ELUCIDATION OF MICROBIAL CARBON CYCLING IN CONTAMINATED ENVIRONMENTS USING COMPOUND SPECIFIC ISOTOPE ANALYSIS

By

BENJAMIN R. COWIE, B.Sc.

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AUTHOR: Benjamin R. Cowie, B.Sc. (McMaster University)

SUPERVISOR: Dr. Gregory F. Slater

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ABSTRACT

The development of novel bioremediation systems has widespread benefits for human health and natural ecosystems. Optimization of such systems is only possible with a thorough understanding of the processes that drive bioremediation. This thesis developed novel understanding of carbon sources and cycling relationships for microbial communities that are integral in controlling contaminant fate in two contaminated environments. In the first case (Chapter 2), biodegradation in the soil microbial community was determined to be the primary pathway for recalcitrant petroleum pollutant removal. Microbial uptake and metabolism of petroleum hydrocarbons was conclusively demonstrated via ^{14}C analysis of their PLFA biomarkers. This microbial community was the most ^{14}C depleted bacterial system detected in an environmental system to date. In addition, complete mineralization of petroleum carbon was demonstrated with ^{14}C analysis of soil CO_2. The second paper (Chapter 3) identified unique Phospholipid Fatty Acid (PLFA) biomarkers and stable carbon isotopic fractionation patterns for heterotrophic and autotrophic bacterial communities of an acid mine drainage (AMD) system. The characteristic isotopic fractionations observed during biosynthesis of PLFA biomarkers in autotrophic versus heterotrophic metabolic pathways provided the basis for a model capable of elucidating the relative roles of these members of the microbial community in the environment. The major implications of the knowledge developed in this thesis, are two new methods to

identify microbial carbon cycling pathways and processes in contaminated environments. These advances may lead to new methods for mitigating the effects of contamination in environmental systems through better understanding of the microbial processes at the contaminated sites.

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PREFACE

This thesis is the result of research carried out by the author under the supervision of Dr. Greg Slater in partial fulfillment of the degree Master of Science (M.Sc.). While Dr. Slater guided the research process and provided editorial commentary for this thesis, the research was carried out solely by the author. All research in this thesis, including literature review, field sample collection, laboratory analysis and manuscript preparation was conducted by the author, with the exception of the microbial cultures in Chapters Two and Three that were generously provided by Dr. Bruce Greenberg (University of Waterloo) and Dr. Luc Bernier and Dr. Lesley Warren (McMaster University) respectfully. Dr. Luc Bernier and Dr. Lesley Warren also contributed relatively minor genetic analysis in Chapter Three.

Chapter One of this thesis consists of an introduction to microbial carbon cycling and isotope analysis. Chapter Two (Identification of biodegradation pathways in a multi-process phytoremediation system using natural abundance ^{14}C analysis) and Chapter Three (Carbon Isotope Fractionation in Lipid Biomarkers of Bacteria Native to an Acid Mine Drainage Lake) represent manuscripts for submission to peer-reviewed journals. The final Chapter summarizes major findings and conclusions from the research contained in the thesis.

CHAPTER 1

Introduction

Environmental pollution is a serious concern for human health and the function of natural ecosystems. Many pollutants, including organic compounds and heavy metals, that are released into the environment by human activity are acutely toxic, have long-term health effects, or are known carcinogens. Novel, efficient methods are required for remediation of contaminated sites, and for monitoring increasing levels of environmental contamination caused by growth in resource exploitation and economic development. Understanding carbon cycling pathways of microbial communities at contaminated sites will allow 1) enhancement and optimization of existing remediation techniques, 2) development of novel methods for removal and mitigation of environmental pollution and 3) effective monitoring of the degradation and removal of undesired compounds from environmental systems. This thesis developed understanding of microbial carbon cycling and makes new contributions to remediation and monitoring at two sites: a petroleum contaminated land farm site, and an acid mine drainage (AMD) site.

Petroleum contamination is widespread in terrestrial environmental systems including soils, sediments, and aquifers. Petroleum includes many known

or suspected carcinogens, therefore mitigating hydrocarbon contamination and developing new remediation techniques is an important goal to manage sustainable resource development and exploitation. At many sites, engineered remediation systems are being developed that employ multiple techniques to remove target pollutants from a system. However the micro-scale processes that actively degrade hydrocarbons in these systems are often not fully understood.

Phytoremediation, the use of plants to enhance the removal of environmental pollutants, is often employed as a remediation technique for *in situ* remediation of contaminated soils because it is more economical than *ex situ* techniques (Pilon-Smits, 2005). In phytoremediation systems, there are several potential pathways by which remediation may occur, in some cases plants are uptaking target pollutants and either metabolizing or storing them, conversely in other systems degradation is actively occurring in the rhizosphere by soil bacteria (Cohen, 2002). Soil microbial metabolism is a major factor in phytoremediation of many petroleum-contaminated systems, and the diverse metabolism of soil microbial communities are capable of degrading most petroleum-derived pollutants (Atlas, 1981; Vidali, 2001). Often this is due to co-metabolic processes where the plants provide nutrients, stimulating the microbial community which in turn are capable of degrading the organic pollutants in the contaminated soil (Cohen, 2002). However, directly identifying microbial uptake and metabolism of contaminants is challenging due to the complexity of hydrocarbon compounds

and many potential degradation products in a remediation system (Sutton et al., 2005). Furthermore, degradation can often be heterogeneous; rates and degradation pathways vary with physical and chemical factors such as redox, moisture availability and temperature, and many compounds are recalcitrant under certain geochemical conditions (Atlas, 1981; Vidali, 2001). Differentiation of plant versus microbial degradation processes and carbon cycling in phytoremediation systems will lead to optimized efficiency, identify novel applications for phytoremediation and will permit better management of contaminated sites in general.

Acid mine drainage (AMD) is another type of environmental contamination that is caused by the oxidation of waste rock deposits. AMD is a major water quality concern, leading to metal loading and acidification in lakes and rivers. Remediation systems that reduce and monitor AMD discharge are required to diminish the levels of acidity and heavy metals that are released into the natural environment. In many acid mine systems, bacteria are responsible for accelerating the rate of mineral oxidation, therefore contributing to environmental pollution (Baker and Banfield, 2003). Because microbial processes are responsible for generating a large portion of acidity in AMD systems, new knowledge about the microbial communities that inhabit these systems is a necessary step to reduce AMD discharge to the environment. Many studies have shown the iron-sulphur oxidizing bacteria *Acidithiobacillus ferrooxidans* is

capable of accelerating acidification (Druschel et al., 2004; Bernier and Warren 2005), however very little is known about the interactions of this bacterium with other parts of the microbial community in environmental systems (Johnson, 1998). Recently a study by Bernier and Warren (2007) identified substantial differences between the rates of sulphur oxidation in pure strain *A. ferrooxidans* and *A. thiooxidans* compared to natural environmental enrichment cultures from Moose Lake, ON, containing these bacteria and a species of acid-tolerant heterotrophic bacteria. The relationship between the autotrophic and heterotrophic communities at AMD sites is unconstrained although it has been suggested that the heterotrophic organisms may play a role in acidity generation (Fournier et al., 1998; Johnson, 1998; Marchand and Silverstein, 2002; Baker and Banfield, 2003; Baker et al., 2003). Developing knowledge of the carbon cycling relationships within AMD microbial ecosystems may provide a foundation for the development of novel remediation strategies with benefits for management of acid mine waste sites and preservation of natural environmental systems.

The major common theme of this thesis was developing new applications for compound specific isotope analysis of Phosopholipid Fatty Acids (PLFA) in order to constrain microbial carbon sources and cycling in contaminated environments. While this approach has been used in many previous studies, unique developments and applications of this methodology were made in this thesis. Chapter Two of this thesis was the first study to use compound specific

radiocarbon analysis of PLFA to directly demonstrate petroleum uptake and degradation in the soil microbial community of a phytoremediation system, and to identify rhizosphere degradation as the primary pathway for removal of petroleum hydrocarbons. Previously, only two other studies have used radiocarbon analysis of PLFA to directly demonstrate petroleum uptake in an environmental system (Slater et al., 2006; Wakeham et al., 2006). The research in Chapter Three is the first to use the differential isotopic fractionation in PLFA biosynthetic pathways to identify autotrophic versus heterotrophic carbon cycling pathways in an AMD ecosystem. This is also the first study to assess the role of heterotrophic organisms in acid mine drainage environments using isotopic analysis.

Multiple Carbon Isotope Analysis

Analysis of the ratios of isotopes of carbon in organic compounds is a powerful tool to detect environmental processes. There are two stable isotopes of carbon (^{12}C, 98.9% abundance, ^{13}C, 1.1% abundance) and one radiogenic isotope (^{14}C, 1 part per trillion abundance) found in nature. Ratios of $^{13}C/^{12}C$ are variable in natural systems and can be used to identify the sources of carbon and/or changes in biogeochemical cycles. Many processes, such as photosynthesis, change the ratio of $^{13}C/^{12}C$ due to faster reaction rates of ^{12}C compared to ^{13}C, causing what is known as an isotopic fractionation effect (O'Leary, 1981; Farquhar, 1989). When a process causes the ratio of $^{13}C/^{12}C$ to increase and the

resulting product of the reaction is isotopically heavy compared to the reactant, it is termed an isotopic enrichment. Conversely, when a process creates a lower $^{13}C/^{12}C$ ratio, it is termed an isotopic depletion.

In addition to the stable isotopes, modern carbon contains approximately one part per trillion ^{14}C, derived from ^{14}N degradation in the upper atmosphere. Radiocarbon (^{14}C) decays with a half-life of 5730 years, and using current methods, is not detectable in materials older than 60,000 years (Hughen et al., 2006). Similar to ^{13}C, ^{14}C is fractionated by natural processes, however as explained below, this fractionation is corrected during data processing to eliminate fractionation effects, thereby allowing ^{14}C to be used directly for comparisons between systems.

Throughout this thesis, conventional delta notation will be used to report isotopic values. Delta notation compares the ratio of heavy to light isotopes in a sample, relative to an international standard, and is expressed as deviations from the standard ratio in parts per thousand (‰ = per mille). Equation 1-1 expresses $\delta^{13}C$ relative to the Pee Dee Belemnite international standard, and Equation 1-2 expresses $\Delta^{14}C$ as the relative activity (A = counts per minute) of a sample compared to the NBS Oxalic Acid standard, normalized to $\delta^{13}C$ of -25‰ (Stuiver and Polach 1977).

$$\delta^{13}C\ (‰) = [(^{13}C/^{12}C_{sample})/(^{13}C/^{12}C_{standard}) - 1] \times 1{,}000 \quad [1\text{-}1]$$

$$\Delta^{14}C\ (‰) = [(A_{SN})/(A_{ON}) - 1] \times 1000 \quad [1\text{-}2]$$

Northern hemisphere atmospheric CO_2 currently has a $\delta^{13}C$ of -8‰ (Allison et al., 2003) and a $\Delta^{14}C$ of +55‰ (Turnbull et al., 2007). Biological carbon fixation by C_3 photosynthesis produces a strong depletion in ^{13}C relative to atmospheric carbon, averaging a $\delta^{13}C$ value of -27‰ and a $\Delta^{14}C$ value close to atmospheric CO_2. Petroleum hydrocarbons are derived from ancient photosynthesis, and therefore retain the $\delta^{13}C$ signature of photosynthetically derived carbon ($\delta^{13}C$ = -27‰). However, over millions of years, all ^{14}C has decayed in petroleum, producing a $\Delta^{14}C$ value of -1000‰. Because of the large differences between sources, the use of ^{13}C and ^{14}C isotopic measurements is a powerful tool for identifying carbon cycling processes and sources in the environment. The use of ^{13}C to determine fractionation and thereby elucidate processes and the use of ^{14}C to delineate carbon sources is an especially powerful tool for studying petroleum carbon cycling in contaminated environments.

Table 1-1: ^{13}C and ^{14}C isotope composition of carbon in environmental systems.

	$\delta^{13}C$ (‰)	$\Delta^{14}C$ (‰)	References
C_3 Organic Material	-27 (-25 to -29)	+55	O'Leary 1988
C_4 Organic Material	-14 (-12 to -16)	+55	O'Leary 1988
Petroleum Hydrocarbons	-27 (-25 to -35)	-1000	Stahl, 1977
Atmosphere	-8 (-7.8 to -8.2)	+55	Allison et al., 2003 Hughen et al., 2006 Turnbull et al., 2007

PLFA as a tool in Microbial Ecology

Phospholipid Fatty Acid (PLFA) analysis is an established tool for the investigation of microbial community composition in soils and sediments. PLFA are found in all cell membranes of eukaryotic and bacterial organisms, they decay rapidly upon cell death, thereby profiling the active microbial community, and can be characteristic of groups of bacteria (e.g. methanotrophs) (White, 1979). Two recent reviews by Green and Scow (2000) and Kaur et al. (2005) have described the capabilities and limitations of PLFA to characterize microbial communities. Benefits of PLFA analysis include: rapid laboratory analysis when compared to molecular techniques such as DNA profiling; direct sampling from the entire *in situ* microbial community; no influence of culturing biases; and the ability to provide an estimate of total biomass in soils or sediments.

Because PLFA can be extracted and purified effectively, they are ideal candidates for stable isotope analysis. This approach is frequently used to

determine carbon cycling and metabolic relationships within an active microbial community. This technique and its application was reviewed by Boschker and Middelburg (2002) and Meier-Augustine (2002) in papers that describe theory, methodology and applications of compound specific PLFA isotope analysis. The technology that enabled these advances is gas-chromatography combustion isotope ratio mass spectrometry (GC/IRMS), which permits very precise measurements of $^{13}C/^{12}C$ isotope ratios in individual compounds at nanomolar concentrations from an environmental sample. As described above, stable isotope analysis is a powerful tool for elucidating carbon cycling processes in the environment. The capability to analyze individual PLFA from an environmental matrix enables sourcing of carbon sources and identifying carbon cycling pathways for specific components of the microbial ecosystem. Variable isotopic fractionation occurs for these processes in biological systems and, if characterized, can be used to identify these processes in nature.

Stable isotope analysis has been successfully used to measure natural abundance $\delta^{13}C$ of PLFA and in studies that introduced a ^{13}C labeled tracer into the environment to measure incorporation into PLFA (Boschker et al., 1998; Pelz et al., 1998; Hanson et al., 1999; Johnsen et al., 2002; Zhang et al., 2002; Petsch, 2003; Crossman et al., 2004; Fang et al., 2004; Treonis et al., 2004; Crossman et al., 2005; Glaser, 2005; Ziegler et al., 2005; Maxfield et al., 2006; Kreuzer-Martin, 2007). The major drawback for natural abundance ^{13}C measurements

occurs when two carbon sources with indistinguishable $\delta^{13}C$ values exist in an environmental system (e.g. Table 1, petroleum hydrocarbons and C_3 natural organic carbon). In these cases, it is impossible to directly determine uptake of one carbon source over another, necessitating other methods to reveal carbon source information. The addition of ^{13}C labeled compounds to soil may elucidate certain processes and pathways, however this approach can be prohibitively expensive, difficult to constrain in the field, and the labeled compounds added to the system may be consumed preferentially relative to the existing recalcitrant target compounds (Steinberg et al., 1987). Therefore, it is beneficial to develop a non-invasive sampling technique that can resolve carbon cycling relationships in the microbial community of petroleum-contaminated environments.

Recently, compound specific radiocarbon analysis of PLFA was developed as a method to detect petroleum incorporation in the microbial community (Eglinton et al., 1996; Slater et al., 2006; Wakeham et al., 2006). Crude petroleum and all chemicals derived from petroleum hydrocarbons contain no detectable ^{14}C due to radioactive decay over millions of years. Therefore because modern carbon contains ^{14}C at the part per trillion level, it is possible to discriminate between modern and petroleum based carbon sources using ^{14}C as an inverse tracer of petroleum uptake (Slater et al., 2005; Slater et al., 2006; Wakeham et al., 2006). Accelerator Mass Spectrometry (AMS) is capable of analyzing the ^{14}C content of 2.0μmol C or more (Eglinton et al., 1996), and using Preparative Capillary Gas

Chromatography (PCGC), PLFA can be cryogenically isolated in sufficient quantities for ^{14}C analysis. This technique is a new and powerful tool for identifying carbon cycling pathways in environmental systems, since it does not requires site disturbance by the addition of a labeled compound, it samples the whole microbial community *in situ,* and has a detection limit of approximately 3% petroleum hydrocarbon incorporation relative to modern organic matter (Slater et al 2005).

The first paper in this thesis uses the ^{13}C and ^{14}C composition of PLFA in addition to other geochemical techniques to determine the degradation pathway of recalcitrant petroleum hydrocarbons in a Multi-Process Phytoremediation System (Huang et al., 2005). This paper contributes novel understanding of *in situ* phytoremediation processes and provides insight into the carbon cycling pathways involved in organic contaminant degradation in phytoremediation systems.

PLFA Biosynthesis

Isotopic fractionation occurs due to kinetic isotope effects in biological processes such as photosynthesis where the chemical reaction rates of ^{12}C are slightly faster than that of ^{13}C. Variations in the relative extent of this biosynthetic fractionation can be related to specific processes. For example, fixation of inorganic carbon results in discrimination of up to 30‰ for the Rubisco Type I enyzyme found in C_3 green plants, algae, and many chemoautotrophic bacteria

(O'Leary, 1988), whereas for C_4 plants and organisms using the PEP Carboxylase pathway the discrimination is only 2‰ (O'Leary, 1981; Farquhar et al., 1989; Hayes, 2001). Further, these fractionation factors can be limited under stresses such as carbon limitation or water depletion (Farquhar et al., 1989). In contrast, incorporation of organic carbon via heterotrophic metabolism typically does not induce a fractionation effect, and thus heterotrophic bacteria will have a $\delta^{13}C$ value similar to their carbon source.

Intracellularly, fractionation occurs at branch points where carbon is separated into two different pathways (e.g. Acetyl-coA is transferred both to the Kreb's cycle and used to produce lipids, including PLFA). Differential reaction rates and fractionation factors can generate substantial isotope effects between these different reservoirs. Furthermore, isotope fractionation is enzyme specific and position specific within a molecule, therefore individual compounds are not isotopically homogenous, and certain atoms within a molecular structure can be consistently depleted relative to the bulk $\delta^{13}C$ of a compound (Monson and Hayes, 1982; Hayes, 2001). If intracellular fractionation does occur, isotopic mass balance must be maintained for the entire cell, such that the sum of all components equals the bulk $\delta^{13}C$ value of the cell (Equation 1-3, Hayes, 2001, X = mole fraction of a class of compounds, $X_{\text{protein}} + X_{\text{lipid}} + X_{\text{nucleic acid}} + X_{\text{carbohydrate}} = 1$)

$$\delta^{13}C_{cell} = \delta^{13}C_{protein}X_{protein} + \delta^{13}C_{lipid}X_{lipid} + \delta^{13}C_{nucleic\ acid}X_{nucleic\ acid} + \delta^{13}C_{carbohydrate}X_{carbohydrate} \quad [1\text{-}3]$$

Typically, aerobic heterotrophic organisms produce PLFA biomarkers that are between 0-3‰ depleted in $\delta^{13}C$ relative to total biomass (Monson and Hayes, 1982; Blair et al., 1985; Teece et al., 1999; Hayes, 2001; Abraham and Hesse, 2003). In contrast, autotrophic and anaerobic organisms often express greater isotopic fractionation between biomass and PLFA (Summons et al., 1994; Sakata et al., 1997; Hayes, 2001; Londry et al., 2004). These differences in isotopic fractionation between autotrophic and heterotrophic bacteria can provide insight into metabolic activities of microbes, and are potentially useful for elucidation of carbon sources and cycling within a microbial community. If unique PLFA biomarkers can be identified for a class of microorganisms in an environmental system, *in situ* analysis of PLFA can provide information about carbon source and potentially ecological relationships between members of the microbial community.

In the second paper of this thesis (Chapter Three), fundamental biosynthetic isotope fractionation factors were measured under controlled conditions for unique autotrophic and heterotrophic PLFA biomarkers enriched from an AMD environment. Using these unique fractionation factors, a model was constructed to discern the ecological relationships within this microbial

community *in situ*, specifically to determine the role of heterotrophic fungi found in association with the autotrophic community.

Research Objectives

The objective of the first study (Chapter Two), was to identify a biodegradation pathway for Total Petroleum Hydrocarbons in a novel Multi-Process Phytoremediation System. While this system has proved to be efficient at petroleum clean up, the relative contribution of the plants and soil microbial community to observed petroleum mass loss is currently unknown. As outlined above, developing process level understanding of phytoremediation methods is important for optimizing remediation systems and providing new biodegradation applications that are beneficial to human health and for restoring natural ecosystems.

The objective of the second paper (Chapter Three), was to identify unique PLFA biomarkers and biosynthetic isotope signatures for autotrophic and heterotrophic microbial communities native to an AMD system, and to use these distinct metabolic processes to develop a model for studying the microbial ecology of AMD systems *in situ*. Because the microbial communities of AMD systems are often more efficient at generating acidity than pure strains of sulphur oxidizing bacteria, understanding the relationships between autotrophs and

heterotrophs in these systems may reveal ecological conditions that enable more favourable acid generation pathways in the environment.

The goal of each study was to develop process-level understanding of carbon cycling and sources in the microbial communities that inhabit contaminated systems. Fundamental understanding of these microbial systems provide valuable insight and/or new tools for the developed of improved mitigation strategies for contaminated environments.

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Chapter 2

Identification of biodegradation pathways in a multi-process phytoremediation system using natural abundance ^{14}C analysis

Abstract

Optimizing remediation of petroleum-contaminated soils requires thorough understanding of the mechanisms and pathways involved in a proposed remediation system. In many engineered and natural attenuation systems, multiple degradation pathways may contribute to observed contaminant mass losses. In this study, biodegradation in the soil microbial community was identified as a major pathway for petroleum hydrocarbon removal in a novel Multi-Process Phytoremediation System (MPPS) using natural abundance ^{14}C analysis. In contaminated soils, the $\Delta^{14}C$ of Phospholipid Fatty Acids (PLFA) of less than -800‰ directly demonstrated microbial uptake and utilization of petroleum derived carbon during bioremediation. Mass balance indicated that more than 80% of microbial carbon was derived from petroleum hydrocarbons and a maximum of 20% was produced from metabolism of modern carbon sources. In contrast, the nearby uncontaminated control microbial community maintained a nearly modern ^{14}C signature. Mass balance using $\delta^{13}C$ and $\Delta^{14}C$ of rhizosphere CO_2 demonstrated that mineralization of petroleum carbon contributed 60-65% of

soil CO_2 at the contaminated site. The remainder was derived from the atmosphere (27-30%) and decomposition of non-petroleum natural organic carbon (5-10%). The clean control exhibited substantially lower CO_2 concentrations that were derived from atmospheric (55%) and natural organic carbon (45%) sources. This study highlights the value of using multiple carbon isotopes to identify degradation pathways in petroleum-contaminated soils undergoing phytoremediation.

Introduction

Identifying degradation mechanisms and pathways during bioremediation of contaminated environments is integral for optimizing and assessing the efficiency of remediation schemes. However, in many systems, multiple pathways and mechanisms may be involved, particularly when more than one method is being applied (Bamforth 2005, Cohen 2002, Atlas 1995, Vidali 2001). While traditionally employed to remediate heavy metals, phytoremediation is a useful tool to enhance the remediation of organic contaminants (Pilon-Smits, 2005). Phytoremediation of organic contaminants uses plants to accelerate the removal of contaminants from surface soils to take advantage of enzymatic activity in the plant roots or to generate mutually beneficial interactions between plants and rhizosphere bacteria (Cohen 2002). Recently, a Multi-Process Phytoremediation System (MPPS) was developed to remove recalcitrant total petroleum

hydrocarbons (TPH) from a land-farm soil at the Imperial Oil Refinery complex in Sarnia, Ontario (Huang et al., 2005). In MPPS, several practices including phytoremediation and the addition of plant growth-promoting rhizobacteria (PGPR) are combined to remediate soils that were contaminated with high concentrations of TPH from the refining process. While the MPPS was demonstrated to be effective for hydrocarbon removal, the active microbial mechanisms and degradation pathways remained unconstrained (Huang et al., 2005).

A central question raised within MPPS, and phytoremediation systems in general is the role of soil microorganisms versus the role of plants in the removal of TPH. Processes that occur in the rhizosphere, the fraction of the soil most closely associated with root exudates, including interactions between rhizobacteria and contaminants are potentially responsible for driving remediation in these systems, however differentiating the relative roles of soil microbes and plants is often impossible using conventional approaches. Concentration measurements of TPH in soils can provide information about the levels of contamination in a soil, however assessing the occurrence and extent of remediation of petroleum hydrocarbons is difficult due to the complexity of compounds in a hydrocarbon mixture and their frequently heterogeneous distribution within a matrix. In addition, dissolution or volatilization can remobilize and/or remove hydrocarbons in soil without any active degradation

processes occurring. Further, while genetic profiling and microbial culturing methods can monitor changes in the microbial community, and identify the presence of microbes capable of degrading a contaminant, they do not demonstrate that degradation processes are actively occurring *in situ*.

Compound specific stable isotope analysis has emerged as a new tool to investigate and trace pathways of organic contaminant degradation (Slater, 2003; Schmidt et al., 2004). A challenge of using stable isotope methods is that frequently the $\delta^{13}C$ of natural organic matter ($\delta^{13}C_{NOM}$ = -25 to -29) overlaps with that of petroleum ($\delta^{13}C_{TPH}$ = -25 to -35) preventing resolution of these carbon sources (Slater et al., 2006). Petroleum hydrocarbons contain no detectable ^{14}C due to radioactive decay over millions of years. When modern organic carbon is fixed from atmospheric CO_2, it retains a modern ^{14}C signature due to production of this isotope in the upper atmosphere. When heterotrophic organisms consume recently produced organic carbon, these modern levels of ^{14}C are transferred to their metabolic products including cellular components and byproducts such as CO_2. In contrast, when bacteria metabolize petroleum-derived carbon the ^{14}C content of their cellular material and metabolic byproducts decreases due to incorporation of these ^{14}C depleted carbon source (Petsch et al., 2001; Slater et al., 2005; Slater et al., 2006; Wakeham et al., 2006).

Multiple isotope measurements of soil CO_2 using ^{13}C and ^{14}C have been used to distinguish between atmospheric, natural organic carbon and ancient

petroleum derived CO_2 in contaminated systems (Aelion et al., 1997; Kirtland et al., 2005) thereby directly demonstrating mineralization of petroleum. Detection of mineralization alone does not conclusively indicate biodegradation because abiotic degradation processes, or those occurring in plant roots will produce identical ^{13}C and ^{14}C values to microbial breakdown of petroleum hydrocarbons.

In a petroleum bioremediation system, natural abundance radiocarbon profiling of phospholipid fatty acid biomarkers (PLFA) is the optimal method to elucidate microbial carbon sources, and to therefore constrain major degradation pathways. PLFA are integral components of cell membranes that can be characteristic of classes of microbes. Further, because PLFA degrade quickly upon cell death (White et al., 1979) they provide a snapshot of the viable microbial community at a site. Stable isotope measurements of PLFA have been previously used to elucidate microbial processes and carbon cycling pathways (Boschker et al., 1998; Hanson et al., 1999; Petsch, 2003; Fang et al., 2004), however the limited range of $\delta^{13}C$ in petroleum and natural organic carbon can prevent the identification of biodegradation using ^{13}C (Slater et al., 2005). By exploiting the large disparity in ^{14}C between modern and ancient carbon, it is possible to distinguish between ancient petroleum and modern carbon sources, and therefore delineate degradation pathways in the microbial community, via natural abundance compound specific radiocarbon analysis of PLFA (Petsch et al., 2001; Slater et al., 2005; Slater et al., 2006; Wakeham et al., 2006). This novel ^{14}C

approach overcomes the limitation of using the $\delta^{13}C$ signature of PLFA to trace carbon sources and pathways in environmental systems when the indistinguishable nature of natural soil organic matter and petroleum hydrocarbons precludes utilization of this approach to demonstrate biodegradation of TPH.

The objective of this study was to demonstrate the contribution of rhizosphere bacterial biodegradation to a phytoremediation system, thereby characterizing the processes that promote effective and rapid degradation of total petroleum hydrocarbons in the rhizosphere of MPPS, using multiple carbon isotope analysis of PLFA and soil CO_2. By demonstrating the contribution of rhizosphere bacteria to petroleum removal, this study constrains the degradation pathways in a Multi-Process Phytoremediation System.

Methods

Site Description

Two experimental phytoremediation plots and two control sites at the Imperial Oil land farm site in Sarnia, ON were selected for this study. Experimental plots were created with the following MPPS design: Barley, Wild Rye and Tall Fescue and treated with Plant Growth Promoting Rhizobacteria, PGPR ("BRF+") (Huang et al., 2004b, a; Huang et al., 2005); Wild rye and Tall Fescue and no PGPR ("RF-"). The PGPR treatment substantially increased plant

growth relative to sites that did not receive treatment. The untreated control was a petroleum-contaminated site that had not received treatment and the clean control was a nearby natural site, with a local grass cover, that was never contaminated with petroleum hydrocarbons.

Soil Gas Collection and Analysis

Soil gas was collected by installing a porous plastic 60mL syringe to create a void space in the soil at 10cm depth. The syringe was attached to Tygon, clamped at the surface to prevent atmospheric mixing with subsurface gases. Gas was sampled after a one-month equilibration period by attaching a 60mL syringe to the surface tubing, releasing the clamp and drawing one pore volume. The contents of the syringe were transferred into an evacuated 60mL serum bottle for later analysis. Soil CO_2 concentrations were analyzed on an SRI 8610C gas chromatograph (3' silica gel column) isothermal at 120°C. Precision on CO_2 concentration standards was better than 1%. Stable carbon isotope ratios of CO_2 were determined using an Agilent 6890 GC equipped with a GS/Q column, coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. The GC temperature program was set to 120°C isothermal. Reproducibility of CO_2 isotopic standards was 0.2‰ or better. CO_2 for radiocarbon analysis was purified cryogenically on a vacuum line, sealed in quartz tubes and shipped to the National Ocean Sciences Accelerator Mass Spectrometer (NOSAMS) facility at Woods Hole Oceanographic Institution,

Woods Hole, MA for radiocarbon analysis. Data is expressed in $\Delta^{14}C$ notation, normalized to $\delta^{13}C$ of -25‰, in per mille (‰) deviation relative to the ^{14}C Standard Reference Material for 4990B, oxalic acid (Stuiver and Polach, 1977), and are age corrected to the year of collection (2006). Soil CO_2 $\delta^{13}C$ values were independently verified at NOSAMS within 0.5‰ of the reported values in this study.

Soil Sampling and Bulk Organic Carbon Isotope Analysis

Bulk soil samples (500mL) were obtained from each MPPS treated site and the two control sites in August 2006 from a soil depth of 5-10cm. Soils were sampled directly into pre-combusted 500mL glass jars with Teflon faced lids, and frozen at -20°C until analysis. Prior to analysis, roots were removed, soils were homogenized using a mortar and pestle, acidified three times with 1N HCl to remove carbonates, washed three times with distilled, deionized water, and dried at 50°C for 24h. Triplicate samples were combusted and analyzed online by EA/ IRMS, with standards IAEA 600, IAEA CH-7, NBS 21, USGS 24 and USGS 40. Precision for replicate analysis of standards was a standard deviation of 0.2‰ or better. BRF+ and Clean Control Soils were analyzed for bulk organic ^{13}C and ^{14}C at NOSAMS. The $\delta^{13}C$ values measured at NOSAMS were within 0.5‰ of the values measured at McMaster.

PLFA Extraction

Soil samples, approximately 150g wet weight, were homogenized using a mortar and pestle, and sonicated with 2:1:0.8 methanol:dichloromethane:phosphate buffer in centrifuge tubes using the modified Bligh and Dyer extraction (White, 1979, Slater 2005). Soils were centrifuged for 10 minutes at 2000rpm (Sigma 4-15C), and solvent was decanted through a filter to a separatory funnel. Distilled, de-ionized nanopure water was added to separate aqueous from organic phase, and the organic phase was drained for analysis. Polar lipids were separated from the total lipid extract on one hundred grams of fully activated silica gel (100-200 mesh, 60Å) by elution with 1L dichloromethane (f_1, non-polar lipids), 1L acetone (f_2, neutral lipids) and 1L methanol (f_3, polar lipids including phospholipids). Solvent was removed from the phospholipid fraction (f_3) via a rotary evaporator, and the sample was re-dissolved in dichloromethane. Due to the high levels of petroleum hydrocarbons present in the samples, a secondary silica gel purification was required using 3g fully activated silica gel (100-200 mesh, 60Å) and eluted with 30mL dichloromethane (f_1), acetone (f_2) and methanol (f_3 PLFA) to ensure a clean baseline during chromatographic analysis. The secondary f_3 fraction was evaporated to dryness under a stream of nitrogen gas, and reacted to Fatty Acid Methyl Esters (FAME) via the mild alkaline methanolysis reaction (White et al., 1979). FAME $\delta^{13}C$ and $\Delta^{14}C$ values were corrected during data analysis for the addition of methanol carbon. FAME were further purified using a third silica gel chromatography step,

on 0.5 grams of fully activated silica gel and eluted with 5 mL hexane, 5 mL dichloromethane (FAMEs), and 5 mL methanol.

Pure cultures of PGPR bacteria (*UW4,* Huang et al., 2005) were obtained from Bruce Greenberg at the University of Waterloo (Waterloo, ON, Canada). Cultures were filtered through a 0.2μm filter and extracted as described above. The first (100g) silica gel column was not necessary for separation of PLFA from the culture media, and was therefore omitted from this procedure.

Identification and Quantification of PLFA

An Agilent 6890 GC (30 m x 0.25 mm DB-5 MS column, 0.25 μm film thickness) coupled to a 5973 quadrupole mass spectrometer was used for identification and quantification of FAME. The temperature program was 40°C hold 1 min, ramp to 130°C at 20°C/min, to 160°C at 4°C/min and finally to 300°C at 8°C/min.. FAME were quantified using external calibration standards methyl tetradecanoate and methyl eicosanoate (Supelco Inc.). FAME were identified using several bacterial reference standards (Bacterial Acid Methyl Esters CP Mix, Matreya Inc., Fatty-Acid Methyl Ester Mix, Supelco Inc.), mass-fragmentation patterns, and retention times. Double bond positions were determined by analysis of DMDS adducts (Nichols et al., 1986).

GC-C-IRMS analysis of PLFA

Stable carbon isotope ratios of individual PLFA were determined using an Agilent 6890 GC (30 m x 0.25 mm DB-5 MS column, 0.25 μm film thickness)

coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. The GC program was 50°C for 1 min; 10°C/min to 150°C; 1.5°C/min to 180°C for 20 minutes; 10°C/min to 280°C; 15°C/min to 320 for 15 minutes. Data is reported in delta notation ($\delta^{13}C$) relative to the PeeDee Belemnite standard reference material. Reproducibility for isotopically characterized hexadecane, octacosane and m-terphenyl laboratory standards was better than 0.3‰ (1σ) and accuracy was always within 2σ of expected values. Precision for analysis of microbial PLFA was better than 1.5‰ (1σ) for minimum of three analyses.

Compound Specific Radiocarbon Analysis

Concentrated PLFA extracts were collected cryogenically on a Preparative Capillary Gas Chromatograph (PCGC) to obtain greater than 2.0 μmolC required for ^{14}C analysis (Eglinton et al., 1996; Pearson et al., 1998). Due to extensive time and resource requirements for ^{14}C analysis, only the Clean Control and BRF+ soil PLFA were analyzed. PLFA were separated on an Agilent gas chromatograph (60 m x 0.5 mm DB-5 column, 0.25 μm film thickness) interfaced with a Gerstel preparative fraction collection (PFC) system. Samples were collected cryogenically in glass u-tubes at -20°C using chilled methanol to condense PLFA. GC temperature program was 50°C; 10°C/min to 160°C; 1.5°C to 180°C for 20 minutes; 1.5°C/min to 220°C; 10°C/min to 300°C. The transfer line between the GC and PFC was set isothermal at 310°C, and the PFC interface was set isothermal at 320°C. Glass u-traps were eluted with dichloromethane and

transferred to GC vials. The total area of non-target peaks in all samples was less than 0.5% of the collected target, and total target recovery was better than 80%. Compound specific AMS analysis to determine $\Delta^{14}C$ was performed at the NOSAMS via previously described methods (Pearson et al., 1998). Data is reported in $\Delta^{14}C$ notation, as described above. Collection of PLFA on a PCGC can induce isotope fractionation effects if only a partial peak is collected (Eglinton et al., 1996) therefore verification of collected PLFA is necessary to ensure that no fractionation occurred at this step. The mean $\delta^{13}C$ fractionation measured before and after collection was 0.7‰ (n=11) and the maximum observed fractionation was 2.5‰.

Results

Bulk Organic Carbon

The total organic carbon (TOC) $\delta^{13}C$ of the contaminated soil was -28.8‰ (Table 2-1), and the $\Delta^{14}C_{TOC}$ of the BRF+ MPPS treated soil was -941‰ (Table 2-2). Extracted TPH from the contaminated site was more depleted ($\Delta^{14}C_{TPH}$ = -992‰) than the bulk TOC of the contaminated soils. The Clean Control soil had a $\Delta^{14}C_{TOC}$ of -148‰ and a $\delta^{13}C_{TOC}$ of -27.8‰, that were typical of uncontaminated C_3 plant soils (Rethemeyer, 2007). The Clean Control values were assumed to be representative of natural organic matter that would have been present in the contaminated soils prior to petroleum contamination. Atmospheric

CO_2 $\delta^{13}C$ was -8‰ (Allison et al., 2003) and $\Delta^{14}C$ was +55‰ (Turnbull et al., 2007).

Soil gas analysis

Soil gas CO_2 composition is shown in Table 1. CO_2 comprised 0.4-0.6% of the total gas composition in the contaminated soils and 0.1% in the Clean Control.

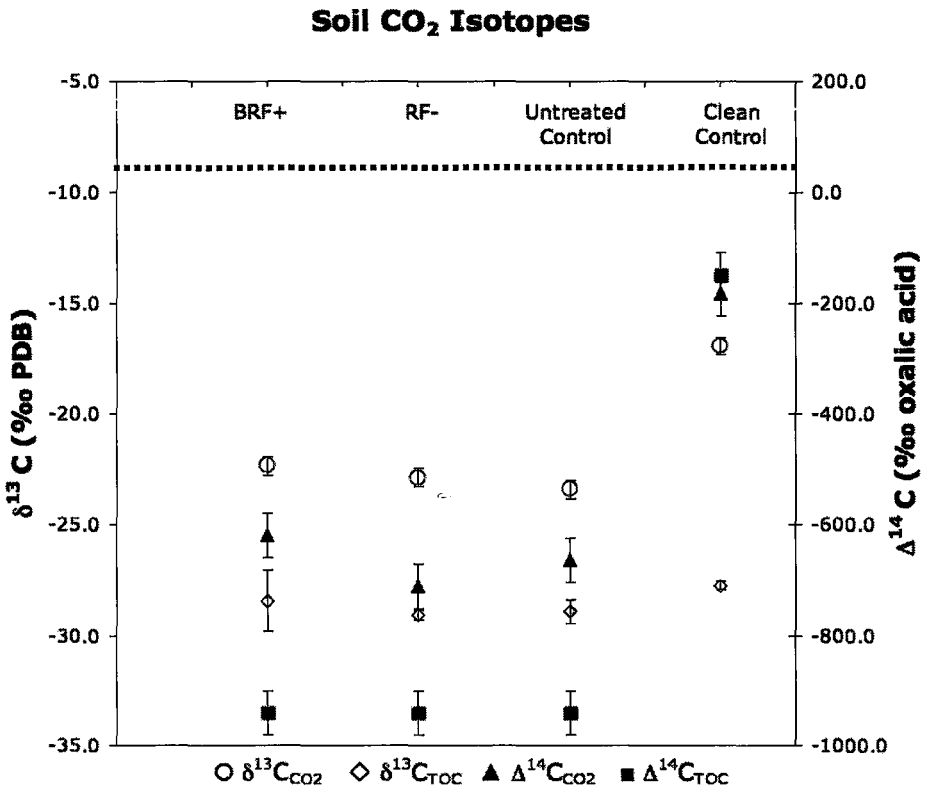

Figure 2-1: $\delta^{13}C$ and $\Delta^{14}C$ of soil gas and TOC from contaminated and uncontaminated sites. Error bars represent 40‰ (two times standard ^{14}C age error) for $\Delta^{14}C_{CO2}$ measurements, 2σ for $\delta^{13}C_{CO2}$, and $\delta^{13}C_{TOC}$. Dotted line represents atmospheric $\delta^{13}C$ (-8‰) and $\Delta^{14}C$ (+55‰) values.

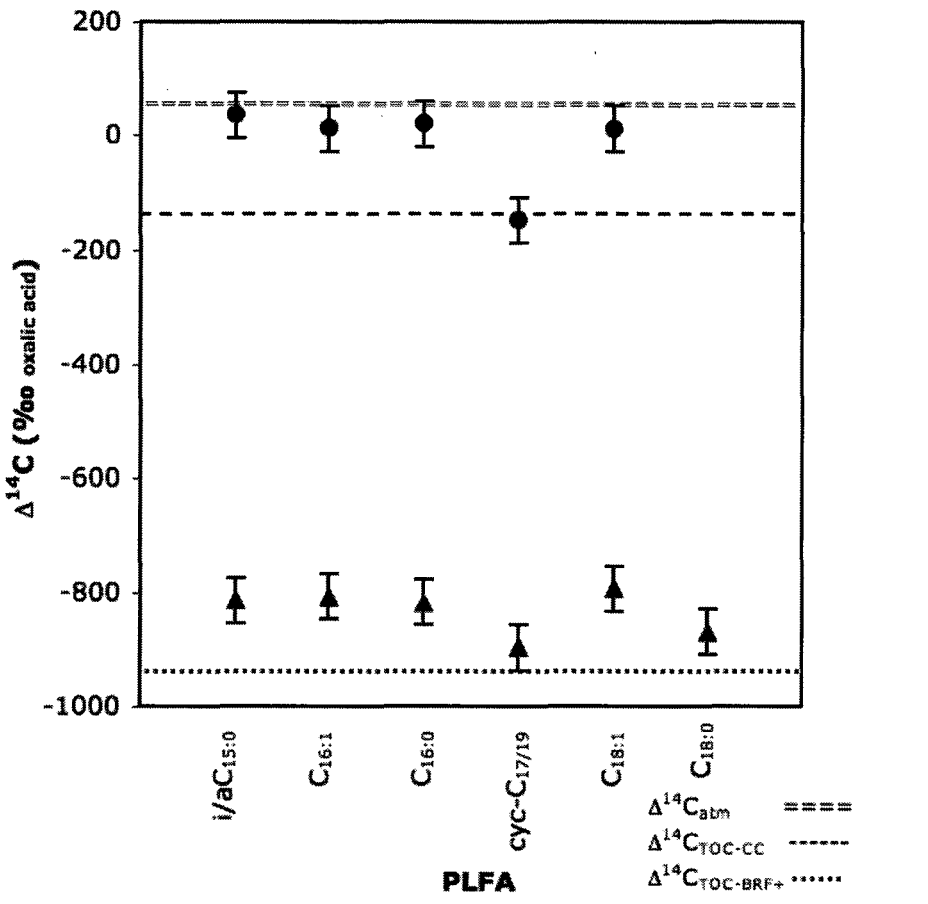

Figure 2-2: ^{14}C results for PLFA from the BRF+ treated soil (triangles) and the Clean Control soil (circles). Error bars represent two times the standard radiocarbon age error (±40‰) for $\Delta^{14}C$. PLFA in this figure represent >50% of all detected PLFA by mole percent.

In addition to CO_2, a substantial O_2 peak was detected in all samples. O_2 concentrations were not precisely quantified due to an interfering N_2 peak, however, the presence of O_2 in the surface soils indicates that aerobic conditions prevail in the bulk soils. Stable isotope analysis of the soil CO_2 revealed isotopic depletions in $\delta^{13}C_{CO2}$ in the contaminated soils relative to the Clean Control (Figure 2-1). The BRF+, RF- and contaminated control soils did not vary greater than instrument reproducibility. Soil $\Delta^{14}C_{CO2}$, also shown in Figure 2-1, indicates that CO_2 was substantially depleted ($\Delta^{14}C$ = -621‰ to -712‰) relative to the Clean Control ($\Delta^{14}C$ = -148‰) in all contaminated soils.

PLFA

PLFA analysis of the Clean Control and contaminated soils demonstrated the presence of distinct microbial communities (Table 2-3). More than sixty different PLFA were detected in contaminated sites, and over forty different PLFA were detected in the Clean Control. The most substantial difference in PLFA distribution was the large abundance of unsaturated PLFA in the Clean Control soil, and a corresponding greater proportion of saturated and branched PLFA in the contaminated soils. Analysis of pure cultures of PGPR did not produce unique biomarker lipids, and the treated sites were not dominated by their PLFA (data not shown).

Table 2-1: Results of $\delta^{13}C$ measurements of soil CO_2, and resulting mass balance results for determining the contributions of atmospheric and respired carbon to soil CO_2

Treatment Type	$\%CO_2$ ± 0.10%	$\delta^{13}C_{TOC}$ ±0.5‰	$\delta^{13}C_{CO2}$ ±0.2‰	f_{atm} ± 5%	f_{TOC} ± 5%
BRF+PGPR	0.42%	-28.5‰	-22.4‰	30%	70%
RF-	0.55%	-29.1‰	-22.9‰	29%	71%
Untreated Control	0.44%	-28.9‰	-23.4‰	27%	73%
Clean Control	0.11%	-27.8‰	-16.9‰	55%	45%

Table 2-2: Results of $\Delta^{14}C$ measurements of soil CO_2, and resulting mass balance results for determining soil CO_2 sources. n.d. indicates not determined. f_{atm} was determined from ^{13}C mass balance.

Treatment Type	$\Delta^{14}C_{TOC}$ ±20‰	$\Delta^{14}C_{CO2}$ ±20‰	f_{atm} ± 5%	f_{NOC} ± 5%	f_{TPH} ± 5%
BRF+PGPR	-941‰	-620‰	30%	7%	63%
RF-	n.d.	-712‰	29%	-2%[a]	73%
Untreated Control	n.d.	-664‰	27%	7%	66%
Clean Control	-148‰	-182‰	55%	45%	0%[b]

a. within error, this represents 0% modern carbon respiration
b. it was assumed that petroleum hydrocarbon degradation does not contribute to soil CO_2 in the Clean Control

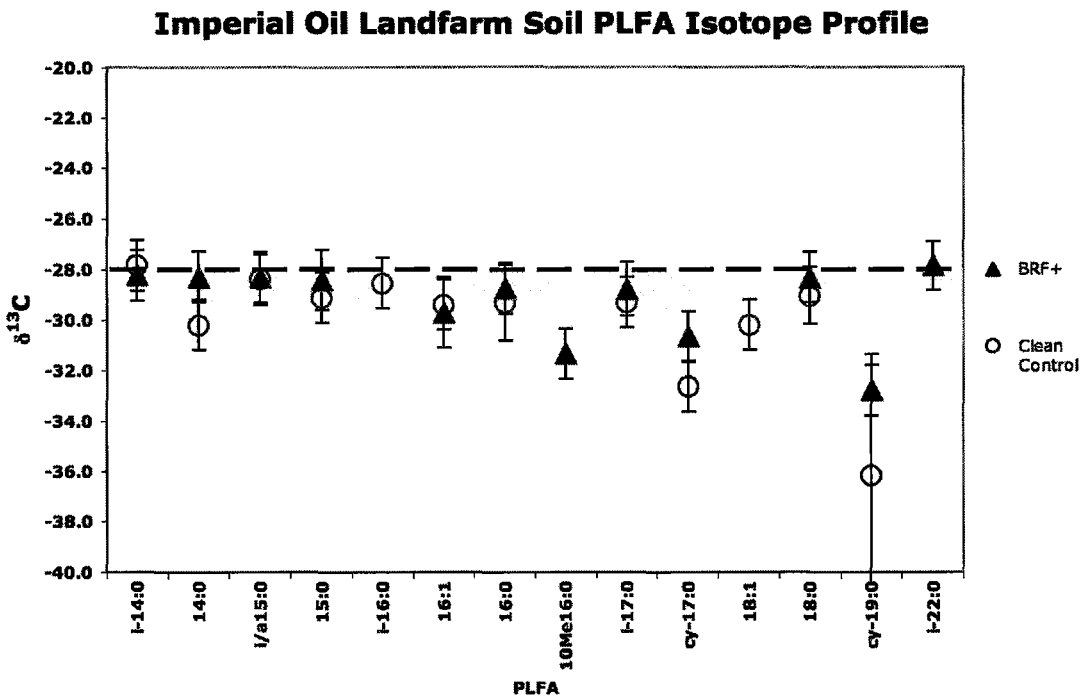

Figure 2-3: $\delta^{13}C$ distribution of PLFA from the MPPS treated BRF+ site and the Clean Control. Shaded area represents 0-3‰ fractionation expected in aerobic heterotrophic microbial communities, dashed line represents $\delta^{13}C_{TOC}$ of the Clean Control Error bars represent 2σ of triplicate analyses reproducibility of GC-IRMS analysis. In the clean Control cy-19:0 co-eluted with an interfering peak, generating poor reproducibility.

Radiocarbon analysis of PLFA (Figure 2-2) revealed a very depleted microbial community in the BRF+ site, and a relatively modern community at the Clean Control. In the BRF+ soil, PLFA ($\Delta^{14}C_{PLFA}$ = -793 to -897‰) were slightly enriched relative to TOC. In the Clean Control soil, $\Delta^{14}C_{PLFA}$ were also enriched relative to $\Delta^{14}C_{TOC}$, and range between +36‰ and -147‰. Stable isotope analysis of the PLFA in all three contaminated soils and the Clean Control were virtually indistinguishable from each other (Figure 2-3). In both soils, PLFA were depleted in $\delta^{13}C$ between 0-3‰ relative to $\delta^{13}C_{TOC}$ and there was little variability within the community of the clean or contaminated sites.

Table 2-3: PLFA Distributions for contaminated and uncontaminated soils. TerBrSat = terminally branched saturates, Sat = saturates, MidBrSat = Mid-chain branched saturates, Unsat = unsaturates.

Treatment Type	TerBrSat	Sat	MidBrSat	Unsat
Untreated Control	24.6%	24.0%	14.5%	30.1%
BRF+	24.7%	25.8%	16.3%	26.0%
RF-	22.8%	24.9%	18.1%	33.2%
Clean Control	11.5%	16.9%	6.3%	62.9%

Discussion

Relative concentration of petroleum hydrocarbons

To constrain the relative amount of petroleum ($\Delta^{14}C_{TPH}$ = -992‰) relative to modern organic carbon in the contaminated BRF+ soil, a mass balance was derived in Equation 2-1 using the Clean Control ($\Delta^{14}C_{TOC}$ = -148‰) as the natural background organic carbon, as described above.

$$\Delta^{14}C_{BRF+\,TOC} = \Delta^{14}C_{TPH} \times (f) + \Delta^{14}C_{CC\,TOC} \times (1-f)$$

f = fraction of organic material in soil derived from TPH degradation [2-1]

Using this equation, it was calculated that 94% of organic carbon in the BRF+ soil is comprised of petroleum hydrocarbons, indicating extensive contamination of the soils.

Petroleum hydrocarbon mineralization

Increased concentrations of CO_2 in soil gas relative to the Clean Control suggested increased respiration of organic material is occurring in the subsurface of the contaminated sites, relative to the Clean Control. This observation was supported by ^{13}C results that indicated the elevated CO_2 levels are derived from the degradation of ^{13}C depleted organic carbon (either NOM or TPH) in these soils, rather than by advective transport or atmospheric mixing. The presence of near atmospheric O_2 concentrations in the soil gas indicated that aerobic

conditions prevail, and therefore aerobic oxidation is likely a major pathway of petroleum removal in the soil.

In order to constrain the relative contribution of organic carbon respiration to soil gas, a two-member mass balance was derived using Equation 2-2 to determine the fraction of CO_2 derived from atmospheric ($\delta^{13}C_{atm}$ = -8‰) and TOC ($\delta^{13}C_{TOC}$ = -28‰) sources. Because the natural organic matter and petroleum hydrocarbons have similar $\delta^{13}C$ values, this mass balance cannot differentiate between petroleum mineralization and NOM. However, it can identify the relative contribution of atmospheric CO_2 versus respiration of soil organic carbon.

$$\delta^{13}C_{CO2} = \delta^{13}C_{TOC} \times (f) + \delta^{13}C_{atm} \times (1\text{-}f)$$

f = fraction of CO_2 derived from TOC degradation [2-2]

For the contaminated soils, this mass balance indicated that between 27-30% of CO_2 was derived from the atmosphere, and 70-73% was derived from TOC degradation. Whereas, in the Clean Control soil, 55% of the CO_2 was atmospheric and only 45% was derived from TOC respiration (Table 2-1). The elevated CO_2 concentrations and increased fraction of TOC-derived CO_2 in soils indicated that the differences between the clean and contaminated sites are caused by increased respiration of ^{13}C depleted organic carbon, as opposed to carbonate dissolution, increased atmospheric flux or advective transport processes.

Natural abundance radiocarbon analysis is capable of overcoming the limitations of the stable isotope approach and can directly identify petroleum derived carbon in the soil gas. Depleted $\Delta^{14}C_{CO2}$ in contaminated soils provides direct evidence for *in situ* petroleum mineralization. In order to differentiate the component of soil gas generated by TPH mineralization from NOM, the three member mass balance presented in Equation 2-3 was used. This mass balance used the following values to determine the fraction of soil CO_2 derived from the degradation of TPH and NOM in contaminated soils ($\Delta^{14}C_{atm}$ = +55‰ (Turnbull et al., 2007), f_{atm} derived from $\delta^{13}C$ mass balance in Table 2-1, $\Delta^{14}C_{TPH}$ = -992‰, $\Delta^{14}C_{NOM}$ = -148‰, from Clean Control $\Delta^{14}C_{TOC}$).

$$\Delta^{14}C_{CO2} = \Delta^{14}C_{atm} \times f_{atm} + \Delta^{14}C_{TPH} \times f_{TPH} + \Delta^{14}C_{NOM} \times f_{NOM},$$

$$(f_{atm} + f_{TPH} + f_{NOM} = 1) \text{ [2-3]}$$

f_{atm} = fraction of CO_2 derived from atmospheric mixing

f_{TPH}= fraction of CO_2 derived from petroleum degradation

f_{NOM} = fraction of CO_2 derived from natural organic carbon degradation

For the contaminated soils, $\Delta^{14}C$ mass balance estimated that 63 to 73% of CO_2 in the contaminated soils is derived from petroleum (Table 2-2). Within error, these values cannot be distinguished from each other. This mass balance indicates that a maximum of 7% of total soil CO_2 was derived from background natural

organic matter at the contaminated sites. Complete mineralization of TPH is the optimal end-product in MPPS, and these results indicate that CO_2 in soil is derived largely from petroleum hydrocarbons. However, it does not specifically identify a degradation pathway since abiotic degradation and biodegradation of TPH will produce the same isotopic signature in soil CO_2.

Because CO_2 in the Clean Control soil was more depleted ($\Delta^{14}C_{CO2}$ = -182‰) than NOM ($\Delta^{14}C_{NOM}$ = -148‰), another depleted source of CO_2 must contribute to soil gas composition. This depleted source could potentially be contributed by advective fluxes from the subsoil or contributed from soil carbonate dissolution. However, because the CO_2 of the contaminated soils was enriched compared to TOC in ^{13}C and ^{14}C, reflecting modern atmospheric contributions, it is unlikely that carbonate dissolution contributes a major amount of CO_2 to the contaminated systems.

Microbial biodegradation of petroleum hydrocarbons

PLFA composition indicated that different microbial communities exist at the contaminated sites compared to the Clean Control. Greater abundance of unsaturated PLFA in the Clean Control are indicative of a community comprised mainly of Gram negative bacteria (Green and Scow, 2000). In contrast, the contaminated sites were dominated by branched and saturated PLFA indicating a community comprised mainly of Gram positive bacteria (Green and Scow, 2000).

No unique biomarkers were present in PGPR cultures to provide evidence that PGPR bacteria were a major component of the soil microbial community.

Multiple isotope analysis of PLFA directly demonstrated petroleum uptake and metabolism in the soil microbial community. The depleted $\Delta^{14}C_{TOC}$ at the BRF+ treated site ($\Delta^{14}C$ = -941‰) compared to the clean control ($\Delta^{14}C$ = -148‰) indicates that the majority of organic carbon at the contaminated sites is derived from petroleum hydrocarbons. Figure 2-2 depicts microbial PLFA in the BRF+ soil, and indicates that they are derived from 80-90% petroleum hydrocarbons ($\Delta^{14}C_{PLFA}$ = -793‰ to -897‰), compared to the nearly modern ^{14}C signature detected in the PLFA of the Clean Control soil. The large difference between clean control and contaminated PLFA indicated that the majority (>50% of PLFA by mol% were analyzed for ^{14}C content) of the soil microbial community in MPPS is actively consuming petroleum hydrocarbons as opposed to primarily natural organic carbon. In addition, these $\Delta^{14}C_{PLFA}$ values in the BRF+ treated soil are the largest $\Delta^{14}C$ depletions ever detected for an *in situ* microbial community.

Compound specific ^{14}C analysis overcame the limitations of stable isotope measurements of PLFA that were incapable of resolving differences in carbon source between the Clean Control and MPPS treated microbial communities (Figure 2-3). PLFA were depleted in $\delta^{13}C$ 0-3‰ relative to $\delta^{13}C_{TOC}$ in both contaminated soils and Clean Control. This $\delta^{13}C_{PLFA}$ signature indicates that the soil microbial community is consuming organic matter *in situ*, but it does not

discriminate between natural organic matter and petroleum hydrocarbons. This range of depletion from carbon source to PLFA has previously been identified as a signature of aerobic, heterotrophic microbes, however this signature alone is not a unique indicator of this type of community (Blair et al., 1985; Hayes, 2001). The use of this multiple isotope approach, particularly the addition of ^{14}C analysis, conclusively demonstrated petroleum uptake and metabolism in the BRF+ soil microbial community, and establishes biodegradation as a principal degradation pathway in MPPS.

Preferential degradation of modern soil carbon sources

In the BRF+ treated soil, $\Delta^{14}C_{CO2}$ and $\Delta^{14}C_{PLFA}$ correlate closely. Removal of the atmospheric CO_2 component from the mass balance in Equation 2-2 calculates that 89% of respired CO_2 in contaminated soils is derived from TPH, while only 11% is respired from NOM sources. This indicates that microbial respiration of petroleum is the primary production pathway for CO_2 production in the rhizosphere of MPPS treated soils. In contrast, the PLFA in the Clean Control soil are enriched relative to the bulk $\Delta^{14}C_{TOC}$ value. These results suggest that the microbial community in the Clean Control soil is preferentially degrading recent, more labile organic carbon as a nutrient source in the rhizosphere, as opposed to the older, more recalcitrant soil carbon. This is expected based on previous work by Rethemeyer et al., (2004) who demonstrated preferential microbial metabolism of younger soil carbon sources. Furthermore, because the PLFA are enriched in

$\Delta^{14}C$ relative to TOC, microbial respiration cannot be the primary source of depleted CO_2 observed in these soils.

Biosynthetic variation in PLFA production

Isotopic compositions of PLFA were consistent within the BRF+ site and the Clean Control site with the exception of cy-$C_{19:0}$ and cy-$C_{17:0}$. These PLFA were depleted in $\delta^{13}C$ greater than 3‰ relative to $\delta^{13}C_{TOC}$. This was attributed primarily to a biosynthetic mechanism that is discussed in greater detail in Chapter Three. During the synthesis of the cyclopropyl group in the fatty acid chain, a ^{13}C depleted methyl group from the amino acid methionine is added to an unsaturated fatty acid chain (Grogan and Cronan, 1997; Sacks and Brenna, 2003), resulting in a slightly more depleted $\delta^{13}C$ value for the cyclopropyl fatty acids. This biosynthetic effect explains the observed depletions for the depleted cyclopropyl fatty acid chains observed in both the clean and contaminated soils.

Conclusions

Radiocarbon analysis of soil PLFA, combined with ^{13}C and ^{14}C soil gas analysis identified a major petroleum degradation pathway in this Multi-Process Phytoremediation System and conclusively demonstrated petroleum uptake and metabolism in the soil microbial community. Elevated CO_2 concentrations and $\delta^{13}C_{CO2}$ depletions in soil gas is evidence for increased organic carbon respiration was occurring in contaminated soils relative to the Clean Control. Soil CO_2 in contaminated soils was measured to be depleted in $\Delta^{14}C$ relative to the Clean

Control, indicating degradation of petroleum constituted a major source to soil gas. Finally, ^{14}C depleted PLFA biomarkers directly indicated uptake and metabolism of TPH in the soil microbial community, determining that the primary carbon source to the microbial community was indeed petroleum hydrocarbons. Therefore , the soil microbial community were determined to be responsible for a large proportion of TPH degradation in this remediation system.

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Chapter 3

Carbon Isotope Fractionation in Lipid Biomarkers of Bacteria Native to an Acid Mine Drainage Lake

Abstract

This study identifies unique isotope signatures associated with autotrophic and heterotrophic microbial communities that may be used to determine carbon cycling relationships *in situ* for acid mine drainage (AMD) sites. Stable carbon isotope ratios ($\delta^{13}C$) of carbon sources, bulk cells, and membrane phospholipids (PLFA) were measured for autotrophic and heterotrophic enrichment cultures from an AMD system at Moose Lake, Ontario, Canada, and for pure strains of the sulfur oxidizing bacteria *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*. The autotrophic enrichments and pure strains were found to have indistinguishable PLFA distributions and cyc-$C_{19:0}$ was determined to be a unique biomarker in this system for these sulfur oxidizing bacteria. In contrast, $C_{18:2}$ was uniquely produced by heterotrophic enrichments. Genetic information and PLFA distributions for the heterotrophic cultures were consistent with communities primarily composed of fungi.

Biosynthetic carbon isotope fractionation factors were determined for these unique PLFA biomarkers. Bulk cellular material in all autotrophic cultures

was depleted in $\delta^{13}C$ by 5.6‰ to 10.9‰ relative to their dissolved CO_2 carbon source, indicating that inorganic carbon fixation in these cultures are carbon limited. Individual PLFA in autotrophs were further depleted 8.2‰ to 14.6‰ compared to the bulk cell $\delta^{13}C$, which are among the largest biosynthetic isotope fractionation factors reported in the literature. Heterotrophic bulk cells did not significantly fractionate $\delta^{13}C$ relative to their carbon source. Heterotrophic PLFA ranged from 4‰ depleted to 3‰ enriched relative to their overall biomass. These unique PLFA biomarkers and isotopic fractionations associated with autotrophic and heterotrophic activity in this laboratory study provide a means to delineate autotrophic and heterotrophic carbon cycling in AMD environments.

Introduction

Acid generation from the oxidation of mine tailings poses a significant environmental and economic challenge worldwide. Microbial communities native to AMD systems thrive in an environment unfavorable to most organisms, encountering stresses such as very low pH (pH =< 4), and high loads of heavy metals (Baker and Banfield, 2003a; Johnson 1998). Notwithstanding these unfavorable conditions, AMD microbial ecosystems are complex, and the carbon cycling and ecological interactions are poorly understood. It has been suggested that the minor heterotrophic components of AMD ecology play an influential role in acid generation processes by removing organic carbon, producing CO_2 and

providing surfaces for biofilm formation (Fournier et al., 1998; Baker and Banfield, 2003a,b; Bernier and Warren, 2005, 2007). In contrast, it has been reported that the presence of glucose and heterotrophic bacteria significantly reduced the rate of pyrite oxidation for *A. ferrooxidans* in laboratory cultures (Marchand and Silverstein, 2002). A better fundamental understanding of the role of heterotrophic organisms and their contribution to acid generation in AMD environments will help elucidate the partially understood microbial ecology of these systems, and provide tools for improved biogeochemical modeling of these systems.

Compound specific $\delta^{13}C$ (Equation 3-1) analysis of phospholipid fatty acid (PLFA) biomarkers can elucidate carbon cycling pathways in microbial ecosystems and have brought valuable insight to microbial ecology of environmental systems by relating substrate usage to specific components of a microbial community (Boschker and Middleburg, 2002; Pancost and Damste, 2003).

$$\delta^{13}C = [R_{sample} / R_{standard} - 1] \times 1000 \text{ ‰},$$

where R is the measured ratio of $^{13}C/^{12}C$ [3-1]

PLFA degrade within days to weeks after cell death and therefore represent an in situ snapshot of the viable microbial community (White et al., 1979). Signature PLFA have been used as biomarkers of classes of organisms in

many systems (Boschker et al., 1998; Pelz et al., 1998; Pelz et al., 2001; Zhang et al., 2002; Slater et al., 2005) and they can be analyzed for $\delta^{13}C$, at natural abundance levels. Comparison of $\delta^{13}C$ of PLFA, bulk cells and carbon source can provide insight into carbon sources and/or metabolic processes.

In prokaryotes, carbon isotope fractionation among carbon source, bulk organic matter and PLFA biomarkers is wide-ranging and metabolically influenced. Isotope fractionation occurs at carbon branch points during biosynthesis of many cellular components including PLFA (Hayes, 2001). Typically, heterotrophic organisms have bulk cell $\delta^{13}C$ values similar to their carbon sources, and produce PLFA that are approximately 3‰ depleted relative to biomass (Monson and Hayes, 1982; Blair et al., 1985; Teece et al., 1999; Hayes, 2001; Abraham and Hesse, 2003). In contrast, autotrophic organisms often express greater isotopic fractionation, primarily due to effects generated by the fixation of inorganic carbon (Summons et al., 1994; Sakata et al., 1997; Hayes, 2001; Londry et al., 2004). Identifying fundamental biosynthetic isotope fractionation factors for biomarkers of autotrophs and heterotrophs in the laboratory may enable their carbon sources and ecological relationships to be evaluated *in situ*.

To gain insight into carbon cycling in complex AMD communities, this study characterized biosynthetic isotope fractionation for PLFA biomarkers of two pure strains of sulfur oxidizing bacteria, and two naturally occurring sulfur

oxidizing communities enriched from an AMD system at Moose Lake, ON, Canada (Bernier and Warren, 2007). In addition to these chemoautotrophic cultures, isotopic fractionation factors were measured for the *Acremonium* enrichment cultures from the AMD ecosystem to delineate the role of these organisms in the environment. Based on these results, a model was developed to elucidate microbial community structure and carbon cycling relationships in AMD environments.

Materials and Methods

Environmental Enrichments

Autotrophic enrichment cultures, ML2002 and ML2003, from Moose Lake (Onaping, Ontario, Canada), have been previously described (Bernier and Warren, 2007). These two cultures are comprised of up to 95% *Acidithiobacillus sp.*, as determined by by whole cell fluorescence hybridization. Pure strains of *Acidithiobacillus ferrooxidans* (ATCC 19859), and *Acidithiobacillus thiooxidans* (ATCC 19377) were cultured in identical conditions to the autotrophic enrichments. The remaining 5% of cells in ML2002 and ML2003 were characterized by 18S rRNA sequencing, and through BLAST were identified as the fungus *Acremonium sp* KR21-2 (data not shown). These fungi were enriched from ML2002 and ML2003 by replacing the S_4O_6 with glucose and yeast extract and identified as H2002 and H2003 respectively. It is interesting to note that while

the heterotrophic cultures rapidly assimilated complex carbon sources consisting of glucose, fructose, lactose, maltose and manitol, they are incapable of growth on simpler substrates, acetate or formate (data not shown). A total of four ML2003, four *A. ferrooxidans*, one ML2002, one *A. thiooxidans*, two H2002 and two H2003 cultures were analyzed for PLFA and $\delta^{13}C$.

Culture Conditions

Autotrophic cultures were grown on a basal medium modified from ATCC 125: $(NH_4)_2SO_4$ 1.5 mM; $MgSO_4$ 2.1 mM; $CaCl_2*2H_2O$ 2.5 mM; KH_2PO_4 22 mM; $FeSO_4*$ $7H_2O$ 36 μM and $S_4O_6^{2-}$ ($Na_2S_4O_6$ * $7H_2O$, 20 mM). A 10% inoculum (v/v) was used in 250 mL of 0.2 μm filter-sterilized culture medium in sterile 500 mL Erlenmeyer flasks, and sealed with a thin aluminum film which allowed gas exchange with the atmosphere. Two drops of sterile bromophenol blue 0.02% were added as a pH indicator (blue → yellow, pH 4.6 → 3.0), and pH was adjusted after inoculation under sterile conditions to 4.0. Heterotrophic cultures were grown on a similar basal medium, modified from ATCC 125: $(NH_4)_2SO_4$ 1.5 mM; $MgSO_4$ 2.1 mM; $CaCl_2*2H_2O$ 2.5 mM; KH_2PO_4 22 mM; $FeSO_4*7H_2O$ 36 μM, glucose ($C_6H_{12}O_6$, 20 mM), and 100mg yeast extract. Flasks were left at 20°C under static conditions until harvested. Cultures were harvested during early stationary phase, as determined by pH growth curves established previously (Bernier and Warren, 2007). One set of cultures was harvested during exponential growth phase, however these cultures did not exhibit significant

differences outside the variability of stationary phase replicates for PLFA distribution or isotopic composition. Because there was no significant difference between exponential and stationary phase cultures, data from both growth phases were aggregated.

PLFA Analyses

Liquid cultures were harvested by centrifugation and subsequently freeze-dried for 48 hours prior to analysis. Lipid extraction was performed using the modified Bligh and Dyer method as previously described (White et al., 1979) using a 2:1:0.8 methanol-dichloromethane-phosphate buffer solution. Polar lipids were separated from the total lipid extract on two grams of fully activated silica gel (100-200 mesh, 60Å) by elution with 20 mL dichloromethane (f_1, non-polar lipids), 20 mL acetone (f_2, neutral lipids) and 20 mL methanol (f_3, polar lipids including phospholipids). The phospholipid fraction (f_3) was evaporated to dryness under UHP N_2 and reacted to Fatty Acid Methyl Esters (FAMEs) via the mild alkaline methanolysis reaction (White et al., 1979). Methanol used in this reaction was characterized for its $\delta^{13}C$ value (-37.7‰) and PLFA isotope values were corrected during data analysis for this addition. FAMEs were further purified using a secondary silica gel chromatography step, on 0.5 grams of fully activated silica gel via elution with 5 mL hexane, 5 mL dichloromethane (FAMEs), and 5 mL methanol.

Identification and Quantification of PLFA

An Agilent 6890 GC coupled to a 5973 quadrupole mass spectrometer was used for identification and quantification of FAMEs. The GC was equipped with a 30 m x 0.25 mm DB-5 MS column with a 0.25 μm film thickness. The temperature program used was 40°C hold 1 min, ramp to 130°C at 20°C/min, to 160°C at 4°C/min and finally to 300°C at 8°C/min. FAMEs were identified using several bacterial reference standards (Bacterial Acid Methyl Esters CP Mix, Matreya Inc., Fatty-Acid Methyl Ester Mix, Supelco Inc.), mass-fragmentation patterns, and retention times. Double bond positions were determined by analysis of DMDS adducts described previously (Nichols, 1985). FAMEs were quantified using external calibrations, and reproducibility on replicate measurements was better than 10%. PLFA were not observed in laboratory blanks.

PLFA are identified as Cn:mωx where n is the number of carbon atoms and m is the number of double bonds and ωx is the position of the double bond, if known (e.g. $C_{16:1\omega9}$ indicates a sixteen carbon chain, with one double bond occurring at the ninth carbon from the carboxyl end of the fatty acid). Methyl branching is indicated by i-Cn:mωx for branching on the penultimate carbon atom, a-Cn:mwx for branching on the ante-penultimate carbon atom, or yMeCn:mωx where y represents the methyl group position on the carbon chain. Cyclopropyl PLFA (CFA) are represented by cycCn:mωx.

Bulk Isotope Analysis

Centrifuged cell pellets were washed three times with distilled, deionized water, freeze dried and homogenized. Triplicate samples were subsequently combusted and analyzed online by EA/IRMS. Standards analyzed with samples included: IAEA 600, IAEA CH-7, NBS 21, USGS 24 and USGS 40. Precision for replicate analysis of standards was a standard deviation of 0.2‰ or better.

GC-C-IRMS analysis of PLFA

Stable carbon isotope ratios of individual PLFA were determined using an Agilent 6890 GC coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. The GC program was 50°C for 1 min; 10°C/min to 150°C; 1.5°C/min to 180°C for 20 minutes; 10°C/min to 280°C; 15°C/ min to 320 for 15 minutes. Reproducibility for isotopically characterized hexadecane, octacosane and m-terphenyl laboratory standards was better than 0.3 permil (1σ) and accuracy was always within 2σ of expected values. Precision for analysis of microbial PLFA was better than 1.0‰ (1σ) for minimum of three analyses. Replicate samples from the same culturing flask produced insignificant differences relative to precision. Variability of the same bacterial culture, grown in two adjacent flasks was similar to previously reported environmental samples (Cifuentes and Salata, 2001), with less than 2.0‰ variability (1σ) for PLFA between replicate cultures.

Results

PLFA Distribution

Five PLFA accounted for 90% of the total PLFA in all autotrophic cultures (n = 5 enrichments, n=5 pure strains) (Table 3-1). The PLFA distribution of the pure cultures was similar to previous results for *A. thiooxidans* (Knief et al., 2003; Kerger et al., 1986) and *A. ferrooxidans* (Knief et al., 2003) that found the same five lipids comprising more than 65% of the PLFA fraction. The primary PLFA for the autotrophic cultures were $C_{16:1\omega9}$, $C_{16:0}$, cyc-$C_{17:0}$, $C_{18:1\omega11}$ and cyc-$C_{19:0}$. Several other $C_{16:1}$ and $C_{18:1}$ isomers were detected, but they represented less than 2% of total PLFA, and double bond position was not determined by DMDS. The two autotrophic enrichment cultures, ML2002 and ML2003 exhibited highly similar PLFA distributions to the pure strains *A. ferrooxidans and A.thiooxidans*. Of the PLFA in the autotrophic cultures, cyc-$C_{17:0}$ and cyc-$C_{19:0}$ were found to be unique identifiers and were not present in the heterotrophic cultures.

The heterotrophic cultures displayed very different distributions of PLFA compared to the autotrophic enrichments. Heterotrophic enrichments produced very high percentages of only three PLFA: $C_{18:2}$, $C_{18:1w9}$, and $C_{16:0}$. Several previous studies have associated $C_{18:2}$ and $C_{18:1\omega9}$ as biomarkers produced by fungi (Vestal and White, 1989; Green and Scow, 2000), and these two lipids comprise 70% of the total lipid fraction in the heterotrophic enrichment cultures from this study.

Table 3-1 PLFA Distribution of enrichment cultures and pure strain bacteria. PLFA structure is indicated by $C_{n:m\omega x}$ where n represents the number of carbon atoms in the chain, m represents the number of double bonds and ωx is the position of the double bond, if known. Cyclopropyl PLFA are indicated by a cyc- prefix. PLFA comprising less than 1% of total distribution in all cultures were omitted. n.d. denotes PLFA was not detected in any replicate of the culture.

PLFA	A.ferrooxidans (n=4)	A.thiooxidans (n=1)	ML2003 (n=4)	ML2002 (n=1)	AVG Auto (n=10)	H2003 (n=2)	H2002 (n=2)
$C_{16:1\omega7}$	14%	11%	10%	13%	12%	n.d.	n.d.
$C_{16:1\omega9}$	1%	0%	1%	1%	1%	n.d.	n.d.
$C_{16:0}$	7%	9%	20%	7%	12%	25%	30%
$cycC_{17:0}$	7%	16%	3%	2%	6%	n.d.	n.d.
$C_{17:0}$	3%	2%	1%	1%	2%	n.d.	8%
$C_{18:2}$	n.d.	n.d.	n.d.	n.d.	n.d.	29%	28%
$C_{18:1\omega9}$	n.d.	n.d.	n.d.	n.d.	n.d.	43%	34%
$C_{18:1\omega11}$	23%	11%	31%	33%	26%	n.d.	n.d.
$C_{18:1}$	3%	1%	n.d.	2%	2%	n.d.	n.d.
$C_{18:0}$	3%	2%	2%	1%	2%	n.d.	n.d.
$cycC_{19:0}$	29%	43%	32%	34%	33%	n.d.	n.d.

Isotopic fractionation

The $\delta^{13}C$ values for bulk cells and carbon sources are presented in Table 3-2. Autotrophic cultures were grown with atmospheric CO_2 ($\delta^{13}C$ = -10‰ +/- 1‰) as their carbon source. The isotopic discrimination due to carbon fixation exhibited by these autotrophic cultures, $\Delta^{13}C_{bulk\ cell\text{-}CO2}$, ranged from -5.6‰ to -10.9‰. Heterotrophic cultures showed no significant isotopic discrimination between their glucose carbon source ($\delta^{13}C$ = -10.0‰, Table 3-2) and bulk cells ($\delta^{13}C$ = -9.9‰).

The $\delta^{13}C$ of individual PLFA are presented in Figure 3-1. For the autotrophic cultures, the range of $\Delta^{13}C_{biomass\text{-}PLFA}$ was -7.9‰ to -15.6‰. In all cases, the least depleted PLFA relative to biomass was $C_{16:0}$. In almost all cases, cyc-$C_{19:0}$ was the most depleted PLFA, with the exception of one sample of *A. thiooxidans* that produced a more depleted cyc-$C_{17:0}$. Growth phase did not affect the isotopic composition of bulk cells or PLFA. Autotrophic cells harvested during exponential growth phase did not vary in $\delta^{13}C_{biomass}$ or $\delta^{13}C_{PLFA}$ greater than 1σ from the mean $\delta^{13}C$ for cultures harvested at stationary phase. This finding is consistent with the observations by Londry et al. (2004) that growth phase does not influence biosynthetic isotope fractionation. Heterotrophic PLFA ranged in $\Delta^{13}C_{bulk\ cell\text{-}PLFA}$ from +3.0 to -4.0‰. The results for $\delta^{13}C$ of bulk cells and PLFA are consistent with previous reports of aerobic heterotrophic assimilation of organic carbon (Blair et al., 1985; Teece et al., 1999; Hayes, 2001;

Londry and Des Marais, 2003; Londry et al., 2004), with the exception of $C_{16:0}$ in the H2003 culture, that was enriched relative to total biomass.

Table 3-2: Isotopic composition ($\delta^{13}C$) of individual PLFA from pure strains and Moose Lake enrichment cultures. C_{16} and C_{18} unsaturated isomers presented as a single isotopic value, because these compounds were not fully baseline resolved on the GC trace. Not all compounds were analyzed for $\delta^{13}C$ in each replicate due to low cell densities. PLFA denoted n.d. were not analyzed for $\delta^{13}C$ in any replicate of the culture.

PLFA	ML2003 (n=4)	ML2002 (n=1)	A.Ferrooxidans (n=4)	A.Thiooxidans (n=1)	Avg Autotrophs (n=10)	H2003 (n=2)	H2002 (n=2)	Avg. Heterotrophs (n=4)
$C_{16unsaturated}$	-25.3	-29.7	-28.5	-27.1	-27.4	n.d.	n.d.	n.d.
$C_{16:0}$	-24.4	-25.1	-26.2	-25.5	-25.0	-6.8	-10.8	-8.8
$cycC_{17:0}$	n.d.	n.d.	n.d.	-32.0	-32.0	n.d.	n.d.	n.d.
$C_{18unsaturated}$	-26.2	-28.1	-29.3	-30.3	-28.4	-9.1	-13.0	-11.1
$cycC_{19:0}$	-28.8	-28.7	-29.5	-29.2	-29.8	n.d.	n.d.	n.d.
Bulk Cell	**-18.8**	**-15.1**	**-17.8**	**-16.5**	**-16.1**	**-9.9**	**-9.9**	**-9.9**
Carbon Source	**-10.0**	**-10.0**	**-10.0**	**-10.0**	**-10.0**	**-10.0**	**-10.0**	**-10.0**

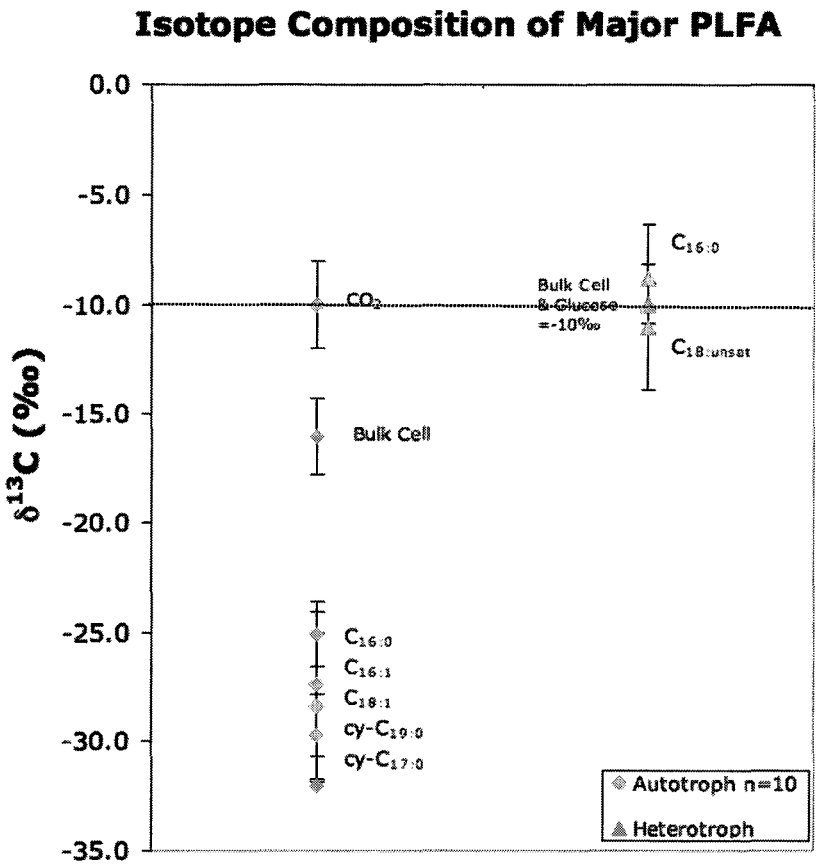

Figure 3-1: Isotopic fractionation observed in autotrophic and heterotrophic enrichment cultures and autotrophic pure strains. Dark line denotes microbial carbon source for autotrophs (CO_2) and heterotrophs (glucose). Error bars represent two standard deviations of all replicate analyses.

Discussion

Biomarker PLFA

PLFA distributions from these laboratory cultures identified unique biomarkers for the autotrophic and heterotrophic components of this replicated AMD system. The autotrophic Moose Lake enrichments and pure strains produced consistent PLFA distributions comprised of five major lipids of which cyc-$C_{19:0}$ and cyc-$C_{17:0}$ made up nearly 40 percent of the lipids (Table 3-2). These cyclopropyl PLFA (CFA) were found to be unique to the autotrophic enrichments. Production of CFA is associated with response to stresses such as dessication, starvation, stationary phase stasis or acid shock (Kieft et al., 1994; Grogan and Cronan, 1997; Chang and Cronan, 1999; Kim et al., 2005). The large proportions of cyc-$C_{19:0}$ observed in all autotrophic cultures was significant as this PLFA was not present in the heterotrophic cultures. Therefore cyc-$C_{19:0}$ is a biomarker for autotrophic acid generating bacteria in this study, and potentially for AMD systems in general. In contrast, the heterotrophic fungi produced a distinct PLFA distribution comprised of only three major PLFA (Table 3-2). Of these three PLFA, $C_{16:0}$ was common to the autotrophs, as was expected due to its ubiquitous nature, but $C_{18:2}$ and $C_{18:1\omega9}$ were unique to the heterotrophs. The two PLFA, $C_{18:2}$ and $C_{18:1\omega9}$, have been previously proposed as biomarkers of fungi in other systems (Green and Scow, 2000). These two PLFA are considered biomarkers for the fungal community in the Moose Lake system, as they were unique to the

heterotrophic cultures. *Acidithiobacillus* comprises up to 45% of the actual microbial community at Moose Lake (Bernier and Warren, 2005), therefore a large component of cyc-$C_{19:0}$ should be present in an environmental sample. Fungi have been previously identified and enriched from AMD environments (Johnson, 1998; Baker et al., 2004), therefore biomarkers consistent with fungal populations were expected. The unique observation of these PLFA in the enrichments and pure cultures implies that they can be used as biomarkers of the sulphur-oxidizing and heterotrophic components of the microbial communities in the AMD environmental system.

In addition to the sulfur oxidizing and heterotrophic microbial communities, euglena are photosynthetic protists known to inhabit AMD systems and possibly contribute to acid generation by producing O_2 to support Fe and S oxidation (Bernier and Warren, 2005; Johnson, 1998, Rowe, 2007). Their conspicuous absence in either enrichment culture was attributed to laboratory low light conditions in which the cultures were grown. Previous studies of *Euglena sp.* did not detect the cyc$C_{17:0}$, cyc$C_{19:0}$ or $C_{18:2}$ biomarkers in their samples (Barsanti et al., 2000), therefore the presence of *Euglena* in the Moose Lake AMD site will not compromise the use of these biomarkers.

Carbon isotope discrimination: evidence of CO_2 limitation

The isotopic discriminations between carbon source and bulk cells expressed by the autotrophic AMD cultures, and pure strains *A. ferrooxidans* and

A. thiooxidans are smaller (-5.6‰ to -10.9‰, Table 3-2) than previously reported for sulfur oxidizing bacteria, indicating that the bacteria in this study are carbon limited. In the previous study by Ruby et al (1987) that was conducted at circum-neutral conditions, isotopic discrimination between carbon source and bulk biomass of other related sulfur oxidizing bacteria *Thiomicrospira sp.* and *Thiobacillus neapolitanus* was -24.6 to -25.1‰ (Ruby et al., 1987). Depletion of the magnitude described by Ruby et al., (1987) is indicative of carbon fixation using the Rubisco form I enzyme and Calvin cycle, which generally produces very depleted organic carbon (-25‰ to -30‰) relative to carbon source (Farquhar, 1989; Hayes, 2001; O'Leary, 1981). *Acidithiobacillus ferrooxidans* is known to possess genes for Rubisco and its carbon metabolism has been previously characterized (Heinhorst et al., 2002; Appia-Ayme et al., 2006). The small depletions observed in this study indicate that isotopic discrimination during autotrophic growth in our system is carbon limited. This is consistent with the acidic conditions (pH <4) present in these enrichment cultures and at an AMD site that result in dissolved inorganic carbon (DIC) in the form of $(CO_2)_{aq}$. These low concentrations of DIC would limit isotopic discrimination in the Moose Lake cultures (O'Leary, 1988).

An alternate explanation of this limited isotopic fractionation that must be considered is that the Moose Lake cultures could potentially utilize a different biosynthetic pathway to fix carbon. *A. ferrooxidans* possesses two copies of

Rubisco Type I, and one copy of Rubisco Type II (Shivley et al., 1993; Heinhorst et al., 2002).Carbon fixation via the type II Rubisco enzyme can decrease $\Delta^{13}C_{\text{carbon source-bulk cell}}$ by up to 12‰ (Robinson et al., 2003) and would result in similar fractionations to those observed in this study. However previous studies indicate that Type II is likely used only as an electron acceptor by *A. Ferrooxidans*, and not for carbon fixation (Appia-Ayme et al., 2006). Therefore, Rubisco II would not be expected to explain the observed discrimination effects in this study.

Isotopic composition of Biomarker PLFA

Individual PLFA were more depleted relative to total cell biomass ($\Delta^{13}C_{\text{PLFA-biomass}}$ = -9.2‰ to -15.9‰) than in previous reports (Blair et al., 1985; Sakata et al., 1997; Hayes, 2001; Londry et al., 2004). The least depleted PLFA relative to biomass in all autotrophic cultures was $C_{16:0}$ ($\Delta^{13}C_{C16:0\text{-biomass}}$ = -9.2‰ to -10.8‰). The biomarker, cyc-$C_{19:0}$, was consistently the most depleted PLFA relative to biomass ($\Delta^{13}C_{\text{cyc-C19:0-biomass}}$ = -12.4‰ to -15.3‰) in all autotrophic cultures except *A. thiooxidans* in which cyc-$C_{17:0}$ was more depleted ($\Delta^{13}C_{\text{cyc-C17:0-biomass}}$ = -15.5‰). The reason for the extreme depletion of CFA is likely related to their biosynthetic production pathway. As previously indicated above, CFA are produced in response to stress. They are formed when a methyl group from S-adenosyl-L-methionine (SAM) reacts with the double bond of a monounsaturated PLFA (Law, 1971). The methyl group that becomes a component of the

cyclopropyl PLFA is derived from methionine. Position specific isotope analysis by Sacks and Brenna (2003) demonstrated that this methyl group is depleted up to 30‰ relative to the total methionine molecule. Isotopic mass conservation requires that inclusion of this depleted methyl group will result in an isotopic depletion of the product. In the autotrophic cultures, cyc-$C_{19:0}$ was depleted by an average of 1.7‰ relative to its $C_{18:1}$ precursor. A mass balance calculation between cyc-$C_{19:0}$ and precursor $C_{18:1}$ determined that the $\delta^{13}C$ of the donated methyl group was -49‰, which is within the expected range given by Sacks and Brenna (2003). Since the bulk isotopic composition of methionine is enriched relative to the methyl carbon ($\Delta^{13}C_{\text{methionine-methylcarbon-}} = +30‰$), methionine in the autotrophic cultures is calculated to be -19‰. The fractionation between bulk cells and methionine ($\Delta^{13}C_{\text{methionine-biomass}} = -2‰$) is within the expected isotopic range relative to biomass for individual microbial amino acids (Hayes, 2001). This position specific isotope effect gives sufficient explanation for the depletions observed in the CFA biomarkers, relative to other autotrophic PLFA.

In contrast to the fractionation observed in the autotrophic cultures, isotopic discrimination from carbon source to bulk cells ($\Delta^{13}C_{\text{bulk cell - carbon source}} = 0.1‰$), and bulk cells to PLFA ($\Delta^{13}C_{\text{PLFA - bulk cell}} = 0.1‰$) was insignificant for the heterotrophic cultures as expected based on previous studies (Monson and Hayes, 1982; Blair et al., 1985; Hayes, 2001). The only exception observed in this study was for the $C_{16:0}$ PLFA in the H2003 heterotrophic cultures, that were enriched

relative to biomass and carbon source. The reason for the enrichment in this study is unclear, however it may reflect a difference in biosynthetic pathway. In certain cases, bacteria using the reverse TCA cycle have been reported to produce PLFA that are enriched relative to bulk cells (van der Meer et al., 1998; Londry et al., 2004).

Implications for Studying Microbial Ecology in situ: a model AMD ecosystem

The isotopic composition of unique PLFA biomarkers in this study can be used to discern the carbon cycling pathways of heterotrophic and autotrophic organisms *in situ* for AMD environments. Figure 3-2 illustrates a model of the relationships between $\delta^{13}C$ of carbon sources, total cellular biomass and biomarker PLFA that can be used to investigate and constrain the carbon source for the heterotrophic component of the microbial community. Determining the relationship of the heterotrophs relative to the autotrophs and directly probing the extent to which the heterotrophs are using the autotrophs as a carbon source will help determine the community relationship between these parts of the microbial community. In this model, two hypothetical systems are proposed, the first where the heterotrophic community is directly consuming carbon from the autotrophs, and the second where allochthonous organic carbon contributes to heterotrophic metabolism.

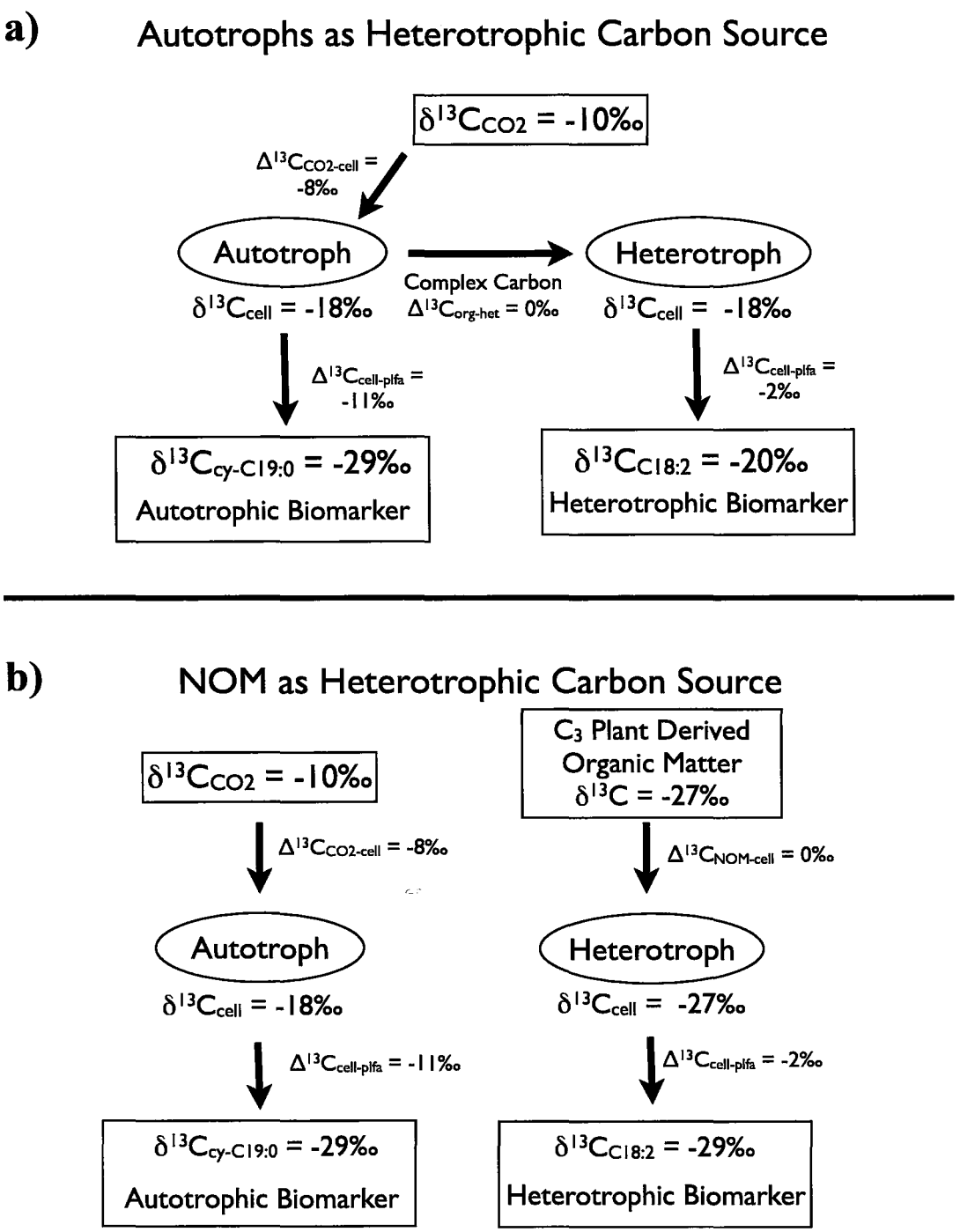

Figure 3-2: Two potential carbon cycling models for a hypothesized AMD microbial ecosystem. In (a), the only source of organic carbon to the heterotrophic community is the autotrophic bacteria. In (b), the heterotrophs consume allochthonous organic carbon from runoff derived terrestrial photosynthesis.

In the first system (Figure 3-2a), autotrophic carbon fixation by sulphur oxidizing bacteria is the only source of organic carbon. As per the average of the results of this study, this organic matter is isotopically depleted with respect to atmospheric CO_2 by 8‰ due to the carbon limitation observed in this study. Therefore the bulk cells of this autotrophic community will be approximately -18‰, relatively enriched compared to organic matter produced under non-limiting conditions. The model then assumes that the PLFA of these autotrophs are depleted relative to the bulk cells by the same amount as was observed in the culture experiments. For the biomarker cyc-$C_{19:0}$, this results in a $\delta^{13}C_{PLFA}$ of -29‰, as observed in the experimental cultures. For this model where the autotrophs are the primary source of carbon for the heterotrophic community, the heterotrophs will then have a bulk carbon isotope signature identical to the autotrophs ($\delta^{13}C$ = -18‰) consistent with the lack of fractionation between the heterotrophs and carbon source observed in this study. Because there is only a small fractionation between bulk cells and biomarker PLFA in heterotrophs, the $C_{18:2}$ biomarker will then have an isotopic signature within 2‰ ($\delta^{13}C$ = -20‰) of the bulk cell. In this scenario therefore, the heterotrophic utilization of the autotrophic cell mass generates an enriched heterotrophic biomarker relative to the depleted $\delta^{13}C$ observed in the autotrophic cyc-$C_{19:0}$ biomarker. Such an observation would provide direct evidence of the heterotrophic utilization of autotrophic carbon.

In the second scenario (Figure 3-2b), the isotopic patterns are the same for the autotrophic community. However, in this case the heterotrophs are assumed to be consuming only allochthonous organic carbon that is produced via non-limited terrestrial C_3 plant photosynthesis. In this case the lack of isotopic fractionation during heterotrophic carbon metabolism means that the bulk cells for the heterotrophs will be have a $\delta^{13}C$ of -27‰, significantly depleted relative to the autotrophic bulk cells. The limited fractionation between heterotrophic bulk cells and the biomarker $C_{18:2}$ mean that the $\delta^{13}C$ of this biomarker would then be -29‰. Interestingly in this scenario, this biomarker $\delta^{13}C$ is now comparable to the autotrophic biomarker $\delta^{13}C$ signature. Therefore, observation of the same $\delta^{13}C$ for the heterotrophic and autotrophic biomarkers would demonstrate that in fact the heterotrophs are not using the autotrophs as a direct carbon source, and are co-existing with rather than consuming, the sulphur oxidizing bacteria. This scenario is comparable to the hypothesis of Marchand and Silverstein (2003) that suggests heterotrophic bacteria consume organic material such as glucose that may inhibit iron oxidation for *A. ferrooxidans* and provide a CO_2 source for iron oxidation through respiration of natural organic carbon.

Potential Implications for Environmental AMD Systems

Use of the model developed in this study to identify autotrophic and heterotrophic processes *in situ* may be complicated by many factors including a more diverse microbial community and more complex carbon cycling

relationships in natural systems. While this laboratory study demonstrated fractionation factors and unique biomarkers for bacteria native to AMD, there may be other bacteria that contribute to carbon fixation and other heterotrophic bacteria consuming organic material to convolute this model in a natural system. Other autotrophic microbes may be present along with *Acidithiobacillus*, however carbon limitation, and associated carbon isotope effects, will apply to all photosynthetic and chemoautotrophic bacteria. In addition to *Acremonium* fungi, heterotrophic bacteria may be present, therefore to extend this laboratory study into a field setting, a biomarker for these bacteria must be established to determine the role of the entire heterotrophic community. The biomarker identified for the *Acremonium* fungal enrichments remains a useful biomarker for fungi in these, and other previously described communities (Green and Scow, 2000) and indicates a signature of decomposers in a natural AMD system. Complex carbon re-cycling within an Acid Mine Drainage lake may influence isotopic signatures of heterotrophic and autotrophic organisms, however bulk cell to PLFA fractionation factors will not change. Therefore if recycling of carbon is occurring, the system will appear as Figure 3-2a where heterotrophs are consuming organic carbon from the autotrophs, because autotrophy will be the primary source of organic carbon to the system.

Summary and Conclusions

In this study we have identified biomarker PLFA for autotrophic sulfur oxidizing bacteria and heterotrophic fungi enriched from an acid mine drainage site. The bulk biomass of the autotrophic community was determined to be carbon limited due to enriched $\delta^{13}C$ values relative to other autotrophic organisms. The depleted $\delta^{13}C$ of the PLFA biomarkers relative to the bulk cell can be explained by biosynthetic effects. In particular cyc-$C_{19:0}$ is depleted, relative to its precursor PLFA $C_{18:1}$, due to depletion induced during methylation by s-adenosyl-methoionine. The differences in biosynthetic fractionation discussed in this study are sufficient to allow for these PLFA biomarkers to be used *in situ* to probe carbon cycling and to discriminate between heterotrophic and autotrophic metabolic activity in AMD environments.

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Chapter 4

Summary of Findings and Conclusions

This thesis contributed novel understanding of carbon cycling in microbial communities native to petroleum-contaminated and AMD environments. These developments in the understanding of complex microbial ecosystems and carbon cycling can be expected to contribute to the development of more efficient remediation methods, and to provide tools for new monitoring techniques for environmental pollution, thereby providing benefits for human health and environmental protection.

The common theme in this thesis was the use of compound specific isotope analysis of PLFA in order to constrain microbial carbon sources and cycling, and thus their role, in two contaminated systems. While this approach has been applied in many studies, the unique developments of this thesis were the use of compound specific radiocarbon analysis of PLFA to directly demonstrate petroleum degradation in a phytoremediation system and the use of metabolic differences in PLFA biosynthesis to identify autotrophic versus heterotrophic pathways.

In Chapter two, compound specific ^{14}C analysis of PLFA biomarkers determined that mineralization by rhizosphere bacteria is the major degradation

pathway in a novel Multi-Process Phytoremediation System. This technique also determined that the microbial community is comprised mainly of hydrocarbon degrading bacteria, and that only a small fraction of modern carbon is consumed in the soil relative to petroleum hydrocarbons. The microbial community in MPPS is the most ^{14}C-depleted community reported for an environmental system in the literature to date. This conclusively demonstrated that microbial uptake and metabolism of ^{14}C depleted petroleum compounds was occurring. Mass balance using $\delta^{13}C$ and $\Delta^{14}C$ of soil CO_2 calculated the contributions of petroleum derived, atmospheric and natural organic carbon components to soil gas. Soil CO_2 data correlated well with the PLFA results, suggesting the majority of CO_2 in soil is derived from microbial respiration of petroleum hydrocarbons. This study is the first to illustrate the capability of ^{14}C to identify degradation pathways in multi-component remediation systems. Future work should include a combination of isotopic measurements and soil gas fluxes to enable precise calculation of degradation rates in soils contaminated with a petroleum-derived compound. Carbon-14 will continue to develop as a monitoring tool for remediation of contaminated systems, and to trace microbial carbon cycling within these environments. Identifying a major degradation pathway in an efficient remediation system will serve to optimize and derive new applications for phytoremediation of organic pollutants in soils.

In Chapter Three, the role of heterotrophic and autotrophic microorganisms in an acid mine drainage microbial enrichment culture was delineated using PLFA analysis. Unique PLFA biomarkers and biosynthetic isotope fractionation effects were identified for the autotrophic and heterotrophic components of the simulated AMD microbial ecosystem. Biosynthetic fractionation in autotrophs identified carbon limitation for this part of the community. For the heterotrophs, a model of two potential carbon cycling pathways was developed to elucidate carbon source using PLFA analysis in the field. This study provides a framework for studying the ecological relationships of AMD bacteria *in situ* and for elucidating the carbon source of heterotrophic and autotrophic communities in AMD systems. This method for acquiring a more thorough understanding of the microbial ecology in AMD environments may lead to new technologies for slowing acidity generation and removing heavy metals through biological processes in acid mine systems.

The development of new knowledge and understanding of microbial carbon cycling pathways and metabolic processes in contaminated systems will lead to benefits for human health and natural and impacted ecosystems. This thesis demonstrated novel approaches to attain these objectives, and overcome limitations of traditional approaches for understanding microbial processes in contaminated environments. The use of novel methods to investigate contaminated systems may identify new techniques to preserve and remediate the

natural environmental systems that are essential to sustainable development of future generations.

Appendix

Supplementary Data - Chapter 2

Total Organic Carbon (TOC) $\delta^{13}C$ analyses. NA denotes sample not analyzed.

	$\delta^{13}C_{TOC}$					
Sample	R1	R2	R3	R4	Mean	St Dev
BRF+	-29.03	-28.62	-27.71	NA	-28.45	0.7
RF-	-29.12	-29.03	-29.05	NA	-29.07	0
DC	-29.12	-28.73	NA	NA	-28.92	0.3
CC	-27.72	-27.73	-27.9	-27.77	-27.78	0.1

Soil gas $\delta^{13}C_{CO2}$ analyses at Imperial Oil Landfarm

	$\delta^{13}C_{CO2}$				
Sample	R1	R2	R3	Mean	St. Dev
BRF+	-22.3	-22.4	-22.5	-22.4	0.1
BRF+	-22.4	-22.3	-22.5	-22.4	0.1
Untreated Control	-23.3	-23.4	-23.5	-23.4	0.1
RF-	-22.9	-22.9	-22.9	-22.9	0.0
Clean Control	-16.9	-16.9	-16.9	-16.9	0.0

Soil gas $\Delta^{14}C_{CO2}$ analyses at Imperial Oil Landfarm

Sample	$\Delta^{14}C_{CO2}$
BRF+	-620.8
Untreated Control	-664.2
RF-	-712.3
Clean Control	-181.9

$\delta^{13}C_{PLFA}$ for RF- . Standard deviation is reported for triplicate analyses.

PLFA	$\delta^{13}C$	Std. Deviation
i-C14:0	-28.2	0.3
C14:0	-28.0	0.4
i/a-C15:0	-28.4	0.2
C15:0	-28.0	0.6
C16:1	-28.4	0.4
C16:0	-29.0	0.2
10MeC16:0	-31.1	0.3
cy-C17:0	-30.1	0.1
C18:0	-27.1	0.5

$\delta^{13}C_{PLFA}$ for BRF+. Standard deviation is reported for triplicate analyses.

PLFA	$\delta^{13}C$	Std. Deviation
i-C14:0	-28.2	0.1
C14:0	-28.3	0.3
i/a-C15:0	-28.3	0.3
C15:0	-28.4	0.6
C16:1	-29.7	0.7
C16:0	-28.7	0.3
10MeC16:0	-31.3	0.2
i-C17:0	-28.8	0.5
cy-C17:0	-30.7	0.2
C18:0	-28.3	0.4
cy-C19:0	-32.8	0.1
i-C22:0	-27.8	0.5

$\delta^{13}C_{PLFA}$ for Clean Control. Standard deviation is reported for triplicate analyses.

PLFA	$\delta^{13}C$	Std. Deviation
i-C14:0	-27.8	0.4
C14:0	-30.2	0
i/aC15:0	-28.4	0.1
C15:0	-29.1	0.3
i-C16:0	-28.6	0.1
C16:1	-29.4	0.1
C16:0	-29.3	0.7
i-C17:0	-29.3	0.4
cy-C17:0	-32.6	0.1
C18:1	-30.2	0.2
C18:0	-29.0	0.6
cyc-C19:0	-36.2	2.4

$\delta^{13}C_{PLFA}$ for Contaminated Control. Standard deviation is reported for triplicate analyses.

PLFA	$\delta^{13}C$	Std. Deviation
i-C14:0	-28.8	0.3
C14:0	-28.6	0.4
i/aC15:0	-30.3	0.9
C15:0	-30.4	1.8
i-C16:0	-30.5	1.2
C16:1	-31.2	0.5
C16:0	-31.0	0.5
10MeC16:0	-33.9	0.9
cy-C17:0	-31.5	0.3
C18:1	-27.7	1.7
C18:0	-28.7	0.4
cy-C19:0	-33.2	0.5
i-C22:0	-27.8	0.8

$\Delta^{14}C_{PLFA}$ for BRF+ . Measurement error = 20‰

PLFA	$\Delta^{14}C$
$i/aC_{15:0}$	+36
$C_{16:1}$	+12.5
$C_{16:0}$	+21.4
$cycC_{17/19}$	-147.4
$C_{18:1}$	+12.5

$\Delta^{14}C_{PLFA}$ for Clean Control. Measurement error = 20‰

PLFA	$\Delta^{14}C$
$i/aC_{15:0}$	-814.6
$C_{16:1}$	-807.4
$C_{16:0}$	-818.3
$cycC_{17/19}$	-897.6
$C_{18:1}$	-793.5
$C_{18:0}$	-870.4

UW4 Pure Culture PLFA Distribution (n=6 replicate cultures). N.A. Indicates PLFA was only detected in one replicate

PLFA	Mole %	Std. Deviation
$C_{14:0}$	0.4	0.2
$C_{16:1}$	41.9	0.9
$C_{16:0}$	35.6	1.9
$cycC_{17:0}$	6.1	1.4
$C_{18:1}$	9	6.2
$C_{18:0}$	0.8	N.A.

Supplementary Data - Chapter 3

Isotopic Composition ($\delta^{13}C‰$) of ML 2003 Autotrophic Enrichment Cultures, St. Phase and Exp. Phase denotes whether cells were harvested in stationary or exponential growth phase. n.d. denotes compound was below the analytical limit of quantification. Analytical precision is 0.5‰.

	ML2003			
	St. Phase	St. Phase	Exp. Phase	St. Phase
C16:1	-25.7	-24.8	n.d.	n.d.
C16:0	-24.0	-24.4	n.d.	-24.7
cy-C17:0	n.d.	n.d.	n.d.	n.d.
C18:1	-26.2	-26.3	n.d.	-26.2
cy-C19	-30.6	-28.4	-28.0	-28.2
Bulk Cell	-15.6	n.d.	-19.8	-20.9
Carbon Source	-10.0	-10.0	-10.0	-10.0

Isotopic Composition ($\delta^{13}C‰$) of *Acidithiobacillus ferrooxidans* pure cultures, St. Phase and Exp. Phase denotes whether cells were harvested in stationary or exponential growth phase. n.d. denotes compound was below the analytical limit of quantification. Analytical precision is 0.5‰.

	A. ferrooxidans (ATCC 19859)			
	St. Phase	St. Phase	Exp. Phase	St. Phase
C16:1	-26.9	n.d.	-30.1	n.d.
C16:0	-25.5	n.d.	-26.9	n.d.
cy-C17:0	n.d.	n.d.	n.d.	n.d.
C18:1	-29.0	n.d.	-29.6	n.d.
cy-C19	-30.6	-27.2	-31.1	-29.0
Bulk Cell	-17.0	n.d.	-19.0	-17.4
Carbon Source	-10.0	-10.0	-10.0	-10.0

Isotopic Composition ($\delta^{13}C‰$) of ML Heterotrophic Enrichment Cultures. All cultures were harvested during stationary phase. Analytical precision is 0.5‰.

	H2003		H2002	
C16:0	-5.8	-7.8	-11.0	-10.7
C18:2/1	-7.2	-11.0	-14.2	-11.8
Bulk Cell	-8.8	-11.0	-10.2	-9.7
Carbon Source	-10.0	-10.0	-10.0	-10.0