MICROBIAL DEGRADATION OF PETROLEUM HYDROCARBONS

ASSESSING IN SITU DEGRADATION OF PETROLEUM HYDROCARBONS BY INDIGENOUS MICROBIAL COMMUNITIES

By

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ABSTRACT

Biodegradation of petroleum hydrocarbons by microorganisms is one of the most effective methods used to remediate environmental systems. However, much of what is known is based on the ability of (mostly bacterial) species to degrade hydrocarbons under enrichment conditions in a laboratory setting. In order to refine biodegradation as a remediation method, there is a critical need to understand the dynamics and mechanisms of microbial communities under *in situ* conditions. The goal of this dissertation was to provide insight and knowledge into the function of microbial communities in petroleum-contaminated environments using a combination of DNA, lipid and isotopic analyses. Microbial biomass, community structure, carbon sources were assessed at two study sites: (1) a former industrial facility contaminated by PAHs and (2) coastal salt marshes impacted by the *Deepwater Horizon* oil spill.

Isotopic analyses of soils collected from the PAH-contaminated site revealed that microbial carbon sources were derived from vegetation and/or natural organic matter present in soils matter rather than PAHs. Similarly, microbial community structure remained consistent across samples and there were no observed shifts in phylotype diversity with increasing levels of PAHs. Bioaccessibility assays revealed that a large fraction of soil-borne PAHs at the site are not bioavailable to microorganisms; thus, highlighting the importance of environmental factors to *in situ* biodegradation.

Biodegradation of *Deepwater Horizon* spilled oil was detected in salt marsh sediments such that petroleum-derived carbon was a primary carbon source for indigenous microbial communities in the months following the spill. Likewise,

pyrosequencing of all three microbial domains showed an increase in the relative of abundance of taxonomic groups known to include hydrocarbon-degrading species, such as *Sphingomonadales*. These results suggest that Gulf of Mexico marsh sediments have considerable biodegradation potential and that natural attenuation may be feasible remediation strategy in this region.

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PREFACE

This dissertation is a comprised of four peer-reviewed research papers which are presently published (Chapters 2 to 4) or under review (Chapter 5). Chapter 1 describes the connections between these papers as well as the objectives for this dissertation and Chapter 6 summarizes the major findings and includes recommendations for future research. The first author on these research papers (dissertation author) carried out literature reviews, laboratory analyses, data interpretation, writing and revisions of these papers. Dr. Greg Slater was closely involved with this dissertation by providing guidance and direction on the research objectives, discussion of the results and detailed editorial comments during preparation of manuscripts. Dr. Roberta Fulthorpe co-supervised this dissertation by providing significant input and direction on experimental design, research methods and interpretation of results. In addition, Dr. Fulthrope provided lab facilities for all of the DNA analyses (Chapter 2, 3 and 5).

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concentrations. Co-author T.M. Porter assisted with statistical and bioinformatic analyses (Chapter 5). The individual research papers presented herein include:

Chapter 2: Mahmoudi N., Slater, G.F., Fulthorpe, R.R. (2011) Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soils. *Canadian Journal of Microbiology*, 57(8):623-8.

Chapter 3: Mahmoudi, N., Fulthorpe, R.R., Burns, L., Mancini, S., Slater, G.F. (2013) Assessing microbial carbon sources and potential PAH degradation using natural abundance radiocarbon analysis. *Environmental Pollution*, 175: 125-130.

Chapter 4: Mahmoudi, N., Slater, G.F., Juhasz, A.L. (2013) Assessing limitations for PAH biodegradation in long-term contaminated soils using bioavailability assays. *Water, Air, & Soil Pollution*, 224: 1411.

Chapter 5: Mahmoudi N., Porter, T.M., Zimmerman A.R., Fulthorpe, R.R., Kasozi, G.N., Silliman, B.R., Slater, G.F. (2013) Rapid degradation of Deepwater Horizon spilled oil by indigenous microbial communities in Louisiana salt marsh sediments. Submitted to *Environmental Science & Technology*, April 2013.

"The role of the infinitely small in nature is infinitely great."

- Louis Pasteur

CHAPTER 1

INTRODUCTION

Throughout the last century, significant amounts of natural and synthesized chemical compounds have been released into the environment as a result of the demand for agricultural, industrial and therapeutic products. Reports estimate approximately 60,000 chemicals in current commercial production and 800 to 1000 chemicals are being added to production each year (Cairns, 1980; Mackay et al. 1997, 2006). The large-scale impact of chemical usage and disposal has become recognized over the last few decades (Hemond and Fechner-Levy, 2000). For example, many chemical compounds have been found to have adverse ecological and human health effects (Carson, 1962; Thakker et al., 1985; Harvey, 1996; Lamb and Hentz, 2006). It is estimated that these chemical compounds, or environmental contaminants, are placing at least 125 million people at risk of death or disease; the majority of these people residing in low to middle income countries which do not have the resources to remediate polluted sites (Yanez et al., 2002; Prüss-Ustün et al., 2011; Blacksmith Institute and Greencross, 2012). One primary group of environmental contaminants of considerable concern is hydrocarbons due to the widespread use of petroleum and petroleum-derived products. Hydrocarbon contamination can cause extensive and even permanent damage to local ecosystems due to accumulation in plants and animals which may result in mutations or death (Speight and Arjoon, 2012). Although most chemicals such as petroleum have enabled great

technological advances, it is clear that even 'contained' applications of petroleum will result in a certain level of discharge into the environment (Schwarzenbach et al., 1993). Thus, developing remediation approaches that are efficient, economical and that can be rapidly applied to a wide range of environmental systems is important.

Biodegradation of contaminants to non-toxic end products by microorganisms, referred to as bioremediation, is one of the most effective methods for remediating environmental systems in both engineered and *in situ* remediation schemes. This approach is often more cost-effective and environmentally-friendly than traditional detoxifying methods such as excavation or incineration (Singh and Ward, 2004). This dissertation seeks to further our knowledge and understanding of biodegradation in petroleum-contaminated environments. The following sections discuss the biodegradation potential of indigenous microbial communities, environmental factors that can affect biodegradation, as well as challenges in assessing and characterizing microbial communities and degradative processes in the natural environment.

1.1 Petroleum Hydrocarbons

Petroleum (or crude oil) is a naturally occurring mixture of hydrocarbons which is produced by the thermal decay of buried organic material over millions of years. The chemical composition of petroleum is highly complex and can include hundreds to thousands of distinct compounds (Marshall and Rogers, 2004). Once extracted from the subsurface, petroleum is transported to refineries where it undergoes distillation to produce various products such as gasoline, asphalt, kerosene and lubricants that are

widely used for industry and daily life (Speight, 2007). Leaks, improper disposal and accidental spills can occur often during exploration, production, storage and transport of petroleum and petroleum-derived products. Many constituents of petroleum have been found to be highly toxic due to their carcinogenic, teratogenic and mutagenic properties (Guerin et al., 1981; Thakker et al., 1985; Leighton, 1993; Harvey, 1996; Xue and Warshawsky, 2005). Consequently, contamination of water and soil by hydrocarbons has become a major environmental problem. Depending on the contamination source, petroleum hydrocarbons can be present as either individual compounds, classes of compounds (e.g. alkanes or aromatics) or a mixture of a wide array of compounds referred to as unresolved complex mixture (UCM). UCM describes a baseline hump that is often observed in gas chromatograms of petroleum due to the presence of thousands of different compounds which are structurally similar and cannot be resolved by traditional gas chromatography (Frysinger et al., 2003) (Figure 1.1). It is thought that UCM is the result of microbial degradation of petroleum in reservoirs and at the surface which leads to a relative increase in aromatic and polar compounds and loss of lighter compounds (Head et al., 2003). UCM is considered to be an indicator of petroleum contamination and can persist in the environment for decades (Reddy et al., 2002; Peacock et al., 2007). It is not uncommon for coastal sediments to contain large quantities of UCM due to oil spills, urban runoff and waste treatment plants (Wakeham et al., 1981, O'Connor et al., 2000; White et al., 2012). For example, the 2010 *Deepwater Horizon* oil spill released approximately 5 million barrels of crude oil in the Gulf of Mexico waters (Crone et al., 2010; Atlas and Hazen, 2011). The majority of compounds in UCM are considered non-

toxic; however, some studies have shown that some compounds have detrimental effects to benthic organisms (Smith et al., 2001; Rowland et al., 2001, Donkin et al., 2003; Crowe et al., 2004; Booth et al., 2007, 2008).

Accidental spills of crude oil or improper disposal of petroleum waste generated from refineries can also release significant amounts of alkanes into the environment. Alkanes are saturated hydrocarbons that make up to 60% w/w of petroleum oil (Speight and Arjoon, 2012) and can be found in linear, branched or cyclic arrangements. The linear alkanes are the most abundant and are classified as either short-chain (C_1 to C_9) or long-chain (C_{10} or higher). Short-chain alkanes can rapidly enter the vapour phase and are considered more toxic than long-chain alkanes due to their ability to penetrate into the cell membrane and dissolve within the cell (Sikkema et al., 1995).

Polycyclic aromatic hydrocarbons (PAHs) are well known components of petroleum and are ubiquitous in the environment. PAHs are generally classified as either petrogenic or pyrogenic depending on their origin (Douben et al., 2003). Petrogenic PAHs are found naturally in petroleum and can enter the environment through accidental oil spills or natural oil seeps. Pyrogenic PAHs are formed during the incomplete combustion of organic matter such as such as diesel, coal and wood and primarily enter the environment via atmospheric deposition. Due to their hydrophobic structure, PAHs have low water solubility and are readily absorbed onto sediments where they can persist for many years until they are degraded (Neely et al., 1974; Southworth et al., 1978; Wild et al., 1991; Jones and De Voogt, 1999).

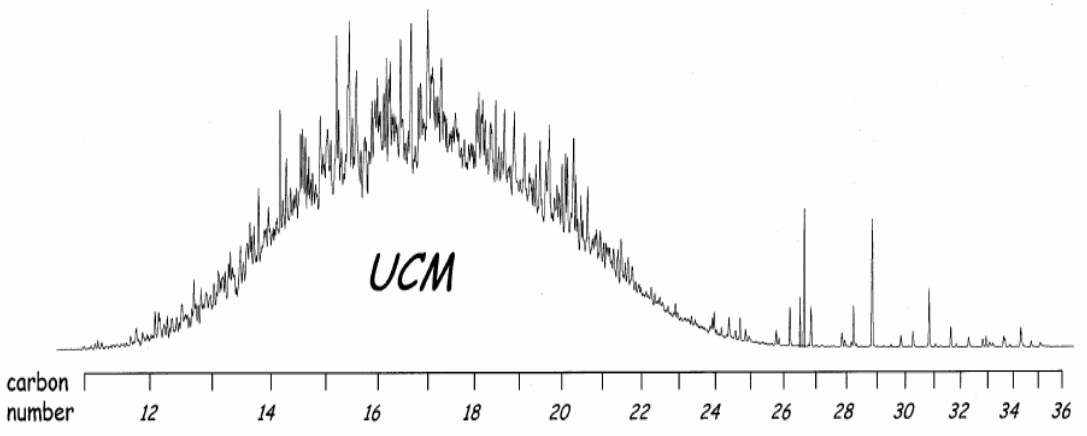

Figure 1.1 Gas chromatogram of UCM hump extracted petroleum-contaminated marsh sediments. Adapted from Reddy et al., (2002); Frysinger et al., (2003).

1.2 Biodegradation of Petroleum Hydrocarbons

Biodegradation is a natural process by which petroleum hydrocarbons are transformed into less harmful compounds through the metabolic or enzymatic activity of microorganisms. Petroleum hydrocarbons may be mineralized to carbon dioxide, water and cellular biomass or degraded to smaller products that can undergo successive degradations until the compound is fully mineralized (Kissin, 1987; Mango, 1997). Biodegradation of hydrocarbons is typically 'energetically favourable', meaning that microorganisms gain energy during degradation that can be utilized for cellular functions (Speight and Arjoon, 2012). In addition to serving as an energy source, microorganisms can also gain carbon, an essential component of all cellular constituents. Some microorganisms possess the ability to degrade alkanes, others aromatics, and others both (Atlas, 1981; Atlas and Bartha, 1992; Whyte et al., 1997; Obayori et al., 2009; Das and Chandran, 2010). The metabolic capability (i.e. genes and enzymes) necessary for utilizing petroleum hydrocarbons as a carbon and energy source is widespread among bacteria and fungi (Peng et al., 2008). As a result, employing the genetic potential of the indigenous microbial community is a favourable approach to remediation of petroleum-impacted sites. The ability to degrade hydrocarbons is thought to arise from the utilization of naturally occurring hydrocarbons produced by plants, algae and other organisms as well as those released from natural oil seeps (Atlas, 1995; MacDonald 1993, 1996; Orcutt, 2010). Based on their chemical properties, the general order-of-preference for biodegradation are alkanes, which are the most readily degraded, followed by

branched alkanes, monoaromatic hydrocarbons, cyclic alkanes, and polynuclear aromatic hydrocarbons (Van Hamme et al., 2003).

1.2.1 Biodegradation as a 'microbial community process'

Microorganisms with the metabolic capability to degrade hydrocarbons have been well studied and to date there are over 500 microbial species that have been recognized to be capable of degrading hydrocarbons (Head et al., 2006; Yakimov et al., 2007). However, much of what is known is based on the ability of bacterial species to degrade hydrocarbons in a laboratory setting where they are isolated from other species. It is thought that organic contaminants in the environment are often degraded in a step-wise process such that some microbial community members degrade one or more compounds to smaller substrates which are then utilized by other members. Consequently, a consortia of species that are highly interdependent develops and biodegradation is carried out by a number of microbial groups working in cooperation with each other (Haack and Bekins, 2000; Eylers et al., 2004). The widespread co-existence of bacteria, eukarya and archaea in the natural environment suggest that physical interactions and catabolic cooperation between these groups may play an important role in biodegradation. Laboratory co-cultures of bacterial and fungal species have shown improved degradation rates of diesel oil and many PAHs (Boonchan et al., 2000; Chavez-Gomez et al., 2003; Kim and Lee, 2007; Li et al., 2008; Wang et al., 2012). These findings emphasize the importance of catabolic cooperation between these groups during biodegradation. Therefore, it is crucial to assess biodegradation in the context of a multi-domain

community in order to fully understand the metabolic potential of the indigenous microbial community.

Degradation of petroleum hydrocarbons by bacterial species has been well-documented and the metabolic pathways have been elucidated (Atlas, 1984; Cerniglia, 1984, 1993; Menn et al., 1993; Kiyohara et al., 1994; Marin et al., 2001; Tropel et al., 2004; Wentzel et al., 2007) (Figure 1.2). Bacterial degradation of hydrocarbons occurs in an assimilatory manner such that bacteria gain energy as well as carbon from the breakdown of hydrocarbons. Aerobic biodegradation of aliphatic and aromatic compounds is initiated by a monooxygenase or dioxygenase system that incorporates both atoms of molecular oxygen into the aromatic rings producing intermediates that are cleaved and further converted to tricarboxylic acid cycle (TCA) intermediates such as pyruvate, acetate, succinate and aldehydes (Bamforth and Singleton, 2005). Likewise, n-alkanes are degraded in a similar fashion through a series of oxidation reactions catalyzed by oxygenases which convert alkanes into fatty acids which are then converted to acetyl-coenzyme A's (CoAs) by a process known as β-oxidation (Rojo, 2009). Oxygen can quickly become depleted in petroleum-contaminated environments due to the activity of aerobic hydrocarbon-degrading bacteria which can lead to anaerobic conditions. Anaerobic degradation of hydrocarbons is a much slower process and less is known about the genes and enzymes involved in these pathways (Heider et al., 1998). Degradation of hydrocarbons in the absence of oxygen has been shown to occur in the presence of other electron acceptors such as Fe^{3+}, SO_4^{2-} and NO_3^- (Foght, 2008). Subsequently, a succession of different microbial populations driven by the availability of terminal

electron acceptors can arise (Wang and Stout, 2007). This has been well demonstrated in BTEX-contaminated aquifers (Bekins et al., 1999; Haack and Bekins, 2000; Anderson and Lovley, 2000; Chapelle et al., 2002); however, it is less likely to occur in surface soils and sediments where oxygen can be quickly replenished.

The majority of biodegradation studies have focused on the degradation potential of bacteria although archaea and eukarya are commonly found in contaminated environments. Archaea were previously thought to only be present in extreme environments; however, molecular approaches have demonstrated that archaea are found in almost all environments (DeLong, 1998), including petroleum-contaminated aquifers and soils (Gieg et al., 1999; Röling et al., 2004; Kleikemper et al., 2005; Dojka et al., 1998; Liu et al., 2009; Wang et al., 2011). Furthermore, studies carried out in enrichment cultures and amended aquifers have observed decreases in BTEX compounds and PAHs under methanogenic conditions (Weiner and Lovley, 1998; Ficker et al., 1999; Reinhard et al., 2005; Chang et al., 2006). Methanogens cannot use long-chain fatty acids or aromatic compounds and are restricted to only a few substrates such as CO_2, formate, methanol and acetate, all of which are converted to methane as the main product (Schink, 2002). As a result, methanogens depend on the activity of other microorganisms to break down complex organic compounds into more simple substrates. Studies in marine environments have found that archaeal community composition between petroleum-contaminated and clean control samples was similar even when the samples contained different bacterial communities, which suggests that archaea may not be playing a primary role in biodegradation in marine environments (Röling et al., 2004; Redmond and

Valentine, 2012). Little is known about the function and ecology of archaea in the natural environment which makes it is difficult to discern their role in hydrocarbon degradation.

Fungi, another key microbial group, are eukaryotic microorganisms that comprise a large portion of the microbial biomass in soils and sediments. The ability of fungi to rapidly adapt their metabolism to varying carbon sources allows them degrade a wide array of environmental pollutants including PAHs, chlorinated solvents, polychlorinated biphenyl (PCBs) and pesticides (Roberts, 1998). There are two different known mechanisms that fungi utilize for degradation. The first mechanism is the nonspecific, extracellular enzymes lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases which can degrade lignin as well as a broad spectrum of environmental contaminants (Clemente et al., 2001; Tortella et al., 2005). The breakdown of contaminants by these enzymes whose normal function is to breakdown lignin is thought to be initiated by nutrient limitations (Hammel et al., 1992; Reddy, 1995). The second degradation mechanism utilized by fungi is the intracellular cytochrome P450 system which carries out a wide range of biocatalytic conversions (Van den Brink et al., 1998). It is hypothesized that fungi have greater degradation potential than other species because they can reach contaminants immobilized in micropores using their multicellular mycelium (Bennet et al., 1997, 2002; Cerniglia, 1997).

Numerous studies have demonstrated an array of fungi species that have the ability to oxidize petroleum hydrocarbons including high molecular weight (MW) PAHs which are thought to be resistant to biodegradation (Cerniglia and Gibson, 1979; Hammel et al, 1992; Yadav and Reddy, 1993; Weber et al., 1995; Bogan et al., 1996; Prenafeta-

Boldu et al., 2001; Da Silva et al., 2004). Most fungi do not utilize high MW PAHs as sole sources of carbon and energy but instead co-metabolize the compound to hydroxylated products (Cerniglia, 1997). The water soluble products can then be excreted out of the cell into the environment for subsequent attack by another organism (Keck et al., 1989). It has been suggested that in PAH-contaminated soils bacteria live on PAH-metabolites produced by fungi instead of parent PAHs since the metabolites are water soluble and therefore more bioavailable (Johnsen et al. 2005). However, a recent study demonstrated that the fungal PAH-metabolites were much more resistant to degradation by soil microorganisms compared to the parent PAHs (Schmidt et al., 2010). Aside from laboratory and bioaugmentation studies, little is known about the actual contribution of fungi to the degradation of petroleum hydrocarbons in natural environment.

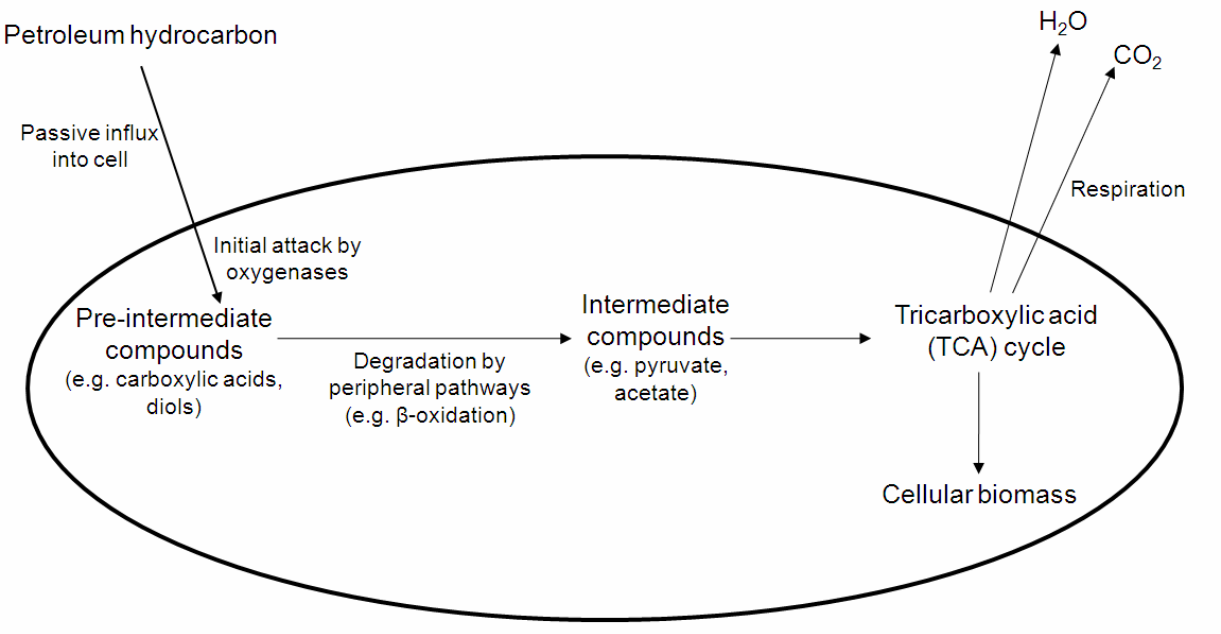

Figure 1.2 Overview of aerobic hydrocarbon degradation pathways in microorganisms. Adapted from Das and Chandran, (2011).

1.2.2 Environmental factors affecting biodegradation

Microbial communities are highly sensitive to their environment and can rapidly respond to changes in their surrounding conditions including available sources of carbon and energy. In pristine environments, it is estimated that hydrocarbon-degrading microorganisms comprise <1% of the total microbial population (Atlas, 1981). A large input of petroleum hydrocarbons will increase the proportion of hydrocarbon-degrading microorganisms and lead to a shift in the overall structure of the microbial community. Previous studies have observed an enrichment of hydrocarbon-degrading microorganisms and a decrease in microbial diversity in a number of petroleum-contaminated environments (Juck et al., 2000; Röling et al., 2002; Brakstad and Lodeng, 2005; Yakimov et al., 2005; Hazen et al., 2010; Kostka et al. 2011; Beazley et al., 2012, Bik et al. 2012; Redmond and Valentine, 2012). Despite this knowledge, our ability to understand and predict biodegradation under *in situ* conditions is limited. Microbial degradation in the natural environment is highly variable and involves complex interactions between species and the geochemical properties of the surrounding environment. Environmental factors such as nutrients, temperature, salinity and the presence of oxygen can significantly affect the occurrence and rate of biodegradation (Figure 1.3). For example, temperature can affect the chemistry and solubility of hydrocarbons as well as the physiology and metabolic rate of the microbial community (Foght et al., 1996; Venosa and Zhu, 2003).

Laboratory experiments can overestimate biodegradation potential in the natural environment because biodegradation is a function of the physical and chemical properties

of the compound as well as the surrounding environment (Loehr and Webster, 1996; Posada-Baquero and Ortega-Calvo, 2011; Wild and Jones, 1993). As residence time in soils and sediments increases, a greater proportion of petroleum hydrocarbons diffuse into micropores and partition onto organic matter thereby decreasing their bioavailability to microorganisms and limiting their degradation in the environment. Consequently, the extent of biodegradation observed in field studies is often lower than observed in laboratory studies (Wild et al., 1990, 1991; Huesemann, 1997; Gallego et al., 2011). Furthermore, carbon sources that are more labile than petroleum hydrocarbons are often present in the natural environment and microorganisms may utilize these natural organic matter (NOM) components preferentially over contaminants resulting in the persistence of petroleum hydrocarbons. Such behaviour was observed in salt marsh sediments contaminated by the *Florida* oil spill of 1969 in which the microbial community was utilizing NOM as opposed to petroleum hydrocarbons (Slater et al., 2005).

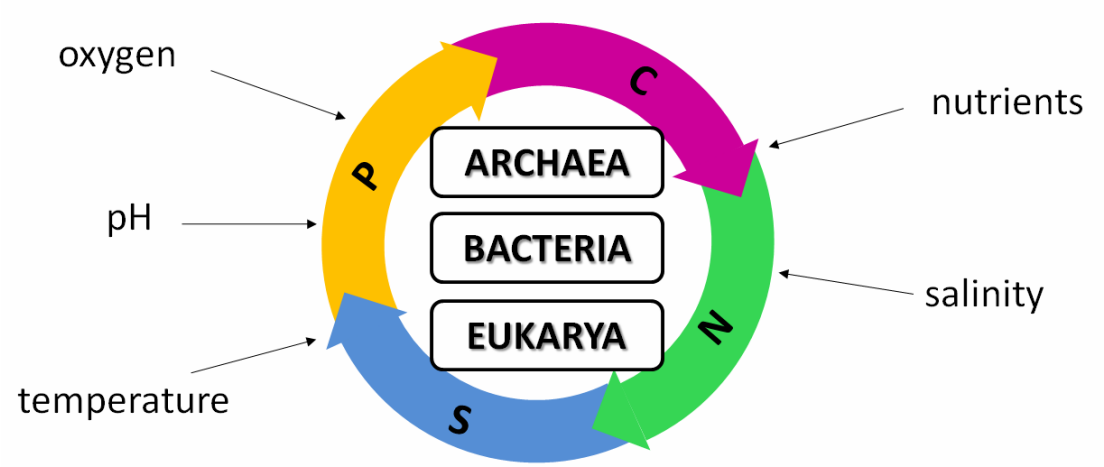

Figure 1.3 Main environmental factors that can affect the occurrence and rate of biodegradation in the environment. *In situ* microbial communities are composed of species from all three domains – bacteria, archaea, eukarya – which can result in competition, synergistic interactions and cycling of nutrients (N- nitrogen; S- sulfur; P- phosphorus) and carbon (C) compounds.

1.3 Assessing Biodegradation in the Natural Environment

While the potential for microorganisms to degrade almost all petroleum hydrocarbons has been widely shown in a laboratory setting, demonstration of biodegradation under *in situ* conditions is more challenging. Traditional culture-based methods can be problematic because they only reveal the presence of a small percentage microorganisms that are culturable and secondly, the presence of microorganisms does not reveal whether they will degrade the contaminant under *in situ* conditions (Torsvik et al., 1990; Pace, 1999). Therefore, utilizing approaches that involve direct extraction of cellular constituents or biomarkers such as nucleic acids and lipids can further our understanding of microbial communities in the natural environment.

1.3.1 Characterizing in situ microbial communities using DNA and lipid analyses

DNA analysis has been able to address previous issues with traditional methods and provide a simple, fast and efficient way to characterize specific microorganisms or community structure (Amann et al. 1995). Characterizing microbial communities and assessing phylogenetic diversity involves sequencing small subunit ribosomal RNA (rRNA) genes (16S in prokaryotes and 18S in eukaryotes) which are present in all organisms and highly conserved due to their role in protein synthesis (Pace et al., 1987; Olsen and Woese, 1993). There is a wide variety of molecular techniques available; however, amplification of 16S rRNA genes or other genes using polymerase chain reaction (PCR) is often the first step (Amann et al., 1995). Subsequently, PCR products can be gel-purified, cloned into a vector and sequenced, or directly sequenced using next-generation sequencing approaches. Recovered sequences can then be compared to public

databases such as Ribosomal Database Project (RDP) or GenBank in order to identify microbial groups within a sample (Cole et al., 2005). The amplified 16S rRNA and 18s rRNA genes can also be analyzed using denaturing gel gradient electrophoresis (DGGE) which separates DNA fragments of the same length but different nucleotide sequence by electrophoresis in a gradient of increasing denaturing strength (Muyzer et al., 1993). This technique allows for the estimation of community diversity, since the number of bands serves as a measure of richness of species and the intensity of the bands can provide a measure of evenness of the species (Muyzer et al., 1993). Moreover, DNA can be extracted and sequenced from individual DGGE bands which allows for identification of dominant species. Compared to next-generation sequencing, PCR-DGGE analysis has low coverage and poor resolution. Bands can often co-migrate and there are also questions of the 'one band one species' principle behind this approach (Vallaeys et al., 1997). Next-generation sequencing approaches have higher coverage and thereby provide more comprehensive information regarding the diversity and composition of the microbial community including low-abundance groups which have been largely unexplored (Hill et al., 2002; Sogin et al., 2006; Roesch et al., 2007). Although next-generation sequencing may be preferred, community fingerprinting using DGGE analysis is an still adequate method when comparing patterns and variations of the abundant phylotypes across samples (Hanning and Ricke, 2011; Chu et al., 2011; Gilbert et al., 2012; Ling et al., 2012; Yang et al., 2012; Vaz-Moreira et al., 2013).

Microbial lipids are relatively sensitive cellular constituents that allow for the study of biomass, community structure, metabolic status and activity of microbial

communities (Vestal and White, 1989). There are several classes of important lipids found within the cell including glycerides, fatty alcohols, phospholipids, waxes, glycol-, sulfo- and peptido-lipids (Lechevalier and Moss, 1977; Ratledge and Wilkinson, 1988). Cellular membrane lipids, specifically phospholipid fatty acids (PLFA) are commonly used to analyze microbial community structure and biomass. PLFA are primary components of cell membranes in bacteria and eukarya where they form a lipid bilayer that provides a semi-permeable barrier to keep molecules from diffusing into and out of the cell. PLFA are rapidly turned over in metabolically active cells and degraded within days to weeks after cell death (White et al., 1979). Thus, the total phospholipid fatty acid (PLFA) composition can provide a viable microbial community profile as well as an estimate of cell density (Guckert et al., 1985). Compared to DNA analysis, microbial lipids are more advantageous for estimating cell density because PCR amplification of DNA can be biased due to non-uniform amplification resulting from differences in the G+C content of species (Spiegelman et al., 2005). Thus, lipids provide a more accurate estimate of microbial biomass because they can be extracted and measured directly via gas chromatography-mass spectrometry (GC-MS) without the need for amplification. Using PLFA to estimate cell density relies on a conversion factor from mass of PLFA to mass of microorganisms (Green and Scow, 2000). It is important to note that conversion factors may provide inaccurate estimates of microbial cell density because PLFA concentrations vary in response to community makeup and physical conditions such as pH (Haack et al., 1994; Steward et al., 1996; Frostegård and Bååth, 1996). Many individual PLFA can be used as biomarkers for the presence of specific microbial groups

based on the assumption that all members contain that specific lipid and members of other groups do not (Kaneda, 1991; Zelles, 1999; Green and Scow, 2000). Unfortunately, the phylogenetic resolution of PLFAs is fairly low and provides limited taxonomic information; therefore, identification of microbial groups based on DNA analysis is much more appropriate.

1.3.2 Demonstrating in situ biodegradation of petroleum hydrocarbons

While culture-independent methods such as DNA and lipids analyses have provided a wealth of knowledge regarding phylogenetic diversity and structure of microbial communities, these methods do not adequately describe the function of microbial communities in the environment. Demonstrating microbial degradation of petroleum hydrocarbons under *in situ* conditions is a key challenge. Heterogeneous contaminant distributions across a site and slow degradation rates pose significant challenges when biodegradation is based on measurement of concentration changes alone (Bombach et al., 2010). Compound specific stable isotopic analysis (CSIA) can overcome these challenges by measuring the $\delta^{13}C$ signature of contaminants and their potential products. Biodegradation of petroleum hydrocarbons, specifically BTEX compounds, alters the isotopic signatures of these compounds in a recognizable pattern which can provide information regarding the extent of biodegradation in the environment (Dempster et al., 1997). CSIA has been successfully used to monitor *in situ* biodegradation of BTEX compounds in contaminated aquifers (Dempster et al., 1997; Griebler et al., 2004; Meckenstock et al., 2004; Fischer et al., 2009). Unfortunately, this approach is not successful in many environments due to variations in isotopic fractionation by

microorganisms as well as signal dilution as the number of carbon atoms in a molecule increases (Mazeas et al., 2002; Slater 2003; Schmidt et al., 2004; Sun et al., 2004). For example, there is no isotopic fractionation associated with biodegradation of *n*-alkanes and PAHs which limits the use of this approach in petroleum-contaminated sites containing alkanes, aliphatic or aromatic compounds.

Microbial lipid analysis combined with isotopic analysis can link microbial groups with degradation of specific compounds and provides insight into the function of microbial communities in the environment. This approach is based on the assumption that heterotrophic microorganisms will display similar carbon isotopic ratios as their carbon source; thus incorporation of an isotopic label from a substrate into microbial cellular components provides direct evidence for metabolism of that compound (Boschker and Middelburg, 2002; Evershed et al., 2006). Because PLFA are rapidly turned over during metabolism and degraded after cell death, the isotopic composition of microbial PLFA can provide a snapshot of the carbon source of the active microbial population. PLFA analysis can be combined with ^{13}C- or ^{14}C-labeled substrates in order to track of substrate usage and carbon flow during biodegradation of contaminants (Fang et al., 2004; Dumont and Murrell, 2005). This technique is referred to as stable isotope probing (SIP) and has been applied to assess biodegradation of toluene in soil (Hanson et al., 1999) and aquifer samples (Pelz et al., 2001), as well as phenanthrene in soil (Johnsen et al., 2002). However, SIP experiments are often carried out in the laboratory under controlled conditions using microcosms which do not resemble natural conditions. Field-based SIP, which involves adding a labeled tracer into groundwater or sediments, can

overcome this challenge but they can require long incubation times in order to produce a resolvable signal (Padmanabhan et al., 2003). Nevertheless, SIP can be a useful approach for understanding the degradation of a specific contaminant because theoretically any contaminant can be labeled and added to the community of interest.

Variations in the natural abundance of ^{13}C also have the potential to be used to elucidate microbial carbon sources and assess biodegradation of petroleum hydrocarbons (Boschker and Middelburg, 2002). However, this approach is often unsuccessful in terrestrial environments that are dominated by C_3 vegetation due to the overlapping isotopic signatures between crude oils ($\delta^{13}C$ = -26 to -28‰) and C_3-dervied organic matter ($\delta^{13}C$ = -25‰). In addition, there is an the expected fractionation of 4 to 6‰ between heterotrophic PLFA and their carbon source due to lipid synthesis (Hayes, 2001) which further confounds the use of ^{13}C as a tool for resolving microbial carbon sources in C_3-domainted environments (Ahad et al., 2010; Cowie et al., 2010). In contrast, coastal marshes are dominated by grasses, specifically *Spartina sp.*, which have $\delta^{13}C$ values of -12 to -14‰ and sedimentary organic matter in these marshes have $\delta^{13}C$ values ranging -14.4 to -17‰ (Chmura et al., 1987; Natter et al., 2012). This isotopic difference was used to demonstrate that *Spartina*-derived organic matter, rather than petroleum hydrocarbons, was the primary source of carbon for microbial communities in other oil-impacted salt marsh sediments (Slater et al., 2005; Pearson et al., 2008).

Natural abundance compound specific radiocarbon (^{14}C) analysis of microbial lipids has become a useful tool that can confirm biodegradation of petroleum hydrocarbons in a wide variety of environments without the need for culturing or adding a

labeled tracer. This technique is based on the fact that petroleum hydrocarbons are millions of years old and will have no detectable ^{14}C due to radioactive decay ($\Delta^{14}C$= -1000‰). In contrast, natural organic matter will contain components with variable radiocarbon ages including recently photosynthesized material which will have a $\Delta^{14}C$ value of approximately +50‰ (Figure 1.4). Thus, microbial metabolism of hydrocarbons will reduce the ^{14}C content of their membrane lipids relative to the surrounding natural organic matter. Moreover, any isotopic fractionation affects are removed from ^{14}C data during processing (Stuiver and Pollack, 1977); as a result, ^{14}C can be used as a direct tracer of microbial carbon sources (Slater et al., 2005). This approach has elucidated microbial carbon sources and confirmed *in situ* biodegradation of petroleum hydrocarbons in a number of complex environmental systems including soils, marsh sediments and shallow groundwater systems (Ahad et al., 2010, Cowie et al., 2010; Slater et al., 2005, 2006; Wakeham et al., 2006; Mahmoudi et al., 2013).

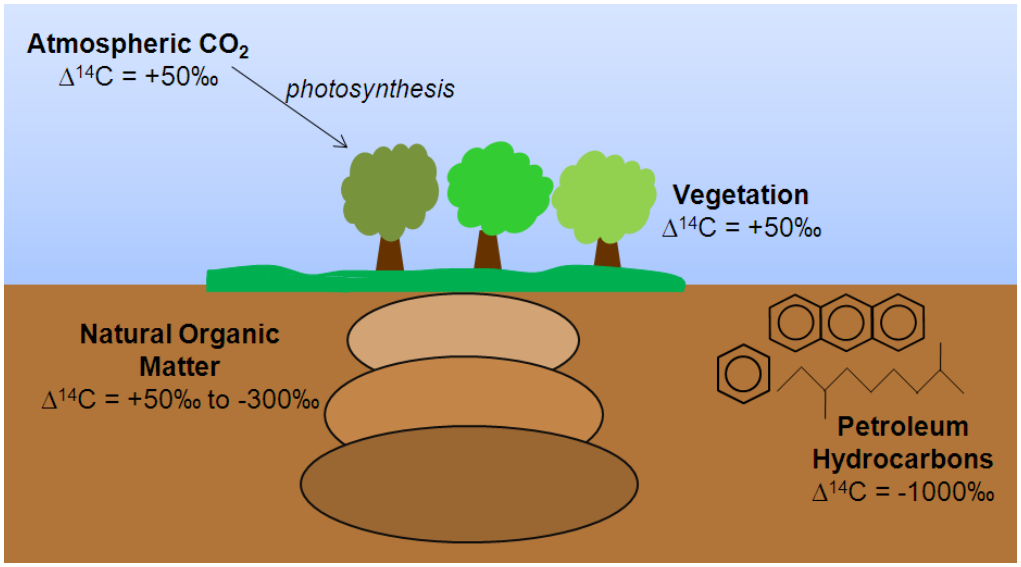

Figure 1.4 $\Delta^{14}C$ signatures of potential microbial carbon sources in terrestrial environments. In soils, natural organic matter is comprised of multiple carbon pools with variable radiocarbon ages including younger, labile compounds derived from recently photosynthesized material as well as older, recalcitrant compounds (Trumbore, 2000). The radiocarbon ages for natural organic matter differ due to the rate of organic matter cycling, resulting in considerable variability across sites.

1.4 Research Objectives

In order to refine our understanding of biodegradation as a remediation method, there is a critical need to assess the dynamics and mechanisms of microbial communities under *in situ* conditions. The objective of this doctoral dissertation was to integrate genetic and geochemical approaches in order to provide new insight and knowledge into the function of microbial communities in petroleum-contaminated environments. Within this overall objective, specific goals of this research were to assess the response of all three microbial domains to petroleum contamination and to focus on PAHs which can be strongly affected by their inherent chemical properties as well as environmental factors. In order to address these goals, this dissertation focused on two study sites: (1) a former industrial facility in southern Ontario contaminated with PAHs for many decades and (2) salt marshes in Louisiana recently impacted by the *Deepwater Horizon* oil spill. A combination of DNA, lipid and isotopic approaches were applied at each site to assess microbial biomass and community structure as well as microbial carbon sources and cycling (Figure 1.5). In the case of the former industrial facility, PAH bioavailability was also assessed since bioavailability is considered to be a major limitation in remediating PAH-contaminated soils and sediments.

Molecular characterization of the indigenous microbial community in petroleum-contaminated soils and sediments is often a first step in assessing the potential for intrinsic biodegradation. Extraction and purification of high quality, purified DNA is an essential requirement for successful PCR amplifications that underlie most subsequent procedures such as DGGE and next-generation sequencing. Petroleum hydrocarbons such

as PAHs have the potential to interfere with DNA quantification and amplification. Chapter 2 evaluated four different commercial DNA isolation kits to determine which kit is most efficient at extracting high quality bacterial and eukaryotic DNA from PAH-contaminated soils. The results of Chapter 2 revealed that PowerSoil DNA Isolation kit was the most effective and reliable kit for PAH contaminated soils because it provided the highest quality DNA that was consistently amplifiable using both eukaryotic and prokaryotic primers. Furthermore, it was found that commercial kits differ with respect to their degree of cell lysis, and so, observed phylogenetic diversity depends greatly on the extraction kit being used. These results highlight biases that can be introduced during DNA extraction and emphasize the importance of selecting an appropriate DNA extraction kit.

Chapter 3 assessed microbial carbon sources and the occurrence biodegradation in soils collected from a former industrial site contaminated with PAHs. Natural abundance ^{14}C analysis of microbial PLFA revealed that microbial carbon sources were derived primarily from vegetation and other types of natural organic matter rather than PAHs. DNA fingerprints of the bacterial, archaeal and eukaryotic communities were generated using PCR-DGGE to assess changes in microbial community structure across soil samples. However they were not included in the final version of this published manuscript and are included here in Appendix A. Microbial structure remained consistent across soil samples and there were no observed shifts in phylotype diversity with increasing levels of PAHs. The results of Chapter 3 indicate that there is minimal or

negligible PAH biodegradation at this site emphasizing the importance of *in situ* conditions to microbial degradation.

Chapter 4 addressed questions raised in Chapter 3 regarding the lack of observed PAH biodegradation in contaminated soils. Specifically, Chapter 4 investigates the bioavailability of PAHs at this site using a number of bioaccessibility assays designed to estimate the fraction of PAHs available for biological uptake. The results of these assays indicate that a large fraction of the soil-borne PAHs at the site are not bioavailable to microorganisms and that these bioavailability limitations may be a primary cause for the lack of observed biodegradation at this site. This chapter validates the importance of environmental factors, particularly bioavailability, to PAH biodegradation in the natural environment.

Chapter 5 synthesizes approaches described in previous chapters such that DNA, lipid and isotopic analyses are integrated to assess microbial degradation of *Deepwater Horizon* spilled oil in Louisiana salt marshes over the first 18 months of the spill. Microbial carbon sources and biodegradation were assessed using both natural abundance ^{13}C and ^{14}C analysis of microbial PLFA in order to confirm whether biodegradation was a contributing factor to the observed petroleum mass loss in impacted marsh sediments. Concurrently, the response of all three microbial domains to oil contamination was assessed via pyrosequencing to correlate biodegradation with changes in microbial community structure. The results of Chapter 5 reveal rapid degradation of petroleum by the indigenous microbial community such that petroleum-derived carbon was the primary carbon source for microbial communities 5 months after oil intrusion. Also at this time

point, pyrosequencing of all three microbial domains show an increase in the relative of abundance of taxonomic groups known to include hydrocarbon-degrading bacteria, such as *Sphingomonadales*. Chapter 5 successfully correlates temporal changes in microbial carbon sources with shifts in the community structure and highlights the considerable biodegradation potential of indigenous microbial communities within coastal ecosystems in the Gulf of Mexico.

Chapter 6 summarizes the significant findings and conclusions that arose from this body of work and includes recommendations for future work.

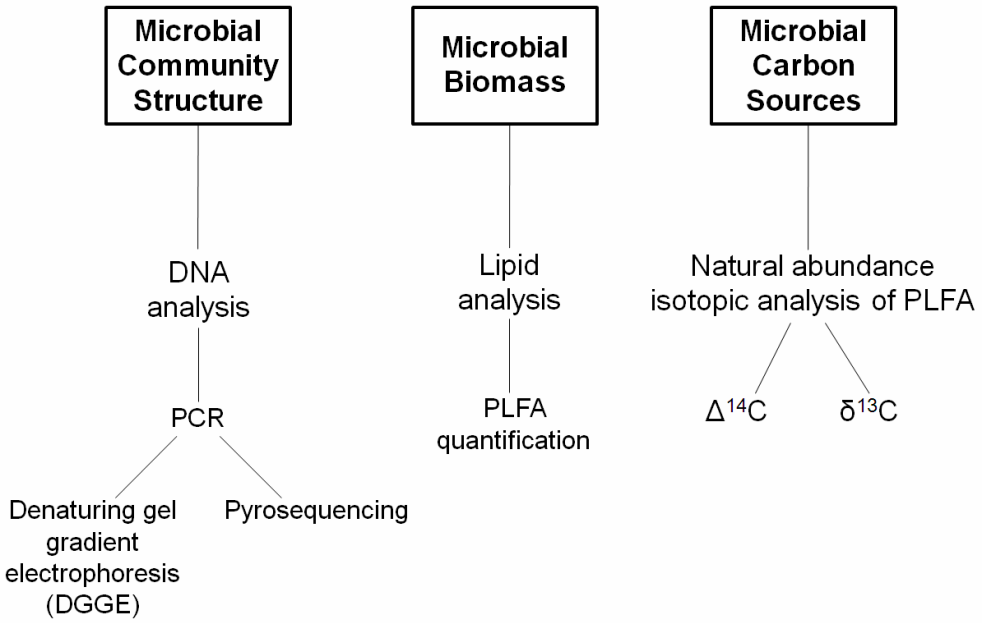

Figure 1.5 Main approaches applied to each study site in order to assess *in situ* biodegradation of petroleum hydrocarbons.

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CHAPTER 2

COMPARISON OF COMMERCIAL DNA EXTRACTION KITS FOR ISOLATION AND PURIFICATION OF BACTERIAL AND EUKARYOTIC DNA FROM PAH-CONTAMINATED SOILS

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Abstract

Molecular characterization of the microbial populations of soils and sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) is often a first step in assessing intrinsic biodegradation potential. However, soils are problematic for molecular analysis owing to the presence of organic matter, such as humic acids. Furthermore, the presence of contaminants, such as PAHs, can cause further challenges to DNA extraction, quantification, and amplification. The goal of our study was to compare the effectiveness of four commercial soil DNA extraction kits (UltraClean Soil DNA Isolation kit, PowerSoil DNA Isolation kit, PowerMax Soil DNA Isolation kit, and FastDNA SPIN kit) to extract pure, high-quality bacterial and eukaryotic DNA from PAH-contaminated soils. Six different contaminated soils were used to determine if there were any biases among the kits due to soil properties or level of contamination. Extracted DNA was used as a template for bacterial 16S rDNA and eukaryotic 18S rDNA amplifications, and PCR products were subsequently analyzed using denaturing gel gradient electrophoresis (DGGE). We found that the FastDNA SPIN kit provided significantly higher DNA yields for all soils; however, it also resulted in the highest levels of humic acid contamination. Soil texture and organic carbon content of the soil did not affect the DNA yield of any kit. Moreover, a liquid-liquid extraction of the DNA extracts found no residual PAHs, indicating that all kits were effective at removing contaminants in the extraction process. Although the PowerSoil DNA Isolation kit gave relatively low DNA yields, it provided the highest quality DNA based on successful amplification of both bacterial and eukaryotic DNA for all six soils. DGGE fingerprints among the kits were dramatically

different for both bacterial and eukaryotic DNA. The PowerSoil DNA Isolation kit revealed multiple bands for each soil and provided the most consistent DGGE profiles among replicates for both bacterial and eukaryotic DNA.

2.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment through petroleum refining and transport, the use of coal tar and creosote, and the incomplete combustion of organic matter (Cerniglia 1984; Freeman and Cattell 1990; Lijinsky 1991; Lim et al. 1999). PAHs have gained widespread attention because some of them are carcinogenic, teratogenic, and mutagenic (Harvey 1996; Xue and Warshawsky 2005). Due to their hydrophobic structure, PAHs have low water solubility and are readily absorbed onto soils and sediments where they persist until they are degraded (Cerniglia 1992). Microbial degradation of PAHs has been well documented under in situ and laboratory conditions and is thought to be an important process in remediating contaminated sediments and soils (Cerniglia 1984, 1992; Peng et al. 2008). Molecular characterization of the natural microbial population in PAH-contaminated soils and sediments is often a first step in assessing the potential for intrinsic biodegradation. The amplification of ribosomal RNA genes using domain-specific primers, followed by analysis of the ribotypes present via cloning, fragment analysis, or denaturing gel electrophoresis methods is routine for community characterization. Regardless of downstream applications, high-quality, purified DNA is an essential requirement for the successful DNA amplifications that underlie all subsequent procedures. Over the years,

several different DNA extraction and purification methods have been developed specifically for soils, and a variety of commercial extraction kits are available that provide consistent solutions for the central problems — cell lysis and humic acid removal (Tsai and Olson 1992; Young et al. 1993; Harry et al. 1999; Varanini and Pinton 2001). Commercial DNA extraction kits are widely available and have become favorable because they are often cheaper and faster than traditional extraction methods. Many popular commercial DNA extraction kits lyse microbes in the soil by a combination of heat, detergent, and mechanical force against specialized beads (Roose-Amsaleg et al. 2001). While the effectiveness of these kits has been examined in various soils, the efficiency and ability of various commercial kits to extract high-quality DNA from contaminated soils has yet to be investigated. Furthermore, the observed microbial community structure and diversity has shown to be impacted by the mechanism used to isolate and purify DNA (Krsek and Wellington 1999; Martin-Laurent et al. 2001; Maarit Niemi et al. 2001). This can be a significant issue, especially for contaminated soils where inaccurate community analysis can lead to potentially erroneous estimations regarding the biodegradation capability of the natural microbial population.

The goal of our study was to compare the effectiveness of commercial soil DNA extraction kits to extract pure, high-quality bacterial and eukaryotic DNA from PAH-contaminated soils. The importance of eukaryotic species, especially fungi, in the degradation of PAHs has been demonstrated in recent years. Fungi have greater degradation potential than bacterial species because they can reach PAHs immobilized in micropores because of their multicellular mycelium (Cerniglia 1997; Bennett et al. 2002).

Thus, any kit used to obtain microbial community DNA must also successfully extract eukaryotic DNA to accurately assess the degradation potential of the natural microbial community. Six different contaminated soils were used to determine if there were any biases among the kits due to soil properties or level of contamination. DNA yield was measured after completion, and the extracted DNA was used as a template for bacterial 16S rDNA and eukaryotic 18S rDNA amplification. PCR products were analyzed using denaturing gradient gel electrophoresis (DGGE) to determine the effect of the extraction kit on microbial diversity.

2.2 Materials and methods

2.2.1 Soil samples

Samples were collected from the upper 0.5–0.6 m of surface soil at an industrial site in southern Ontario where soils were contaminated with varying levels of PAHs. Soils were transported to McMaster University upon collection and stored at –20 °C prior to DNA analysis. PAH concentrations were assessed using the EPA method 8270. Grain size was determined using the Beckman Coulter LS 230 laser diffraction particle size analyzer (Brea, California), and soil textures were assessed using the standard textural triangle. Finally, organic carbon content was analyzed using a continuous flow system consisting of a Costech 4010 elemental combustion system (Milan, Italy) with peak intensities being measured using a Delta Plus XP isotope ratio mass spectrometer (Thermo Finnigan, Herts, UK) (Table 2.1).

2.2.2 DNA isolation

The soil nucleic acids were extracted using the UltraClean Soil DNA Isolation kit, PowerSoil DNA Isolation kit, Power-Max Soil DNA Isolation kit (all from MoBio Laboratories Inc., Carlsbad, California), and FastDNA SPIN kit (MP Bio-medicals, Solon, Ohio), according to the manufacturer's protocol. Triplicate DNA extractions of each soil using each kit were completed, and the maximum amount of soil as suggested by the manufacturer was used for all kits. In the case of the PowerMax kit, the eluted DNA was concentrated to 200 µL, according to the manufacturer's protocol. A total of 5 µL of each DNA extract was run on 1% agarose gel with a 1 kb DNA ladder (GeneRuler). Gels were stained with ethidium bromide and photographed with the G-Box gel documentation system (Syngene, Cambridge, UK). However, since many of DNA extracts were below the detection limit (1 ng/µL), the final DNA yield was quantified using a Nano-drop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware), and the purity of DNA was determined by the value of OD_{260}/OD_{280} and the value of OD_{260}/OD_{230}.

2.2.3 PCR

Amplification of bacteria 16S rDNA within the V3 region was done using the eubacterial-specific universal primers 341F-GC (5′-CGCCCGCCGCGCGCGGCGG-GCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′, which includes a 40 bp GC clamp on its 5′ end; Invitrogen Canada) and 534R (5′-ATTACCGCGGC-TGCTGG-3′; Invitrogen Canada) (Muyzer et al. 1993). The universal eukaryotic primers forward 1427–1453 (5′-GCCCGCCGCGCCCCGCGCCCGGCCCGCCG-

CCCCCGCCCCTCTGTGATGCCCTTAGATGTTCTGGG-3′, which includes a 40 bp GC clamp on its 5′ end) and reverse 1616–1637 (5′-GCGGTGTGTACA-AAGGGCAGGG-3′) were used to amplify eukaryotic 18S rDNA within the V4 region (van Hannen et al. 1998). PCR reactions were 50 µL in total and contained approximately 50 ng of template DNA, 1 µmol/L (each) forward and reverse primers, and 2.5 U of HotStarTaq DNA polymerase (Qiagen, Valencia, California). PCR cycling was done using a PTC-100 thermal cycler (MJ Research Inc., Waltham, Massachu-setts). Bacterial 16S rDNA fragments were amplified using the following conditions: initial enzyme activation of 95 °C for 5 min; 34 cycles of denaturation at 94 °C for 1 min, denaturation at 55 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. Eukaryotic 18S rDNA fragments were amplified using the following conditions: 95 °C for 5 min; 30 cycles at 94 °C for 30 s, 52 °C for 1 min, and 68 °C for 1 min; followed by a final extension at 68 °C for 10 min. PCR products were run on a 1.2% agarose gel (m/v) stained with ethidium bromide prior to DGGE analysis to confirm successful amplification. PCR amplification was subsequently replicated to assess reliability and consistency of the extracted DNA.

2.2.4 DGGE

Bacterial and eukaryotic PCR amplicons were applied onto 8% polyacrylamide gels with a denaturing gradient of 40%– 70% (bacterial) or 30%–55% (eukaryotic) (100% denaturant contains 7 mol/L urea and 40% (v/v) formamide). Ten microlitres of a sample was mixed with 5 µL of loading dye and loaded onto wells. Electrophoresis was performed in 0.5× Tris–acetate–EDTA buffer at 70 V at 60 °C for 16 h using a DGGE-

2401 apparatus (C.B.S. Scientific, DelMar, California). Gels were stained with ethidium bromide and visualized with the G-Box gel documentation system (Syngene, Cambridge, UK). The DGGE profiles were normalized and compared using GelCompar II version 6.5 (Applied Maths, Belgium).

2.2.5 Presence and effect of PAHs on extracted DNA

To determine if there were any residual PAHs in the extracted DNA using the commercial kits, a liquid–liquid extraction was performed on the remaining DNA extracts after amplification. Nanopure water was added to the DNA extract to bring the volume to 500 µL. An equal volume of dichloromethane was added to the extract, and the resulting solution was vortexed for several minutes. The organic phase was extracted using a Pasteur pipette and concentrated to 20 µL using N_2 gas. The concentrated organic phase was then run on an Agilent 6890 gas chromatograph coupled to a 5973 quadruple mass spectrometer to identify PAHs.

2.3 Results and discussion

2.3.1 DNA yield and quality

The DNA yields, as determined by UV absorbance, were consistently low for the UltraClean and PowerMax kits but were consistently high for the FastDNA SPIN kit, which pro-vided significantly higher DNA yields for all soils based upon an ANOVA analysis of yield measurements, $p < 0.05$, (Fig. 2.1). The PowerSoil kit gave intermediate DNA yields for most soils and was equivalent to the FastDNA SPIN kit for soil 1. Across the soils, DNA extraction yield was not significantly affected by the soil texture or the

organic carbon content of the soil (Pearson's r values were not significant).

One of the most important requirements for all kits is high-quality DNA free of contaminants, which allows for successful amplification of the extracted DNA. The level of contamination can be determined by examining absorbance ratios, since DNA has an absorption peak at 260 nm. DNA purity can be assessed for contamination from residual proteins using a ratio of A_{260}/A_{280}, where ratios lower than 1.7 reflect protein contamination and ratios greater than 1.7 reflect pure DNA. Similarly, purity from humic compounds can be determined using a ratio of A_{260}/A_{230}, where ratios <2 reveal humic acid contamination and ratios >2 are characteristic of pure DNA. All four kits were roughly equivalent with respect to A_{260}/A_{280} ratios (Fig. 2.2a).

However, A_{260}/A_{230} ratios greatly varied among kits (Fig. 2.2b). Although none of the extracts were visibly coloured, the FastDNA SPIN kit gave the lowest A_{260}/A_{230} ratios and, therefore, had the most residual humic acid contamination. DNA yields estimated by band intensities on agarose gels support that the FastDNA SPIN kit yields were overestimates caused by high levels of UV absorbant compounds (Table 2.2). The UltraClean kit gave the lowest DNA yields, having both low absorbance and relatively low A_{260}/A_{230} ratios, resulting in amounts not detectable in 5 µL of DNA extract on agarose gel. The highest A_{260}/A_{230} ratios and, therefore, the cleanest DNA, was provided by the PowerMax or PowerSoil kits, depending on the soil. The higher yield given by the PowerSoil kit versus the PowerMax kit makes the former a better choice by providing the greatest amount of high-quality DNA.

Low A_{260}/A_{230} ratios can be caused by humic acids but also by other aromatic

compounds such as residual PAHs. This was illustrated by adding several different concentrations of naphthalene (ranging from 6 to 102 ppm) to DNA oligomer mixtures (consisting of 17–57 bp primers) of known concentrations and reassessing DNA concentration. The addition of low concentrations of naphthalene, up to 13 ppm, did not give significantly higher absorbance readings; however, mixtures containing amounts above 51 ppm were detectable on the Nanodrop instrument. To determine whether PAH contaminants were interfering with either ex-traction efficiency or assessment of DNA yields, a liquid-liquid extraction of the DNA extracts was performed. The resulting extract was run on a gas chromatography – mass spectrometer with a detection limit of 1 ppm for PAHs; there were no PAHs found in any of the DNA extracts tested. Therefore, DNA yields and concentrations presented are not biased by any PAHs found in the soils because PAHs found below 1 ppm would not have any significant effect on absorbance readings.

2.3.2 PCR amplification

The PowerSoil kit provided the highest quality DNA based on successful amplification of both bacterial and eukaryotic DNA for all six soils (Tables 2.3 and 2.4). The FastDNA SPIN kit extracted high-quality DNA, as demonstrated through successful eukaryotic amplifications of all six soils; however, amplification of prokaryotic DNA was not successful. DNA extracted from both the UltraClean and PowerMax kits was unreliable for PCR and lead to some successful PCR reactions depending on the soil. Specifically, the PowerMax kit produced DNA from which amplification was possible for soils 1, 2, and 3; however, amplification was problematic for soils 4, 5, and 6. It was

much less successful at providing high-quality DNA from soils with higher levels of contamination, such as soils 5 and 6.

2.3.3 Phylotype diversity

Products from successful PCR reactions were analyzed via DGGE to assess the impact of the extraction kit on the rDNA fingerprints, i.e., the perceived phylogenetic diversity of the samples. Replicability varied between kits (Table 2.5), and they gave dramatically different fingerprints, ranging from 2% to 10% similarity between kits for the 16S profiles and from 10% to 25% for the 18S profiles. This result indicates that the kits differ with respect to degree of cell lysis, and so, observed phylogenetic diversity depends greatly on the extraction kit being used (Fig. 2.3). For bacterial diversity, the PowerSoil, PowerMax, and FastDNA SPIN kits displayed the greatest number of bands. However, the PowerSoil kit was the most consistent and revealed the greatest number of bands for all six soils, whereas the PowerMax kit and the FastDNA SPIN kit varied and would often reveal fewer bands than the PowerSoil kit, depending on the soil. The UltraClean kit displayed very few or almost no bands for all soils, which may reflect the poor quality of the extracted DNA.

Apparent eukaryotic diversity also varied greatly between kits. Similar to bacterial diversity, the UltraClean kit revealed very few or almost no eukaryotic bands for all six soils. The FastDNA SPIN kit revealed the greatest number of bands for all soils; however, this was not consistent, and replicates would often reveal significantly fewer bands, indicating that this kit is not the most reliable for estimating eukaryotic diversity. On the other hand, the PowerSoil kit revealed multiple bands for each soil and was very

consistent between replicates.

In conclusion, the results clearly demonstrate that commercial DNA extraction kits can be used on a wide variety of soils, including heavily contaminated soils, and residual PAHs do not co-extract with the DNA. In our hands, the PowerSoil kit was the most effective and reliable kit for contaminated soils because it provided the highest quality DNA that was consistently amplifiable using both eukaryotic and prokaryotic primers.

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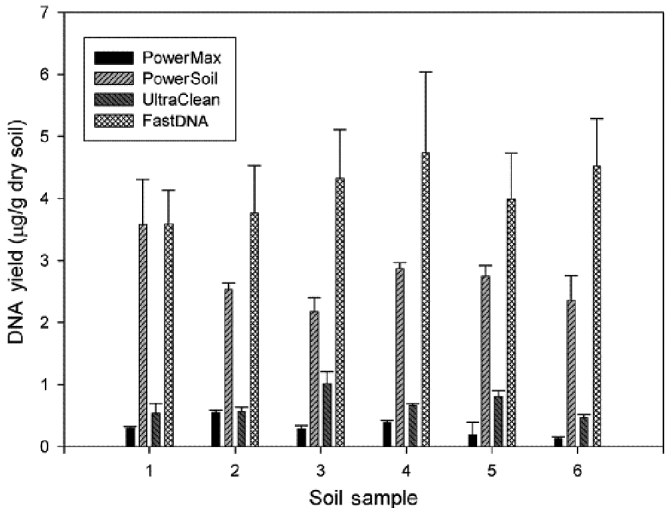

Figure 2.1 Final yield of extracted DNA from commercial DNA kits, as determined by UV absorbance.

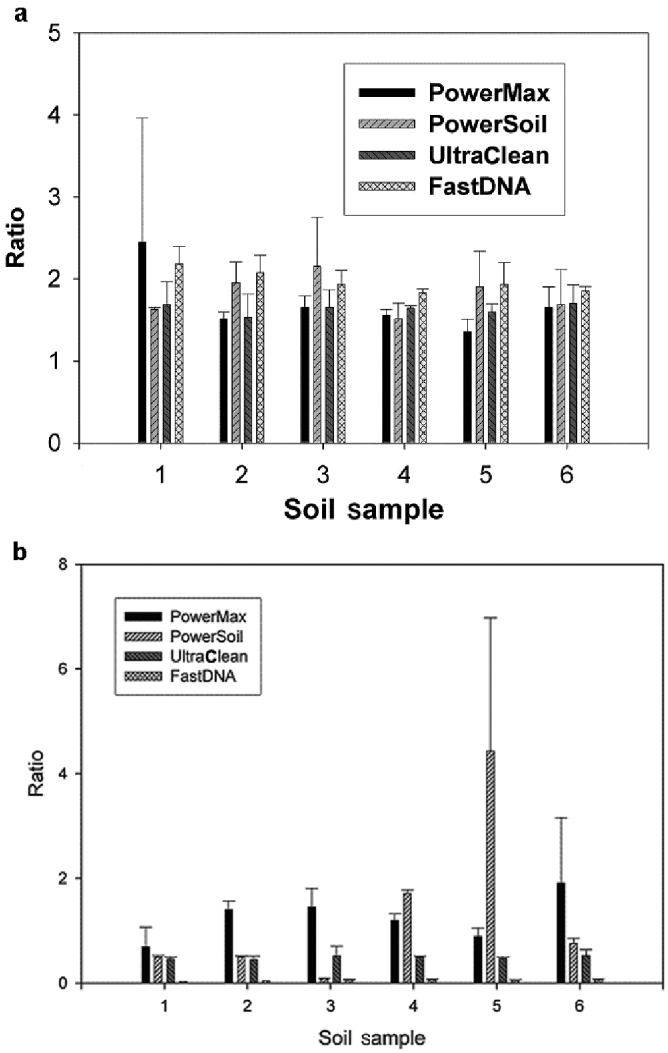

Figure 2.2 Efficiency of commercial kits at removing (a) residual proteins using an absorbance ratio of A_{260}/A_{280} (ratios <1.7 reflect protein contamination, >1.7 pure DNA) and (b) humic acids using an absorbance ratio of A_{260}/A_{230} (ratios <2 reveal humic acid contamination, >2 pure DNA).

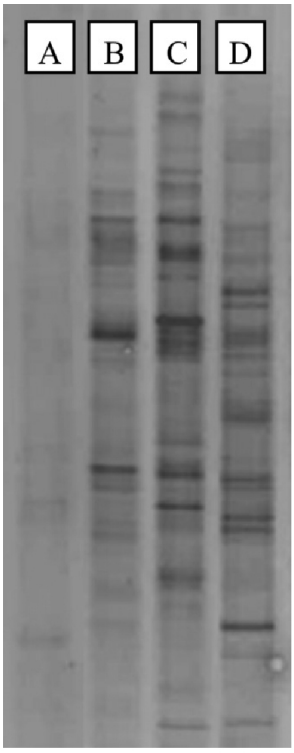

Figure 2.3 Denaturing gradient gel electrophoresis (DGGE) profiles of 16S rDNA of the same soil sample (soil 2) extracted using four different commercial DNA isolation kits: UltraClean (lane A), FastDNA SPIN (lane B), PowerMax (lane C), PowerSoil (lane D).

Table 2.1 Analysis of soil samples used for DNA extraction.

Soil Sample	% clay	% silt	% sand	Soil Texture	Total [PAH] (ug/g)	Organic carbon (%)
1	23	77	0	Silt loam	<0.25	0.5
2	27	73	0	Silt loam	161	0.4
3	22	78	0	Silt loam	1299	3.0
4	4	25	70	Sandy loam	1079	2.4
5	26	74	0	Silt loam	3552	0.4
6	2	13	84	Loamy sand	4802	2.0

Table 2.2 DNA yields of six soil samples, obtained from four commercial DNA extraction kits.

Soil Sample	PowerMax soil DNA isolation kit	PowerSoil DNA isolation kit	Ultraclean soil DNA isolation kit	FastDNA SPIN kit for soil
1	0.21±0.02 µg/g	4.80±0.50 µg/g	<1 ng/µL	1.52±0.30 µg/g
2	0.18±0.02 µg/g	7.20±0.70 µg/g	<1 ng/µL	3.20±0.40 µg/g
3	<1 ng/µL	<1 ng/µL	<1 ng/µL	<1 ng/µL
4	0.06±0.01 µg/g	4.24±0.40 µg/g	<1 ng/µL	2.40±0.20 µg/g
5	<1 ng/µL	3.60±0.40 µg/g	<1 ng/µL	1.20±0.10 µg/g
6	0.12±0.01 µg/g	<1 ng/µL	<1 ng/µL	6.00±0.40 µg/g

Note: DNA yields were determined using agarose electrophoresis assay. Values are the means ± standard deviations.

Table 2.3 16s PCR amplification results*

Soil Sample	PowerMax soil DNA isolation kit	PowerSoil DNA isolation kit	Ultraclean soil DNA isolation kit	FastDNA SPIN kit for soil
1	++/++	++/+	-/++	-/+
2	++/++	++/++	+/++	++/+
3	++/++	++/++	-/-	++/-
4	++/-	++/++	++/-	++/-
5	+/+	++/++	-/-	++/++
6	-/+	++/++	++/+	++/+

(++ indicates successful amplification, + indicates partial amplification, - indicates no amplification)
*amplification results include sample and replicate

Table 2.4 18s PCR amplification results*

Soil Sample	PowerMax soil DNA isolation kit	PowerSoil DNA isolation kit	Ultraclean soil DNA isolation kit	FastDNA SPIN kit for soil
1	++/-	++/++	+/+	++/++
2	++/++	++/++	+/+	++/++
3	++/++	++/++	-/+	++/++
4	-/++	++/++	+/-	+/++
5	-/+	++/++	+/+	++/++
6	++/++	++/++	+/++	++/++

(++ indicates successful amplification, + indicates partial amplification, - indicates no amplification)
*amplification results include sample and replicate

Table 2.5 Percent similarity of replicate samples based on normalized denaturing gradient gel electrophoresis (DGGE) profiles.

Soil Sample	PowerMax soil DNA isolation kit	PowerSoil DNA isolation kit	Ultraclean soil DNA isolation kit	FastDNA SPIN kit for soil
1	50.0%	75.0%	55.0%	0.0%
2	50.0%	65.0%	50.0%	70.0%
3	80.0%	70.0%	0.0%	0.0%
4	40.0%	72.5%	0.0%	0.0%
5	30.0%	80.0%	0.0%	80.0%
6	40.0%	20.0%	35.0%	55.0%

Note: A value of 0 indicates that replication of the DNA was unsuccessful resulting in no replicate for comparison.

CHAPTER 3

ASSESSING MICROBIAL CARBON SOURCES AND POTENTIAL PAH DEGRADATION USING NATURAL ABUNDANCE ^{14}C ANALYSIS

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Abstract

Natural abundance ^{14}C analysis was applied to PLFAs collected from an industrial site in southern Ontario in order to assess microbial carbon sources and potential PAH biodegradation in soils. $\Delta^{14}C$ of microbial phospholipid fatty acids (PLFA) at the site ranged from +54‰ to -697‰. Comparison of these values to surrounding carbon sources found that microbial carbon sources were derived primarily from vegetation and/or natural organic matter present in the soils rather than PAHs. This study highlights that microbes are able to utilize almost all available pools of organic matter including older pools which are thought to contain recalcitrant compounds. Furthermore, it shows that even with the presence of an active microbial community, there may be little biodegradation of PAHs. This study illustrates challenges in assessing microbial activity in the environment and the advantage of using natural abundance ^{14}C analysis as a tool to elucidate microbial carbon sources.

3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic contaminants found in petroleum and petroleum-derived products such as coal tar, asphalt and creosote and are associated with spills or improper disposal of these materials. PAHs are also ubiquitous in the environment due to atmospheric deposition resulting from the incomplete combustion of organic matter such as diesel, coal and wood. PAHs have gained widespread attention because many of them are classified as carcinogens and mutagens (Harvey, 1996; Mastrangelo et al., 1996). Due to their structure, PAHs have

low water solubility and are readily absorbed onto soils and sediments where they persist unless they are degraded (Bamforth and Singleton, 2005; Cerniglia, 1992; Neely et al., 1974; Southworth et al., 1978).

Biodegradation of petroleum hydrocarbons and related compounds by microorganisms can be an effective method to remediate contaminated environments (e.g. Atlas and Hazen, 2011). Laboratory studies have revealed that most PAHs are susceptible to degradation by either bacteria, fungi or algae (Cerniglia, 1997; Da Silva et al., 2003; Juhasz and Naidu, 2000; Peng et al., 2008; Potin et al., 2004). However, the extent of degradation observed in field studies is generally much lower than observed in laboratory studies (Wild et al., 1990, 1991; Huesemann, 1997; Gallego et al., 2011). Laboratory biodegradation experiments are often conducted with clean soils that have been amended with PAHs (Maliszewska-Kordybach, 1993; Muckian et al., 2009; Shuttleworth and Cerniglia, 1995). This often results in overestimation of the degradation potential in the natural environment because PAH biodegradability is a function of soil type (Loehr and Webster, 1996; Posada-Baquero and Ortega-Calvo, 2011; Wild and Jones, 1993) and aged chemicals have been shown to be more resistant to degradation compared to freshly added compounds (Allard et al., 1994; Fu et al., 1994; Scribner et al., 1992; Steinberg et al., 1987) As soil residence time increases, aging of contaminants occurs through processes such as partitioning onto soil organic matter and diffusion into soil micropores; this ultimately reduces rates of biodegradation (Alexander, 2000; Hatzinger and Alexander, 1995). Furthermore, carbon sources that are more labile than PAHs are often present in the natural environment and microorganisms may

preferentially utilize natural organic matter (NOM) resulting in the persistence of contaminants. Such behavior was observed in salt marsh sediments contaminated by the *Florida* oil spill of 1969 in which the microbial community was utilized NOM as opposed to petroleum hydrocarbons (Slater et al., 2005). Variations in microbial metabolic pathways and rates under laboratory versus in situ conditions may also be responsible for differences in laboratory studies and field observations. In situ studies can overcome these differences, but the heterogeneous distribution of PAHs and geologic conditions across a site combined with relatively slow degradation rates make assessment based on observed mass loss difficult.

Compound-specific ^{14}C analysis of phospholipid fatty acids (PLFA) has become a useful tool that can overcome these challenges by elucidating microbial carbon sources and confirming biodegradation in situ (Ahad et al., 2010; Cowie et al., 2010; Slater et al., 2005, 2006; Wakeham et al., 2006). Because they hydrolyze rapidly after cell death, PLFA are biomarkers for active microbial populations (Vestal and White, 1989; Frostegard and Bååth, 1996). As a result, many studies utilize the fact that incorporation of an isotopic label from a potential substrate into microbial cellular components, such as PLFA, provides direct evidence for metabolism of that compound (Boschker and Middelburg, 2002; Evershed et al., 2006). Since ^{14}C decays over time, petroleum derived contaminants have no detectable ^{14}C due to the geologic age of the carbon source. This lack of ^{14}C provides a natural abundance label for petroleum derived contaminants. When in the presence of more modern (higher ^{14}C content) carbon sources,

biodegradation and utilization of petroleum derived carbon will result in a decrease in the ^{14}C content of the cellular components, such as PLFA.

This study utilized natural abundance ^{14}C analysis to assess microbial carbon sources and potential PAH biodegradation at a PAH contaminated site in southern Ontario. PLFA concentrations were used to assess changes in microbial abundance with changes in PAH concentrations and microbial carbon sources were elucidated via ^{14}C analysis. To the best of our knowledge, this is the first attempt to apply this approach to an environmental system that is primarily contaminated with PAHs.

3.2 Materials and Methods

3.2.1 Sampling

Six soil samples were collected in May 2010 from an industrial site in southern Ontario which operated from the 1950s to the mid 1990s and used PAH compounds as part of its manufacturing process. More detailed information regarding the nature of the industrial processes which took place at the site can be found in Supporting Information. The site is approximately 11 acres in size and generally level with a gentle slope to the north. At the time of operation, the site consisted of a number of buildings which had been demolished at the time of sampling. The site includes regions with established vegetation, as well as unpaved areas with little vegetation such as hard-packed gravel road ways.

All samples were collected from the upper 50 to 60 cm of soil. Figure S1 depicts the site at the time of sampling and locations for sampling collection. Soil 1 was collected

from a vegetated, non-contaminated area, with negligible PAH concentrations near the site as a control for comparison with other soils. Soil samples 3, 4 and 6 were collected from areas where there was minimal or no vegetation present. Soil samples 3 and 6 were collected from underneath the former building footprint (land surface area covered by the building) and soil 4 was collected from underneath a well worn gravel driveway. Soils 2 and 5 were collected from areas with higher vegetation (based on areal coverage by plants) that were known to be former industrial storage and staging areas. It is important to note that some soils were not of natural origin, since construction of certain areas of the site involved the use of fill materials such as gravel. Moreover, in certain areas (such as from where soil 6 was collected), fill material included semi-solid coal tar residues which were a by-product of industrial processing. Given the history of the site as an industrial property, the site has been filled and re-graded multiple times and a layer of fill material is present across the production areas of the site (with the exception of the sampling area for soil 1). However, the majority of coal tar residues and PAH contamination was present beneath the eastern-most production building formerly present at the site (Figure 3.S1). The thickness of the fill materials is variable and ranges from 0.2 to 1.2 m in the vicinity of the soil sampling areas. After collection, all samples were transported to McMaster University immediately following collection and stored at -20°C. If present, any visible plant debris and pebbles were removed with tweezers from the soil prior to any analysis. As the water table at this site is typically present from 2.5 to 3.2 m below ground surface (mbgs), collected soils were assumed to be aerobic.

3.2.2 Soil analysis and PAH quantification

Soil grain size distributions were determined using a Beckman Coulter LS 230 laser diffraction particle size analyzer (Brea, CA) and soil textures were assessed using the standard textural triangle (Table 3.1). Samples were treated with 1M HCl to remove carbonate and percentages of total organic carbon (% TOC) were determined using continuous flow system consisting of a Costech 4010 elemental combustion system (Milan, Italy) with peak intensities being measured using a Delta plus XP isotope-ratio mass spectrometer (Thermo Finnigan, Herts, UK). Soil samples were submitted to Maxxam Analytics Inc. for PAH analysis and concentrations were assessed following EPA method 8270.

3.2.3 Microbial PLFA analysis

Approximately 170 to 280g of freeze-dried soil was extracted using a modified Bligh and Dyer method (White et al., 1979) as per Ahad et al., 2010. The exact amount of soil required in order to obtain sufficient mass for ^{14}C analysis was determined based on final PLFA yields of previous extractions of these samples. Briefly, samples were extracted with 2:1 methanol:dichloromethane and the organic fraction of the extract was collected and separated by silica gel chromatography to isolate the phospholipids. PLFA were obtained via mild alkaline hydrolysis of phospholipids and transesterification to fatty acid methyl esters (FAME) and quantified via GC/MS. Details of phospholipid extraction, purification and identification are described in the Supporting Information.

3.2.4 ^{14}C analysis

PLFAs were sent as a bulk fraction for ^{14}C analysis because collection of sufficient mass of individual PLFAs would have required impractically large sample sizes (at least 12kg for some soils). Furthermore, previous contaminated systems where sufficient time has elapsed for an isotopic label to be recycled through the microbial community have shown little variation in $\Delta^{14}C$ between PLFAs from the same site (Cowie et al., 2010; Slater et al., 2005; Wakeham et al., 2006). Process blanks which were exposed to the same solvents and procedures as soil samples were also found to be free of any contaminants in the PLFA fractions.

In addition to PLFA, the ^{14}C content of TOC and solvent extracted residue (EXT-RES) carbon pools for each soil were also measured. $\Delta^{14}C$ of TOC was determined by decarbonating oven dried soil (50 ºC for 48 h) using HCl and subsequently submitting for ^{14}C analysis. $\Delta^{14}C$ of EXT-RES (residue remaining after solvent extraction) was also determined. This EXT-RES, which had any solvent soluble PAHs as well as any other solvent extractable organic components removed, was taken as a proxy for the natural organic matter as per White et al., (2005) and Ahad et al., (2010). PAHs and other solvent extractable organic materials were removed by carrying out a total lipid extraction (TLE). This was done by extracting approximately 8g of oven-dried soil using 1:1 hexane:acetone along with a microwave accelerated reaction system (MARS, CEM Corporation). Subsequently, organic compounds extracted by solvent (referred to as TLEs) were filtered using burned glass fiber filters to remove soil particles (GF/G, Whatman) and treated with activated copper to remove elemental sulfur. Residual soil

collected by filters (defined as EXT-RES) was decarbonated, analyzed for total organic carbon (% TOC) and sent for ^{14}C analysis. ^{14}C measurements were determined by accelerator mass spectrometry (AMS) at the National Ocean Sciences Accelerator Mass Spectrometry Facility (NOSAMS) at Woods Hole Oceanographic Institute after conversion of CO_2 to graphite and normalized to $\delta^{13}C$ values of -25‰ (McNichol et al., 1994). ^{14}C values are expressed in $\Delta^{14}C$ notation, in per mille (‰) deviation from the ^{14}C Standard Reference Material 4990B "O*xalic Acid*" (Stuiver and Polach, 1977). In this context, petroleum-derived carbon such as PAHs have a value of -1000‰ since they contain no detectable amounts ^{14}C whereas carbon derived from recently photosynthesized materials will have values of approximately +50‰. The accuracy and reproducibility for $\Delta^{14}C$ analysis was ±10‰ (for TOC and EXT-RES) and ±20‰ for PLFA, these values include the accuracy and precision of the AMS as well as the limitations of the method (Pearson et al., 1998; Ingalls and Pearson, 2005; Santos et al., 2010).

3.3 Results

3.3.1 PAH concentrations

The total PAH concentration for the soil samples ranged from <0.25 µg/g to 4802 µg/g dry weight (Table 3.1). Table S1 shows the concentrations of individual PAHs for soil samples collected from the site. Soil 1 was collected from a non-impacted area and had negligible PAH concentrations. Soil samples 2-6 were contaminated by both low and high molecular weight (MW) PAHs. However fluoranthene and pyrene (which are both

high MW PAHs) had the highest concentrations for all soils with the exception of soil 4 which had a higher concentration of phenanthrene (which is a low MW PAH). PAH concentrations across soils 2-6 exceeded the Ontario Ministry of Environment's standards for industrial/commercial land use in a non-potable groundwater condition (Ministry of Environment, Soil, Ground Water and Sediment Standards, 2011) by amounts ranging from a few µg/g to several orders of magnitude.

3.3.2 Microbial community structure and cell density

PLFA concentrations varied greatly between soils and ranged from 255 to 1141 µg/kg (dry weight) (Table 3.1). The highest total PLFA concentration (1141 µg/kg) was found in the uncontaminated clean soil (soil 1) and the lowest total PLFA concentration (255 µg/kg) was found in the soil with the highest PAH concentrations (soil 6). Moderately contaminated soils (3 and 4) also had relatively low PLFA concentrations; however soil 5 which contained high levels of contamination (>3500 µg/g) had the second highest PLFA concentrations. Soils with higher % TOC were found to have lower PLFA concentrations (Table 3.1). Using an average conversion factor of 4×10^4 cells $pmol^{-1}$ of PLFA (Green and Scow, 2000) these PLFA concentrations correspond to cell densities of 1.6×10^8 cells g^{-1} in the uncontaminated soil and 3.7×10^7 to 1.5×10^8 cells g^{-1} in the contaminated soils (Table 3.1), consistent with previously reported values for contaminated environments (Green and Scow, 2000). In all samples, the distribution of PLFA was dominated by n-saturated and monounsaturated PLFA as expected for surface soils (Table 3.S2) (Zelles, 1999). There was no relationship between PLFA distribution and PAH concentration (Table 3.1); this is consistent with previous work (Ahad et al.,

2010; Slater et al., 2005). However other studies have observed a relationship between the presence of hydrocarbons and increases in the proportion of terminally branched, mid-chain branched, cyclopropyl and odd-numbered straight chained saturated PLFA (Aries et al., 2001; Doumenq et al., 2001; Greenwood et al., 2009; Hallmann et al., 2008; So and Young, 1999). PLFA diversity (i.e. the total number of individual PLFAs extracted from each soil) was lowest in soils with lower cell density (soils 3 and 4) and was highest in soils with higher cell density (soils 1, 2 and 5) (Table 3.1).

3.3.3 ^{14}C measurements

The $\Delta^{14}C$ content of the TOC, EXT-RES (representing naturally occurring organic matter) and PLFA were measured for each soil (Figure 1 depicts $\Delta^{14}C$ signatures across all soils). The $\Delta^{14}C_{TOC}$ ranged from -159 to -758‰ across the six soils and the $\Delta^{14}C_{EXT-RES}$ ranged from -174 to -983‰. The $\Delta^{14}C_{EXT-RES}$ for soils 1, 5 and 6 was lower than TOC (by at least 100‰), whereas soils 2, 3 and 4 had similar EXT-RES and TOC values. In soils where $\Delta^{14}C_{TOC}$ is higher than $\Delta^{14}C_{EXT-RES}$ the TOC must contain contributions of higher $\Delta^{14}C$ inputs such as recently photosynthesized material.

The $\Delta^{14}C$ value for PLFAs ranged from +54 to -697‰ which indicated that microbial carbon sources across soils differed (Figure 3.1). The $\Delta^{14}C_{PLFA}$ values reported here (with the exception of soil 6, $\Delta^{14}C$= -697‰) are higher compared to other petroleum-contaminated sites (Ahad et al., 2010; Cowie et al., 2010). In addition, the $\Delta^{14}C_{PLFA}$ values are higher relative to $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{EXT-RES}$. The extent of microbial metabolism of PAHs and/or natural organic carbon based on $\Delta^{14}C$ analysis is discussed in detail in the following sections.

3.4 Discussion

3.4.1 Are PAHs a major source of microbial carbon?

If the microbial community at this site was utilizing petroleum-derived carbon such as PAHs as major carbon source, it would be expected that $\Delta^{14}C$ of PLFA would be lower (i.e. ^{14}C depleted) relative to the surrounding carbon sources. Such depletion in $\Delta^{14}C$ of microbial PLFA has been observed at other sites such that biodegradation of petroleum derived hydrocarbons could be clearly demonstrated (Ahad et al., 2010; Cowie et al., 2010; Wakeham et al., 2006). However, such depletion in PLFA was not observed at this site (Figure 3.1), indicating that microbial communities at this site were not utilizing PAHs as a major carbon source. In soils, microbial utilization of multiple carbon sources of varying ages, and thus ^{14}C contents, can result in potentially mimicking or masking the use of one carbon source by the use of a combination of other carbon sources. While it may be challenging to precisely resolve the use of multiple carbon sources by the microbial community, an isotope mass balance approach can be applied to constrain the extent to which particular carbon sources are being used.

3.4.2 Isotope mass balance of potential microbial carbon sources

In order to constrain the potential for PAH degradation in these soils, we applied an isotopic mass balance (as per eq. 1) between the potential carbon pools that could be contributing to the $\Delta^{14}C$ of PLFA.

$$\Delta^{14}C_{PLFA} = f_{PAH}\,(\Delta^{14}C_{PAH}) + f_{modern}\,(\Delta^{14}C_{modern}) + f_{EXT\text{-}RES}\,(\Delta^{14}C_{EXT\text{-}RES}) \qquad (1)$$

The carbon pools included in this mass balance were chosen to represent potential end-member carbon sources that the microbial community may be utilizing. Since this site

contains only petrogenic PAHs, the $\Delta^{14}C_{PAH}$ can be assigned a value of -1000‰ representing the lowest $\Delta^{14}C$ carbon source at this site. At the other end of the spectrum, the highest $\Delta^{14}C$ carbon source was assumed to be recently photosynthesized carbon originating from the modern atmosphere; $\Delta^{14}C_{modern}$= +55‰ (Turnbull et al., 2007). While the presence of photosynthesized material with higher ^{14}C values due to inputs of carbon originating from atmospheric weapons testing ("bomb carbon", $\Delta^{14}C$ = +64‰, Levin et al., 1985) may be present, the similarity between the expected value for bomb carbon influenced soils and modern atmosphere resulted in no significant change in isotopic mass balance. The third component of the mass balance, $\Delta^{14}C_{EXT\text{-}RES}$, was measured and assumed to represent the oldest naturally occurring carbon in the sample which has been diagenetically altered such that it is no longer extractable and provides a representative $\Delta^{14}C$ value for natural organic matter as shown by White et al., (2005) and Ahad et al., (2010). Since soil organic matter is not homogenous and is comprised of assorted organic material in varying stages of decomposition with different turnover times (Trumbore, 2000, 2009), the EXT-RES pool could potentially include some older carbon cycling at millennial timescales, but based on this slow cycling such carbon would be expected to contribute little to the active microbial community. The material removed during solvent extraction (referred to as TLEs) would be expected to contain a mixture of modern carbon, PAHs and natural carbon younger or equivalent to EXT-RES. In our study, the TLEs contained predominantly more modern carbon as evidenced by the lower $\Delta^{14}C$values observed for the EXT-RES relative to the TOC.

Using these three carbon pools, and the requirement that the fraction of each in eq. 1 must add up to 100%, we can investigate the extent to which PAH degradation could be contributing to the observed $\Delta^{14}C_{PLFA}$ values by iteratively varying the contributions of each pool (Table 3.2). By setting the fraction of EXT-RES to zero, the maximum contribution of PAH derived carbon to microbial PLFA ranged from 12 to 71% across soils. Alternatively, we can assume that no PAH carbon is being utilized and that all ^{14}C depleted carbon in the PLFA is derived from the EXT-RES pool. Setting the PAH fraction to zero resulted in maximum contributions of the EXT- RES pool which ranged from 30 to 100% (Table 3.2). Based on the results of previous studies (Cowie et al., 2010; Ahad et al., 2010, Wakeham et al., 2006), the preponderance of evidence is that the EXT-RES pool, or naturally occurring carbon makes at least some contribution to microbial PLFA. Thus, the contribution of EXT-RES to PLFA reduces the maximum contribution that PAH derived carbon can make to the observed $\Delta^{14}C_{PLFA}$. The estimated contributions of carbon pools to microbial PLFA are shown in Table 3.2 and discussed in detail for each soil below.

3.4.3 Assessing microbial utilization of carbon sources

The clean control (soil 1) had the highest $\Delta^{14}C_{PLFA}$ value of +54‰ which corresponds to utilization of a modern source of carbon (recently photosynthesized organic matter has $\Delta^{14}C$ value of approximately +55‰, Turnbull et al., 2007) and is consistent with values observed by Cowie et al., (2010) for similar clean controls.

For soil 2, assuming that modern inputs is a primary carbon source as is the case for soil 1, inputs of carbon with lower ^{14}C values (either from the EXT-RES pool or PAH

pool) are required in order to account for the observed $\Delta^{14}C_{PLFA}$ = -70‰. Based on the isotopic mass balance, the maximum possible input of PAH derived carbon to the PLFA at this site is 12%. However, to the extent that any carbon being used by the microbial community lies between the modern and PAH end-members, for instance the EXT-RES pool has a $\Delta^{14}C$ value of -589‰, this reduces the maximum possible input of the PAH derived carbon to the observed $\Delta^{14}C_{PLFA}$ value. Therefore, in order for the mass balance to hold true based on the observed $\Delta^{14}C_{PLFA}$, the contributions of the three pools to PLFA must be 79 - 100% modern carbon, 0 - 21% for EXT-RES and 0 - 12% for PAHs.

In the case of soil 3, the mass balance between modern and PAH carbon result in the maximum contribution of PAH derived carbon to the observed $\Delta^{14}C_{PLFA}$ value (-192‰) being 23%. Alternatively, if there are no PAH inputs, the contribution of EXT-RES ($\Delta^{14}C$ = -174‰) cannot be less than 99% assuming that the remaining fraction comes from modern carbon. As this sample was collected from underneath the footprint of a former building and thus had no surface vegetation present, it may be expected that modern inputs may be limited. The agreement between $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{EXT-RES}$ within analytical error for this sample further supports limited inputs of modern carbon. These two factors, in combination with the fact that the $\Delta^{14}C_{PLFA}$ agrees very closely with the $\Delta^{14}C_{EXT-RES}$ in this sample suggest that modern inputs to this sample are in fact limited. If this is assumed to be true, then 96 - 100% of the carbon in the PLFA is coming from the EXT-RES pool and less than 4% of a contribution is coming from the PAH pool.

Soil 4 is similar to soil 3 in that it was collected from an area underneath a well worn gravel driveway and had no vegetation present. Assuming a mass balance with

modern carbon, the maximum contribution of PAH derived carbon to the observed $\Delta^{14}C_{PLFA}$ (-336‰) value can be 37%. But, in the absence of PAH inputs, the EXT-RES must contribute at least 94% to the PLFA (modern carbon = 6%). However, as for soil 3, there are likely minimal inputs of modern carbon based on the area from which the soil was collected. In addition, the observed $\Delta^{14}C_{EXT\text{-}RES}$ (-341‰) and $\Delta^{14}C_{TOC}$ (-273‰) values are fairly close. This implies that a large input of modern carbon is not likely. If we assume no input of modern carbon to this soil, the estimated contributions of the remaining two pools are 98 - 100% EXT-RES and 0 - 2% PAHs.

The observed$\Delta^{14}C_{PLFA}$ value (-175‰) for soil 5 is much higher than the $\Delta^{14}C_{EXT\text{-}RES}$ (-693‰) which indicates that the microbial community is using a relatively large component of modern carbon. Assuming a mass balance between atmospheric carbon and PAH derived carbon, the maximum contribution of PAH derived carbon to PLFA can be 22%. If the mass balance is done using only EXT-RES and modern pools the contribution of modern carbon must be at least 69%. Thus the contributions of the three potential carbon pools must be 69 - 78% modern carbon, 0 - 30% EXT-RES and 0 - 22% PAHs. This is consistent with the fact that soil 5 was collected from an area with higher vegetation (relative to the other soils) which would enable input of recently photosynthesized modern $\Delta^{14}C$ material.

In the case of soil 6, the $\Delta^{14}C_{PLFA}$ value (-697‰) is higher than the $\Delta^{14}C_{EXT\text{-}RES}$ (-983‰) indicative of more modern inputs of carbon along with inputs of older carbon. However, in this case, the low $\Delta^{14}C$ values for all carbon pools suggest that modern contributions are expected to be lower and in fact the input of modern carbon to PLFA in

this soil can be a maximum of only 29%. Thus, in the absence of any contribution from the EXT-RES pool, the maximum contribution of PAH derived carbon to PLFA can be 71%. Similarly, the maximum contribution of EXT-RES to PLFA will be 71% if the remaining carbon is solely from modern inputs. The $\Delta^{14}C$ values for EXT-RES and PAHs are the same within error and therefore make it impossible to differentiate between these two potential carbon sources in this sample. This will generally be true for systems where such highly depleted EXT-RES is present.

3.4.4 Implications to organic matter cycling and the role of microorganisms

Microorganisms play an important role in the cycling of organic matter in soils; however it is not clear whether they ultimately controlling the turnover rate of organic carbon since the stabilization of the organic matter depend on many factors including the structural and chemical properties of the compounds. Each pool of organic matter is made up of different chemical compounds and the mechanisms of stabilization may differ due to differences in charged surfaces and mineral interactions (Sollins et al., 1996). The mechanisms of stabilization can then in turn affect the availability of carbon to the microorganisms which would normally utilize carbon that is most available to them. It is generally thought that there is a pool of recalcitrant organic compounds in soil that is biologically inert (Falloon et al., 2000) and microbes will utilize the younger pool with more labile compounds. Accordingly, in this study soils 1, 2 and 5 show evidence of relatively young carbon as a dominant microbial carbon source which is consistent with previous work on microbial carbon sources (Cowie et al., 2010; Rethemeyer et al., 2005). However, the microbial communities in soils 3, 4 and 6 are relying on a much older

carbon suggesting that microbes are able to utilize pools of organic matter that contain significantly older carbon. Similarly, Rethemeyer et al., (2004) found that microbial communities were utilizing old, refractory carbon found within the soil. We also found that soils with the highest TOC (soils 3, 4 and 6) had the lowest microbial biomass although other studies have shown TOC and microbial biomass to be positively correlated (Anderson and Domsch, 1989; Piao et al., 2001). This may be due to the nature of the carbon in these soils and/or stabilization mechanisms which result in making the organic carbon less available to microorganisms. In contrast, soils which had lower TOC (soils 1, 2 and 5) had higher microbial biomass suggesting that the carbon present in these soils may be more available for microbial uptake relative to the other soils. This is consistent with the fact that soils 1, 2 and 5 came from areas which had higher vegetation cover and may have had inputs of carbon derived vegetation which can lead to bioavailable carbon in the form of DOC that can be utilized by the microbial community. Some of this carbon derived from DOC may turn over rapidly such that it may not be captured by our sediment-based sampling approach. Our study raises questions regarding the influence of microbes on soil organic matter stabilization and the availability of different carbon pools with varying ages.

3.4.5 Implications for assessing degradation and remediating PAH-contaminated soils

Direct demonstration of PAH degradation in situ remains a challenge. The results of this study found limited evidence for PAHs as a major microbial carbon source at this site. Notwithstanding the limitation on our ability to constrain microbial carbon sources to specific contributions, our results indicate that if any degradation is occurring it is a

relatively small component of microbial carbon pools. This may be interpreted to mean that PAH degradation is currently occurring relatively slowly. However, our assessment is only valid for this specific time point and the site was in operation for over 40 years. Thus, PAH degradation may have occurred more rigorously early on when PAHs were initially deposited and more readily available for biological uptake.

In-situ biodegradation of PAHs by microorganisms is a more environmentally friendly and cost effective strategy than other commonly employed intrusive remediation strategies such as excavation and active in-situ chemical oxidation. However, the presence of an active microbial population at a contaminated site may not ensure degradation of contaminants of concern. Where this is the case, a change in geochemical conditions such as the addition of electron acceptors or surfactants may be required to stimulate biodegradation in order to achieve site remediation. Unfortunately, the use of bioremediation at many contaminated sites may not be feasible due to high concentrations of contaminants, slow degradation rates or other factors. Continued research on microbial degradation of PAHs in the natural environment will improve our ability to assess the potential for intrinsic remediation and optimize the use of microorganisms in remediation strategies.

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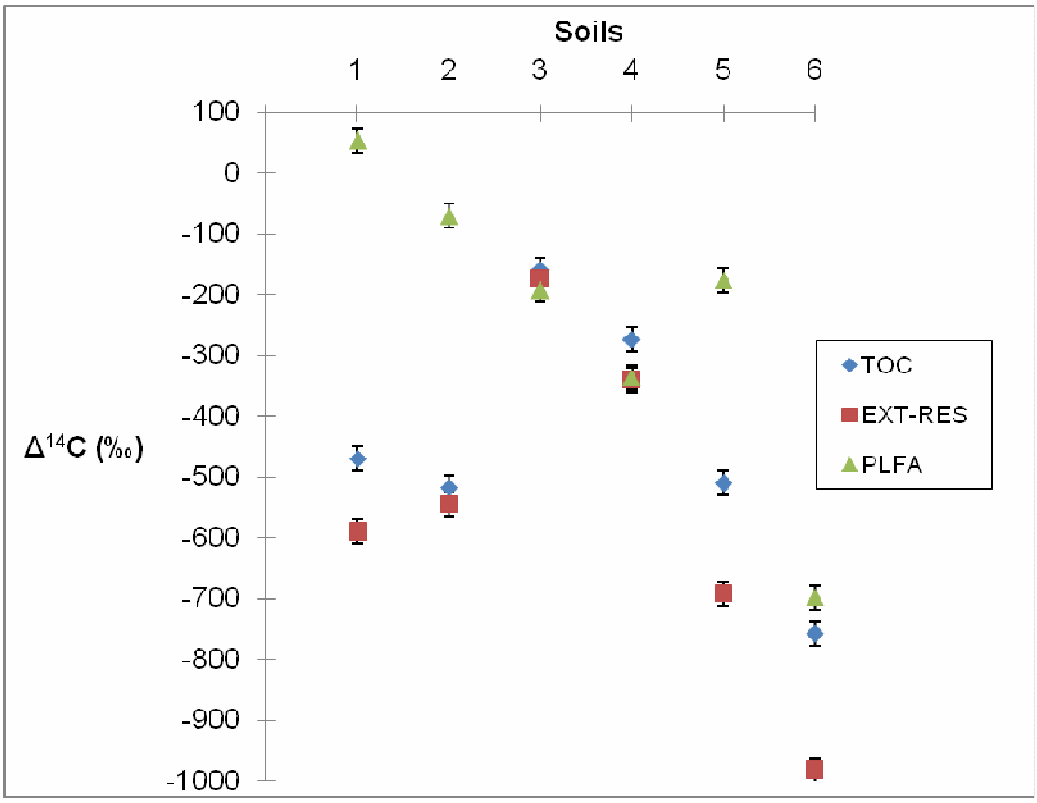

Figure 3.1 $\Delta^{14}C$ results for TOC, EXT-RES and PLFA for all six soils. Error bars on TOC and EXT-RES represent 10‰ accuracy and reproducibility. Error bars on PLFA represent 20‰ accuracy and reproducibility.

Table 3.1 PLFA results and analysis of soil samples.

	Total PLFA (ug/kg)	PLFA diversity (no. of PLFAs)	Cells g^{-1}*	Total organic carbon (%)	Total [PAH] (µg/g)	Soil Texture	Presence of vegetation**
Soil 1	1141	51	2×10^8	0.5	<0.25	Silt loam	high
Soil 2	975	59	1×10^8	0.4	161	Silt loam	high
Soil 3	376	29	5×10^7	3.0	1299	Silt loam	none
Soil 4	385	33	5×10^7	2.4	1079	Sandy loam	none
Soil 5	1088	67	2×10^8	0.4	3552	Silt loam	high
Soil 6	255	35	4×10^7	2.0	4802	Loamy sand	low

* cells per gram estimate was estimated from PLFA concentrations
** based on areal coverage by plants

Table 3.2 Estimated contributions (%) of carbon pools to observed microbial $\Delta^{14}C_{PLFA}$ values based on three end member mass balance.

	Modern Carbon	EXT-RES (representative of natural organic matter)	PAHs
Soil 1	100	0	n/a
Soil 2	79-100	0-21	0-12
Soil 3	n/a	96-100	0-4
Soil 4	n/a	98-100	0-2
Soil 5	69-78	0-30	0-22
Soil 6	28-29	0-71	0-71

3.5 Supporting Information

3.5.1 Details on Study Site

The site was used from the 1950s and involved industrial processing with PAHs, including liquid coal tar. The use of coal tar terminated in the 1970s. The process water and liquids used in the coal tar processing were reportedly collected in unlined trenches on the building floor prior to off-site disposal. Manufacturing ceased at the site in the 1990s. The spent chemicals, including excess solid coal tar, were sporadically spilled on the ground surface outside the plant and pieces of semi-solidified coal tar can be observed on the ground. Soil and groundwater sampling at the site has included analysis for PAHs, metals, inorganics, semi-volatile organics, and petroleum hydrocarbons. Results of the sampling have identified PAHs and metals as the primary contaminants in soil and groundwater.

3.5.2 PLFA extraction and analysis

Approximately 170 to 280g of freeze-dried soil was extracted using a modified Bligh and Dyer method (White et al., 1979) as per Ahad et al., 2010. Soils were extracted using 2:1 methanol/DCM and the resulting soil/solvent mixtures were centrifuged in solvent-rinsed centrifuge tubes (10 min, 2000 rpm). Subsequently, samples were filtered into separatory funnels using 0.45µm pre-combusted glass fiber filters (GF/G, Whatman). The organic phase was drained and collected for analysis following separation of aqueous and organic phases by the addition of deionized, nanopure water. The organic extract was separated into three fractions using fully activated silica (precombusted at 450°C for 8 h) and dicholormethane (DCM), acetone and methanol to elute non-polar, neutral and polar

fractions, respectively. The polar fraction containing the phospholipids was then evaporated to dryness under a stream of nitrogen gas and subjected to a mild alkaline methanolysis and converted to fatty acid methyl esters (FAME). FAMEs were purified using a secondary silica gel step and eluted in dichloromethane. Identification and quantification of FAMEs utilized an Agilent 6890 gas chromatograph coupled to an Agilent 5973 quadrupole mass spectrometer (equipped with a 30 m x 0.25 mm DB-5 MS column). The temperature program for the GC oven was 40 ºC for 1 min, ramp to 130 at 20 ºC/min, to 160 at 4 ºC/min and then to 300 at 8 ºC/min, with a final hold time of 5 min. Finally, FAMEs were identified using a bacterial reference standard (Bacterial Acid Methyl Esters CP, Mix, Matreya Inc), mass-fragmentation patterns and retention times and quantified using external calibration standards (which contained FAMEs of various chain length).

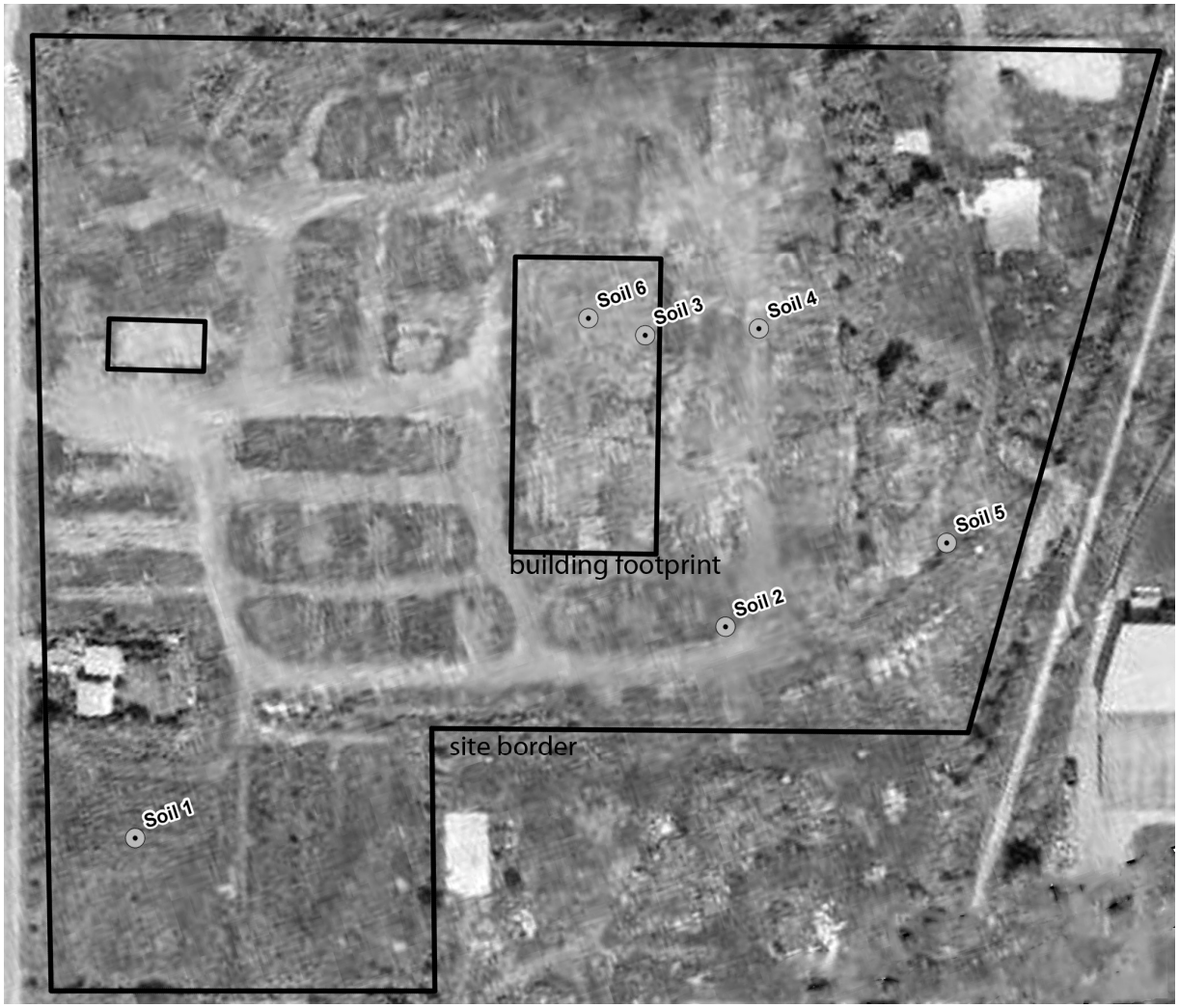

Figure 3.S1 Sampling locations for soils 1 to 6 from industrial processing site. Former building footprints are outlined in black.

Table 3.S1 PAH concentrations for soil samples collected from contaminated site, µg/g

	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6
Acenaphthene	<0.01	*<2*	20	110	200	130
Acenaphthylene	<0.005	*<2*	1.7	*<10*	*<50*	*<10*
Anthracene	<0.005	3	62	32	400	220
Benzo(a)anthracene	0.02	13	110	31	150	420
Benzo(a)pyrene	0.016	14	64	12	<50	290
Benzo(b/j)fluoranthene	0.03	19	95	23	87	420
Benzo(g,h,i)perylene	<0.02	8	39	*<10*	*<50*	140
Benzo(k)fluoranthene	<0.01	8	39	11	55	150
Chrysene	0.01	12	97	24	160	340
Dibenz(a,h)anthracene	<0.02	*<2*	13	*<10*	*<50*	39
Fluoranthene	0.038	33	390	220	1300	920
Fluorene	<0.005	*<2*	12	86	70	120
Indeno(1,2,3-cd)pyrene	<0.02	9	40	*<10*	<50	150
1-Methylnaphthalene	<0.005	*<2*	0.2	*<10*	<50	7
2-Methylnaphthalene	<0.005	*<2*	0.2	*<10*	<50	6
Naphthalene	<0.005	*<2*	0.3	*<10*	*<50*	10
Phenanthrene	0.01	13	46	350	180	630
Pyrene	0.032	29	270	180	950	810
Total [PAH]	<0.25	161	1299	1079	3552	4802

Adapted from Mahmoudi et al., 2013

Table 3.S2 Grouped distribution of PLFA expressed as mole percentage

	Soil 1	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6
Monounsaturated FA	26.3	16.3	17.5	15.7	24.9	28.4
n-Saturated FA	22.3	25	38.4	35.2	25.7	39.2
Terminally branched saturated FA[a]	18.3	13.6	22.9	18.1	11.8	16.6
Mid branched saturated FA	18.1	34.1	14.5	22.0	22.1	12.7
Cyclopropyl FA[b]	10.8	11.0	6.7	9.1	10.8	3.1
Polyunsaturated FA	4.5	0	0	0	4.5	0

[a]iso- and anteiso- PLFA
[b]cyc 17:0 and cyc 19:0

CHAPTER 4

ASSESSING LIMITATIONS FOR PAH BIODEGRADATION IN LONG-TERM CONTAMINATED SOILS USING BIOACCESSIBILITY ASSAYS

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are generated by a range of industrial processes including petroleum and gas production and are often found in high concentrations at industrial sites. Once PAHs enter the environment, the predominant mechanisms for removal are biological via microbial activity. However, PAHs have the potential to partition onto soil organic matter thereby decreasing their bioavailability to microorganisms and limiting their degradation. This explanation was felt to be the reason for a lack of evidence of PAH biodegradation in a study of long-term contaminated soils. To test the hypothesis that bioavailability was a limiting factor for biodegradation in theses soils, PAH bioavailability was determined using non-exhaustive extraction (propanol, butanol, hydrooxypropyl-β-cyclodextrin) and oxidation (persulfate) methodologies designed to determine the fraction of contaminants within soil which are available for biological uptake. The assays gave varying results for each soil and no specific trends across all soils were observed. PAH bioaccessibility, derived from the HP-β-CD assay which has been the most extensively tested in the literature, was estimated to be between 0 to 10% for most soils, with the exception of pyrene, indicating that a large fraction of the soil-borne PAHs at the site are not available to microorganisms and that bioavailability limitations may be a primary cause for the lack of observed biodegradation at this site. These results highlight the importance of bioavailability to PAH degradation as well as the relevance of utilizing an assay that has been evaluated across many soil conditions and parameters.

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous organic contaminants that are associated with accidental spills, improper disposal and incomplete combustion of petroleum and petroleum-derived products. They are also generated by a range of industrial processes including oil and gas production and are often found in high concentrations at industrial sites. PAHs are of concern because many of them are classified as carcinogens and mutagens (Harvey, 1996; Pavanello and Lotti, 2012). Although immobilization can be a desired fate for some contaminants, removal of PAHs from environmental systems is preferable due to exposure hazards and the potential for migration with organic particles (Cachada et al., 2012; Fiala et al., 1999; Jones et al., 1989; Wang et al., 2012).

Once PAHs enter the environment, the predominant mechanisms for removal are biological via microbial activity, although physiochemical processes such volatilization can reduce the concentration of some PAHs. Laboratory studies have revealed that most PAHs are susceptible to degradation/transformation by either bacteria, fungi or algae (Cerniglia 1992; Cerniglia and Sutherland, 2010; Juhasz & Naidu, 2000), however, bioremediation of PAH-contaminated sites often fails to achieve target clean up levels (Erickson et al., 1993; Haritash and Kaushik, 2009; Weissenfels et al., 1992). The extent of biodegradation in the environment depends on the nature, concentration and chemical properties of the PAH as well as soil residence time. As PAHs 'age' and soil residence time increases, a greater proportion of PAHs diffuse into soil micropores and partition onto organic matter thereby decreasing their bioavailability to microorganisms and

limiting their degradation in the environment. Bioavailability, in the context of bioremediation, is defined as the extent to which a contaminant may be available for biological conversion and is considered to be a major limitation in remediating contaminated soils (Juhasz et al., 2000; Katayama et al., 2010; Maier, 2000). Consequently, microorganisms may preferentially utilize carbon sources that are more labile than PAHs such as natural organic matter (NOM), leading to the persistence of contaminants over time.

Although biodegradation is affected by a number of factors including pH, moisture content and nutrients, the fraction of contaminants available to microorganisms is crucial and can dictate the end-point for bioremediation. The term 'bioaccessibility' has been put forth to describe the amount of contaminant that is not only available a given point in time but the total fraction potentially available over time (Semple et al, 2004). In the last decade, a number of assays have been developed to measure PAH bioaccessiblity including exhaustive and nonexhaustive extraction and oxidation methods designed to remove the bioaccessible fraction from the soil in order to estimate the fraction which is potentially biodegradable. Exhaustive methods utilize solvents to extract total contaminant concentrations and have been shown to overestimate the bioaccessible fraction (Reid et al., 2000). In contrast, nonexhaustive extractions may use low-molecular-weight primary alcohols (such as propanol and butanol) which allow for mild extraction of hydrophobic compounds from soil (Semple et al., 2007). This approach has been successfully used to estimate the availability of pyrene and phenanthrene in spiked soils (Kelsey et al., 1996; Liste and Alexander, 2002). In recent years, aqueous extraction

techniques have also been developed, specifically hydroxypropyl-β-cyclodextrin (HP-β-CD) which has been shown to provide a good estimate of PAH bioaccessibility in a wide variety of spiked and contaminated soils (Reid et al., 2000; Cuypers et al., 2002; Juhasz et al., 2005, Stokes et al., 2005; van der Heijen and Jonker, 2009). Another developed assay is based on the partial selective chemical oxidation of soil organic matter by persulfate ($S_2O_8^{2-}$) (Cuypers et al., 2000). In limited studies, this assay has been shown to successfully predict the biodegradability of three and four ring PAHs (Cuypers et al., 2000; Juhasz et al., 2005).

In a previous study, natural abundance radiocarbon analysis of microbial phospholipid fatty acids (PLFA) was applied to soils collected from a former industrial site in southern Ontario contaminated with PAHs (Mahmoudi et al., 2013). By relating the ^{14}C contents of microbial lipids to surrounding carbon sources, microbial carbon sources were elucidated and found to be primarily derived from vegetation and/or natural organic matter present in the soils rather than PAHs. It was hypothesized that limited PAH bioavailability resulted in the lack of PAH metabolism observed at this site. As a consequence, the aim of the current study was to assess PAH bioavailability to determine whether this was a limiting factor for biodegradation in these long-term contaminated soils. Bioavailability was assessed using surrogate bioaccessibility assays included a variety of non-exhaustive extraction (propanol, butanol, hydrooxypropyl-β-cyclodextrin) and oxidation (persulfate) methodologies. The secondary aim of this study was to compare and contrast PAH bioaccessibility data across methodologies to determine

whether the predicted endpoints of biodegradation (based on PAH bioaccessibility) are similar irrespective of the assay used for their derivation.

4.2 Materials and Methods

4.2.1 Contaminated Soils

Soils were collected from the upper 0.5 to 0.6 m of surface soil at an industrial site in southern Ontario which operated from the 1950s to the mid 1990s. A detailed description of these soils can be found in Mahmoudi et al., 2013. Briefly, the total organic carbon content ranged from 0.4 to 3.0% and the total PAH concentration ranged from 161 to 4802 µg/g dry weight (Table 4.1).

4.2.2 Bioaccessibility Assays

Four non-exhaustive extraction methods and one oxidation method was used to assess PAH bioaccessibility. Bioaccessibility assays were performed on the < 2 mm soil particle size fraction following freeze-drying to remove residual moisture. All bioavailability assays were performed in triplicate. Bioaccessibility was based on total PAH concentration measured prior to the assay and the residual concentration following the assay, as shown in eq 1.

$$\text{PAH Bioaccessibility (\%)} = \frac{PAH_{soil} - rPAH_{soil}}{PAH_{soil}} \times 100 \qquad [1]$$

Where:

PAH_{soil} = the initial concentration of individual PAHs in soil prior to bioaccessibility assessment (mg kg^{-1}).

$rPAH_{soil}$ = the residual concentration of individual PAHs in soil following bioaccessibility assessment (mg kg^{-1}).

Non-exhaustive solvent extractions

Low-molecular weight primary alcohols (100% 1-butanol, 100% 1-propanol and 50% (v/v) 1-propanol in water) were used to extract PAHs from contaminated soils (Tang and Alexander, 1999; Kelsey et al., 1996; Liste and Alexander, 2002). Five grams of dry soil was added to glass Schott bottles containing 50mL of the low-molecular weight primary alcohol. Samples were shaken end over end (150 rpm) for 60 minutes. Immediately following extraction, samples were transferred to Teflon centrifuge tubes and centrifuged (3074*g*, 5 minutes) to remove suspended particles. Subsequently, the alcohol extract was decanted and soil samples were retained for analysis of residual PAHs following freeze-drying.

Hydroxypropyl-β-cyclodextrin extraction

Hydroxypropyl-β-cyclodextrin (HP-β-CD) extraction of contaminated soil was performed according to the method of Reid et al., 2000. Five grams of soil was added to glass Schott bottles containing 100mL HP-β-CD solution (40mM). Samples were shaken end-over-end for 20 hours at room temperature. Following extraction, samples were centrifuged (3074*g*, 5 min), supernatants discarded and soils recovered for residual PAH analysis following freeze-drying.

Persulfate oxidation

Persulfate oxidation of contaminated soils was performed according to the method of Cuypers et al., 2000. Five grams of dry soil was mixed with potassium persulfate and

deionized water to achieve a perfulfate to organic matter ratio of 12 g g^{-1} equivalent to an aqueous persulfate concentration of 35.7 g L^{-1}. The soil-persulfate slurry was incubated at 70°C, shaking end to end (150 rpm) for 3 hours. Subsequently, slurries were centrifuged (3074*g*, 5 min), supernatants discarded and soils recovered for PAH extraction following freeze-drying.

PAH quantification

Recovery of PAHs from contaminated soils was achieved using an accelerated solvent extraction method (ASE200 Accelerated Solvent Extraction System, Dionex Pty Ltd., Lane Cove, NSW, Australia). Prior to use, 1g of solvent washed silica gel (Davisil, Sigma-Aldrich Pty Ltd, Sydney, Australia) was sandwiched between two cellulose filter circles and added to 11mL ASE extraction cells in order to assist in sample clean-up prior to GC analysis of PAH extracts. Approximately 2g of freeze-dried soil was ground with diatomaceous earth (Dionex), weighed into extraction cells (on top of the silica layer) and surrogate standard (benzo[*b*]fluorene 100µl: 1mg ml^{-1}) added prior to sealing. Soils were extracted using standard conditions (150°C, 10.34 MPa, static time 5 min) and a solvent mixture consisting of hexane:acetone (1:1 v/v). Subsequently, soil extracts were concentrated to dryness under a steady flow of nitrogen gas and resuspended in 2 mL of hexane:acetone (1:1 v/v) prior to filtering through 0.45µm Teflon syringe filters into 2mL GC vials prior to analysis.

Gas chromatographic analysis of extracts and PAH standards were performed on an Agilent Technologies 7890A gas chromatograph with flame ionization detector. Samples were separated using a 30 m x 0.25 mm x 0.25 µm Zebron ZB-50 column

(Phenomenex Australia, Lane Cove, NSW, Australia). The oven temperature was programmed at 80°C for 2 min followed by a linear increase to 290°C at 5°C min^{-1} followed by an increase of 20°C min^{-1} to a final temperature of 330°C which is held for 15 min once reached. Injector and detector temperatures were maintained at 320°C and 330°C, respectively. Surrogate recovery during PAH quantification ranged from 96 to 107% while results of duplicate analysis of the same sample showed a standard deviation of less than 9%. An uncontaminated soil was also used as a reference blank for each assay and PAH concentrations were found to be less than the limits of quantification.

4.3 Results and Discussion

4.3.1 Comparison of PAH bioaccessibility estimates according to assays

PAH bioavailability is a critical parameter influencing the extent of biodegradation (Pignatello & Xing, 1995). As a result, assays that can estimate PAH bioavailability (i.e. bioaccessibility assays), in the context of biodegradation, and therefore predict the endpoints of bioremediation are valuable assessment tools. In our previous study (Mahmoudi et al., 2013), it was hypothesised that the lack of biodegradation in long-term contaminated soils was a result of limited PAH bioavailability. This study aimed to test that hypothesis through the use of non-exhaustive extraction and oxidation methodologies. Although these assays have previously been used to measure PAH bioaccessibility (Cuypers et al., 2000; Juhasz et al., 2005; Kelsey et al., 1996; Liste and Alexander, 2002; Breedveld and Karlsen, 2000), data for some assays is limited to a narrow number of spiked soils and therefore the strength of

their predictive capabilities is open to discussion. As a result, a comparative study was undertaken (as these are rare) to determine if variability in PAH bioaccessibility data existed when different assays were applied to different soils from a field site environment where PAHs have undergone aging under in situ conditions for an extended period of time (up to 40 years). Data for alcohol-based extraction methods and persulfate oxidation were compared to HP-β-CD bioaccessibility data as numerous studies have demonstrated the robustness and reproducibility of this assay for estimating PAH bioavailability across a variety of soil types, PAH concentrations and PAH sources (Cuypers et al., 2002; Stokes et al., 2005; Juhasz et al., 2005; Allan et al., 2006; Doick et al., 2005, 2006; Papadopoulos et al., 2007a, 2007b; Hickman et al, 2008; Stroud et al., 2009).

For the five PAH contaminated soils used in this study, the assays gave varying results for each soil and no specific trends across all soils were observed suggesting that assessment of bioaccessibility was assay dependent. PAH bioaccessibility values based on persulfate oxidation varied from HP-β-CD values and the relationship was poor (slope= 0.72, r^2= 0.37). In most cases, persulfate oxidation overestimated HP-β-CD bioaccessibility for three and five ring compounds and underestimated HP-β-CD bioaccessibility for four-ring compounds (Figure 4.1D). Likewise, the relationship between HP-β-CD and alcohol-based PAH bioaccessibility values were poor, with alcohol-based extractions producing values that were up to 3.2, 3.8 and 2.7-times greater for 3, 4 and 5 ring PAH compounds respectively (Figure 4.1A, B, C). Similar results were observed by Juhasz et al., 2005 where propanol and butanol-based extraction of creosote-contaminated soil gave PAH bioaccessibility values that were up to 3 times greater than

HP-β-CD and persulfate methodologies. Higher estimates of PAH bioaccessibility is likely due to the cosolvency power of low-molecular weight alcohols which leads to an increased amount of PAHs being desorbed from soils resulting in lower residual concentrations. Moreover, the cosolvency power increased as the cosolvent carbon length and composition increased. This trend would explain the observation that butanol-based extraction provided higher estimates of PAH bioaccessibility compared to propanol. Although the relationship was poor (r^2= 0.38), the 50%-propanol-based assay provided the best correlation with HP-β-CD bioaccessibility values (slope = 0.77) since it had a lower cosolvency power and decreased potential for PAH desorption from soils. These results emphasize the variability in PAH bioaccessibility results obtained for different assays and highlight the importance of utilizing an assay that has been evaluated across many soil conditions and parameters. Assessment of PAH bioaccessibility can provide insight into the predicted endpoints of biodegradation however selecting an appropriate method is critical. Certain methodologies, such as propanol and butanol-based assays, may significantly underestimate the residual fraction and overestimate endpoints in PAH biodegradation leading to inaccurate assessments of overall bioremediation potential. In the following sections, we focus on PAH bioaccessibility data for the five soils derived from the HP-β-CD assay because the effectiveness and reproducibility of this method has been demonstrated across a number of soil types.

4.3.2 Three-ring PAHs

When assessing bioaccessibility using HP-β-CD extraction, phenanthrene bioaccessibility was low in four out of five soils (2, 3, 5 and 6) and was estimated to be

0.0 - 20.7% ± 4.2% (Figure 4.2A). In contrast, phenanthrene bioaccessibility was 74.3% ± 5.7% in soil 4 which suggest that the majority of this PAH is available for biodegradation. Similar results were obtained for anthracene where bioaccessibility was estimated to be 0.0% in soils 2, 3, 5 and 6 whereas in soil 4 bioaccessibility was 44.4% ± 1%. The biodegradation of phenanthrene and anthracene by bacterial and fungal species has been well demonstrated (Cerniglia, 1992, 1997; Samanta et al., 1999; Jacques et al., 2005; Hinga, 2003) and the degradative pathways have been elucidated (Menn et al., 1993; Kiyohara et al., 1994; Pinyakong et al., 2003; Habe & Omori, 2003). Moreover, the metabolic capability to degrade three-ring compounds is thought to be widespread among bacteria and fungi (Peng et al., 2008). Thus, the biodegradation of three-ring compounds is often influenced by environmental factors (including bioavailability) rather than metabolic potential. DNA fingerprinting of these soils revealed the presence of active microbial communities (composed of both bacteria and eukarya) with cell densities ranging from 3.7×10^7 to 1.5×10^8 (Mahmoudi et al., 2011), which is consistent with previously reported values for contaminated environments (Green and Scow, 2000). In addition, natural abundance radiocarbon analysis of microbial lipids and surrounding carbon sources revealed that microbial communities were preferentially utilizing other carbon sources such as NOM rather than PAHs (Mahmoudi et al., 2013). Although we cannot rule out the influence of factors such as nutrients, redox potential, etc. for the lack of biodegradation, previous analyses demonstrate that these soils have metabolically active microbial communities that are utilizing non-dervied PAH carbon from their surrounding environment. Similar behaviour was observed in salt marsh sediments

contaminated by the *Florida* oil spill of 1969 in which the microbial community was preferentially utilizing natural organic matter as opposed to petroleum hydrocarbons (Slater et al., 2005).

4.3.3 Four-ring PAHs

Some variability in bioaccessibility was observed between four-ring PAHs and contaminated soils. Fluoranthene and chrysene bioaccessibility was less than 10% ± 3.4% for soils 2, 3, 5 and 6 with higher values (44.8% ± 3.4% and 19.7± 13.3%, respectively) observed in soil 4 (Figure 4.2B). Greater variability between soils was observed for pyrene with bioaccessibility ranging from 0.0% for soils 3 and 6, 21.6% ± 12.0% for soil 4 to 100.0% ± 1.0% for soils 2 and 5. Likewise, Papadopoulous et al. 2007a found pyrene bioaccessibility to vary across contaminated soils collected from a gas plant and Juhasz et al., 2005 noted variability in bioaccessibility of four-ring compounds in creosote-contaminated soil. Similar to three-ring compounds, the biodegradation of four-ring compounds has been well studied and the genetic capability to degrade these compounds is thought to be common among many bacteria and eukarya (Caldini et al., 1995; van Herwijnen et al., 2003 Kim et al., 2007; Peng et al., 2008; Xu et al., 2010; Ting et al., 2011). This supports the notion that bioaccessibility is a limiting factor for some four-ring compounds although there is some variability between soils which may be due to the source or timing of PAH deposition.

4.3.4 Five-ring PAHs

Bioaccessibility of benzo(b)fluoranthene and benzo(a)pyrene was less than 10% for all soils indicating that only a small fraction of five-ring compounds are available for

biodegradation (Figure 2C). Other studies have reported five-ring PAH bioaccessibility in soils to be 10.3 and 12.0% (Papadopoulos et al., 2007a; Juhasz et al., 2005) however biodegradability of five-ring PAHs tends to be over-estimated based on bioavailability estimates. Previous studies have observed limited degradation of five-ring compounds which were thought to be bioavailable to soil microorganisms (Cornelissen et al., 1998; Huesemann et al., 2002, 2003, 2004; Juhasz et al., 2005). Five ring PAHs are more resistant to microbial attack due to biological factors such as high activation energies, unfavorable Gibbs free energy, or slow transport across the cell membrane (Bonten, 2001). Therefore, the lack of observed microbial degradation for five-ring compounds likely depends on multiple factors and cannot be accounted for solely by low bioaccessibility.

4.3.5 PAH bioaccessibility at this site

The bioaccessiblity of all PAHs at this site was estimated to be between 0 to 10% for most soils, with the exception of pyrene, indicating that a large fraction of the soil-borne PAHs at the site are not available to soil microorganisms and therefore may not undergo significant biodegradation. The degradation of lipophilic organic contaminants is thought to be controlled by microbial activity (specifically the rate of transfer to the cell and the rate of uptake and metabolism by PAH-degrading microorganisms), as well as the extent of contaminant sequestration by binding processes inherent to the soil. Microorganisms have the potential to degrade most PAHs dissolved in soil solution, however, degradation of sequestered contaminants may occur very slowly (due to the presence of a slowly desorbable fraction) or not at all (Alexander, 2000; Pignatello and

Xing, 1995, Zhang et al., 1998). In 'aged' soils, the rate and extent of biodegradation is thought to be controlled primarily by the rate of desorption or dissolution of contaminants from the sorbed phase into solution where they will be available to soil microorganisms for uptake and degradation (Alexander, 2000). Therefore, bioavailability is determined by the rate of mass transfer relative to microbial activity and is cited as an important factor for biodegradation in contaminated soils (Bosma et al., 1996). In this study, we found low PAH bioaccessibility which is not surprising since the industrial site from which soils were collected was in operation for over 40 years and PAHs were likely deposited over time and aged. Although limited PAH biodegradation was observed to be currently occurring in these soils (Mahmoudi et al., 2013), it is likely that degradation occurred in the past when PAHs were initially deposited onto soils and bioavailability constraints were limited. It is generally thought that biodegradation rates decrease over time as a greater percentage of PAHs become less bioavailable and the recalcitrant fraction is left behind (Shuttleworth & Cerniglia, 1995). Laboratory studies have demonstrated that PAH biodegradation rates in aged and freshly spiked soils slow down in a matter of days or weeks (Lee et al., 2001; Yuan et al., 2001; Huesemann et al., 2002).

Environmental factors such as pH, temperature, organic matter content and quality/type of clay materials can influence the sorption and desorption of PAHs which ultimately reduces their bioavailability. Mechanisms which can occur during 'aging' and lead to sorption include a number of soil-PAH interactions such as van der Waals forces, cation bridging, hydrogen bonding, ion exchange or covalent bonding (Khan & Ivarson, 1982; Isaacson & Frink, 1984). Soil organic matter is the primary sorbent for

hydrophobic contaminants however many other soil properties such as cation-exchange capacity, texture and nanoporosity can also influence sequestration (Amellal et al., 2001; Chung & Alexander, 2002). The presence of nanopores with diameters of 0.3 – 1.0 nm can lead to localized sequestration since contaminants may diffuse into pores and voids and become entrapped in soil aggregates (Alexander, 2000). In addition, if pores contain hydrophobic surfaces, desorption will be exceedingly slow thereby resulting in the contaminant being poorly available (Nam & Alexander, 1998). Although PAH bioaccessibility was found to be low in most soils, soil 4 had consistently higher PAH bioaccessibility ranging from 44.0 to 74.3% for three-ring PAHs and 19.7 to 44.8% for four-ring PAHs. Based on soil analysis (Table 4.1), soil 4 has a greater sand content relative to soils 2, 3 and 5 which may result in greater release of PAHs due to weaker PAH-soil interactions and/or lower nanonporosity compared to soils with higher clay and silt content. This is consistent with the notion that the propensity for PAH sequestration is less in soils with higher sand content due to greater flux of PAHs from the sorbed to solution phase compared to soils with higher clay content. Although soil 6 also contains a high sand content PAH bioaccessibility was reduced for this soil. However our knowledge regarding the nature and chemical properties of the organic matter present in these soils is limited and perhaps the chemical and physical properties of the organic matter in soil 6 may be leading to greater sorption and therefore reduced bioaccessibility of PAHs. It may also be the case that PAHs in soil 4 were deposited at later time relative to the other soils and less 'aging' occurred. Resolving differences in bioaccessibility

across soils is difficult since many factors influence sorption and mechanisms underlying this process are poorly understood.

4.3.6 Implications for site remediation

As contaminants 'age' they become less available to soil microorganisms, less readily assimilated by earth worms (Kelsey & Alexander, 1997) and continue to persist in the environment. Concurrently acute and chronic toxicity of harmful contaminants also declines as they become increasingly sequestered over time (Alexander, 2000). Many contaminants including benzo[a]pyrene have been shown to become less hazardous to test mammals after they are added to soil for a short period of time (Abdel-Rahman et al., 1992; Yang et al., 1989, Roy & Singh, 2001; Rostami & Juhasz, 2012). Similarly, the toxicity of herbicides such as simazine and atrazine is greatly reduced over time and has less impact on the growth of seedlings and plants (Bowmer, 1991; Scribner et al., 1992). Insecticides including DDT and dieldrin have also shown to be less toxic to insects once the contaminant had been in soil for a number of days to weeks (Robertson & Alexander, 1998; Morrison et al., 2000). However a recent study found that residual PAHs which are thought to be non-bioavailable may still be bioavailable to important receptor organisms such as earthworms and plants (Juhasz et al., 2010).

Although toxicity is thought to be reduced with aging, there is continued risk for exposure hazards and leaching to drinking water sources. In addition, contaminants may become released following physical disturbance if they are present in pockets in nonaqueous-phase liquids within soil (Alexander, 2000). For soils 2, 3, 5 and 6, it is likely that the toxicity of PAHs in these soils is low since they have been increasingly

sequestered from plants and insects. However based on government standards, these soils will still require remediation. Surfactants and other organic solvents may be used to enhance bioavailability of PAHs to microorganisms if bioremediation is the selected as the appropriate remediation strategy (Tiehm, 1994; Volkering et al., 1995; Thibault et al., 1996; Zhu & Aitken, 2010). The use of surfactants for bioremediation has produced variable results and in some cases it has stimulated biodegradation while in others it was found to inhibit biodegradation (Singh et al. 2007). Variable results with surfactants may be due to differences in the nature of the contaminants, characteristics of the contaminated medium, surfactant properties and the physiology of the organisms involved (Van Hamme et al., 2003). Therefore, further investigation of these characteristics would be useful prior to adding surfactants in order to enhance PAH bioavailability. In the case of soil 4, bioremediation may still be a relevant and more appropriate option since PAH bioaccessibility is estimated to be higher and perhaps stimulation of the microbial community may reduce the concentration of three and four-ring compounds. However, reducing concentrations of five-ring compounds is much more challenging and may require alternative treatment strategies.

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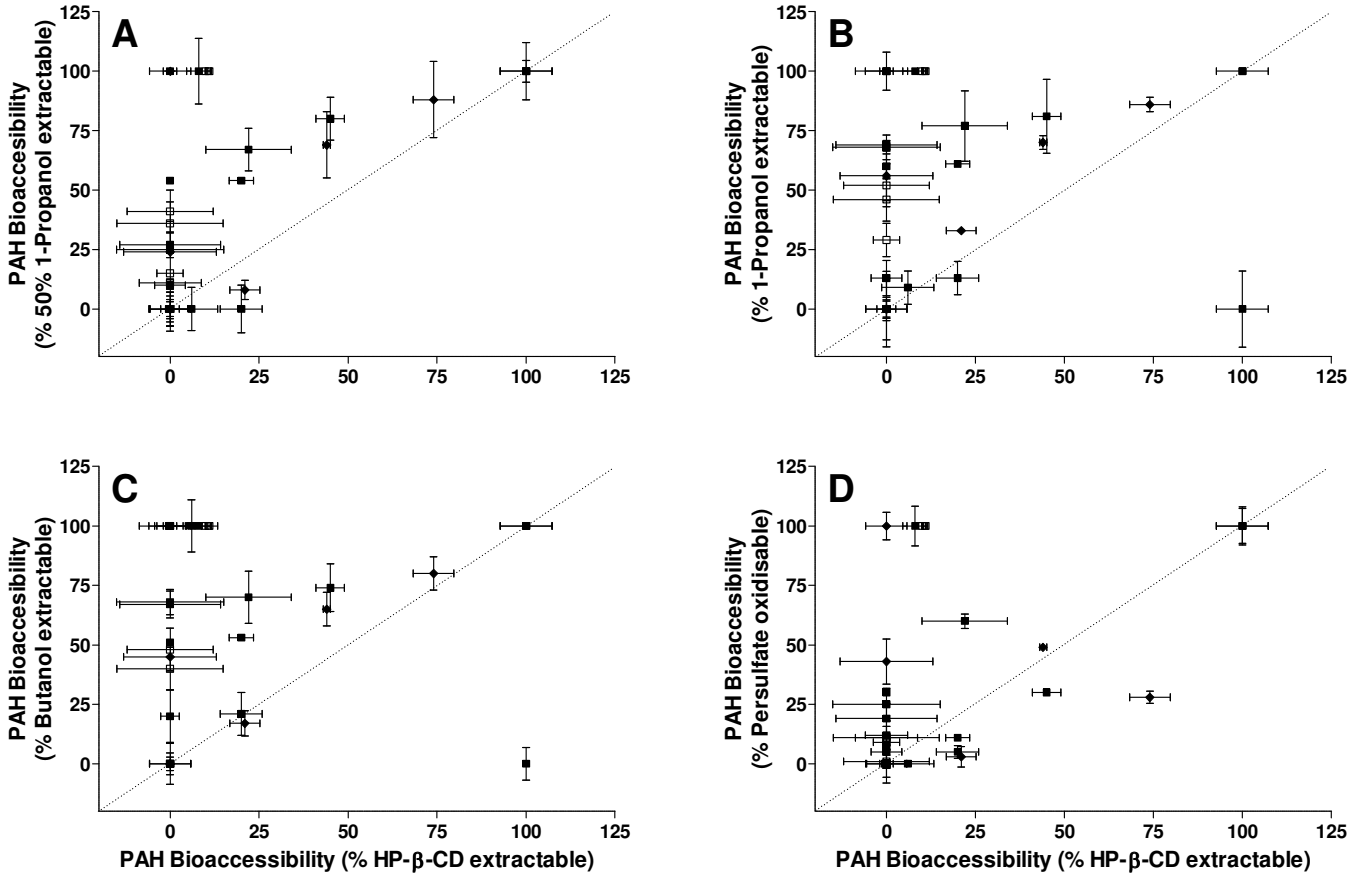

Figure 4.1 PAH bioaccessibility (%) estimated across soils using HP-β-CD extraction versus PAH bioaccessibility (%) estimated across soils using (A) 50% propanol extraction (B) 100% propanol extraction (C) 100% butanol extraction (D) persulfate oxidation. The dotted line represents a hypothesized 1:1 correlation.

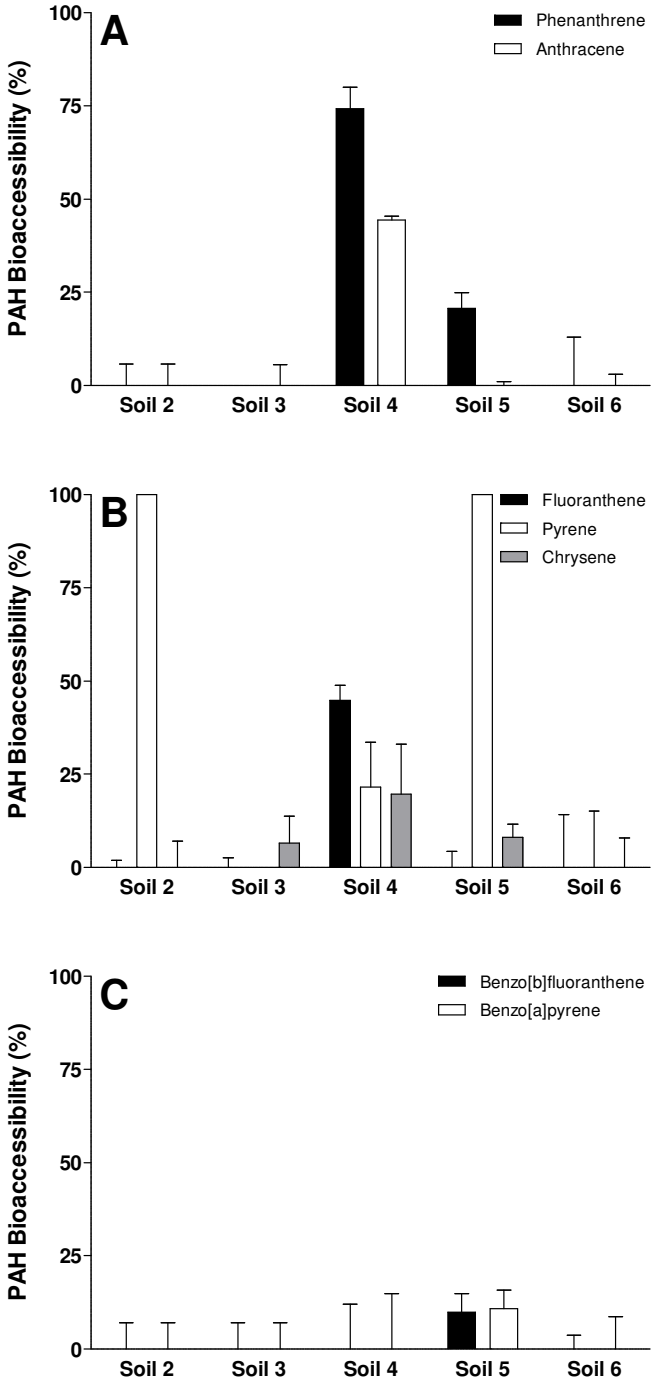

Figure 4.2 Bioaccessibility of representative three- (A), four- (B) and five-ring PAHs (C) across all soils following HP-β-CD extraction. Error bars represent the standard deviation of triplicate analysis.

Table 4.1 Characteristics of contaminated soils used in this study*

	Soil 2	Soil 3	Soil 4	Soil 5	Soil 6
Polycyclic aromatic hydrocarbons (µg/g)					
Acenaphthene	*<2*	20	110	200	130
Anthracene	3	62	32	400	220
Benzo(a)anthracene	13	110	31	150	420
Benzo(a)pyrene	14	64	12	<50	290
Benzo(b/j)fluoranthene	19	95	23	87	420
Benzo(g,h,i)perylene	8	39	*<10*	*<50*	140
Benzo(k)fluoranthene	8	39	11	55	150
Chrysene	12	97	24	160	340
Dibenz(a,h)anthracene	*<2*	13	*<10*	*<50*	39
Fluoranthene	33	390	220	1300	920
Fluorene	*<2*	12	86	70	120
Indeno(1,2,3-cd)pyrene	9	40	*<10*	<50	150
Phenanthrene	13	46	350	180	630
Pyrene	29	270	180	950	810
Total [PAH]	161	1299	1079	3552	4802
Organic carbon (%)	0.4	3.0	2.4	0.4	2.0
Soil texture	Silt loam	Silt loam	Sandy loam	Silt loam	Loamy sand
Clay (%)	27	22	4	26	2
Silt (%)	73	78	25	74	13
Sand (%)	0	0	70	0	84

*adapted from Mahmoudi et al., 2011

CHAPTER 5

RAPID DEGRADATION OF *DEEPWATER HORIZON* SPILLED OIL BY INDIGENOUS MICROBIAL COMMUNITIES IN LOUISIANA SALT MARSH SEDIMENTS

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Abstract

The *Deepwater Horizon* oil spill released almost 5 million barrels of oil into Gulf of Mexico waters leading to severe contamination of surrounding coastal environments. A previous study detailed coastal salt-marsh erosion and recovery in a number of oil-impacted and non-impacted reference sites in Barataria Bay, Louisiana over the first 18 months after the spill (Silliman et al., 2012). Concentrations of alkanes and polyaromatic hydrocarbons (PAHs) at oil-impacted sites significantly decreased over this time period. Here, a combination of DNA, lipid and isotopic approaches confirm that microbial biodegradation contributed to the observed petroleum mass loss and led to changes in community structure of the indigenous microbial communities. Natural abundance ^{14}C analysis of microbial phospholipid fatty acids (PLFA) reveal that petroleum-derived carbon was a primary carbon source for microbial communities at impacted sites several months following oil intrusion, while ^{13}C analysis of lipids was ineffective at resolving petroleum biodegradation Pyrosequencing of all three microbial domains at this time show an increase in the relative abundance of taxonomic groups known to include hydrocarbon-degrading bacteria, such as *Sphingomonadales*. These results suggest that Gulf of Mexico marsh sediments have considerable biodegradation potential and that natural attenuation is playing a role in impacted sites.

5.1 Introduction

The April 2010 explosion on the *Deepwater Horizon* drilling platform resulted in the largest accidental marine oil spill in recorded history. Over the course of three

months, approximately 5 million barrels of light crude oil (MC252) were released into Gulf of Mexico waters leading to the contamination of hundreds of kilometers of shoreline (Camilli et al., 2010; Crone et al., 2010; Atlas and Hazen, 2011; Thibodeaux et al., 2011). Coastal ecosystems provide important ecological and economic services such as storm protection, carbon sequestration and nurseries for fish and shellfish (Mitsch and Gosselink, 2000; Engle, 2011; Deegan et al., 2012). Salt marshes are particularly vulnerable to oil contamination due to low wave action and high organic matter content of sediments which lead to greater sorption of organic contaminants (Venosa et al., 2002). Previous studies have shown that spilled oils can persist in salt marshes for years to decades (Reddy et al., 2002; Peacock et al., 2005, 2007; Slater et al., 2005; Oudot et al., 2010).

In situ biodegradation by indigenous microbial communities is one of the most effective methods to remediate oil spills, particularly in salt marsh environments where physical clean-up activities can cause further damage to vegetation (Hoff, 1995; Pezeshki et al., 2000). Although microorganisms with the metabolic capability to degrade hydrocarbons have been well-studied, the majority of biodegradation studies have focused mainly on bacterial degradation of hydrocarbons under homogenous enrichment conditions in laboratory settings (Carmen et al., 1996; Coates et al., 1997; Cui et al., 2008; Teramoto et al., 2009; Jung et al., 2010; Lekunberri et al., 2010; Dash et al., 2013). However, the natural environment is highly variable and microbial degradation can be affected by a number of factors including temperature, nutrients, oxygen, salinity and the physical state of the oil (Atlas, 1981; Leahy and Colwell, 1990). Thus, our ability to

discern the response of microbial communities to petroleum contamination under *in situ* conditions is limited. Knowledge of microbial community structure and the response of crucial hydrocarbon-degrading groups can provide insight into the dynamics and mechanisms controlling the growth and activity of microorganisms in contaminated environments (Head et al., 2006). The *Deepwater Horizon* oil spill has provided a unique opportunity to investigate the response of all three microbial domains to severe petroleum contamination under *in situ* conditions and to assess which members actively degrade hydrocarbons. To date, studies have observed enrichment of hydrocarbon-degrading bacteria and fungi in a number of marine environments following the *Deepwater Horizon* oil spill including the water column, beach sands and marsh sediments (Hazen et al., 2010; Kostka et al. 2011; Lu et al., 2011; Beazeley et al., 2012; Bik et al.,2012; Mason et al., 2012; Redmond and Valentine, 2012).

Beyond identifying hydrocarbon-degrading microbial groups, demonstrating biodegradation under *in situ* conditions is challenging. Due to the heterogeneous distributions of contaminants, high levels of oil sorption to organic matter and minerals, and dynamic conditions of coastal environments, it is often difficult to confirm that mass loss of petroleum is due to biodegradation rather than abiotic weathering processes such as photooxidation and hydrolysis. Compound specific radiocarbon (^{14}C) analysis of microbial phospholipid fatty acids (PLFA) has become a powerful tool for elucidating microbial carbon sources thus confirming *in situ* biodegradation in complex environmental systems (Slater et al., 2005, 2006; Wakeham et al., 2006; Ahad et al., 2010, Cowie et al., 2010; Mahmoudi et al., 2013). This technique is based on the million

year age ranges of petroleum which will therefore have no detectable ^{14}C due to radioactive decay ($\Delta^{14}C$= -1000‰). In contrast, sedimentary organic matter contains carbon with variable radiocarbon ages (Trumbore, 2000) including recently photosynthesized material with a $\Delta^{14}C$ value of approximately +50‰, consistent with a modern atmosphere source (Czimczik and Welker, 2010). Thus, microbial uptake and incorporation of petroleum carbon will reduce the ^{14}C content of microbial membrane lipids, such as PLFAs, relative to the surrounding natural organic matter (Slater et al., 2005). In addition, salt marshes provide the opportunity to use variations in the natural abundances of stable carbon (^{13}C) to identify microbial carbon sources (Boschker and Middelburg, 2002). Coastal marshes are dominated by grasses, specifically *Spartina sp.*, which have $\delta^{13}C$ values of -12 to -14‰ and sedimentary organic matter in these marshes have $\delta^{13}C$ values ranging -14.4 to -17‰ (Chmura et al., 1987; Natter et al., 2012). In contrast, crude oils are more depleted in ^{13}C (MC252 oil, $\delta^{13}C$ = -27‰). This isotopic difference was used to demonstrate that *Spartina*-derived organic matter, rather than petroleum hydrocarbons, was the primary source of carbon for microbial communities in other oil-impacted salt marsh sediments (Slater et al., 2005; Pearson et al., 2008).

In a previous study, salt-marsh erosion and ecological impacts and recovery were detailed at a number of oil-impacted sites in Barataria Bay, Louisiana, which experienced some of the most extensive oil contamination due to the *Deepwater Horizon* oil spill (Silliman et al., 2012). Marsh sediments collected at 5, 11 and 18 months following oil intrusion showed significant decreases in hydrocarbon concentrations such that by 18 months (October 2011), oil-impacted sites had hydrocarbon concentrations comparable to

those of non-impacted reference sites. The study reported here used the same samples to assess changes in microbial community structure and carbon sources over the first 18 months after the spill. Both natural abundance ^{13}C and ^{14}C analyses of microbial PLFA were used to confirm whether biodegradation was a process contributing to the observed petroleum mass loss. Concurrently, changes in community structure in all three microbial domains due to oil contamination was assessed via pyrosequencing in order to identify the taxonomic groups that may be involved in petroleum biodegradation.

5.2 Materials and Methods

5.2.1 Study Site and Sample Collection

All sampling sites were located in *Spartina alterniflora*-dominated salt marshes in Barataria Bay, Louisiana. Samples were collected from two "impacted" sites and two "reference" sites (Figure 5.S1, Table 5.S1). A detailed description of these sites can be found in Silliman et al., 2012. Briefly, impacted sites had evident oil residues and oil-covered dead and decaying grass stems whereas reference sites had no visible oil residues on substrates or marsh plants. Sediment samples were collected 3 m from the marsh platform edge at each site in October 2010, April 2011 and October 2011 (approximately 5, 11 and 18 months after initial oil intrusion). For each sample, four cores, 5 cm in depth, were homogenized in the field and placed in precombusted glass jars and maintained in a cooler until reaching the laboratory, at which point they were stored at -80ºC.

5.2.2 Quantification of hydrocarbons

Methods used to analyze sedimentary hydrocarbon compound concentrations in marsh sediments are detailed elsewhere (Silliman et al., 2012). Briefly, 0.05–2.5 g wet sediment samples were spiked with a range of deuterated PAH and alkane standards. The samples were extracted three times by accelerated solvent extraction (ASE) using a hexane and acetone mixture (50:50, v/v), concentrated, then back-extracted three times into a 50:50 (v/v) mixture of aqueous sodium chloride and hexane to remove the remaining water. Samples were purified and separated into alkane and PAH compound classes using activated silica open-column chromatography. Alkanes and their associated 'unresolved complex mixture' (UCM), were analyzed by gas chromatography (Shimadzu GC-2010 coupled with an FID). The alkanes were quantified using alkane standard compound added just prior to GC injection and the UCM was quantified assuming the average relative response factor for C16-C32 alkane standards. Polyaromatic hydrocarbons (PAHs) were identified and quantified by GC-mass spectrometry (Shimadzu GC-2010 coupled with a MS GCMS-QP2010S).

5.2.3 Microbial PLFA analysis

For each sediment sample, 40 to 100g of sediment was extracted using a modified Bligh and Dyer method (White et al., 1979) as per Slater et al. (2005). Briefly, lipids were separated into non-polar, neutral and polar fractions using silica gel chromatography. The polar fraction, which contained phospholipids, was subjected to mild alkaline methanolysis and converted to fatty acid methyl esters (FAMEs). Details of phospholipid extraction, purification and identification are described in the Supporting Information.

5.2.4 Compound specific stable carbon and radiocarbon analysis

Stable carbon and radiocarbon isotopes were measured in microbial PLFA and total organic carbon pools in sediments collected 5 and 18 months after initial oil contact. Comparison between isotope signatures of PLFA and the surrounding carbon sources identifies uptake and incorporation of petroleum carbon by the indigenous microbial community.

Bulk PLFAs were used for $\delta^{13}C$ and $\Delta^{14}C$ analyses since there was insufficient sample to analyze individual PLFAs. However, bulk PLFA analysis still allows for accurate assessment of microbial carbon sources since there is little variation between the isotopic signature of PLFA compounds from the same site (Slater et al., 2005; Wakeham et al., 2006; Cowie et al., 2010). PLFA fractions were confirmed to contain only FAMEs by GC/MS analysis and therefore to be free of any background lipid contamination. Process blanks which were exposed to the same solvents and procedures as sediment samples were also found to be free of contaminants.

Bulk TOC-$\delta^{13}C$ and of TOC-$\Delta^{14}C$ of each sample was determined after decarbonating oven dried sediment (50 ºC for 48 hours) using HCl. In addition, $\delta^{13}C$ and $\Delta^{14}C$ of residual sediment remaining following solvent extraction, defined as solvent-extracted residues (EXT-RES), was also determined. Based on White et al. (2005), this EXT-RES was taken to represent the natural organic matter since any petroleum hydrocarbons are removed during extraction. Details of the total lipid extraction procedure used can be found in the Supporting Information.

Stable and radiocarbon isotope signatures of PLFA, TOC and EXT-RES were measured at the Centre for Applied Isotope Studies at University of Georgia. Samples were combusted to carbon dioxide (CO_2) and approximately 10% of the CO_2 was reserved for $\delta^{13}C$ analysis which was performed on a dual inlet Finnigan MAT 252 Isotope Ratio Mass Spectrometer (IRMS). The remaining CO_2 was converted to graphite for $\Delta^{14}C$ analysis by accelerator mass spectrometry (AMS). Radiocarbon values were normalized to a $\delta^{13}C$ of -25‰ and expressed in $\Delta^{14}C$ notation as per mille (‰) relative to deviation from the ^{14}C Standard Reference Material 4990B oxalic acid (Stuiver and Polach, 1977). This normalization removes the effects of isotopic fractionation, thereby allowing for interpretation of $\Delta^{14}C$ as a direct tracer of microbial carbon sources (Slater et al., 2005). In this context, petroleum will have a $\Delta^{14}C$ value of -1000‰ since it contains no detectable ^{14}C whereas carbon derived from recently photosynthesized materials will have values of approximately +50‰.The accuracy and reproducibility for $\Delta^{14}C$ analysis was ±10‰ for TOC and EXT-RES and ±20‰ for PLFA; and ±0.5‰ for $\delta^{13}C$ analysis. These errors include the accuracy and precision of the instrument as well as the limitations of the preparation method (Cherkinsky et al., 2013; Culp et al., 2013).

5.2.5 Microbial DNA analysis

Genomic DNA was extracted from two replicate sediment samples collected in October 2010 and October 2011 time points (5 and 18 months after oil intrusion) using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA) according to manufacturer's protocol. Three DNA extractions per replicate sediment sample was pooled and sent to Molecular Research LP (Lubbock, TX, USA) for tag-encoded GS FLX

Titanium amplicon pyrosequencing (Roche 454). The 16S ribosomal DNA (rDNA) was targeted using primers 27F-530R for Bacteria and Arch344F-Arch915R for archaea (Lane, 1991; Raskin et al., 1994). The fungal internal transcribed spacer (ITS) region of rDNA was targeted using primers ITS1F-ITS4R (White et al., 1990; Gardes and Gruns, 1993; Manter et al., 2007). Details of microbial DNA analysis including primer sequences and bioinformatic analyses can be found in the Supporting Information.

5.3 Results

5.3.1 Hydrocarbons

Five months after the spill the impacted sites had UCM concentrations of 26,465 to 50,380 mg/kg, total alkane concentrations of 1303 to 6987 mg/kg and PAH concentrations of 16.2 to 99.4 mg/kg (Figure 5.1). These concentrations were 100 times higher than those of the reference sites which had UCM concentrations of 18 to 280 mg/kg, total alkane concentrations of 17 to 52 mg/kg and total PAH concentrations of 1.1 to 1.5 mg/kg. Following the 5 month time point, UCM, alkane and PAH concentrations at impacted sites rapidly decreased and by 11 months, concentrations had been reduced by 80-90%. By 18 months, PAH, alkane and UCM concentrations at impacted sites were almost equivalent to those at reference sites.

5.3.2 Microbial PLFAs

Microbial PLFA concentrations were greater at reference sites at 5 and 11 months after oil intrusion compared to those of impacted sites (Figure 5.1, Table 5.S2). However, by 18 months, PLFA concentrations at impacted sites were comparable to those at

reference site 1. PLFA concentrations at reference site 2 decreased over time whereas PLFA concentrations at the other three sites remained relatively consistent or increased slightly. Using an average conversion factor of 4 x 10^4 cells $pmol^{-1}$ of PLFA (Green and Scow, 2000), these PLFA concentrations correspond to cell densities of 4.6 x 10^8 to 3.6 x 10^9 cells g^{-1} at the reference sites and 2.1 x 10^8 to 5.3 x 10^8 cells g^{-1} at the impacted sites (Table 5.S2), which are within the range of previously reported cell densities for salt marshes and contaminated environments (Green and Scow, 2000; Slater et al., 2005; Wakeham et al., 2006). The PLFA distribution at all sites was dominated by monounsaturated and *n*-saturated PLFAs, as expected for surface sediments (Table 5.S3) (Zelles, 1999). Consistent with previous findings (Slater et al., 2005; Ahad et al., 2010; Mahmoudi et al., 2013), there was no relationship between PLFA distributions and hydrocarbon concentrations in sediments (Table 5.S2).

5.3.3 Compound-Specific Radiocarbon

Impacted sites sediments had dramatically lower $\Delta^{14}C_{TOC}$ values 5 months after oiling relative to reference sites due to the presence of highly depleted petroleum carbon (Figure 5.2, $\Delta^{14}C$ = -1000‰). By 18 months, $\Delta^{14}C_{TOC}$ values at impacted and reference sites were comparable, consistent with the similar hydrocarbon concentrations found across sites at this later time point. There was less variation in $\Delta^{14}C_{EXT\text{-}RES}$ values across sites; this is expected since EXT-RES represents naturally occurring organic matter and does not include the contribution of petroleum carbon (White et al., 2005). Reference sites had similar $\Delta^{14}C_{EXT\text{-}RES}$ and $\Delta^{14}C_{TOC}$ values (within error) confirming the minimal presence of petroleum at these sites. Temporal variations in $\Delta^{14}C_{EXT\text{-}RES}$ and $\Delta^{14}C_{TOC}$

values were observed across all sites over the 18 months of this study; however these variations were fairly small (~20 - 30‰) and may be due to natural variability in the age of organic matter in this system.

The $\Delta^{14}C_{PLFA}$ at impacted sites after 5 months ($\Delta^{14}C_{PLFA}$ = -815 and -882‰) were highly depleted in ^{14}C relative to TOC and EXT-RES. These depleted $\Delta^{14}C_{PLFA}$ values are consistent with previous studies in which microbial degradation of petroleum was observed (Slater et al., 2006; Ahad et al., 2010; Cowie et al., 2010). By 18 months, $\Delta^{14}C_{PLFA}$ values at impacted sites were considerably less depleted relative to TOC and EXT-RES and comparable to $\Delta^{14}C_{PLFA}$ values at reference sites.

5.3.4 Compound-Specific Carbon Stable Isotopes

The $\delta^{13}C_{TOC}$ of the impacted site sediments 5 months following oil intrusion were 4-5‰ lower compared to reference sites; consistent with the presence of ^{13}C- depleted petroleum carbon (Figure 5.3, BP crude oil; $\delta^{13}C$ = -27‰). By 18 months, $\delta^{13}C_{TOC}$ values at impacted sites were comparable to that of references sites, consistent with minimal hydrocarbon concentrations measured at this later time point. The observed sediment $\delta^{13}C_{TOC}$ values were similar to other Gulf of Mexico salt marsh environments (~18-20‰) (Wang et al., 2003; Wakeham et al. 2006; Natter et al., 2013). The $\delta^{13}C_{EXT\text{-}RES}$ values were similar across all sites and comparable to the expectation for *Spartina*-dominated environments (Slater et al., 2005).

The $\delta^{13}C_{PLFA}$ values of impacted sites ranged from -28.7 to -31.5‰ while the reference sites ranged from -26.2 to -29.2‰. Unlike for $\Delta^{14}C$, the $\delta^{13}C_{PLFA}$ values at

impacted sites were indistinguishable from reference sites and there was little temporal variation (less than 2‰) in $\delta^{13}C_{PLFA}$ values.

5.3.5 Microbial community structure

Pyrosequencing-based analysis of 16S rDNA and fungal ITS amplicons recovered a total of 66,129 bacterial 16S, 85,645 archaeal 16S and 99,013 fungal ITS raw pyrotag sequences. Following quality control, a total of 9,090 bacterial 16S, 23,170 archaeal 16S and 39,866 fungal ITS reads remained for community analysis. Sequence alignment and clustering based on 97% sequence similarity yielded a total of 3,338 bacterial, 3,611 archaeal and 948 fungal operational taxonomic units (OTUs).

The archaeal pyrosequencing data revealed the presence of two phyla across all sites and timepoints; *Crenarchaeota* and *Euryarchaeota* (65.1% and 34.9%, average classified sequences, respectively) and three dominant classes; *Thermoprotei* (65.1%), *Methanomicrobia* (24.3%) and *Methanobacteria* (7.2%) (Figure 5.4a). Impacted sites had higher relative abundance of *Methanomicrobia* and lower relative abundance of *Thermoprotei* compared to reference sites. Non-metric multi-dimensional scaling (NMDS) analysis of the archaeal community based on the mean relative abundance of taxonomically assigned OTUs classified to the class rank showed clustering of impacted sites of both time points (Figure 5.5a, 2D stress: 0.1, $r^2 = 0.95$). However, there was no significant correlation ($p > 0.05$) between the observed pattern of taxonomic clustering and total alkane, PAH, or UCM concentrations. Thus, the archaeal communities at impacted sites were more taxonomically similar to each other than to reference sites, regardless of the presence of petroleum

The dominant bacterial phylum across all sites was *Proteobacteria* (63.4% of classified sequences on average), although *Firmicutes* (10.8%), *Bacteroidetes* (6.3%), *Chloroflexi* (5.2%) and *Acidobacteria* (4.3%) were also always present. Among the *Proteobacteria*, the predominant classes were the *Alphaproteobacteria* (29.4%), *Gammaproteobacteria* (21.6%) and *Deltaproteobacteria* (8.7%) (Figure 5.4b). *Alphaproteobacteria* were the dominant taxa at impacted sites at 5 months whereas *Gammaproteobacteria* and *Bacilli* were dominant at reference sites. By 18 months, relative abundances of *Alphaproteobacteria* decreased at impacted sites such that the relative abundance of dominant taxa across sites became similar. Order-level identification of *Alphaproteobacteria* showed higher relative abundance of *Sphingomonadales* and *Rhodobacterales* at impacted sites at 5 months compared to all other sites and time points (Figure 5.S2). NMDS analysis of the bacterial community based on the mean relative abundance of taxonomically assigned OTUs classified to the class rank showed that 5 month-impacted sites clustered together whereas 18 months-impacted sites were clustered more closely with the reference sites (Figure 5.5b, 2D stress: 0.2, $r^2 = 0.82$). Fitting of hydrocarbon concentrations to the NMDS ordination revealed a significant relationship ($p < 0.05$) between the observed pattern of taxonomic clustering of impacted sites at 5 months with alkane and PAH concentrations (Alkanes, $r^2 = 0.64$, $p < 0.05$; PAH $r^2 = 0.64$, $p < 0.05$).

The fungal communities across all sites were dominated by the phylum *Ascomycota* (56.1% of classified sequences on average), though *Basidomycota* (25.2%), and *Chytridiomycota* (12.0%) were also prevalent. The most abundant fungal class was

Sordariomycetes (32.5%) followed by *Agaricomycetes* (21.0%) and *Dothideomycetes* (18.1%) (Figure 5.4c). Five months after oil intrusion, impacted sites were dominated by *Dothideomycetes* while reference sites were dominated by *Sordariomycetes*. By 18 months, the relative abundance of *Dothideomycetes* decreased at impacted sites such that both impacted and reference sites were dominated by *Sordariomycetes* and *Agaricomycetes* as well as *Chytridiomycetes,* in one case. NMDS analysis of fungal communities based on the mean relative abundance of taxonomically assigned OTUs classified to the class rank show minimal clustering of impacted sites at either time point (Figure 5.5c, 2D stress: 0.3, r^2= 0.6). Fitting of hydrocarbon concentrations to the NMDS ordination found no significant correlation ($p > 0.05$) between the observed pattern of fungal taxonomic clustering and total alkane, PAH, or UCM concentrations.

5.4 Discussion

5.4.1 Presence of petroleum oil in marsh sediments

An isotopic mass balance approach was used to estimate the fraction of TOC made up of petroleum carbon ($f_{petro.}$) in each sample:

$$\Delta^{14}C_{TOC} = f_{EXT\text{-}RES} (\Delta^{14}C_{EXT\text{-}RES}) + f_{petro.} (\Delta^{14}C_{petro.}) \quad (1)$$

where $\Delta^{14}C_{petro.}$ was assumed to be -1000‰, $\Delta^{14}C_{EXT\text{-}RES}$ was assumed to be natural sediment organic matter containing no petroleum contribution and assuming $f_{EXT\text{-}RES} + f_{petro} = 1$. Using this approach, petroleum was estimated to make up 56% and 74% of the carbon in marsh sediments at impacted sites 1 and 2, respectively, 5 months after oil intrusion and 1% and 9% of the carbon, 18 months after oil intrusion (Table 5.S5). These

contributions were not inconsistent with measured PAH, alkane and UCM concentrations measured at these sites. Similarly, using an isotopic mass balance as per eq (1) but using $\delta^{13}C$ values, petroleum was estimated to make up 54% and 74% of the carbon at impacted sites 1 and 2, respectively, 5 months after oil intrusion.

5.4.2 Microbial incorporation of Deepwater Horizon oil in marsh sediments

The highly depleted $\Delta^{14}C_{PLFA}$ values observed at impacted sites 5 months after oil intrusion demonstrate that *in situ* biodegradation by the indigenous microbial communities was contributing to the loss of petroleum organic components at these sites. Using an isotopic mass balance similar to eq (1), it was estimated that 86% and 78% of the carbon in microbial PLFAs was derived from petroleum at impacted sites 1 and 2, respectively, at this time. This high proportion of petroleum carbon present in PLFA, which are produced by both bacteria and fungi, suggest that petroleum hydrocarbons were the primary microbial carbon source at impacted sites at this time through direct degradation by hydrocarbon-degrading microbes and/or recycling of microbial organic compounds to non-hydrocarbon-degrading microbes.

By 18 months, $\Delta^{14}C_{PLFA}$ values were significantly less depleted indicating that the relative contribution of petroleum carbon to microbial PLFAs decreased at the impacted sites. $\Delta^{14}C_{PLFA}$ values at impacted sites at 18 months were depleted relative to the TOC and EXT-RES values by up to 174‰. With one exception, $\Delta^{14}C_{PLFA}$ values at reference sites at all time points were similarly depleted by approximately 100 to 200‰ with respect to TOC and EXT-RES values (Figure 5.2, Table 5.S4). This depletion in microbial PLFA relative to TOC and EXT- RES implies that there was some ^{14}C depleted

carbon source being metabolized by these microbial communities and incorporated into PLFA. Assuming a fossil carbon source such as petroleum, the input of highly depleted carbon ($\Delta^{14}C$= -1000‰) to microbial PLFAs was estimated to be 9-18% at impacted sites at 18 months and 5 to 22% at reference sites. Given that the Gulf of Mexico experiences frequent inputs of petroleum hydrocarbons via natural hydrocarbon seeps as well as smaller scale spills, the presence of hydrocarbons in sediments may be a common occurrence. Indeed, hydrocarbon concentrations and estimates of petroleum carbon in TOC even at some our reference sites were non-zero (Table 5.S6); this may represent a potential source of highly depleted carbon to microbial PLFAs in sediments with no visible oil. However, the highly depleted $\Delta^{14}C_{PLFA}$ values observed at impacted sites at 5 months coupled to the decreases in hydrocarbon concentrations demonstrate that biodegradation was contributing to mass loss of petroleum in these sediments. It is important to note that additional processes such as abiotic oxidation and export via sediment erosion were also likely contributing to mass loss of petroleum in these sediments (Silliman et al., 2012).

The relatively large offset between the $\delta^{13}C$ of the natural organic matter, represented by EXT-RES, and petroleum make this one of the most favorable systems for applying $\delta^{13}C$ analysis as a tool for differentiating microbial carbon sources (Slater et al., 2005). Despite this, microbial incorporation of petroleum carbon could not be resolved based on $\delta^{13}C_{PLFA}$ values. The $\delta^{13}C_{PLFA}$ values at impacted sites were expected to be more depleted relative to reference sites at 5 months due to the utilization and incorporation of $\delta^{13}C$-depleted petroleum carbon into microbial lipids. However, no such depletion was

observed at 5 months and $\delta^{13}C_{PLFA}$ values at impacted and reference sites differed by only 1 to 3‰. At 18 months, $\delta^{13}C_{PLFA}$ values between impacted and reference sites varied by 2 to 4‰ and $\delta^{13}C_{PLFA}$ at all sites were more depleted than TOC and EXT-RES (by 7 to 12‰), greater than the expected 4 to 6‰ fractionation between heterotrophic microbes and their carbon source (Hayes, 2001) (Table 5.S6). This result is very likely due to variation in fractionations during lipid synthesis as depletions of 3 to 14‰ between microbial lipids and carbon sources have been previously observed (Teece et al., 1999; Cifuentes and Salata, 2001; Londry et al., 2004). These results demonstrate that natural abundance $\Delta^{14}C$ analysis can overcome these factors and resolve microbial carbon sources in order to assess *in situ* petroleum biodegradation in salt marsh environments.

5.4.3 Impact of petroleum oil on microbial community structure

Biodegradation of petroleum involves complex interactions between microbes and the geochemical properties of the surrounding environment (Atlas, 1981). Consequently, linking the identity of microorganisms to the degradation of hydrocarbons in the natural environment is challenging because these interactions are difficult to replicate within a laboratory setting. Studies investigating the *in situ* response of microbial communities to released MC252 oil are providing valuable information regarding the biodegradation potential of indigenous microbial communities in the Gulf of Mexico (Hazen et al., 2010; Valentine et al., 2010; Edwards et al., 2011; Kostka et al. 2011; Beazeley et al., 2012; Bik et al., 2012; Mason et al., 2012; Redmond and Valentine, 2012).

Although archaea have been detected in many petroleum-impacted environments including aquifers (Dojka et al, 1998; Haack et al., 2004; Kleikemper et al., 2005), soils

(Liu et al., 2009; Wang et al., 2011) and petroleum reservoirs (Magot et al., 2000; Jones et al., 2007), the effect of petroleum on archaea and their role in hydrocarbon degradation is not well understood. Redmond and Valentine, (2012) found that archaeal community compositions were similar in oil plume and non-plume samples over a period of five months, even while the samples contained different bacterial communities, suggesting that presence of petroleum does not have a large impact on the archaeal communities. In this study, the archaeal communities at impacted sites were found to be taxonomically more similar to one another than to that of the reference sites even when petroleum concentrations in the sediments were minimal. This may be because the effects of the oil on community composition remained even after the oil was largely degraded, or because the impacted sites happened to be more similar sedimentary environments, even prior to the oiling event. For example, the greater abundance of *Methanomicrobia* at impacted sites may have resulted from anoxic conditions induced by the aerobic degradation of petroleum by bacteria such as *Sphingomonadales*. However, methanogenic archaea such as *Methanomicrobia* have been found to be prevalent in salt marsh sediments (Oremland et al., 1982; Franklin et al., 1988; Munson et al., 1997). Due to our limited knowledge of the metabolic capability of archaea, it is difficult to discern whether the composition of the archaeal community at impacted sites resulted from their role in petroleum degradation, from an indirect impact of petroleum on the sedimentary environment or from other environmental factors inherent to each site.

The relative abundance of dominant bacterial taxa shifted in the presence of petroleum such that the abundance of reputed hydrocarbon degraders increased.

Specifically, the relative abundance of *Sphingomonadales* and *Rhodobacterales* was higher in impacted sediments 5 months after oil intrusion. As the hydrocarbon concentrations decreased, the relative abundance of these groups also decreased and the taxonomic composition of impacted and reference sites became similar by 18 months after oil intrusion. Based on their frequent occurrence in contaminated sites and their known metabolic capability, members of *Sphingomonadales* are considered to be dominant PAH degraders in soils and sediments (Leys et al., 2004; Alonso-Gutierrez et al., 2009). Recent metagenomic analyses from Elmer's Island, Louisiana show enrichment of *Rhodobacterales* in oil impacted samples (Chakraborty et al., 2012). Likewise, Kostka et al. (2011) found a significant community shift toward members of *Gammproteobacteria*, and specifically *Rhodobacterales*, in oil-contaminated beach sands in Florida. Although we observed a shift in the microbial community structure representing an increase in the relative abundance of known hydrocarbon-degrading bacteria, no concomitant increase in microbial biomass in oil-impacted sediments, as measured by total PLFA concentrations, was observed. Similarly, surface waters near the *Deepwater Horizon* site showed comparable microbial biomasses inside and outside the oil slick (Edwards et al., 2011).

One might expect fungi to play an important role in the breakdown of the higher molecular weight components of petroleum due to their production of non-specific phenol oxidase enzymes such as laccase and peroxidase (Peng et al., 2008; Qasemian et al., 2012). Our results point to the particular involvement of *Dothideomycetes* based on the dominance of this class at impacted sites 5 months after oil intrusion. Likewise, Bik et al.

(2012) found post-spill beach sediments impacted by MC252 oil to be dominated by the ascomycete genera *Cladosporium* and *Alternaria*, both of which belong to the class *Dothideomycetes*. Species belonging to this class have been shown to degrade hydrocarbons and thrive in polluted environments (Colfone et al., 1973; Okpokwasili and Okorie, 1988). In addition, some species within this class (*Dothideomycetes*) have increased activity of lignin-degrading enzymes such as laccases that are capable of degrading PAHs (Clemente et al., 2001; Alcade et al., 2002; Atalla et al., 2010). Many studies of marine fungi tend to focus on taxonomic novelty and phylogenetic diversity, but it is recognized that this is an understudied field (Richards et al., 2012). Additional functional studies of marine fungi are needed to elucidate the role that fungi may play in the breakdown of petroleum in natural settings.

The highly depleted $\Delta^{14}C_{PLFA}$ values coupled to the increase in the relative abundance of known hydrocarbon-degrading bacteria show that *in situ* biodegradation by the indigenous microbial communities contributed to degradation of MC-252 oil in the severely oiled marsh sediments of Barataria Bay. These results suggest that the Gulf of Mexico coastal systems have considerable biodegradation potential and that natural attenuation may be feasible remediation strategy in this region. Future studies which link specific microbial groups and metabolisms to the degradation of specific hydrocarbon compounds are needed to further understand the dynamics of petroleum biodegradation in the natural environment and better guide future remediation strategies.

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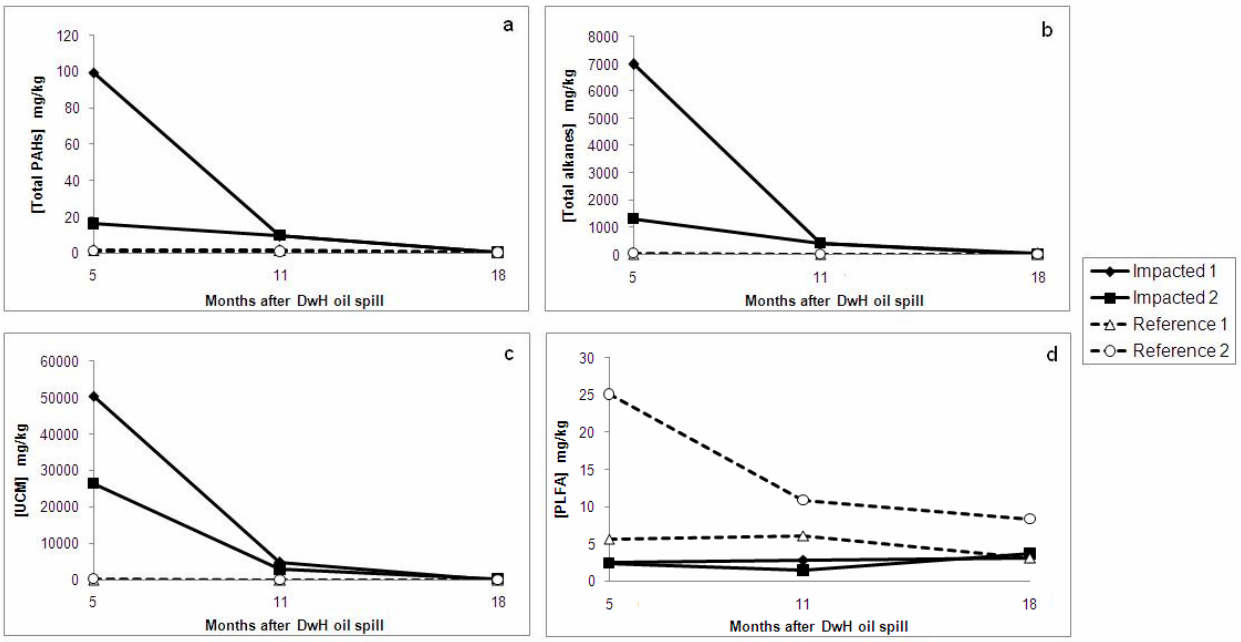

Figure 5.1 Sediment compound class concentrations at 5, 11 and 18 months after initial oil contact at two oil-impacted and two reference sites for (a) PAH (b) alkane (c) UCM (d) PLFA concentrations

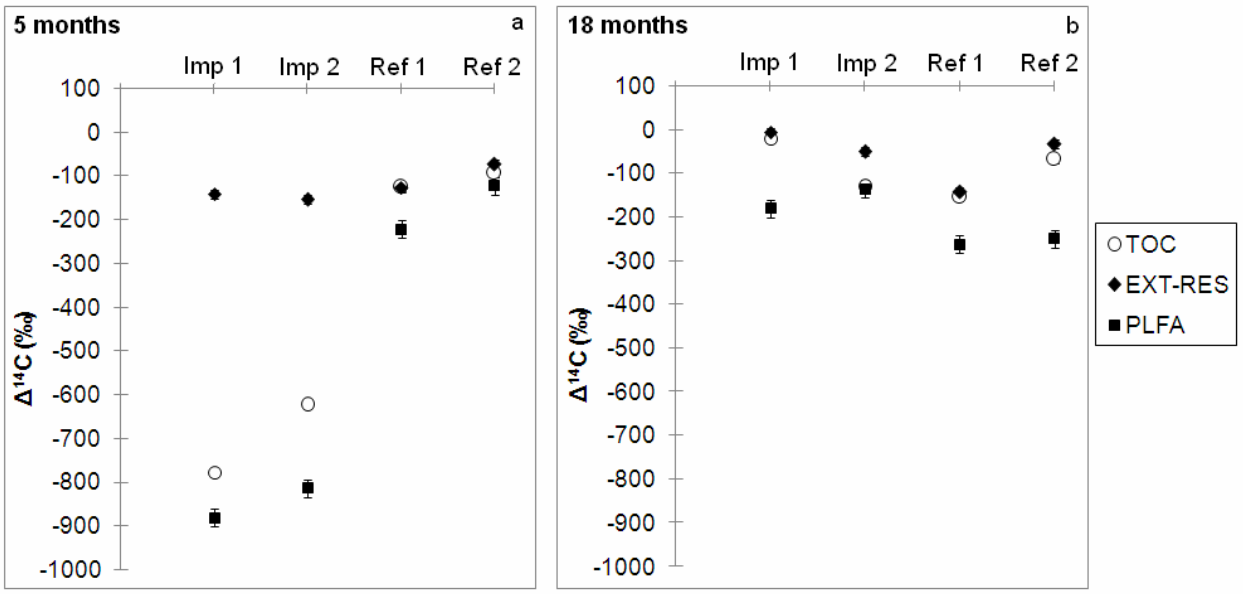

Figure 5.2 Radiocarbon ($\Delta^{14}C$) values for TOC, EXT-RES and PLFA for oil-impacted and reference sites (a) 5 months after oil intrusion (October 2010) (b) 18 months after oil intrusion (October 2011). Error bars represent accuracy and reproducibility of the analyses.

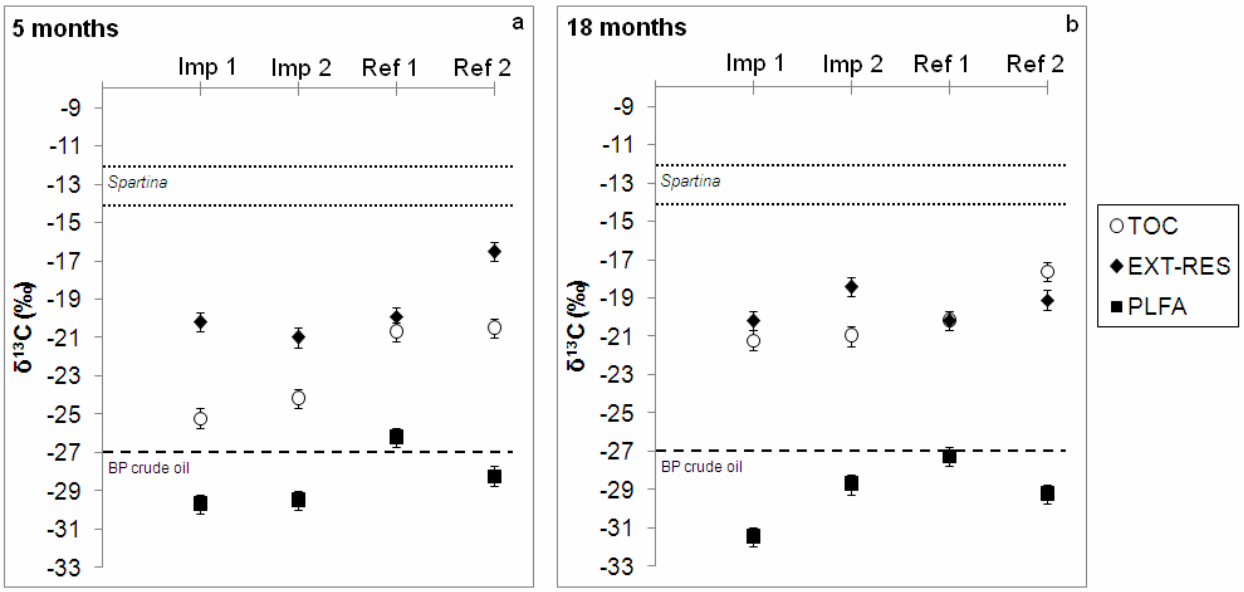

Figure 5.3 Stable carbon ($\delta^{13}C$) values for TOC, EXT-RES and PLFA for oil-impacted and reference sites (a) 5 months after oil intrusion (October 2010) (b) 18 months after oil intrusion (October 2011). Error bars represent the accuracy and reproducibility of the analyses.

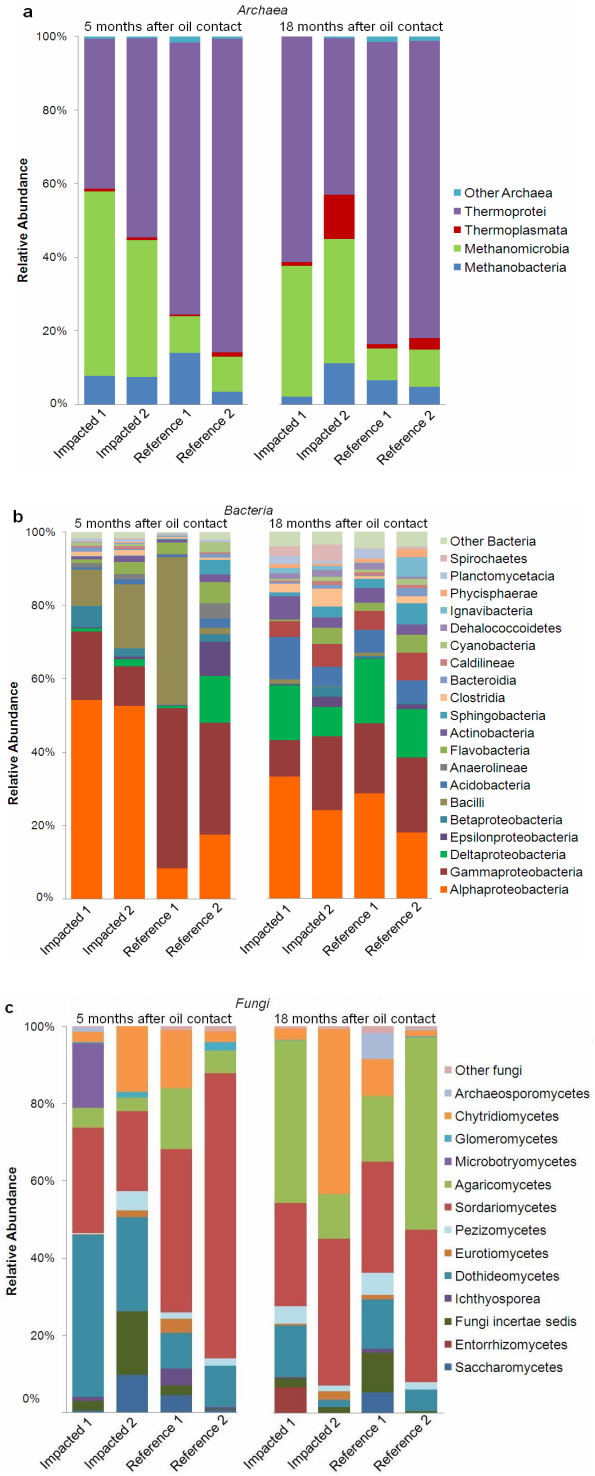

Figure 5.4 Relative abundances of dominant (a) archaeal, (b) bacterial, and (c) fungal classes in sediments collected from two oil-impacted and two reference sites 5 and 18 months after oil contact. Relative abundances are based on the frequencies of sequences that could be assigned at the class level.

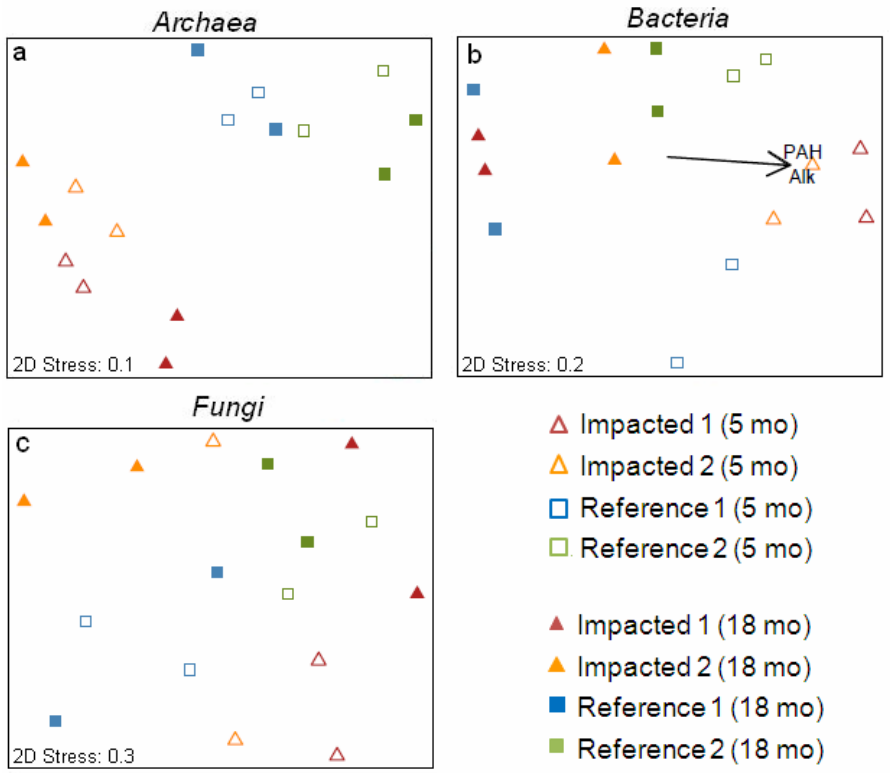

Figure 5.5 Non-metric multi-dimensional scaling (NMDS) ordination of (a) archaeal, (b) bacterial, and (c) fungal communities in sediments at 5 months (open symbols) and 18 months (closed symbols) after oil intrusion. Two data points shown for each sediment sample represent environmental replicates. Distance matrices were generated using Chi squared distances and is based on the relative abundance of taxonomically assigned OTU sequences by BLAST + MEGAN. Only statistically significant fitted ($p < 0.05$) hydrocarbon concentrations (alkanes, PAHs, UCM) are shown as vectors (arrows).

5.5 Supporting Information

5.5.1 Total Lipid Extraction

$\Delta^{14}C$ of EXT-RES (residue remaining after solvent extraction) was used as a proxy for natural organic matter based on White et al., (2005), Ahad et al., (2010) and Mahmoudi et al., (2013). Petroleum hydrocarbons and solvent extractable organic materials were removed by carrying out a total lipid extraction (TLE). This was done by extracting approximately 5g of oven-dried sediment using 1:1 hexane:acetone along with a microwave accelerated reaction system (MARS, CEM Corporation). Subsequently, organic compounds extracted by solvent (referred to as TLEs) were filtered using burned glass fiber filters to remove sediment particles (GF/G, Whatman) and treated with activated copper to remove elemental sulfur. Residual sediment collected by filters (defined as EXT-RES) was decarbonated, analyzed for total organic carbon (% TOC) and sent for ^{14}C analysis.

5.5.2 PLFA extraction and analysis

Approximately 40 to 100g of freeze-dried sediment was extracted using a modified Bligh and Dyer method (White et al., 1979) as per Slater et al., (2005). Using 2:1 methanol/DCM, sediments were extracted and the resulting sediment/solvent mixture was centrifuged in solvent-rinsed centrifuge tubes (10 min, 2000 rpm). Following centrifugation, samples were filtered into separatory funnels using 0.45µm pre-combusted glass fiber filters (GF/G, Whatman). Nanopure water was added to separatory funnels in order to separate aqueous and organic phases. Subsequently, the organic phase was

collected and separated into three fractions by gravity column chromatography using fully activated silica (precombusted at 450°C for 8 h) and dicholormethane (DCM), acetone and methanol to elute non-polar, neutral and polar fractions, respectively. The polar fraction which contained phospholipids was evaporated to to dryness under a stream of nitrogen gas and reacted to fatty acids methyl esters (FAMEs) via mild alkaline methanolysis reaction. Subsequently, FAMEs were further purified using a secondary silica gel step (hexane/DCM 4:1, DCM, methanol) and eluted in dichloromethane. Identification and quantification of FAMEs utilized an Agilent 6890 gas chromatograph coupled to an Agilent 5973 quadrupole mass spectrometer (equipped with a 30 m x 0.25 mm DB-5 MS column). The temperature program for the GC oven was 40 ºC for 1 min, ramp to 130 at 20 ºC/min, to 160 at 4 ºC/min and then to 300 at 8 ºC/min, with a final hold time of 5 min. Lastly, FAMEs were identified using a bacterial reference standard (Bacterial Acid Methyl Esters CP, Mix, Matreya Inc), mass-fragmentation patterns and retention times and quantified using external calibration standards (which contained FAMEs of various chain length).

5.5.3 Microbial DNA and Bioinformatics Analysis

16S rRNA gene amplicons for bacteria were created using primers 27F-530R (27F: 5'-AGRGTTTGATCMTGGCTCAG-3'; 530R: 5'-CCGCNGCNGCTGGCAC-3') and Arch344F-Arch915R for archaea (Arch344F: 5'-ACGGGGYGCAGCAGGCGCGA-3'; Arch915R: 5'GTGCTCCCCCGCCAATTCCT-3') (Lane, 1991; Raskin et al., 1994). Bacterial and archaeal 16S rRNA gene amplicons were analyzed using the Ribosomal

Database Project (RDP) pyrosequencing pipeline (http://pyro.cme.msu.edu/) to remove low quality sequences, align sequences, cluster reads into operational taxonomic units (OTUs) based on 97% sequence similarity, and provide putative/tentative classifications for all taxa. For bacteria and archaea, sequences were discarded if they had an average phred quality score less than 20, were shorter than 150 bp, had more than 2 primer mismatches, or contained any unidentified bases (Ns). After quality filtering, there was a total of 9090 bacterial reads with an average length of 462bp and 23170 archaeal reads with an average length of 510 bp. Bacterial and archaeal sequences were submitted to the RDP Naïve Bayesian Classifier using 16S rRNA training set 9 for taxonomic classification using the default 80% bootstrap confidence estimate threshold (Wang et al., 2007).

Fungal ITS amplicons were created using primers ITS1F-ITS4 (ITS1F: 5'CTTGGTCATTTAGAGGAAGTAA; ITS4: 5'CCTCCGCTTATTGATATGC-3') (White et al., 1990; Gardes and Bruns, 1993). ITS amplicons were processed using a custom semi-automated pipeline as follows: Reads were sorted by barcode, zero mismatches allowed, and the barcodes were removed. Reads were checked for the presence of the ITS1F forward primer, one mismatch allowed. Reads were quality trimmed using SeqTrim (Falgueras et al., 2010) using a sliding window size of 10bp, windows were discarded if the average phred quality score was less than 20, and trimmed sequences were discarded if they were less than 80 bp in length. After quality filtering, a total of 39,866 ITS reads with an average length of 442bp were retained. Primers sequence, including any trailing small or large subunit rDNA sequences were removed

using the Fungal ITS Extractor (Nilsson et al., 2010). Trimmed reads were subjected to BLAST 2.2.26+ (Altschul et al., 1997) searches using the blastn (megablast) algorithm against a local copy of the GenBank nucleotide database [April 2013] with an e-value cutoff of 1e-10. BLAST results were imported into MEGAN 4.70.4 (Huson et al., 2011) for automated taxonomic assignments. MEGAN uses a lowest common ancestor (LCA) algorithm to parse through the best hits of a BLAST report to make assignments to variable taxonomic ranks by summarizing the taxonomic lineages associated with these hits. The parameters we used to define the best set of BLAST hits used by the LCA parser are as follows: minimum support = 1, minimum score = 50, top percent = 1, win score = 0.0, minimum complexity filter = 0.44. Since taxonomic assignments based on the ITS1 region only (70% of the raw data) resulted in 75% of sequences with no blast hits or no taxonomic assignment (data not shown), we focused our analyses on the sequences covering the ITS1-5.8S-ITS2 region (40% of the raw data) where nearly all the sequences were taxonomically assigned by MEGAN. To avoid over-assigning our partial ITS sequences, we summarized taxonomic assignments to the class rank.

Distance matrices for the archaeal (class), bacterial (class), and fungal (class) community samples were created in MEGAN using normalized OTU/read counts and the Chi-squared metric. Non-metric multidimensional scaling plots were calculated using the ecodist package in [R] (Goslee and Urban, 2007).

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Figure 5.S1 Location of sediment samples collected from impacted and reference salt marshes. Impacted sites were located in the northeast corner of Barataria Bay on St. Mary Island which received heavy oil coverage due to prevailing winds and currents after the Deepwater Horizon spill. Reference site 1 was located in the northwest corner of the bay, east of Hackberry Bay. Reference site 2 was located on the south side of the bay in Grand Isle State Park (Silliman et al., 2012).

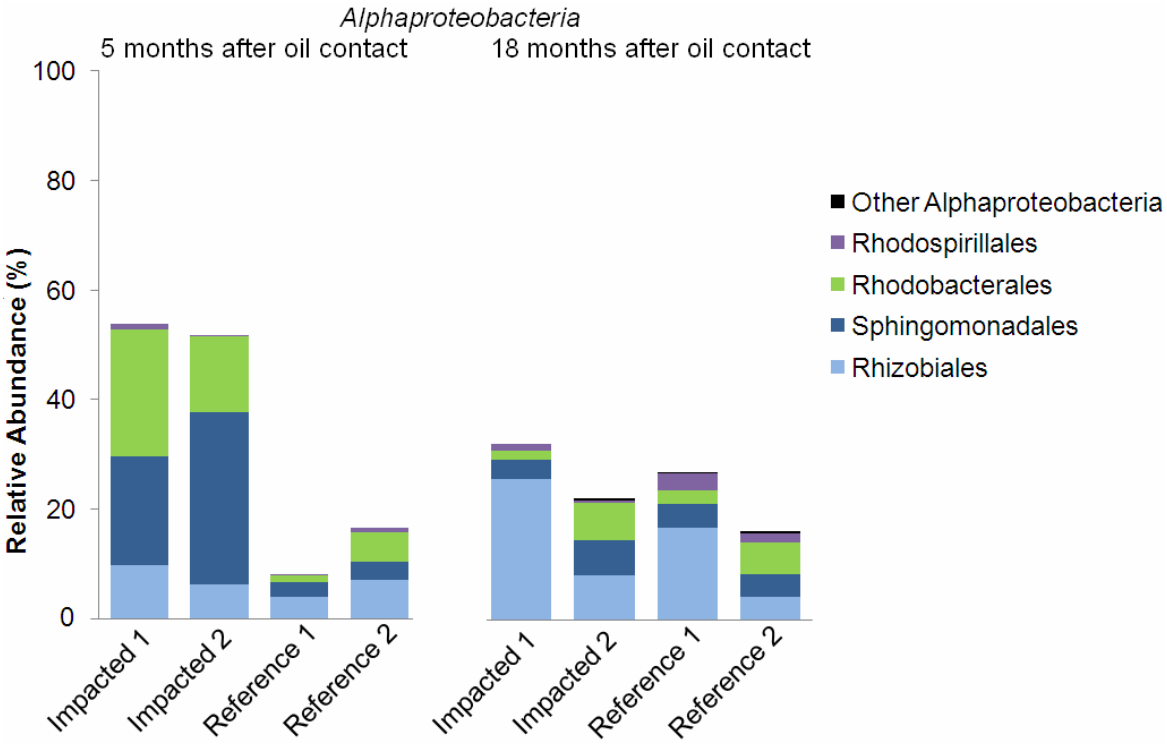

Figure 5.S2 Relative abundances of bacterial orders within Alphaproteobacteria class at impacted and reference sites in Barataria Bay at 5 and 18 months after oil contact. Relative abundances are based on the frequencies of sequences that could be assigned at the order level.

Table 5.S1 Coordinates of impacted and reference sites sampled in Barataria Bay, Louisiana.

Sites	Latitude	Longitude
Impacted 1	29º26'819"N	89º56'264"W
Impacted 2	29º26'196"N	89º54'681"W
Reference 1	29º24'535"N	89º59'239"W
Reference 2	29º13'402"N	90º00'443"W

Adapted from Silliman et al., 2012

Table 5.S2 Sediment PAHs, alkane, UCM and PLFA concentrations (mg/kg) at 5, 11 and 18 months after initial oil contact at two impacted and two reference sites.

Sites	Months following oil intrusion	Date of Sample Collection	TOC (%)	Total PAHs (mg/kg)	Total alkanes (mg/kg)	UCM (mg/kg)	Total PLFA (mg/kg)	Cells g^{-1}*
Impacted 1	5	Oct 2010	38.6	99.4	6987	50380	2.5	3.6E+08
Impacted 2	5	Oct 2010	14.7	16.2	1303	26465	2.4	3.3E+08
Reference 1	5	Oct 2010	4.4	1.5	17	18	5.7	8.2E+08
Reference 2	5	Oct 2010	3.9	1.1	52	280	25.1	3.6E+09
Impacted 1	11	Apr 2011	10.1	9.5	413	4673	2.9	4.1E+08
Impacted 2	11	Apr 2011	8.6	10.0	415	2875	1.4	2.1E+08
Reference 1	11	Apr 2011	5.1	1.5	19	51	6.2	9E+08
Reference 2	11	Apr 2011	5.1	1.0	25	41	10.8	1.6E+09
Impacted 1	18	Oct 2011	5.7	0.7	29	42	3.1	4.6E+08
Impacted 2	18	Oct 2011	6.9	0.8	19	282	3.7	5.3E+08
Reference 1	18	Oct 2011	8.2	0.4	20	43	3.2	4.6E+08
Reference 2	18	Oct 2011	5.3	0.4	14	24	8.4	1.2E+09

*cells per gram estimate was estimated from PLFA concentrations

Table 5.S3 Distribution of PLFA classes expressed as mole percentage of the total.

Sites	Months following oil intrusion	Monoun-saturated FA	n-Saturated FA	Terminally branched saturated FA[1]	Mid branched saturated FA	Cyclopropyl FA[2]	Polyun-saturated FA
Impacted 1	5	28	49	8	16	0	0
Impacted 2	5	26	42	12	17	4	0
Reference 1	5	39	32	12	8	8	2
Reference 2	5	24	32	14	19	7	4
Impacted 1	11	29	28	14	14	12	3
Impacted 2	11	34	28	27	0	11	0
Reference 1	11	28	29	17	15	5	7
Reference 2	11	22	23	15	33	7	1
Impacted 1	18	21	33	16	21	7	1
Impacted 2	18	28	33	15	15	5	3
Reference 1	18	19	38	16	20	3	4
Reference 2	18	24	31	18	16	8	4

[1]iso- and anteiso- PLFA
[2]cyc 17:0 and cyc 19:0

Table 5.S4 $\delta^{13}C$ and $\Delta^{14}C$ signatures of PLFA, Total Organic Carbon (TOC), Extracted-Residue (EXT-RES) at impacted and reference sites following oil intrusion.

Sites	Months following oil intrusion	$\Delta^{14}C_{PLFA}$ ± 20‰	$\Delta^{14}C_{TOC}$ ± 10‰	$\Delta^{14}C_{EXT-RES}$ ± 10‰	$\delta^{13}C_{PLFA}$ ± 0.5‰	$\delta^{13}C_{TOC}$ ± 0.5‰	$\delta^{13}C_{EXT-RES}$ ± 0.5‰
Impacted 1	5	-882	-778	-142	-29.7	-25.2	-20.2
Impacted 2	5	-815	-625	-155	-29.5	-24.2	-21
Reference 1	5	-222	-127	-127	-26.2	-20.7	-19.9
Reference 2	5	-123	-94	-73	-28.2	-20.5	-16.5
Impacted 1	18	-181	-21	-7	-31.5	-21.2	-20.2
Impacted 2	18	-136	-132	-52	-28.7	-21	-18.4
Reference 1	18	-263	-156	-144	-27.2	-20.2	-20.2
Reference 2	18	-250	-69	-34	-29.2	-17.6	-19.1

Table 5.S5 Estimated fraction of TOC made up of petroleum carbon based on isotopic mass balance using $\Delta^{14}C$ values.

Sites	Months following oil intrusion	Date of Sample Collection	TOC (%)	Fraction of TOC made up of petroleum carbon (%)*
Impacted 1	5	Oct 2010	38.6	74
Impacted 2	5	Oct 2010	14.7	56
Reference 1	5	Oct 2010	4.4	0
Reference 2	5	Oct 2010	3.9	2
Impacted 1	18	Oct 2011	5.7	1
Impacted 2	18	Oct 2011	6.9	8
Reference 1	18	Oct 2011	8.2	1
Reference 2	18	Oct 2011	5.3	4

*as per eq (1)

Table 5.S6 $\delta^{13}C$-depletion of PLFAs relative to TOC, EXT-RES and BP crude oil.

Months following oil intrusion	Sites	TOC	EXT-RES	BP crude oil (-27.2‰)
5	Impacted	4-6‰	8-10‰	2-3‰
5	Reference	5-8‰	6-12‰	0-1‰
18	Impacted	7-11‰	10-12‰	1-5‰
18	Reference	7-12‰	7-10‰	0-2‰

CHAPTER 6

CONCLUSIONS & FUTURE RESEARCH

This dissertation sought to integrate genetic and geochemical approaches to assess biodegradation of petroleum hydrocarbons in the natural environment in order to better understand the growth and activity of microorganisms in contaminated environments. Addressing this goal at two very different contaminated study sites provided insight into the importance of environmental factors such as bioavailability (Chapter 4) as well as the dynamics of biodegradation in long-term (Chapter 3) versus recently contaminated sites (Chapter 5). Because biodegradation is significantly affected by the surrounding environment as well as the source and history of the contamination, the occurrence of biodegradation at these two sites differed greatly and contrasted one another. This research represents the first application of natural abundance ^{14}C analysis to the biodegradation of PAHs as well as to oil related to the *Deepwater Horizon* spill. The latter is particularly unique because ^{14}C analysis was applied at two different time points and changes in microbial carbon sources could be observed over time. Integrating natural abundance ^{14}C analysis of microbial lipids with molecular techniques represents another novelty of this dissertation. Molecular characterization of all three microbial domains is seldom performed and the papers presented here include some of the only studies that have assessed all three microbial domains simultaneously in a petroleum-contaminated environment. This work furthers our understanding of biodegradation by indigenous

microbial communities particularly with respect to fungi and archaea whose presence in contaminated environments is often neglected from consideration. The integration of multiple methods at two different sites contributes to our broader knowledge of microbial carbon sources and cycling in soils and sediments particularly in the case of the PAH-contaminated soils where microbes are utilizing older pools of organic matter which are thought to contain recalcitrant compounds. Collectively, the results of this dissertation aid in assessing the potential for intrinsic biodegradation at contaminated sites and optimize the use of microorganisms as a natural remediation tool.

6.1 Research Summary

Biodegradation in the natural environment is highly variable and involves complex interactions between species as well as the chemical and physical properties of the environment. Consequently, no two sites are ever alike and biodegradation can vary from site to site. This dissertation focused on two study sites: (1) a former industrial facility in southern Ontario primarily contaminated with PAHs and (2) salt marshes in Louisiana impacted by the *Deepwater Horizon* oil spill. A combination of DNA, lipid and isotopic approaches were applied at each site to assess microbial biomass and community structure as well as microbial carbon sources and cycling.

One of the challenges in characterizing microbial communities using molecular techniques is the ability to extract, high-quality, purified DNA. Soils are often problematic for molecular analysis due to the presence of organic matter such as humic acids which can inhibit PCR reactions and interfere with DNA quantification because

they absorb as the same wavelengths as DNA (Yeates et al., 1998). Likewise, some PAHs absorb at similar wavelengths as DNA, this can lead to overestimations of DNA yield and concentration. Chapter 1 compares the effectiveness of four commercial soil DNA extraction kits to extract pure, high quality bacterial and eukaryotic DNA from six different PAH-contaminated soils. The results of this chapter found that the PowerSoil DNA Isolation kit provided the highest quality DNA based on successful amplification of both bacterial and eukaryotic DNA for all six soils. In addition, commercial kits differed with respect to degree of cell lysis, and so, observed phylogenetic diversity depends greatly on the extraction kit being used. These results highlight biases that can be introduced during DNA extraction and emphasize the importance of selecting an appropriate DNA extraction kit particularly in light of next-generation sequencing approaches which are more sensitive than DGGE and cloning techniques. This can be a significant issue especially for contaminated soils where inaccurate community analysis can lead to potentially erroneous estimations regarding the biodegradation capability of the natural microbial population.

The dynamics of PAH biodegradation in long-term contaminated soils collected from a former industrial site was assessed in Chapters 3 and 4. Natural abundance ^{14}C analysis of microbial PLFA was used to elucidate microbial carbon soils and confirm whether PAH biodegradation was occurring in Chapter 3. This approach revealed that microbial carbon sources were derived primarily from vegetation and other types of organic matter present in the soils rather than PAHs. Similarly, DNA fingerprinting and PLFA analysis showed that microbial structure and diversity remained consistent across

soils and there was no observed enrichment for hydrocarbon degraders across all three domains. These results demonstrate that even with the presence of an active microbial community, there may be little biodegradation of PAHs. Furthermore, these results provide insight into the soil organic matter cycling and the role of microbes. Soil organic matter is not in a single pool but rather in multiple carbon pools with different intrinsic turnover times that can be on annual to decadal and even millennial timescales (Trumbore, 2000). It is generally thought that there is a pool of recalcitrant organic compounds in soil that is biologically inert (Falloon et al., 2000) and microbes will utilize the younger pools with more labile compounds. However, the results of this chapter indicate that microbes are able to utilize almost all available pools of organic matter including older pools which are thought to contain recalcitrant compounds.

Bioavailability, in the context of bioremediation, is defined as the extent to which a contaminant may be available for biological conversion and is considered to be a major limitation in remediating PAH-contaminated soils (Juhasz et al., 2000). Further investigation of these PAH-contaminated soils in Chapter 4 revealed that only 0 to 20% of PAHs at this site are bioavailable to soil microorganisms. This suggests that bioavailability is likely the primary cause for the lack of observed biodegradation at this site. The combined results of Chapters 3 and 4 contrast demonstration of PAH degradation in laboratory studies and emphasize the importance of *in situ* conditions to biodegradation.

Biodegradation of *Deepwater Horizon* spilled oil in salt marsh sediments was assessed over the first 18 months after the spill in Chapter 5. Natural abundance ^{14}C

analysis of microbial phospholipid fatty acids (PLFA) revealed that petroleum-derived carbon was the primary carbon source for indigenous microbial communities at impacted sites 5 months after oil intrusion. Also at this time, pyrosequencing of all three microbial domains show an increase in the relative of abundance of taxonomic groups known to include hydrocarbon-degrading species, such as *Sphingomonadales*. These results confirm that biodegradation was a contributing factor to the rapid petroleum mass loss over this time period. Surprisingly, stable carbon isotopic analysis of microbial PLFA could not resolve incorporation of petroleum carbon into microbial lipids. The results of Chapter 5 indicate that the Gulf of Mexico has considerable biodegradation potential and that natural attenuation may be feasible remediation strategy in this region.

6.2 Future Directions

Over the last few decades, bioremediation of petroleum-contaminated sites through the activity of microorganisms has gained widespread attention as a favourable remediation strategy. However, microbial processes that remediate contaminants in laboratory settings may not function as well in full-scale field applications and bioremediation strategies that proved successful in one site may not work in another (Lovley, 2003). This is largely due to a lack of predictability and understanding of fundamental microbial processes that occur in the natural environment (Speight and Arjoon, 2012). Because microbial and chemical processes interact to control the fate of petroleum hydrocarbons, a single method can hardly distinguish complex interactions between microbial metabolic activity and the observed geochemistry. Therefore, a multi-

disciplinary approach which integrates microbiology with geochemistry is necessary to overcome limitations associated with a particular method (Weiss & Cozzarelli, 2008). Some of the most insightful studies in contaminated environments have combined multiple methods to provide a more robust characterization of *in situ* microbial communities (Geyer et al., 2005; Pombo et al., 2005; Hendrickx et al., 2005; Lin et al., 2005, 2007; Eriksson et al., 2006; Carney et al., 2007; Shrestha et al., 2008). Future studies should continue to combine complementary approaches in order to advance our understanding of microbial processes and create a stronger basis for assessing biodegradation in the natural environment (Bombach et al., 2010).

While the development of new methodologies such as next-generation sequencing have greatly increased our ability to characterize *in situ* microbial communities, linking the identity of microorganisms to a specific set of metabolic processes in the natural environment continues to be difficult. To date, there is no approach or technique which can directly link the *in situ* degradation of compound to a specific microbial group without the addition of a label or tracer. Natural abundance ^{14}C analysis of microbial lipids has brought us closer to such an approach with respect to biodegradation of petroleum hydrocarbons; however, the phylogenetic resolution of this approach remains low. Additional research that was conceptualized during early stages of this dissertation to characterize the roles of all three microbial domains included natural abundance ^{14}C analysis of lipids with higher phylogenetic resolution such as phospholipid ether lipids (PLEL) which are specific to archaea (Mancusco et al., 1985) and ergosterol which synthesized solely by fungi and microalgae (Newell et al., 1987). Unfortunately, initial

exploration of these ideas revealed that much work needed to be done in order to develop this method. Because few studies have extracted, identified and quantified PLEL, there is currently no standard method and further development of this technique is required. With respect to ergosterol, the primary challenge is not necessarily extraction and quantification but rather purification of ergosterol from other organic compounds found in soils and sediments. Continued development on the isolation and purification of lipids with higher specificity to certain microbial groups would greatly enhance our understanding of microbial carbon sources in petroleum-contaminated environments.

In recent years, natural abundance isotopic ^{14}C analysis has been applied to microbial DNA rather than lipids in order to elucidate microbial carbon sources in surface water and groundwater samples (Cherrier et al., 1999; Hansman et al., 2009; Mailloux et al., 2013). Currently, the phylogenetic resolution of this approach remains at the domain level, providing similar taxonomic resolution to that achievable by lipid based approaches. Recently, DNA was extracted from the seafloor near a natural oil seep and magnetic beads were used to separate archaeal and bacterial rDNA and subsequently subjected to stable carbon isotopic analysis (MacGregor et al., 2002, 2012). It would of great interest to see this approach expanded to ^{14}C analysis and also to develop the use of magnetic beads for isolating more specific DNA sequences for isotopic analysis. This would potentially allow us to link the degradation of petroleum hydrocarbons to specific microbial groups. However, the obvious challenge is adequate purification of DNA particularly in samples with high humic acid content. In this case, the use of a spectrophotometer is not a viable method for validating the purity of DNA and the use of

gel electrophoresis can cause further contamination to DNA (Trembath-Reichert et al., 2008). Another challenge for natural abundance ^{14}C analysis is the amount of mass needed for analysis via accelerator mass spectrometer (AMS) which currently requires at least 30µg or more of carbon for measurement of ^{14}C (Pearson et al., 2006; Santos et al., 2007; Cherkinsky et al., 2013). Consequently, isolation and collection of DNA (and sometimes individual lipids) requires prohibitively large sample sizes which may not be feasible for some studies. Further development of AMS analytical capabilities and improved recovery of DNA and lipids from environmental samples will increase the applicability of this approach.

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APPENDIX A

DGGE ANALYSIS OF PAH-CONTAMINATED SOILS

In Chapter 3, microbial carbon sources and the occurrence PAH biodegradation was assessed in PAH-contaminated soils using natural abundance $\Delta^{14}C$ analysis of microbial PLFA. Concurrently, PCR-DGGE analysis was used to investigate changes in microbial community structure with increasing levels of PAHs to observe selection for PAH degrading microorganisms across all three microbial domains. DGGE profiles revealed no observed enrichment for hydrocarbon-degrading organisms in contrast to studies that have observed reductions of bands and the emergence of strong bands corresponding to hydrocarbon-degrading microorganisms (Juck et al., 2000; Röling et al., 2002; Brakstad & Lodeng, 2005). Initially, the DGGE results were included in the manuscript, however, the reviewers selected for the submitted manuscript felt that the DGGE analysis was unnecessary and did not add support to the radiocarbon findings. In addition, they felt that DGGE analysis greatly under represents the diversity of soil microorganisms and suggested using next-generation sequencing approaches. It appears that the scientific community is somewhat divided on the need for in depth sequencing versus approaches that focus on the major trends in the microbial community. Although next-generation sequencing may be preferred and provides greater coverage as seen in Chapter 5, community fingerprinting using DGGE analysis is an adequate method when comparing patterns and variations on the abundant phylotypes across samples (Hanning

and Ricke, 2011). We note that while the reviewers of the initial version of this paper did not feel DGGE was sufficient, a number of recent papers have used the same approach to assess the major changes in community structure (Chu et al., 2011; Gilbert et al., 2012; Ling et al., 2012; Yang et al., 2012; Vaz-Moreira et al., 2013). Due to time and budget constraints, sequencing of these soils was not done for re-submission to *Environmental Pollution* and ultimately these DGGE results were removed from the final published version of this paper. A brief summary of the DGGE methods and results included in the initial draft of the manuscript is presented here.

A.1 Materials and Methods

The soil nucleic acids were extracted in triplicate using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA) since it effectively removes humic acids and other aromatic contaminants such as PAHs from soil (Mahmoudi et al., 2011). Amplification of bacteria 16S rDNA within the V3 region was done using eubacterial specific universal primers, 341F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCG GGGGCACGGGGGGCCTACGGGAGG CAGCAG -3'; which includes a 40-bp GC clamp on its 5' end; Invitrogen Canada) and 534R (5'- ATTACCGCGGCTGCTGG-3'; Invitrogen Canada) (Muyzer et al. 1993). Universal eukaryotic primers, forward 1427-1453 (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCTCTGTG ATGCCCTTAGATGTTCTGGG - 3'; which includes a 40-bp GC clamp on its 5' end) and reverse 1616-1637 (5'-GCGGTGTGTACAAAGGGCAGGG - 3') were used to amplify eukaryotic 18S rDNA within the V4 region (van Hannen et al. 1998). The V3

region of the archaeal 16S rDNA was amplified by nested PCR using archaea-specific forward primer Arch21F (5'- TTCCGGTTGATCCTGCCGGA - 3') and A934R (5'-GTGCTCCCCCGCCAATTCCT-3') for the first PCR reaction, and archaea-specific forward primer A344F-GC (5'- CGGGCGGGGCGGGGGCACGGGGGGCGCG GCGGGCGGGGCGGGGGACGGGGTGCAGCAGGCGCGA - 3'; which includes a 40-bp GC clamp on its 5'end) and universal reverse primer 534R (5'- ATTACCGCGG CTGCTGG-3'; Invitrogen Canada) for the second PCR reaction (DeLong, 1992; Raskin et al., 1994; Muyzer et al. 1993).

PCR reactions were 50µl in total and contained 50ng of template DNA, 1 µM of each forward and reverse primers and 2.5U of HotStarTaq DNA polymerase (Qiagen, Valencia, Ca) and were subsequently duplicated. PCR cycling was done using a PTC-100^{TM} thermal cycler (MJ Research Inc., Waltham, Massachusetts). Bacterial 16S rDNA fragments were amplified using the following conditions: initially 95°C for 5 min; followed by 34 cycles of 94°C for 1 min; 55°C for 1 min; 72°C for 1 min; followed by a final extension of 72°C for 10 min. Eukaryotic 18S rDNA fragments were amplified using following conditions: initially 95°C for 5 min; followed by 30 cycles of 94°C for 30 sec; 52°C for 1 min; 68°C for 1 min; followed by a final extension of 68°C for 10 min. Archaeal 16S rDNA fragments were amplified using the following conditions for the first reaction: initially 95°C for 5 min; followed by 34 cycles of 94°C for 1 min; 53°C for 1 min; 72°C for 1 min; followed by a final extension of 72°C for 10 min. The second reaction for amplifying archaeal 16S rDNA fragments used template from the first reaction and the following conditions: initially 95°C for 5 min; followed 34 cycles of

94°C for 1 min; 53°C for 1 min; 72°C for 1 min; followed by a final extension of 72°C for 7 min. PCR products were run on 1.2% agarose gel (wt/vol) stained with ethidium bromide prior to DGGE analysis to confirm successful amplification.

Bacterial, eukaryotic and archaeal PCR amplicons were separated on 8% polyacrylamide gels with a denaturing gradient of 40% to 70% (bacterial and archaeal) or 30% to 55% (eukaryotic) (100% denaturant contains 7M urea and 40% (vol/vol) formamide). Ten uL of sample was mixed with five uL of loading dye and loaded onto wells. Electrophoresis was performed in 0.5 x Tris-acetate-EDTA (TAE) buffer at 70V at 60 ºC for 16h using a DGGE-2401 apparatus (C.B.S Scientific, DelMar, Ca). Gels were stained with ethidium bromide and visualized with a G-Box gel documentation system (Syngene, Cambridge, UK). The DGGE profiles were normalized and compared using GelCompar II version 6.5 (Applied Maths, Belgium).

A.2 Results and Discussion

The phylotype richness (as determined by the number of bands observed in the DNA fingerprints) did not vary across soils and there seemed to be consistent levels of diversity among soil samples (Figure A.1, A.2, A3; Table A.1), with the exception of soil 6 which had a lower bacterial diversity. Based on previous reports (Juck et al., 2000; Röling et al., 2002; Brakstad & Lodeng, 2005), a decrease in microbial diversity and selection for PAH degraders (as seen by reductions of bands and the emergence of strong bands) with increasing levels of contamination was expected in these soils. Although phylotype diversity for each domain (bacteria, archaea, and eukarya) was fairly similar

across the soils, diversity between the three domains within the same soil varied for all soils with the exception of soil 6. Specifically, bacteria had highest diversity, followed by eukarya and finally archaea which had the lowest diversity. Even though phylotype diversity did not vary between soils, phospholipid fatty acid (PLFA) results demonstrate significant differences in microbial abundance and PLFA diversity (i.e. the total number of individual PLFAs extracted from each soil). PLFA diversity also correlated with microbial abundance; PLFA diversity was lowest in soils with low cell density (soils 3 and 4) and was highest in soils with high cell density (soils 1, 2 and 5) (Table 3.1). However, no trend was observed between PLFA diversity and actual phylotype diversity in any soils which supports that the notion that PLFA diversity should not be interpreted to actual species diversity (Frostegård et al., 2011).

Natural abundance radiocarbon analysis of microbial PLFA found negligible evidence of PAH biodegradation in these contaminated soils. Microbial carbon sources were found to be derived from vegetation and/or natural organic matter present in soils matter rather than PAHs. Likewise, no evidence for enrichment of PAH degrading organisms was observed in all three microbial domains based on DGGE fingerprints. These findings indicate that while microorganisms capable of PAH degradation may be expected to be present in soils, this degradation cannot be assumed to be occurring.

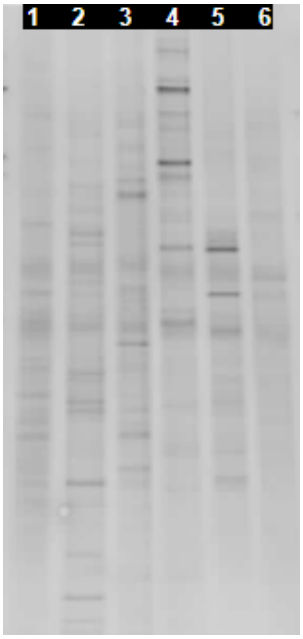

Figure A.1 Denaturing gradient gel electrophoresis (DGGE) profiles of 16S bacterial rDNA of all six soil samples.

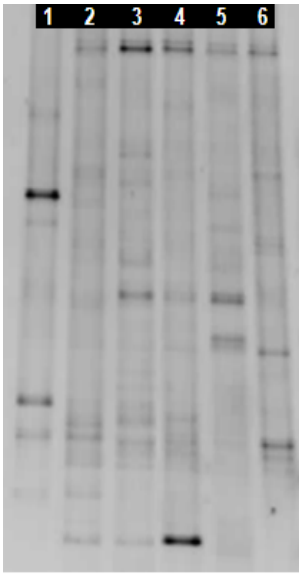

Figure A.2 Denaturing gradient gel electrophoresis (DGGE) profiles of 18S rDNA of all six soil samples.

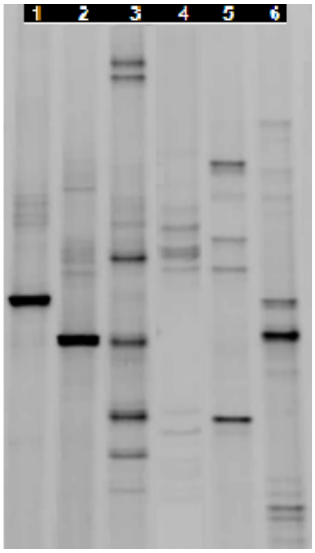

Figure A.3 Denaturing gradient gel electrophoresis (DGGE) profiles of 16S archaeal rDNA of all six soil samples.

Table A.1 Phylotype richness as determine by number of bands observed in DGGE profiles.

	Bacteria	Eukarya	Archaea
Soil 1	14	8	5
Soil 2	19	12	6
Soil 3	17	12	8
Soil 4	17	9	7
Soil 5	12	10	6
Soil 6	7	9	11

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