BIODEGRADATION AND PHYTOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBONS USING MUSHROOM COMPOST

by

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(Under the Direction of VALENTINE ASONGU NZENGUNG)

ABSTRACT

Soils contaminated with Polycyclic Aromatic Hydrocarbons (PAHs) are commonly found in petroleum, gas-work and wood-impregnation sites. Interest in the biodegradation and environmental fate of PAHs is motivated by a worldwide ubiquitous distribution, low bioavailability and prolonged environmental persistence. PAHs are toxic and present both human and environmental health hazards; therefore they need to be mineralized to harmless products such as carbon dioxide (CO_2), methane (CH_4) and water (H_2O). Due to high hydrophobicity, however, PAHs tend to interact with solid phases that reduce their availability for microbial attack - the principal mechanism for mineralization.

In this research, laboratory and greenhouse studies were conducted to determine the effectiveness of using natural organic compost amendments to increase the bioavailability, degradation rates, and PAH concentrations in contaminated soils. The results indicate that Organic Compost Tea®, a mushroom compost extract (MCE) rich in dissolved organic carbon (DOC), is capable of increasing the solubility and bioavailability of PAHs. High microbial respiration rates, measured by CO_2 production, and greater microbial numbers in soils treated with MCE, which were > 4 orders of magnitude higher than in the no amendment controls,

indicated enhanced microbial degradation of PAHs. Biodegradation in MCE treated soils was confirmed by the identification of key PAH metabolites such as carboxylic acid, 3,4-dihydroxybenzaldehyde, and 1-benzopyran-2-one. Faster degradation rates, well described by first order kinetics, were also realized with the application of MCE. This was in contrast to the bi-phasic kinetic models determined in the control soils representing initial rapid PAH loss due to sorption followed by a slow transformation phase.

In greenhouse experiments, high PAH concentrations were decreased from 2100 mg/kg to <100 mg/kg within 6 weeks of MCE pre-treatment. This allowed for successful plant establishment while residual PAH concentrations (> 200 mg/kg) in the controls were shown to be toxic to plants. This research indicates that MCE acts as a natural co-solvent to increase PAH solubility in soil matrices, increases microbial degradation in contaminated soils, and decreases PAH concentrations. Highly contaminated sites can therefore be rapidly remediated by the application of compost extracts as pre-treatment with the potential for phytoremediation as a polishing tool.

INDEX WORDS: biodegradation, polycyclic aromatic hydrocarbons (PAHs), mushroom compost extract, phytoremediation, metabolites.

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DEDICATION

This dissertation work is dedicated to my family: my parents Emmanuel and Elizabeth Kodjo-Wayo, my sister Pia, brother-in-law Rupert, and nephew Selorm. Thanks for your unwavering love and support!

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Chapter 1 : INTRODUCTION

1.1 Problem Statement

Soils contaminated with hydrocarbons represent an important environmental problem worldwide. It is estimated that 1.7 to 8.8 million metric tons of petroleum hydrocarbons are released annually into the global environment through spills, leaks, natural seeps, offshore production, transportation, industrial wastewater, and urban runoff (Leahy and Colwell, 1990). The high molecular weight and more recalcitrant fraction of hydrocarbons are dominated by polycyclic aromatic hydrocarbons (PAHs). Polycyclic aromatic hydrocarbons are undoubtedly the most widespread carcinogens in the environment (Harms et al., 2003) and are commonly associated with the disposal of combusted materials or petroleum residues (Suess, 1976).

Polycyclic aromatic hydrocarbons are a group of ubiquitous hydrocarbon compounds that impact both terrestrial and aquatic ecosystems. They exist as two or more fused benzene rings and have low solubilities in water, which results in PAHs having high octanol-water partition coefficients (K_{ow}). This physico-chemical property accounts for their preferential partioning to natural organic matter, limited availability to microbial interaction, and long environmental persistence (Mackay et al., 1999). Polycyclic aromatic hydrocarbons are highly toxic and pose considerable human health risks, thus have generated significant interest worldwide. There are more than one hundred known PAHs, sixteen of which are listed by the United States Environmental Protection Agency (USEPA) as priority pollutants with carcinogenic potential (Keith and Telliard, 1979). They are therefore considered a serious pollution problem (Douben,

2003). In addition, many PAHs had been identified at up to 50% of the 1430 National Priority List (NPL) sites as of 1999 (Olsen et al., 2003).

A number of approved technologies are currently used to remediate PAHs at hazardous waste sites. Biological technologies have been shown to be effective and less costly for *in-situ* remediation of PAHs in comparison to conventional remediation methods such as excavation, incineration, thermal desorption, soil vapor extraction, and chemical oxidation. Additionally, biological technologies are generally non-intrusive and aesthetically pleasing. Some of the conventional treatment technologies may result in the release of high concentrations of hydrocarbons into the atmosphere, further compounding the risk to human health if inhaled. For example, incineration used for the treatment of PAH-contaminated soils, not only causes soils to become sterile due to loss of organic matter but also results in the release of toxic contaminants into the atmosphere. Although soil vapor extraction and chemical oxidation have received increased interest, the total remedial cost of applying these methods is still high. Some of the advantages of using biological processes in site cleanup include cost-effectiveness and wide acceptability by the public.

Biological remediation technologies interchangeably referred to as 'bioremediation technologies' pertain to all types of biologically mediated remediation techniques that employ a range of plant and microbial activities. These techniques include bioaugmentation (i.e., the addition of microbes capable of degrading a contaminant of interest), phytoremediation, and biostimulation (i.e., the addition of suitable amendments to stimulate degradation). Some drawbacks of applying bioremediation technologies are the time it takes to achieve clean-up goals and the limited applicability at highly contaminated sites. This explains why the more expensive and intrusive conventional methods are selected for aggressive cleanup.

Bioremediation of PAHs is impaired by low contaminant availability to microbial metabolism (bioavailability). This results in the low biodegradation rates in soils and groundwater (Cerniglia, 1992). *Bioavailability* is defined as the as the amount of dissolved contaminant available to microbes in the aqueous phase. Sorption of PAHs to sediment and particulate material such as soil organic carbon further decreases PAH bioavailability (Scow, 1993). Also, the increase in toxicity at high PAH concentrations results in a decrease in microbial numbers in the contaminated media and presents additional challenges to site cleanup by bioremediation. The toxic and hazardous nature of PAHs, as well as their general low solubility thus presents a justifiable need to develop new methods to overcome the limitations of bioremediation technologies.

1.2 Polycyclic Aromatic Hydrocarbons

1.2.1 Sources of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons may be naturally formed by pyrogenesis, petrogenesis, and diagenesis, while human activities such as wood, coal, and diesel burning are sources of anthropogenic releases. Forest fires contribute significantly to the pyrogenic release of PAHs due to the incomplete combustion of carbon-containing material or organic matter. This occurs at high temperatures between 500 – 700˚C. Petrogenic PAH releases are globally on the rise due to increased petroleum recovery, production, transport, and use of a broad range of products ranging from light gasoline to heavy industrial oils. In addition, petrogenic PAHs in petroleum escape into the environment as complex mixtures of thousands of aromatic and aliphatic compounds (Burgess et al., 2003). Diagenetic PAHs refers to the formation of these compounds

from biogenic precursors such as plants. Diagenetic processes occur at relatively low temperatures over geologic time scales to produce oil seeps and fossil fuel and commonly form derivatives of PAH compounds such as phenanthrene. Thus, geologic rock units such as oily shale naturally contain detectable and measurable amounts of diagenetic phenanthrene.

Natural sources and some human activities, such as urban runoff and summer barbeques, contribute relatively small amounts of PAHs into the environment. Conversely, industrial activities such as gas manufacturing, wood-impregnation, and petroleum production, transport and storage, are responsible for the major incidents of PAH releases into the environment. Through these sources, the concentrations of PAHs released into the environment have continued to increase over the past 100 years (Jones et al., 1989). Generally, the distribution of PAHs into the environment is very heterogeneous. In wastewater from North America and European municipalities the concentration of total PAHs range from < 1 $\mu g\ l^{-1}$ to over 625 $\mu g\ l^{-1}$ (Yilmaz et al., 1998). The range of PAH contamination in soils is from 5 $mg\ kg^{-1}$ in an undeveloped area to 1,790 x 10^3 $mg\ kg^{-1}$ at a spillage site in an oil refinery (Juhasz and Naidu, 2000), while the concentrations of total PAHs from an industrial creosote production site has been measured as high as 5,863 $mg\ kg^{-1}$ (Ellis et al. 1991).

In petroleum products, PAHs are often present as the residual fraction in lighter weight gasoline range organics and increase in concentration as the fraction of petroleum distillate increases. Diesel is a complex mixture of petroleum hydrocarbons containing volatile, low molecular weight alkanes, heterocyclic compounds, and PAHs and their alkylated derivatives. The latter is also a strong indicator of a petroleum source signal (Hoffman et al., 1984). Diesel has the highest content of total aromatics and PAHs in the medium distillate fuel used in terrestrial environments (Wang et al., 1990). The PAH concentrations in diesel fuel could be

1,500 mg l^{-1} or more (Verschueren, 1996), representing up to 60% of the total hydrocarbon composition (Block et al., 1991).

1.2.2 Structure and Physico-Chemical Properties

Structurally, PAHs consist of carbon atoms that are arranged in a series of adjoining or fused benzene rings (Figure 1-1). As derivatives of the benzene ring, PAHs are thermodynamically stable due to their large negative resonance energies (Mueller et al., 1996). The stability of these multi-ring compounds increases as the number of benzene rings increase. Polycyclic aromatic hydrocarbons are also referred to as polynuclear aromatic hydrocarbons (PAHs) or polycyclic aromatic compounds (PACs), and are generally classified into low molecular weight (e.g. naphthalene) and high molecular weight (e.g. pyrene) compounds.

Table 1-1 summarizes some of the important physico-chemical properties influencing the environmental fate and transport of the sixteen PAHs on the USEPA priority list. There is a distinct variability in the physico-chemical properties of the individual compounds, which corresponds to an increase in the number of benzene rings. Naphthalene (a.k.a. tar camphor), for example, is a 2-ring compound and is also the most soluble (31 mg l^{-1}) PAH compound. This 2-ring low molecular weight PAH is also the volatile of the PAHs, while the remaining compounds are semi to non-volatile and become increasingly insoluble as the number of benzene rings increases. Benzo[ghi]perylene is a 6-ring high molecular weight PAH compound and is the least soluble (0.00026 mg l^{-1}) and a non-volatile (vapor pressure, 1.01×10^{-10} Pa) of the compounds presented in Table 1-1.

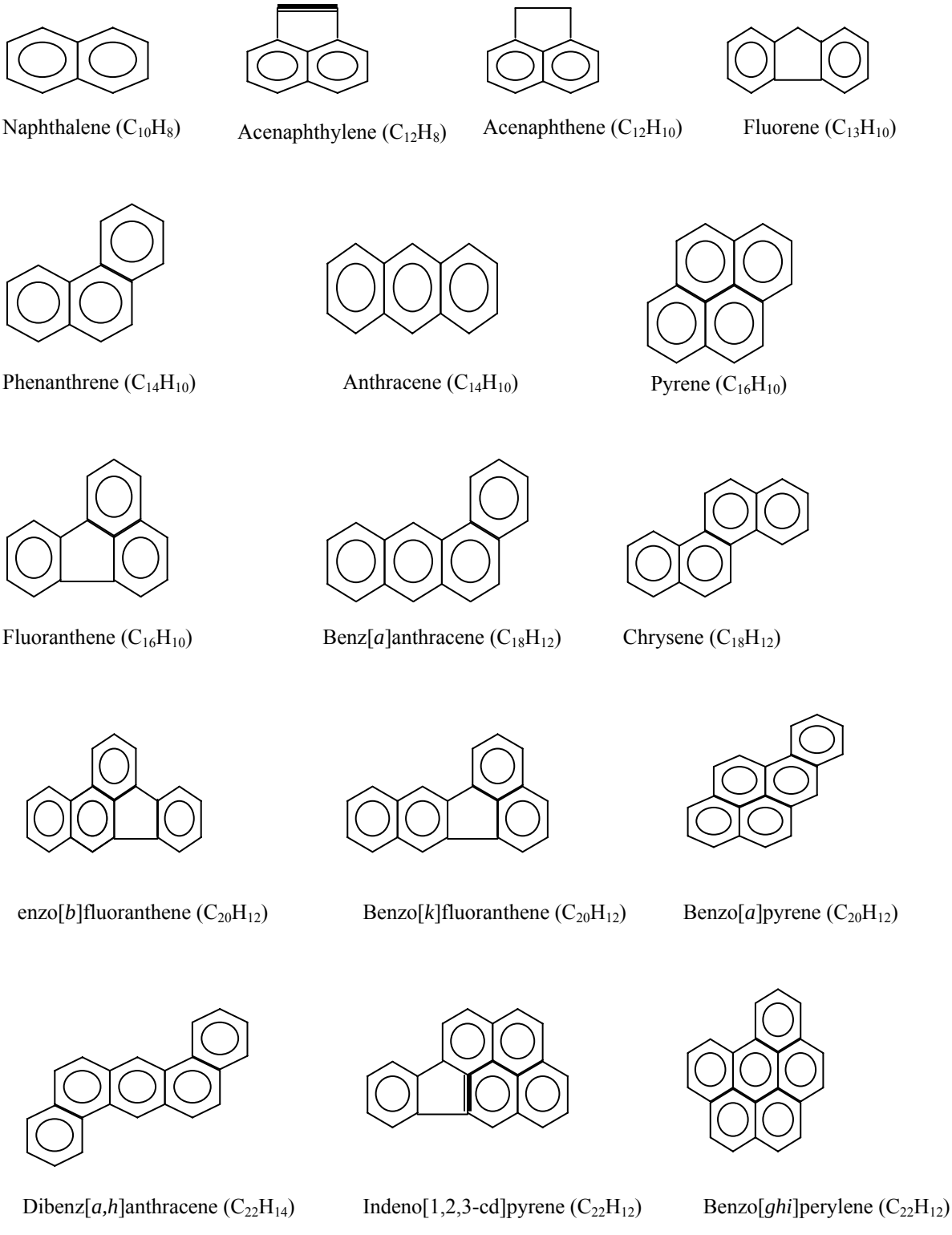

Figure 1-1 Chemical structures of polycyclic aromatic hydrocarbons on US EPA priority pollutant list

Table 1-1 Physico-chemical properties and structure of the 16 PAHs on the USEPA priority pollutant list

	Number of Rings	Molecular weight	Aqueous solubility (mg L^{-1})	Log K_{ow}	Vapor Pressure (Pa, solid)
Naphthalene	2	128	31	3.37	10.4
Acenaphthylene	3	152	16.1	4.00	0.9
Acenaphthene	3	154	3.8	3.92	0.3
Fluorene	3	166	1.9	4.18	0.681
Phananthrene	3	178	1.1	4.57	0.09
Anthracene	3	178	0.0045	4.54	0.02
Pyrene	4	202	0.13	5.18	0.001
Fluoranthene	4	202	0.26	5.22	0.00123
Benzo[*a*]anthracene	4	228	0.011	5.91	0.0006
Chrysene	4	228	0.006	5.91	-
Benzo[*b*]fluoranthene	5	252	0.0015	5.80	-
Benzo[*k*]fluoranthene	5	252	0.0008	6.00	5.20E-08
Benzo[a]pyrene	5	252	0.0038	6.04	7.00E-07
Dibenzo[*a,b*]anthracene	6	278	0.0006	6.75	3.70E-10
Indeno[*1,2,3-cd*]pyrene	6	276	0.00019	7.66	-
Benzo[*ghi]*perylene	6	276	0.00026	7.23	1.01E-10

Source: Mackay et al., 1992; Sims and Overcash, 1983.

Due to their very low aqueous solubility, PAHs are considered lipophilic (hydrophobic) and have a strong affinity for organic matter. Their measured log Kow values, which is the coefficient measuring as the partioning of PAHs between organic phases (e.g., octanol) and aqueous phases (e.g., water), is high.

1.2.3 Sorption and Biodegradation Kinetics

A good understanding of soil-PAH interactions is needed to develop and implement a successful bioremediation system. The high log K_{ow} of PAHs (values ≥ 3) means that this group of compounds is strongly sorbed and not readily available in the aqueous phase. Sorption of PAHs to solids involves both *ab*sorption and *ad*sorption mechanisms (Alexander, 1994).

Absorption occurs when PAHs are transported into the mineral or solid particles by diffusion, advection and dispersion. Adsorption occurs when PAHs adhere to the two-dimensional surfaces of solid particles through physical electrostatic or chemical electron sharing interactions (Evangelou, 1998). Adsorption requires an attraction between the charged mineral surface of the adsorbent and the charged ionic ends of the adsorbate through short-range electrostatic interactions. When mineral surfaces and contaminants react chemically through bond sharing covalent bonds, the mechanism is referred to as *chemi*sorption. Polycyclic aromatic hydrocarbons are primarily adsorbed to mineral surfaces through hydrophobic interactions and/or physical precipitation because they are non-ionic (non-polar).

Bioavailability is of extreme importance because it frequently accounts for the persistence of compounds that may be biodegradable and that might otherwise be assumed to be readily decomposed (Alexander, 1994). There is a need for non-polar and hydrophobic organic compounds partitioning from the solid into the aqueous phase to be microbially degraded. However, strong sorption to the solid phase has generally accounted for the inability of soil microorganisms to metabolize a significant number of PAHs in the environment (Weissenfels et al., 1992). Polycyclic aromatic hydrocarbons can become bioavailable if they are dissolved in the interstitial water between soil particles while being fractionally associated with the rapidly desorbing linear domain portion of organic carbon present (Harms and Bosma, 1997; Burgess et al., 2003). In other words, sorption is reversible when organic compounds associated with particulate matter are re-dissolved into the aqueous phase or if a change in the sorption-desorption equilibrium occurs.

Since sorption, bioavailability, and biodegradation of PAHs are interrelated, a sound knowledge of their contribution in any soil system enables the quantitation of the contaminant

biodegradation kinetics and the determination of the contaminant fraction remaining at a given time. This also allows assessments to be made about the potential degradation of contaminants before they are transported off-site and cause exposure to susceptible organisms (Alexander, 1994). A number of kinetic models are used to describe the degradation of organic contaminants. Some models incorporate the sorption-desorption kinetics of the contaminant compound, and bioavailability to determine the biodegradation rate constants. In an earlier model for example, Furmidge and Osgerby (1967) made use of sorption (k_1), desorption (k_2) and biodegradation rate constants (k_3) to model the biodegradation kinetics of sorbed hydrophobic compounds (C):

$$\text{Sorbed C} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Aqueous C} \xrightarrow{k_3} \text{Products} \qquad (1).$$

The model presented in Equation 1 has been used to show that the partioning and subsequent biodegradation of non-ionic organic compounds (C) between the solid and liquid phases in soils is dependent on the equilibrium sorption-desorption rate constants k_1 and k_2, as well as the actual biodegradation rate constant k_3.

The characteristics of the sorption rate constant (k_1) and the extent of uptake of non-ionic contaminants varies with the soil/sediments composition (i.e., organic-matter and mineral content), particle size fractions, moisture and the medium from which the contaminant is being sorbed (Chiou et al., 1985). For example, the differences in sorption within the silt and clay fractions are largely the result of differences in organic carbon (OC) content (Karickhoff et al., 1979). The clay fraction generally contains a higher concentration of OC than the silt fraction. Thus, high concentrations of soil organic carbon (>0.1%) increase PAH sorption in soils and sediments. Chiou and others (2000) have also suggested that small amounts of high surface-area

carbonaceous material (HSACM) (e.g., wood chars or soot) may significantly change the sorption behavior of soils/sediments for organic contaminants. The use of organic carbon-normalized sorption coefficients, log k_{oc}, has therefore been adopted to account for non-ionic compound partitioning from soil to organic carbon fraction (f_{oc}). In dry soils, sorption is significant and occurs mainly by adsorption to mineral sites. When soils are fully hydrated, adsorption of organic solutes by soil minerals becomes relatively insignificant compared to the uptake by partitioning into soil organic matter, presumably because water is preferentially adsorbed by minerals (Chiou et al., 1985).

Desorption accounts for the dissolution of hydrophobic contaminants from the solid into the aqueous phase. The desorption rate (k_2) of organic contaminants from soil and sediments (Equation 1) can be considered to be a two-stage process, with a labile fraction that desorbs quickly and a refractory fraction that desorbs much more slowly (Reeves et al., 2004). Hysteresis occurs when sorption and de-sorption kinetics are not in equilibrium and limits the reversibility of sorbed contaminants from soil matrices. The hysteresis effect is also affected by the length of time a contaminant is in contact with the contaminated media, and increases with time.

The first-order biodegradation kinetic model is commonly used to estimate the biodegradation kinetics of environmental contaminants in natural systems. In this model (Equation 2), the degradation rate constant (k) is proportional only to the change in contaminant concentration (C) over time (t):

$$-dC/dt = kC \qquad (2).$$

Also, the first order model generally assumes that microorganisms are not increasing in numbers and makes no assumptions about the limiting carbon substrates essential for microbial growth. Other models incorporate microbial growth-linked processes. Examples of such kinetic models

are the Michaelis-Menton and Monod models, which use bacterial enzyme catalysis and microbial growth parameters in the presence of a limiting C-substrate, respectively, to estimate biodegradation rates. The Monod model is mathematically described as:

$$\mu = \mu_{max}\, S/ (K_s + S) \qquad (3).$$

where μ is the specific growth rate of the microorganism, μ_{max} is the maximum growth rate, S is the substrate concentration, and K_s is a constant that represents the substrate concentration at which the rate of growth is half the maximum rate. All kinetic models (Equations 1, 2 and 3) are used to determine the rates at which biodegradation occurs, whether it is linked to the growth of microbial organisms and the use of available C-substrates or not.

1.2.4 Degradation and Transformation Pathways

The ubiquity of PAHs explains the presence of a wide range of microbial consortia: bacteria and fungi that are capable of catalyzing the transformation of PAHs under both aerobic and anaerobic conditions. Common PAH degrading bacteria of the genus *Pseudomonas* and *Mycobacterium* (aerobic bacteria capable of anaerobic denitrification) are also ubiquitously present in pristine environments (Sims and Overcash, 1983). Higher numbers of these bacterial species are found in most PAH-contaminated soils (Carmichael and Pfaender, 1997). It has also been found that microbial metabolism is the most effective mechanism of PAH degradation in soils (Cerniglia, 1992; Wilson and Jones, 1993; Duoben, 2003). Beside the transformation into intermediate products, PAHs can be also be completely transformed or degraded into inorganic CO_2, H_2O, and organic acids by a process known as mineralization.

The mineralization of PAHs requires the presence of an appropriate electron acceptor (Volkering and Beure, 2003). Under aerobic conditions, oxygen is the preferred terminal electron

acceptor (TEA) in the microbial electron transport chain. Microorganisms transport electrons as part of a series of complex metabolic activities that result in the production of energy. Simply, electrons are transferred from reduced organic compounds or inorganic carbon substrates such as PAHs or labile C to the highest oxidized compound (O_2,) on the electron transport chain. This process occurs either by direct metabolism of labile C substrates or by indirect co-metabolism of less labile C-substrates and results in the oxidation of the substrates and simultaneous reduction of the T.E.A. Microorganisms use the energy that is generated from electron transport for high-energy catabolic functions such as growth and reproduction. In the absence of oxygen (O_2), i.e., anoxic conditions, alternate terminal electron acceptors, for example iron (Fe^{3+}), nitrate (NO_3^-), fumarate ($C_4H_2O_4^{2-}$), sulfate (SO_4^{2-}), and carbon dioxide (CO_2) are sequentially utilized to generate energy for microbial catabolism. The use of alternate TEA under anaerobic conditions, however, generates lesser amounts of energy and is therefore not a preferred mechanism for most microorganisms. Also, the decrease in energy generation is particularly lower when less oxidized compounds lower down the electron transport chain are used for microbial catabolic functions. As a result, bacteria preferentially metabolize the PAHs aerobically or anaerobically using NO_3^- as the TEA rather than using CO_2.

The bacterial pathway is the most prominent for PAH transformation under both aerobic and anaerobic conditions. Eukaryotic microorganisms such as the fungus *Phaenerochaete chrysosporium* have also been shown to be significantly involved in PAH transformation, especially in the topsoil (Cerniglia et al., 1992; Kästner et al., 1994). Bacteria and fungi, however, metabolize PAHs differently. Bacteria use PAHs as the sole carbon and energy source. Two atoms of oxygen are incorporated into the PAH molecule in an initial hydroxylation attack by dioxygenase enzyme to form *cis*-dihydrodiols (Figure 1-2). Catechol is subsequently formed

in a second stage that involves the re-aromatization of cis-dihydrodiols by the dehydrogenase enzyme. Fungi, on the other hand, utilize PAHs by metabolizing them into more soluble compounds such as phenols with the help of cytochrome P-450 monooxygenase or lignin peroxidase enzymes. In the fungal pathway B (Figure 1-2), only one atom of the oxygen molecule is incorporated into the PAH to destabilize the aromatic ring and subsequently form a *trans*-dihydrodiol. The second oxygen atom is reduced to water. Fungal metabolism can also generate quinone compounds in reactions that are catalyzed by lignin peroxidase enzymes (Volkering and Beure, 2003). The transformation of PAHs by yeasts, cyanobacteria and algae has not as yet been shown to be important in the fate of PAH degradation in soil systems.

Polycyclic aromatic hydrocarbons are generally considered to be resistant to microbial attack under strictly anaerobic conditions and their limited biodegradation is not well documented (Pothuluri and Cerniglia, 1994). Overall, the reduced state of the un-substituted benzene ring and high thermodynamic stability results in the persistence of PAHs in anaerobic environments. Also, the absence of highly oxidized O_2 to initiate ring cleavage of the reduced compounds explains the unfavorable microbial transformation and persistence of PAHs in anaerobic environments. However, certain chemo-organotrophic bacteria have been shown to obtain energy from the transport of electrons from reduced organic substrates, including low molecular weight PAHs, to alternate TEA under methanogenic and sulfate-reducing conditions (Langenhoff et al., 1996; Zhang and Young, 1997). Unlike aerobic degradation, anaerobic PAH degradation is not ubiquitous in soil systems (Volkering and Buere, 2003).

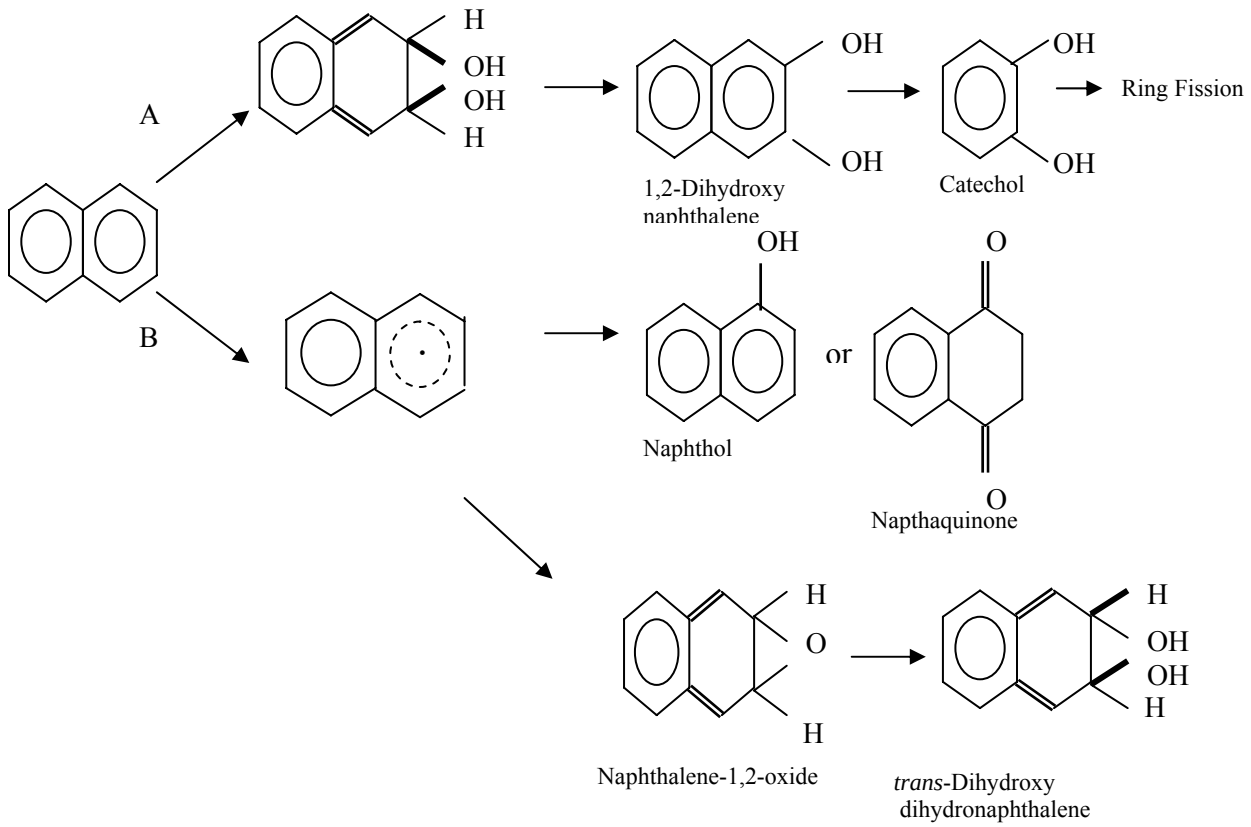

Figure 1-2. General microbial degradation pathway PAHs, e.g. naphthalene; (A) Bacterial pathway - *cis*-dihydrodiol, dehydrogenase enzyme; (B) Fungal pathway – Cyt P-450 monooxygenase (phenol) or lignin peroxidase (quinone). Adapted from Cerniglia and Heitcamp, 1989.

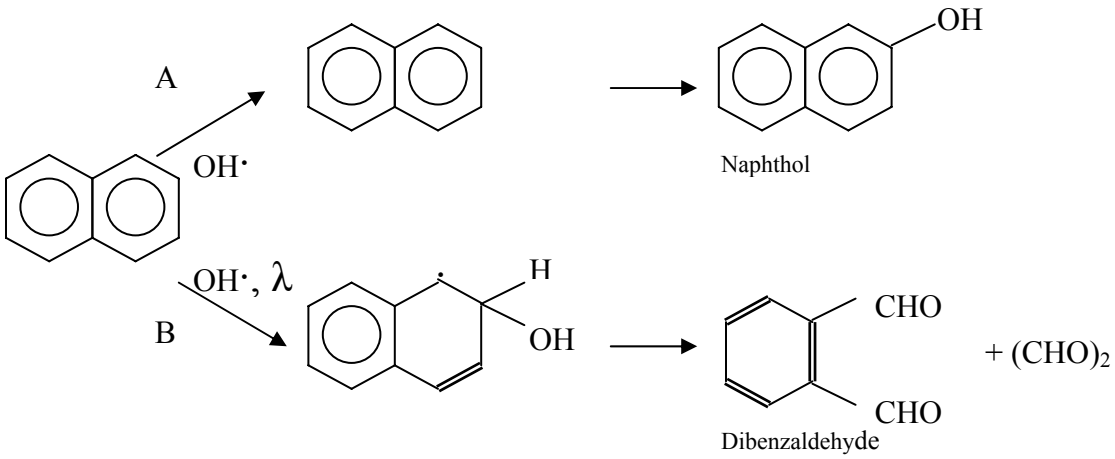

Figure 1-3. Photochemical transformation of PAHs, eg. naphthalene; (A) in the presence of nitrate (NO_2); (B) in the presence of oxygen O_2. Adapted from Arey and Atkinson, 2003.

Polycyclic aromatic hydrocarbons are also transformed and degraded via chemical and photochemical pathways. Similar to the biological reactions, chemical transformations predominantly occurs in the presence of O_2. These reactions are generally referred to as hydroxylation mechanisms and vary in the products formed, depending on the degradation pathway taken. Photochemical reactions transform gas-phase PAHs into polar derivatives in the atmosphere (Figure 1-3). Polycyclic aromatic hydrocarbons are attacked by free hydroxyl radicals ($OH^{\cdot}$) in reactions that may be catalyzed by sunlight as shown in pathway B. This results in relatively short atmospheric lifetimes of about two days (Wania and Mackay, 1996; Arey, 1998). The addition of the hydroxyl (-OH) groups increases the solubility of PAHs and appreciably enhances their degradability. The difference in hydroxylation mechanisms and the metabolites formed under aerobic conditions via the bacterial, fungal, and photochemical pathways, presented in Figures 1-2 and 1-3 can be used to establish the dominant pathways for the degradation of individual PAHs.

1.2.5 Toxicity

All PAHs are toxic and pro-carcinogens that are metabolically activated within the tissues of organisms. Once absorbed, PAHs direct their activity towards the nucleophilic groups of cellular macromolecules (Akcha et al., 2003). Simply, PAHs are absorbed dermally and activated in the organism's cells to cause cell mutations and carcinogenicity. The earliest record of PAH carcinogenicity was of skin cancers documented in London's chimney workers and Germany's coal tar workers in the early 1700s (Eisler, 1987). Currently, benzo[a]pyrene is a confirmed carcinogen and its maximum contaminant level (MCL), set by the USEPA, in drinking water is as low as 0.002 ppb (ASTDR, 1998). Due to the severe human health risks associated with this

ubiquitous and often persistent group of compounds, studies have focused on developing technologies to degrade PAHs in soils and water. Attainment of the cleanup goals for PAH-contaminated sites is often based on the total petroleum hydrocarbons (TPH) remaining in the media or the residual concentration of benzo(a)pyrene.

1.3 Overview of Biological Remediation Technologies

Biological remediation techniques have been applied at numerous sites for the treatment of several classes of organic and inorganic contaminants. Bioremediation technologies involve a series of biologically catalyzed reactions, involving plants, macro-organisms and microorganisms (Table 1-2). Plant catalyzed reactions are referred to as phytoremediation while reactions involving macro- or microorganisms are generally referred to as bioremediation. All three biological systems (plants, macro- and micro-organisms) rely on specific groups of microbial consortia and enzyme systems to effect the degradation of the contaminant of concern.

1.3.1 Phytoremediation

Phytoremediation is the use of plants and the associated root zone microorganisms to decontaminate and manage contaminants in the environment. McCutcheon and Schnoor (2003) broadly define phytoremediation as the use of green plants, fungi, algae, bacteria, and microbial mats in processes that involve three vital plant processes in waste management. These are: (1) phototrophic conversion of sunlight to useful energy and use of atmospheric carbon dioxide to synthesize new biomass, fuel plant, rhizosphere microbial control and metabolism of contaminants; (2) green-liver metabolisms involving transformation, conjugation, and

sequestration of contaminants and the resulting by-products (e.g., fungal lignification); and (3) plant transpiration to control the movement of contaminants in water, soil, and air.

Phytoremediation is cost-effective in comparison to other biological technologies that require the use of deliberately cultured microorganisms (Cunningham et al., 1996). Usually applied *in situ*, phytoremediation has several other advantages including being energy efficient because it is solar driven, highly accepted by the public due to its aesthetic value, and the low amounts of secondary wastes that is generated.

Table 1- 2. Bioremediation Technologies

In-situ	
Biostimulation	The addition of nutrients to stimulation indigenous microbial populations in soils and/ groundwater; *in situ* or *ex situ*
Bioventing	Method of treating contaminated soils by providing oxygen to the soil to stimulate microbial growth and activity
Composting	Aerobic, thermophilic treatment process in which contaminated material is mixed with a bulking agent; can be done using static piles, aerated piles, or continuous fed reactors, *in situ* or *ex situ*
Landfarming	Solid-phase treatment systems for contaminated soils; may be done *in situ* or in a constructed soil treatment cell
Ex-situ	
Bioaugmentation	Addition of bacterial cultures to a contaminated medium; frequently used in bioreactors and other *ex situ* systems. Also applied *in situ* for the decontamination of groundwater systems
Bioreactors	Biodegradation in a container or reactor; used to treat liquids or slurries
Biofilters	Use of microbial stripping columns to treat air emissions
Prepared Bed Reactor	Similar to landfarming but with more engineering controls; *in situ* or *ex-situ*

Phytoremediation is limited by the depth and lateral extent of plant roots. When phreatophytic trees such as poplars and willows are used, plant roots have been shown to reach depths of 5 - 15 m in some specialized systems (Quinn et al., 2001). Other limitations of phytoremediation include contaminant phytotoxicity. As a result, phytoremediation has been used as a polishing tool following source removal by another technology. Phytoremediation is not just one technology but a multitude of biological technologies used to treat a wide range of contaminants. These phytoremediation technologies are listed in Table 1-3 and are briefly described below:

Phytoaccumulation /Phytoextraction – is the ability of plants to uptake unusually large amounts (> 0.1% by dry weight of plant) of contaminants into their above ground biomass (Susarla et al., 2002). Phytoaccumulation is applicable to recalcitrant and non-degradable contaminants such as metals. Hyper-accumulation can also occur when more than 1% of the element or compound, by dry weight basis, is accumulated within the plant tissue; for example, iron, manganese, and arsenic (Dushenkov et al., 1995)

Phytodegradation/ Phytotransformation – refers to the uptake and metabolism or transformation of degradable contaminants mediated by plant enzyme or enzyme co-factors (Dec and Bollag, 1994). Examples of common plant enzymes are peroxidase and dioxygenase, which catalyze oxidation-transformation and reductases which catalyzed reductive-transformation reactions.

Phytostabilization/ Phytosequestration – Involves the immobilization of contaminants of interest by the reduction in their transport in the contaminated media. The main outcome is a

reduction in contaminant availability by immobilizing toxic contaminants in soils or using plants as hydraulic controls to reduce transport from the site of contamination.

Phytovolatilization – refers to the uptake and volatilization of volatile or semi-volatile contaminants by plants. The chemicals are removed from soil or groundwater and transferred into the vapor phase via plant leaves.

Rhizodegradation– Involves the degradation or transformation of contaminants by microorganisms within the plant root's zone of influence, also referred to as the *rhizosphere*. Rhizodegradation is achieved through a symbiotic relationship that exists between plants and soil microorganisms thereby making the rhizosphere an area of very high microbial activity. Microorganisms in the rhizosphere have been shown to be several orders of magnitude higher than those in the bulk soil (Brady, 1990). This phytoremediation process is also called Plant-assisted bioremediation.

Table 1-3. Phytoremediation Technologies

Type	Contaminants Treated	Media
Phytoaccumulation/ Phytoextraction	Arsenic, cadmium, zinc and other heavy metals; radionuclides	Soils
Phytodegradation/ Phytotransformation	PAHs, Munitions (nitrobenzene, RDX, TNT), atrazine; chlorinated solvents (chloroform, polychlorinated biphenyls (PCBs); pesticides	Surface water Groundwater Soils
Phytostabilization	Heavy metals in mine tailings ponds, phenols and chlorinated solvents (tetrachloromethane)	Soils Groundwater Mine tailings
Phytovolatilization	Chlorinated solvents (tetrachloroethane), mercury and selenium	Soils Groundwater
Rhizodegradation	Polycyclic aromatic hydrocarbons, BTEX, Inorganic compounds (nutrients)	Soils Groundwater

1.3.2 Bioremediation

The USEPA has estimated that 30% of polluted sites are currently using bioremediation (Chaparian, 1995). Subject to the treatment location, bioremediation can be either applied *in situ* or *ex situ*. *In situ* applications involve the treatment of the contaminated media in place. Examples of *in situ* treatments are bioventing of hydrocarbon contaminated soils, and bioaugmentation of contaminated soils using microbial cultures or nutrient amendments. *Ex situ* treatments, such as slurry bioreactors, require physical removal by excavation and the transportation of the contaminated soil to an on-site or off-site location for treatment. The latter treatment thus involves higher operation and maintenance costs and is strictly regulated due to the increased exposure of the contaminants and the associated risks to human health. *Ex situ* treatments are therefore not particularly favored or recommended.

Each of these bioremediation technologies involves the stimulation of microbial activity and requires that suitable microbial nutrients and environmental conditions be available. These conditions include optimum pH and temperature, the presence of electron acceptors, availability and sufficiency of inorganic nutrients (nitrogen and phosphorous) and a labile carbon source. Both environmental and nutrient conditions can be optimized to enhance bioremediation. Other factors, such as the physico-chemical properties of the contaminant, will determine the contaminant interactions with the contaminated media and influence their availability for microbial metabolism.

1.3.3 Bioremediation of PAHs in Vegetated Soils

Microorganisms and vegetation have long been used for the treatment of organic contaminants in soils. Most organic contaminants are, however, toxic to soil microorganisms and

plants. The contaminant toxicity to the soil ecosystem is evidenced by stunted growth of plants and a severe reduction in microbial numbers in vegetated soils. Thus, it is always a challenge to grow healthy plants in highly contaminated soils and sediments. Some plant species, for example grasses, may grow at contaminated sites only after natural attenuation has reduced the contaminant concentrations to less toxic levels. For this reason, plants and microorganisms have been advantageously used as sentinel species for the detection of contamination in the environment (Stephenson et al., 1997). Germination tests may also be valuable as rapid assays to determine toxicity of specific contaminants to plant growth (Kapustka, 1997).

The efficacy of biodegradation and transformation of organic contaminants in planted ecosystems depends on the physico-chemical properties of the contaminant and soil, and the health of the microbial community in the root-zone. The selection of a suitable plant species is also important to minimize the toxicity effects to the plant. The octanol/water partitioning coefficient (K_{ow}) of the contaminant is important in that it influences the phytoremediation mechanisms and the biodegradation rate of the contaminant as a whole. Because PAHs are hydrophobic (log K_{ow} values >3), their uptake into the upper plant biomass is limited (Burken, 1996). Hydrophobic PAHs partition into the soil media and accumulate around the roots of plants through sorption. For this reason, the applicable phytoremediation mechanisms are limited to rhizodegradation and rhizostabilization with minimum uptake and phytodegradation. Thus, it is important that the environmental conditions within the soil and the area surrounding the plant's root system are optimized to enhance PAH degradation. This can be achieved by: a) applying nutrients to enhance growth and a healthy development of the root mass, b) increasing the bioavailability of the PAHs by enhancing desorption out of the soil matrices, and c) stimulating or increasing the microbial activity in the root zone of plants in contaminated soils.

Microbial stimulations increase the total microbial numbers and the rate of PAH biodegradation or rhizodegradation (Olsen et al., 2003).

The interaction between plant roots and soil microorganisms can also change the soil environment and increase PAH bioavailability and subsequent degradation rates in the plant rhizosphere. For example, plant roots are able to increase microbial access to the sorbed PAHs by carrying microbes on fine root hairs. Regardless of the increased microbial access, PAH contaminants still need to be in a soluble or aqueous phase to be metabolized by the microorganisms (Harms and Bosma, 1997). Through root turn over or *rhizodeposition*, annual plants such as corn are able to release up to 90% of stored biomass carbon in the form of a wide variety of plant-derived organics into the surrounding soil (Whipps and Lynch, 1985; Lynch and Whipps, 1990). These organics are classified into root exudates, lysates, secretions, plant mucilage and mucigel (Rovira et al., 1979). Root exudates, such as organic acids and carbohydrates provide labile C-substrates for microbial catabolic functions such as respiration and growth. Mucilage is released into the surrounding soil when younger roots slough off C-rich root material (Brady, 1990). These compounds increase the dissolved organic carbon content in soil pore water and are able to decrease the surface tension between contaminants and the surrounding soil medium (Burken, 1997). The solubility of initially hydrophobic PAHs is therefore increased as the contaminants dissolve from the soil matrix into the surrounding pore spaces.

In addition to increasing contaminant bioavailability, rhizodeposits also increase microbial biomass around the roots (Rovira et al., 1979). Soil microorganisms use plant-derived organics for growth and developmental functions. The root-zone microorganisms (bacteria and fungi) have also been shown to be beneficial in catalyzing the degradation of PAHs (Schnoor et

al., 1995). The symbiotic association of fungi with plant roots is known as plant mycorrhizae – meaning "fungi root". It is widespread and affects up to 80% of plant species (Burken, 1996). In the plant mycorrhizae, plants provide root exudates as food for fungi while the fungi make essential nutrients such as nitrates, magnesium, phosphorous and chelated metals such as iron available for plant growth. During rhizodegradation, the fungi provide unique enzymatic pathways to degrade PAHs that cannot be solely degraded by bacteria (Schnoor et al., 1995). More specifically, fungi can break down less labile carbon substrates, such as lignin, into soluble quinone (Cerniglia, 1997). This capability allows fungi to break down high molecular weight PAHs (e.g. pyrene) into soluble quinone compounds.

In summary, the combined influence of bacteria, fungi, and plants may greatly enhance the degradation of PAHs (Canet et al., 2001) and such microbial interactions may be stimulated in the rhizosphere of select plant species (Olsen et al., 2003). Providing abundant nutrient and suitable carbon sources for plant and microbial biomass production, through the application of mushroom compost for example, could facilitate overcoming low microbial numbers and PAH bioavailability limitations. Phytoremediation alone is slow in achieving desirable cleanup goals and many plant species are highly affected by PAH toxicity. *In situ* phytoremediation may therefore be successfully applied along with an appropriately selected bioremediation treatment process that reduces soil toxicity and renders the soil amendable to polishing by phytoremediation.

1.4 Mushroom Compost as a Potential Bioremediation Tool

Mushroom production is the biggest solid-state-fermentation industry in the world (Moore and Chiu, 2001). China and the United States of America (U.S.) are the world's largest producers of mushrooms (Figure 1-4). In the U.S. alone, the consumption of all mushrooms totaled 1.13 billion pounds in 2001, i.e. 21% greater than in 1991 (Lucier et al., 2003). The increase in mushroom consumption has resulted in an equal increase in its cultivation to meet consumer needs. The cultivation of mushrooms involves several different operations, the first stage being the laboratory generation of a pure mycelium of a specific mushroom strain or from several germplasm provided by commercial vendors such as the National Center for Agricultural Utilization Research or Sylvan. The generated mycelium is subsequently grown on a specific bulk-growth substrate, usually a compost, which is prepared from agricultural waste materials such as wheat-straw, hay, horse and poultry manure, cottonseed meal, cocoa shells and gypsum.

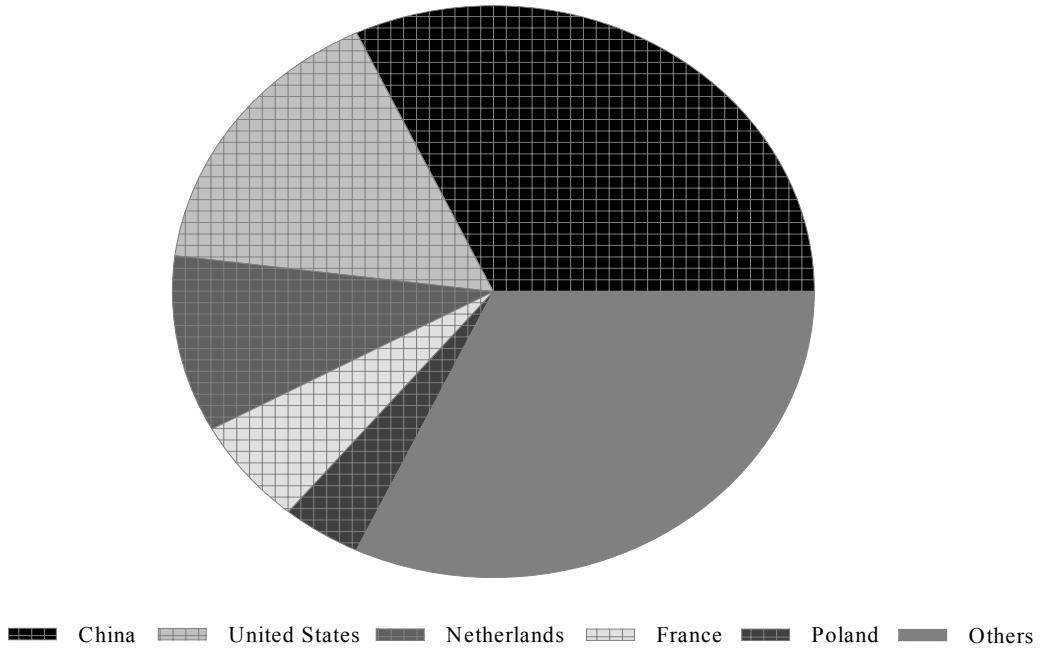

Figure 1- 4. Global mushroom production, 2001. Source: United Nations Food and Agriculture Organization.

Agaricus bisporus, also called the white button mushroom, and its related species are the most commonly cultivated mushrooms throughout the world. *A. bisporus* is typically grown on a substrate in which straw and hay are major components. The enzymes associated with *A. bisporus* have been shown to degrade both natural and

synthetic lignin (Durrant et al., 1991), and also demonstrated to have a lignin-degrading enzyme system similar to that of the wood-rotting fungi (Boonen et al., 1994).

A large amount of by-product, known as spent mushroom compost or substrate, is generated following the harvest of mushrooms. The American Mushroom Institute defines the spent mushroom compost as: "… a composted growing medium that results from the mushroom growing process after harvest". Mushroom compost is a readily available byproduct of the mushroom industry, with 400,000-500,000 (Semple and Fermor, 1997) tons produced per year in the UK, and > 1,000,000 tons in the U.S. alone (American Mushroom Institute). This is approximately 5 times the yearly mushroom production of each country. Accordingly, 5 kg of mushroom compost will be generated from the production of 1 kg of mushrooms (Semple et al., 2001). This requires proper disposal, which is often costly because the substrate is bulky (Chui et al., 2000).

The spent mushroom compost can be re-cycled (Sharma et al., 1999) and used in many applications including re-cultivation of mushrooms, as animal feed, soil conditioner, and as a nutrient amendment in the bioremediation of contaminated soil (Sanchez, 2004). High levels of residual nutrients, for example nitrogen (N) and phosphorous (P) make the mushroom compost a good soil conditioner and fertilizer for stimulating seed germination (Chiu et al., 1998; Semple et al., 2001). On the other hand, the lignolytic enzymes remaining in the substrate after the harvest

of mushroom crops release abundant extracellular and lignocellulolytic enzymes capable of digesting complex carbon substrates, and biodegrading xenobiotics or persistent pollutants including polyaromatic hydrocarbons (Hammond, 1981; Fermor et al., 2000). The mushroom compost waste substrate also has a good pH buffering capacity, since lime is added during preparation of the compost. Moreover, the mushroom compost also harbors a diverse bacteria and fungi consortium, which together with the enzymes help the biodegradation of organic pollutants. Thus, mushroom composts are potentially useful and effective in bioremediation of contaminated soils and sediment (Semple et al., 2001; Lau et al., 2003; Xawek et al., 2003).

Two types of mushroom compost were used in this research: (1) Organic Mushroom Compost®, which is the solid spent mushroom compost substrate used to grow white button mushrooms (*Agaricus bisporus*), and (2) Organic Compost Tea®, which is a liquid extract made from rainwater run-off from the Organic Mushroom Compost stockpile. The compost substrate was prepared from a mixture of turkey and chicken litter, cottonseed meal, lime, wheat straw and Canadian Peat Moss. Both solid and liquid mushroom composts were obtained from Advantage Compost, Duncanville, TX.

1.5 Research Objectives

A review of the published literature reveals the importance of bioavailability of PAHs on the successful application of bioremediation of sorbed contaminants. If advances in research can provide low-cost approaches to overcome this limitation, then bioremediation and phytoremediation should be significantly more cost-effective and desirable than the more invasive conventional treatment technologies currently applied.

The overall goal of the current research was to demonstrate that low cost and widely available agricultural waste products are effective in rapidly decreasing the toxicity of highly contaminated PAHs in aged soils to enable polishing using phytoremediation. In phase one of the treatment process, aged PAH contaminated soils were remediated using solid and liquid mushroom compost as a pretreatment step to enhance the PAH bioavailability and biodegradation. The bioremediated soils pretreated using intrinsic biodegradation were further treated by phytoremediation using corn plants (*Zea Mays*) to polish up any residual contamination. Corn is fast growing and is planted globally. Its root exudates, rich in organic acids and C-substrates, have also been used in phytoremediation of PAHs (Yoshitomi and Shann, 2001). The specific objectives of this research were:

1. To enhance the bioavailability and biodegradation of PAHs in soil (Chapter 3).
2. To determine the optimum concentration of the liquid mushroom compost required to biodegrade PAHs in diesel contaminated soils. This included the determination of appropriate microbial kinetic models to determine growth parameters, as well as propose the degradation pathway of target PAH compounds using identified transformation products (Chapter 4).
3. To determine the relative effectiveness of solid and liquid mushroom compost extract as pretreatments of PAHs in aged diesel-contaminated soils to improve polishing with phytoremediation (Chapter 5).

To achieve these objectives, PAHs in aged diesel-contaminated soils were treated in laboratory and greenhouse studies. The laboratory experiments were conducted in microcosms under aerobic and anaerobic conditions. In the greenhouse, PAH contaminated soils were

bioremediated with mushroom compost amendments and subsequently planted with corn. Laboratory instrumental analysis included the use of the gas chromatograph with a mass selective detector (GC/MS), and gas chromatograph with thermal conductivity and electron capture detectors (GC/TCD and GC/ECD). The GC/MS analysis provided quantitative and qualitative results of the target parent PAHs in the contaminated soils as well as the identity of individual PAHs and their metabolites. The GC/TCD and ECD were used to monitor gaseous by-products (CO_2, CH_4 and N_2O) formed from the metabolic activities of microorganisms in the laboratory slurry reactors. A determination of the microbial population numbers was conducted using the plate pour method. This is a simple microbial enumeration method that was coupled with serial dilution. Other microbial growth parameters such as biomass were established using a Total Organic Carbon (TOC) analyzer. The TOC analyzer was able to provide values to estimate microbial respiration; specifically, inorganic carbon (IC) determined as the difference of total carbon (TC) and dissolved organic carbon (DOC) values. For the establishment of degradation pathways, transformation products were identified and specific genes encoding PAH degradation enzymes present in the soil systems were used to confirm biodegradation and outline pathways based on existing information. A relatively fast and inexpensive microbial fingerprinting method was used in the identification of PAH degrading genes, e.g. *nah* and *phen* genes associated with catabolic enzymes that contribute to the breakdown of the naphthalene and phenanthrene, respectively. Standard analytical methods were used to monitor parent PAH compounds, their transformation products, gases, microbial activity, and nutrients present in the contaminated soils. A quality assurance/quality control method was also adopted which included the analysis of replicates samples, blanks, calibrations check standards and calculation of percent recovery where applicable.

1.6 Research Hypothesis

The bioremediation of PAH-contaminated soils using compost amendments has been the focus of many studies (McFarland and Qiu, 1995; Kästner and Mahro, 1996; Wischmann and Steinhart, 1997; Parrish et al., 2004). Other than the high microbial numbers found to be present in compost materials, studies have shown that compost extracts contain dissolved organic C capable of increasing the solubility of hydrophobic organic contaminants (Janzen et al., 1996). Recent studies also show that the chemicals derived from compost waste materials are rich in humic acids (Quagliotto et al. 2006) and that these humic acids possess surfactant properties with high extraction efficiencies for hydrophobic contaminants (HOCs) similar to commonly used synthetic ionic surfactants (Conte et al. 2005).

Senesi and Miano (1994) and Stevenson (1994) have characterized humic substances in compost extracts and have shown that they are composed of a complex mixture of proteins, carbohydrates, lipids, waxes, aliphatic, and aromatic moieties containing functional groups such as carboxylic acids, phenols, alcohols, amines, amides, Schiff bases, esters, and ethers present in the millimolar (mM) concentration range. These natural acids also contain S- and P-bearing compounds present in the micromolar (μM) to nanomolar (Nm) concentration range (Stevenson, 1994). The practical application of using compost wastes is that the humic acids they contain, at relatively low concentrations, can strongly enhance the solubility of hydrophobic compounds by micelles formation in the bulk water phase (Quagliotto et al. 2006). Solid fractions of humic acids such as those found in soil organic matter and solid compost, have in some research been shown to be responsible for the formation of non-extractable or bound PAH residual in bioremediated soils (Semple et al., 2001; Hartlieb et al, 2003).

In the current research, it is hypothesized that natural mushroom compost extracts, commercially marketed as Organic Compost Tea®, will: (a) enhance the desorption of aged

PAHs in soil through the action of natural biosurfactants in the compost, and (b) enhance biodegradation rates and reduce the toxicity of PAHs through oxidation reactions that are coupled with denitrification. In addition, planting the pretreated soils with corn significantly reduces the residual fraction of PAHs.

Chapter 2: LITERATURE REVIEW

2.1 Assessment of PAH Contamination in Soils

The methods used to determine polycyclic aromatic hydrocarbon (PAH) pollution at contaminated sites have evolved over the years. Currently, the remediation of contaminated soils is conducted based on Clean-up Standards established by the US EPA under the Resource Conservation and Recovery Act (RCRA) of 1976. Under RCRA, for instance, the Superfund Amendments and Reauthorization Act (SARA) 1986, Section 121 established standards to provide rules and specific approaches for remediation (Balba, 1991). The extent of hydrocarbon contamination at sites was traditionally monitored using total petroleum hydrocarbons (TPH) or monoaromatic hydrocarbons such as benzene, toluene, ethyl benzene and xylene (BTEX) (Wilson and Jones, 1993). These target hydrocarbons were also used in contaminant assessment for site remediation, and were composed of the more degradable light molecular weight aliphatic and monoaromatic hydrocarbons only. This conventional monitoring approach thus failed to detect the presence of specific hydrocarbons such as the higher molecular weight and carcinogenic PAHs. Thus, the more recalcitrant PAHs were seldom used as a basis for site remediation (Wilson and Jones, 1993).

In 1978, sixteen PAHs were included on the list of priority hazardous materials contaminants regulated by State and Federal regulatory agencies. With the inclusion of PAHs on the EPA priority list came the need for the identification and degradation of these compounds as a separate group at hydrocarbons at contaminated sites. The remediation of PAHs thus began to

be more carefully reviewed. It was found that PAHs were more difficult to remove using treatments that were successful for the treatment of the more degradable light molecular weight hydrocarbon compounds such as the BTEXs and aliphatic hydrocarbons (Manilal and Alexander, 1991; McGinnins et al., 1988; Wild et al., 1990; Weissenfels et al., 1990). The remediation of PAHs in contaminated soils thus became more aggressive in an attempt to restore sites impacted by these hydrocarbons. The more aggressive remedial approaches, for example combustion and chemical oxidation (refer to Chapter I), tend to be expensive, labor intensive and very intrusive to the environment (Volkering and Beure, 2003). In response to growing concerns over PAH ubiquity and recalcitrance in the environmental and the high costs associated with site cleanup, innovative strategies involving low-cost technologies began to be explored.

In the late 1980s and early 1990s, biological remediation methods involving the use of alternate electron acceptors (Milhelic and Luthy, 1988), plant rhizodegradation (Aprill and Simms, 1990), and application of soil amendments to enhance microbial activity (Liebeg and Cutright, 1999) were considered emerging and innovative. In 1993, the bioremediation of PAH compounds was extensively reviewed by Wilson and Jones. The authors concluded that bioremediation by microorganisms in situ was among the most effective approaches for the degradation of low molecular weight (LMW) PAHs although provision of oxygen and nutrients was important but limiting in the enhancement of degradation. Some important research needs that came out of Wilson and Jones' review were: 1) the need for technologies to increase degradation rates in contaminated soils particularly for high molecular weight (HMW) PAHs, 2) research to investigate and enhance the factors controlling PAH availability and toxicity, and 3) further investigation of innovative bioremediation methods and polishing techniques. Current research studies continue to focus on the research needs identified by the Wilson and Jones

review. There is also an increase in the studies involving the application of microbial remediation (bioremediation) and phytoremediation, sometimes combined in a complementary manner to enhance plant-microbe interactions for the degradation of PAHs (Olsen et al., 2003).

2.2 Biodegradation Kinetics, Sorption and Bioavailability

2.2.1 Biodegradation kinetics

The influence of physico-chemical properties of PAH degradation has been studied and documented in published literature. Park et al. (1990) investigated the aerobic biodegradation rates of PAHs in two soil types and found that the LMW PAHs were degraded faster than the HMW compounds. Park's findings were consistent with earlier studies conducted by Tabak et al., (1981), and Herbes and Schwall (1978) in which both groups concluded that the biodegradability of two- and three-ring PAHs was extensive whereas that of four-, five-, and six-ring PAHs was considerably less significant. More specifically, Sims et al. (1988) conducted laboratory studies that showed that the degradation of two-ring naphthalene in sandy soils was extensive with half-life values of approximately two days. Comparatively, the half-lives for the three-ring PAHs (anthracene and phenanthrene) were 16 and 134 days, respectively. Four-, five-, and six-ring PAHs generally exhibited half-lives of greater than 200 days. McGinnis et al. (1988) performed laboratory-treatability studies on creosote-waste constituents in soil from wood-treatment sites. They found that PAHs with two rings generally exhibited half-lives of <10 days and those with three rings had half-lives of <100 days. However, most four- and five-ring PAHs generally exhibited half-lives of >100 days. Thus, there is general agreement between PAH structure and the rate of biodegradation in soils.

Maliszewska-Kordybach (1993, 1998) correlated the physico-chemical properties of four PAHs with their biodegradation rates in ten different soil types having varying pH and organic matter content at different temperatures. Initially, volatilization accounted for PAH loss in the soils. However, other properties such as PAH solubility in soil pore water (bioavailability) and sorption became more important in determining the degradation of individual PAHs over time. It was found that sorption had the strongest influence on the persistence of PAHs in soils.

Mackay et al. (1992) reviewed and compiled the biodegradation half-lifes of several PAHs from the published literature. Their studies included both laboratory and field investigations of biodegradation rates in different soil types. Generally, the aerobic biodegradation rates have been found to be higher in contaminated soils than in pristine soils with no previous history of hydrocarbon contamination (Cerniglia, 1992). With respect to sandy loam soils, Mackay et al. (1992) established a conservative estimate of mean PAH biodegradation half-lifes that falls within the range of 12 – 3048 days. Half-life values were significantly higher in soils that had an increased organic carbon content. For example, the mean half-life estimated by Wild et al. (1991) was 3176 days for benzo[k] fluoranthene in agricultural soils amended with sewage sludge compared with the significantly lower values of 912 - 2154 days estimated for the same compound by Coover and Sims (1987) in organic-poor soils. The Office of Environmental Health and Hazards Assessment (OEHHA, 2000) recommends that the half-live of all PAHs be set at 570 days, based on the various estimates of biodegradation kinetics.

2.2.2 Effects of Sorption on Biodegradation Kinetics

Following the compilation of PAH biodegradation kinetic parameters (rates, half-lives) by Mackey et al. (1992), many future studies were directed at overcoming biodegradation limitations due to sorption as well as enhancement of PAH bioavailability. The results of many sorption studies have found that PAH partitioning into the soil medium increased in systems rich in organic matter content of soils and sediment (Swarzenbach and Westall, 1981; Alberts et al., 1994; Chiou, 1998; Carmo et al., 2000). Other studies have shown that sorption of PAHs varies with the inter-particle size and soil structure (Nam and Alexander, 2001) and determines the entrapment of PAHs within these micropores (Steinberg et al., 1987). Chiou and Kile (1998) identified that multiple mechanisms were responsible for the partitioning of PAHs (naphthalene and pyrene) to soils and sediments. McCarthy and Jimenz (1985) showed that unlike the partioning to solid organic matter, sorption of PAHs to some fraction of naturally occurring humic substances was reversible. These findings suggest that a change in bioavailability can be achieved depending on the type and nature of organic material present in the soil.

The sorption and biodegradation kinetics studies indicated that PAHs needed to be in the aqueous phase to become available for microbial access (Miller and Alexander, 1991) and increase the rate of degradation. The importance of hydrophobic contaminant (PAH) solubility and desorption from the soil microsites was confirmed by Harms and Bosma (1997) after conducting a study on contaminant mass transfer limitations to microbial activity. Subsequent work by Nam et al. (1998) also showed that increased contaminant bioavailability could be achieved if PAHs were reversibly desorbed out of soil micropores. However, sorption and desorption hysteresis have been shown to be a major limitation for a range of persistent organic compounds including the PAHs (Kan et al., 1998). Thus, desorption of hydrophobic contaminants from aged soils relates to the two-phase sorption model. The model generally

describes a fast initial desorption followed by a slower phase to release the sequestered fraction that is limited by hysteresis (Cornelissen et al., 1997; Williamson et al., 1998). Reid et al. (2000) reviewed many sorption/desorption and bioavailability studies of PAHs and other similar hydrophobic organic contaminants. It was the authors' conclusion that mobility and transport of organic contaminants are important to the enhancement of biodegradation rates. Huessemann et al. (2003), however, proposed that the main limitation to PAH biodegradation in aged soils was not due to mass transfer or bioavailability limitations but rather due to microbial limitations. These microbial limitations could be due to either the absence of specific PAH degraders or cometabolic substrates.

The literature shows that both mass transfer kinetics related to sorption/desorption, bioavailability and microbial numbers are important for the enhancement of PAH biodegradation rates. Few biodegradation kinetic studies (Volkering et al., 1992; Al-Bashir et al., 1994; Traux et al. 1995) have highlighted the direct link between PAH bioavailability and degradation rates to substrate availability and microbial growth kinetics. In Al-Bashir's work, for example, the degradation kinetics of aminonaphthalene was linked with microbial growth kinetics using the Michaelis-Menton model. The results of this study identified three types of PAH recalcitrance resulting from: a) contaminant physico-chemical properties, b) limitations due to enzyme catalysis, and c) limited substrate availability. The important implication of the work conducted by Al-Bashir's group was that an investigation into PAH degradation kinetics and its simultaneous microbial growth kinetics would help distinguish between the three types of recalcitrance that renders PAHs biologically unavailable. Also, the kinetic study involving both contaminant degradation and microbial growth parameters would have important implications for PAH mineralization in contaminated soils.

2.3 Innovative Bioremediation Techniques

2.3.1 Application of Soil Amendments

Earlier studies indicated that the addition of soil amendments enhanced the biodegradation reactions of high molecular weight (Park et al. 1990) and low molecular weight (Volkering et al., 1992) PAHs. Many researchers (Jones et al., 1996; Liebeg and Cutright, 1999; Admon et al., 2001) followed up on this finding by conducting studies that were directly aimed at increasing PAH degradation rates through the application of soil amendments. Different classes of biogenic and non-biogenic materials are currently applied through techniques that are aimed at improving the physical property of the soil or the physico-chemical property of PAHs within the soil matrix. Non-ionic and ionic surfactants, for example, are used in soil bioremediation treatments to enhance desorption of PAHs from the soil matrix and increase biodegradation rates. Non-ionic synthetic surfactants such as Triton X-100 (Rouse et al., 1994; Allen et al., 1999), ionic surfactants such as alkylphenol ethoxylate (Garon et al., 2002), hydroxypropyl-β-cyclodextrins (Cypers et al., 2002), and dialkylated disulfonated diphenyl oxide (Chun et al., 2002), have been used to improve the solubilization of PAHs during bioremediation. It has been observed that synthetic surfactants, including Triton X-100, are toxic to soil microorganisms and lead to the inhibition of biological activity (Sandbacker et al., 2000). In a related study, it was also found that surfactants are limited by clay and silt-sized soil texture (Lee et al., 2002). In a recent study, Conte et al. (2003) compared the effectiveness of biogenic organic surfactants (humic acids) with common synthetic surfactants (sodium dodecylsulfate and Triton X-100), in the washing of two highly polluted PAH contaminated soils of different textures. It was found that the organic humic acid surfactants were equally efficient as the synthetic surfactants and able to remove up to 90% PAHs from both coarse and fine-textured contaminated soils. Conte's group also hypothesized that natural humic acids are able to improve

the soil biomass activity and further contribute to the natural attenuation of washed soils unlike the toxic synthetic surfactants.

Besides increasing desorption rates, many other studies have used soil amendments in techniques to increase the nutrient supply to soil microbes and increase the overall microbial activity during bioremediation. Liebeg and Cutright (1999) recognized two methods to increase microbial activity in PAH contaminated soils: i) biostimulation to increase the activity of indigenous microbial populations by adding nutrients (or carbon substrates) and/ or a terminal electron acceptor (TEA), and ii) Bioaugmentation, which is the addition of foreign or cultured microbial strains. Generally, cultured microbial strains have been effectively used in laboratory experiments for the degradation of PAHs but have not been very successful in field studies. In a bibliographic review, Alexander (1994) gave the following reasons to explain the failures of cultured microbial inoculum in bioaugmentation: a) poor survival in foreign environments due to the presence of predators and parasites, b) inability to compete with the indigenous microbial consortia for available food and energy sources, and c) generally high cost of application especially on large scales. Genetically engineered microorganisms have been used to overcome the adaptation problems associated with inoculated microorganisms. However, the cost of application is still high. Inorganic nutrients such as nitrates, commonly applied as ammonium-N fertilizers, have been used in biostimulation techniques to increase the biomass activity of indigenous microorganisms (Lin and Mendelssohn, 1998; Admon et al., 2001; Sarkar et al., 2005). Nitrates are also are used to increase the oxidative degradation of PAHs. In this case, the degradation of PAHs is coupled with a series of energy-efficient microbial reactions involving the use of nitrate as the TEA. A number of studies have shown PAH degradation under nitrate reducing conditions (Lin and Mendelssohn, 1998). In a related study, Boopathy (2003) showed

the effectiveness of mixed electron acceptors to remediate hydrocarbons in soils contaminated with diesel oil under anaerobic conditions. The author observed 81% removal within 300 days.

2.3.2 Compost Addition and Composting Systems

The activities of the heterotrophic microbial community in soils are driven primarily by the oxidation of organic carbon that enters the soil ecosystem as root exudates, plant litter, manure, compost, or industrially produced waste materials (Tate, 1997). For this reason, many studies focusing on biostimulation and bioaugmentation of contaminated soils use animal biosolids, manure, and compost to increase the degradation soil contaminants. For example, in bioaugmentation studies using animal biosolids, it has been found that at least 60% decontamination efficiency of PAH can be achieved in contaminated soils (Langbehn and Steinhart, 19956; Atagana, 2004; Sakar et al., 2005). Compost and compost materials have also been advantageously used in inexpensive bioremediation applications such as windrow turning and landfarming (Semple et al., 2001). Additionally, the rich microbial consortia found in mature compost have made their application highly favorable and cost-effective as amendments for soil bioremediation, especially in large-scale applications (Martens, 1982). In bioremediation, compost has generally been used either in *in-situ* compost amendment additions or in *ex-situ* composting systems. The dissimilarity between compost and composting is well discussed in a review by Semple et al. (2001). In the former, mature compost is directly added to contaminated soils to biostimulate soil microorganisms or bioaugment the soil with nutrients inherently present in the compost. In contrast, composting is an *ex-situ* processes by which compost is produced from raw materials such as straw and manure.

Composting systems and compost additions are relatively new bioremediation strategies used for the decontamination of PAH contaminated soils. Adenuga et al. (1992) showed that pyrene could be degraded in the composting of soil/sludge mixtures although the rate and extent were not mentioned in this study. In a similar study conducted by Mahro and Kästner (1993), the fate of pyrene in soil and soil composting systems was investigated over a period of 100 days. It was observed that the degradation of pyrene was significantly enhanced by composting with >80% removed after 20 days, while <5% removal was determined in the absence of compost. Another major finding was that 23% anthracene was mineralized to CO_2 while 43% was bound within the compost matrix as non-extractable residues after 103 days. As the bioremediation of contaminated soils amended with compost began to be investigated, it became more uncertain as to whether the decrease in contaminant concentration was actually a result of biodegradation or due to the formation of non-extractable residues (Wild and Jones, 1989). In attempts to reduce the formation of PAH residues, a study was conducted by McFarland and Qiu (1995) in which a fungal inoculum, *Phanerochaete chrysporium*, was added to a composting system made up of household garbage and benzo[a]pyrene contaminated soil. This *ex-situ* study showed that although benzo[a]pyrene appeared to be removed, there was no appreciable difference in final contaminant concentrations between the un-inoculated and inoculated systems. Similar amounts of benzo[a]pyrene were removed during the 95 days of the study (67 and 63%) with the only difference being the faster initial rates measured in the inoculated incubations. The authors therefore concluded that compost additions and composting systems in general could be used as an entrapment mechanism for PAHs, although the long-term environmental fate of benzo[a]pyrene was generally unknown. In another study by Lau et al. (2003), a mushroom-degraded paddy straw substrate previously used as composting material for the cultivation of

mushrooms was used to completely degrade (100% removal efficiencies) naphthalene, phenanthrene, benzo[a]pyrene, and benzo[g,h,i]perylene. In this study, the solid compost material was ground into a powder and used in composting of PAH contaminated soils at 80°C. Similar investigations of PAH degradation in soils amended with mature compost had been conducted earlier and the formation of PAH degradation products were identified (Zink and Lorber, 1995; Wischman et al., 1996; Wischmann and Steinhart, 1997). These metabolites were used to confirm PAH degradation in soils to which mature compost was applied. However, in these latter studies also, high concentrations of residual compounds up to 54% of high molecular weight (HMW) PAHs including benzo[a]pyrene were observed (Wischmann and Steinhart, 1997).

The association of residual compounds as parent PAHs and metabolites with the soil humic fraction was well described by Semple et al. (2001). In their review, the formation of residual fractions was attributed to PAH ageing caused by the entrapment of the hydrophobic contaminants within humic complexes present in compost and compost materials. All of the above findings were later confirmed in a study by Hartlieb et al. (2003) in which it was concluded that the progressive formation of non-extractable residues during bioremediation composting was due to covalent binding of PAHs to solid humic fractions.

The formation of bound residues has been shown to be more extensive for the heavier PAHs. The matrix with which PAHs are associated may also influence their bioavailability. The amount and nature of soil organic C has been proposed by many workers as being one of the most significant factors dominating organic compound interactions within soil (Brusseau et al., 1991; Hatzinger and Alexander, 1995; Cornelissen et al., 1998). In very recent studies, it has also been proposed that soluble compost extracts could be effectively used as biosurfactants to

improve PAH bioavailability and degradation (Quagliotto et al., 2006). These studies are based on previous research that show that compost materials are high in humic acid content therefore capable of acting as natural surfactants to improve the desorption of PAHs from soil matrices (Janzen et al., 1996; Conte et al., 2005). The studies also demonstrate the great potential of using compost extracts to improve desorption rates and increasing microbial degradation. In Quagliotto et al. (2006), a direct confirmation of the reversible sorption mechanics of hydrophobic contaminants attached to natural humic acid compounds, which was initially proposed by McCarthy and Jimenz in 1985, is presented.

2.3.3 Phytoremediation of PAHs

The observation of the ability and the role of natural organic carbon in degrading hydrophobic xenobiotics compounds had earlier on spurned many research speculations on the use of plants and their root exudates to remediate PAH contaminated soils (Hsu and Bartha, 1979, Burken and Schnoor, 1996). Many of the earlier studies on the plant capabilities for treatment of hydrophobic organics were centered on root exudates and their ability to provide available substrates for microbial growth (Banks et al., 1999; Olsen et al., 2003b; Muratova et al., 2003; Rentz et al., 2003, Joner et al., 2003) These studies were based on studies in which it was established that up to 40% of the net carbon fixed during photosynthesis could be released into the plant rhizosphere (Martin 1977; Lynch and Whipps, 1990). Many plants were investigated, including maize, which was found to exude a rich combination of organic acids, sugars and amino acids that were used to support a diverse group of rhizosphere microorganism (Kraffczyk et al., 1984). In a phytoremediation study by Yoshitomi and Shann (2001), for example, it was shown that organic compounds from plant root exudates increased the

mineralization of pyrene. Many studies, including the work by Yoshitomi and Shann (2001) have confirmed that plant exudates are able to degrade PAHs. Yet, the findings in the latter study were in contrast to the increase in the number of xenobiotics degraders observed by Haby and Crowley (1996). Both groups of researchers however agreed that the results from the studies would depend on the microbial community present, soil and plant type, existing environmental conditions and the physico-chemical character of the xenobiotics present. Despite the many advances in root-zone phytoremediation (rhizodegradation), many PAHs remain highly persistent in soil environments (Olsen et al., 2003). It is the suggestion of Olsen et al. (2003) that the goal of supporting rhizosphere bioremediation by providing stimulatory environments for enhanced biodegradation of pollutants by root-associated microorganisms should be considered further. Recently, plants have also been used together with compost bioremediation treatments in an effort to improve the soil conditions, increase the number of xenobiotics degrading microbes, and utilize plants as a polishing tool for the reduction of residual PAHs (Parrish et al., 2004).

2.4 Establishing Biogeochemical Degradation Pathways

2.4.1 Aerobic Degradation

Microbial degradation pathways for PAHs have been elucidated over the years, given that under favorable conditions even recalcitrant compounds will be eventually biodegraded in the environment (Olsen et al., 2003a). The bacterial degradation of low molecular weight PAHs under aerobic conditions has been well established and is the subject of many reviews (Cerniglia, 1984; Gibson and Subramanian, 1984; Cerniglia and Heitkamp, 1989; Cerniglia, 1992; Mackay et al., 1992; Wilson and Jones, 1993; Shutttleworth and Cerniglia, 1995; Sutherland et al., 1995). Bacteria break down PAHs either by direct use of the contaminants as substrates via metabolic or

by co-metabolic transformations leading to final degradation or mineralization. In 1992, Cerniglia et al. conducted an extensive study of aerobic microbial degradation of PAHs. It this study, it was shown that the metabolism of low molecular weight (LMW) PAHs, 2 – 3 ring compounds, was catalyzed by the dioxygenase enzymes of bacteria. The bacterial pathway was found to be very important, as bacterial species such as the *Pseudomonads* were found to be ubiquitously present in the environment (Kästner et al, 1994). In a study of high molecular weight (HMW) PAHs, Bouchez et al. (1995) found that bacterial growth on LMW naphthalene supported the degradation of HMW fluoranthene. The findings by Bouchez et al. (1995) supported earlier works by Gibson et al. (1975) and Gibson and Subramanian (1984) in which the microbial degradation of benzo[a]pyrene, benzo[a]anthracene and fluoranthene was attributed to co-metabolism.

In a pioneering work by Cerniglia et al. (1988), it was shown that the extensive degradation of four-ring PAHs was possible by a group or consortia of microbial species including fungi. In the same year, Mahaffey et al. presented a direct demonstration of ring cleavage in the degradation of high molecular weight PAHs. It was therefore established that bacterial degradation of higher molecular weight PAHs occurred by co-metabolism. The researchers also proposed a direct ring-fission where other substrates such as LMW naphthalene and phenanthrene were used to provide the energy for the breakdown of HMW benzo[a]pyrene (Heitcamp and Cerniglia, 1987; Juhasz and Naidu, 2000; Kanaly and Bartha, 1999). Both metabolic and co-metabolic pathways are however very similar and form the same key intermediate metabolites such as salicylate. Currently, there is only limited information on bacterial-mediated biodegradation of PAHs of five or more rings in both environmental samples, and pure or mixed cultures (Kanaly and Harayama, 2000).

Unlike the bacterial pathway, direct oxidation of PAHs via fungal transformation forms arene oxides or phenols, which undergo further transformation into *trans*-dihydrol end- products (Cerniglia et al., 1992; Müncnerová and Augustin, 1994). The major works in fungal oxidation of selected PAHs were initially described by Cerniglia et al. in 1979, and followed by more extensive work involving fungal consortia such as non-ligninolytic *Cunninghamella elegans* and *Penicillium janthinelum* (Cerniglia et al., 1980; Cerniglia and Crow, 1981; Cerniglia et al., 1982; Cerniglia et al., 1985; Boonchan et al., 2000). Other fungal metabolic reactions were found to occur through a detoxification process in which PAHs are oxidized to epoxides by cytochrome P-450 monooxidase. It was also found that the epoxides were either transformed to phenols through non-enzymatic re-arrangement or enzymatically converted to *trans*-dihydrodiols (Cerniglia, 1984). In 1992, work by Cerniglia elucidated PAH transformation by another important microbial group – a variety of white rot fungal genera. *Phaenerochaete chrysosporium* and the *Bjerkandera* spp. are examples of fungal genera associated with termites and rotting wood, using lignin and lignin-like compounds as their primary food substrate. Ligninolytic fungi oxidize lignin extracellularly by the action of the unspecific enzymes: lignin peroxidases, Mn-dependent peroxidases and laccases (Johnson et al., 2005). *P. chrysosporium* fungi were found to produce ligninolytic enzymes with the capability to degrade high molecular weight PAHs (Cerniglia et al., 1992; Müncnerová and Augustin, 1994). For example, Hammel (1992) found that the products of the peroxidase-catalyzed PAH-oxidations are PAH-quinones. Laccases use molecular oxygen to oxidize phenolic compounds to very reactive, free radicals (Bollag, 1992). The degradation of structurally different PAHs, acenaphthene, phenanthrene, anthracene, 2-methylanthracene, 9-methylanthracene and benzo(a)pyrene, was also found to be catalyzed by laccase purifed from *Coriolopsis gallica* fungi (Pickard et al., 1999). As a result of these

lininolytic enzymes and the mobility of fungal enzymes, the initial attack on HMW PAHs in soil by fungi appears to be more likely than attack by bacterial intracellular enzymes. Fungal enzymes are external and have been shown to advantageously diffuse to highly immobile HMW PAHs. This is in contrast to bacterial PAH-dioxygenases, which are generally cell-bound because they require NADH as a co-factor (Johnson et al., 2005).

2.4.2 Anaerobic Degradation

Unlike aerobic degradation, the anaerobic degradation of PAHs is not well described. Initially, the information on anaerobic degradation of PAHs was limited and unsubstituted PAHs were thought to be resistant to microbial attack under strictly anaerobic conditions (Pothuluri and Cerniglia, 1994). The degradation pathways for 2-ring naphthalene and substituted PAHs were generally well described and new degradation pathways were proposed for naphthalene, 2-methyl naphthalene and tetralin (Annweiler et al., 2002). However, it was not until work conducted by Coates et al. (1996) and thereafter by many other researchers (Coates et al., 1997; Zhang and Young, 1997; Rockne and Strand, 2001; Chang and Yuan, 2002; Boopathy, 2003) that the potential of anaerobic PAH degradation began to be realized. Naphthalene was found to degrade under nitrate-reducing (Bregnard et al., 1996) are under sulfate-reducing conditions (Coates et al., 1996) although the transformation pathways were not described. In further studies, the degradation of other PAHs, phenanthrene, acenaphthalene, anthracene, fluorene and pyrene, was also observed under nitrate-, iron-, sulfate- and methanogenic-reducing conditions as well (Zhang and Young, 1997; Rockne and Strand, 2001; Chang et al., 2001; Chang et al., 2002). The range of PAH degradation when oxygen is not used as the terminal electron acceptor is estimated to vary between 8 –96%. The results of published research generally suggest that the potential of

PAH degradation under anaerobic conditions may be greater than previously recognized (Johnson et al, 2005).

BIOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN COMPOST EXTRACT- TREATED SOILS[1]

[1] Wayo, L.K., Nzengung, V.A., and S. Hassan, 2005. *Proceedings of the 8th International In-situ and On-site Bioremediation Symposium,* Paper F-27.

Abstract

The biodegradation of a mixture of five polycyclic aromatic hydrocarbon (PAH) compounds, naphthalene, phenanthrene, anthracene, fluoranthene and pyrene, was studied in laboratory batch microcosms and mesocosms at 25°C. The PAH-contaminated soils were amended with organic mushroom compost 'tea' (OCT) rich in nutrients. This is the first in a series of studies aimed at increasing bioavailability and biodegradation of high concentration (~100 μg PAH/g soil) PAH-contaminated soils. Contaminated sandy loam soils were treated with 100% OCT for approximately 50 days. The OCT contains very high concentrations of labile dissolved organic compounds, nitrogen, sulfur, and phosphorous. The OCT increased the bioavailability of PAHs in the soil pore water by decreasing sorption. The pyrene degradation rate was 0.0197 day^{-1} in OTC treated soil compared to 0.0024 day^{-1} in the unamended control. Up to 95% naphthalene was degraded after 49 days and up to 75% of the remaining compounds versus 60% in the unamended controls. Evidence of biodegradation of PAHs was supported by observed increase by greater than four orders of magnitude in the microbial count (1.73×10^2 - 2.72×10^6 $CFUg^{-1}$) in OCT-amended soils. We envision the use of nutrient rich OCT as an advance rapid treatment of PAH-contaminated soils followed by a phytoremediation polishing step, especially for highly contaminated soils, to achieve shorter clean-up times.

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous group of organic compounds classified as priority pollutants with carcinogenic and mutagenic potential (Pereira et al., 1999). They are an environmentally persistent group of compounds, especially when the number of rings increases and the aqueous solubility decreases. The presence of PAHs in many ecosystems has been linked to both natural and anthropogenic sources, including the exploitation, refining, storage and transport of petrochemicals by the gas and oil industry.

Over the years, biological remediation methods have been found to be the most effective for organic contaminant degradation (Bento et al., 2005). These include addition of microorganisms (bioaugmentation), providing suitable nutrient amendments (intrinsic bioremediation) and/or the use of plants (phytoremediation). Bioremediation methods are cost-effective with relatively minimal impact on the environment. Some of the more cost-effective bioremediation methods include land farming and biopiles. To date, most studies have focused on the use of solid compost (Parrish et al., 2004), fertilizer (Wilson and Jones, 1993), and/or bacteria inoculations (Boonchan et al., 2000) in bioremediation of PAHs in soils, often producing variable success in reducing the concentrations of the higher molecular weight (four- and five-ring) compounds. Microbial degradation was found to represent the major pathway responsible for the ecological recovery of PAH contaminated soils (Volkering and Breure, 2003). However, relatively long microbial lag phases tend to increase contaminant sorption within soil micropores (Manilal and Alexander 1991). Often, little or no attention is given to the effect of additives, such as fertilizers and compost, on bioavailability and the microbial population – two very important factors that affecting biodegradation. Particularly for hydrophobic contaminants, such as PAHs, reduction in bioavailability increases with increased residence time in the soil (Harms

and Bosma, 1997). Nam and Alexander (1998) directly correlated surface hydrophobicity in soil micropores to reduced microbial bioavailability. This study evaluated the use of liquid nutrient extracts (100% mushroom compost tea) to enhance bioavailability and biodegradation of PAHs in soil. Mushroom farming is a gigantic and thriving industry that generates organic leachate (tea) and compost as byproducts used in landfarming and agriculture. Compost is also rich in a diverse population of microorganisms including pseudomonas, bacilli bacteria and lignin-degrading *Phanerochaete chrysosporium* (white-rot) fungi (Cerniglia et al., 1992). The primary objective of this study was to increase contaminant bioavailability in PAH spiked soils through the addition of liquid mushroom extracts. Secondly, the addition of organic compost tea to stimulate microbial activity and enhance biodegradation of PAH-contaminated soils was determined. The batch samples used in this study were aerated but not actively stirred or mixed. This was done in an attempt to more closely simulate field conditions.

3.2 Materials and Methods

3.2.1 Chemicals, Media, and Nutrient Amendment

Naphthalene in crystal form was purchased from J.T. Baker (Phillipsburg, NJ). Phenanthrene, fluoranthene, anthracene and pyrene at 1000 μg/ml in dichloromethane (DCM) and a 16-compound Polycyclic Aromatic Hydrocarbon standard mixture, 100-1000 μg/ml in DCM, were obtained in analytical grade from Ultra Scientific (North Kingstown, RI). Dichloromethane and methanol were purchased from Fisher Scientific (Pittsburgh, PA). All solvents were of >99% chemical purity.

Bacteriological media, Nutrient Agar (NA) was purchased from Difco Laboratories (Detroit, MI). The composition of NA is described as 3.0 g beef extract, 5.0 g peptone and 15.0 g Agar in 1L. A nutrient amendment, marketed as 100% Organic Compost Tea (OCT) is supplied by Advantage Compost (Duncanville, TX). It is a liquid extract from a mushroom compost pile. The chemical components of OCT are given in Table 3-1. This amendment, rich in major trace elements and organic matter, is used to increase access of nutrients into soil microsites.

3.2.2 Soil

Local sandy clay loam obtained from an uncontaminated site (Riverbend Road; Athens, GA) with no history of hydrocarbon contamination was used in all experiments. The soil was collected from the Ah horizon, dried for 2 hours at 35°C, and run through a #10 sieve (<2 mm mesh size). A particle size distribution of 64% sand, 12% silt and 24% clay was obtained. The soil pH (in 0.01 $CaCl_2$) was 4.8 and the organic matter content was 4%. The soil properties are listed in Table 3-2. Plant roots were removed prior to the measurement of the soil physical-chemical properties and the batch transformation experiment. The dried soil was manually homogenized before use.

Table 3-1. Characterization of 100% Organic Mushroom Compost (Tea) Extract

Analysis	Concentration (mg/l)	Analysis	Concentration (mg/l)
K	2924.00	$NO_3^- + NO_2^-$	0.75
Na	533.20	NO_2^-	0.74
Mg	233.00	SO_4^{2-}	2256.31
Ca	78.21	Total N	346.90
P	8.61	Total P	3.90
Fe	2.60	TOC	2408.21
NH_4^+	361.35	TIC	735.47

Table 3-2. Selected Properties of the Soil used in this Study

Analysis	Value
pH (in 0.01 $CaCl_2$)	4.80
Organic Matter (%)	4.04
Sand (%)	64.0
Clay (%)	24.0
Silt (%)	12.0

3.2.3 Experimental Protocol

The same mass of soil (1 kg) was weighed into a 5-L glass reactor. The soil was spiked with a predetermined volume of a mixture of naphthalene, phenanthrene, fluoranthene anthracene, and pyrene of 100 µg/ml standard solutions in dichloromethane to achieve concentrations of 100 µg PAH/g of soil. To ensure a uniform distribution of the contaminants, the soil was continuously mixed while the PAHs were added. Dichloromethane was then allowed to completely evaporate overnight. 300 g of contaminated soil was subsequently transferred into three batch reactors set up in 1.5-L wide-mouth fruit jars at 25°C.

For abiotic controls, two separate 300 g soils were twice sterilized in their respective 1.5-L jars with a Sterilmatic Autoclave (Market Force Industries Inc.) at 121°C for 15 min. The soils were spiked in their respective glass jars as described above. Two uncontaminated controls were set-up as biotic controls to monitor biological activity. Each jar was wrapped with aluminum foil to minimize PAH losses due to photodegradation and remained partially open throughout the experiment to allow aeration. The reactors were incubated for seven days at 25°C and were subsequently treated with de-ionized water (DI) or nutrient amendment. A 100% organic compost tea was applied to the spiked soil at full strength and as 50% (v/v) aqueous solution. Both liquids, OCT and de-ionized water were applied up to 60% of the soil water holding capacity determined in an earlier experiment using the procedures described in Forster (1995).

A total of seven 1.5 L jars were set up as follows: (A) Contaminated soil + 100% OCT, (B) Contaminated soil + 50% OCT, (C) Contaminated soil + de-ionized water, (D) sterile contaminated soil + 50% OCT, (E) sterile contaminated soil + DI water, (F) non-contaminated soil + DI water, (G) non-contaminated soil + 50% OCT. Samples were taken and analyzed for parent compound and microbial numbers after 0, 7, 21, 35, and 49 days.

3.2.4 Microbial Enumeration.

Viable plate counts of bacteria were determined using 1 g soil sub-samples. These were performed in triplicate using serial dilution pour plates in order to determine the colony forming units (CFU) per gram of soil. A sterile liquid solution was used for making dilutions. This was prepared using de-ionized water and 0.005% Triton X-100 surfactant. The addition of surfactant is required to yield higher recoveries by reducing cell clumping (Danova et al., 1988). Plates were incubated in the dark up to 48 hours at 30°C in a Thelco Model 6M Incubator (GCA/Precision Scientific). Triplicate agar blanks were used as controls.

3.2.5 Analytical Procedures

The concentrations of PAH components were determined by soil extractions and gas chromatography with a mass spectrometer. A relatively simple extraction method based on a shaking procedure developed by Schwab et al. (1999) was used. A 2-g soil mass was weighed into a 20-ml glass vial and 10-ml 50:50 (v/v) dichloromethane: acetone mix added. The vial was covered in aluminum-faced septa and mechanically shaken for 2-h and centrifuged for 15 min at 3000 rpm. Soil sample extractions were carried out in triplicate. The entire extraction procedure was carried out with vials covered with aluminum foil to avoid photodegradation. The clear

liquid supernatant was then quantified. PAH concentrations were quantified by gas chromatography with a mass selective detector (GC/MS). An HP 6890 GC fitted with an ALS 7673 Autosampler and Injector unit set up in splitless injection mode was employed. Injection volume was 1 µl. A 5% phenyl methyl siloxane HP-5MS column (30m, i.d. 0.25mm, film thickness 0.25 µm) was used. Helium was used as the carrier gas at 9.35 psi. The oven temperature program was set at 50°C for 1 min, ramped at rate of 8°C/min up to 200°C and maintained for 5 min before increasing to 250°C at a rate of 20°C/min with a final holding time of 10 min. The injector and transfer lines were heated at 250°C and 280°C respectively. The SIM program of channels was composed of ten molecular ions of PAHs. Initial calibration was performed using a 16-compound PAH standard mixture.

3.3 Results and Discussion

3.3.1 Attenuation of PAH in Soil

The concentration of naphthalene, anthracene, phenanthrene, fluoranthene and pyrene remaining in soil was monitored over a period of 49 days. The results are summarized below in Figures 3 –1 and 3-2. After seven days, the total PAHs in the nutrient treated soils (A and B) decreased by up to 80%. When the experiment was terminated after 49 days, up to 95% of naphthalene and 75% of the remaining compounds were lost. Comparing the nutrient treated soil to the abiotic controls (D and E), there is clear evidence that biotic factors are responsible for the decrease in PAH concentrations. After day 7, an average of 20% PAHs were lost in D and E in contrast to the almost 80% PAH loss in A. After 49 days, 40% of the initial PAHs were still recoverable in the abiotic controls. The decrease in PAH compounds from the controls (D and E)

is attributed mainly to physical and abiotic processes while biological mechanisms contributed to losses in the amended samples (A and B).

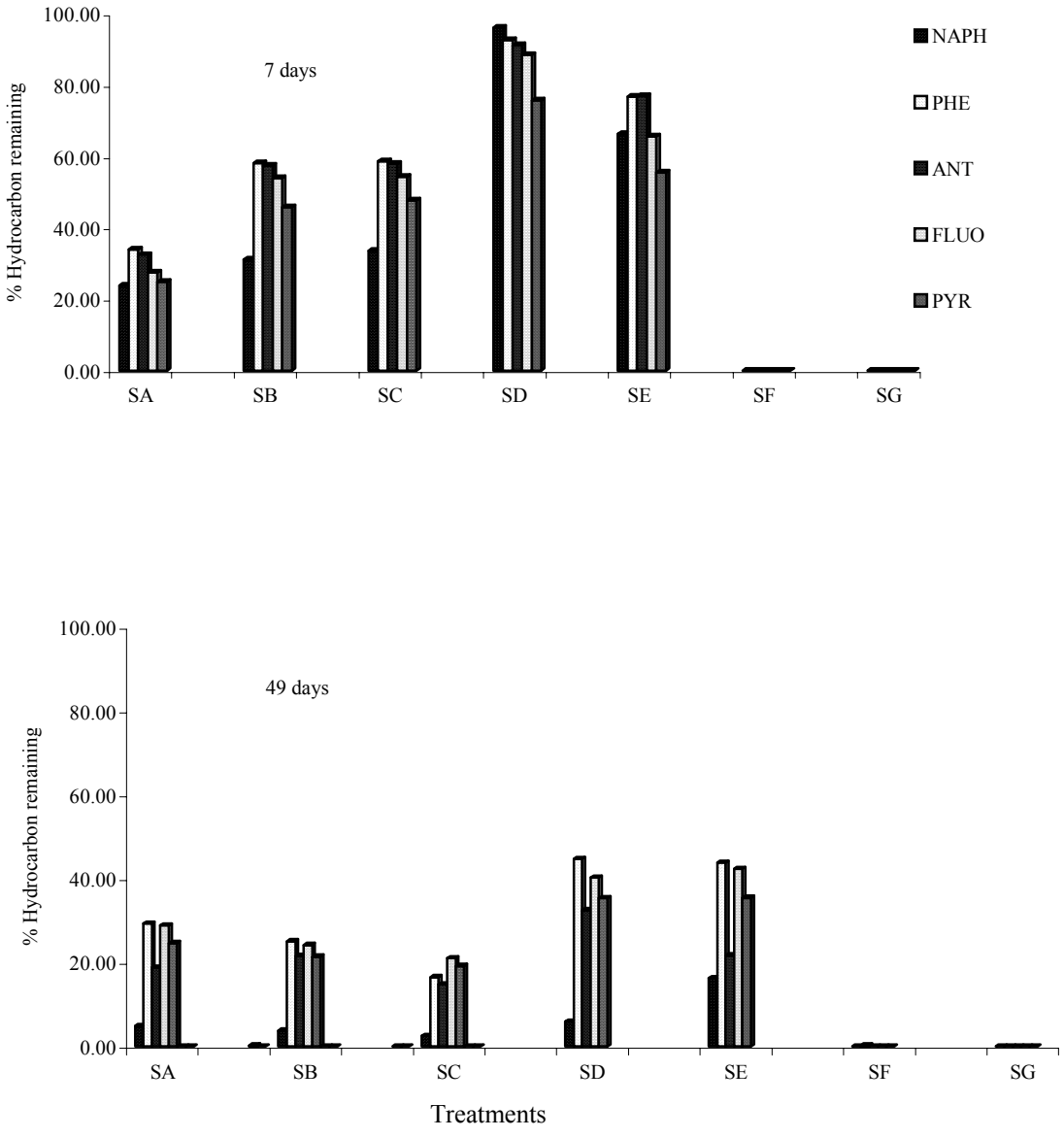

Figure 3-1 and 3–2. Hydrocarbons remaining after 7 and 49 days after treatment using different: 100% mushroom compost (A), 50% mushroom compost (B), de-ionized water (C), sterile + 50% mushroom compost (D), sterile + DI water (E), uncontaminated + DI water (F), and uncontaminated + 50% mushroom compost (G). Legend applies to both graphs.

Table 3-3. Degradation Rate Constants in Microcosm SC[2]

Contaminant	k_1 (day $^{-1}$)	R^2	k_2 (day $^{-1}$)	R^2
Naphthalene	0.0752	0.9433	-	-
Anthracene	0.0761	1.0000	0.0110	0.0995
Phenanthrene	0.0778	1.0000	0.0108	0.1279
Fluoranthene	0.0869	1.0000	0.0050	0.2619
Pyrene	0.1057	1.0000	0.0024	0.0923

Kinetic analysis of the time course data for OCT treatment and de-ionized water treatment is presented in Table 3-3. The decrease in the extractable PAH compounds from the contaminated soil were described by pseudo first-order kinetics as follows:

$$\ln C = \ln Co - kt$$

where C and Co are the initial and final concentrations respectively, t is time and k is the rate constant. The results of OCT-amended soil B (Figure 3-3a) are described by first-order kinetics. Regression values, R^2, are 0.9609 and 0.8342 for naphthalene and pyrene respectively. Figure 3-3b shows that the kinetic data for the PAH-contaminated soil control (C – only de-ionized water added) was not described by pseudo first-order kinetics.

Except for naphthalene, a rapid decrease in concentration of the PAH components was followed by the leveling of the data. Rate constants (k_1) in C, were higher than the corresponding values obtained in treatment B. Specifically, phenanthrene, anthracene, fluoranthene, pyrene concentrations were initially rapidly lost, followed by a leveling off after 14 days of incubation. This observation is characteristic of sorption losses. The complete removal of naphthalene from the unamended controls is attributed to adsorption, biodegradation and volatilization losses. In

[2] Refer to Table A-1 in the appendices for a comparison of the kinetic data in OCT-treated vs. control soils.

previous research (Harms and Bosma, 1997; Admon et al., 2001) naphthalene was observed to apparently degrade at a faster rate than the higher molecular weight PAHs, attributable to greater influence of abiotic factors such as sorption. It should be noted that the soil had about 4% organic carbon, which could support biological activity. The pseudo first-order rate constant estimated for the different compounds ranged from 0.1057 for pyrene to 0.0752 for naphthalene in C and 0.0197 to 0.0528 in OTC soil B. Microbial analysis data presented below was used to confirm that while biotic processes may have contributed to the rapid decrease in the PAH compounds in the OCT-amended soils, abiotic processes likely predominated in the de-ionized water treated controls. In the absence of DOC provided by the OCT, sorption rapidly binds these hydrophobic hydrocarbons to the soil matrix. As the hydrophobicity increases with increasing number of rings, from naphthalene to pyrene, the amount that will be available for biological action decreases. This is supported by kinetic (Figures 3-3a and b) and biological data (Figure 3-4).

The extractability and thus bioavailability of PAHs decreases with increased incubation time (Pavlosthatis and Gossett, 1986). The organic compost tea decreases the propensity of naphthalene, anthracene, phenanthrene, fluoranthene and pyrene to partition into the soil matrix as the solution phase is enriched with high DOC, which acts more like an organic cosolvent; a favorable media for dissolution of the PAHs. The mushroom compost extract as shown below does not only increase bioavailability, but also establishes microorganism populations to the contaminated soil.

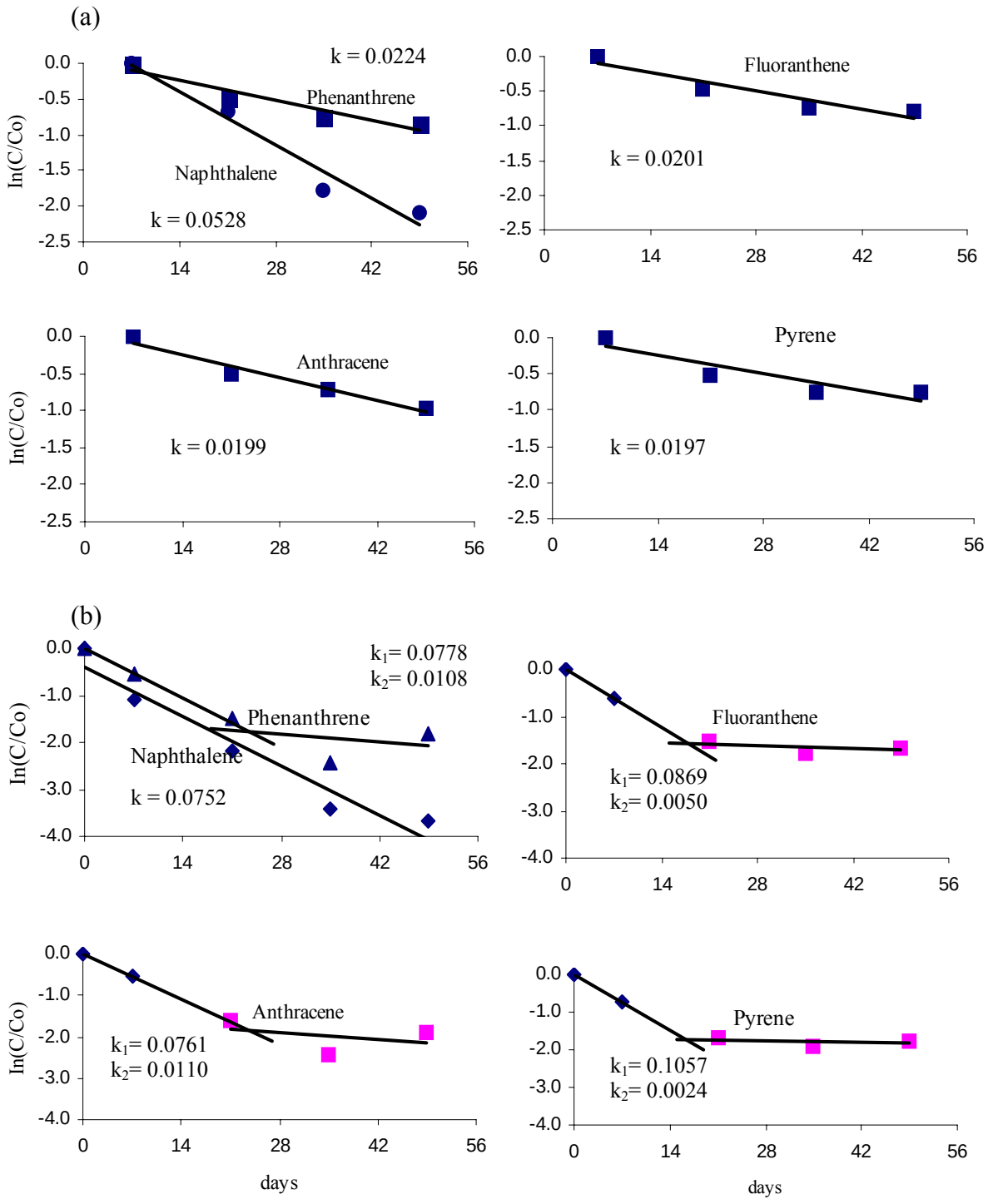

Figure 3-3. Kinetic data depicting: (a) first-order degradation of PAHs in treatment B, and (b) biphasic degradation of PAHs in treatment C, where k_1 and k_2 (day^{-1}) represents the rate constants of two separate degradation stages.

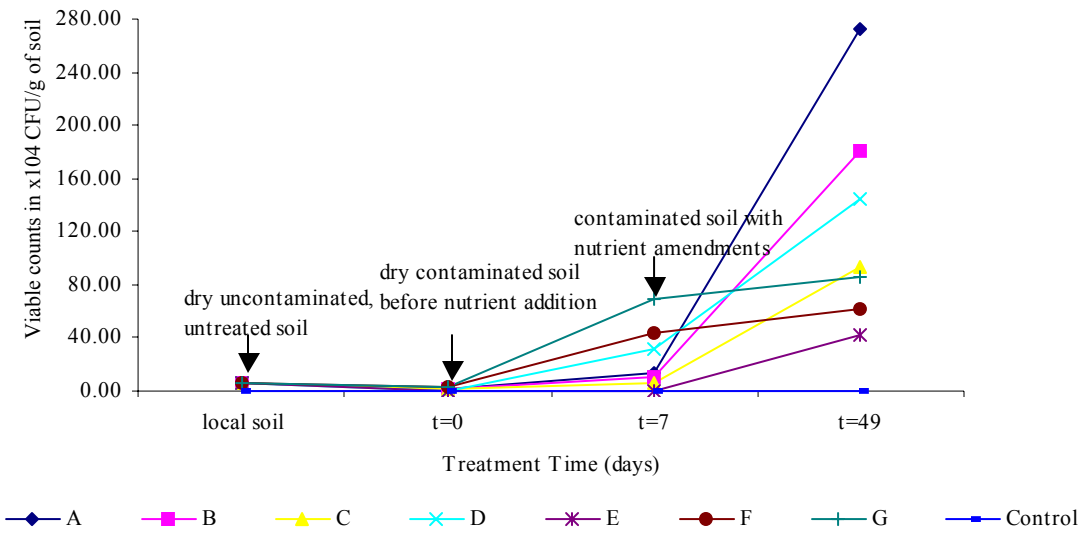

Figure 3-4. Direct measurement of microbial biomass in reactors treated with compost extract and water (control).

3.3.2 Microbial Populations

Counts of viable microbial populations were conducted at the beginning, after treatment, and at the end of the experiment. Initial investigations of microbes grown on the nutrient agar indicated that the PAH concentrations used to spike soils were toxic to the indigenous microorganisms. As a result, the reduction in microbial populations from 5.5×10^4 $CFUg^{-1}$ to 1.73×10^2 $CFUg^{-1}$ was observed (Figure 3-4). After amendment of the contaminated soil with OCT (A, B and D), microbial numbers increased by four orders of magnitude. Soil A had the highest microbial counts of 2.72×10^6 $CFUg^{-1}$ (Figure 3-4). The inference is that more microbial growth was achieved with higher concentration (100%) of the organic compost tea. The observed trend: 2.72×10^6 $CFUg^{-1}$ > 1.80×10^6 $CFUg^{-1}$ > 1.45×10^6 $CFUg^{-1}$ > 9.40×10^5 $CFUg^{-1}$ corresponds to 100% OCT (A), 50% OCT (B), autoclaved soil plus 50% OCT (D) and de-

ionized water (C) treatments, respectively. Generally, contaminated soils with OCT addition had the highest microbial numbers relative to contaminated soils treated with DI water only or the uncontaminated controls. In the uncontaminated soils G and F, the microbial numbers rapidly increased and reached an asymptote as the available organic carbon and nutrients were completely consumed.

These observations suggest that the organic compost tea: 1) stimulated and enhanced microbial growth through nutrient and dissolved organic carbon additions, and 2) enhanced biodegradation as the increased microbial populations metabolized the PAHs. The microbial data provides additional evidence that the losses in amended samples in Figures 3-1 and 3-2 were due to biodegradation and not simply sorption. Only the loss of naphthalene in the unamended control appears to be due in part to microbially mediated transformation.

3.4 Conclusion

The biodegradation of polycyclic aromatic hydrocarbon decreases as the residence time of the contaminant in soils increases. This study has shown that amendment of PAH-contaminated soils with organic compost tea decreases sorption of even the highly hydrophobic PAHs. The OCT does not only increase bioavailability, but also provides nutrients utilized by the soil microorganisms and results in orders of magnitude increases in the soil microbial numbers. Interestingly, the 100% OCT treatment of soils achieves about the same rate of degradation of the high molecular weight PAHs as naphthalene. Our research shows that compost additions to contaminated soils are more effective than adding high quantities of cultured PAH-degrading bacteria. The use of compost has also proven to be an inexpensive intrinsic bioremediation

option. Finally, toxicity to microorganisms and plants at highly contaminated sites will be reduced. Following the more rapid treatment of soils with suitable soils amendments that enhance biodegradation of PAHs, polishing technologies such as phytoremediation can be applied with greater success.

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ANAEROBIC BIODEGRADATION OF PAHs IN DIESEL-CONTAMINATED SOILS USING MUSHROOM COMPOST EXTRACTS[2]

[2] Wayo, L.K., V.A. Nzengung, and S.M. Hassan. To be submitted to *Soil and Sediment Contamination*.

Abstract

The optimum concentration of mushroom compost extract (MCE) for biodegradation of aged diesel-contaminated soils was evaluated in anaerobic batch microcosms. The highest fraction removal of hexane extractable materials (HEM), at an initial concentration of 7684 mg kg^{-1}, was observed in the diesel contaminated soils amended with 50% diluted and undiluted (100%) MCE. The highest percent removal of 79% of the initial HEM in the 50% diluted MCE treatments corresponded with a higher microbial growth rate ($\mu = 0.0496\ d^{-1}$) than the undiluted (100%) MCE treatments ($\mu = 0.0114\ d^{-1}$). Only 4% removal was achieved in the untreated controls. Four PAHs identified in the diesel contaminated soil, acenaphthene, anthracene, pyrene and fluoranthene, were degraded by approximately 95% in the 50% MCE treatments. The decrease in concentration of the PAHs in samples treated with 100% MCE was attributed to sorption. High concentrations of anaerobic gases (CH_4, N_20) detected in the headspace of the reactor vials, together with low concentrations of HEM and PAHs in the 50% diluted MCE treated diesel contaminated soils suggested that the diesel hydrocarbons were removed under anaerobic oxidation. The formation and subsequent reduction of nitrous oxide (N_2O) gas and increase in ammonium (NH_4^+) suggested that MCE-enhanced degradation occurred under denitrifying conditions. Identified PAH metabolites, such as 1-benzopyran-2-one, 1,4-naphthoquinone, carboxylic acid and anthranol, provided evidence that PAH was mostly degraded. The results of this study indicate that aged diesel contaminated soils can be rapidly bioremediated using about 50% strength liquid mushroom compost extract.

Key words: diesel hydrocarbons, PAHs, compost extract, degradation, microbial growth

4.1 Introduction

Biodegradation of monoaromatic hydrocarbons has been shown to occur under both aerobic and anaerobic conditions (Pardieck et al., 1992; Vogel and Grbić-Galić, 1986; Kaiser and Hanselmann, 1982). The aerobic biodegradation of polycyclic aromatic hydrocarbons (PAHs) is thermodynamically favored and has been studied in great detail (Cerniglia, 1993, Heitcamp and Cerniglia, 1987). Meanwhile, anaerobic oxidation of PAHs is less efficient and the process has received less attention (Lei et al., 2005). Many studies reported that PAHs were persistent under anaerobic conditions (Heitcamp and Cerniglia, 1987; Milhecic and Luthy, 1988), yet more recent studies have demonstrated PAH biodegradation in anaerobic soils, sediments, and wastewater systems (Cerniglia and Heitcamp, 1989; Coates et al., 1997). For example, anaerobic biodegradation of lower molecular weight PAHs, such as naphthalene, fluorene, and phenanthrene has been observed under denitrifying (Rockne and Strand, 1998) and sulfate-reducing conditions (Zhang and Young, 1997).

Recent research shows that anaerobic microbial transformation of PAHs is potentially effective and is especially important in the field application of bioremediation, as most subsurface soil systems are anaerobic. The effectiveness of anaerobic biodegradation of PAHs is largely dependent on the availability of carbon substrates, which could be soil organic matter or the PAHs, and the availability of suitable terminal electron acceptors (Pothuluri and Cerniglia, 1994; Hueseman, 1995; Paul and Clark, 1996). In many bioremediation approaches, organic amendments, such as compost, are used to provide labile organic carbon substrates and nutrients (nitrogen, N, and phosphorous, P) to stimulate microbial activity and catalyze hydrocarbon degradation (Alexander, 1994). Although successful mineralization of PAHs in contaminated soils amended with compost has been reported (Lau et al., 2003), other studies have reported

significant sorption of PAHs, due to their high hydrophobicity, creating persistent non-extractable residual fractions in both the compost matrix and the soil (Mahro and Kästner, 1993; Hartlieb et al. 2003). Quagliotto et al. (2006) proposed that soluble compost extracts could be effectively used as biosurfactants to improve PAH bioavailability and degradation. In Chapter 3, it was shown that mushroom compost extracts increased the bioavailability and biodegradation of aged PAHs in soil (Wayo and Nzengung, 2005). By enhancing the bioavailability of PAHs, the liquid mushroom compost extract reduced the pore water surface tension; exerting the properties of organic solvents and surfactants. Although these studies have described the feasibility of PAH mineralization in the presence of compost extracts, no study has focused on microbial utilization of mushroom compost extracts and the extent of anaerobic biodegradation of PAHs in diesel-contaminated soils.

The objectives of this study were: (a) to determine the optimum concentration of organic mushroom compost extract (MCE) required to anaerobically metabolize PAHs in diesel-contaminated soil, (b) to estimate the microbial growth rate for diesel-contaminated soils treated with different concentrations of MCE, and (c) to identify metabolites of the probe PAHs and propose a biodegradation pathway. The characterization of the liquid MCE showed that it was rich in organic carbon (3900 mg L^{-1}) and had moderate concentrations of nitrogen (N, 15 mg L^{-1}). Diesel fuel was chosen because it is a common source of soil contamination and has significant concentration of PAHs (ASTDR, 1990). Diesel fuel is a complex mixture of intermediate distillates composed of approximately 40% n-alkanes, 40% iso- and cycloalkanes, and 20% aromatic hydrocarbons. The lower molecular mass compounds tend to evaporate and degrade more readily during contaminant aging or soil weathering leaving the higher molecular mass components such as PAHs as the residual fraction (Lee et al., 1992). In all experiments,

MCE was provided as the sole substrate or co-substrate to biostimulate the degradation of PAHs in the aged diesel contaminated soil. Microbial growth parameters and respired gases, carbon dioxide (CO_2) and methane (CH_4), were measured and used to estimate microbial utilization rates of MCE. In addition to measuring the loss of extractable PAHs in soil, the production of ammonium-nitrogen (NH_4^+) in solution nitrous oxide (N_2O) in headspace gas and organic carbon utilization were also monitored and used to infer the redox condition for PAH biodegradation. An analysis of PAH transformation products (metabolites) was used to confirm biodegradation in the contaminated soil.

4.2 Materials and methods

4.2.1 Chemicals

A 16 analyte PAH standard mix in dichloromethane was purchased from Ultra-Scientific (North Kingstown, RI). Hexane, dichloromethane and acetone were purchased as HPLC grade from Fisher Scientific (Fair Lawn, NJ). Ether was obtained from Sigma-Aldrich (Milwaukee, WI). Silylating agents used for metabolite derivatization, trimethylchlorosilane (TMCS) and N-methyl-trimethylsilyltri-fluoroacetamide (MSTFA) were purchased from Supelco (St. Louis, MO).

4.2.2 Soil and Mushroom Compost Extract (MCE)

A local loamy sand soil, with no prior history of petroleum hydrocarbon contamination, was collected from the Ah horizon of an uncontaminated site in Athens, NE Georgia, USA. The soil was dried at 35ºC, and run through a #10 sieve (<2 mm mesh size). The particle size

distribution and other physico-chemical characteristics of the soil and MCE are given in Table 4-1. The soil was manually homogenized, contaminated with commercially available diesel, and air-dried to remove the volatile and lighter hydrocarbon fraction, leaving a predominantly heavier and more recalcitrant fraction rich in PAHs. The dried soil was subsequently "aged" for 6 months in a covered steel barrel, after which the concentration was gravimetrically determined as approximately 7684 mg kg^{-1} hexane extractable diesel hydrocarbon material including PAHs. The total extractable PAH concentration in the soil was determined by gas chromatography with mass selective detector as 419 mg kg^{-1}, of which acenaphthene had the highest concentration of approximately 270 mg kg^{-1}. Alkyl-PAHs, such as 2,4-dimethyl naphthalene and 2-methyl phenanthrene were also present and qualitatively analyzed (Figure 4-1).

Table 4-1 Chemical composition of MCE, and soil after initial diesel contamination.

Mushroom Compost Extract (mg l^{-1})	Value[a]	Soil	Value
Fe	1.2	Sand (%)	66.0
Na	1012	Silt (%)	11.0
NH_4^+	31.4	Clay (%)	23.0
SO_4^{2-}	202.0	Equivalent water pH	5.4
TC/DOC	3876/36 05	OM (%)	3.9
Ca	78	Ca (mg kg^{-1})	232.4
K	3534	K (mg kg^{-1})	119.8
Mg	343.1	Mg (mg kg^{-1})	61.6
		Mn (mg kg^{-1})	17.8
Total P	14.8	P (mg kg^{-1})	9.8
Total N	10.3	Zn (mg kg^{-1})	1.6
Equivalent water pH[b]	8.4	Hydrocarbons in mg kg^{-1}	
		Total extractable diesel hydrocarbon material (HEM)	7684
		PAHs in mg kg^{-1}	
		acenaphthene	270.9
		anthracene	29.7
		pyrene	74.9
		fluoranthene	43.5

[a] mean values of duplicate analysis; [b] no units

A VG Elemental Plasma Quad III Inductively Coupled Plasma-Mass Spectrometer was used to conduct elemental analysis of the MCE. The total carbon (TC) determined on a Shimadzu 5050A Total Carbon analyzer at 680ºC without samples acidification was 3876± 41 mg l^{-1}. The same instrument was also used to measure inorganic carbon (IC) and dissolved organic carbon (DOC) concentrations. Samples analyzed for DOC were dosed with 1% HNO_3 by volume to remove inorganic carbon. Blanks and check standards were included for QA/QC.

4.2.3 Batch Microcosms

In an initial microcosm study conducted at 25°C for 168 hr, 30 g of uncontaminated soil was weighed into 100 ml glass vials that had been previously autoclaved for 15 min at 121ºC in a Sterilmatic Autoclave (Market Forge Industries Inc.). The autoclaving of the vials ensured that the indigenous microbes in the soil and MCE amendment would dominate in the treated samples. Aqueous solutions (30 ml) of MCE were added to uncontaminated soil as 100%, 50%, 25%, 10%, 5%, and 0% dilutions. The 0% dilution represented the untreated control, which consisted of 30 ml de-ionized water added to the uncontaminated soil. A second batch of samples was set-

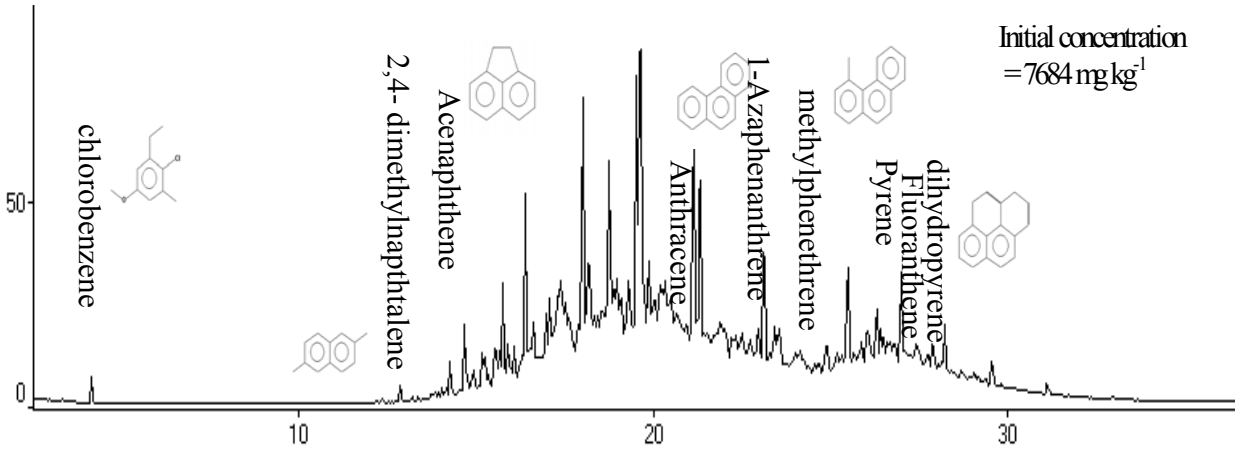

Figure 4-1. GC-MS scan of initial diesel-contaminated soil. The names and structures of the identified hydrocarbons are directly above or immediately adjacent to the top of peaks.

up similarly in duplicate and used to monitor the change in microbial biomass in the presence of aged diesel-contaminated soil. The bacterial enumeration method was by plate count. This was conducted every 12 hr from 0 – 168 hr. At each 12 hr interval 0.5 ml aqueous samples were collected from both the initial and second batch of microcosms for DOC analysis. The measurement of DOC was used to determine changes in MCE concentrations due to microbial metabolism during the incubation of the samples. Each sample was diluted, filtered and acidified to remove any inorganic carbon.

A third set of batch vials was similarly prepared as above with contaminated soils. The purpose was to measure biochemical parameters such as CO_2 and CH_4 production, which is an indicator of microbial respiration, and changes in DOC concentrations to estimate microbial utilization of MCE in long-term (42 days) experiments. Prior to adding the amendment, the diesel-contaminated soil in each vial was purged with argon (Ar) gas to remove any residual volatile hydrocarbons in the soil. The vials were crimp sealed with Teflon-lined caps. Altogether, 6 treatments were set-up in triplicate to obtain a total number of 18 batch reactors. Periodic sampling was conducted by withdrawing 5 ml headspace gas with a gas-tight syringe. One to two milliliters of the solution phase were sampled for carbon (TC and IC) and nutrient (NH_4^+) analysis on days 3, 8, 11, 15, 22, 29, 36, and 40. After each gas and solution sampling, an equal volume (5 ml) of laboratory grade standard atmospheric air was used to replace the withdrawn headspace gas to maintain a constant gas volume and delay the on-set of anaerobic conditions.

At the termination of each set of experiments, the batch vials were sacrificed, completely extracted, and gravimetrically analyzed for petroleum hydrocarbons following EPA Method 1664. This method extracts total petroleum hydrocarbons and their polar phases (Huesemann, 1995). The vials were centrifuged at 2500 rpm for 20 min to separate the liquid and solid phases.

Exactly 20 ml of the supernatant was extracted three times to give 60 ml n-hexane (3 x 20 ml). The hexane phase was collected into a weighed conical flask (W_1) and evaporated to dryness under a fume hood. The final weight of the conical flask containing the dried hydrocarbon residue was recorded (W_2). The difference in weight ($W_2 - W_1$) and the supernatant volume (20 ml) were used to gravimetrically determine the mass of n-hexane extractable material (HEM), which is an estimated concentration of diesel-hydrocarbons in the liquid phase. The soil phase (~ 30 g) was air-dried and mixed with 3 g anhydrous Na_2SO_4 then extracted three times with 60 ml hexane (3 x 20 ml) with sonication for 30 minutes. The concentration of diesel-hydrocarbons was determined as HEM mass (mg) per weight (kg) of soil as described above. Residues recovered from both liquid and soil phases were re-dissolved in 10 ml hexane and analyzed for PAHs by gas chromatography with mass selective detector (GC/MS). The PAHs in the liquid phase proved to be negligible and, therefore the fraction in the soil phase only was used to determine PAH removal by bioremediation.

4.2.4 Gas Chromatography Analysis

A Shimadzu QP5000 GC/MS was used to identify and quantify the PAHs. A 16 compound PAH standard (100 – 1000 mg l^{-1}) in dichloromethane obtained from Ultra Scientific (North Kingstown, RI) was used for instrument calibration. The separation column was a 30 m x 0.32 mm (i.d.) HP-5MS column (J&W Scientific, Folsom, USA) with a 0.25 μm film thickness. The oven temperature was initially set at 50°C for 1 min, increased at a rate of 8°C min^{-1} to 220°C and held for 5 min, and then increased at a rate of 20ºC/min to a final temperature of 270°C and held for 10 min. The total run time was 39.75 minutes. The injector and transfer lines were heated at 250ºC and 280ºC, respectively. For identification, the GC was tuned to the scan

mode and to the selective ion mode (SIM) for PAH quantitation. A 1µl sample was injected in splitless mode using an AOC-17A autosampler with He carrier gas at 9.35 psi.

The headspace gas samples (5 ml) in each vial were analyzed by gas chromatography (GC) with both thermal conductivity detector (TCD) and micro electron conductivity detectors (µECD). The GC was fitted with 2 ml sampling loops in series attached by two ten-port valves that separated flow to: (a) a HaySep DB stainless-steel packed column (30 ft x 1/8 in o.d., 80/100 mesh) connected to the TCD, and (b) a 5 ft HaySep N column (6 ft x 1/8 in o.d., 80/100 mesh) connected to the µECD. All runs were carried out under the following conditions: TCD and µECD detectors were set at 150°C and 350°C, respectively; the injector at 70°C; and column at105°C. Helium was the carrier gas to the TCD with a flow rate of 28.8 ml min^{-1} while 95% Ar with 5% CH_4 set at a flow rate of 16.7 ml min^{-1} was the carrier gas to the µECD. The oven temperature of 70ºC was held for 8 min before ramped at a rate of 10ºC per min to a final temperature of 150ºC. The CO_2 and CH_4 concentrations were measured by TCD while N_20 was measured by µECD. A detailed description of the gas analytical method has been described by Washington et al. (2004).

Standard calibration curves and check standards consisted of Scotty standard mixes from Supelco (Bellefonte, PA) and custom air blends from Scott-Marrin (Riverside, CA). These were used to construct 4 – 6 point calibration curves and multiple check standards that were periodically injected during a sample run for QA/QC. Low standard air concentrations of CO_2 (357 ppm), CH_4 (1.79 ppm), and N_2O (0.320 ppm) were also used as QA/QC blanks.

4.2.5 Ammonia Analysis

Composite liquid samples of treated and control batch microcosms were analyzed for

ammonia (NH_4^+) by the phenate method (Clesceri et al., 1998). The NH_4^+ was reacted with hypochlorite and phenol to form indophenol blue. The intensity was measured spectrometrically (HACH DR/2010; Loveland, CO, USA), at λ = 640 nm. Fresh calibration standards and blanks were prepared and analyzed for each NH_4^+ analysis.

4.2.6 Microbial Enumeration

The number of total heterotrophic bacteria was determined using the pour-plate method. A suspension was prepared by mixing 1 ml of soil-slurry from the microcosm vials with a 9 ml sterile solution. The solution was prepared by adding 0.005% Triton-X 100 to de-ionized water, to prevent cell clumping (Danova et al., 1988), and autoclaved for 15 min at 121ºC. A 10-fold serial dilution was carried out to determine the number of colonies forming units (CFU). The bacteria were cultured on nutrient agar broth (Difco Laboratories, Detroit, MI) incubated in the dark at 36°C for 48 h in a Thelco Model 6M Incubator (GCA/Precision Scientific). The number of colony-forming units per ml (CFU ml^{-1}) of MCE solution or soil-slurry suspension was counted in duplicate plates of each MCE concentration. Triplicate analysis and agar blanks were also used for quality assurance/quality control (QA/QC).

4.2.7 Data Analysis

The Monod microbial kinetic model was used to estimate microbial growth parameters. This model was chosen because it incorporates both the microbial growth rate and the degradation of available substrates (Alexander, 1994). Additionally, this model can be extended to sole- and multi-substrate systems, assuming a common enzyme system is used by a mixed microbial culture system (Guha et al., 1999). In the Monod model, the specific growth rate

(μ, h^{-1}) is related to the concentration of the substrate (S, mg l^{-1}) through the relationship:

$$\mu = (\mu_{max} S) / (K_S + S)$$

where μ_{max} is the maximum specific growth rate (h^{-1}) of microorganisms on the substrate, and K_S is the half-saturation constant (mg L^{-1}). The experimental observations for the microcosm study conducted for 168 h were compared to the 42 d batch reactor study.

The values are presented as means of duplicates or triplicates of each treatment, with standard error values calculated for triplicate treatments. A pair-wise comparison of some of the treatments was conducted using the Student t-test ($p < 0.05$) in SigmaPlot 9.0 (Systat Software, Inc, Point Richmond, CA, USA).

4.2.8 Analysis of PAH degradation products

A separate set of experiments was conducted to identify PAH biodegradation products in the diesel hydrocarbon samples treated with 50% MCE only. Three soil samples, a 50% MCE treatment and control, were set up in parallel for metabolites analysis. Prior to analysis, the polar metabolites were derivatized following the method of Zink and Lorber (1995). At the predetermined time intervals, a 2 g soil sample was taken from each vial and extracted with 3 ml of hexane in a 1:1 extraction to remove the non-degraded parent PAH compounds. The residual soils were then extracted with (1 x 3 ml) ethyl ether by unltrasonication, after 1 ml 6N HCL had been added to increase the polarity of the target compounds. The collected extracts were evaporated to dryness and the dried residues were dissolved and silylated by adding 100 µl TMCS and 200 µl MSTFA in a 10 ml screw cap glass tube and placed in a water bath at 65°C for 15 min. The derivatized samples were transferred into hexane and analyzed by GC/MS.

Table 4-2. Characterization of the diesel-contaminated soil at the beginning and end of 42-d incubation with mushroom compost extract (MCE).

	pH		Extractable Diesel-Hydrocarbons[a]				
	Initial	Final	Carbon Substrate (mg L^{-1})[c]	Soil phase (mg kg^{-1})	Liquid phase (mg L^{-1})	∑ HEM in both phases (mg)	% Loss of diesel - hydrocarbons[d]
Initial soil	5.4[b]	-		7684±5.0	-	230	-
Treatments							
MCE- 0%	5.4	6.7	193±8	7133±1.0	342±0.8	221	4.0
MCE- 5%	5.6	6.8	398±14	5917±3.0	202±0.4	182	20.9
MCE- 10%	6.0	6.7	528±41	4053±5.0	305±0.5	128	44.3
MCE- 25%	6.6	7.1	1113±17	4483±1.0	420±0.4	143	37.8
MCE- 50%	6.9	7.7	1810±21	1470±3.0	275±0.6	51	79.1
MCE-100%	7.2	7.4	3876±40	3331±2.0	95±0.1	102	55.7

[a]Solid and liquid phase diesel hydrocarbon concentration as the mean ± standard errors of three replicates (three different vials).
[b]Determined in the solid phase only
[c]Initial carbon concentration at the beginning (day 3) of the batch reactor treatment and measured as total carbon (TC)
[d] Percent of total mass of hydrocarbon partitioned in both liquid and solid phases of each treatment relative to the initial HEM

4.3 Results and Discussion

4.3.1 Biodegradation of Diesel-Hydrocarbon

The average initial hexane extractable material (HEM) recovered from aged diesel-contaminated soil was 7684 mg kg^{-1}. Figure 4-1 is a representative chromatogram of the hydrocarbon constituents in the diesel-contaminated soil. The identified constituents included monoaromatics (e.g, chlorobenzene), alkyl-substituted PAHs (e.g., 2,4-dimethylnaphthalene), un-substituted PAHs (e.g., pyrene), and their metabolites (e.g., dihydropyrene). The identified un-substituted PAHs and their respective concentrations in the aged diesel contaminated soil was determined as: acenaphthene, 270.9 mg kg^{-1}; anthracene, 29.7 mg kg^{-1}; pyrene 74.9 mg kg^{-1}; and fluoranthene, 43.5 mg kg^{-1}.

A general decrease in the mass of HEM was observed in both the soil and liquid phase after 42 days of incubation of the diesel-contaminated soil with the MCE (Table 4-2). Overall, more than 55% of the initial HEM was lost from samples treated with 50% and 100% MCE as compared to only 4% loss in the controls (MCE-0%). The data presented in Table 4-2 confirms

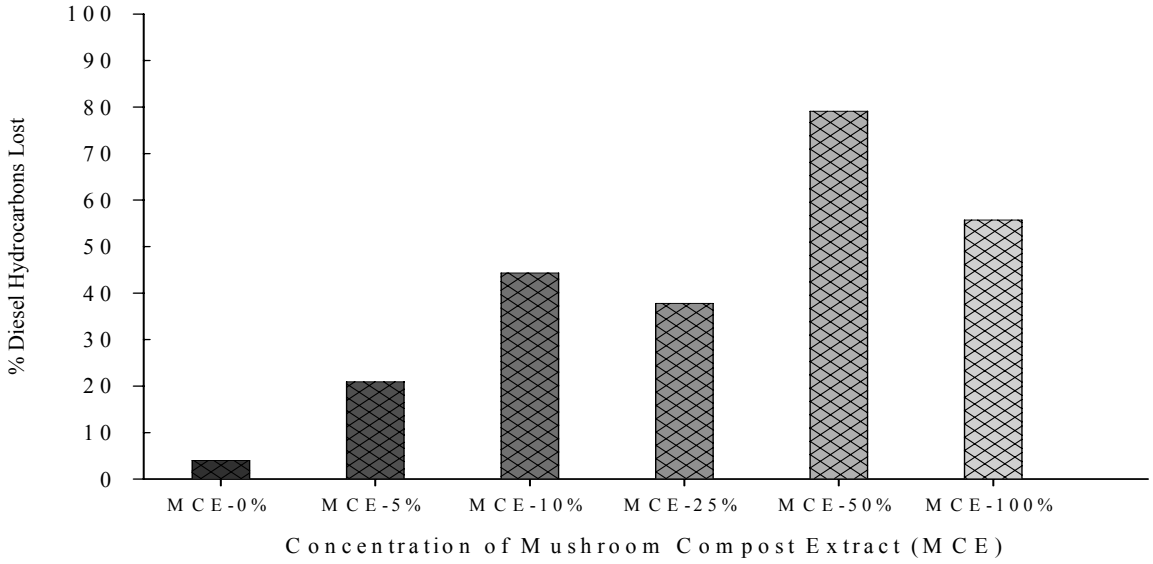

Figure 4-2 Loss of diesel hydrocarbons in the soil phase after 42-d incubation with MCE.

that the decrease in concentrations of extractable diesel-hydrocarbons did not increase with increasing MCE concentrations as predicted. At the termination of the experiments, the loss of diesel-hydrocarbons measured in the soil phase of the different MCE treatments was 0% < 5% < 25% < 10% < 100% < 50% (Figure 4-2). Meanwhile in the aqueous phase, the HEM generally decreased with increasing concentration of MCE added to the soil. The highest concentration of HEM measured in the liquid phase was 420 mg L^{-1} in untreated controls (MCE-0%), which is an order of magnitude higher than detected in the 100% MCE treatments (95 mg L^{-1}).

The observed decrease in the amount of HEM in soils treated with MCE relative to the controls was attributed to the effectiveness of the MCE amendments in enhancing the biodegradation of the hydrocarbon compounds. The maximum loss in diesel hydrocarbons was observed with 50% and not with 100% MCE as predicted. This observation suggested that at high concentration of organic carbon (3876 mg l^{-1}) provided by the MCE-100%, optimum conditions for microbial degradation of the diesel hydrocarbons were not created (Madigan et al.,

2000). Additionally, at the high organic carbon loading with undiluted mushroom compost extract, highly reducing conditions should be rapidly created as shown by the data presented below. It has been shown that very reducing (anaerobic) conditions, for example methanogenic conditions, are generally unfavorable for microbial degradation of hydrocarbons (Cerniglia, 1992). The soil surface may be enriched with the un-metabolized organic carbon, which will render the soil more effective as a sorbent, thereby decreasing the bioavailability of the more hydrophobic compounds in the diesel contaminated soil (Chiou et al. 2000). Therefore the lowest concentration of HEM measured in the liquid phase of MCE-100% treated soils was attributed to sorption. This was confirmed by the higher concentrations of HEM recovered in the soil phase of MCE-100% (3331 mg kg^{-1}) than in the 50% MCE treated soils.

Overall, the highest percent removal of diesel hydrocarbons (HEM) was 80% of the initial HEM in batch microcosms treated with 50% diluted MCE. This is significantly greater than the 56% decrease for the same aged diesel contaminated soils treated with 100% MCE. The

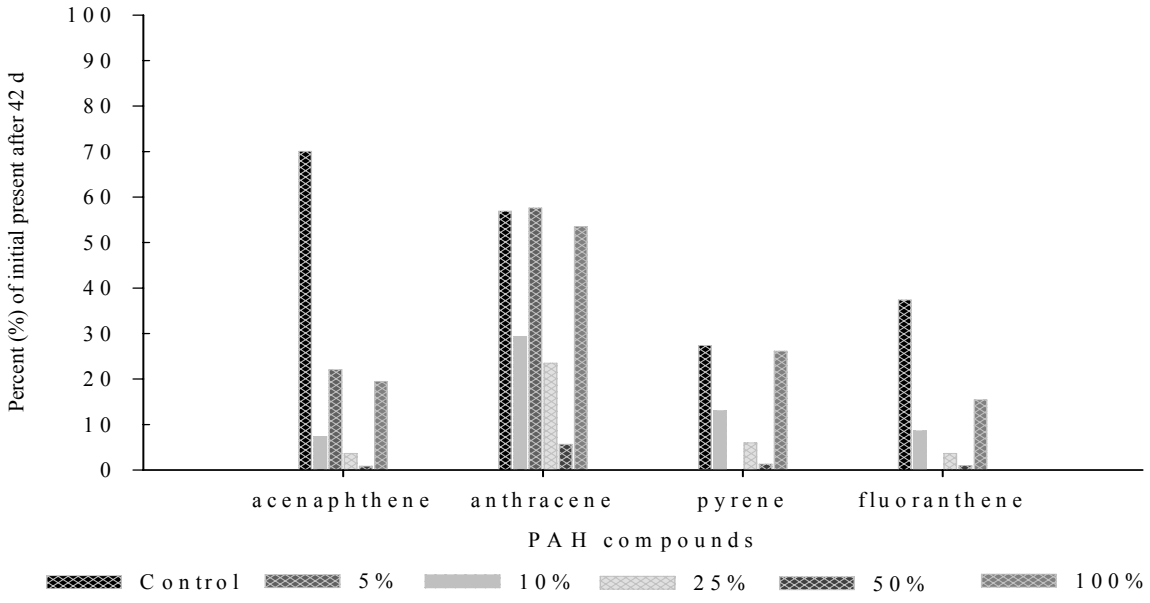

Figure 4-3. Loss of PAHs from diesel contaminated soils at different MCE dilution levels after 42 days of incubation.

decreased concentrations of HEM were similar to the PAHs loss after 42 days (Figure 4-3). The PAH losses for the different treatments were not consistent with the increasing concentrations of MCE added. The highest and lowest PAH losses observed in the 50% MCE treatments and controls, respectively, were consistent with the determinations of total losses in diesel hydrocarbons. The decrease in concentration of DOC and high heterotrophic bacteria growth rates observed after 42-d of incubation provided evidence that biodegradation contributed to the higher rates of PAH and HEM removal in the 50% MCE treatments..

The addition of MCE increased the soil pH from 5.4 to 7.4. The pH generally increased with increasing concentration of MCE added. For example, the pH increased from an initial value of 5.4 in the untreated controls to 7.2 in the soil samples treated with undiluted MCE. At the end of the incubation period, the pH in the control was 6.7 compared to 7.4 in the samples treated with the maximum strength MCE.

4.3.2 Estimation of Monod Kinetic parameters

Monod kinetic parameters were estimated from experimental data of MCE treated samples incubated for 168-hr (Table 4-3) and 42-d (Table 4-4). The parameter estimation was based on microbial utilization of supplied MCE, CO_2 production and the bacteria growth rates.

Table 4-3. Monod kinetic parameters in 168-hr microcosm systems incubated with MCE.

	Uncontaminated Controls			Diesel-Contaminated Treatments		
	Growth rate (h^{-1})	R^2	Substrate[1] (mg L^{-1})	Growth rate (h^{-1})	R^2	Substrate (mg L^{-1})
MCE- 5%	0.010	0.54	213	0.017	0.76	398
MCE-10%	0.010	0.67	439	0.021	0.85	528
MCE- 25%	0.253	0.72	886	0.053	0.95	1113
MCE- 50%	0.032	0.85	1801	0.042	0.93	1810
MCE- 100%	0.043	0.98	3605	0.066	0.09	3876
Growth characteristics						
μ_{max}, maximum growth rate (h^{-1})		0.043			0.066	
K_S, Monod constant (mg L^{-1})		750			1350	

[1]Substrates concentrations are presented as mean of duplicate vials analyzed

The CO_2 and CH_4 formation in the headspace of microcosms were used as indicators of microbial respiration during the incubation period. Except for the uncontaminated controls amended with 25% MCE, the bacteria growth rate was generally higher in the MCE treated aged diesel contaminated soil than the uncontaminated MCE treated soils. A maximum growth rate (μ_{max}) of 0.066 h^{-1} was obtained for MCE amended contaminated soil compared to 0.043 h^{-1} for similarly treated uncontaminated soils. The higher maximum growth rate in the former suggests that MCE was metabolized as a co-substrate during biodegradation of hydrocarbons in aged diesel contaminated soil.

After 42 days of incubation of the aged diesel contaminated soils treated with different concentrations of diluted MCE, the highest microbial growth rate (μ) of 0.064 d^{-1} was obtained in reactors treated with 10% MC. The 50% and 100% MCE treated soils had μ values of 0.050 d^{-1} and 0.011d^{-1}, respectively (Table 4-4). Microbial activity appeared to be higher at 10% MCE than at MCE 100%. A good correlation was observed between the rate of CO_2 production (microbial respiration) and substrate (DOC) utilization in the long-term treatments (42-d batch microcosms). The concentration of CO_2 produced increased with increasing concentration of MCE added to the diesel-contaminated soil (Figure 4-4). The highest CO_2 concentration of 172,000 ppm was measured in the headspace of microcosms treated with 100% MCE. A lag phase of about 8 days generally preceded the progressive increase in respiration rate. The increase in respiration (CO_2 production) was accompanied by a decrease in DOC.

Table 4-4 Monod kinetic parameters in 42-d anaerobic microcosm systems containing MCE-treatment of diesel-contaminated soil

Amendment Added	MCE-0%	MCE-5%	MCE-10%	MCE-25%	MCE-50%	MCE-100%
Growth rate (d^{-1})	0.043	0.046	0.064	0.039	0.050	0.011
R^2	0.93	0.98	0.96	0.95	0.99	0.86
DOC[1] (mg L^{-1})	201	475	575	1078	1742	5374

[1]Substrates concentrations are presented as mean of duplicate vials analyzed

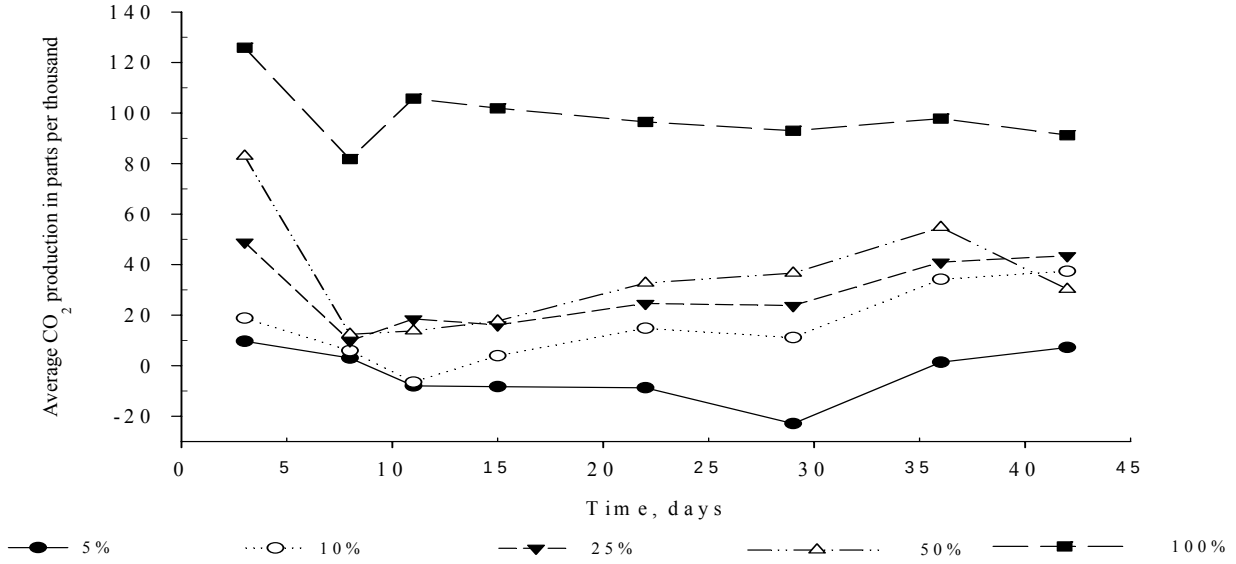

Figure 4-4 Average CO_2 in headspace gas measured in 42-d batch microcosms of diesel-contaminated soil amended with different concentrations of MCE. Plotted values have been corrected for CO_2 measured in control samples in order to determine gas production from the metabolism of MCE added to the soil. Negative values means the measured concentrations were below the control values.

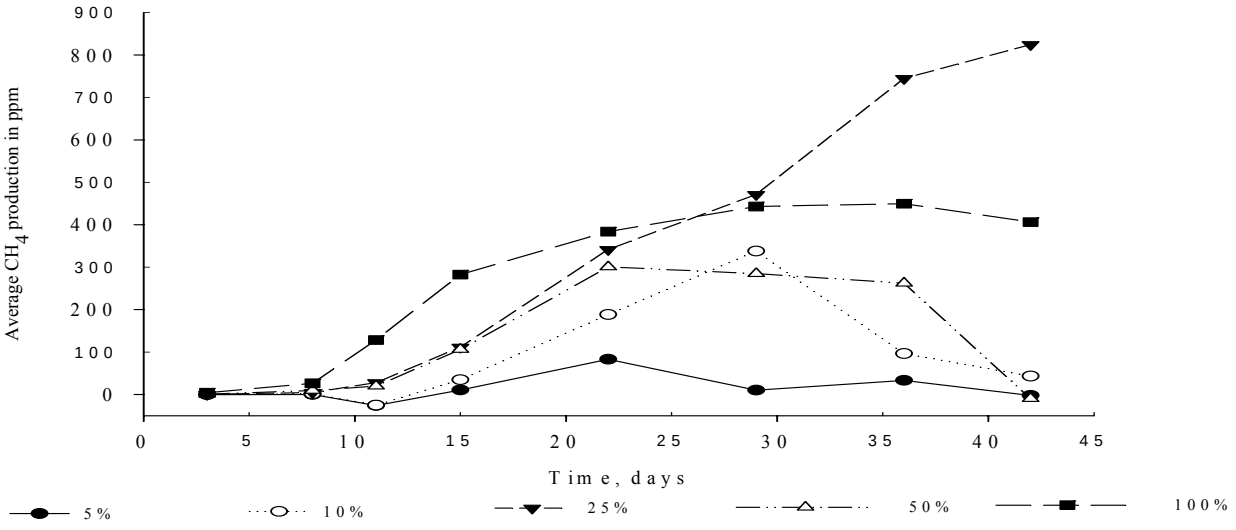

Figure 4-5. Average CH_4 in headspace gas measured in 42-d batch microcosms of diesel-contaminated soil amended with different concentrations of MCE. Plotted values have been corrected for measured in control samples in order to determine gas production from the metabolism of MCE added to the soil. Negative values means the measured concentrations were below the control values.

4.3.3 Evidence of Anaerobic Oxidation

The production of high concentrations of methane (880 – 1238 ppm) at the end of the incubation period suggested that highly reducing and methanogenic conditions were created in the batch microcosms, and more so in vials containing 25% and 100% diluted MCE (Figure 4-5). The highest concentration of CH_4 observed in contaminated soil treated with 25% MCE cannot be explained by these data. The rapid increase in CH_4 production from 4 to 880 ppm in MCE-100% treatments, suggests that highly anaerobic (methanogenic) conditions were created in the vials by the end of the incubation period. The anaerobic oxidation of diesel hydrocarbons under methanogenic conditions would be expected to be very limited (Pothuluri and Cerniglia, 1994).

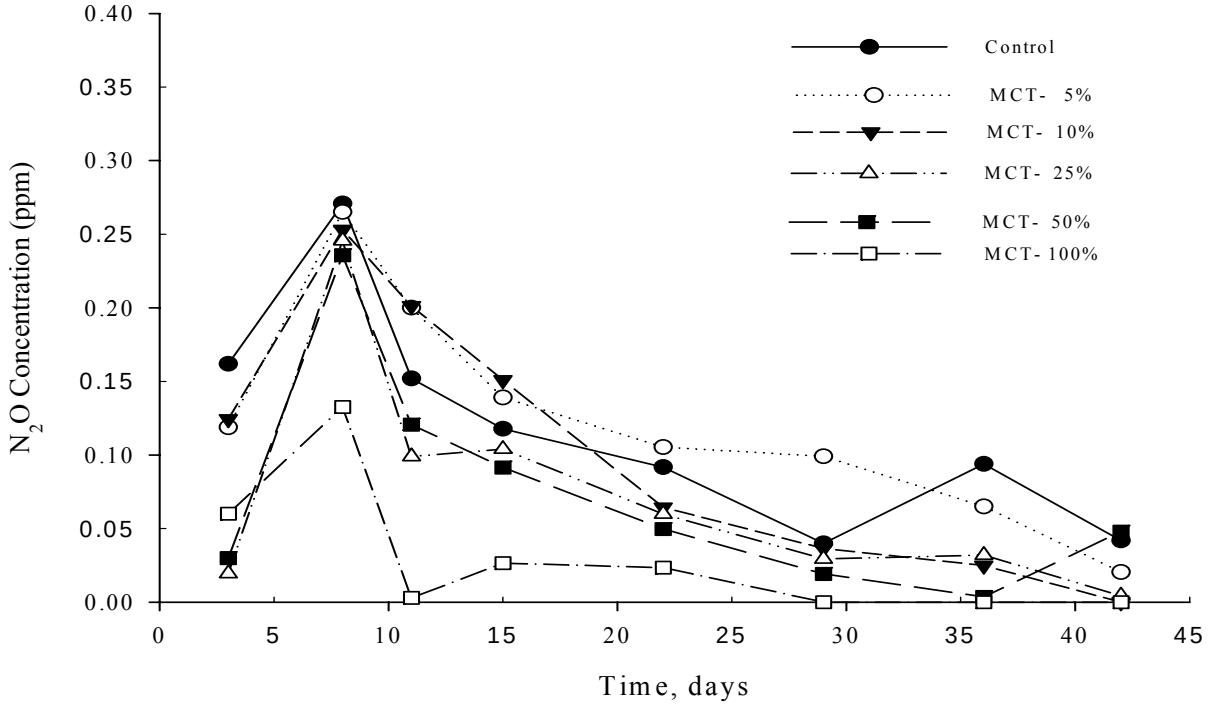

Figure 4-6. Average N_20 in headspace gas measured in 42-d batch microcosms of diesel-contaminated soil amended with different concentrations of MCE.

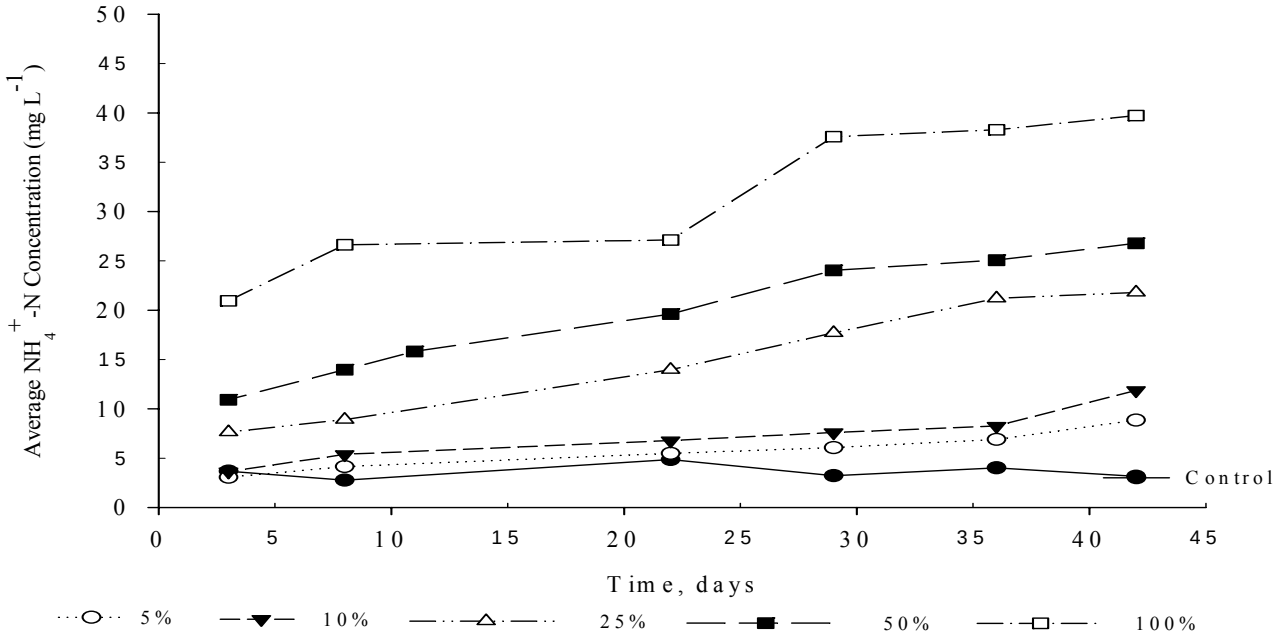

Figure 4-7. Cumulative concentration of ammonium measured as $N\text{-}NH_4^+$ in batch microcosms studies with different concentrations of MCE.

The detection of nitrous oxide (N_2O) in the measured headspace gas provided evidence for the presence of the creation of denitrifying conditions during some stage of incubation. The results presented in Figure 4-6 show an initial increase in N_2O during the first 7 days of incubation, which corresponds to a decrease in CO_2 (Figure 4-4). At the end of the first 7 days of incubation, CH_4 (Figure 4-5) production progressively increased while N_2O production progressively decreased. The concentration of N_2O decreased more rapidly in samples treated with higher concentrations of MCE (Figure 4-6) suggesting that anaerobic conditions were more rapidly formed at those concentrations of MCE. This observation is consistent with highly anaerobic systems, in which organic nitrogen (N) is preferentially reduced to ammonia or ammonium (ammonification) by-passing the formation of N_2O gas (Grundmanis and Murray, 1977). This explains the lowest concentration of N_2O measured in MCE-100% treated samples, while the highest concentrations were detected in the controls and in 5% MCE treatments.

4.3.4 PAH Metabolites and Proposed Biodegradation Pathways

Although a rapid rate of decrease in concentration of extracted diesel hydrocarbons was observed in both MCE treated samples and controls (Figure 4-2 and Figure 4-8), there was a significant difference in the types of PAH metabolites detected (refer to Tables A-3 to A-5 in the appendix). The 50% MCE treated samples generally contained a higher concentration of PAH metabolites than the control (Table 4-5). Metabolites were detected in the MCE treated soils after 1 week of incubation, during which the concentration of the parent PAHs had decreased by approximately 50% (Figure 4-8). The identified metabolites after week 1 were aromatic acids (e.g., benzene di-carboxylic acid, *m/z* 182) and naphthalene acetic acids (*m/z* 179). No other metabolites were identified that could be structurally linked to the biodegradation of the higher molecular weight (> 2-ring) PAHs. The identification of metabolites of only naphthalene after 1 week of liquid compost treatment was attributed to its relatively high bioavailability and degradability (Mackay et al., 1992).

A greater number of metabolites were obtained after 14 weeks of incubation. The identified metabolites included alcohols (trimethoxy phenol, *m/z* 184), organic acids (benzene 2,7- dicarboxylic acid, *m/z* 266), aldehydes (benzaldehyde 3,4-dihydroxy, *m/z* 168), and ketones (benzopyran-2-one, *m/z* 176). The suite of products identified after 14 weeks suggests that the degradation of diesel-contaminated soil by mushroom compost yields more ketones and hydroxylated degradation products than acids. The fungal metabolism of PAHs yields a variety of ketones (Cerniglia et al., 1992). This suggests that the microbial activity of the MCE treated soils is predominantly due to fungal activity in the contaminated soil. Studies by Wischmann and Steinhart (1997) have also indicated that ketones may accumulate and become toxic over time. Except for 1,2-dihydroanthra 1,2-d thiazole-2,6,11-trione (m/z 281), none of the ketones

identified in the liquid MCE (1H-1-benzopyran-2-one *(m/z* 176), hexen-3-one (*m/z* 188), 3,4-dihydro naphthaleneone (*m/z* 146), 1, 2, 3,4-terahydro phenanthrenol (m/z 182), and 1,2-dihydroanthra 1,2-d thiazole-2,6,11-trione (m/z 281)) remained in the treated soils after 14 weeks. This suggests that the ketones formed were microbially degraded and did not persist over time.

Short-lived aromatic acids were also formed and rapidly degraded, as they have been found to be readily susceptible to microbial degradation (Feinberg et al., 1980). By week 19, the probed PAHs had decreased to almost non-detectable levels (Figure 4–8), and subsequent transformation into easily mineralizable product fractions as confirmed by the change in the GC scan of derivatized PAH fraction. (Figure 4–9).

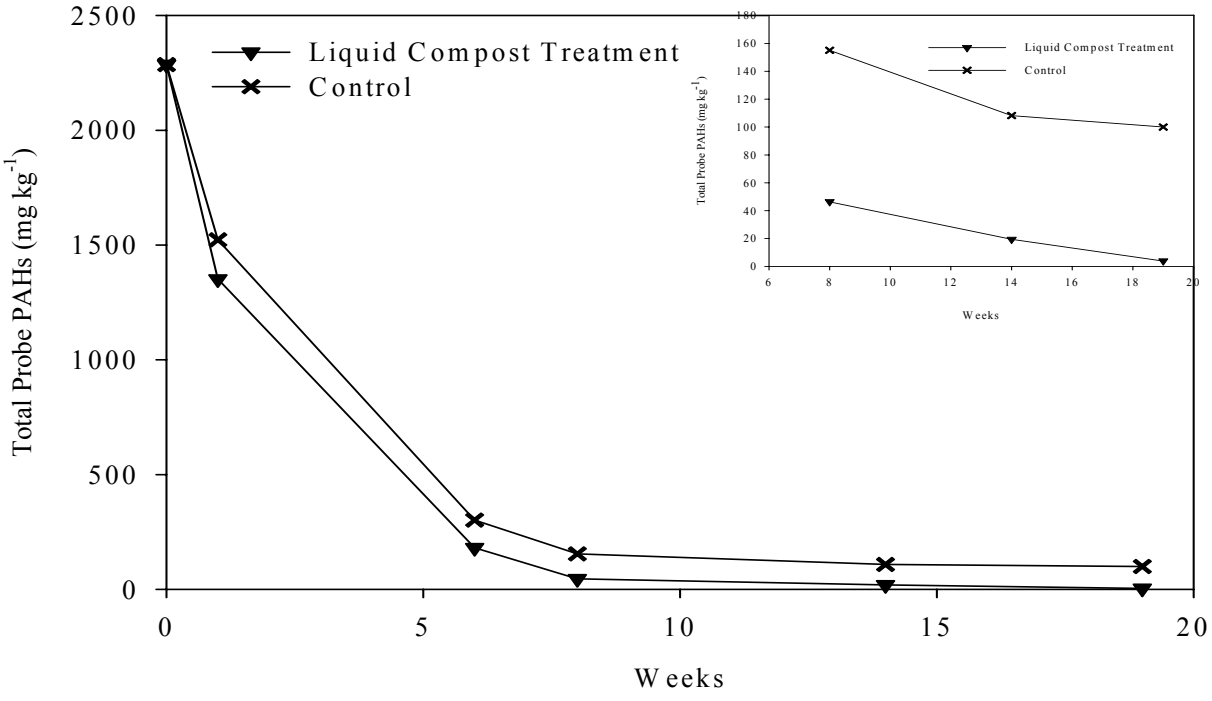

Figure 4-8 Change in the concentration of total probe PAHs: naphthalene, acenaphthylene, acenapthene, fluorene, fluoranthene and pyrene in soils amended with liquid compost and control. Graph insert is a more detailed view of data collected from 8 – 19 weeks.

Table 4-5 A summary of PAH degradation products identified by GC/MS.

Compound [a]	Rt. (min) [b]	m/z ion [c]	Compost [d] Extract	Control [e]
Aromatic Acids				
Benzene acetic acid	12.3	208		x
Benzene acetic acid	22.3	238	x	
Benzene 1,2-dicarboxylic acid	4.9, 8.3	266	x	x
Benzene 2,7-dicarboxylic acid	15.3	268	x	
Naphthalene acetic acid	14.7, 15.4	200	x	x
Benzoic acid, 3,4 dimethoxy	11.9, 8.6	182	x	x
Phthaladehydic acid	11.4	165	x	
Aldehydes				
Benzaldehyde 2,4-dihydroxy	16.3, 17.8	168	x	x
1H-1-Benzopyran-2-one	14.8	176	x	
4-Phenoxybenzaldehyde	17.2	198	x	
Benzaldehyde, 2,4-bis	20.5	282	x	
Benzaldehyde3,4,-dihydroxy	16.2	168	x	
2-quinolinecarboxaldehyde	16.5	188	x	
1-Naphthalenecarboxaldehyde	15.3	171	x	
Alcohols				
1,2,3,4-tetrahydro 4-phenanthrenol	21.8	194	x	
1-Phenanthrenemethanol	20.1, 19.6	183	x	x
Ketones				
Naphthalenenone, 3,4-dihydrol	11.4	164	x	
2(1H)-Naphthalenone, octahydro	20.8	166	x	
1,4 Naphthoquinone	12.8	146	x	
Cyclohexanone	20.9, 20.9	243	x	x
Naphthalenenone, 3,4-dihydrodiol	11.4	146	x	
Quinoline-2-one	15.3	228	x	
Anthranol	24.2	208	x	
Epoxynaphthalene tetrahydro	11.9	346	x	
Hydroxides				
2-hydroxynaphthalene	18.4, 21.3	184	x	x
Tetradecahydroanthracene	21.9	248	x	
Tetrahydropyrene, 1,4,5,9,10-	26.9	206	x	
Dibenzopyran-6,7,8,10 tetrahydro	23.4	386	x	
Phenanthrene, 1,2,3,4-tetrahydro	18.2	182	x	

[a] Compound identified as -trimethylsilyl, -methyl ester-, or -trimethoxy compound.
[b] GC retention time, [c] m/z – mass to charge ratio of identified metabolite (as -trimethylsisyl, -methyl ester-, or -trimethoxy compound).
[d] Samples were treated with 50% liquid mushroom compost extract (MCE); [e] Samples treated with water (control).
"x" indicates the presence of identified metabolite.

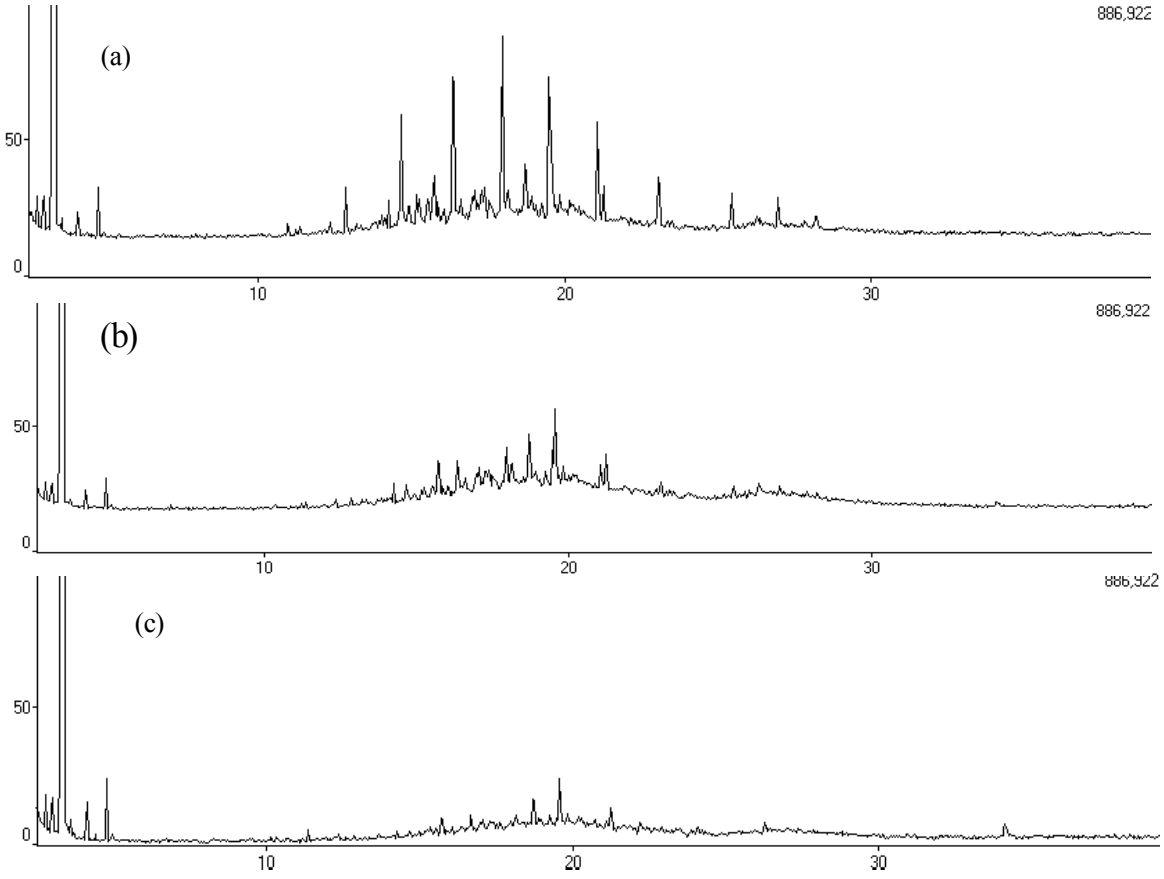

Figure 4-9 GC/MS chromatograms showing the loss of PAHs and their metabolites from diesel-contaminated soils amended with 50% mushroom compost extract: (a) diesel soil after 1 week; (b) diesel soil after 14 weeks; (c) diesel soil after 19 weeks.

Using the suite of identified metabolites, both bacterial and fungal biodegradation pathways were proposed for naphthalene and phenanthrene through the intermediate metabolite 1-benzopyran-2-one (Figure 4-10). The formation the intermediate metabolite indicates either direct microbial metabolism of phenanthrene or co-metabolic transformation of naphthalene and it alkyl-derivatives (Langbehn and Steinhart, 1995, Abbott and Gledhill, 1971). Further degradation of 1-benzopyran-2-one may form benzaldehyde. A pathway for phenanthrene and methyl-phenanthrene degradation that includes both fungal and bacterial metabolism is also proposed in Figure 4-11. The identified metabolites and the change in the GC-MS profiles of the

derivatized soil samples provide strong support for substantial decrease in the PAH concentrations in the MCE treated diesel-contaminated soils (Figures 4-3).

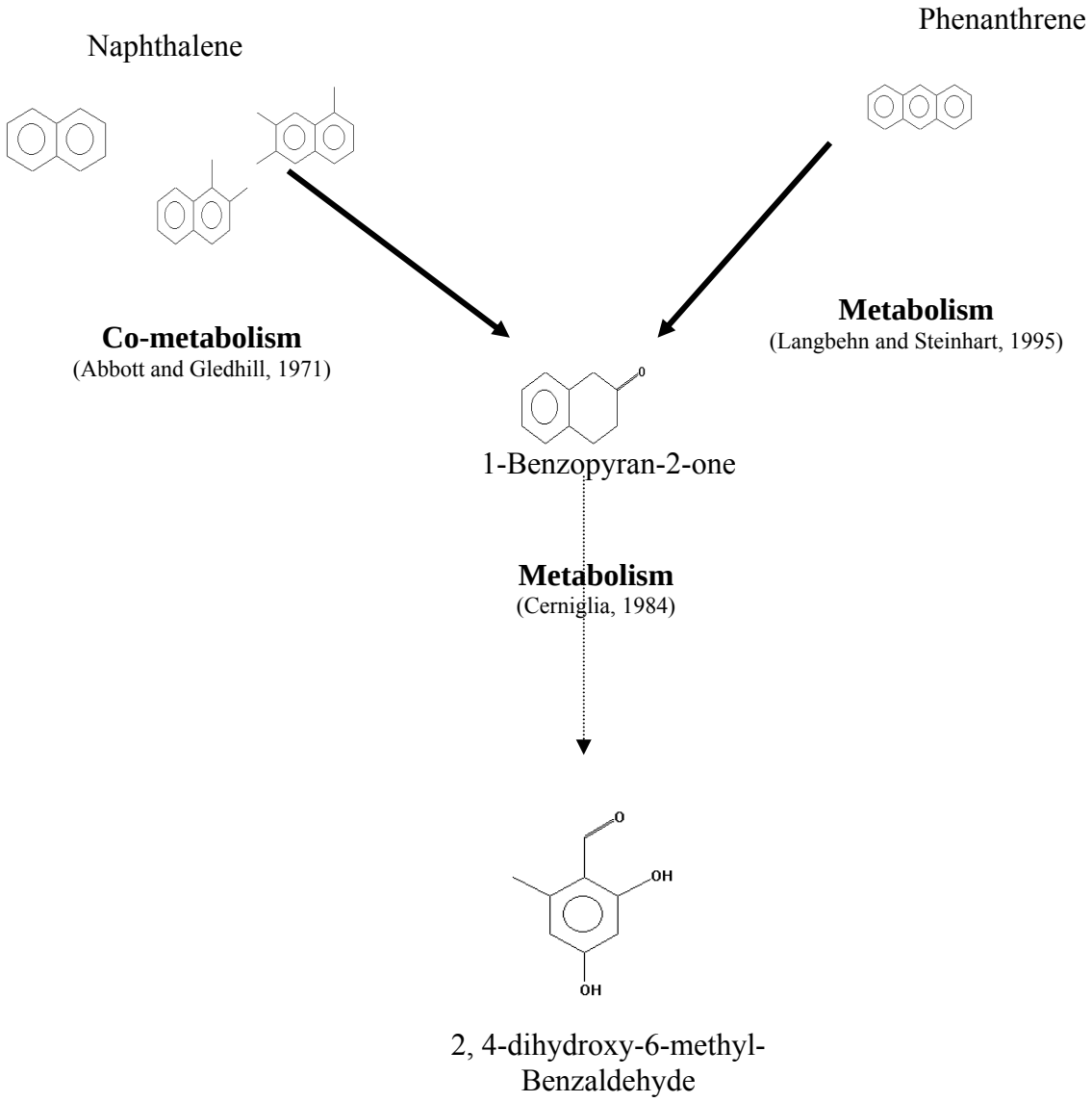

Figure 4-10 Proposed bacterial metabolism of naphthalene (un-substituted or alkylated) and phenanthrene degradation through 1-Benzopyran-2-one in diesel-contaminated soils amended with liquid mushroom compost extracts. The pathway determination is based on the identified metabolites in this study and a review of literature. Established pathways are shown by solid line. Proposed pathways are shown by dashed line.

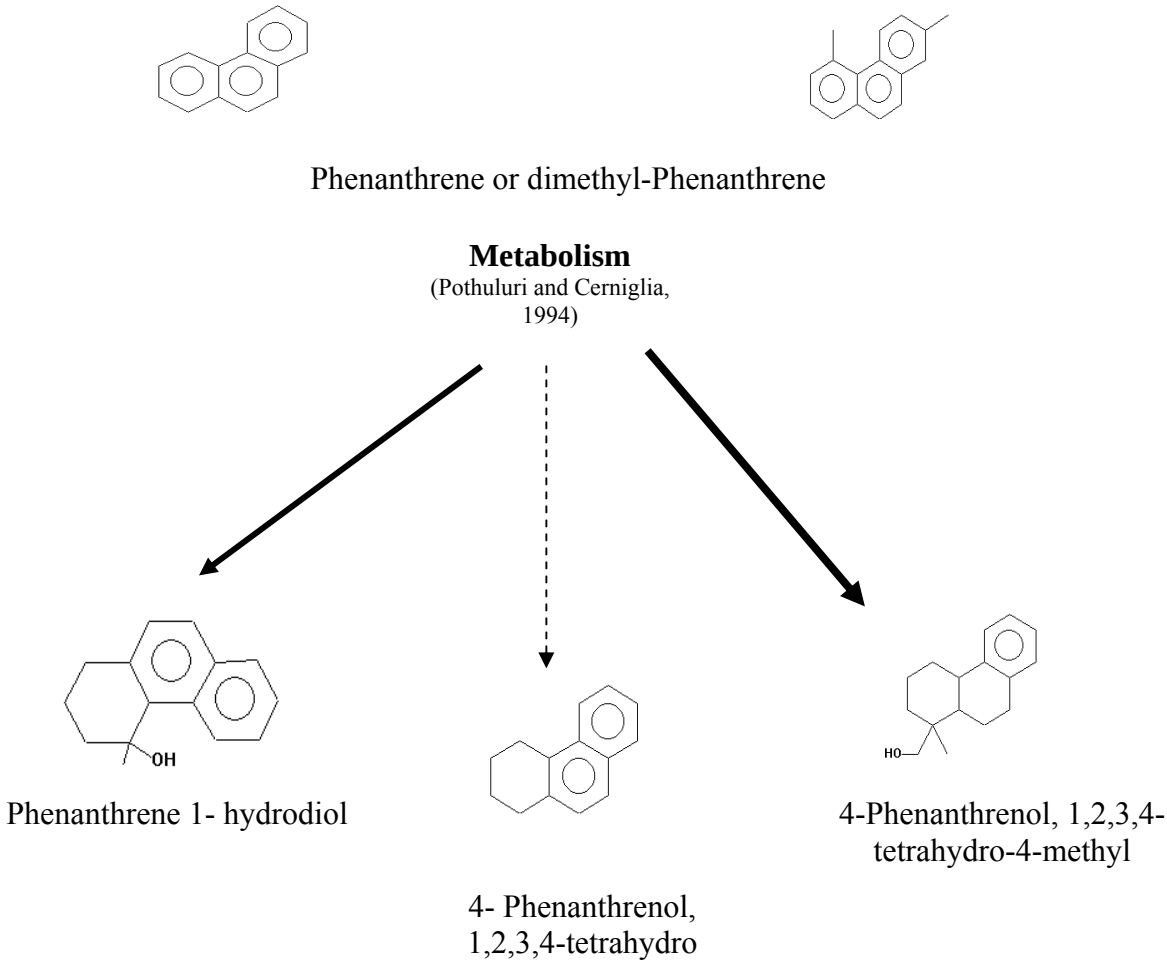

Figure 4-11 Proposed fungal metabolism of phenanthrene in diesel-contaminated soils amended with liquid mushroom compost extracts. The pathway determination is based on identified metabolites and a review of literature. Established pathways are shown by solid line. Unknown pathways are shown by dashed line.

4.4 Summary

The results of this study showed that 50% diluted mushroom compost extracts (MCE) significantly enhanced the bioavailability and biodegradation of a range of diesel hydrocarbons. The organic-C and nutrients provided under these conditions (1796 mg L^{-1} DOC, 5.2 mg L^{-1} N, and 7.4mg L^{-1} P) were in the optimum range for stimulation of high microbial growth, μ =

0.0496 d^{-1}, required to efficiently degrade diesel hydrocarbons; 79% in MCE treatments compared to only 4% in the un-treated controls. The low biodegradability of PAHs observed in soils treated with 100% MCE suggested that the enhanced biodegradation of the aged diesel does not necessarily occur at the highest loading of the compost extract amendment. In addition, the most effective biodegradation tended to occur in samples where nitrate-reducing conditions were likely created.

The addition of MCE resulted in the degradation of 2 – 4 ring PAHs and a reduction of the total PAH concentration in diesel to < 4 mg kg^{-1}. Based on the proposed pathways, the degradation of parent PAHs tended to have occurred along both bacterial and fungal pathways. Many of the identified metabolites: acetic acids and oxidized metabolites (ketones) were further biodegraded and did not accumulate as toxic or dead-end products. This study indicates that soils contaminated by petroleum heavy-ends, such as PAHs, can be rapidly bioremediated using diluted liquid extracts of composted agricultural waste used to cultivate mushrooms.

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EFFECTS OF ORGANIC CARBON AMENDMENTS ON PAH BIOAVAILABILITY, TRANSFORMATION, AND DEGRADATION[1]

[1]Wayo, L.K. and V.A. Nzengung, 2006. Submitted to *Journal of Hazardous Materials*

Abstract

A comparative study was conducted to determine the relative effectiveness of utilizing solid mushroom compost and mushroom compost tea (liquid) to remediate PAHs in aged diesel-contaminated soils and polishing with phytoremediation. Corn (*Zea mays* L.) was selected for the phytoremediation studies. After 10 weeks of pre-treatment, 98% of the total extractable probe PAHs ($TPAH_{ext}$) was removed from liquid compost treated soils. Specifically, initial $TPAH_{ext}$, of 2285.9 mg kg^{-1} reduced to 43 mg kg^{-1} compared to 124.4 mg kg^{-1} and 128.9 mg kg^{-1} in the solid compost and no-amendment control soils, respectively. Following 4 weeks of successful corn growth in liquid compost soils, an additional 55% of $TPAH_{ext}$ was removed to a final 19.5 mg kg^{-1} with phytoremediation. Conversely, plant establishment was unsuccessful in both solid compost and control soils and less than 10% of the $TPAH_{ext}$ was removed giving final residual concentrations of 99.3 mg kg^{-1} and 108.2mg kg^{-1}, respectively, by week-14. The presence of aromatic metabolites, including dicarboxylic acids and dihydro-2, 3-naphthalenedione, was used to confirm PAH degradation in the liquid compost soils. This study provides evidence that liquid organic carbon-rich compost is better suited for rapid bioremediation of high-concentration petrochemical contaminated soils and polishing residual contamination with phytoremediation.

Keywords: compost tea; phytoremediation; PAHs; organic carbon

5.1 Introduction

In-situ biological remediation of contaminated soils is a cost-effective and environmentally friendly alternative to conventional methods of soil decontamination (Volkering and Beure, 2003). Over the years, cultured microorganisms, nutrient amendments and plants have been used with varying degrees of success for the remediation of soil organic contaminants (April and Sims, 1990; Bouchez et al., 1995; Schnoor et al., 1995). However, toxicity resulting from high contaminant concentrations has hindered plant and microbial growth during phytoremediation and bioremediation applications (Huang et al., 2004). Additionally, chemical characteristics of organic contaminants such as the highly hydrophobic nature of polycyclic aromatic hydrocarbons (PAHs) may create contaminant mass-transfer limitations (Harms and Bosma, 1997). This was attributed to sorption of the hydrophobic compounds to soil organic matter and entrapment within soil microsites. Faster sorption and slower desorption rates decrease contaminant bioavailability which presents a major challenge to the successful application of biological remediation methods.

In the remediation of contaminated soils, PAHs are of particular interest due to their toxic, carcinogenic and ubiquitous nature (Wilson anad Jones, 1993). Phytoremediation alone has been found to be relatively less favorable for the restoration of PAH-contaminated sites (Banks et al., 2000). Natural nutrient amendments, such as biosolids (Atanaga, 2004) and compost amendments (Lau et al., 2003) have generally been more successful than phytoremediation in enhancing PAH degradation. For instance, Kästner and Mahro (1996) demonstrated enhanced degradation of low molecular weight PAHs (1 – 3 rings) in composted soils although the concentrations of high molecular weight compounds (≥ 4-ring) reached asymptotic end-points after forty days. Similar degradation results were obtained in a later study

by Wischmann and Steinhart (1997) in which high residual concentrations of chrysene and benzo (a) pyrene (high molecular weight PAH) persisted after composting. In both studies, the enhanced PAH loss following compost addition was due to degradation and the formation of non-extractable bound residues within the soil matrix (Mahro et al., 1994). This rendered the non-degraded but bound PAH fraction unavailable for leaching and transport, minimizing exposure risks. Although it seems unlikely that bound or sequestered residual PAHs may be re-mobilized in the course of humus (compost) turnover (Hartlieb et al., 2003), their environmental persistence especially at residual concentrations >10 mg kg^{-1} may present ecotoxicological risks. More recently, the coupling of remedial technologies, for example the use of compost as pre-treatment to phytoremediation (Parrish et al., 2003), has been adopted to enhance the biological restoration of PAH-contaminated sites. There is an immediate and growing need for research to provide a better mechanistic understanding of the role of organic-rich composts as suitable soil amendments to enhance PAH biodegradation. More specifically, at sites where phytoremediation cannot be applied due to toxicity resulting from high contaminant concentrations, pre-treatment of the soils with suitable amendments could be followed by phytoremediation polishing of the residual contamination.

Organic carbon provided as dissolved organic carbon (DOC) is utilized as an energy source by microorganisms involved in PAH degradation. Additionally, providing organic carbon as DOC could also improve contaminant bioavailability to the targeted microorganisms by decreasing surface tension and enhancing the solubility of PAHs in the treated soils. In this study, the enhancement of in-situ bioremediation of PAHs in contaminated aged soils using liquid mushroom compost rich in dissolved organic carbon was compared to solid mushroom compost to evaluate their suitability for intrinsic bioremediation and polishing by

phytoremediation. The liquid amendment is commercially available as 100% Organic Compost Tea®. It is a natural organic carbon source used as a nutrient supplement for plant growth and is produced as a large volume of leachate run-off from a solid mushroom compost pile. Corn (*Zea mays* L.) was selected for the phytoremediation phase of this study of diesel-contaminated soils. Diesel is a complex mix of aliphatic and aromatic hydrocarbons and contains many PAH compounds that could make up as much as 40% of all aromatic hydrocarbons present (NTP Technical Report, 1986). The solid and liquid mushroom compost products were evaluated by: (1) comparing the extent to which each amendment initially increased PAH bioavailability in the amended soils, (2) the extent of PAH transformation to identifiable metabolites, and (3) the efficacy of compost to reduce the toxicity of PAH-contaminated soils and support plant growth to polish up any residual contamination.

5.2 Materials and Methods

5.2.1 Soil and Compost Amendments

Petroleum contaminated soil was collected from a stream bank downstream of a gasoline station in Athens, Georgia (USA). The selection of this soil was to ensure the presence of indigenous hydrocarbon degrading microorganisms (Mueller et al., 1994). The soil was dried at 25ºC, homogenized and sieved to a 2 mm mesh size fraction. The soil was classified as loamy sand (78% sand, 16% silt and 6% clay) with a low organic matter content of 1.43% (determined by the "loss-on-ignition" method at 360°C). The soil pH was determined in equivalent water as 5.16. Physical and chemical characterization of the soil, except for hydrocarbons, was conducted by the Soil Test Laboratory at the University of Georgia (Table 5-1). After drying and sieving, the soil was artificially contaminated with commercially available diesel fuel (20% v/v) to

Table 5-1 Characterization of soil (in mg kg^{-1}) and mushroom compost amendments (in mg l^{-1})

Loamy Sand Soil			Compost		
Analysis		Hydrocarbons[c]	Analysis	Solid	Liquid
K	26.1	Aromatics	K	6642	3534
Mn	94.8	Benz (0.05)	Na	1334	1012
Mg	31.9	Naph (0.08)	Mg	1036	343.1
Ca	153.5	Phen (1.96)	Ca	6948	1005
P	12.8		P	132.4	16.6
Zn	11.2	Aliphatics	Fe	0.7	1.2
N	0.07 [b]	Phthalate	NH_4^+	4.9	31.4
S	0.02 [b]	Eicosane	NO_3^-	116.4	1.0
C	0.86 [b]	Hexanone	NO_2^-	14.0	0.7
LBC [a]	265.0	decacosane	SO_4^{2-}	2081	202.0
			Total N	132.1	14.8
			Total P	79.4	10.3
			DOC [d]	2358	3004

[a] Lime Buffer Capacity ($CaCO_3$/pH) measured as ppm

[b] Concentration in %

[c] Gas Chromatographic identification of hydrocarbons present prior to diesel contamination: Benz – benzene, Naph – naphthalene, Phen – phenanthrene; ( ) concentrations in µg g^{-1} of soil

[d] Determined after acidification

increase the concentration of parent PAH compounds. The contaminated soil was then air-dried for 2 weeks to allow aging and the evaporation of volatile compounds. During air-drying, the soil was constantly mixed with a stainless steel hand shovel to ensure the homogeneous distribution of contaminants.

The mushroom composts used in this experiment are rich in plant growth nutrients (Table 5-1). The organic compost tea, hereafter referred to as liquid compost, has a higher concentration of DOC (3004 mg l^{-1}) than the solid compost (2358 mg l^{-1}). A Shimadzu TOC-5050A Analyzer was used to determine the DOC concentrations. Elemental analysis of the compost amendments was conducted by VG Elemental Plasma Quad III Inductively Coupled Plasma- Mass Spectrometry. The concentrations of the major plant nutrients were quantified with a Braun-Luebbe Auto Analyzer II Continuous Flow System.

5.2.2 Experimental Design and Sampling

A 14-week greenhouse study was set up in 7.6 liter cylindrical glass containers (27 cm high x 22 cm OD) containing clean sand at the bottom 10 cm and overlaid with dry contaminated soil on the top 15 cm. The jars were wrapped with aluminum foil to prevent PAH photodegradation and algal growth. Triplicate treatments were set up with solid compost, liquid compost, and a no-amendment control. The solid compost was mixed in with the top 3 cm of the contaminated soil. Each week, 100 ml of 50% compost tea was applied to the liquid compost treated soil bioreactors while an equal amount of water was added to the solid compost and control treatments. Additionally, two sets of no-plant controls with contaminated and uncontaminated soils were prepared and handled in parallel.

Soil samples were collected weekly with a stainless steel spatula. Samples were collected as composites at a 5 cm depth from different parts of each jar to make-up approximately 10 g of the analyzed sample. This sampling method was adopted to reduce contaminant variability due to isolated "hot-spots" within each jar. Each sample was subjected to microbial enumeration and extracted for PAHs. All soil samples for analysis were stored in the dark below 4ºC. Analysis of PAH metabolites was conducted at the end of 14 weeks.

5.2.3 Soil Carbon Measurements

The soil organic matter (OM) was estimated by the loss-on-ignition (LOI) method in soil. Simply, the masses remaining after drying 1 g of soil in a muffle furnace (Isotemp®, Fisher Scientific) at 105ºC for 18 h (MT_1) and after incineration at 550ºC for 5 h (MT_2) in a crucible (mass M) were determined. The soil OM was calculated as follows:

$$OM_{LOI} = (M_{T1} - M_{T2}) / (M_{T1} - M) \times 100\%$$

Estimates of soil organic carbon (OC) were obtained by dividing calculated OM values by the conventional 'Von Bemmelen' factor of 1.724 (assuming 58% of OM is OC) (Broadbent, 1953).

Extractable DOC in the different soil treatments was estimated based on previous literature (Bolan et al., 2003). Specifically, a 1g soil mass was equilibrated with 10 ml 0.5 M K_2SO_4 solution in a 15 ml centrifuge tube and shaken for 3 h with an end-over shaker. The mix was then centrifuged for 40 min and filtered through a 0.45 um filter. The filtrate was collected and subsequently analyzed for DOC using a Shimadzu TOC-5050A Analyzer after making correction for soluble inorganic carbon.

5.2.4 Plant Growth Experiments

Corn (*Zea mays* L.) was selected primarily due to the ability of its root exudates to increase soil microbial communities capable of degrading xenobiotics and stimulating rhizodegradation of PAHs (Yoshitomi and Shann, 2001; Kraffczyck et al., 1984; Haby and Crowley, 1996). The germination and growth of corn was used to establish the reduction in toxicity of the PAH-contaminated soils pretreated by intrinsic bioremediation. In an initial study, corn was grown in the greenhouse in uncontaminated soils (clean sand) treated with mushroom compost and commercial fertilizer (Miracle-Grow®) as the sole source of plant nutrients. Specifically, two corn seeds were sown in each 500 ml glass beaker filled with an equal mass (500 g) of clean sand. Triplicate treatments were set up using solid compost, liquid compost, Miracle Growth® solution or water only for the no amendment control. This preliminary experiment was conducted to evaluate the performance of corn plants in soils amended with different nutrient sources. Corn growth was subsequently initiated in the contaminated soils following pretreatment by intrinsic compost bioremediation and using corn phytoremediation as

the polishing step. The rate of seed germination and upper plant biomass production were monitored for 4 weeks during the preliminary experiment and phytoremediation phase of the study.

5.2.5 Extraction of PAHs and Metabolites

The polycyclic aromatic hydrocarbon compounds of concern were extracted with organic solvent using a simple mechanical shaking method (Schwab et al, 1999). Briefly, a known mass of soil sample was dried for 24 h in an oven set at 28ºC. Two grams of dried soil were weighed into a 15 ml glass centrifuge tube to which 10 ml dichloromethane: acetone (50:50, v/v) was added. The solvents were purchased as HPLC grade from Fisher Scientific (Fair Lawn, NJ). The added samples were sealed with aluminum-lined septa, wrapped in aluminum foil, and mechanically shaken for 24 h at 18 rpm. The resulting extract was centrifuged for 30 min and filtered through glass wool for cleanup prior to analysis.

After 14 weeks of treatment, PAH metabolites were identified in the derivertized extracts. The polar PAH metabolites were extracted and derivertized as follows: 2 g of dried soil sample was extracted once with 5 ml hexane to remove the non-degraded PAH compounds. Then 1 ml of 6 M HCL was added to the soil and sonicated for 10 min. The mixture was extracted with 15 ml (3 x 5 ml) ether. The organic phases of the three extracts were combined and anhydrous Na_2SO_4 was added to ensure the removal of soil moisture. The organic phase was completely evaporated to a residue using a water bath. The remaining residue was silylated by adding 100 µl trichlorosilane (TMCS) and 200 µl N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) in a screw cap glass tube and placed in a water bath at 65°C for 20 min. Both silylating agents were purchased from Supelco (St. Louis, MO).

5.2.6 GC-MS Analysis

A gas chromatograph with a mass selective detector (GC-MS) (Shimadzu QP5000) was used for the quantitation and qualitative identification of the PAHs and their metabolites. The GC was fitted with a 30 m x 0.32 mm i.d. HP-5MS column (J&W Scientific, Folsom, USA) with a 0.25 um film thickness. The oven temperature was initially set at 50°C for 1 min, increased at a rate of 8°C/min to 220°C and held for 5 min, and then increased at a rate of 20ºC/min to a final temperature of 270°C and held for 10 min. The injector and transfer lines were heated at 250ºC and 280ºC, respectively. For parent compound quantitation, the mass spectrometer was tuned to the selective ion mode (SIM). A 16 PAH compound standard mix (100 – 1000 mg l^{-1} in dichloromethane) was obtained from Ultra Scientific (North Kingstown, RI) and used for calibration of the target compounds at various levels. The 1µl injection was performed in splitless mode using an AOC-17A autosampler with helium as the carrier gas at 9.35 psi.

Identification of metabolites was achieved by comparism of spectral peak patterns to the fragmentation programs of the NIST 62 and NIST 12 libraries as well as published literature with the MS set in the scan mode. Spectral peak patterns and GC retention times of standard compounds were also used for the identification of parent PAHs.

5.2.7 Microbial Enumeration

A simple dilution plate count method was used to determine the number of aerobic heterotrophic bacteria only in the different treatments. A 1 g soil sample was measured into a 50 ml sterile 0.01% Triton X- 100 solution. The suspension was shaken with a vortex mixer for 60 s. Decimal dilutions up to 10^{-9} were incubated on nutrient agar (Difco Scientific; Detroit, MI) for 48 h at 35°C. Triplicate counts of colony forming units per gram (cfu g^{-1}) were conducted.

5.3 Results and Discussion

5.3.1 PAH Removal from Compost Treated Soils

The initial (day 0) concentrations of the six probe PAHs monitored in the aged diesel contaminated soils were: naphthalene - 650 mg kg^{-1}, acenaphthylene - 476 mg kg^{-1}, acenaphthene - 610 mg kg^{-1}, fluorene - 120 mg kg^{-1}, fluoranthene - 122 mg kg^{-1}, and pyrene - 305 mg kg^{-1} (Table 5-2). After week-1, the l total extractable polycyclic aromatic hydrocarbons ($TPAH_{ext}$) concentration of the six probe PAHs of 2286 mg kg^{-1} decreased by 36%, 41% and 33% in the solid compost, liquid compost and control treated soils respectively. These reductions were significant, and greatest in the liquid compost treated soils. However, it is unclear whether the observed decreases after week-1 were as a result of physical losses such as sorption and volatilization or actual degradation. After week-6 the extractable PAH concentrations had decreased by 87%, 92%, 86.8% in the solid compost, liquid compost and control treatments respectively. At the end of six weeks none of the treatments supported the growth of corn plants, an indication that the soils still contained high concentrations of the residual PAHs toxic to corn seeds. After week-6 the $TPAH_{ext}$ for solid compost, liquid compost and control soils was determined as 300 mg kg^{-1}, 181 mg kg^{-1} and 302 mg kg^{-1} respectively. After week-8, the $TPAH_{ext}$ had reduced by 95% in both solid compost and control soils to 133 mg $kg^{-1}$and 115 mg kg^{-1} respectively. Higher concentrations of fluorene (> 40 mg kg^{-1}) and pyrene (> 35 mg kg^{-1}) still remained in the solid compost treatments and control soils than in the soils treated with liquid compost (5.6 mg kg^{-1} fluorene and 17.8 mg kg^{-1} pyrene) where the $TPAH_{ext}$ had reduced by 98%. The significantly lower concentration of the total extractable polycyclic aromatic hydrocarbons

Table 5-2 Concentrations of extractable PAHs (in mg kg^{-1}) in compost amendment and no-amendment control soils[a]

	Day-0	Week-1			Week-6			Week-8			Week-14		
		Solid	Liquid	Control	Solid	Liquid	Control	Solid	Liquid	Control	Solid	Liquid	Control
NAPH	650.5	455.8	357.4	552.6	21.0	3.0	25.9	3.1	0.1	0.2	0.2	0.03	1.0
ACEY	476.8	331.0	348.7	379.5	49.1	6.6	63.6	9.9	1.4	4.6	3.2	1.7	1.3
ACEA	610.3	420.9	345.8	322.2	69.6	30.6	80.7	15.1	5.7	7.9	9.5	2.5	37.4
FLUO	120.7	80.2	66.1	67.2	78.8	55.7	55.2	44.1	15.6	50.9	34.9	6.8	44.6
FLUA	122.1	18.3	17.9	18.4	9.5	7.4	15.3	16.0	5.8	16.0	9.6	2.6	7.9
PYRE	305.5	229.4	215.7	182.5	71.8	77.8	60.9	45.1	17.8	35.4	41.9	5.9	16.0
$TPAH_{ext}$	2285.9	1455.4	1351.6	1522.4	299.8	181.1	301.6	133.3	46.4	155	99.3	19.5	108.2

[a] Values are the means of triplicate analysis (excluding outliers >3s)

Abbreviations: $TPAH_{ext,}$ Total extractable PAHs NAPH, naphthalene; ACEY, acenaphthylene; ACEA, acenaphthene; FLUO, fluorene; FLUA, fluoranthene; PYRE, pyrene.

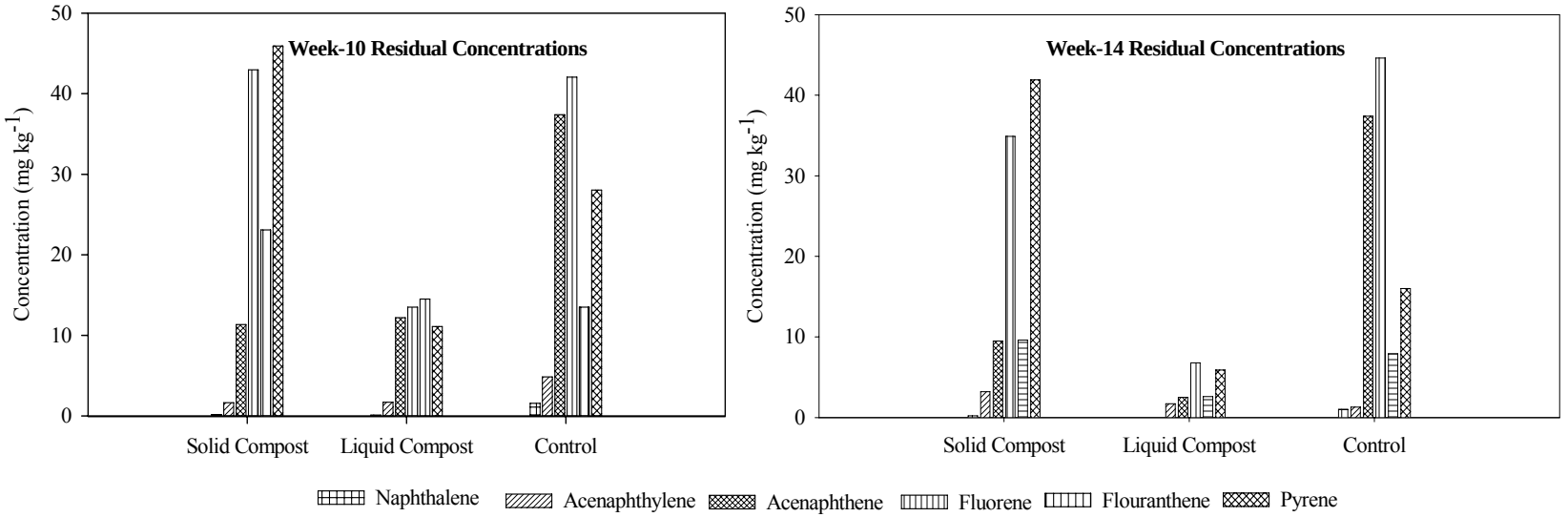

Figure 5-1 Determination of residual PAH concentrations in solid and liquid compost, and control treatments at week-10 and 14. Corn was planted at week-10 following soil pre-treatment with solid and liquid mushroom compost.

($TPAH_{ext}$) in the liquid compost treated soil samples relative to the solid compost and control soils suggest that the liquid compost treatment was more effective at increasing PAH bioavailability and transformation.

At week-10, the $TPAH_{ext}$ from the liquid compost treated soils was about 43 mg kg^{-1}, still much lower than 125 mg kg^{-1} in the solid compost treated soil and 126 mg kg^{-1} in the control samples. The PAH compounds with the highest concentrations in the liquid compost treated soil samples was fluoranthene (14.5 mg kg^{-1}) and for the solid compost and control treatments were pyrene (45.9 mg kg^{-1}) and fluorene (45.1 mg kg^{-1}), respectively (Figure 5-1). As a result, corn plants were successfully established in the liquid compost treatments, further confirming that the liquid compost is more effective than solid compost at treating PAH-contaminated aged soils. Residual PAH concentrations in soil < 20 mg kg^{-1} appear to be non-toxic to plants, allowing for the successful germination and growth of corn. The residual concentrations of fluorene, fluoranthene and pyrene in soils treated with solid compost were as high as or higher than their corresponding concentrations determined in the control samples. This suggests an accumulation rather than degradation of the PAHs and is indicated by the slight concentration changes between week-10 and week-14 in both solid compost and control soils.

5.3.2 Organic Carbon Content in Treated and Untreated Soils

Comparing the organic carbon content of solid mushroom compost and compost tea treated soils, it was observed that soils amended with solid compost had the highest TOC concentrations (3.52 ± 0.13% to 4.03 ± 1.99%) and the lowest concentration of DOC at 40.53 ± 5.41mg l^{-1} to 68.75 ± 21.01 mg l^{-1} (Figures 5-2 and 5-3). As expected, the highest DOC concentrations were measured in soils amended with liquid compost. The higher amounts of non-

dissolved OC in solid compost treated samples explained the high concentrations of residual PAHs sorbed and recovered during extraction of the treated soils. These results are in agreement with earlier studies that showed a significant decrease in bioavailability of hydrophobic PAHs that bind strongly to the soil organic matter in soils rich in humus (Chiou et al., 1989). The solid organic compost therefore increased the fraction of bound residual PAH in the solid compost treated soils, which limits their bioavailability and biodegradation.

The measured concentration of DOC in the "uncontaminated" or control soil increased from 9.4 mg l^{-1} at the beginning of the experiment to >25 mg l^{-1} when contaminated with diesel. The nearly linear increase in concentration of DOC in the compost amended and control soils was attributed to biodegradation of the soil organic matter and solubilization of some diesel compounds with increased incubation. Following this line of reasoning it can be inferred, from Figures 5-2 and 5-3 that transformation of the diesel compounds, including PAHs, into more soluble compounds occurred progressively.

The high rate of increase in DOC concentration in liquid compost treatments of aged diesel contaminated soils shown in Figure 5-3 corresponded to the greatest decrease in PAH concentration (Table 5-2) and lower concentrations of residual PAH compounds (Figure 5-1) in the latter samples. The liquid compost was predicted to increase the soluble (bioavailable) PAH fraction because it behaves similarly as a co-solvent. The attenuation of PAH concentrations and identification of metabolites as discussed below provides additional support to our hypothesis that pretreatment of aged PAH-contaminated soils with liquid organic compost significantly increases bioavailability and degradation of the probe PAHs.

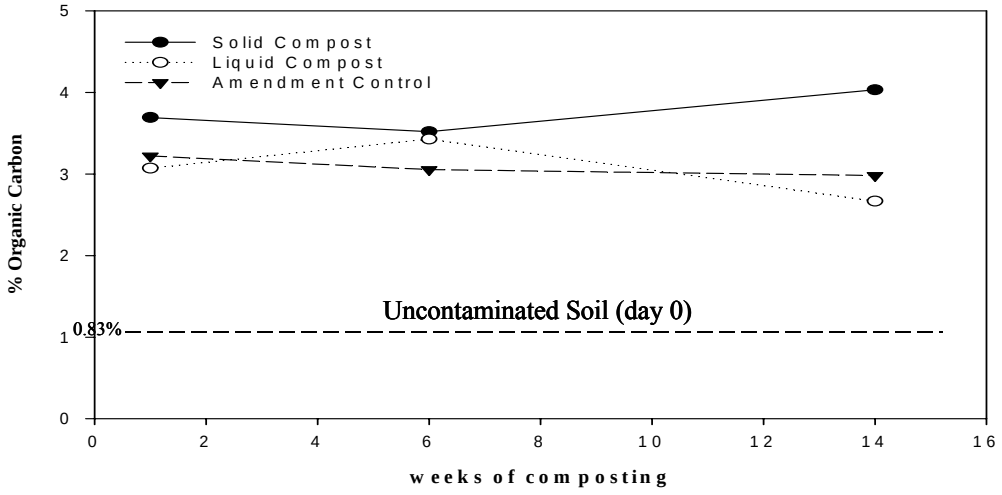

Figure 5-2. Changes in percent total organic carbon (TOC) content during 14 weeks of composting. Horizontal dashed line represents initial soil TOC concentration before contamiantion with diesel.

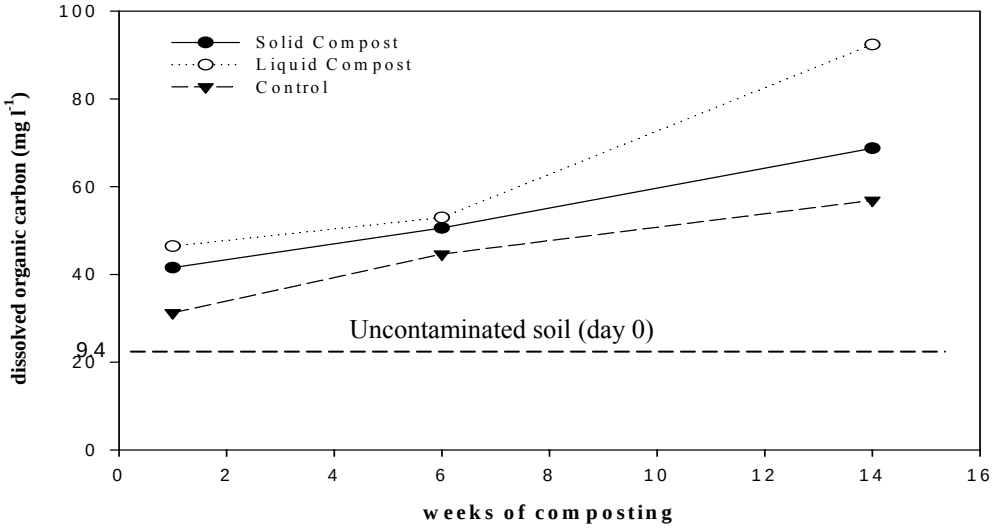

Figure 5-3. Changes in soil DOC concentrations during 14 weeks of composting followed by planting with corn. Horizontal dashed line represents initial soil DOC concentration before contamination with diesel.

5.3.3 PAH metabolism

The results of qualitative analyses to identify key PAH metabolites during biodegradation of the parent compounds in the compost amended soil showed the formation of aromatic acid, alcohol, aldehyde and ketone fractions after 14 weeks of treatment (Table 5-3). Compared to the baseline concentrations (Table 5-1), it is clear that the aromatic metabolites were not original components of the soil. Additionally, the presence of aromatic dicarboxylic acids indicates that these compounds are not naturally occurring soil acids. The metabolites identified in this study are similar to confirmed products of PAH transformation in compost treated soils reported in other studies on the biodegradation of diesel hydrocarbons (Langbehn and Steinhart, 1995).

A qualitative examination of the transformation products shows that more metabolites were identified in soils amended with liquid mushroom compost than in solid compost treatments and controls. There is support for PAH biodegradation given by the identification of known metabolites in both compost treatments. However, greater peak areas of the metabolites were observed in soils treated with liquid compost (not shown here). In addition, key metabolites identified in the liquid compost soils, specifically tetrahydrophenanthren-4-ol (m/z 212) and hydroxymethoxyanthen-9-one (m/z 302) could have formed from the degradation of 3- and 4-ring compounds such as phenanthrene, anthracene, fluorene and pyrene. The mixture of mono- and di-aromatic acids such as benzoic acid (m/z 224), hydrophenanthrene carboxylic acid (m/z 342), soluble and easily degraded 2-naphthalenol (m/z 174), and hydroaromatic compounds such as tetradecahydroanthracene (m/z 360) also confirmed the degradation of the parent PAHs. Readily mineralizable metabolites such as dihydro-2,3-naphthalenedione (m/z 216) and hydroxy benzaldehyde (168), are confirmed products of bacterial breakdown of naphthalene (Eaton and Chapman, 1992), and suggest end-stage degradation of PAH compounds in the liquid compost

Table 5-3 Identified metabolites of PAH in solid and liquid mushroom compost treated soils and untreated controls handled in parallel. (Identified as TMS-derivaties and esters)

Compound	Rt.[a] min.	MW[b]	Spectral[c] Masses	Solid Compost	Liquid Compost	Control
Aromatic Acids						
Benzoic acid	3.5	224	207, 211,224	x	x	x
Benzoic acid	15.7	370	370,281,297	x	x	
Hydroxybenzenedicarboxylic acid	17.9	182	165,137,120		x	
4-Hydroxy-3-methoxy benzoic acid	18.2	168	139,124,169	x		
Benzo-pyran-2-carboxylic acid	16.6	390	391,364,393		x	
hydrophenanthrene carboxylic acid	16.6	342	267,327,283	x	x	
Tetrahydrocarbazole-carboxylic acid	16.3	344	285,286,168	x		x
Methoxy-Benzoic acid	24.8	212	137,179,197		x	
Indole propanoic acid	27.4	202				
Alcohols						
Dimethyl-1-benzopyran-3-ol	16.4	346	328,167,180		x	
Tetrahydrophenanthren-4-ol	21.1	212	194,179,165	x	x	
2-naphthalenol	16.1	174	131,159,145		x	
Aldehydes						
3,4-dihydroxy-benzaldehyde	16.4	168	167,125,139	x		x
Benzenedicarboxyaldehyde	17.7	134	105,77,106		x	
Hydrophenanthrenecarboxyaldehyd	16.5	290	257,275,229		x	
Benzaldehyde	5.5	194	179,151,161	x		
Acetyl-hydroxy-benzaldehyde	24.3	210	195,181,153		x	
Ketones						
Hydroxymethoxyanthen-9-one	16.3	302	287,288,259		x	
Cyclohexadien-1-one	18.3	198	155,170,183	x		x
Dihydro-2,3-Naphthalenedione	14.6	216	145, 216,160	x	x*	
dihydronaphthoquinone	15.3	322	307,323,289		x	
dihydrotetralindione	14.7	145	160,173,216	x		x
Dihydroxy-1-benzopyran-4-one	27.8	276	220,189,276		x	
Hydroxy-2-quinoline carboxamide	23.8	354	235,339,353	x	x	
Oxygenates						
Dibenzo b,d pyran-1-oxy	14.7	372	357,329,301		x	
Hydroaromatics						
Dihydro-1-Indene	14.9	208	193,178,130		x	
Dodecyl-Tetradecahydroanthracene	25.9	360	191,264,303		x	
Tetrahydrophenanthrene	18.2	182	154,165,141	x		x

x - Identified in the treatment

[a] Retention time in minutes, *identified at retention times 14.6 and 16.8 min

[b] MW: molecular weight of target compound

[c] Spectral mass pattern of associated with the base peak

treated soils. Although direct confirmation of mineralization of the PAH was not an objective of this study, it can be inferred from the suite of metabolites identified and amount of PAHs removed (Figure 5-1) in the compost tea treated samples that transformation and mineralization were achieved. Fungi were not cultured in the microbial analysis. However, the detection of dihydro-napthoquinone (m/z 322) suggests fungal metabolism under anaerobic conditions (Cerniglia et al., 1985). It thus appears that there was aerobic metabolism at the upper levels of the soils and anaerobic metabolism in the lower levels involving bacteria and fungal co-cultures.

In the control samples, the amounts of the identified metabolites (e.g., carbozole-carboxylic acid) estimated from the size of the chromatogram peak were relatively low. Thus, losses due to biodegradation by indigenous soil microorganisms were minimal. The dominant mechanisms of PAH loss in the control soils may be attributed to natural attenuation by physical processes such as sorption, entrapment within the soil matrices, and volatilization.

5.3.4 Soil Bacteria

The results of bacterial counts determined at multiple points during the biotreatment of the aged diesel contaminated soils with liquid and solid mushroom compost treated soils are compared to controls in Figure 4. The number of culturable bacteria in the compost treated contaminated soils at week-10 increased by five orders of magnitude compared to the uncontaminated soils used as controls. Although the number of colony forming units decreased slightly at week-14, the number of culturable bacteria was still higher than in the uncontaminated soils. The decrease in the bacterial counts at week-14 may be attributed to the formation of potentially toxic metabolites from PAH transformation (Lundstedt et al., 2003) or a decease in the available PAH substrates. The high microbial activity (Figure 5-4) and the PAH metabolites

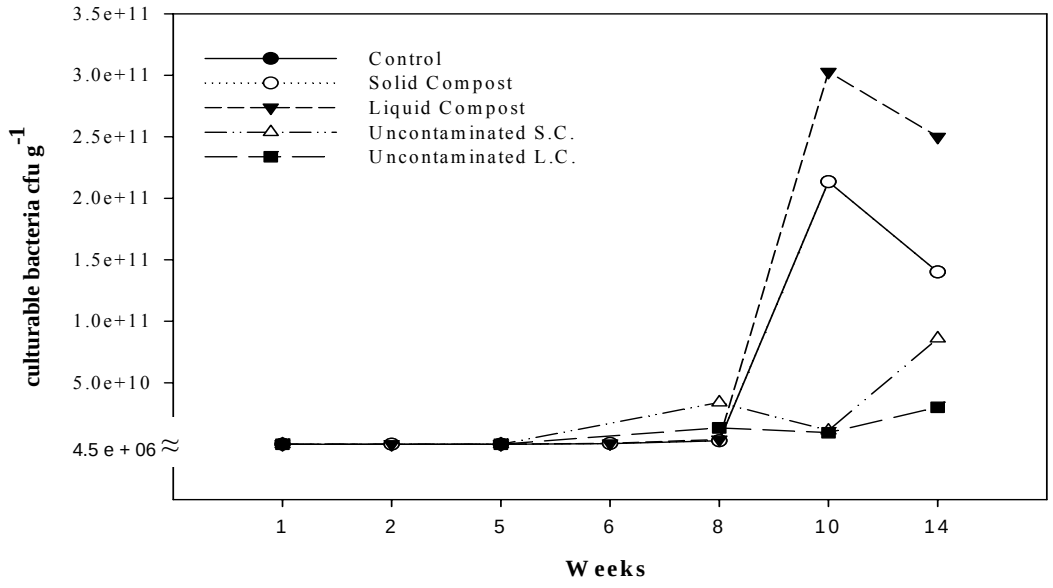

Figure 5-4. Changes in the number of culturable bacteria in diesel-contaminated and uncontaminated soils during 10 weeks of compost pre-treatment. Abbreviations: SC, solid compost; LC, liquid compost.

(Table 5-3) after week-10 corresponded with the treatment points at which plant growth was successful and a good indication of decrease in PAH toxicity.

5.3.5 Plant Growth

The survival and growth corn plants in the aged contaminated soils treated with mushroom compost signified the reduction of soil toxicity by biodegradation of strongly sorbed PAH components. In the uncontaminated soils, the corn germinated and grew better in the mushroom compost amended soils than in the same soil treated with commercial fertilizer. For the corn germination experiment in uncontaminated soil, the following results were obtained: solid compost (4 d, 100%) ≡ liquid compost (4 d, 100%) > fertilizer (4 d, 83%) > control (6 d, 67%); representing the number of days, d, it took for two planted seeds to germinate and 100%

germination rate equals all six planted seeds germinating in all three replicate nutrient amendments. The growth heights of the upper plant biomass after 4 weeks were as follows: solid compost (38 ± 1.53 cm) > liquid compost (36 ± 8.66 cm) > fertilizer (24 cm) ≡ water only (22 ± 10.54 cm) (based on the average of triplicates, where available). These results show that both solid and liquid composts provided sufficient nutrients to support corn germination and growth. Although the seed germination was faster in the fertilized soil than the water control, both treatments performed poorly compared to the compost amended soils.

Phytoremediation as a polishing tool of the mushroom compost treatment of aged diesel contaminated soil was attempted at weeks 6, 8, and 10, respectively. The ability of corn seeds to germinate, survive and grow for more than 4 weeks was used to assess phytoremediation success. No germination was observed in any treatment at week-6. At week-8, germination was observed in the liquid compost treatments only. Plant survival, however, was temporary as the corn mortality occurred after six days. The third and final planting, at week-10 resulted in no mortality in the liquid compost samples, with up to 22 cm increase in plant height after 28 days of plant growth. During the same period (4 weeks), only one out of three soils amended with solid compost supported plant growth and seed germination was only 50%. Nevertheless, the corn plant survival was relatively short (8 d) with only 3 cm increase in plant height. Similar to the solid compost treatments, only one of the three unamended control jars had successful corn germination and growth of 42 cm. The unexpected growth success in only one of the triplicate unamended controls may be attributed to decreased contaminant toxicity from reduced contaminant bioavailability (Hübner et al., 2000).

Contaminant toxicity to plants may explain the inability to grow corn in the aged diesel contaminated soil at weeks 6 and 8. Ten weeks of pre-treatment with mushroom compost tea was

sufficient to reduce the concentration of PAH and its metabolites to non-toxic levels that would support germination of corn seeds. This observation was supported by a 43% decrease in residual PAH concentration that occurred between week-10 and 14 compared to less than 1% decrease in the solid compost treated soils and controls.

5.4 Conclusion

The application of liquid mushroom compost as suitable amendments for the pretreatment and detoxification of highly contaminated aged diesel soils was achieved in about 10 weeks. Unlike the solid mushroom compost, the liquid compost increases bioavailability and aerobic/anaerobic biodegradation of the adsorbed PAHs. Contrarily, for the solid compost treated soils, physical processes such as sorption of the probe PAHs onto humus fractions and into soil microsites predominantly accounts for the reduction in concentration of the bioavailable fraction of the compounds. The residual PAHs bound to the soil organic matter may continue to persist in the environment and pose ecotoxicological risks.

The results of this study have indicated that pre-treatment of aged diesel contaminated soils using liquid organic carbon and nutrient rich compost extracts enhances rapid removal of PAHs from the soil matrices by increasing bioavailability and biodegradation. This enabled for polishing of the residual parent compounds and metabolites using phytoremediation. If grain crops such as corn are used in polishing of residual contamination remaining in soils pre-treated to reduce its toxicity, the harvested grain could be used in the production of bioenergy, for example production of ethanol used as gasoline additive.

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Chapter 6: CONCLUSION

6.1 Summary of Research Results

Generally, the biodegradation rate of many organic compounds thought to be readily degradable in natural systems is much lower in solid than in the liquid phase. This is especially true for hydrophobic compounds, such as the polycyclic aromatic hydrocarbons (PAHs), which due to their hydrophobic nature, partition strongly into solid phases such as organic matter and the mineral surfaces of soils. Thus, PAHs are environmentally persistent in soil systems. In addition, PAHs are environmentally ubiquitous due to human activities such as the manufacture, transportation, and storage of petroleum products. Their toxicity to living organisms and potential carcinogenicity, together with their widespread distribution, has resulted in a global contamination problem that needs to be addressed.

Thus, it is important to investigate new and cost-effective remediation methods to improve the methods currently being used in the remediation of PAHs from contaminated soils. Bioremediation and phytoremediation are innovative remediation techniques that are capable of transforming, or completely degrading PAHs into harmless by-products. These processes are dependent on the metabolic functions of soil microorganisms and/plants. Biodegradation is limited by the partioning of PAHs to solid phases as well as high total PAH concentrations > 100 mg kg^{-1}. The growth of plants during phytoremediation thus becomes a major challenge at highly contaminated sites.

In this research study, the overall goal was to improve bioavailability and enhance the biodegradation of highly-impacted PAH contaminated soils to enable the application of

phytoremediation to further remove residual PAHs that often remain following the application of bioremediation treatments. Using laboratory and greenhouse experiments, this research study was able to decrease PAH sorption and enhance their bioavailability through the application of a liquid and natural amendment collected as run-off from a spent mushroom compost pile. The mushroom compost extract (MCE) is rich in dissolved organic carbon (> 3500 mg l^{-1}) and of medium nutrient (N: 5 mg l^{-1}, P: 7.5 mg l^{-1}) range. The MCE provided a liquid carbon substrate that acted as a natural co-solvent and improved PAH solubility out of the soil microsites. The increased bioavailability of PAHs was confirmed in a biodegradation kinetic analysis, in which the MCE amended soils demonstrated an adherence to the first-order biodegradation kinetic model. This observation sharply contrasted the initially fast PAH decreases due to sorption, followed by slow transformation in the bi-phasic model established in the absence of MCE (Chapter 3). In addition, the organic carbon and nutrients in the compost extract provided a readily available food source for growth of both plants and soil microorganisms. The latter was confirmed by up to four orders of magnitude increase in microbial numbers following the application of MCE to highly contaminated soils.

The increase in soil microorganisms, either in the indigenous consortia or provided by the MCE was effective in decreasing PAH concentrations up to 95% in approximately 50 days (Chapter 4). At different dilutions of MCE, the highest anaerobic degradation of diesel hydrocarbons (70%) and individual PAHs (90%) was at 50% MCE concentrations (Chapter 5). Complete mineralization of 1 – 3 ring PAHs (naphthalene, acenaphthene, phenanthrene and anthracene) was observed in both aerobic and anaerobic systems and was established by the identification of a suite of metabolites formed as a result of bacterial and fungal metabolisms. Overall, metabolites such as: 3,4 hydroxy benzaldehyde, dicarboxylic acids, phenanthrene

carboxylic acid, benzopyran-2-one, tetrahydrophenanthrene, quinolin-2-one, and 1,4-napthoquinoe, 1,2,3,4-tetrahydrophenanthren-4-ol were identified. The abundance of ketones indicates fungal degradation, which may have originated from the mushroom compost or formed as a result of the activity of ligninolytic enzymes remaining in the compost after mushroom harvests. Additionally, the identification of the *phen*-gene, responsible for phenanthrene metabolism, in the MCE-treated soils yet absent in the control soils provided further support for a feasibility of enhancing PAH degradation with a liquid mushroom compost amendment (refer to Figure A-3 in the appendices).

This research study also shows that 50% MCE can be affectively applied as bioremediation treatment of diesel contaminated soils with a total PAH concentration of ~ 2200 mg kg^{-1} as a pre-treatment to reduce initial total PAH concentration to < 100 mg kg-1 within 6 weeks. Using corn crops, plants were successfully established within 8 weeks of beginning the experiment and resulted in an additional 55% reduction in residual PAHs. The total PAH concentration was reduced to < 20 mg kg^{-1} at the end of 14 weeks of biodegradation followed by phytoremediation. In contrast, corn crops failed to establish in the contaminated soils that had been previously treated with solid compost amendment; indicating a sequestration of toxic residual PAHs.

6.2 Implications for Bioremediation

This research provides a strong basis for field application of liquid extracts from the mushroom compost industry or similar natural organic carbon amendments containing microbial growth nutrients, PAH degrading enzymes as well as diverse microbial consortia. The high

production, > 1 million tones per year, of spent mushroom compost as released as agricultural waste in the United States alone can be effectively re-cycled and used in the bioremediation of highly toxic and ubiquitous organic contaminants in the environment. This bioremediation method is cost-effective and sustainable (Chui et al., 2000) and can be effectively applied in either *in-situ* (landfarming) or *ex-situ* (bioslurry) soil remediation processes. As shown by this research study, mushroom compost extracts have an enormous potential to be used to decrease contaminant toxicity at sites where phytoremediation cannot be applied, and allow for the application of phytoremediation as a polishing tool at a latter stage. Although contaminant degradation rates are generally site specific, this study has shown that the application of dual remediation methods (biodegradation and phytoremediation) will also results in faster clean-up times and enhanced mineralization of contaminants.

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APPENDICES

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Table A -1. Bi-phasic kinetic model of SC (amendment control) compared to the first-order model of SB (50% compost amendment) microcosms[a,b]

Contaminant	Non Sterile Control (SC)				50% Compost Amendment (SB)	
	k_1	$t_{1/2}$	k_2	$t_{1/2}$	k	$t_{1/2}$
Naphthalene	0.0752	9.2	-	-	0.042	16.6
Anthracene	0.0761	9.1	0.0110	63.0	0.020	34.8
Phenanthrene	0.0778	8.9	0.0108	64.2	0.022	30.9
Fluoranthene	0.0869	8.0	0.0050	138.6	0.019	36.5
Pyrene	0.1057	6.6	0.0024	288.8	0.018	38.5

[a] k is the rate constant, units = day-1
[b] $t_{1/2}$ is the half-life, units = days

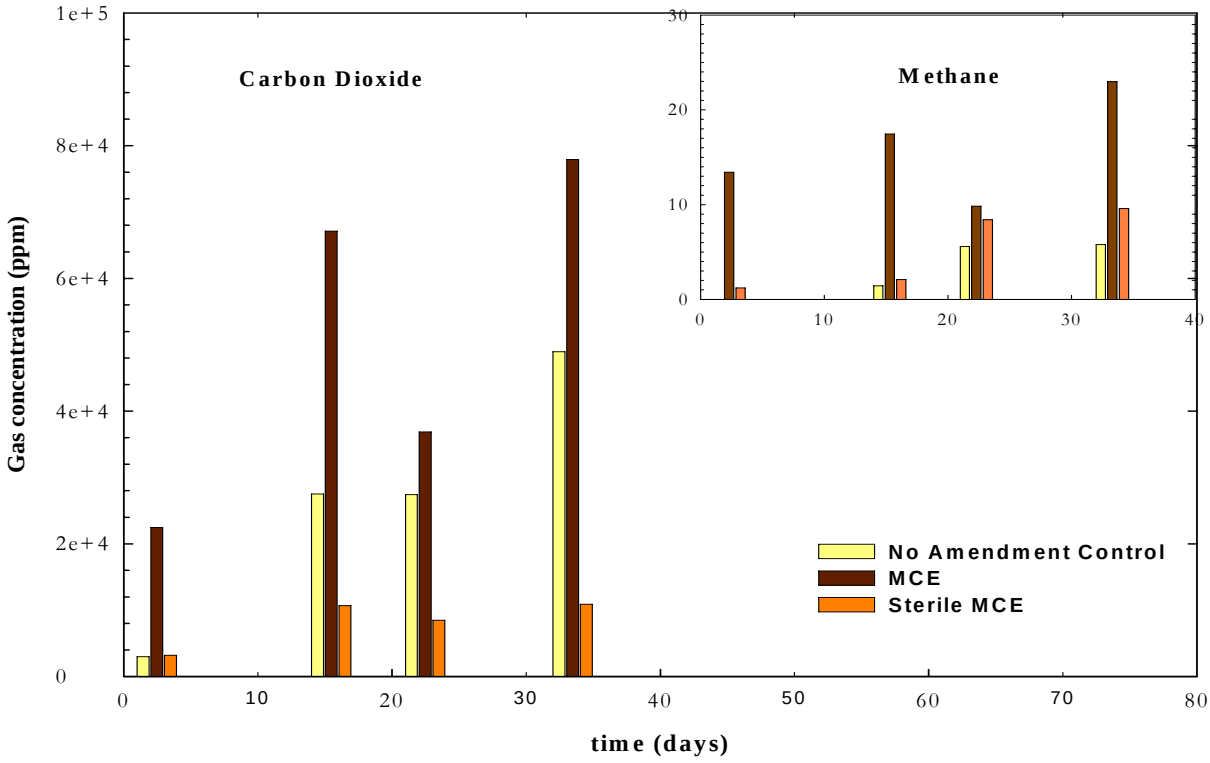


Figure A-1. Mean carbon dioxide and methane (insert) gas produced in headspace of bioreactors containing PAH-contaminated soils incubated for 32 days with organic mushroom compost, sterilized organic mushroom compost extract, and water only.

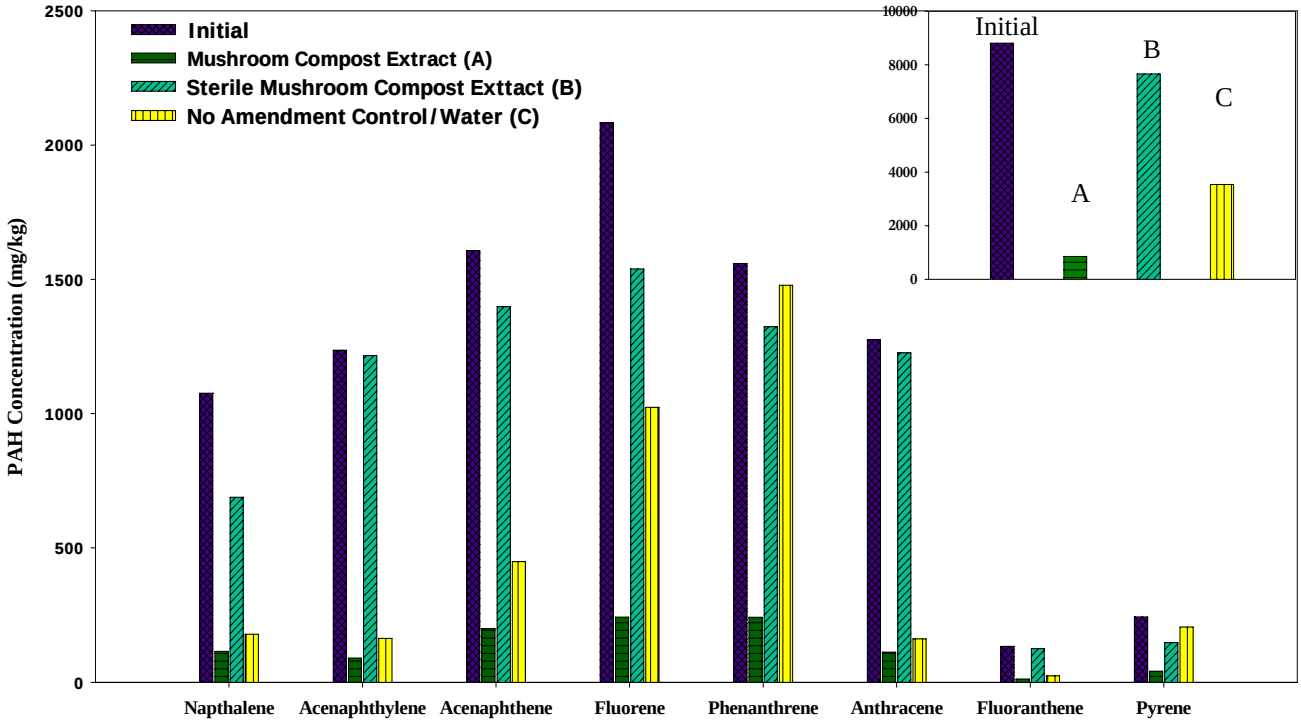
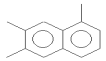
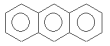
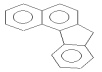


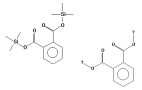
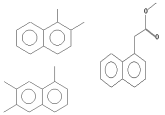
Figure A-2. Concentration of 8 PAH compounds remaining in the different bioreactors containing PAH-contaminated soil incubated with: A, organic mushroom compost extract; B, sterilized organic mushroom compost extract; and C, water only; after 32 days of treatment. The graph insert shows the total PAH concentrations at the beginning of the experiment and after 32 days of incubation with treatments A, B, and C.

Table A-2. GC-MS identification of PAH parent compounds and their alkylated-derivatives in MCE-treated soil

Parent Compound	Alkyl Derivative	Retention [a] time (min)	Molecular [b] Weight (m/z)	Molecular Formula	Structure
Naphthalene		10.80	128	$C_{10}H_8$	
	1,2-dimethylnaphthalene	15.22	156	$C_{12}H_{12}$	
	1,6,7-trimethylnaphthalene	17.43	170	$C_{13}H_{14}$	
Acenaphthylene		15.72	152	$C_{13}H_8$	
Acenaphthene		16.32	154	$C_{12}H_{10}$	
Fluorene		17.93	166	$C_{13}H_{10}$	
Anthracene		21.083	178	$C_{14}H_{10}$	
	2-methylphenanthrene	24.07	192	$C_{17}H_{12}$	
Fluoranthene		26.69	202	$C_{16}H_{10}$	
Pyrene		27.45	202	$C_{16}H_{10}$	
3,4 Benzofluorene		29.42	216	$C_{17}H_{12}$	

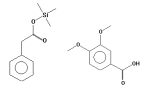
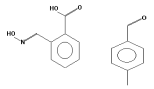
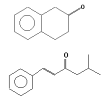
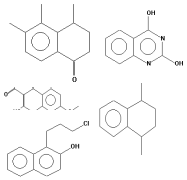
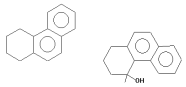
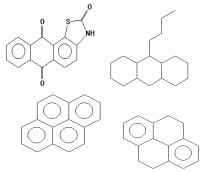
[a] GC retention time; [b] mass/charge ratio of fragment ion

Table A-3. GC- MS identification of PAHs and metabolites determined after 1-week treatment with MCE

Group/ Parent	Compound Identified [a]	R. time (min) [b]	Molecular Weight (m/z) [c]	Molecular Formula	Structure
Alcohol	trimethyl, 5-methyl, 2-phenoxy	4.13	222	$C_{13}H_{22}OSi$	
Organic Acids	Benzene 1,2-dicarboxylic acid	4.97, 7.10	286,310	$C_{13}H_{22}O_4Si_2$	
Naphthalene		10.8	128	$C_{14}H_8$	
	Naphthalene acetic acid	14.73	200	$C_{13}H_{12}O_2$	
	Naphthalene 1,2-dimethyl	14.83	156	$C_{13}H_{12}$	
	Naphthalene 1,6,7-trimethyl	16.98	156	$C_{13}H_{14}$	
Acenaphthylene		15.72	152	$C_{13}H_8$	
Acenaphthene		16.32	154	$C_{12}H_{10}$	
Fluorene		17.93	166	$C_{13}H_{10}$	
Phenanthrene					
	Phenenthrene 2.5-dimethyl	26.283	206	$C_{14}H_{16}$	
Anthracene					
	Anthracene 9,10 dimethyl	26.00	206	$C_{14}H_{16}$	
Fluoranthene		26.69	202	$C_{16}H_{10}$	
Pyrene		27.45	202	$C_{16}H_{10}$	

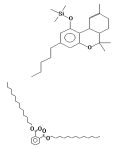
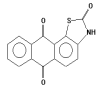
[a] as trimethyl derivatives or esters; [b]GC retention time; [c] mass/charge ratio of fragment ion

Table A-4. GC- MS identification of PAHs and metabolites products determined after 14-weeks of MCE-treatment

Group/ Parent	Products Identified [a]	R. time [b] (min)	Molecular [c] Weight (m/z)	Molecular Formula	Structure
Acids	Benzeneacetic acid	12.25	208	$C_{11}H_{16}O_2Si$	
	Benzoic acid	11.88	182	$C_9H_{10}O_4$	
Alcohol	3,4,5-trimethoxyphenol	19.37	184	$C_9H_{12}O_4$	
Aldehydes	Benzaldehyde 2,4-dihydroxy	6.317	152	$C_8H_3O_3$	
	Benzaldehyde	14.37	120	C_9H_7NO	
Ketones	1H-1-Benzopyran-2-one	14.78	176	$C_9H_{12}O_2$	
	Hexen-3-one	11.05	188	$C_9H_{12}O_4$	
Naphthalene					
	Naphthalenenone, 3,4-dihydrol	11.40	146	$C_{10}H_{10}O$	
	Quinazolinedione	11.88	162	$C_8H_6N_2O_2$	
	1,4 Naphthoquinone	12.75	306	$C_{15}H_{14}O_7$	
	Tetrahydronaphthalene	13.35	160	$C_{12}H_{16}$	
	2-hydroxynaphthalene	18.48	184	$C_{13}H_{13}ClO$	
Phenanthrene					
	Phenanthrenol, 1,2,3,4-tetrahydro	18.15	182	$C_{14}H_{14}$	
	4-Phenanthrenol, 1,2,3,4-tetrahydro	21.82	194	$C_{15}H_{16}O$	
Anthracene					
	1,2-Dihydroanthra 1,2-d thiazole-2,6,11-trione	22.23	281	$C_{15}H_7NO_3S$	
	9-butyl tetradecahydroanthracene	21.96	248	$C_{18}H_{32}$	
Pyrene		27.45	202	$C_{16}H_{10}$	
	4,5,9,10-tetrahydropyrene	25.98	206	$C_{16}H_{14}$	

[a] as trimethyl derivatives or esters; [b] GC retention time; [c] mass/charge ratio of fragment ion.

Table A-5. GC- MS identification of PAHs and metabolites products determined after 19-weeks of MCE treatment

Group/ Parent	Products Identified [a]	R. time [b] (min)	Molecular [c] Weight (m/z)	Molecular Formula	Structure
Alcohol	trimethoxyphenol	4.13	222	$C_{13}H_{22}OSi$	
Acids	Benzene 1,2-dicarboxylic acid	4.97, 7.10	310	$C_{13}H_{22}O_4Si_2$	
	Phthaladehydic acid	11.40	165	$C_8H_7NO_3$	
Phenanthrene	dibenzopyran-6,7,8,10-tetrahydro	23..37	386	$C_{13}H_{22}O_4Si_2$	
Anthracene	1,2-Dihydroanthra 1,2-d thiazole-2,6,11-trione	22.23	281	$C_{15}H_7NO_3S$	
Pyrene		27.45	202	$C_{16}H_{10}$	
	4,5,9,10-tetrahydropyrene	26.96	206	$C_{16}H_{14}$	

[a] as trimethyl derivatives or esters; [b] GC retention time; [c] mass/charge ratio of fragment ion

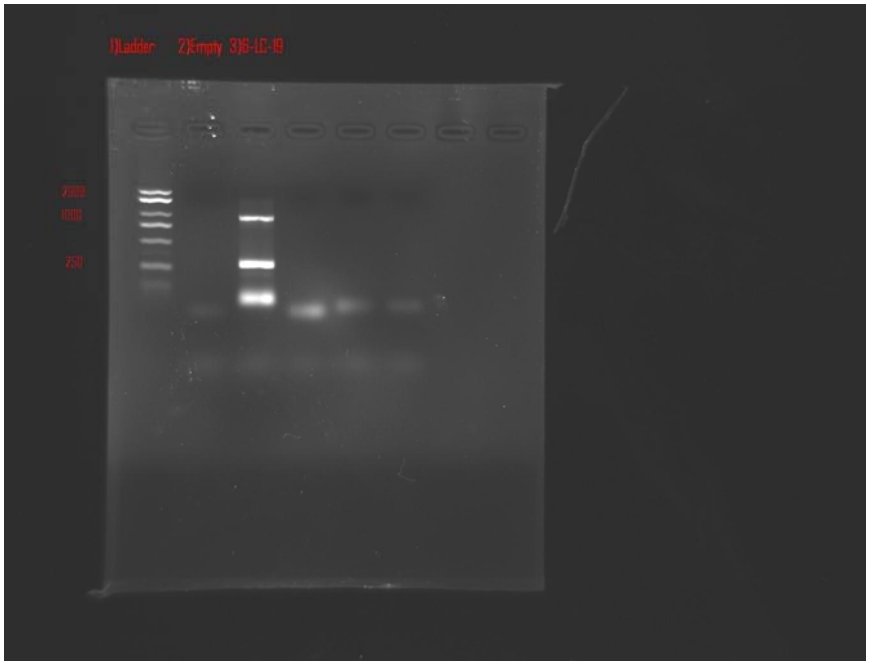


Figure A-3. Polymerase Chain Reaction (PCR)-amplification identifying the *phen*-gene in the 993 region of a DGGE run (Lane 3) from soils treated with liquid mushroom compost extract (LC).

DNA was extracted from five samples using the Mo Bio UltraClean™ Soil DNA Kit. The extracted DNA was further purified using the QIAquick® PCR Purification Kit. The primer sequence for the phenanthrene-degrading gene was:

5´–TTCGAGCTGGAATGTGAGC 3´– CAAACCTTAGCGGCCAATAA

This gene amplifies in the 993-bp region. The gel was then observed using the BioChemi System UVP BioImaging System and photographs were taken. Detailed description of the methods used has been described by Lloyd-Jones et al. (1999).