhibited by high concentration of secondary metabolites that accumulated during atrazine degradation.

In all soils that showed substantial atrazine mineralization, the amount of $^{14}$C evolved in 10 days of incubation was between about 45 to 55\% of that added. Assuming that some portion of $^{14}$C released from uniformly $^{14}$C-labeled s-triazine ring would be incorporated into cell mass, the extent of atrazine mineralization in these soils is probably much higher than measured.

In general, atrazine degradation (defined as disappearance of parent atrazine) was easily enhanced or activated in typical agricultural soils. In contrast, complete mineralization did not routinely occur, especially where a high rate of atrazine contamination occurred. However, it seems there exists in activated soil, microbial populations capable of complete mineralization of atrazine that can mineralize atrazine provide suitable conditions exist to remove the inhibitory parameters associated with initial high levels of atrazine. In general, disappearance of the parent compound is considered a positive first step in detoxification of atrazine (i.e. the conversion of the parent material to a secondary metabolite) and this did not occur rapidly in all the activated soils.
4.4.7 Compost as an inoculum carrier

Inoculant carriers are known to greatly affect the survival and activity of microorganisms introduced to soil, such as those involved in nitrogen fixation (Van Elsas and Heijnen 1990, Smith 1992). Therefore, inoculum carrier is thought to be one of the important factors determining success of remediation using bioaugmentation, and there have been numerous reports about materials used as inoculum carriers. Microorganisms used to bioaugment remediation of contaminated sites have been introduced to the environment as a liquid culture, immobilized inside or on the surface of solid materials, or by directly enriching in natural medium. Materials used for inoculum carriers include mainly agricultural byproducts such as manure, poultry litter, corncobs, compost, commercial resins, etc. Several advantages of compost as an inoculum carrier have been postulated. Besides serving as supporting materials for the microorganisms inoculated, compost may contribute to survival of inoculated microorganisms by providing them with microniche, thereby preventing them from predation. It may also serve as source of nutrients required for survival and/or for degradation of other compounds.

Compost was inoculated with 1% (w/w) ACTIVATED soil containing enriched numbers of atrazine-degrading microorganisms. The inoculated compost was then further enriched by repetitive applications of atrazine at 4 mg/kg rate. After the third treatment, the atrazine applied to ACTIVATED compost at an initial concentration of 4, 40, or 400 mg atrazine/kg compost was rapidly degraded (Figure 4.9). The extractable atrazine dropped to below 10% of the initially recovered within 3, 6, and 9 hours of incubation when atrazine was applied at rates of 4, 40 and 400 mg/kg soil, respectively. Note that the
Figure 4.9. Atrazine degradation in ACTIVATED compost as a function of initial atrazine concentration. Note that the unit of time here is not ‘days’ but ‘hours’. Error bars represent Least Significant Difference (LSD) among treatment means, excluding those of “Control,” at given incubation time.
degradation patterns, as a function of atrazine rate, is very similar to those found for ACTIVATED soil, but the unit of time here is ‘hour’ and not ‘day.’ Thus, in comparison with ACTIVATED soil, the rate of degradation in ACTIVATED compost was much faster. These observations imply that enriched microorganisms capable of atrazine degradation and complete mineralization had been successfully transferred and their population sizes increased in the compost. Also, based on degradation rate, the number of atrazine degraders that was supported in ACTIVATED compost, compared to ACTIVATED soil, could be much greater.

The atrazine concentration in non-activated compost remained almost constant, and non-inoculated compost was not activated by repetitive application of atrazine at the same rate. Complete mineralization of the atrazine ring to $^{14}$CO$_2$ was also enhanced in ACTIVATED soil (Figure 4.10), similar to results for ACTIVATED soil.

Inoculation of contaminated soils with ACTIVATED compost accelerated degradation of atrazine in soils (Figure 4.11), similar to findings for ACTIVATED soil inoculation. Inoculation of atrazine-contaminated soil with ACTIVATED compost at rates ranging from 0.05%, 0.5%, and 5% resulted in slightly higher activity compared to the ACTIVATED soil, although lower bioavailability of atrazine in compost-amended soil was expected due to sorption of atrazine by the organic matter in compost. Much better results were observed in Soil IV inoculated with compost as compared to soil. This soil has a low pH of 4.5, and the compost inoculant may have increased efficiency by providing buffering capacity. This is another advantage of compost inoculant over soil inoculant.
Figure 4.10. Degradation and mineralization of atrazine in non-activated- and activated compost as measured by disappearance of parent compound and appearance of $^{14}$CO$_2$, respectively. Atrazine was added at a rate of 4 mg/kg soil. Error bars represent Least Significant Difference (LSD) between treatment means at given incubation time. Means of degradation and mineralization were compared separately.
Figure 4.11. Effect of inoculation of ACTIVATED compost on degradation of atrazine in soils. Each target soil was treated with atrazine at a rate of 4 mg/kg soil. Incubation temperature was $25 \pm 2^\circ$C. Error bars represent Least Significant Difference (LSD) between treatment means at given incubation time.
4.4.8 Cross enhancement

Behki and Khan (1994) documented that the strain *Rhodococcus* B-30, previously isolated as an EPTC-degrader by enrichment technique (Assaf 1991), degraded atrazine, propazine, and simazine. It has been reported that either addition of thiocarbamates in the medium or preincubation of the bacteria with EPTC resulted in accelerated rate or stimulatory effect on atrazine degradation by *Rhodococcus* TE1 in liquid culture (Behki 1995). According to this report, the addition of EPTC enhanced the degradation of atrazine by *Rhodococcus* TE1 initially by almost threefold, and the enhancement lasted until the bacterial culture attained its own full degradative capability. In contrast, addition of atrazine had no effect on EPTC degradation. In addition, it has been documented that a single cytochrome P-450 is involved in degradation of EPTC and atrazine by *Rhodococcus* sp. strain NI86/21 (Nagy et al. 1995a). These previous studies imply that cross enhancement of degradation between atrazine and EPTC might be possible.

In order to investigate the possibility of cross enhancement of degradation between atrazine and EPTC, the rates of atrazine or EPTC degradation were compared in non-activated, atrazine-activated, and EPTC-activated Soil I. As shown in Figure 4.12, although it was possible to activate this soil both for atrazine and EPTC degradation, there was no cross enhancement between atrazine and EPTC. There was no effect of EPTC-activation on atrazine degradation, and no effect of atrazine-activation on EPTC degradation. According to previous reports, EPTC-degrading microorganisms primarily from the Genus *Rhodococcus*, and the same gene is involved in both atrazine and EPTC
Figure 4.12. Degradation of EPTC and atrazine in cross-activated soil. (A) EPTC degradation; and (B) atrazine degradation. Application rates of EPTC and atrazine were 20 and 4 mg/kg soil, respectively. Error bars represent Least Significant Difference (LSD) among treatment means at given incubation time.
degradation (Nagy et al. 1995a, b). In addition, a single species can degrade both atrazine and EPTC. However, it seems that cross enhancement of degradation between atrazine and EPTC is not common. The population enhanced in the ACTIVATED soil and compost were very specific in their ability to degrade either EPTC or atrazine.

4.4.9 Effect of storage condition on stability of ACTIVATED soil and compost

Readily usable inoculum is required for immediate availability of inoculant for bioaugmentation and thus successful bioremediation. For practical use of inoculum for bioaugmentation, the inoculum must be stable during long periods of storage. The stability, in terms of atrazine degrading activity, of ACTIVATED soil and compost during storage under various conditions was investigated. The conditions tested for storage included (1) at room temperature without moisturizing, (2) at room temperature with moisture adjustment of 15 - 25% and 30 – 40 % for soil and compost, respectively, (3) at low temperature (10 and 4 °C), and (4) in a frozen state.

The results, both for ACTIVATED soil and compost, were almost exactly the same as that for EPTC-activated soil discussed in Chapter 3 of this dissertation (refer to Figure 3.7). Microbial consortia of atrazine-degraders carried in either ACTIVATED soil or compost were found to be very stable, in terms of degrading activity, during storage at low temperature (10 °C, 4 °C, and -20 °C) for at least for 6 months. In contrast, those microbial consortia were relatively unstable at room temperature, regardless of the moisture content of ACTIVATED soil and compost. According to Barriuso and Houot (1996), moisture content of the soil is one of the factors affecting atrazine mineralization activity: air-drying in laboratory conditions until a residual moisture of 2% caused a loss of atrazine mineralization capability.
4.5 SUMMARY AND CONCLUSION

The rates of atrazine degradation (defined as disappearance of parent atrazine) and mineralization (defined as $^{14}$CO$_2$ evolution from uniformly $^{14}$C-labeled s-triazine ring of atrazine) in natural soils were found to be relatively slow. Depending on soil type and properties, only about 30 to 50% of atrazine added to and initially recovered from non-activated natural soils (4 mg/kg) was degraded after 10 days of incubation at 25 ± 2°C. Based on captured $^{14}$CO$_2$, the amount of atrazine mineralized in non-activated soils during the same period varied from 0 to 25% of radio-labeled atrazine initially added to soil. On the contrary, the rate of atrazine degradation in atrazine-spiked and aged soil was very high, indicating that microorganisms capable of atrazine degradation were enriched or stimulated during aging. However, no mineralization was observed in this soil.

It was found that atrazine degrading microorganisms in Soil I (a Wooster silt loam) are readily enriched during repetitive application of atrazine at a rate of 4 mg/kg soil. After three treatments, the number based on MPN, of atrazine-utilizing bacteria as C- or N-source, increased by about 3 logs or 1 log of magnitude, respectively. Atrazine degradation was accordingly accelerated. Increased levels of atrazine degradation were observed in other soils when treated with atrazine three times at a rate of 4 mg/kg soil. Exception was observed in Soil IV (a forest soil), and this might be attributed to the low pH of this soil (pH of 4.5), resulting in inhibited microbial growth or activity.

Directly enriched soil microbial populations, both atrazine-degrading and mineralizing, were successfully transferred to compost, and inoculation of atrazine-contaminated soil with ACTIVATED soil or compost, resulted in significant
increase in degradation of atrazine in contaminated soil. This result implies that the
ACTIVATED soil and compost are effective inoculants to bioaugment atrazine
degradation in the environments. Also, the use of compost as an inoculum seems
especially promising as it could support much greater activity than soil when compared on
an equal mass basis. The ACTIVATED soil and compost were stable at temperatures
below 10 °C and moisture content above 15% and 30% (w/w, for soil and compost,
respectively) further suggesting the potential usefulness of these materials to bioaugment
the remediation of atrazine-contaminated environments.
REFERENCES


Wang, Y. 2002. Characterizing the structure and diversity of the EPTC-degrading microbial community in soils. Ph.D. dissertation submitted to the Ohio State University, Columbus, Ohio.


