I, Katherine E. Gómez, hereby submit this as part of the requirements for the degree of:

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It is entitled:

Phytoremediation of contaminated soil from a petroleum refinery land treatment unit

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PHYTOREMEDIATION OF CONTAMINATED SOIL
FROM A PETROLEUM REFINERY LAND TREATMENT UNIT

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ABSTRACT

Phytoremediation, the use of plants to remove contaminants from soil, has attracted a lot of attention because of its low cost relative to other remediation strategies and its non-invasive approach to returning land to a more pristine state. Application of phytoremediation to field sites is hampered by the lack of knowledge concerning which types of sites may be effectively treated by phytoremediation. There may be a wide array of contaminants present, and there are many variables associated with a specific site’s history that could influence remediation efforts.

The distribution of PAHs and certain heavy metals were measured at 2 different depths and from unplanted and planted plots across a petroleum refinery landfarm. The contaminant data was related to microbial biomass, activity and community structure. Large differences existed between the upper and lower soil layers in terms of contaminant levels, and microbial population. The differences between the planted and unplanted plots, however, were not significant probably because the unplanted plots had only been plant free for a period of 1 year prior to sampling.

Plants of several species were grown in the laboratory on the contaminated soil to evaluate the potential of phytoremediation to treat the soil and return it to an acceptable level of contamination. After 4 months of growth the concentration of PAHs was significantly lower, while the heavy metal concentration was unchanged. Microbial changes had occurred in the soil as evidenced by changes in microbial activity, and there was a plant species effect on the soil microbial community structure.
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TABLE OF CONTENTS

ABSTRACT

ACKNOWLEDGEMENTS

LIST OF TABLES 2

LIST OF FIGURES 3

CHAPTER 1: LITERATURE REVIEW 5
   a. Bioremediation
   b. Phytoremediation
   c. Bioavailability of inorganic and organic contaminants
   c. Polycyclic aromatic hydrocarbons
   e. RTDF studies
   f. Justification of research

CHAPTER 2: THE INFLUENCE OF PAHS AND HEAVY METALS ON MICROBIAL ACTIVITY AND COMMUNITY STRUCTURE IN SOIL FROM A PETROLEUM REFINERY LAND TREATMENT UNIT 25

CHAPTER 3: THE INFLUENCE OF PLANTING ON PAHS, HEAVY METALS AND MICROORGANISMS IN AGED PETROLEUM CONTAMINATED SOIL 53

BIBLIOGRAPHY 82
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Chapter 1:</th>
<th>Table 1. Physical properties of polycyclic aromatic hydrocarbons</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>

| Chapter 2: |
|------------|---------------------------------------------------------------|------|
| Table 1. Sequential extractants and shake times | 31   |
| Table 2. Characteristics of LTU soil samples | 35   |
| Table 3. Relative mole percentages of phospholipid in the upper and lower layers of LTU soil | 42   |
| Table 4. Rate constants and maximum mineralization extents as a function of soil layer and plot | 45   |

<p>| Chapter 3: |
|------------|---------------------------------------------------------------|------|
| Table 1. Characteristics of soil samples from the upper and lower layers of the LTU | 62   |
| Table 2. Sequential extractants and shake times | 65   |
| Table 3. Percentage of total root biomass found in the upper and lower soil layers of column-grown plants | 69   |
| Table 4. Total germination of rye, fescue, and legume mix out of 20 seeds on soil from the upper, 50/50, and lower layers after 30d | 70   |
| Table 5. Tissue concentrations of specific heavy metals in plants grown in soil from the upper or lower layers on the LTU, and in a mixture of both layers | 71   |
| Table 6. Mean PAH concentration in soil before and after pot study | 74   |
| Table 7. Rate constants and maximum mineralization extents as a function of soil layer and plant species | 77   |
| Table 8. Relative percentages of the total PLFAs for each plant treatment | 79   |</p>
<table>
<thead>
<tr>
<th>Chapter 1:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 1. Plan of the Remediation Technologies Development Forum (RTDF) sites</td>
<td>18</td>
</tr>
<tr>
<td>on the Chevron Refinery Land Treatment Unit.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 2:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 1. Total extractable PAH concentrations in the upper and lower</td>
<td>36</td>
</tr>
<tr>
<td>soil layers.</td>
<td></td>
</tr>
<tr>
<td>Fig 2. Concentrations of bioavailable PAHs.</td>
<td>37</td>
</tr>
<tr>
<td>Fig 3. Total metal concentrations in upper and lower soil layers.</td>
<td>37</td>
</tr>
<tr>
<td>Fig 4. Concentration of bioavailable metals in upper and lower soil layers.</td>
<td>38</td>
</tr>
<tr>
<td>Fig 5. Principle component analysis of PAH and heavy metal concentrations</td>
<td>40</td>
</tr>
<tr>
<td>in upper and lower layer field site soil samples</td>
<td></td>
</tr>
<tr>
<td>Fig 6. Field soil microbial biomass measurements for vegetated and</td>
<td>41</td>
</tr>
<tr>
<td>unvegetated, upper and lower soil layers.</td>
<td></td>
</tr>
<tr>
<td>Fig 7. Results of principle component analysis of FAME data from field</td>
<td>44</td>
</tr>
<tr>
<td>site soil samples. Plot of FAME PC1 versus FAME PC2 showing upper and</td>
<td></td>
</tr>
<tr>
<td>lower layer soil samples.</td>
<td></td>
</tr>
<tr>
<td>Fig 8. Cumulative percent mineralization of $^{14}$C-pyrene from upper</td>
<td>45</td>
</tr>
<tr>
<td>and lower layer field soil samples.</td>
<td></td>
</tr>
<tr>
<td>Fig 9. Cumulative percent mineralization of $^{14}$C-pyrene in upper and</td>
<td>46</td>
</tr>
<tr>
<td>lower layer soil that was planted for 4 months with fescue, rye, or a legume mix.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 3:</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig 1. Mean total plant biomass per pot of rye, fescue, and legume mix</td>
<td>70</td>
</tr>
<tr>
<td>In upper layer, 50/50 and lower layer soil after 30 days of growth.</td>
<td></td>
</tr>
<tr>
<td>Fig 2. Mean total metal concentrations of soil analyzed after 4 months</td>
<td>72</td>
</tr>
<tr>
<td>Fig 3. Concentration of bioavailable heavy metals in soil from rye, mix,</td>
<td>73</td>
</tr>
<tr>
<td>and unplanted pots.</td>
<td></td>
</tr>
</tbody>
</table>
Fig 4. Scatter plot of soil samples separated along factor 1 and 2 of the principle component analysis of heavy metal and PAH data after the experimental period.

Fig 5. Cumulative percent mineralization of $^{14}$C-pyrene in soil removed from pots planted with the mixture.

Fig 6. Cumulative percent mineralization of $^{14}$C-pyrene in soil removed from unplanted pots.

Fig 7. Microbial biomass measured by total extractable lipid phosphate

Fig 8. Plot of FAME component 1 versus component 2
Over the last century, a lifestyle has evolved in developed nations that generates a huge amount of waste, exploits natural resources, and alters our environment through loss of habitat and by polluting water, air and soil. It is a challenge for policy makers to limit future environmental impact and to compensate for previous impacts without making demands that are perceived as too high for government, industry or the general public. Pollution by point sources such as wastewater flowing into rivers from sewage treatment plants can be addressed at the source by strict limitations on specific harmful contaminants and enforcement of these limitations. Non-point sources such as deposition onto soil of contaminants carried down-wind from industrial centers is less easily regulated and controlled.

Polluted soil is a difficult and expensive medium to remediate due to the heterogeneous nature of soil and the volume of material that must be treated. If a site contains hazardous compounds and there is a risk of leaching into ground water or harmful exposure to humans or animals, then removal of the soil to an area where it can be contained is essential. Soil removed from the original site of contamination may be transported long distances to incineration sites where conditions must be carefully managed to ensure that hazardous by-products are not produced (Hitchman et al., 1995). Few states will accept and incinerate wastes because of public concern over the risks associated with incineration and the air pollution that is generated. Soil that necessitates removal from its original location may also be treated by less aggressive means such as
landfarming, which involves transportation to a local site where biological remediation processes are employed.

In cases where contaminants are stationary on the site and do not pose a risk of migrating, soil excavation and removal is not required and alternative, passive in situ remediation strategies [various aspects of two in situ approaches, bioremediation and phytoremediation, are discussed below] may be possible. The desire to eliminate the costs and risks of excavation and transportation of contaminated soil and to ensure that industries can afford to clean up their waste, has led to research and development of a variety of in situ remediation techniques.

**Bioremediation**

Bioremediation has been researched in the lab and successfully implemented at contaminated field sites to reduce chemical concentration, mobility and toxicity (Loehr and Webster, 1996). Bioremediation takes advantage of the ability of soil microorganisms to metabolize organic compounds and use them as energy sources. In order to take in and utilize the compounds needed for essential life processes, microorganisms have receptors, enzymes, and reaction pathways specific to particular compounds. Because many soil contaminants have similar structures to these carbon sources, they can fit into the same sites on receptors and enzymes and be partially or fully metabolized, or changed into different compounds in a non-energy producing process called cometabolism (Alexander, 1999).

Mineralization, the biotic or abiotic breakdown of an organic substrate into an inorganic substrate (usually CO₂) and energy, is very important in removing hazardous
substances from the environment (Alexander, 1999). The number of different species of biota (mainly bacteria and fungi) living in an area of soil is such that there will probably be several species able to completely mineralize or partially degrade a particular soil contaminant that may be present. Contaminated sites frequently have more populations of microorganisms that can degrade organic pollutants than uncontaminated sites because these microorganisms are selected for by chronic exposure to a stressful environment (Heitkamp et al., 1998). Although native or introduced soil microorganisms may have the ability to degrade organic compounds, bioremediation (as a technology) also involves the optimization of conditions for maximum microbial activity. These conditions include regulating pH, redox potential, nutrient content, oxygen concentration, temperature, and water content (Rulkins et al., 1998). For those contaminants that are not easily or quickly degraded, genetically engineered microorganisms may be developed for introduction into impacted soils (Wackett et al., 1994).

Bioremediation may also be considered for sites contaminated with inorganic compounds. Unlike organic contaminants which are generally broken down into simpler organic molecules or volatilized as CO₂, inorganic contaminants may be modified to change their ionic speciation (Alexander, 1999). Metals may also be immobilized in soil or biomass through uptake into living or non-living microbial cells or by precipitation reactions with microbial products (Alexander, 1999). A reduction in the toxicity of inorganic ions to microbes and plants by modification or immobilization can permit bioremediation of organic contaminants to continue even in heavily metal-contaminated soils.
The presence and relative abundance of microbial species in a soil community is affected by the type and concentration of contaminants in the soil (Frostegard et al., 1993). Disturbances such as the addition of heavy metals to soils have been shown to adversely affect microbial biomass, activity, and diversity (Frostegard et al., 1993; Landmeyer et al., 1993). Upon exposure to a stressor, sensitive species may die off leaving available niches that can be filled by indigenous tolerant species or by those that are able to adapt to the new environment (Landmeyer et al., 1993). Analysis of the microbial community can be very helpful in predicting the potential for degradation of organic pollutants. Characterization of the microbial community is not, however, easily done. The vast majority of microorganisms are not cultureable, and many techniques are insensitive to the viability of the cells being detected. Microbial activity and biomass measurements, as well as Biolog and phospholipid fatty acid (PLFA) analyses are techniques that are frequently utilized to quantify soil microorganisms and to track changes in community structure and function. Biolog can provide information on substrate utilization, which can be assessed within certain microbial groups, but it is an indirect measure of community structure (Pennanen, 2001). Analysis PLFAs, which are found in the membranes of all living cells, enables direct characterization of the microbial cells present. Because phospholipids are rapidly degraded upon the death of an organism by the enzymatic release of the phosphate group from the diglyceride (White et al., 1979), PLFA analysis separates live biomass from dead. PLFA analysis also has the advantage of examining the entire community, not just those organisms that are cultureable. Following extraction of PLFAs from soil, they are transmethylated to create fatty acid methyl esters (FAMEs), which can then be detected by gas chromatography. A
few “biomarker” fatty acids have been identified for particular microorganisms, but because organisms have membranes composed of a mixture of fatty acids, most characterizations can only identify broad groups of bacteria and fungi (i.e. gram positive, or sulfate reducing bacteria).

**Phytoremediation**

Phytoremediation takes bioremediation one-step further by adding the effects of plants to the potential for microbial degradation of organic pollutants. Phytoremediation is the use of plants to remediate contaminated soils, sediments, and waters. Plants have many beneficial effects on both the stabilization and remediation of soil pollutants. Roots serve to anchor soil contaminants by preventing lateral dispersion of contaminants by erosion and also by preventing leaching into the groundwater (Ernst, 1996). Shoots and leaves provide shade to soil, protecting it from drying out during hot summer months and maintaining moist conditions for optimum microbial degradation of organic contaminants. Roots break apart soil aggregates, exposing new surfaces and making available nutrients, organic matter, and pollutants for microbial degradation or plant uptake. In addition, roots also provide a surface for attachment of microorganisms.

The area surrounding plant roots, known as the “rhizosphere”, is enriched in carbon compounds released in the form of plant root exudates, or resulting from the sloughing off of cells and their contents as roots penetrate through soil. Plants have been shown to spend between 5 and 20% of their energy from photosynthesis on the production of exudates (polysaccharides, organic acids, lipids, vitamins, and enzymes) (Morel, 1999) given off in response to nutrient deficiency (Marschener, 1998) or other
types of stress. Exudates may alter conditions in the rhizosphere, or have an antibiotic effect on soil organisms, presumably to improve the soil environment for plant growth (Hornby, 1990). Plants also form relationships with fungi, which benefit from plant root exudates, thus effectively extending their root system for nutrient acquisition (Marschener, 1998).

This area of enrichment (i.e. the rhizosphere) in an otherwise nutrient poor soil allows for the growth of a much larger microbial population than is present in bulk soil (Siciliano and Germida, 1999; Banks et al., 2000). The rhizosphere, with its enhanced microbial community, is also more effective at remediating and mineralizing soil contaminants than bulk or unplanted soil (Boyle and Shann, 1995; Lee and Banks, 1993; Siciliano and Germida, 1999; Liste and Alexander, 2000). Knaebel and Vestal (1992) observed mineralization rates of surfactants in the rhizosphere to be increased by a factor of 1.1 to 1.9 over non-rhizosphere soils. Schwab and Banks (1994) saw 36% greater mineralization of $^{14}$C-pyrene in soils amended with organic acids typically found in the rhizosphere than in unamended soils. The composition of root exudates is different for various plant species; therefore the influence on the microbial population size and community structure (and thus on their ability to degrade soil contaminants) is also thought to be species specific (Alexander, 1999; Liste and Alexander, 2000).

As well as enhancing bioremediation in the rhizosphere, plants species such as hybrid poplars, are also capable of remediating organic contaminants by taking them up into their biomass (Gordon et al., 1998). Once inside the plant tissue the organic compounds may be metabolized and used as energy sources, sequestered in cell walls or vacuoles, or volatilized.
The behavior of organic compounds in soils and plants is generally dependent upon whether they are hydrophobic or hydrophilic. Uptake of organic compounds by roots and their mobility within plants is strongly correlated with the log of their octanol to water partition coefficient or “log $K_{ow}$”, which is an indicator of hydrophobicity (Kömives and Gullner, 2000). Compounds with very low log $K_{ow}$ values are hydrophilic and soluble in the polar soil solution. These can diffuse through water but don’t easily pass through phospholipid bilayer membranes. Compounds with a high log $K_{ow}$ are hydrophobic and do not readily diffuse into plant roots but tend to adsorb to surfaces of roots or soil particles. Over time hydrophobic organic compounds can become sequestered in soil micropores or tightly bound to soil particles. Uptake and translocation to shoots is most effective for compounds with intermediate polarities with log $K_{ow}$ values around 1.8 (Cunningham et al., 1997). Uptake of organic contaminants is also greatest in soils with low organic matter content, as this provides a strong sorptive surface.

Since all plants must acquire essential (nutritional) inorganic ions from the environment, they may also alter the availability and remove inorganic contaminants from soil. This is a valuable characteristic, as few options exist for stabilization and remediation of metal contaminated soils. Many plant species have evolved extensive root systems with large surface areas for adsorption of water and nutrients; heavy metals may be stabilized and prevented from leaching by binding to these plant roots or to the organic matter in the soil that is generated by the decomposition of plant roots and shoots. Just as plant exudates have constituents that affect rhizosphere microorganisms, they also produce compounds that have a role in mobilization of compounds such as nutrients.
Phytosiderophores chelate essential nutrients, such as zinc and iron, which are carried towards plant roots by bulk flow and into roots by diffusion (Khan et al., 2000; Hopkins, 1995). Organic acids are another group of exudates that assist in the mobilization of nutrients (Marschener, 1998). Plants can also alter the availability of adsorbed ions by changing the pH of the rhizosphere through the pumping of protons and phenolic compounds out of root cells (Kömives and Gullner, 2000). All of these mobilization mechanisms may also increase the availability of soil metal contaminants (Ernst, 1996).

Because metals are immutable, that is they cannot be changed into different elements, remediation of metal contaminants from soil must include plant extraction. Heavy metal soil contaminants (indistinguishable from essential elements) may be taken up into a plant along with inorganic nutrients. In order to use plants for the purpose of metal extraction, it is essential that they be able to translocate the metal into the above ground biomass, which can be harvested and incinerated.

Plants species may exclude metals from their roots or take them up, while others may not be able to grow in metal-contaminated soils at all. Uptake of metals into roots may occur passively from the soil solution or actively using specific transporters across membranes (Kömives and Gullner, 2000). If a plant can take up and transport metals through the xylem into the shoots, then the above-ground tissue may be harvested, and the metal economically extracted and recycled in a process known as phytomining (Brooks et al., 1998). Plants that do take up metals may avoid toxicity by immobilization in the roots, or by accumulation in leaves followed by detoxification through leaf fall. Non-essential metals in shoot or root tissue may be prevented from interacting with metabolism by the addition of an organic molecule that facilitates transport into vacuoles.
where they are sequestered (Hopkins, 1995). Metal ions may also be incorporated into non-essential metabolic pathways that produce non-functional (but non-toxic) compounds. Plants that (using one of these tolerance mechanisms) have the ability to accumulate certain metals, such as nickel or copper, to greater than 1% of their biomass are known as hyperaccumulators (Brooks et al., 1998). Hyperaccumulators would seem to have potential for use in remediation of heavy-metal contaminated soils, but they are frequently specific for only one metal and generally have low biomass (Dahmani-Muller et al., 2000; Ernst, 1996).

**Bioavailability of Inorganic and Organic Contaminants**

Phytoremediation is limited by the availability of pollutants for microbial degradation or plant uptake. When contaminants are initially applied to soil they are readily available and there is a rapid loss due to microbial degradation of organic compounds or plant uptake of metals. As time passes the portion of the contaminants that were not removed interact physically or chemically with soil particles in a process known as “aging” (Reid et al., 2000). These aged contaminants are less accessible to microorganisms and plant roots, and the rate of degradation slows. Contaminants may sorb to soil particles, become sequestered in soil micropores where they are inaccessible to microorganisms, or they may form complexes with organic soil fractions (Alexander, 1999; Maliszewka-Kordybach, 1999; MacLeod and Semple, 2000). Sorption increases with the concentration of contaminant, the amount of organic matter present in the soil, and the soil-pollutant contact time (Reid et al., 2000). Frequently, the rate of transfer from unavailable to bioavailable is the limiting factor in the rate of bioremediation (Berg
et al., 1998), although Laor et al.(1999) saw enhanced biodegradation of sorbed phenanthrene perhaps due to the presence of sorbed microorganisms.

Methods to Evaluate or Increase Bioavailability. Many single and sequential extraction procedures have been developed to approximate the amount of metal present in different soil fractions and the portion that is available for uptake by plants or by microorganisms (Wenzel and Jockwer, 1998). Sposito et al.(1982), developed a sequential extraction method that involved extraction of an individual soil sample with different extractants that removed metal fractions roughly equivalent to exchangeable, adsorbed, organically bound, carbonate bound, and sulfide or residually bound. The exchangeable and adsorbed fractions represent the metal concentration that is readily available for plant uptake; the metal in the organic and carbonate bound fractions could slowly become available over time; and the residual fraction is considered unavailable for plant uptake or microbial use. A single extraction procedure for extraction of bioavailable PAHs was developed by Tang and Alexander(1999). Tang and Alexander found that extraction of soil with n-butanol was positively correlated with the fraction that was bioavailable and represented a toxicological risk to plants and animals. Soil extraction with mild n-butanol has also been used to estimate the plant or microorganism bioavailable fraction of organic contaminants (MacLeod and Semple, 2000).

Polycyclic Aromatic Hydrocarbons

One group of organic compounds, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous hydrophobic contaminants that are frequently found as soil, water, and air pollutants. They are formed by natural process such as volcanic eruptions and forest
fires, but the major portion is formed during incomplete combustion of fossil fuels.

Sixteen PAHs are on the EPA priority pollutants list because they are known to be carcinogenic, mutagenic, or teratogenic (Environment Canada, 1994). They have very stable structures made up of two or more benzene rings that are not easily broken down or metabolized by microorganisms. Table 1 shows the physical properties of several common PAHs.

Table 1: Physical Properties of Polycyclic Aromatic Hydrocarbons

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular Weight</th>
<th>log $K_{ow}$</th>
<th>Water Solubility 25°C (mg/L)</th>
<th>Melting Point</th>
<th>Vapor Pressure 25°C (mPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>128.16</td>
<td>3.50</td>
<td>31.700</td>
<td>80.5</td>
<td>11960</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>154.21</td>
<td>4.33</td>
<td>3.420</td>
<td>95.0</td>
<td>594</td>
</tr>
<tr>
<td>Fluorene</td>
<td>166.00</td>
<td>4.18</td>
<td>1.980</td>
<td>116.5</td>
<td>95</td>
</tr>
<tr>
<td>Anthracene</td>
<td>178.24</td>
<td>4.50</td>
<td>0.045</td>
<td>216.0</td>
<td>25</td>
</tr>
<tr>
<td>Pyrene</td>
<td>202.26</td>
<td>4.90</td>
<td>0.135</td>
<td>156.0</td>
<td>91 x 10^{-6}</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>202.26</td>
<td>5.10</td>
<td>0.260</td>
<td>111.0</td>
<td>1328</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>252.32</td>
<td>6.00</td>
<td>0.0038</td>
<td>179.0</td>
<td>37 x 10^{-8}</td>
</tr>
</tbody>
</table>

Source: Environment Canada (1994)

PAHs belong to a category of chemicals known as “persistent organic pollutants” that are resistant to degradation and can remain in the environment causing adverse effects for long periods of time (Maliszewska-Kordybach, 1999). The log $K_{ow}$ values of PAHs are between 3.5 and 7.66, indicating their moderate to highly hydrophobic behavior (Schwarz and Jones, 1997). The parameter found to have the most influence on PAH retention in soil is the amount of organic matter present, which controls soil sorption affinity towards PAHs (Maliszewska-Kordybach, 1999). The smaller ring PAHs may be volatilized from soil or rapidly biodegraded within several months of application.
especially if the soil is moist, leaving behind the larger PAHs that are more persistent and resistant to degradation (Sayles et al., 1999; Hawthorne and Grabanski, 2000). Higher molecular weight PAHs with more than three benzene rings are less water soluble, have little vaporization, and are more hydrophobic than the smaller PAHs (Maliszewka-Kordybach, 1999). Juhasz et al. (1997) found that for soils contaminated with mixtures of PAHS, the high molecular weight PAHs might be degraded more rapidly in the presence of low molecular weight PAHs, which may serve as carbon sources for soil microbes. Many contaminated field sites contain mixtures of PAHs along with other organic and inorganic pollutants (Alexander, 1999). One of the PAHs frequently found on petroleum-contaminated sites is pyrene, with four benzene rings and the formula C_{16}H_{10}.

Phytoremediation has shown to be effective at degrading some PAHs in the lab and for some sites contaminated with PAHs (Aprill and Sims, 1990, Banks et al., 2000; Liste and Alexander, 2000), but few researchers have looked at mixtures of PAHs and inorganic contaminants and their effects upon each other.

RTDF Studies

Phytoremediation has been extensively researched in the lab (Aprill and Sims, 1990; Gordon et al., 1998; Khan et al., 2000), several using soils from actual contaminated field sites. The results of field studies, however, may take years to gather and may be highly site specific. Any rates generated in the lab neglect the impact of a variable environment and may not extrapolate to field scale. To minimize the limitations of conducting industrial field studies (using various experimental designs) while retaining the reality of the environmental effects, the Remediation Technologies Development
Forum (RTDF) has initiated a national investigation of phytoremediation. Eleven field sites across North America have been selected to examine the efficacy of plants in remediating soil contaminated with aged petroleum hydrocarbons. Replicate plots across the sites were planted with trees, a grass, a mixture of rye, fescue, and legumes, or were cleared of all vegetation and maintained as unplanted controls. Soil core sampling has been performed at regulated time intervals to evaluate agronomic conditions and contaminant concentrations. One test site is the Chevron Refinery Land Treatment Unit (LTU). The LTU is a 5.5-acre site located on a hilltop approximately 15 miles west of Cincinnati in Hooven, Whitewater Township. From 1982 until 1989 the site received approximately 2,838,116 gallons of sludges, hazardous and non-hazardous wastes from the nearby petroleum refinery. For the past 11 years landfarming methods (disking to a depth of 18 inches, fertilizing, planting, and maintenance of pH and moisture) have been employed to optimize microbial degradation of organic contaminants. The large amount of carbon present in petroleum wastes can potentially support a large microbial biomass, but nitrogen or phosphorus are frequently limiting nutrients, and the oxygen supply may be depleted by aerobic microbes. Fertilization and tilling help to alleviate these problems. Perennial rye grass was sown on the site along with willow trees in the moister areas. The contaminants still present on the site are high molecular weight PAHs and some heavy metals including lead, nickel, copper, chromium, and zinc. The “soil” is divided into 2 distinct layers, an upper layer and a lower layer. The upper layer is dark brown in color and extends from the surface down to between 7 and 16 inches across the site. The lower layer extends approximately from a depth of 16 inches down to 21 inches and is dark brown to black and smells strongly of petroleum. Beneath the lower layer is a
natural clay lining that has effectively prevented leaching of contaminants into the groundwater. The RTDF study has divided the site into 4 quadrants, each containing a control, grass, hackberry, and a willow plot (Figure 1).

Figure 1. Plan of the Remediation Technologies Development Forum (RTDF) test plots

Land Treatment Unit in Hooven, OH.

Justification of Research

Phytoremediation, as an inexpensive alternative to more invasive remediation techniques, has generated a lot of interest and research. Laboratory experiments using...
soils spiked with PAHs or heavy metals have confirmed the potential of plants to enhance
degradation or to extract contaminants from soil. There is a growing awareness that lab
results on single organic or inorganic contaminants may not extrapolate to soils
contaminated with mixtures (Yuan and Xing, 2001) - as they often are on actual sites. It
is essential, then, to study phytoremediation in field experiments and to evaluate its
potential in soils in need of remediation. Therefore, the goal of my research was to assess
the status of an actual site; in particular one where phytoremediation and bioremediation
has likely been a factor. Further, I wanted to evaluate the ability of plants and their
associated rhizosphere microorganisms to remediate soils removed from the site. For this
work, the petroleum contaminated land treatment unit (LTU) in Hooven was used. Since
it contained mixtures of heavy metals and organic pollutants including PAHs, these were
the contaminants that my studies focused on.

Following this literature review, chapter 2 covers the biological and chemical
characterization of the Chevron Refinery LTU. Soil samples from several site locations
and from both soil layers were extracted and analyzed to evaluate in situ community
structure, microbial biomass, and degradative ability. These measurements indicate
which microorganisms are present, in what numbers, and how active they are at
degrading added PAHs. FAME and phospholipid phosphate analyses were conducted to
evaluate the composition and size of the microbial community. Radiorespirometry was
used to measure the ability of the microbial community to degrade a typical site PAH
(pyrene). In separate laboratory studies, microbial parameters after planting were
measured to assess the influence of several plant species on degradative ability.
As little is known about the amounts or distribution of contaminants on the site, soil samples from grass and control plots were extracted and analyzed to determine variations in the total amounts of several heavy metals and PAHs laterally and vertically. Because the site has been a LTU for over 15 years, aging processes may have reduced the bioavailability of contaminants to plants. The soils were sequentially extracted to determine heavy metal availability and mildly extracted to measure the portion of PAHs that are plant available. Differences in microbial distribution, activity, and numbers across the LTU were evaluated in regards to the measured levels of heavy metals and PAHs.

Chapter 3 examines the ability of several plant species to remediate soil from the LTU. Plants were grown on several combinations of the upper and lower soil layers to evaluate whether deep tilling on the site to combine the layers would allow for greater remediation. Concentrations of PAHs and several heavy metals from soil were measured after planting and metal concentrations in the plant tissue were recorded. The influence of plants on soil microbial populations and on the behavior of contaminants in soil may differ because of variance in their root morphologies and exudates. The bioavailable fraction of metals and PAHs after planting was assessed to examine the effect of various plant species on bioavailability. Changes in the soil microbial community due to planting were measured through microbial biomass measurements, FAME analysis, and the mineralization of $^{14}$C-pyrene.
References


CHAPTER 2: THE INFLUENCE OF PAHS AND HEAVY METALS ON MICROBIAL ACTIVITY AND COMMUNITY STRUCTURE IN SOIL FROM A PETROLEUM REFINERY LAND TREATMENT UNIT

Abstract

Biodegradation is a fundamental process operating in natural and contaminated environments. For natural attenuation and site remediation, it is important that the spacial distribution is characterized and the factors driving it be identified. Microbial community structure was determined by phospholipid fatty acid (PLFA) analysis of soil collected from vegetated and unvegetated plots located on a petroleum refinery land treatment unit. Communities were examined in relation to soil concentrations of total and bioavailable heavy metals and PAHs. Microbial biomass and ability to mineralize $^{14}$C-pyrene were also assessed. PAH and heavy metal contamination on the site was influenced by the plot (vegetated versus unvegetated), but varied much more significantly with the depth in the soil profile. Microbial biomass, activity, and community structure also varied by depth. Although little difference in concentrations of PAHs and heavy metals existed between soils collected from vegetated and unvegetated field plots, pyrene mineralization rates following planting of soil in the greenhouse, indicate that soil microbial activity increases with planting. This study suggests that while differences were present after four months in the lab, one year in the field was not enough time to create a difference between vegetated and unvegetated plots.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are released into the environment by natural fires and volcanic eruptions, but more frequently they are anthropogenically
evolved during the incomplete combustion of fossil fuels. A major source of PAHs is the petroleum refining process, which concurrently releases long chain aliphatic hydrocarbons and heavy metals. Refinery wastes are frequently remediated by landfarming, a process in which optimum conditions in soil are maintained for biodegradation of the organic contaminants. The PAHs with greater than 4 benzene rings are slow to degrade, and tend to persist in the environment due to their structural stability and low water solubility (Heitkamp, et al. 1988). These larger PAHs are also of greater concern due to their potential toxicity and carcinogenicity. Remediation of PAH contamination is therefore critical.

As previously noted, petroleum refinery wastes may contain heavy metals including Cr, Cu, Ni, Pb, and Zn. The presence of heavy metals on a site that is also contaminated with organic compounds may add complicating factors to bioremediation efforts. Frostegard et al.(1993) reported adverse effects on microbial biomass and activity, and changes in the microbial community structure in heavy metal-contaminated soil. The effect of heavy metals on indigenous microorganisms may decrease their potential for bioremediation of organic contaminants.

Immediately following an exposure event to heavy metals or PAHs, sensitive members of a microbial community may die off. Over time, however, populations may recover and return to their original size or in some cases become even larger as empty niches are filled by metal-tolerant species and by those that are able to use organic contaminants as energy sources (Federle et al., 1990). Shifts in community structure in PAH contaminated soils are reflected by faster PAH mineralization rates as species that can tolerate or metabolize PAHs proliferate (Madsen et al., 1992). Changes in
community structure to more PAH or heavy metal tolerant species may also be accompanied by a decrease in biodiversity, including the loss of species that play an important role in degradation or soil nutrient cycling (Langworthy et al., 1998; Landmeyer et al., 1993; Federle et al., 1990; Megharaj et al., 2000). Sensitive microbial species may be less affected by PAHs and heavy metals as the contaminants age in the soil and become less bioavailable.

In aged contaminated soils, processes such as sorption to soil minerals and organic matter reduce the bioavailability of PAHs and heavy metals to plants and microorganisms. Sorption of hydrophobic PAHs to soil organic carbon has been shown to decrease the bioavailability of PAHs and reduce mineralization rates (Grosser et al., 1991). Decreased bioavailability of inorganic contaminants by sorption to soil particles may reduce their toxicity, enabling sensitive microbial species to survive even when total metal concentrations are high.

Bioavailability may also be influenced by the presence of plants, which release a variety of root exudates. The complex mixture of compounds secreted by plants into soil alters the rhizosphere environment and helps to mobilize bound organic and inorganic contaminants for plant uptake (Khan et al., 2000). These mobilized organic and inorganic contaminants would also become available to microbial communities in the rhizosphere. In addition to affects on the bioavailability of contaminants, root exudates may directly impact microorganisms by acting as growth substrates, essential nutrients, or even antibiotic compounds (Hornby, 1990).

This study examined the distribution and availability of PAHs and heavy metals on a closed petroleum refinery landfarm. The structure, status, and degradative ability of
the microbial community was then characterized and correlated with the levels of total
and bioavailable contaminants. Finally, in greenhouse studies the influence of planting
on contaminant fate and microbial degradation was investigated. Collectively, these
studies provide insight into the factors that have produced the current site conditions and
may clarify the effectiveness of any further remediation.

Materials and Methods

Soil Collection and Characterization

Soil samples were collected in spring of 1999 from a petroleum refinery landfarm,
or land treatment unit (LTU), located in southwestern Ohio. The 5-acre site received
perennial rye grass (*Lolium perenne*) was planted on the site. The LTU was irrigated,
fertilized and disked monthly to a depth of 18 " to promote microbial degradation of
organic compounds. Vegetated and unvegetated plots were established on the LTU by
the USEPA in 1999 as part of a Remediation Technology Development Forum (RTDF)
evaluation of phytoremediation at several locations across North America. This study
took advantage of the established fixed design, sampling from four RTDF plots planted
with rye grass and four unvegetated control plots that had remained plant free for one
year.

The LTU is composed of refinery wastes mixed with local soil, which was
classified as a Switzerland silt loam. No natural soil profile, however, was retained while
the LTU was filled with refinery wastes. During sample collection a distinct transition
from a light brown granular upper soil layer to a black, more aggregated lower layer was
observed approximately 7 to 16" from the surface. The black layer, which released a strong odor of petroleum, extended down to the clay liner at a depth of about 22 inches. Soil samples were taken from the upper and lower layers for laboratory analysis and study. Visible roots were removed, and soils were homogenized, sieved (2 mm mesh) and stored at 4°C until analyzed. General soil characteristics (pH, cation exchange capacity (CEC), total organic carbon (TOC), organic matter (OM), N, P, K) were determined by Spectrum Analytic, Inc. (Washington C.H., OH).

Chemical Analyses

Fresh soils were extracted for determination of total and available PAHs. Total PAHs were extracted using an accelerated solvent extraction system (Dionex ASE 200; Sunnyvale, CA) and Richter’s (2000) method for extraction of hydrocarbons. Extraction cells (22 ml) were filled with a mixture of 4g soil and 2g diatomaceous earth. The extraction solvent was dichloromethane-acetone (1:1,v/v). Cells were heated for 8 min, to reach 175°C. Extraction pressure was 1500 p.s.i., static time was 5 min, flush volume was 70%, and purge was 60 sec with 150 p.s.i. N₂. Samples were dried to 10 ml and then centrifuged for 30 min at 220g. Bioavailable PAHs were extracted with n-butanol using a mild extraction procedure (Tang and Alexander, 1999). The extractant (25 ml of n-butanol) was added to 2g fresh soil in a 50 ml Teflon tube. Tubes were vortexed then centrifuged at 17200g for 10 min at 4°C. The supernatant was decanted, dried by passing through an anhydrous sodium sulfate column, and evaporated to 10 ml. Total and bioavailable PAHs were separated on a gas chromatograph (GC 14A; Shimadzu, Columbia, MD) in the split mode with a flame ionization detector (FID). The column
was a HP-1 (Hewlett Packard, Palo Alto, CA) cross-linked methyl silicone column (60m x 0.25mm internal diameter x 0.25μm film thickness). The injection port temperature was 290°C, column temperature was static at 70°C for 2 min, it then increased by 15°C per min to 150°C, and then increased by 6°C per min to a final temperature of 290°C, which was held for 30 min. All solvents and chemicals for chemical analyses were of reagent grade. Three replicates of each soil sample were run for each extraction.

Soils were also extracted to determine the total amount of metal in the soil (EPA procedure 3050B), and sequentially (Sposito et al., 1982) to determine the amount of metal associated with each soil fraction. For total metal extractions, 10 ml of 1:1 HNO₃:H₂O was added to 1g of dry soil and heated (95°C) for 15 min. After cooling, 5 ml of HNO₃ was added and the sample was heated for 2.5 h. When cool, 2 ml of dH₂O and 3 ml of 30% H₂O₂ were added, and the sample was heated for 2 h, after which time 10 ml of HCl was added, and the test-tube was heated for a further 15 min. Extracts were filtered (Whatman No.41) and made up to 100 ml in nalgene bottles. For sequential metal extractions, 25 ml of an extractant was added to a single 2g sample of dry soil in a centrifuge tube, which was shaken for a specified amount of time (Table 1), after which it was centrifuged at 12000g for 15 min in a Sorvall RC-5B Plus centrifuge (Dupont, Newtown, CT). The supernatant was collected, filtered, and refrigerated until analysis. The remaining soil pellet was treated with 25 ml of the next extractant, and the process repeated.
Table 1. Sequential extractants and shake times.

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>EXTRACTANT</th>
<th>SHAKE TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exchangeable</td>
<td>0.5M KNO₃</td>
<td>16 hours</td>
</tr>
<tr>
<td>Adsorbed</td>
<td>DI H₂O</td>
<td>2 hours</td>
</tr>
<tr>
<td>Organically Bound</td>
<td>0.5M NaOH</td>
<td>16 hours</td>
</tr>
<tr>
<td>Carbonate Bound</td>
<td>0.05M Na₂EDTA</td>
<td>6 hours</td>
</tr>
<tr>
<td>Residual</td>
<td>4M HNO₃</td>
<td>16 hours *</td>
</tr>
</tbody>
</table>

*At 80°C

Detection of Cr, Cu, Zn, and Ni were performed using an ICAP 61E Thermo Jarrell Ash (Franklin, MA) inductively coupled plasma atomic emission spectrometer operated at 1150W. Lead concentrations were measured on a Perkin-Elmer atomic absorption spectrometer (model 3110; Norwalk, CT) with an EDL system 2. Matrix differences were corrected by making standards for each set of sequential extracts using the extractant for each step.

Microbial Biomass and FAME Analyses

Fresh soil (4-6g) was extracted for 24 h in a dichloromethane-methanol-phosphate buffer solution (15 ml DCM, 15 ml MeOH, 5 ml 50 mM phosphate buffer). Dichloromethane and water were then used to partition the aqueous and organic fractions of the extract. After 24 h, samples were centrifuged, and the organic fraction containing the lipid material was recovered. The organic fraction was dried under N₂ and made up to 2 ml with chloroform. A sub-sample was taken for total microbial biomass measurements using the phospholipid phosphate method (Dobbs and Findlay, 1993). The remaining lipid was separated into 3 fractions by solid phase extraction through silicic acid columns. The phospholipid fraction was recovered in methanol. PLFAs were transmethylated to fatty acid methyl esters (FAMEs) by the addition of 0.5 ml of 0.2 N KOH in methanol (Findlay and Dobbs, 1993). FAMEs were purified using reverse-phase
SPE column chromatography, and analyzed by GC using the standard EUKARY chromatographic program (MIDI software, Microbial ID, Newark, DE). FAMEs were separated on a Hewlett Packard 5890 Series II GC in the splitless mode, using a nonpolar cross-linked 5% phenyl methyl siloxane column (25m x 0.25mm internal diameter) and a flame ionization detector (FID). The injection port temperature was 250°C; column temperature started at 70°C then increased by 3°C per min to a final temp of 300°C, which was held for 10 min. Solvents and chemicals were of optima grade.

*FAME Nomenclature*

FAMEs were designated by the total number of carbon atoms in the fatty acids, followed by a colon and the number of double bonds from the aliphatic (ω) end of the molecule. The number after the ω corresponds to the carbon atom before the double bond from the methyl end. “C” or “t” indicate cis or trans configurations, anteiso and iso are represented by “a” and “i” and “cy” stands for cyclopropyl fatty acids.

*Mineralization Assays*

Soil samples from the control and grass plots on the LTU were used in a serum bottle radiorespirometry study (Knaebel and Vestal, 1988) to evaluate the ability of the soil microorganisms to metabolize 14C-pyrene to 14CO2. Fresh soils (5g wet weight) were placed into autoclaved 50 ml serum bottles and brought to approximately 80% water holding capacity with sterile dH2O. Radioactive pyrene (specific activity 58.7 mCi/mol; Sigma, St. Louis MO) in acetone was added as a 20 µl spike (33910 DPM) to each serum bottle. Paper wicks (Whatman No.1) saturated with 70 µl of 0.5M KOH were suspended
in the serum bottles to capture evolved $^{14}$CO$_2$. Bottles were vortexed and sealed. 

Mineralization was measured for 47 d by periodically removing wicks and determining the radioactivity on them by liquid scintillation analysis (TRI-CARB 2200CA; Packard Instrument Co., Downer’s grove, Il). Sterile autoclaved soil was used as an abiotic control.

**Planting Study**

Plants were grown in the LTU soil to determine if they altered the ability of the soil microfauna to mineralize $^{14}$C-pyrene. Large plastic columns (10 cm diameter) were constructed, and soil was added to simulate the same depth profile as on the site (0-16" upper light brown layer, 16-21" lower black layer). Rye, fescue, and a legume mix (either purchased from a local garden store or provided by the EPA) were selected for this study. Species were chosen for their root characteristics and because they were typical of the LTU history or are being used in the RTDF study. Grasses (rye and fescue), grow rapidly and have extensive root systems. They would be expected to provide a large amount of surface area and exudates for microorganisms. Legumes have mechanisms that enable them to live in nitrogen deficient soils, and they have known interactions with microorganisms. Plants were germinated in the columns, thinned to provide more than 80% surface coverage, and grown for four months in a controlled environment. After this time the columns were disassembled and the soil within was used in a mineralization study (as described previously). Mineralization was evaluated for both upper and lower soil layers.
Statistical Analyses

ANOVA and principle component analyses (PCA) were performed using SYSTAT 9 (Systat, Evanston, IL). PCA was performed separately on the contaminants (total PAHs and metals) and on the lipid profiles of samples. This allowed samples from different soil layers and plots to be separated (or clustered) based on soil abiotic and biotic characteristics. Analysis of FAME data was performed on mole percentages after removal of the fatty acids 16:0 and 18:0 because they are ubiquitous in all microbial communities and found in large amounts (Findlay et al., 1990). Individual fatty acids that did not comprise 1% of the total phospholipid phosphate for at least one sample were also eliminated. Mineralization rate constants were determined using the non-linear regression model of Systat. The first order production model (Larson, 1984), $P_t = A (1 - e^{(-kt)})$ was used; where $P_t$ is cumulative percent mineralization, $A$ is the maximum mineralization, $k$ is the rate constant, and $t$ is time in days. Sample C12 did not fit this model due to a long lag period. Rather, it fit the Bolzmann sigmoidal curve model, where $P_t = (A_1 - A_2) / (1 - e^{(x-x_0)/dx}) + A_2$. $A_1$ is the initial percent mineralization, $A_2$ is the final percent mineralization, $x_0$ is the center of the curve, $dx$ is the width of the curve.

Results

Soil Characterization

Upper and lower layers differed in the soil characteristics measured, but no significant difference was found between vegetated and unvegetated control plots (Table 2). Potassium, calcium, percent organic matter (OM), and percent total organic carbon (TOC) were all significantly higher in the upper layer than in the lower layer. In the
lower layer magnesium and cation exchange capacity (CEC) levels were higher than in the upper layer.

Table 2. Characteristics of LTU soil samples

<table>
<thead>
<tr>
<th>Plot</th>
<th>Layer</th>
<th>pH</th>
<th>TOC %</th>
<th>OM %</th>
<th>CEC MEQ</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>K % Base Saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unvegetated</td>
<td>Upper</td>
<td>7.8</td>
<td>3.0</td>
<td>5.2</td>
<td>18.4</td>
<td>15</td>
<td>23</td>
<td>13595</td>
<td>727</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>7.6</td>
<td>2.2</td>
<td>3.8</td>
<td>20.1</td>
<td>17</td>
<td>128</td>
<td>10590</td>
<td>1165</td>
<td>0.9</td>
</tr>
<tr>
<td>Vegetated</td>
<td>Upper</td>
<td>7.5</td>
<td>2.7</td>
<td>4.7</td>
<td>18.2</td>
<td>15</td>
<td>235</td>
<td>12069</td>
<td>695</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>7.2</td>
<td>1.6</td>
<td>2.6</td>
<td>21.6</td>
<td>16</td>
<td>133</td>
<td>9794</td>
<td>1141</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Each value is the mean of 2 replicates of 2 different soil samples.

Soil Contamination

PAHs. The four-ring PAHs (pyrene, chrysene, and benz[a]anthracene) were present in the highest concentrations (Figure 1). After removing outliers from the data set, only the soil layer from which the sample was taken was found (by ANOVA) to be a significant determinant of the fluorene, anthracene, phenanthrene, pyrene, chrysene, and benz[a]anthracene levels in the soil. Neither soil layer nor the plot significantly explained the levels of the larger ring PAHs: benzo[b,k]fluoranthene, dibenzo[ah]anthracene, and benzo[ghi]perylene. Whereas, both layer and plot were significant for acenaphthylene and benzo[a]pyrene, with layer being much more significant than plot. For all PAHs that differed significantly between layers, the lower layer had a higher concentration than the upper layer.

Mild butanol extractions were performed to measure the concentration of bioavailable PAHs (Figure 2). The variance in this data was high and the independent variables used in ANOVA (soil layer and plot) were not significant for most of the individual PAHs. Layer was found to be a significant determinant for phenanthrene,
chrysene, and pyrene concentrations, but the $r^2$ values were very low indicating that soil layer and plot accounted for very little of the variation in the concentration of bioavailable PAHs. For chrysene and benz[a]anthracene the interaction of layer and plot was significant, but again the $r^2$ values were very low. The percentage of the total PAHs in the bioavailable fraction was higher for the lower soil layer than the upper layer. The actual concentration of bioavailable (i.e. butanol-extractible) PAHs paralleled the total concentration of PAHs in the soil, with more PAHs available in the lower layer than in the upper layer for those that differed significantly between layers. The total amount of chrysene in the soil was higher than any other PAH, and correspondingly chrysene was the most bioavailable PAH. However, the portion of the total 3 and 4 ring PAHs that was bioavailable was much higher than the 5 ring PAHs. Overall, very little of the total PAH in soil was bioavailable.

Figure 1. Total extractable PAH concentrations in the upper and lower soil layers. Each value is the mean (± 1SD) of 8 samples with 3 replicate extractions of each.
The trend for soil metal concentration was opposite that for PAH. Concentrations of all metals were significantly higher in upper layer soil than lower layer (Figure 3). For some metals, a plot effect was observed. Ni was higher in the vegetated plot, while Pb was higher in the unvegetated control plot.

**Metals.** The trend for soil metal concentration was opposite that for PAH.

Concentrations of all metals were significantly higher in upper layer soil than lower layer (Figure 3). For some metals, a plot effect was observed. Ni was higher in the vegetated plot, while Pb was higher in the unvegetated control plot.

**Figure 2.** Concentrations of bioavailable PAHs. Each value is the mean of 8 soil samples with 3 replicates of each. Standard deviations exceed bar size and are therefore not shown.

**Figure 3.** Total metal concentrations in upper and lower soil layers. Each value is the mean (± 1SD) of 8 samples each replicated 3 times.
Sequential extractions were performed to determine the soil fractions with which the metals were associated. The metal fraction removed by KNO$_3$ is considered exchangeable, and that removed by dH$_2$O is adsorbed to soil surfaces. These two fractions make up the portion of the total heavy metal concentration in the soil that is readily available for interaction with plants and microorganisms or for leaching from the system. The NaOH and Na$_2$EDTA extractable metals represent respectively, the organically bound and carbonate bound fractions. These are potentially available and may become available over time. The HNO$_3$ extractable metals are considered residual and sequestered or unavailable. The amount of metal present in the residual fraction of the LTU soil was higher than all other fractions for all metals (Data not shown). Very small concentrations of metals were present in the bioavailable fractions represented in Figure 4 by the sum of metals in the exchangeable and adsorbed extracts.

![Figure 4. Concentration of bioavailable metals in upper and lower soil layers. Values are the mean (± 1SD) of 8 soil samples each extracted 3 times.](image)

Relative to the total metal extraction procedure, sequential extraction recovered 93% of soil Pb and 80% Cr. The percent recovery for the other elements was lower (49%
for Ni, 64% for Cu, and 69% for Zn). The decrease in the extraction efficiency of the sequential procedure may be due to error introduced by calculations and multiple handling steps, or may be a function of differential metal-soil interactions. The first two steps of the procedure are likely the least impacted by error. The magnitude of the difference between the amount extracted by water and KOH and that removed by HNO₃ is very large, even if extraction efficiency of the sequential procedure was less than 100%.

Concentrations of bioavailable metals were slightly but significantly higher in the upper layer than in the lower layer, corresponding to the higher overall concentration of heavy metals in the upper layer. Only a very small percentage of each metal was available for plant uptake. Six percent was the highest percentage of bioavailable metal.

**PCA of Contaminants.** PCA of soil samples based on their total heavy metal and total PAH concentrations, reduced multiple variables down to 3 axes that explained 72% of the variation in the data. Component 1 explained 49% of the variation, 2 explained 14%, and 3 explained 9% of the variation. Principle component 1 (PC1) had high negative loadings for the heavy metals and high positive loadings for the two through four ring PAHs. Component 2 (PC2) had large positive loadings for the four and five ring PAHs, and small negative loadings for anthracene and dibenzo[a,h]anthracene. Component 3 (PC3) had large positive loadings for the five ring PAHs (benzo[a]pyrene and dibenzo[a,h]anthracene), and small negative loadings for nickel and pyrene. PC1 separated the upper and lower soil layers (Figure 5). The upper layer soil samples were on the negative side of PC1 because they had relatively higher concentrations of heavy metals; in contrast to lower layer soil samples that had relatively higher concentrations of
the 3 and 4 ring PAHs. There was no separation by whether the soil came from a vegetated or unvegetated plot.

**Microbial characterization.**

**Biomass.** Microbial biomass in the upper layer ranged from 119.30 to 214.11 nmols of phospholipid phosphate per gdw of soil. The range for the lower layer was significantly lower than the upper layer, from 11.27 to 91.00 nmols per gdw soil. The layer accounted for 78% of the variation in biomass ($r^2 = 0.779$), with the amount of microbial biomass in

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**Figure 5.** Principle component analysis of PAH and heavy metal concentrations in upper and lower layer field site soil samples.
the upper layer roughly equal to twice the amount in the lower layer (Figure 6). There was no significant difference between vegetated and unvegetated plots.

There was a strong negative correlation (data not shown) between the microbial biomass present in the soil samples and the first and third principle component (PC1 and PC3) for the total PAH and heavy metal concentration. This correlation implies that there was a negative relationship between microbial biomass and the 3, 4, and 5-ring PAHs that were separated out by these components. Microbial biomass was also strongly negatively correlated with bioavailable Cr, Cu and Ni.

![Phospholipid Phosphate Concentration](image)

**Figure 6.** Soil microbial biomass in vegetated and unvegetated plots, upper and lower soil layers. Each value is the mean of 6 soil samples ± 1SD.

**FAME.** The mean percentages of the phospholipids found in the upper and lower layer LTU soils are shown in table 3.
Table 3. Relative mole percentages of phospholipid in the upper and lower layers of LTU soil.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Upper Layer</th>
<th>Lower Layer</th>
<th>Upper Layer</th>
<th>Lower Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unvegetated</td>
<td>Vegetated</td>
<td>Unvegetated</td>
<td>Vegetated</td>
</tr>
<tr>
<td>i15:0</td>
<td>1.04</td>
<td>0.80</td>
<td>1.52</td>
<td>1.47</td>
</tr>
<tr>
<td>a15:0</td>
<td>1.16</td>
<td>0.99</td>
<td>3.55</td>
<td>3.10</td>
</tr>
<tr>
<td>15:0</td>
<td>0.51</td>
<td>0.59</td>
<td>1.86</td>
<td>1.67</td>
</tr>
<tr>
<td>i16:0</td>
<td>1.99</td>
<td>1.31</td>
<td>4.23</td>
<td>3.32</td>
</tr>
<tr>
<td>16:1 ω7c</td>
<td>4.72</td>
<td>6.63</td>
<td>4.86</td>
<td>4.63</td>
</tr>
<tr>
<td>16:1 ω6c</td>
<td>0.61</td>
<td>0.67</td>
<td>0.50</td>
<td>0.18</td>
</tr>
<tr>
<td>i17:1</td>
<td>10.28</td>
<td>6.44</td>
<td>4.68</td>
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</tr>
<tr>
<td>a17:1</td>
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<td>a17:0</td>
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<td>0.65</td>
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<tr>
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<td>0.74</td>
<td>3.62</td>
<td>3.27</td>
</tr>
<tr>
<td>cy17:0</td>
<td>10.44</td>
<td>10.75</td>
<td>7.26</td>
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</tr>
<tr>
<td>17:0</td>
<td>0.91</td>
<td>0.71</td>
<td>2.70</td>
<td>2.36</td>
</tr>
<tr>
<td>18:1 ω9t al</td>
<td>0.00</td>
<td>0.45</td>
<td>1.95</td>
<td>1.43</td>
</tr>
<tr>
<td>i18:0</td>
<td>0.00</td>
<td>0.00</td>
<td>1.53</td>
<td>0.54</td>
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<tr>
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<td>9.78</td>
<td>1.95</td>
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<tr>
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<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>1.03</td>
</tr>
<tr>
<td>19:1 al</td>
<td>2.70</td>
<td>4.43</td>
<td>4.34</td>
<td>3.59</td>
</tr>
<tr>
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<td>0.00</td>
<td>0.23</td>
<td>0.52</td>
</tr>
<tr>
<td>18.537</td>
<td>0.00</td>
<td>0.00</td>
<td>0.41</td>
<td>0.80</td>
</tr>
<tr>
<td>18.708</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.07</td>
</tr>
<tr>
<td>cy19:0</td>
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<td>20.52</td>
<td>7.71</td>
<td>7.83</td>
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<tr>
<td>20:3 ω6c</td>
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<td>0.00</td>
<td>1.54</td>
<td>1.50</td>
</tr>
<tr>
<td>18:0e9 10 x</td>
<td>0.00</td>
<td>0.19</td>
<td>1.97</td>
<td>2.21</td>
</tr>
<tr>
<td>19:0 3oh</td>
<td>0.00</td>
<td>0.21</td>
<td>2.09</td>
<td>2.59</td>
</tr>
<tr>
<td>20.698</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.31</td>
</tr>
<tr>
<td>21.087</td>
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<td>21.238</td>
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<tr>
<td>21.687</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Specific PLFAs, including i17:1, cy17:0, 18:2 ω6c, and cy19:0, were relatively higher in the upper layer than the lower layer. The PLFAs: a15:0, i16:0, 17:1 ω8c, 17:0, 18:1 ω9t al, i18:0, and 18:1 ω9t/7c were present as higher percentages in the lower layer than the upper layer. Many of the PLFAs found in the lower layer were not present in the
upper layer and the overall number of different PLFAs in the lower layer was greater than in the upper layer. The upper soil from the unvegetated plot had 15 phospholipids, the upper vegetated had 18, the lower unvegetated had 24, and the lower vegetated had 30. For each layer the grass plots had a greater variety of fatty acids than the control plots.

Principle component analysis of samples based on their individual phospholipid fatty acid profiles, identified 3 principle components that accounted for 91% of the variation in the data set. Principle components 1, 2, and 3, accounted for: 67%, 15%, and 9% of the variation, respectively.

FAME principle component 1 (FAME PC1) had high positive loadings for the fatty acids: 18:1 ω9t/7c, 17:1 ω8c, 19:0 3OH, i16:0, 18:0c9 10x, a15:0, 20:3 ω6c and 17:0. High negative loadings were for: cy19:0, cy17:0, 18:1 ω9c, i17:1, and 18:2 ω6c.

FAME principle component 2 (FAME PC2) had high positive loadings for the fatty acids: 21087, cy17:0 and 18:1 ω9t/7c. High negative loadings were for: cy 19:0, 18:2 ω6c, i17:1, 18:1 ω9c, and 19:1 al.

FAME principle component 3 (FAME PC3) had high positive loadings for the fatty acids: 18:0c9 10x, 21087, a17:1, 20698, and 21238. High negative loadings were for: cy 19:0, 18:2 ω6c, 18:1 ω9t/7c, cy17:0, and 18:1 ω9c.

ANOVA's of the three components indicated that only soil layer was significant for FAME PC1 with the lower layer having positive values, and the upper layer having negative values (Figure 7). Neither layer nor plot was significant for FAME PC2 or PC3.

Pearson correlations between the FAME principle components and the factors for the PAH and heavy metal data (Contaminant PCAs), the bioavailable heavy metals and PAHs, and microbial biomass were also performed (data not shown). FAME PC1 was
highly positively correlated with Contaminant PC1 and PC3. It was also positively correlated with the bioavailable fractions of Cu, Ni, and Cr. FAME PC1 was highly negatively correlated with FAME PC2 and PC3, and microbial biomass. FAME PC2 and PC3 were both negatively correlated with everything that was positively correlated with FAME PC1. FAME PC2 was positively correlated with FAME PC3, and microbial biomass. FAME PC3 was also positively correlated with microbial biomass.

Figure 7. Results of principle component analysis of FAME data from field site soil samples. Plot of FAME PC1 versus FAME PC2 showing upper and lower layer soil samples.

Mineralization in Field Collected Soils. Forty-seven days after $^{14}$C-pyrene was added to field-collected site soil, samples from the upper soil layers had mineralized between 39
and 43% of the added pyrene whereas the lower layer had mineralized only 2 to 13% (Figure 8). Whether the soil came from a vegetated or unvegetated plot was not significant, but which layer it came from was (P<0.05). Rate constants for the mineralization curves were determined using first order regression models (Table 4).

![Figure 8. Cumulative percent mineralization of $^{14}$C-pyrene from upper and lower layer field soil samples. Each value is the mean (± 1SD) of 3 replicates.](image)

Table 4. Rate constants and maximum mineralization extents as a function of soil layer and plot. N=3

<table>
<thead>
<tr>
<th>PLOT</th>
<th>LAYER</th>
<th>$k$†</th>
<th>MAXIMUM‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetated</td>
<td>Upper</td>
<td>0.16 (0.01)</td>
<td>40.37 (0.35)</td>
</tr>
<tr>
<td>Vegetated</td>
<td>Lower</td>
<td>0.02 (0.00)</td>
<td>3.64 (0.55)</td>
</tr>
<tr>
<td>Unvegetated</td>
<td>Upper</td>
<td>0.15 (0.02)</td>
<td>36.43 (3.31)</td>
</tr>
<tr>
<td>Unvegetated</td>
<td>Lower</td>
<td>0.08 (0.01)</td>
<td>14.49 (2.27)</td>
</tr>
</tbody>
</table>

†Rate ($k$) is expressed as % d$^{-1}$ (Standard error of the estimate)
‡Estimate of maximum percent mineralization

Studies were also performed in the lab to determine if planting of the upper and lower soil layers could impact pyrene mineralization in the LTU soil. After planting the ability to mineralize pyrene in the lower layer increased relative to the indigenous levels previously found in the field-collected soil (Figure 9). After planting the upper layer
mineralization was less than the lower layer. It was also less than the cumulative percent mineralization of the field soil from planted plots. In all cases the cumulative percent mineralization of $^{14}$C-pyrene after planting was higher in the lower layer than in the upper layer.

Figure 9. Cumulative percent mineralization of $^{14}$C-pyrene in upper and lower layer soil that was planted for 4 months with fescue, rye, or a legume mix. Each value is the mean (± 1SD) of 2 samples.

Discussion

Differences in soil characteristics between the upper and lower layers confirm visual observations that the layers are distinct. The higher concentration of most nutrients in the upper layer was expected as the surface was seasonally fertilized and tilled to a depth of 18" over the past decade. Lower total organic carbon and organic matter in the lower soil layer is probably due to fewer plant roots, root exudates, and plant residues reaching that depth. Although it might be anticipated that planting would
significantly affect soil characteristics, no differences were reported for vegetated versus unvegetated plots. This may be because the unvegetated plots were only cleared one year prior to sampling, and differences had yet to become significant.

The concentration of PAHs in the lower layer was higher than in the upper layer, possibly due to many factors, including those found here to be correlated with layer, microbial biomass and activity. Both microbial biomass and activity were reduced in the lower soil layer, which was characterized by a decrease in nutrients. This and an increase in anaerobic conditions are typically seen as depth in soil increases (Federle, 1990; Hurst, 1996). The difference between the layers for the 4-ring PAHs is, then, probably due to increased microbial degradation in the upper layer. The larger PAHs were not significantly different between layers because they are difficult for microorganisms to metabolize; the greater number of microorganisms in the upper soil layer would have little effect on the pool of these highly recalcitrant PAHs.

Polycyclic aromatic hydrocarbons are hydrophobic, so only a small fraction of the total PAHs would likely be found freely available in the aqueous soil solution. It follows that the larger PAHs, which are more hydrophobic than the smaller PAHs, would be even less available. This proved to be the case in the LTU soil. Mild butanol extraction removed an average of 66% of the 3-ring, 30% of the 4-ring, and 5% of the 5-ring PAHs present in soil. The pool of bioavailable PAHs was greater in the lower layer than in the upper layer, reflecting the higher concentration of PAHs in the lower layer. The smaller concentration of bioavailable PAHs in the upper layer may be due to removal by plants or microbial systems.
Unlike PAHs, the upper soil layer had a higher concentration of heavy metals than the lower layer, possibly due to higher metal concentrations of later applications of refinery wastes. Another explanation is that plants may have taken metals up into their biomass, which was then tilled back into the upper soil layer on a regular basis. Although soil metal concentrations were high, the amount available for uptake by plants or for potentially toxic exposure of microbes was small. This low bioavailability, 10 years after waste applications ceased, reflects the strong sorption capacity of the soil and the effects of aging. Low bioavailability implies that the potential for phytoremediation of the metals by uptake is low unless plants can increase metal availability. However, the use of plants that may increase heavy metal bioavailability could negatively affect the remediation of the PAHs in the LTU soil as certain metals have been shown to adversely affect plant growth even at these low concentrations (Mulford, 1996).

Soil microbial communities may also be adversely affected by soil metal contaminants. When the petroleum wastes were initially applied to the LTU and mixed with the native soil, there was probably a drop in the number of microorganisms present in the soil as sensitive species died off. Over the past decade the numbers will have recovered and stabilized. While microbial biomass measurements provide a general indication of how many organisms are present, they do not give any information on the community structure or function of microorganisms in the soil.

A more thorough examination of the composition of the microbial community aids in understanding the status of soil microorganisms. FAME analysis of the soil samples from the LTU showed that the communities differed between soil layers. The major difference between the layers was the high relative percentage of the phospholipid
18:2 ω6 (a fungal indicator) in the upper layer. High concentrations of 18:2 ω6 have been reported in heavy-metal contaminated soils (Frostegard, 1993). While fungi are generally considered to be more tolerant of heavy metal contamination than either bacteria or actinomycetes, it is also possible that the higher concentration of 18:2 ω6 in the upper layer is present as a result of plant residues and/or mycorrhizal associations (Saranpaa and Nyberg, 1987).

The gram-negative bacterial markers, cy17:0 and cy19:0, were present in both the upper and lower layers, but both were higher in the upper layer. These phospholipids are known to increase in percentage when soil is contaminated with heavy metals (Frostegard et al., 1993). In general gram-negative markers dominated both soil layers. Gram-negative bacteria are generally present in higher percentages in disturbed soils, but as time passes and the system stabilizes, they tend to be replaced by gram-positive bacteria (Langworthy et al., 1998). The composition of the fatty acids of both layers (cis rather than trans) indicates that there is not much stress in the system. The only definite trans fatty acid in the study, 18:1 ω9t al, was greater in the lower layer, indicative of greater stress possibly due to the higher concentration of PAHs. This is also reflected in lower germination, seedling survival, and plant biomass in the lower soil layer (Chapter 3, this thesis).

The diversity of phospholipids, and therefore the microbial community, is much higher in the lower layer than the upper, suggesting a wider variety or number of microhabitats providing niches for a greater variety of microbial species. For both layers there was higher diversity of the fatty acids from vegetated plots than the unvegetated
control plots, suggesting greater variety of energy sources due to plant roots and exudates.

The mineralization of pyrene added to upper and lower layer soils, (as collected from the field), supports the conclusion that there are microbial differences between the layers. Lower mineralization rates and estimated maxima for the lower layer reflect the smaller microbial biomass found there. Mineralization of pyrene was similar in both upper soils, so the presence of plants in the plot did not make a difference.

After planting in the laboratory, pyrene mineralization in the lower layer soil increased significantly. This increase may reflect the same process that occurred in the field over the last 10 years of planting, resulting in the upper layer as it now exists. Lower mineralization in the upper layer soil after planting could be due to lower soil fertility resulting from nutrient removal by plants, competition for nutrients by plants and microorganisms, or some plant-driven increase in available metals having a toxic effect on microbes.

Microbial and chemical data support the visual observation that the upper and lower layers of the LTU soil are different. The layers may exist as a function of historical site vegetation, as planting in the lab was shown to improve the ability of the lower layer microbial community to mineralize added $^{14}$C-pyrene and thus behave in a similar manner to the upper layer. Given the lack of current mineralization in the lower layer in the field, there could be several factors imposing limitations, including rooting depth and general soil conditions. Altering the soil conditions of the lower layer may permit greater degradation of the PAHs.
References


CHAPTER 3: THE INFLUENCE OF PLANTING ON PAHS, HEAVY METALS AND MICROORGANISMS IN AGED PETROLEUM HYDROCARBON CONTAMINATED SOIL

Abstract

Many contaminated fields have been vegetated through the active sowing of seeds or by encroachment of native vegetation onto the site. These vegetated sites could be substantially cleaner than sites that are maintained without vegetation, as phytoremediation may have been occurring. For this study, soil was taken from an aged petroleum refinery LTU that had been planted with rye grass to evaluate the potential for further phytoremediation of the site. Soil samples taken from two depths were planted in the lab with several plant species to evaluate the historic and potential future effect of planting on total and bioavailable concentrations of specific heavy metals and PAHs. Concurrent with this, the effect of the soil on plant germination, biomass, and rooting depth were measured, as was microbial community structure and activity. The conditions of the study facilitated a decrease in the concentration of PAHs in both planted and unplanted pots, but no difference in the heavy metal concentration. Both the plant species and the soil used in the pot influenced plant germination, biomass, rooting depth, and metal uptake. The soil used in the pot study did not influence the microbial community structure, but there were differences in the relative percentages of specific PLFAs for the various plant species.

Introduction

Plants have been shown to take up heavy metals into their biomass (Wenzel and Jockwer, 1999) and to increase the degradation rate of organic pollutants in soil (Aprill
and Sims, 1990). These plant characteristics are the basis of phytoremediation, a relatively low cost soil remediation strategy that is being used to treat a wide variety of soils contaminated with organic and inorganic pollutants (Liste and Alexander, 2000; Dudka et al., 1998). Although it may take years longer than other methods to reach an acceptable end-point, phytoremediation is non-invasive and can proceed with only occasional monitoring. These benefits make it an attractive alternative to traditional excavation and incineration methods. There are, however, limits to phytoremediation because it is based upon the ability of living organisms to survive in and affect an environment that may not be optimal for their growth. The plant species used must be tolerant of the type and degree of pollution, since they must be able to germinate, grow, and maintain sufficient biomass to influence the soil environment. In addition, plant root morphology must be such that there is contact between the contaminants and the root. The depth and the breadth of rooting could be a significant aspect of effectiveness.

Another factor potentially limiting phytoremediation is the availability of contaminants for plant uptake or microbial degradation. The bioavailable portion of a contaminant pool is a function of the specific organism involved, but it is generally defined as the fraction that may pose an ecological risk (Tang and Alexander, 1999). The bioavailability of soil contaminants decreases with contact time as they sorb to solid particles, form complexes with organic material, or become sequestered in micropores (Alexander, 1999). Contaminants that have “aged” in the soil are more difficult for microorganisms and plant roots to access, thereby reducing their rate of degradation or plant uptake (Berg et al., 1998). Hydrophobic organic contaminants with low aqueous solubilities, such as polycyclic aromatic hydrocarbons (PAHs), sorb tightly to soil
particles leaving only a very small portion of the total concentration in the soil solution. Reduced bioavailability of heavy metals may be an advantage in mixed contaminated soils where metal toxicity could inhibit sensitive microorganisms and plants responsible for organic compound degradation. Lower metal toxicity under these conditions would reduce stress on the biota permitting improved growth and survival and increased degradation rates of organic pollutants. Reduced metal bioavailability might permit greater degradation of organic compounds, but it is only a temporary solution, as the total concentration of metals in the soil remains unchanged.

Plants are known to enhance the bioavailability of organic and inorganic compounds for microbial degradation or plant uptake by altering the rhizosphere through the release of root exudates (Ernst, 1996). These exudates, often a response to environmental stress and nutrient deficiency, function to increase the solubility of essential nutrients for plant uptake. Besides influencing the solubility of nutrients, plant root exudates also influence the bioavailability of contaminants, which can be made more soluble and available for remediation. Plant root exudates and the community of microorganisms that inhabit the rhizosphere benefiting from the carbon source provided by the exudates can be specific to that plant (Fang et al., 2001). Because plant species have different exudate compositions their influence over the rhizosphere and the rhizosphere microbial community may vary, resulting in rates of phytoremediation that are species specifics.

A previous study (Chapter 2, this thesis) indicated a significant effect of planting on mineralization in aged soils. This study further investigates the impact of planting on these aged contaminated soils. The potential of rye (*Lolium perenne*), alfalfa (*Medicago*
sativa), and a mixture of rye, fescue (*Fescue arundinacea*) and legumes (*Medicago sativa, Lotus corniculatus L.*, and *Trifolium repents*) to remediate aged petroleum refinery soil contaminated with both metals and PAHs was evaluated. Changes in overall contamination levels were measured, along with changes in the bioavailable fractions of select heavy metals and PAHs. Differences in microbial biomass, activity, and community structure were also measured after planting to define how the different plant treatments altered the microbial community.

**Materials and Methods**

*Soil Collection and Characterization*

Soil samples were collected from the upper (0-16") and lower (16-22") layers of a petroleum refinery land treatment unit (LTU) as previously described (Chapter 2, this thesis). Table 1 shows general soil characteristics for the upper and lower layer soil samples as determined by Spectrum Analytic, Inc. (Washington C.H., OH)

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>TOC %</th>
<th>OM %</th>
<th>CEC MEQ</th>
<th>P lbs/A</th>
<th>K lb/A</th>
<th>Ca lbs/A</th>
<th>Mg lb/A</th>
<th>K %</th>
<th>Mg %</th>
<th>Ca %</th>
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<tbody>
<tr>
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<td>7.7</td>
<td>2.9</td>
<td>5.0</td>
<td>18.3</td>
<td>14.8</td>
<td>233.3</td>
<td>12831.5</td>
<td>710.5</td>
<td>1.6</td>
<td>16.2</td>
<td>82.2</td>
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<td>Lower Layer</td>
<td>7.4</td>
<td>1.9</td>
<td>3.2</td>
<td>20.9</td>
<td>16.3</td>
<td>130.5</td>
<td>10191.5</td>
<td>1152.8</td>
<td>0.8</td>
<td>23.2</td>
<td>72.3</td>
</tr>
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</table>

Each value is the mean of 2 replicates of 4 soil samples.

Soil pH (following EPA method 9045C), and cation exchange capacity (CEC) (EPA method 9081) were independently determined. These values were similar to those reported by Spectrum Analytic.
Pot Study

The objective of the pot study was to determine how different plant species influence soil contamination and the soil microbial community. Square pots (8 cm x 8 cm) were filled with 400g of LTU soil. Pots received either 400g soil from the upper layer (upper) or lower layer (lower), or 200g upper layer soil mixed with 200g lower layer soil (50/50). The mix of the layers was to simulate a potential site management practice (deep tilling) that would incorporate the layers. Perennial rye, alfalfa, and a mix frequently used in EPA phytoremediation studies consisting of rye, fescue, and a legume mixture (birdsfoot trefoil, alfalfa, and clover) were used. Approximate proportions of each species in the mixture were: 15% rye, 65% fescue, and 20% legumes. Seeds were deposited onto the surface of the pot and allowed to germinate. If little germination occurred, then more seeds were added until the surface was covered with seedlings. Each treatment was replicated 3 times for each plant species. After 4 months of growth, plants were removed from the soil, excess soil was shaken from the roots, and roots were washed in dH2O. Plant shoots and roots were separated and dried in a 55°C oven to constant weight. Dried plants were ground, digested, and analyzed for metals. A sample of each soil was oven dried for metal analysis. Fresh soil samples were immediately used for a mineralization study, microbial biomass measurements, and community analysis (FAME). The remainder of the soil was kept at 4°C until it was analyzed for PAHs.

Plant Performance

Column Study. A study was performed to evaluate the limitations for the same plant species used in the pot study when grown on the soil as it is actually layered in the field.
Large plastic columns with layers equal to those on the site (upper layer soil from the surface down to 16", lower layer soil from 16" down to 21") were constructed. Six columns were germinated with two replicates each of rye, fescue and the legume mixture. Enough seeds were planted to cover the surface with seedlings. After four months of growth the columns were cut at 16" and the roots from both sections of each column were removed from the soil by floatation. Roots and shoots were dried in a 55°C oven to constant weight.

*Plant germination and biomass.* The pot study examined the potential effect of the plants on contaminants on upper, lower, and mixed soil layers from the LTU. As an alternative to this, separate studies were conducted to examine the effects of the contaminants on seed germination and subsequent plant growth. Twenty seeds were added to 8 cm x 8 cm square pots containing the same soil treatments as the pot study. Germination was recorded over 30 days. Seedlings that did germinate were allowed to grow over the 30 d period after which time they were removed from the pots and the soil was washed from the roots. Roots and shoots were dried in a 55°C oven to constant weight, and the plant biomass was determined on a per pot and per plant basis.

*Chemical Analyses*

*Plant Tissue.* To examine plant uptake of heavy metals, plant tissue harvested from the pot study was digested for 12 hours in HNO₃ and H₂O₂, then microwave digested in closed-vessel teflon microwave extraction vessels equipped with safety rupture membranes on a rotating turntable. Microwave digestion was performed according to the Rodushkin et al. (1999) closed vessel plant digestion method using a Microwave
Extraction System 1000 (CEM; Matthews, NC) set at 600W for 1h. The digested tissue samples were diluted with dH₂O to 50 ml after which they were centrifuged at 1900g for 5 min. Detection of Cr, Cu, Zn, and Ni were performed using an ICAP 61E Thermo Jarrell Ash (Franklin, MA) inductively coupled plasma atomic emission spectrometer (ICP-AES) operated at 1150W.

Soil. Soils were extracted with a single step procedure (EPA method 3050B) to determine the total amount of metals in the soil, and sequentially (Sposito et al., 1982) to determine the amount of metal associated with each soil fraction. For total metal extractions, 10 ml of 1:1 HNO₃ was added to 1g of dry soil and heated (95°C) for 15 min. After cooling, 5 ml of HNO₃ was added, and the sample was heated for 2.5 h. When cool, 2 ml of dH₂O and 3 ml of 30% H₂O₂ were added and the sample was heated for 2 h, after which time 10 ml of HCl was added and the test-tube was heated for a further 15 min. Extracts were filtered (Whatman No. 41) and made up to 100 ml in nalgene bottles. For sequential metal extractions, 25 ml of an extractant was added to a single 2g sample of dry soil in a centrifuge tube, which was shaken for a specified amount of time (Table 2), after which it was centrifuged at 12,000g for 15 min in a Sorvall RC-5B Plus centrifuge (Dupont, Newtown, CT). The supernatants was collected, filtered, and refrigerated until analysis. The remaining pellet was treated with 25 ml of the next extractant and the process was repeated.

Table 2. Sequential extractants and shake times.

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>EXTRACTANT</th>
<th>SHAKE TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exchangeable</td>
<td>0.5M KNO₃</td>
<td>16 hours</td>
</tr>
<tr>
<td>Adsorbed</td>
<td>DI H₂O</td>
<td>2 hours</td>
</tr>
<tr>
<td>Organically Bound</td>
<td>0.5M NaOH</td>
<td>16 hours</td>
</tr>
<tr>
<td>Carbonate Bound</td>
<td>0.05M Na₂EDTA</td>
<td>6 hours</td>
</tr>
<tr>
<td>Residual</td>
<td>4M HNO₃</td>
<td>16 hours*</td>
</tr>
</tbody>
</table>

*At 80°C
Detection of Cr, Cu, Zn, and Ni were performed using ICP-AES (ICAP 61E Thermo Jarrell Ash; Franklin, MA) operated at 1150W. Matrix differences were corrected by making standards for each set of sequential extracts using the extractant for each step.

Fresh soils were extracted for determination of total and available PAHs. Total PAHs were extracted using an accelerated solvent extraction system (Dionex ASE 200; Sunnyvale, CA) and Richter’s (2000) method for extraction of hydrocarbons. Extraction cells (22 ml) were filled with a mixture of 4g soil and 2g diatomaceous earth. The extraction solvent was dichloromethane-acetone (1:1,v/v). Cells were heated for 8 min, to reach 175°C. Extraction pressure was set at 1500 p.s.i., static time was 5 min, flush volume was 70%, and purge was 60 sec with 150 p.s.i. N₂. Extracts were dried to 10 ml and then centrifuged for 30 min at 220g. Bioavailable PAHs were estimated using a mild extraction procedure (Tang, 1999). The extractant (25 ml of n-butanol) was added to 2g fresh soil in a 50 ml Teflon tube. Tubes were vortexed then centrifuged at 17,200g for 10 min at 4°C. The supernatant was decanted, dried by passing through an anhydrous sodium sulfate column, and evaporated to 10 ml. Total and bioavailable PAHs were separated on a gas chromatograph (Shimadzu GC 14A, Columbia, MD) in the split mode with a flame ionization detector (FID). The column was an HP-1 (Hewlet Packard, Palo Alto, CA) cross-linked methyl silicone column (60m x 0.25mm internal diameter x 0.25µm film thickness). The injection port temperature was 290°C, column temperature was static at 70°C for 2 minutes then increased by 15°C per min to 150°C, and then increased by 6°C per min to a final temperature of 290°C, which was held for 30 min.
All solvents and chemicals for chemical analyses were of reagent grade. Three replicate extractions of each soil sample were run for each extraction.

**Microbial Analyses**

**PAH Mineralization.** Serum bottle radiorespirometry (Knaebel and Vestal, 1988) was used to evaluate the ability of soil microorganisms to metabolize $^{14}$C-pyrene to $^{14}$CO$_2$. Fresh soils (5g wet weight) were placed into autoclaved 50 ml serum bottles and brought to approximately 80% soil moisture with sterile dH$_2$O. Radioactive pyrene (specific activity 58.7mCi/mol; Sigma, St. Louis MO) in acetone was added as a 20 µl spike (33910 DPMs) to each serum bottle. Bottles were vortexed, and sealed. Paper wicks (Whatman No.1) saturated with 70 µl of 0.5M KOH were suspended in the serum bottles to capture evolved $^{14}$CO$_2$. Mineralization was measured for 47 days by periodically removing wicks and determining the radioactivity on them by liquid scintillation analysis (TRI-CARB 2200CA; Packard Instrument Co., Downer’s grove, IL). Sterile autoclaved soil was used as an abiotic control.

**Microbial Biomass and FAME Analyses.** After planting, fresh soil (4-6g) was extracted for 24h in a dichloromethane-methanol-phosphate buffer solution (15 ml DCM, 15 ml MeOH, 5 ml 50mM phosphate buffer). Dichloromethane and water were then used to partition the aqueous and organic fractions of the extract. After 24h, samples were centrifuged and the organic fraction containing the lipid material was recovered. The organic fraction was dried under N$_2$ and made up to 2 ml with chloroform. A sub-sample was taken for total microbial biomass measurements using the phospholipid phosphate method (Dobbs and Findlay, 1993). The remaining lipid was separated into 3 fractions
by solid phase extraction through silicic acid columns. The phospholipid fraction was recovered in methanol. PLFAs were transmethylated to fatty acids methyl esters (FAMEs) by the addition of 0.5 ml of 0.2 N KOH in methanol (Findlay and Dobbs, 1993). FAMEs were purified using reverse-phase SPE column chromatography, and then analyzed by GC using the standard EUKARY chromatographic program (MIDI software, Microbial ID, Newark, DE). FAMEs were separated on a Hewlett Packard 5890 Series II GC in the splitless mode, using a nonpolar cross-linked 5% phenyl methyl siloxane column (25m x 0.25mm internal diameter), and a flame ionization detector (FID). The injection port temperature was 250°C; column temperature started at 70°C then increased by 3°C per min to a final temperature of 300°C which was held for 10 minutes. Solvents and chemicals were of optima grade.

**FAME Nomenclature.** FAMEs were designated by the total number of carbon atoms in the fatty acids, followed by a colon and the number of double bonds from the aliphatic (ω) end of the molecule. The number after the “ω” corresponds to the carbon atom before the double bond from the methyl end of the molecule. “C” and “t” indicate cis and trans configurations, anteiso and iso are represented by “a” and “i”, and “cy” stands for cyclopropyl fatty acids.

**Statistical Analyses**

ANOVA and principle component analyses (PCAs) were performed using SYSTAT 9 (Systat, Evanston, IL). PCA was performed separately on the contaminants (total PAHs and metals) and on the lipid profiles of the samples. This allowed samples from different soil layers and plots to be separated (or clustered) based on soil abiotic and
Biotic characteristics. Analysis of FAME data was performed on mole percentages after the fatty acids 16:0 and 18:0 were removed because they are ubiquitous in all soil microbial communities in large amounts (Findlay et al., 1990). Individual fatty acids that did not comprise 1% of the total phospholipid phosphate of at least one sample were also eliminated. Mineralization rate constants were estimated using the non-linear regression model of Systat. The first order production model (Larson, 1984) $M_t = A (1 – e^{-kt})$ was used; where $M_t$ is cumulative percent mineralization, $A$ is the maximum mineralization, $k$ is the rate constant, and $t$ is the time in days.

**Results**

*Plant performance*

Plant growth in large columns indicated that some plant roots were able to penetrate deeply enough to reach the lower black soil layer. However, after 4 months of growth less than 5% of the roots were found below 16 inches (Table 3).

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>0-16 inches</th>
<th>16-21 inches</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rye</td>
<td>98.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Fescue</td>
<td>99.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Legume</td>
<td>95.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Germination in potted soil was recorded over a 30d period. For each plant species, germination was lower in the lower soil layer than in the 50/50 soil mixture. Survival of the seedlings in the lower layer after germination was also much lower than
in the other 2 layers (67% versus 92% for the upper layer and 94% for 50/50). The legume mix had the lowest germination in each layer and the lowest percent survival in each layer. The difference between plant species was significant with rye consistently having the greatest germination followed by fescue, and then the legume mixture (Table 4).

<table>
<thead>
<tr>
<th>Species</th>
<th>Upper layer</th>
<th>50/50</th>
<th>Lower layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rye</td>
<td>11</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Fescue</td>
<td>11</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Legume mix</td>
<td>7</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

Pots that were planted with rye also had the greatest average plant biomass per pot after 30 days of growth (Figure 1).

![Figure 1. Mean total plant biomass per pot of rye, fescue, and legume mix in upper layer, 50/50 and lower layer soil after 30 days of growth. Error bars represent 1 SD from the mean, n=3.](image-url)
Both rye and legume biomass were higher in the upper layer than in the other 2 layers, even though rye had more germination in 50/50 than the upper layer. Plant species and soil layer were both significant determinants of the variation in plant biomass. When examined on a per plant basis, average legume biomass per plant was 1.6g, fescue was 1.5g, and rye was only 0.79g. Although there were more rye plants per pot, each individual was smaller than each fescue or legume plant.

*Plant Metal Concentration*

Following the pot study, shoot and root tissue was acid digested to extract any heavy metals that had been taken up into the plant biomass. Concentrations of metals in plant tissue were proportional to initial metal concentrations in soil samples from the upper and lower soil layers (Cr>Zn>Cu>Ni) (Table 5). Higher concentrations of metals were present in ryegrass grown in the upper layer soil than from the other layers. Unlike rye, the mix and alfalfa plant biomass both exhibited higher metal levels when grown in the lower layer soil than in the 50/50 or the upper layer.

<table>
<thead>
<tr>
<th>Species</th>
<th>Layer</th>
<th>Cr</th>
<th>Zn</th>
<th>Cu</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rye</td>
<td>Upper</td>
<td>41.3</td>
<td>25.7</td>
<td>16.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>50/50</td>
<td>33.9</td>
<td>23.2</td>
<td>20.5</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>32.0</td>
<td>23.2</td>
<td>15.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Mix</td>
<td>Upper</td>
<td>27.3</td>
<td>22.8</td>
<td>14.1</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>50/50</td>
<td>38.3</td>
<td>25.6</td>
<td>17.8</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>54.0</td>
<td>35.9</td>
<td>18.6</td>
<td>6.5</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Upper</td>
<td>22.1</td>
<td>18.1</td>
<td>4.7</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>50/50</td>
<td>11.2</td>
<td>13.1</td>
<td>3.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>52.0</td>
<td>31.0</td>
<td>9.8</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Uptake of Cu by alfalfa was much less than Cu uptake by the other species, suggesting a possible exclusion mechanism.

**Soil Metal Concentrations**

Soil metal concentrations following the small pot study are shown in Figure 2. Analysis of variance of the data for planted versus unplanted soil indicated that a significant difference between soil layers existed for Zn, which was higher in the upper layer than the 50/50 or lower layer. Since the layer was otherwise unimportant, Figure 2 gives the mean soil metal concentrations across pots of upper and lower layers. Zn was higher in soil from the unplanted pots. Soils planted with alfalfa had significantly more Cu and Ni than other planted pots and even the unplanted soil treatments. This can only be explained by the heterogeneous distribution of metals in the soil that was measured into the pots, as without the presence of plants there is no mechanism for removal of metals from the soil. The higher concentration of Zn in the upper layer may account for the lower biomass in the upper layer than in the 50/50 layer, implying that deep tilling to incorporate the layers would make the soil conditions more favorable for plant germination and growth.

![Figure 2. Mean total metal concentrations of soil analyzed after 4 months (n=9 ±1SD)](chart)
Sequential extractions were performed to determine the soil fractions with which the metals were associated. The metal fraction removed by KNO₃ was considered exchangeable, and that removed by dH₂O adsorbed to soil surfaces. These 2 fractions comprise the portion that is readily available for plant uptake. Sodium hydroxide and Na₂EDTA extractable metals represent, respectively, the organically bound and carbonate bound fractions, or those that are potentially available for plants and may become available over time. Nitric acid extractable metals are considered residual and unavailable for plant uptake. The amount of metal present in the residual fraction was higher than all other fractions for all metals. Very small concentrations of metals were present in the bioavailable fractions (Figure 3). Analysis of variance using the bioavailable metal fractions as the dependent variable indicated that the species, but not the soil layer, was significant. This was due to the lower bioavailability of metals in pots planted with the mixture. Overall, each pot contained 400g of soil, the concentration of the metal in the pots that would be bioavailable would be anywhere from 800 to 2400 µg per pot. The amount taken up into plant tissues was much less than 1% of the total amount of metal in each pot.

![Figure 3. Concentration of bioavailable heavy metals in soil from rye, mix, and unplanted pots. Each value is the mean ± 1SD of 9 replicates.](image)
Soil PAH Concentration

Following the pot study, the amount of PAHs remaining in the soil was a fraction of the initial concentration (Table 6). There was no consistent trend in terms of which specific PAHs were remediated more than others. The concentration of PAHs was not significantly different between soil layers or plant species, and planted soil PAH levels were no different than unplanted. Standard deviations were large reflecting the heterogeneous distribution of PAHs in the soil.

Table 6. Mean PAH concentration (µg/gdw) in soil before and after pot study. (n=9).

<table>
<thead>
<tr>
<th>PAH</th>
<th>Before study</th>
<th>After study</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenapthylene</td>
<td>0.93</td>
<td>0.06</td>
<td>93</td>
</tr>
<tr>
<td>Fluorene</td>
<td>1.61</td>
<td>1.37</td>
<td>15</td>
</tr>
<tr>
<td>Anthracene</td>
<td>1.77</td>
<td>2.54</td>
<td>NC</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3.92</td>
<td>1.72</td>
<td>56</td>
</tr>
<tr>
<td>Pyrene</td>
<td>18.63</td>
<td>5.01</td>
<td>73</td>
</tr>
<tr>
<td>Chrysene</td>
<td>54.81</td>
<td>12.88</td>
<td>77</td>
</tr>
<tr>
<td>Benz[a]anthracene</td>
<td>12.03</td>
<td>2.21</td>
<td>82</td>
</tr>
<tr>
<td>Benzo[bk]fluoranthene</td>
<td>4.88</td>
<td>0.91</td>
<td>81</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>8.12</td>
<td>6.19</td>
<td>24</td>
</tr>
<tr>
<td>Dibenzo[ah]anthracene</td>
<td>1.70</td>
<td>3.75</td>
<td>NC</td>
</tr>
<tr>
<td>Benzo[ghi]perylene</td>
<td>0.97</td>
<td>4.69</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC = no change.

No bioavailable PAHs were detected in the majority of the soil samples after the pot study. Prior to the study, approximately 40% of the PAHs in the soil were in a bioavailable form. After 4 months, 62% of the total amount of PAHs present had been removed, and only 10% of the PAHs still present were in an available form.

Principle Component Analyses of Contaminant Data

PCA of total heavy metal and total PAH concentrations reduced the many variables down to 3 axes that explained 55% of the variation in the data. Factor 1
explained 23% of the variation, 2 explained 19%, and 3 explained 13% of the variation. Separation along factor 1 was associated with heavy metals, factor 2 separated out anthracene and the 5 and 6 ring PAHs, and factor 3 separation was explained by phenanthrene, the 4 ring PAHs (pyrene, chrysene, benz[a]anthracene), and benzofluoranthene. There was no clustering of soil samples by layer, only by plant species. Factor 1 separated alfalfa from rye and mix. Factor 2 separated rye from unplanted. Figure 4 shows a scatter plot of factor 1 versus factor 2.

Figure 4. Scatter plot of soil samples separated along factor 1 and 2 of the principle component analysis of heavy metal and PAH data after the experimental period. Individual soil samples are identified by treatment; u, unplanted; r, ryegrass; m, mixture; a, alfalfa.

Mineralization

The cumulative percent mineralization of $^{14}$C-pyrene over a 56-day period in soil removed from the mix and unplanted pots are shown in figures 5 and 6, respectively. The
layer of soil in the pot and the interaction between layer and species were significant determinants of the mineralization that occurred. In the planted soils, mineralization was highest in 50/50 soil, followed by the lower layer, and then the upper layer. In unplanted soils mineralization was significantly lower in the upper layer than in the other two layers. Soil layer and plant species explained 80% of the variation in the data.

Figure 5. Cumulative percent mineralization of $^{14}$C-pyrene in soil removed from pots planted with the mixture. Values are the mean (± 1 SD) of 3 replicates.

Figure 6. Cumulative percent mineralization of $^{14}$C-pyrene in soil removed from unplanted pots. Values are the mean (± 1SD) of 3 replicate serum bottles.
Rate constants and maximum mineralization estimates were generated for the soils from the potted study (Table 7). The mineralization rates ($k$) were consistently higher for the planted versus unplanted soils for each soil layer. Although the estimated maximum mineralization was not increased by planting, it was influenced by the soil layer (50/50 soil layer was higher than the upper and lower layers).

Table 7. Rate constants and maximum mineralization extents as a function of soil layer and plant species.

<table>
<thead>
<tr>
<th>SOIL</th>
<th>Upper Layer</th>
<th>k†</th>
<th>MAXIMUM‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unplanted</td>
<td>0.051 (0.004)</td>
<td>19.3 (2.2)</td>
<td></td>
</tr>
<tr>
<td>50/50</td>
<td>0.036 (0.005)</td>
<td>33.9 (3.4)</td>
<td></td>
</tr>
<tr>
<td>Lower Layer</td>
<td>0.054 (0.005)</td>
<td>30.8 (1.1)</td>
<td></td>
</tr>
<tr>
<td>Rye</td>
<td>0.059 (0.005)</td>
<td>21.5 (2.1)</td>
<td></td>
</tr>
<tr>
<td>50/50</td>
<td>0.071 (0.013)</td>
<td>25.9 (2.2)</td>
<td></td>
</tr>
<tr>
<td>Lower Layer</td>
<td>0.090 (0.011)</td>
<td>18.4 (2.6)</td>
<td></td>
</tr>
<tr>
<td>Mix</td>
<td>0.065 (0.013)</td>
<td>16.7 (1.9)</td>
<td></td>
</tr>
<tr>
<td>50/50</td>
<td>0.080 (0.006)</td>
<td>32.7 (0.9)</td>
<td></td>
</tr>
<tr>
<td>Lower Layer</td>
<td>0.056 (0.006)</td>
<td>22.7 (1.0)</td>
<td></td>
</tr>
</tbody>
</table>

†Rate ($k$) is expressed as % d$^{-1}$ (Standard error of the estimate)
‡Maximum percent mineralization (Standard error of the estimate)

Cumulative percent mineralization was strongly positively correlated with microbial biomass. When analyzed in terms of the principle components from the heavy metal and PAH data, there was a strong negative correlation between factor 2 (anthracene and large ring PAHs) and percent mineralization. This suggests that the presence of large ring PAHs has a negative effect on mineralization.

**Microbial Biomass**

Soil layer, whether it was planted or not, species, and the interaction of these, explained 98% of the variation in the microbial biomass data. Soil from the unplanted
and alfalfa pots had approximately the same number of microorganisms, but soil from rye
and the mixture pots was significantly lower. The upper layer had less biomass than the
other soil layers.

Microbial biomass was strongly positively correlated with the bioavailable
fractions of the heavy metals and with cumulative percent mineralization. It was not
highly correlated with any of the PCA factors for the total PAH and heavy metal
concentrations.

FAME

FAME data for rye was omitted from the data set because readings for several
samples were unreliable. Individual fatty acids that did not comprise 1% of the total
phospholipid phosphate of at least one sample were also excluded. Table 8 shows the
average relative percentages of the individual PLFAs for soil planted with alfalfa, or the
mix, or left unplanted.

Table 8. Relative percentages of the total PLFAs for each plant treatment. Each value is
the mean of 9 samples.

<table>
<thead>
<tr>
<th>PLFA</th>
<th>Unplanted</th>
<th>Mix</th>
<th>Alfalfa</th>
</tr>
</thead>
<tbody>
<tr>
<td>i15:0</td>
<td>1.31</td>
<td>1.27</td>
<td>1.24</td>
</tr>
<tr>
<td>15:2 ω6c</td>
<td>1.82</td>
<td>2.00</td>
<td>1.73</td>
</tr>
<tr>
<td>15:0</td>
<td>0.77</td>
<td>0.25</td>
<td>0.62</td>
</tr>
<tr>
<td>i16:0</td>
<td>2.11</td>
<td>1.93</td>
<td>2.08</td>
</tr>
<tr>
<td>16:2 ω6c/a16:0</td>
<td>0.49</td>
<td>0.00</td>
<td>0.24</td>
</tr>
<tr>
<td>16:1 ω11c</td>
<td>0.89</td>
<td>0.00</td>
<td>0.88</td>
</tr>
<tr>
<td>16:1 ω7c</td>
<td>6.19</td>
<td>8.39</td>
<td>5.92</td>
</tr>
<tr>
<td>16:1 ω5c</td>
<td>1.30</td>
<td>1.00</td>
<td>1.57</td>
</tr>
<tr>
<td>i17:1</td>
<td>7.15</td>
<td>5.39</td>
<td>6.56</td>
</tr>
<tr>
<td>i17:0</td>
<td>1.20</td>
<td>0.26</td>
<td>1.15</td>
</tr>
<tr>
<td>a17:0</td>
<td>1.63</td>
<td>0.80</td>
<td>1.59</td>
</tr>
<tr>
<td>17:1 ω8c</td>
<td>1.60</td>
<td>0.68</td>
<td>1.42</td>
</tr>
<tr>
<td>17:0 ω?c</td>
<td>8.30</td>
<td>9.38</td>
<td>7.01</td>
</tr>
<tr>
<td>17:0</td>
<td>1.43</td>
<td>0.95</td>
<td>1.33</td>
</tr>
<tr>
<td>18:1 ω? al</td>
<td>0.18</td>
<td>0.00</td>
<td>0.08</td>
</tr>
<tr>
<td>17.608</td>
<td>0.10</td>
<td>0.00</td>
<td>0.62</td>
</tr>
<tr>
<td>18:3 ω6c</td>
<td>0.04</td>
<td>0.43</td>
<td>0.00</td>
</tr>
<tr>
<td>17.719</td>
<td>1.08</td>
<td>0.00</td>
<td>2.31</td>
</tr>
<tr>
<td>18:2 ω6c</td>
<td>0.00</td>
<td>0.72</td>
<td>0.00</td>
</tr>
<tr>
<td>18:1 ω9c</td>
<td>7.45</td>
<td>9.78</td>
<td>8.11</td>
</tr>
<tr>
<td>18:1 ω9t/7c</td>
<td>12.79</td>
<td>16.64</td>
<td>13.88</td>
</tr>
<tr>
<td>18.081</td>
<td>1.03</td>
<td>0.26</td>
<td>1.12</td>
</tr>
<tr>
<td>19:1 ω8? al</td>
<td>2.43</td>
<td>3.03</td>
<td>2.31</td>
</tr>
<tr>
<td>18.714</td>
<td>0.52</td>
<td>0.00</td>
<td>0.26</td>
</tr>
<tr>
<td>19:0cy ω11c</td>
<td>15.29</td>
<td>15.00</td>
<td>15.11</td>
</tr>
</tbody>
</table>

Those PLFAs that comprised a large percentage of each sample include: the
common gram negative bacteria markers 16:1 ω7c and 18:1 ω9t/7c, the gram positive
bacterial markers i17:1 and 17:0 ω?c, and the aerobic prokaryote markers 18:1 ω9c, and
19:0cy ω11c. The only possible trans PLFA in the samples is 18:1 ω9t which was indistinguishable from 18:1 ω7c by GC analysis due to identical elution times.

Several individual PLFAs were significantly different in soils from pots containing the upper or lower layer. These included i17:1, 17:1 ω8c, and 17:0 ω7c. Plant treatment significantly influenced the PLFA profile for most soil samples.

Principle component analysis of the microbial phospholipids reduced the PLFA variables to 3 components that accounted for 80% of the variation in the data. Components 1, 2 and 3 explained 49%, 22%, and 9% respectively.

FAME PC1 separated 16:1 ω7c, 17:0 ω7c, 18:1 ω9c, 18:1 ω9t/7c, and 19:0 cy ω11c from the fatty acid with an equivalent chain length of 17.719. FAME PC2 separated 16:1 ω7c, 15:2 ω6c, 18:2 ω6c and 18:1 ω9t/7c from the fatty acids i17:1 and 19:0 cy ω11c. FAME PC3 separated 16:1 ω7c, i15:0, 15:2 ω6c, and 16:1 ω5c from the fatty acid with an equivalent chain length of 18430.

Plotting the FAME factors against each other showed a distinct difference between the mix and the other 2 plant treatments. Alfalfa and unplanted were clumped together, whereas mix was spread out (Figure 8).

ANOVA of the FAME factors show that the PLFAs were not different for each soil layer, but plant species did influence the PLFAs for FAME factor 2. FAME factor 2 separated the mix from the rye.

Pearson correlations of the PCA factors from the FAME analysis with the factors from the heavy metals and PAH data showed strong positive relationships between FAME factor 3 and factor 3. This implies that the presence of the PAHs phenanthrene, pyrene, chrysene, benzo[a]anthracene, and benzo[fluoranthene
are positively correlated with the presence of microorganisms containing the phospholipids $16:1 \omega 7c$, $i15:0$, $15:2 \omega 6c$, and $16:1 \omega 5c$. The other FAME factors were not highly correlated with the contaminant factors. Positive correlations also existed between FAME factors 1 and 2 and microbial biomass, suggesting that microbial biomass was positively correlated with the PLFAs: $17.719$, $19:0 \omega 11c$, and $i17:1g$.

Cumulative percent mineralization was positively correlated with FAME factor 1 implying that mineralization was higher in soils that contained higher percentages of the fatty acid with an equivalent chain length of $17.719$. Positive correlations also existed between FAME factor 2 and bioavailable Cr and Cu. It is possible that the presence of

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**Figure 8.** Plot of FAME component 1 versus component 2. Symbols represent plant species: m, mix; a, alfalfa; u, unplanted.
the fatty acids represented by FAME PC 2 were present in microbial species tolerant of the soil concentrations of these heavy metals.

Discussion

For phytoremediation to be effective at treating a contaminated field site, it is essential that plant roots be able to penetrate deeply enough to encounter the contaminants. Plant species selected for mixed contaminant sites should also be able to germinate on the contaminated soil, maintain sufficient biomass to influence the degradative ability of soil microorganisms, and be able to take metals up into their biomass.

Plant roots of each species grown in this study reached below 16" (the depth at which the “black layer” begins). A higher percentage of alfalfa roots was found at this depth than either fescue or rye, but the alfalfa root system had lower biomass than either rye or fescue. The low biomass of the alfalfa may have been due to greater sensitive to the soil contaminants as exhibited by the low germination and survival rates of the legume mixture. All plant species had reduced germination, survival, and biomass in the lower layer possibly due to higher concentrations of petroleum hydrocarbons which are known to decrease germination rates (Banks, 2000). Deep tilling to incorporate the two layers may effectively increase remediation by decreasing the toxicity of the lower layer by diluting the PAH concentration, while at the same time adding nutrients which are present in higher amounts in the upper layer.

Greater total biomass below 16" and the high germination, survival, and biomass of rye per pot suggests that planting rye on the site would result in greater coverage than
either the legume mix or fescue and thus exert a greater influence on soil microbial populations and subsequent PAH degradation. Rye grass would also probably remediate heavy metals from the site more quickly than the other plant species as higher concentrations were found in rye biomass.

Plant removal of heavy metals from the soil may have been limited by low bioavailability. Only the concentration of Zn was significantly reduced in planted versus unplanted pots and the amount of bioavailable Zn was lower than the other metals after planting possibly due to plant extraction. The bioavailable fraction of each metal in the pots that had been planted with the mixture was less than the pots planted with rye and those that were unplanted possibly due to uptake of metals from the bioavailable fraction into the plant biomass.

Unlike the metals, there was a large decrease in the total amount of PAHs remaining in the soil after the planting study. There was no difference between plant species or between planted and unplanted pots, suggesting that the conditions of the study (i.e. small pots, frequent watering, ambient temperature) promoted microbial degradation, or abiotic processes such as volatilization to reduced the amount of PAHs in both the total and bioavailable pools.

Although there were no differences in concentration of soil contaminants between plant species, there were differences in microbial activity in planted versus unplanted pots. Previously planted soil had a faster mineralization rate than unplanted soil, suggesting that the presence of plants influenced the microbial community in such a way that added pyrene was more rapidly mineralized than in unplanted soil.
Soil microbial biomass was approximately equal for alfalfa and unplanted pots, both of which had significantly more microbial biomass than rye or the planting mix. The lower microbial biomass in the mix and rye soils may be due to competition between the plants and the soil microorganisms for nutrients. Because alfalfa is a legume and can form symbiotic relationships with soil microorganisms, it utilizes nitrogen that is unavailable to other types of plants, thus possibly reducing nutrient stress and freeing nutrients for plant or microbial use. Because the planting mix was composed of 15% rye, 65% fescue, and only 20% legumes, it would be expected to influence the soil in much the same way as the rye grass.

Soil from unplanted and alfalfa pots were similar in microbial community composition, but both differed from the planting mix. The differences between the mix and the unplanted and alfalfa suggest that the mix may have more of an influence on the microbial community than alfalfa, possibly due to the different exudate compositions of the three plant species represented in the mix which may provide a greater variety of carbon sources for microorganisms. The PLFA profile from soils planted with the mixture had significantly more gram-negative bacterial markers than either the alfalfa or the unplanted pots, and lower relative percentages of gram-positive bacterial markers. Gram-negative bacteria have rapid growth rates and responses to root exudates, and an increase in gram-negative bacteria has been reported in soils containing plant roots (Grayston, 1998; Steer, 2000). The gram-negative bacteria present may have facilitated degradation of the soil PAHs as several gram-negative bacteria that can use PAHs as energy sources have been identified.
Microbial community structure, biomass, and activity were influenced by the species of plant, and plant biomass, rooting depth and germination were shown to vary by plant species. Although the concentration of contaminants in this study was not shown to be influenced by the plant species, it is evident that soil microbial conditions were. While careful selection of plant species may be important, the results of this study suggest that for this particular site, land management strategies for remediation of aged PAHs may be effective with or without the presence of plants. The likelihood of remediation of the aged heavy metals, however, is slim due to low bioavailability and plants were unable to significantly influence the heavy metal concentration after four months of growth. Phytoextraction of heavy metals may be an option if accompanied by other remediation strategies that increase metal bioavailability.
References


