FATE OF PETROLEUM HYDROCARBONS IN THE ENVIRONMENT

IDENTIFYING THE FATE OF PETROLEUM HYDROCARBONS RELEASED INTO THE ENVIRONMENT AND THEIR POTENTIAL BIODEGRADATION USING STABLE CARBON ISOTOPES AND MICROBIAL LIPID ANALYSIS

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ABSTRACT

Petroleum contamination is ubiquitous worldwide, and poses significant health risks to humans, organisms, and the environment. Understanding the fate and behaviour of these chemicals is extremely important in order to predict and mitigate the effects of spills and accidental releases, and limit the exposure of these contaminants to humans and ecosystems. The physical and biological interactions with various petroleum hydrocarbons released into the environment were examined throughout this thesis in two different environmental settings; offshore bay sediments near Deepwater Horizon oil spill impacted sites, and an experimental aquifer injected with compounds representative of ethanol blended fuels. Stable carbon isotopes were used to identify carbon sources in a given environment as well as utilized by microbial communities during biodegradation of petroleum hydrocarbons.

Patterns of n-alkanes, low levels of UCM and the lack of PAHs suggest hydrocarbons in Barataria Bay sediments were of dominantly terrestrial origin. Stable carbon isotope analysis of microbial lipids and n-alkanes indicate the presence of some petroleum residues, however there is no strong evidence of Deepwater Horizon oil.

Dissolved ethanol, toluene, and MTBE were continuously injected into a pilot-scale laboratory tank simulating an unconfined sand aquifer contaminated with ethanol blended fuel. Ethanol, toluene and MTBE all experienced significant mass loss within the aquifer, which was attributed to biological degradation using stable carbon isotope analysis of residual hydrocarbons. Isotopic analysis of PLFA indicated a strong ethanol sourced signature used in microbial metabolism with some indications of an additional carbon sources such as toluene or MTBE.

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INTRODUCTION TO RESEARCH AND FUNDAMENTAL CONCEPTS

1.1 INTRODUCTION

Environmental contamination is a significant concern to human health and environmental wellbeing worldwide. Many chemicals and compounds commonly released into the environment have associated toxic or carcinogenic properties from acute and/or chronic exposure to humans and other organisms. It is extremely important to understand the chemistry contributing to a substance's fate and transport to develop appropriate mitigation and remediation strategies. Chemical contaminants can enter the environment through a variety of means including accidental spills, leaks from storage sites or industrial facilities or as by-products of industrial activity. A wide variety of physical and chemical remediation techniques are commonly used, and are often tailored to the substrate and site being cleaned such as soil, sediment, water bodies, wastewater, leachate etc., as well as the contaminant involved (Khan, Husain, & Hejazi, 2004). Commonly used *in situ* physical and chemical methods are not always completely effective and often require secondary clean up strategies to completely destroy or remove the contaminant whereas excavation of the contaminated substrate is often not feasible or accessible and would require further remediation (Riser-Roberts, 1998). Biological remediation techniques such as microbial bioremediation and phytoremediation have the potential to completely break down and remove harmful contaminants in the environment

in a non-invasive and effective way requiring less labour intensive and less costly strategies than many physical equipment based techniques. By utilising the natural metabolic capabilities of biota, specifically microorganisms, bioremediation has the potential to completely remove harmful or dangerous compounds by being incorporated into their metabolism.

Petroleum contamination is ubiquitous in the environment. As the world's primary energy source and one of the world's most valuable resources, a necessity to modern culture and the source of numerous economic and political conflicts, it comes as no surprise that the many forms and substituents of crude oil can be found almost anywhere. As an organic contaminant consisting of carbon based compounds, biodegradation is a common process occurring naturally in contaminated environment, as well as actively employed for remediation purposes by techniques such as biostimulation, the addition of nutrients to increase productivity of a microbial community, or bioaugmentation, the addition of a microbial strain or community capable of degrading the target contaminant (Prince, 2010).

The persistence of petroleum residues is of ongoing concern, and can occur in many environments. For example, residues from petroleuem spilled into the Prince William Sound, Alaska following the Exxon Valdez oil spill in 1989 were discovered to persist for many years after the incident (Neff, Owens, Stoker, & McCormick, 1995; Boehm et al., 2008). Tar balls and weathered oil were found on the French coast 8 years after the Amoco Cadiz tanker spill in 1979 (Page, Foster, Fickett, & Gilfillan, 1988).

Sediments near West Falmouth, MA, remained contaminated with oil residues 30 years after the Florida barge spill in 1969 (Reddy et al., 2002). With such harmful compounds persisting in a variety of environments for such long periods of time, it is extremely important to understand the fate of petroleum hydrocarbons and to predict where they will end up after being released.

Similarly, petroleum hydrocarbons released into groundwater can have complex fates and transport processes. Complicating the issue is the addition of fuel oxygenators, commonly ethanol, changing the properties of the overall mixture as well as how it reacts in the environment. The addition of ethanol to gasoline is known to alter its degradation capacity by providing a labile high energy carbon source for microbial metabolisms (D. M. Mackay et al., 2006; Feris et al., 2008; D. Mackay et al., 2007; Corseuil et al., 2011; Ulrich, 1999).

Both chapters of this thesis address the fate of petroleum contamination in two different environmental settings. Chapter 2 analyzed the fate of spilled oil from the Deepwater Horizon oil spill in the Gulf of Mexico, and addressed the question of whether coastal bay sediments were impacted by spilled petroleum. The altered fate of ethanol blended fuels in groundwater is addressed in chapter 3, where compound specific stable isotope analysis (CSIA) was used to trace the degradation and microbial uptake of the various components of blended gasolines.

1.2 PETROLEUM HYDROCARBONS

Petroleum, or crude oil, is a complex mixture of organic compounds, generated through the catagenesis of naturally occurring organic matter over millions of years(Kissin, 1987). Petroleum hydrocarbons exist as a variety of compounds, and can have hundreds of thousands of distinct individual compounds (Marshall & Rodgers, 2004). Several different categories of petroleum compounds exist and commonly react and behave independently.

1.2.1 Chemicals and chemical classes

Benzene, toluene, ethyl-benzene, and xylenes (ortho-xylene, meta-xylene, para-xylene), collectively known as the BTEX compounds are a frequently studied class of petroleum hydrocarbons with toxic and in some cases (eg. benzene) carcinogenic properties making them a concern to human and animal health (Ahmed, 2001; Lovley, 1997). These cyclic hydrocarbons are commonly associated with gasoline, and are the most common aromatics in crude oil, making up 2 to 20 percent of oils (Z. Wang, Fingas, Landriault, Sigouin, & Xu, 1995). These ringed compounds cause concern with their frequent contamination of groundwater due to their relative solubility among petroleum hydrocarbons. Due to the relative solubility of BTEX compounds when these contaminants enter the groundwater phase they often form plumes and can easily move through the subsurface(Lovley, 1997; Roy & Smith, 2007).

N-alkanes are saturated aliphatic hydrocarbons consisting of straight carbon chains, of various lengths depending on the petroleum material. Alkanes are short in

gasoline, and generally longer in diesel. As non-polar compounds these readily sorb to organic matter and while the solubility is dependent on chain length, are overall not highly soluble in water. Shorter chain length often less than 10 carbons long, are more common in crude oil and have also been found to be more toxic and volatile than their longer chained counterparts (Mango, 1997; Sikkema, De Bont, & Poolman, 1995).

Polycyclic aromatic hydrocarbons (PAHs) are toxic aromatics generated through various processes. Pyrogenic PAHs are often generated through industrial processes or burning such as products of incomplete combustion. Sometimes PAHs can be generated diagenically through biological reworking of compounds such as those found in sediments or rock, such as in the specific case of perylene (Lima, Farrington, & Reddy, 2005). Petrogenic PAHs are present in the environment through the release of petroleum via spills or natural hydrocarbon seeps, and are generated during the catagenic process of petroleum synthesis. PAHs range from two to six aromatic rings, with varying size, structure, and properties. Smaller PAHs such as the two ringed naphthalene are volatile and prone to atmospheric depositional pathways, whereas larger structures such as the 4 or 5-ringed PAHs are highly persistent in the environment with a high affinity to organic matter and not likely to volatilize(Lima et al., 2005; Sauer, Michel, Hayes, & Aurand, 1998). Several PAHs are of significant health concern, such as the carcinogenic and mutgenic 5 ringed benzo(a)pyrene (Wild, Obbard, Munn, Berrow, & Jones, 1991).

The detection of these compounds in environmental samples is often done using gas chromatography. Based on the contaminant source, petroleum hydrocarbons can be

detected as individual compounds, or as a mixture of many different compounds which often presents itself as an unresolved complex mixture (UCM) on a gas chromatogram. The presence of a UCM is usually indicative of petroleum contamination and is made up of thousands compounds that have often been partially degraded and are structurally similar, and therefore cannot be separated by traditional gas chromatography (H. K. White, Xu, Hartmann, Quinn, & Reddy, 2013; Frysinger, Gaines, Xu, & Reddy, 2003). In this case a raised baseline hump is apparent among or instead of the expected individual peaks of the chromatogram.

1.2.2 Petroleum fate and transport

A variety of processes occur when petroleum products of any kind (crude oil, gasoline, etc) are released into the environment. These can be physical abiotic processes, or biological through interaction with microorganisms and metabolic pathways. The following are abiotic reactions contributing to the breakdown or movement of various compounds and hydrocarbons found in petroleum products. The fastest physical process is the evaporation of light compounds. The speed and efficiency of this can be influenced by environmental factors such as temperature, wind velocity, water turbulence or surface characteristics (Payne et al, 1991; Fingas, 1998). This is often a key process in the initial stages of petroleum release, and has been a major factor in the removal of volatile components of petroleum during large scale spills. Soon after initial petroleum release lighter or shorter chain hydrocarbons can lose large proportions of their mass to evaporation, as much as 99% or more for light alkanes such as propane, butane and pentane (Mango, 1997).

Photolytic breakdown of hydrocarbons, especially PAHs and BTEX compounds found in petroleum, is another s method of petroleum weathering in the environment. By interacting with the aromatic structures pi-bonded system consisting of delocalized electrons, light can be absorbed to promote a higher energy state. This excited state can facilitate breakdown of the compound leading to the degradation of light absorbing compounds within the petroleum mixture (Schwarzenbach, Gschwend, & Imboden, 2005).

Dissolution of petroleum hydrocarbons can occur based on the solubility of the compounds involved. Solubilities of these hydrocarbons vary based on specific properties including molecular size, structure, and polarity. Low molecular weight aromatics are typically among the most soluble compounds within a petroleum mixture (Boehm, Fiest, Mackay, & Paterson, 1982; Payne et al., 1991). Other compounds such as alkanes are non-polar and thus largely hydrophobic and highly unlikely to dissolve in water. Chemical agents are often used in instances of spills to increase the solubility of petroleum compounds. Surfactants and dispersants can create hydrophilic aggregates or emulsions in water, breaking down the petroleum particle sizes and diluting the hazardous compounds and aiding the potential for biodegradation (Najafi et al., 2010; Zuijdgeest & Huettel, 2012).

Many petroleum hydrocarbons have a high affinity for organic matter due to their hydrophobicity. As such they can easily adsorb to organic matter and will preferentially bind to soil and sediments (Yang, Zhu, Lou, & Chen, 2005). Once sorbed, these

compounds are difficult to remove and can persist for many years before degradation (Jones & De Voogt, 1999). One underlying factor contributing to the persistence of sorbed petroleum hydrocarbons is the limited surface area causing a lack of bioavailability for biodegradation and limited access to the physical processes that may otherwise contribute to their breakdown or dilution (Flenner, Parsons, Schrap, & Opperhuizen, 1991). In cases where large volumes of petroleum are reaching shorelines such as large spills, the presence of petroleum hydrocarbons can increase erosion due to interference with the soil/sediment matrix (Silliman et al., 2012). Once sorbed, transport of these hydrophobic contaminants can occur through erosion or sediment or soil mixing. Erosion of coastal sediments has been known to redistribute petroleum hydrocarbons into the water column allowing for transport and potentially re-exposure to plants or marine life, a process which was further explored in chapter 2 with sediments impacted by the Deepwater Horizon oil spill in the Gulf of Mexico.

1.3 BIODEGRADATION

1.3.1 Microbial communities and metabolisms

Microbial biodegradation of petroleum hydrocarbons is a well studied metabolic process by which various microbial species can use hydrocarbons as a source of energy (Speight & Arjoon, 2012). While breaking down the petroleum compound, microorganisms are simultaneously “cleaning” the contamination by transforming it into less harmful substituents. This is important, as it is one of the few processes that can

degrade these contaminants as opposed to transfer to a different state or less significant threat. Due to the natural presence of hydrocarbons such as plant and algal based alkanes in addition to petroleum released from natural seeps, many microbial species have evolved the ability to utilize hydrocarbons as a source of carbon(Atlas, 1995; Orcutt et al., 2010). Specific species can utilize specific compounds (alkanes, cyclic, aromatics etc.), while some can use a variety of those found within petroleum (Atlas, 1981; Atlas & Bartha, 1992). This can occur by heterotrophic organisms in a variety of conditions using various electron acceptors. The most efficient mechanism is in an aerobic setting using oxygen as a high energy electron acceptor, and can be facilitated by a wide variety of organisms. Anaerobic conditions can be generated when these efficient aerobic conditions become depleted, leading to different metabolisms involving alternative electron acceptors such as nitrate, sulphate, iron, and carbon dioxide(Lovley, 1997; Bethke, Sanford, Kirk, Jin, & Flynn, 2011). These metabolisms are more common and well documented in contaminated aquifers and groundwater systems due the limited availability of oxygen in the subsurface. These anaerobic metabolisms involved in the breakdown of BTEX contaminated groundwater were analysed in chapter 3. This study, as well as others, has brought forth the challenges of specific identification of microbial metabolisms. As an organism's mere presence is not indicative of its activity there a several tool and techniques available to aid in this analysis.

1.3.2 Phospholipid fatty acids

Phospholipid fatty acids (PLFA) are an integral part of the bacterial and eukaryotic lipid membrane. These membrane lipids form a semi-permeable bilayer

protecting the cell from outside molecules and facilitating the entry of essential ones, and are known to degrade rapidly upon cell death, within days to weeks, making them an ideal indicator of live biomass (D. White, Davis, Nickels, King, & Bobbie, 1979). PLFA are made up of a polar phosphate and glycerol hydrophilic headgroup, and two hydrophobic fatty acid tails (Chapelle, 2001). The marker of a bacterial or eukaryotic membrane phospholipid is the ester bond linking the fatty acids to the glycerol molecule (Green & Scow, 2000). Fatty acids making up the PLFA can vary in structure and size, with saturated, mono-unsaturated, poly-unsaturated, and/or branched chains, as well as differing chain lengths. PLFA are synthesised using available carbon transformed to pyruvate followed by acetyl CoA (DeNiro & Epstein, 1977; Hayes, 2001). After this, sequential addition of carbon units creates the stages of the fatty acid. Fatty acids are then added to glycerol phosphate for the final phospholipid (Chapelle, 2001). Various pathways are used by different organisms, and thus can create specific PLFA (Hayes, 2001; Riebesell, Revill, Holdsworth, & Volkman, 2000). Due to the relatively constant size of a microbial cell and cell membrane, and therefore consistent number of PLFA within a microbial cell, mass of PLFA in a sample can be used to estimate approximate bacterial biomass. This can be done with a conversion factor of 2×10^4 cells $pmol^{-1}$ of PLFA (Green & Scow, 2000). This is a useful tool in assessing microbial growth, as cell counts cannot be determined using genetic analysis or typical microbial ecology probes. Certain changes and adaptations to PLFA are known to occur in microorganisms in response to environmental factors such as temperature, pressure, pH, oxygen availability, etc. and therefore may reduce the accuracy of this calculation (J. Wang et al., 2014;

Frostegård, Tunlid, & Bååth, 1993). While specific fatty acids detected in a sample are not explicitly indicative of organisms present in a system, certain lipids are known to be used by groups of organisms undergoing a specific metabolism. For example, the branched PLFA 10me16:0 is indicative of sulfur reducing bacteria (SRB) (Parkes, Dowling, White, Herbert, & Gibson, 1993). Conversely, PLFA such as 16:1 and 18:1 are commonly found in many bacterial species and cannot be used as biomarkers for any specific organism or metabolism (Tunlid & White, 1992). Beyond identifying the presence and indicating changes in a microbial community, PLFA can be analyzed in conjunction with isotopic analysis to provide insight into the carbon sources being used by the organisms. This becomes highly relevant and useful when assessing the degradation of contaminants by microorganisms.

1.4 ISOTOPES

1.4.1 Stable carbon ^{13}C

Carbon is present in the environment as two stable isotopes, ^{12}C and ^{13}C, which make up 98.9% and 1.1% of the earth's carbon pool respectively (Brocks & Pearson, 2005)(Farquhar, Ehleringer, & Hubick, 1989; O'Leary, 1981). Carbon also has one unstable isotope, ^{14}C radiocarbon which accounts for trace amounts of the earth's carbon, and has a half life of 5730 years (Brooks et al., 1987). The ratio between ^{13}C and ^{12}C is represented by the $\delta^{13}C$ of a sample. This is measured by comparison to an internationally established standard, Vienna Peedee Belemnite (Boschker & Middelburg, 2002), calculated using the equation:

$$\delta^{13}C = ((^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{standard}) \times 1000‰) / {}^{13}C/^{12}C_{standard}$$

Carbon isotope signatures in the environment are largely controlled by the kinetic isotope effects of biological reactions, and physical fractionation effects during carbon speciation of the carbon source (Brocks & Pearson, 2005). With respect to biologically induced kinetic isotope effects, bonds formed between the lighter ^{12}C isotope require less energy to break than those of the heavier ^{13}C isotope. As a result of this kinetic inconsistency, biological reactions will naturally utilize ^{12}C preferentially, therefore accumulating a higher proportion of ^{12}C relative to the carbon source, considered to be a $\delta^{13}C$ depletion. Likewise this will cause a $\delta^{13}C$ enrichment, or higher proportion of ^{13}C in the remaining carbon product pool.

1.4.2 $\delta^{13}C$ of Plants

Predictable $\delta^{13}C$ ranges exist for plant species based on the photosynthetic pathway used to fix atmospheric CO_2 (O'Leary, 1981; Hayes, 2001). C3, C4, and CAM photosynthesis differ by the enzymes and substrates used along the respective CO_2 fixation pathway, causing subtle differences in isotope fractionation. The more common C3 plants use the rubisco enzyme, while C4 plants use PEP carboxylase and are associated with dry climates and drought resistance(Tanner, Uhle, Kelley, & Mora, 2007; Hayes, 2001; O'Leary, 1981). Due to the different kinetic effects associated with these photosynthetic pathways, isotopic signatures found in these plants are distinct enough to be used as an identification technique. In general, C4 plants are relatively enriched in ^{13}C

with a $\delta^{13}C$ range of approximately -10 to -20‰ (Tanner et al., 2007; Chikaraishi & Naraoka, 2003) while C3 plants range between -24 to -34‰ (Fang et al., 2014).

1.4.3 $\delta^{13}C$ of Petroleum Hydrocarbons

Petroleum is generated by the thermal degradation of modern carbon material over thousands of years. Stable isotope tracing can be a highly useful tool in the identification and fingerprinting of petroleum in the environment. A typical $\delta^{13}C$ range is expected for petroleum hydrocarbons, approximately -23 to -32‰, but a specific crude oil will often have a precise isotopic signature (Natter et al., 2012; Jackson, Pardue, & Araujo, 1996). This is similar to the isotopic signature seen in C3 plants, as this is largely the source material crude oil. This can therefore cause a challenge in differentiating between crude oil and plants when using stable carbon isotope analysis and at times requires secondary analyses and techniques to constrain this difference.

1.4.4 $\delta^{13}C$ of PLFA

Isotopic analysis of PLFA can indicate source carbon used in the synthesis of a microbial community's membrane lipids. During this process PLFA become $\delta^{13}C$ depleted relative to the carbon source, typically a 3-6‰ difference (Hayes, 2001; Londry, Jahnke, & Des Marais, 2004). Both labelling studies and natural abundance studies can apply these principles in order to better understand the compounds involved in a microbial community's metabolism. Analysing the $\delta^{13}C$ signature of PLFA, either specific PLFAs that may be representative of specific metabolisms or species, or a bulk PLFA sample, can give an indication of the carbon source used for lipid synthesis. This is

a useful tool for understanding metabolisms and community processes in the environment where by nature many energy sources and organisms exist simultaneously. This however presents a difficulty, as in the presence of multiple carbon sources, isotopic signatures can be unclear intermediate values due to incorporation of various forms of carbon. Overall it is an effective method to constrain carbon sources where the isotopic difference is sufficient, for example when assessing uptake of carbon from C3 versus C4 metabolic pathways. These isotopic ranges are typically -24 to -34‰ for C3 plants as opposed to approximately -10 to -20‰ for C4 plants, a noticeable and significant difference (Fang et al., 2014; Tanner et al., 2007; Chikaraishi & Naraoka, 2003). This difference is used in chapter 2 when assessing carbon sources in bay sediments to identify original plant material contributing to hydrocarbon residues.

1.5 RESEARCH OBJECTIVES

This dissertation used the principles outlined above to examine petroleum contamination in various settings and interactions and changes caused by in situ microbial communities.

Chapter 2 focused on the detection and presence of petroleum residues in bay sediment samples following extensive contamination of nearby shorelines due to the Deepwater Horizon Spill in April of 2010. Analysis of specific classes of petroleum compounds including alkanes, PAHs, UCM aided in determining whether petroleum was present in bay sediments in addition to other known contaminated environments. Stable carbon

isotope analysis was used to identify the sources of these compounds and distinguish between terrestrial and petroleum sources, as well as correlate residues with Deepwater Horizon oil or other oil contamination. By determining the fate of petroleum released into the environment during such a disastrous and large scale spill and how it interacted with various physical chemical and biological processes along the way, predictions for future disasters can be more accurate. Preparation strategies and remediation efforts involved in similar situations in the future can build upon the findings of this study and be optimally effective.

Chapter 3 used the principles of microbial biodegradation of petroleum compounds to determine the fate of toluene and MTBE in the presence of a labile non-petroleum carbon source, ethanol. Previous studies have shown that when both compounds are present in a groundwater system, microorganisms will only degrade BTEX compounds and other petroleum constituents once ethanol has been exhausted (D. M. Mackay et al., 2006; Corseuil, Hunt, Ferreira dos Santos, & Alvarez, 1998; Powers et al., 2001; Corseuil et al., 2011; Feris et al., 2008). Conversely, studies have also found that the presence of a labile carbon source has enhanced the capacity for degradation of BTEX compounds (D. Mackay et al., 2007; Beller, Kane, Legler, & Alvarez, 2002). As ethanol blended fuels are becoming more common worldwide, gasoline spills into aquifers and groundwater systems are more likely to be in conjunction with ethanol. It is therefore crucial to understand the behaviour and simultaneous interaction between these two types of compounds and a groundwater microbial community. Using stable carbon isotope analysis of toluene, MTBE, and ethanol as well as PLFA collected from the

aquifer, this study was able to identify the biological fractionation of aquifer hydrocarbons and identify carbon sources used by the microbial community.

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CHAPTER 2:

HYDROCARBONS AND MICROBIAL CARBON SOURCES IN BARATARIA BAY SEDIMENTS 18 MONTHS AFTER THE DEEPWATER HORIZON OIL SPILL

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ABSTRACT

The Deepwater Horizon spill released crude oil into the Gulf of Mexico over a period of 3 months in 2010. Barataria Bay, Louisiana was among the most heavily impacted and extensively oil-contaminated coastlines. Studies conducted in the marshes show significant ecological and geologic effects from oil residues that reached the bay.

Observation of an oil sheen associated with sediment disturbance raised the question as to whether petroleum residues were subsequently present in the sediments of Barataria Bay, and suggested the potential for shoreline erosion as a mechanism for petroleum hydrocarbon removal from shorelines. Sediment samples were collected offshore from non-impacted and impacted locations along Barataria Bay shoreline approximately one year after the Deepwater Horizon oil spill. Distribution of total *n*-alkanes, low levels of unresolved complex material (UCM) and the lack of polycyclic aromatic hydrocarbons (PAH) suggest a predominantly terrestrial source of hydrocarbons in the bay sediments. Specific properties of n-alkanes including stable carbon isotope analysis indicate the presence of some petroleum residues, however there is no strong indication that the source of this petroleum is the Deepwater Horizon spill. Concentrations of phospholipid fatty acids (PLFA) and bacterial cell densities were determined to be within typical uncontaminated sediment values at the time of sampling. Stable carbon isotope analysis and community structure did not indicate consumption of Macondo oil. Carbon used for microbial metabolism in the Barataria Bay sediments are likely from a variety of sources including terrestrial plant material and other petroleum inputs.

2.1 INTRODUCTION

The 2010 *Deepwater Horizon* spill released approximately 4.9 million barrels of crude oil (780 million litres) into the Gulf of Mexico over a period of 86 days. Only 0.8 million barrels of oil were recovered while much of the remaining oil led to the contamination of coastal environments (Atlas & Hazen, 2011); (Crone & Tolstoy, 2010). When oil is released into a marine environment a variety of processes are likely to affect

its eventual fate. Initially, oil is likely to float on the water surface and undergo significant evaporation, photooxidation, dissolution and/or dispersion along with biodegradation by water column microorganisms (Neff, Owens, Stoker, & McCormick, 1995). However, surface and water column advection as well as coagulation of oils can lead to oils being transported and deposited on nearby shorelines. Coastal environments often contain sensitive wetland ecosystems such as those found on the Louisiana shoreline. These wetlands are common habitats for fish and wildlife, and can be especially vulnerable to oil spills and contamination due to their low wave action and thus lack of mixing as well as the presence high amounts of organic matter (Venosa et al., 2002) that can easily sorb organic contaminants such as petroleum hydrocarbons. Shoreline sediments can retain sorbed petroleum hydrocarbons, but large spills are also known to increase erosion of shoreline material no longer protected by plant growth (Silliman et al., 2012). Eroded shoreline material, and associated petroleum residues, can then be deposited in surficial sediments of nearby water bodies. Further, petroleum hydrocarbons can also reach surficial sediments by aggregation and sinking of particles from the water column directly. Such oil residues have been previously detected in seafloor sediments following spills such as the Saudi Arabian coastline after the Gulf War spill in 1991 (Sauer, Michel, Hayes, & Aurand, 1998). With a high affinity for organic matter, petroleum hydrocarbons can be highly persistent in sediments and can be detected for long periods of time following initial exposure. For example, evidence of weathered *Amoco Cadiz* oil and tar balls were found along the coast of Brittanny, France up to 8 years following the tanker spill in 1979 (Page, Foster, Fickett, & Gilfillan, 1988).

Likewise, residual and weathered Alaska North Slope crude oil were found in Alaskan sediments several years after the Exxon Valdez Spill of 1989 (Neff et al., 1995) (P. Boehm et al., 1998) and remaining hydrocarbon contamination was detected in sediments near West Falmouth, MA, 30 years after the Florida barge spill (Reddy, Eglinton, et al., 2002).

When assessing the potential presence of petroleum hydrocarbons in environmental samples it must be taken into account that the chemical composition of petroleum is highly complex and can include thousands of distinct compounds (Marshall & Rodgers, 2004). In general, petroleum hydrocarbons in environmental systems are often observed to be present via analysis of n-alkanes, PAHs and unresolved complex mixture (UCM) (Volkman, Holdsworth, Neill, & Bavor Jr, 1992; Frysinger, Gaines, Xu, & Reddy, 2003; Z. Wang, Fingas, & Page, 1999).

N-alkanes are saturated hydrocarbons that are observed in both pristine and petroleum impacted environment (Venkatesan & Kaplan, 1982). N-alkanes account for up to 60% w/w of petroleum oil (Speight & Arjoon, 2012), and are also present in terrestrial organic matter where they are considered modern plant biomarkers as leaf waxes (Bush & McInerney, 2013). In order to differentiate between petroleum and terrestrially derived alkanes in marine sediments the distribution of odd versus even numbered alkanes (Carbon preference index, CPI) and average chain length (ACL) can be used. Terrestrially derived alkanes such as those from leaf waxes tend to have a predominance of odd numbered chain lengths with disproportionately high concentrations

of odd alkanes in the C_{24}-C_{35} range leading to CPI values of greater than 1. In contrast, this pattern is not observed in alkanes derived from a petrogenic source where CPI values of 0-1 are generally observed, though values between 1 and 2 have been associated with petroleum impacts (Lichtfouse & Eglinton, 1995; Bray & Evans, 1961; Ahad et al., 2011). ACL can also be used to distinguish terrestrial or petrogenic sources. Long chain alkanes are more commonly found in modern and terrestrial plant material which results in an ACL approaching or greater than 30 (Eglinton & Eglinton, 2008; Bush & McInerney, 2013), whereas petroleum derived alkanes generally consist of primarily shorter chain lengths, and ACLs of less than 30 (Eglinton & Eglinton, 2008; Fang et al., 2014).

Variations in the natural abundance of hydrocarbon $\delta^{13}C$ can also be used to elucidate carbon sources and assess the presence of petroleum hydrocarbons in the natural environment (Boschker & Middelburg, 2002). Terrestrial organic matter derived from plants will have specific $\delta^{13}C$ values based on the photosynthetic pathway used to synthesize carbon (Chmura, Aharon, Socki, & Abernethy, 1987; Natter et al., 2012) Alkanes derived from C4 plants will have less negative $\delta^{13}C$ ranges of approximately -10 to -20‰ (Tanner, Uhle, Kelley, & Mora, 2007; Chikaraishi & Naraoka, 2003) while those from C3 plants would have ranges of -24 to -34‰ (Fang et al., 2014). Crude oils are depleted in ^{13}C within a similar range of -23 to -32‰, but a specific crude oil will often have a precise isotopic signature (Natter et al., 2012; Jackson, Pardue, & Araujo, 1996). These isotopic signatures can be measured in environmental samples in order to differentiate between plant based and petroleum based organic carbon.

Polycyclic aromatic hydrocarbons (PAHs) present in petroleum (petrogenic PAHs) can be composed of from 2 to 6 fused benzene rings and can also enter the environment as components of petroleum spills (Sauer et al., 1998). These PAHs often must be differentiated from other potential sources of PAHs, particularly from atmospheric deposition of PAHs derived from incomplete combustion of organic matter referred to as pyrogenic PAHs (P. D. Boehm & Requejo, 1986). PAHs are of concern due to their biological effects, as many are considered toxic or carcinogenic to organisms and humans (H. K. White, Xu, Eglinton, & Reddy, 2005; Act, 1993) . In some cases biological activity can yield PAHs such as the microbial generation of perylene which is often found in marine sediments (Boitsov, Jensen, & Klungsøyr, 2009; White et al., 2005; Slater, Benson, Marvin, & Muir, 2013) . The specific PAHs detected can aid in differentiating their origin, as the formation process of these compounds yields specific structures and molecular signatures (Baumard et al., 1998; Boitsov et al., 2009).

The final parameter used to assess petroleum hydrocarbon presence is the observation of an Unresolved Complex Mixture (UCM). The UCM refers to a raised baseline hump on a gas chromatogram, and consists of many unresolved hydrocarbons emerging in the aliphatic fraction that are unable to be individually distinguished(Farrington & Quinn, 1973; H. K. White, Xu, Hartmann, Quinn, & Reddy, 2013). The presence of UCM is generally considered to be indicative of petroleum contamination and is commonly observed in petroleum-impacted samples that have been heavily biodegraded (Frysinger et al., 2003; Gough & Rowland, 1990). While the precise origin of a UCM is unknown, studies have sought out the specific chemical make-up and

source material using various techniques such as GCxGC analysis or differentiation of compounds based on size to further separate the UCM (Frysinger et al., 2003; Hays, Smith, & Dong, 2004). Radiocarbon analysis of uncharacterized compounds from various coastal sediment samples (H. K. White et al., 2013) demonstrated a predominantly petroleum origins in the UCM, which suggests its diagnostic quality for petroleum contaminated samples.

One of the key processes responsible for removal of petroleum hydrocarbons from environmental systems is biodegradation (Atlas, 1981; Atlas, 1995; Leahy & Colwell, 1990). Different petroleum hydrocarbons can be degraded by microorganisms at different rates and under a variety of environmental conditions. Saturated hydrocarbons such as n-alkanes are often the fastest to biodegrade, whereas large PAHs can be much more resistant to biodegradation and therefore persist longer in the environment(Atlas, 1981; Sauer et al., 1998). Although hydrocarbon degrading organisms may not be a large proportion of the community, changing environmental conditions such as a release of petroleum hydrocarbons can cause for a community shift, allowing hydrocarbon degraders that utilize this newly available source of carbon and energy to thrive (Atlas & Hazen, 2011; Atlas & Hazen, 2011). This has been documented in the response of a number of microbial communities in response to the Deepwater Horizon spill including: the Gulf of Mexico's oil plume waters (Hazen et al., 2010); oiled beach sand (Newton et al., 2013); and oiled salt marsh sediments (Mahmoudi et al., 2013) following the Deepwater Horizon spill.

In some systems, isotopic signatures in microbial lipids such as phospholipid

fatty acids (PLFA) have the potential to identify the source of recently metabolized carbon. PLFA are microbial membrane component that degrades within days to weeks of cell death, can provide a snapshot of the carbon source of the active microbial population approximately at the time of sampling (Frostegård, Tunlid, & Bååth, 1993; Slater, White, Eglinton, & Reddy, 2005). Heterotrophic microorganisms have generally been observed to produce PLFA that are 4-6‰ depleted with respect to their carbon source though variations in microbial metabolisms can result in much larger ranges

2.1.1 Study Overview

Barataria Bay, Louisiana, approximately 80km from the source of the spill, received extensive Macondo oil contamination with significant ecological impacts throughout the bay and shoreline. The Louisiana salt marshes impacted by Macondo well oil released from the Deepwater Horizon wellhead saw significant die-off of grasses and marsh plants, as well as negative effects on organism such as adsorption and detrimental genomic effects in marsh fish (Silliman et al., 2012; Garcia et al., 2012; Whitehead et al., 2012). While it is known that oil reached the coast affecting the shores and salt marshes (Silliman et al., 2012), the extent of petroleum deposition in bay sediments is unknown. Further, while reductions in mass of petroleum hydrocarbons present in shoreline salt marsh sediments were demonstrated to be in part due to biodegradation (Mahmoudi et al., 2013), there was also significant potential for shoreline erosion to transport oil impacted materials to surface sediments. Observation of an oil sheen associated with sediment disturbance in October 2011 raised the question as to whether these mechanisms had resulted petroleum residues being present in the sediments of Barataria Bay. If so, these

sediments would represent a contaminant source that could be remobilized during boating, storm activities or other instances of sediment disturbance. In order to address the persistence of petroleum hydrocarbons potentially related to the Macondo oil released from the Deepwater Horizon spill, six sediment cores were collected from Barataria Bay approximately one year after the oil spill. Concentrations of *n*-alkanes, PAH, and UCM were determined in these upper sediments to assess the overall conditions and hydrocarbons present in the bay, and whether these compounds were potentially related to the Deepwater Horizon spill or to other sources such as chronic exposure to fuels or industrial contaminants. In addition, phospholipid fatty acids (PLFA) were identified to assess variations in microbial community structure and abundance across the bay. Microbial carbon sources and potential uptake of regional terrestrial matter or Macondo oil was investigated using stable carbon isotope analysis of PLFA and n-alkanes from the bay sediments.

2.2 METHODS

2.2.1 Sites and Sample Collection

The upper 6cm of sediment were collected from six offshore sites in Barataria Bay during October, 2011, 18 months after initial oil release and 15 months after capping of the well head (Figure 2.1, Table 2.1). Surface sediment sites were determined based on proximity to known oil impacted shorelines over the 3-month period during which oil

was freely released from the uncapped wellhead. Sites 1 and 2 were offshore from shorelines with no known Macondo oil contamination, and sites 3-6 were offshore from highly impacted shorelines and coastal marshes. Sites 1-4 are loamy sediment laden with rocks, shells and debris, while sites 5 and 6 are finer, more homogeneous sands. Surrounding vegetation in the Barataria Bay area consists largely of salt marshes, including the abundant *Spartina* marsh grass species (Silliman et al., 2012; Mahmoudi et al., 2013). The samples were homogenized and maintained in a cooler until reaching the laboratory, at which point they were stored at -20ºC. S4 was discarded due to insufficient sample mass.

2.2.2 Hydrocarbon analysis

Approximately 45g of freeze-dried sediment was extracted using a microwave accelerated reaction system (MARS, CEM Corporation) at 115°C for 10 minutes (after ramping to 115 for 10 minutes) using 1:1 acetone:hexane. Organic compounds extracted by solvent were filtered through burned glass fiber filters to remove sediment particles (GF/G, Whatman). Samples were then phase separated based on polarity into four fractions ((1) hexane; (2) 2:1 hexane:dichloromethane (DCM); (3) DCM; (4) methanol). Fraction 1 was reduced to a small volume and analyzed for n-alkanes on the GC/MS using Ultra Scientific TRPH (Florida)Alkane Standard of C_8 – C_{40}. Fraction 2 was analyzed for PAH's on the GC/MS using SV calibration mix of 16 PAHs. Samples were cleaned of elemental sulfur by adding solid copper beads activated with nitric acid prior to GC/MS analysis.

After quantification of any UCM present in fraction 1 within the samples, urea aduction was conducted to remove all uncharacterised hydrocarbons for clear and representative analysis of n-alkanes modified from (Ahad et al., 2011) (OBALI, 1989) (Yamamoto & Kawamura, 2012). A saturated solution of urea in methanol was prepared. Solution was added to 2:1 hexane:acetone until a precipitate formed. Precipitate was then dried of all solvents under a N_2 evaporator, and re-dissolved in nano-pure H_2O. Solution was then added to samples and pipette out of the remaining solution. An Agilent 6890 GC coupled to a 5973 quadruple mass spectrometer was used for identification and quantification of hydrocarbons. The GC was equipped with column Agilent DB-5 MS 30m x 0.32mm, with a 0.25μm film thickness. The temperature program for n-alkane analysis of the first fraction was 50°C ramped to 310°C at 10°C/min for a final hold time of 15 minutes. The temperature program PAH analysis of the second fraction was 50°C ramped to 200°C at 10°C/min, then ramped to 250°C at 4°C/min with a hold for 5 minutes, followed by a final ramp up to 300°C at 2°C/min.

Carbon preference index (CPI) and average chain length (ACL) were quantified from the fraction 1 alkanes. CPI is the ratio of total odd versus even numbered alkanes, usually within the longer chain length range of 24 – 35, where a terrestrial odd-over-even preference is represented by a CPI greater than 1 (Bray & Evans, 1961) (Ahad et al., 2011). Conversely petroleum derived alkanes would have a CPI between 0 and 1. CPI= $(\sum \mathrm{odd}_{C_{25-35}})/(\sum \mathrm{even}_{C_{24-34}})$, adapted from (Tanner et al., 2010).

ACLs are calculated as the average number of carbons based on the preference for odd-numbered chain lengths (Ahad et al., 2011; Eglinton & Eglinton, 2008). a high ACL of around 30 can indicate long chain alkanes commonly found in leaf waxes, whereas petroleum derived alkanes generally demonstrate a lower ACL (Eglinton & Eglinton, 2008; Fang et al., 2014). ACL= $(\sum[C_i] * i)/(\sum C_i)$, where i = odd $C_{25\text{-}35}$ adapted from (Ahad et al., 2011).

2.2.3 Microbial PLFA extraction and analysis

Approximately 60 to 400g of freeze-dried sediment was extracted using a modified Bligh and Dyer method (Bligh & Dyer, 1959). Sediments were extracted using 2:1 DCM:methanol and then filtered through burned glass fiber filters to remove sediment particles (GF/G, Whatman). Organic extracts were separated by polarity into three fractions; non-polar, neutral and polar (F1: DCM, F2: acetone, F3: methanol) by silica gel chromatography using combusted and activated silica gel. Phospholipids from the methanol fraction were transesterified to fatty acid methyl esters (FAMEs) by mild alkaline methanolysis(White, Davis, Nickels, King, & Bobbie, 1979). The FAMEs are assumed to be from PLFA exclusively as the methanolysis hyrdrolizes and methylates only esterified fatty acids within the mixture(White et al., 1979). These were then further purified by a secondary silica gel chromatography to ensure no other carbon compounds are present in this fraction by selecting for a limited polarity range. The FAMEs were then analyzed by GC/MS. PLFA are identified based on comparison to bacterial reference standards (Bacterial Acid Methyl Esters CP, Mix, Matreya Inc, Supelco 37 Component FAME Mix), as well as retention times and mass fragmentation patterns. FAMEs were

identified and quantified via GC/MS using an Agilent 6890 GC coupled to a 5973 quadruple mass spectrometer was used for identification and quantification of PLFA FAMEs. The GC was equipped with a 30m x 0.32mm DB-5 MS column with a 0.25µm film thickness. The temperature program was 50°C for 1 minute, then ramped to 130°C at 20 C/min, ramp to 160°C at 4°C/min the ramped to 300°C at 8°C/min with a final hold for 5 minutes. Designation of PLFA is according to number of carbon atoms in the chain and the number of double bonds. Prefixes are representative of structural isomers (br-methyl branching; i-iso; a-anteiso; cyc-cyclopropyl).

2.2.4 Stable carbon isotope analysis

$\delta^{13}C$ analysis on individual PLFAs and n-alkanes was performed. Specific PLFA analyzed ranged from $\delta^{13}C_{12:0}$ to $\delta^{13}C_{22:0}$ depending on the sample and available carbon mass. PLFA was obtained from the FAMEs extracted during PLFA analysis above from Barataria Bay sediments. PLFA were identified as FAMES on GC/MS prior to isotopic analysis. In order to volatilize on the GC/MS FAMEs were created by adding isotopically characterized methyl ester groups to polar phospholipids. The $\delta^{13}C$ values were therefore corrected for a known incorporated methanol $\delta^{13}C$ value of -41.95‰ based on isotopic mass balance using a correction factor (Abraham, Hesse, & Pelz, 1998), yielding an accurate isotopic ratio of the PLFA specifically.

Sediment alkanes were analyzed for $\delta^{13}C$ values as described above. Alkanes were previously identified on GC/MS, and subjected to urea adduction to purify samples and remove UCM.

Compound-specific stable carbon isotope analysis was performed in triplicate on a gas chromatography-isotope ratio mass spectrometry (GC-IRMS) system. This consisted of a Agilent 6890 gas-chromatograph interfaced to a Finnigan-MAT DeltaPlus via a GC Combustion III interface. The GC was equipped with a Agilent DB-5 MS column, 30m x 0.32mm with a film thickness of 0.25μm. The temperature profile for both PLFA and alkane isotopic analaysis was 80 C held for 1 minute, ramp to 280 C at 4 C per minute, ramp to 320 C at 10 C per minute with a final hold for 15 minutes.

2.3 RESULTS

2.3.1 Hydrocarbon residues

2.3.1.1 Alkanes

Total alkane concentrations in each bay sediment sample ranged from 136.1ng/g in S6 to 812.8ng/g in S3 (Table 2). There was no obvious difference in alkane concentrations between samples offshore of impacted versus non-impacted locations. Alkanes present ranged from 12 to 35 carbon units in length and had average chain length (ACL) values that ranged from 29.1 to 38.8 and carbon preference index (CPI) values that ranged from 1.16 to 4.28.

Alkane $\delta^{13}C$ values for measured sediment alkanes are shown in table (Table 3). Values are listed only for baseline resolved GC IRMS peaks of sufficient amplitude to yield accurate isotopic compositions. $\delta^{13}C_{Alk}$ values ranged from -27.2 to -32.5‰, with a

standard deviation of 1.08. Overall alkane $\delta^{13}C$ results were highly consistent across all sampling locations.

2.3.1.2 PAHs

PAHs in the sediment samples were below detection with the exception of S3 which had total PAH concentration of 51.5ng/g of sediment. Only three PAH compounds were present in S3; perylene, fluoranthene and pyrene, in order of decreasing concentration (Table 2).

2.3.1.3 UCM

Uncharacterized complex mixture (UCM) was detected in the non-polar alkane fraction (f1) of all sediment sample extractions. The highest UCM concentrations were found in the sites offshore from non-impacted shoreline (S1 and S2), at 45.3ug/g and 53.4ug/g, respectively. UCM was detected at lower concentrations as a slight hump in the gas chromatogram in three of the sites offshore from impacted sites, with S5 at 3.6ug/g, S3 at 10.6ug/g, and S6 at 12.5ug/g.

2.3.2 PLFA concentrations and distributions

PLFA concentrations ranged from 0.81mg/kg in S6, up to 4.48mg/kg in S5. Cell densities based on PLFA concentrations were calculated using a general conversion factor of 2×10^4 cells $pmol^{-1}$ of PLFA (Green & Scow, 2000). Cell densities ranged from

$9.10x10^7$ cells/g in S6 to $2.7x10^8$ in S5 cells/g demonstrating a typical cell density range for sediment samples (Syakti et al., 2006; Gregory F Slater et al., 2005; Petsch, Edwards, & Eglinton, 2003).

The distribution of PLFA found in the sediment samples was highly similar in all 5 sites. In all 5 samples the most significant PLFA is 16:0, making up between 17.5 (S1) and 19.5 (S5) mol% of the each sample (Figure 2). The largest proportion of the samples in mol % is generally saturated PLFA, with the smallest portion being polyunsaturated such as 20:4 and 20:5, which were not detected in all samples.

2.3.3 PLFA $\delta^{13}C$ measurements

Compound specific $\delta^{13}C_{PLFA}$ analysis of sediment samples was used to determine microbial carbon sources. $\delta^{13}C_{PLFA}$ values across all sites were similar and fall in the general range of $\delta^{13}C$ = -24‰ to -32‰ (Figure 3). In all samples, the $\delta^{13}C$ of 16 carbon PLFAs, including 16:0, 16:1, branched 16:0, were among the lightest isotopic signatures observed with values as depleted as -31.9‰ collected from a branched 16 carbon fatty acid in S2. The relatively isotopically heavier values are consistently 15 carbon PLFA, such as i15:0 in S1 with a value of $\delta^{13}C$ = -24.5

2.4 DISCUSSION

2.4.1 Total hydrocarbons in Barataria Bay sediments

The distribution of n-alkanes, the lack of PAH and the low levels of UCM in these sediments provide evidence that while petroleum derived hydrocarbons are present, they are not the dominant source of hydrocarbons associated with the surface sediments in Barataria Bay at the time of sampling, but rather that sedimentary organic matter is primarily derived from terrestrial sources. Further the n-alkane distributions and isotopic signatures indicate that the petroleum derived hydrocarbons that are present are not likely derived from the deepwater horizon oil spill.

Based on the overall distribution of n-alkanes found in the Barataria Bay sediments, the hydrocarbons observed across all sediment samples were derived primarily from terrestrial sources with some minor inputs from petroleum residues. In these samples ACL ranged from 29.1 to 38.8 values that are consistent with, or higher than, salt marsh plants and surrounding sediments (Tanner et al., 2010; X-C Wang, Chen, & Berry, 2003). While being well within the terrestrial range, it is worth noting that both samples offshore from non-impacted sites are slightly higher ($p>0.05$) in ACL than the three samples collected offshore from oiled portions of the bay. This difference is not statistically significant based on a Mann Whitney U statistical analysis (Kriskal-Wallace One-Way ANOVA), but the resolution of the statistics is limited by the relatively small number of samples. The observed difference may be a result of a slightly higher proportion of short chains alkanes found in petroleum hydrocarbons mixing with the dominant terrestrial long chains yielding a comparatively intermediate value. Despite these slight differences between samples, the ACL indicates a predominance of terrestrially derived alkanes.

The interpretation of largely terrestrial carbon sources is further supported by the CPI values which were calculated to be greater than 1 at all sites. For the samples taken offshore of non-impacted sites, the CPI values (S1=3.96, S2=4.28) are considerably above one and are within reported ranges for aquatic and salt marsh plants native to this coastal Louisiana region(Silliman et al., 2012; Mahmoudi et al., 2013). Further, they are consistent with previously reported alkane distributions associated with C3 and C4 plants and sediment samples from salt marshes suggesting these are likely contributors to the sediment alkanes (Tanner et al., 2010; X-C Wang et al., 2003). In the samples taken offshore from impacted sites, the extent of carbon preference is much less pronounced with CPIs ranging from 1.16 to 1.86. This lower range of calculated CPIs shows evidence of only a weak odd over even preference which has been used previously to categorize polluted samples (Lichtfouse & Eglinton, 1995). These lower CPI values are statistically distinct from those observed offshore of the non-oiled sites within a 90% confidence interval based on a Mann Whitney U statistical analysis (Kriskal-Wallace One-Way ANOVA), indicating the potential impact of oiling on these sites. However, the fact that they remain above 1, and when considered in conjunction with the ACL values, indicates that the predominant source of alkanes to these sediments is terrestrial with some inputs from petroleum hydrocarbons.

The stable carbon isotope signature of the alkanes is also consistent with a terrestrial source, however it cannot preclude contributions from petroleum due to the overlap between $\delta^{13}C$ ranges for terrestrial organic matter and petroleum hydrocarbons. Sediment alkane $\delta^{13}C$ values were similar across all sites with an overall average $\delta^{13}C_{Alk}$

of -29.7‰ (stdev 1.08) for all alkane chain lengths. The consistency of alkane $\delta^{13}C$ values between samples is indicative of a consistent source contributing to these sediments in all sampling locations. The sediment isotopic range is in accordance with potential C3 plant sources, which are known to be common in Louisiana coastal marshes and could be contributing to the sediment alkane $\delta^{13}C$ value (Chmura et al., 1987). There is also potential for input of C3 derived plant material via the Mississippi-Atchafalaya river system (Waterson & Canuel, 2008), which may account for more depleted $\delta^{13}C$ values. Interestingly, it is not consistent with being derived from the abundant *Spartina* marsh plant, as they use a C4 pathway and generally have $\delta^{13}C$ of -19‰.

The sediment alkane isotopic range and observation of relatively low CPI and ACL values offshore from impacted sites highlight the possibility of petroleum hydrocarbon contamination. However, the isotopic compositions of the observed alkanes ($\delta^{13}C$ = -29‰) is approximately 2‰ more negative than Macondo well oil ($\delta^{13}C$ = -27‰) and are not consistent with Macondo oil being the source of these alkanes. The observed alkane $\delta^{13}C$ values do not discount other potential petroleum hydrocarbon inputs however as petroleum hydrocarbons are known to have $\delta^{13}C$ signatures between -23 to -32‰ (Natter et al., 2012; Jackson et al., 1996). Other potential sources of petroleum derived alkanes to Barataria Bay surface sediments include shipping traffic, atmospheric deposition, or petroleum seeps in the Gulf of Mexico.

The presence of a UCM in coastal sediments is often considered strong evidence for petroleum hydrocarbon contamination (Frysinger et al., 2003; Jones et al., 1983; H. K.

White et al., 2013; Atlas, 1981). The observation of a UCM in these samples is indicative of the presence of biodegraded petroleum hydrocarbon inputs (Figure 4). High concentrations of UCM were observed to be present associated with impacted shoreline regions(Mahmoudi et al., 2013; Silliman et al., 2012). This UCM would be expected to sorb strongly to particles and potentially sink to surface sediments if eroded. However, the UCM concentrations observed in the bay sediments were in fact very low compared to those observed on shorelines by Mahmoudi et. al and Silliman et al. and in other contaminated environments. Based on observations made by (White et al., 2013) and (Scarlett, Galloway, & Rowland, 2007) of chronically and acutely contaminated sediments, much higher UCM concentrations (up to 8000ug/g, commonly above 1000ug/g) would be expected than those observed in these sediment samples to confirm the presence of Macondo oil, in some cases orders of magnitude differences. Despite these samples being taken over one year after the time of contamination, UCM is known to be highly persistent at spill adjacent sites, remaining detectable at high concentrations for decades after initial exposure (Reddy, Eglinton, et al., 2002). Considering the volume of oil passing through the bay in 2010, the higher concentrations of UCM found at the non-impacted sites suggests that Macondo oil is not the source of this UCM. Close proximity to anthropogenic inputs including the Gulf Intracoastal Waterway, the Mississippi River Delta and several shipping ports contribute to chronic petroleum inputs that may account for a baseline UCM, and would also yield a petroleum signature. Further, UCM has also been linked to natural hydrocarbon seeps (Volkman et al., 1992;

Page et al., 1996) which are widespread in the Gulf of Mexico and may be contributing to the UCM detected in these sediments.

A lack of detection of PAHs in almost all sediment samples is not only below typical values for a contaminated site, but also below typical coastal sediment values from naturally occurring PAH deposition and baseline values (H. K. White et al., 2005; Martins, B'\icego, Taniguchi, & Montone, 2004) PAHs generally have low aqueous solubility and are hydrophobic (Readman et al., 2002). For this reason the sediments are a likely location to find PAHs that have contaminated a marine system and sorbed to sediment organic matter. Values around or below 50ng/g of PAH is often considered typical of sediments distant from any point sources of petroleum hydrocarbons (Readman et al., 2002; Baumard et al., 1998). The values found in these samples suggest that impacted to petroleum hydrocarbon contamination is minimal with negligible concentrations at 4 of the 5 sites and low concentrations (51.5ng/g) of only 3 PAHs in one sample. The observed PAH concentrations here are also several orders of magnitude lower than those previously observed at other contaminated sites (Lee & Page, 1997;Lima, Eglinton, & Reddy, 2003; H. K. White et al., 2005). S3 was the only sample in which PAHs were detectable, and perylene accounted for almost half of the total PAH concentrations at 24.4ng/g. Perylene is unique as it can be synthesized in situ in sediments by microorganisms from various source material, and like petroleum sources may also be generated from the breakdown of more modern carbon (Slater et al., 2013;Reddy, Pearson, et al., 2002). The concentration of perylene found in this sample is typical of common background levels, and would be consistent with that of diagenic

origin from biodegradation of terrestrial plant material (Venkatesan, 1988; Baumard et al., 1998; Boitsov et al., 2009). Previous analyses of northern Gulf of Mexico sediments identified pyrene and fluoranthene as primary PAHs from chronic and pyrogenic sources (Overton, Ashton, & Miles, 2004). These two PAHs were detected in very low levels in S3, and may be attributed to residual contamination from boat traffic or anthropogenic runoff. (Venkatesan & Kaplan, 1982;Lima, Farrington, & Reddy, 2005).

With respect to Macondo oil, PAHs detected in nearby salt marsh sediments were measured as higher than 10000 ng/g five months after the spill, 3 orders of magnitude higher than the largest concentration found in the bay surface sediments (Mahmoudi et al., 2013; Silliman et al., 2012). Although the only sample displaying detectable concentrations was taken from a location proximal to impacted shoreline, the low hydrocarbon concentrations and specific PAHs observed cannot support Macondo well oil contamination. Characteristic PAHs found to be dominant in Macondo oil as well as many crude oils did not correspond to those found in these sediments, specifically phenanthrene and chrysene (Liu et al., 2012; Zhou, Liu, & Guo, 2012).

Macondo oil is known to have reached the shorelines and salt marshes of Barataria Bay following the Deepwater horizon spill (Mahmoudi et al., 2013; Silliman et al., 2012). The Barataria Bay shoreline is also known to undergo significant erosion due to factors such as waves, currents, or bioturbation (Fitzgerald, Kulp, Penland, Flocks, & Kindinger, 2004; Reide Corbett, McKee, & Allison, 2006). The potential for deposition of Macondo oil to the surface sediments within the bay due to erosion of oil shoreline and

wetlands seems high. Assuming a sedimentation rate of less than 1 cm per year as has been measured in Barataria Bay (Reide Corbett et al., 2006), the top 6cm collected for this analysis should capture all naturally occurring sedimentation or typical shoreline erosion material. Since minimal indices of petroleum were detected by alkane and PAH analysis, we can conclude that erosion of oiled shoreline material was not a major process during the 18 months following the Deepwater Horizon spill. Some indications of petroleum residues however may represent an alternative petroleum source.

2.4.2 Microbial community and carbon sources

The microbial community structure and cell density detected in the top bay sediments using PLFA analysis was diverse, with no strong indicators of a particular organism, metabolism, or carbon source. PLFA distribution, proportions of lipid classes, and carbon isotope signatures can be highly useful in considering whether microbial carbon sources match those detected in the sediments. The intrusion of oil into a marine system leads to shifts in microbial community structure and biomass due to an enrichment of hydrocarbon-degrading microorganisms (Prince, 2010). PLFA distributions can assess whether biodegradation removed any Macondo sourced petroleum residues, as petroleum alkanes are known to be the most susceptible hydrocarbons to microbial degradation and can be degraded within a rapid time frame (Leahy & Colwell, 1990; Greenwood, Wibrow, George, & Tibbett, 2009; Frostegård et al., 1993).

PLFA results were notably similar between all sites, with respect to PLFA concentrations, distribution and specific lipid biomarkers. Microbial cell densities estimated based on PLFA concentrations were from $9.10x10^7$ cells/g to $2.7x10^8$cells/g, which is within a similar range observed in nearby salt marsh sediments by Mahmoudi et al. 15 months after the spill(Mahmoudi et al., 2013). This is also within the range expected for sedimentary environments (Syakti et al., 2006; Slater et al., 2005; Petsch et al., 2003). PLFA distributions were likewise similar across sites. High prevalence of 16:0 and 18:1 is typical of most bacterial communities (Ratledge & Wilkinson, 1988; Pearson, McNichol, Benitez-Nelson, Hayes, & Eglinton, 2001; Slater, Nelson, Kile, & Reddy, 2006) and has been detected in both hydrocarbon degrading and non-degrading species (Aries, Doumenq, Artaud, Acquaviva, & Bertrand, 2001). The similarity in PLFA concentrations and proportions of lipid classes across sites and to the healthy community suggests that microbial communities in the bay sediments were not strongly affected by petroleum contamination at the time of sampling.

Compound-specific $\delta^{13}C$ PLFA values observed in these sediments ranged from $\delta^{13}C$ = -24‰ to -32‰ and were not able to resolve microbial carbon sources with confidence. Variations in $\delta^{13}C$ PLFA between PLFA were suggestive that microbial community members produces particular PLFA were accessing different C sources or utilizing different biosynthetic pathways. Biosynthetic fractionation during PLFA synthesis by heterotrophic organisms is generally 4-6‰, however it has been shown to be as high as 10‰ (Teece, Fogel, Dollhopf, & Nealson, 1999; Boschker, De Brouwer, Cappenberg,et al., 1999; Hayes, 2001). The $\delta^{13}C$ of alkanes present in the sediments (-

29‰) would generally be expected to produce PLFA with signatures more depleted than those that were observed here. This is also generally true of Macondo oil alkanes at -27‰. Microbial utilization of Spartina biomass (-19‰) would produce results consistent with observations, however, the absence of evidence of Spartina derived sources raises questions regarding this interpretation. Microbial use of bulk organic carbon would be an explanation, as could use of marine DOC sources which have been reported to be generally within the range of -20‰ to -28‰ in nearby coastal regions or the Mississippi or Atchafalaya River systems (Xu-Chen Wang, Chen, & Gardner, 2004; Peterson, Fry, Hullar, Saupe, & Wright, 1994) and would result in PLFA with a similar $\delta^{13}C$ to that of these samples. Total organic carbon from the nearby salt marsh sediments had a $\delta^{13}C$ value between -20 and -21‰ suggesting a plausible carbon source by way of erosion into the bay sediments (Mahmoudi et al., 2013). Carbon inputs from these marsh plants as well as other carbon sources including C3 and C4 plants would contribute to this intermediate $\delta^{13}C_{PLFA}$ value.

When looking at specific PLFA, there are consistencies among all sample sites. For example fatty acids with 16 carbons (16:1, 16:0, br16:0) are consistently more depleted at approximately -29‰ to -31‰ and are also within the most plausible range to have degraded Macondo oil. Meanwhile i15:0, which is generally associated with SRB, show lower fractionations than other PLFA suggesting that distinct microbial community components are being sampled here.

In general the $\delta^{13}C$ signatures of Barataria Bay PLFA taken 18 months after initial exposure to oil correspond with data taken from nearby salt marshes at impacted and reference sites. The results can provide insight into carbon sources used in PLFA, but cannot directly determine the use of Macondo well oil as a recent microbial carbon source due to similar $\delta^{13}C$ signatures as some plant material. The data found in these sediments are indicative of varied carbon sources including Spartina marsh grasses and C3 plants contributed from the Mississippi river basin, as well as possible chronic oil inputs from boat traffic and anthropogenic runoff.

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FIGURES AND TABLES

Table 2.1. Coordinates of samples collected from Barataria Bay, Louisiana in October 2011

	Latitude	Longitude
Site 1	N 29.2879	W 90.0072
Site 2	N 29.35881	W 89.9640
Site 3	N 29.4091	W 89.9296
Site 4	N 29.4267	W 89.8675
Site 5	N 29.32742	W 89.92657
SIte 6	N 29.35401	W 89.87798

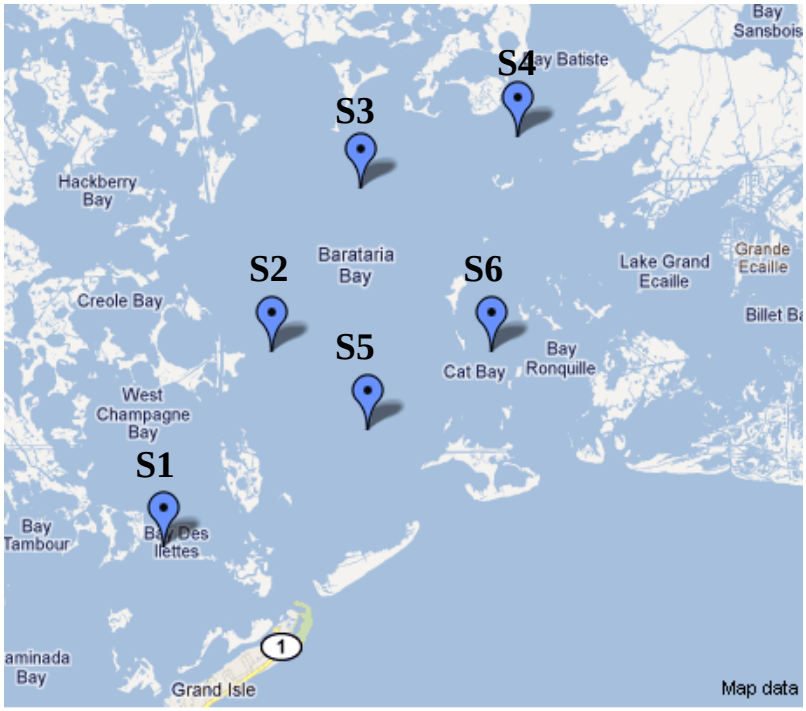

Figure 2.1. Map of core sampling sites in Barataria Bay, Louisiana. Samples were taken October, 2011. Sites 1 and 2 are offshore of non-impacted shoreline, while Sites 3 – 6 are nearby shorelines known to be impacted.

Table 2.2. N-alkane, PAHs, UCM concentrations (mg/kg) as well as Carbon Preference Index (CPI) and Average Chain Length (ACL) of n-alkanes in all five samples

	N-alkanes (ng/g)	Carbon Preference Index (CPI)	Average Chain Length (ACL)	PAHs (ng/g)	UCM (mg/kg)
Site 1	617.7	3.96	38.8	B.D.	45.3
Site 2	663.9	4.28	38.3	B.D.	53.4
Site 3	812.8	1.60	36.1	51.5	10.6
Site 5	586.4	1.16	35.9	B.D.	3.6
Site 6	134.1	1.86	29.1	B.D.	12.5

B.D. = below detection

Table 2.3. N-alkane $\delta^{13}C$ values and standard deviations for all five samples

	S1		S2		S3		S5		S6	
Alkane	δ13C	StDev	δ13C	StDev	δ13C	StDev	δ13C	StDev	δ13C	StDev
C14									-27.9	0.16
C15									-28.5	0.45
C16	-29.5	0.39			-27.2	0.42			-30.3	1.78
C17	-30.8	0.33			-32.0	0.00			-28.6	0.54
C18	-29.9	0.45			-27.9	1.04				
C19	-29.2	0.12			-29.6	0.00			-29.2	0.99
C20	-29.1	0.52	-32.2	0.72	-29.8	0.00	-32.3	1.94	-28.7	1.91
C21	-29.3	0.76	-29.8	0.65	-31.9	0.00	-29.5	1.02	-31.6	0.39
C22	-29.9	0.43	-29.6	1.13	-32.5	0.00	-29.6	1.18	-29.1	1.52
C23	-29.0	0.60	-30.4	0.64	-30.1	0.66	-30.1	0.86	-29.7	0.53
C24	-29.9	0.80	-30.2	0.57	-29.5	0.38	-30.4	1.05	-29.6	0.95
C25	-28.4	0.58	-29.1	0.48	-27.8	0.58	-29.1	0.60	-30.1	0.70
C26	-28.8	0.67	-29.5	0.69	-27.9	0.29	-30.0	0.95	-29.8	1.25
C27	-28.7	0.50	-29.4	0.33	-27.8	0.86	-29.4	0.71	-30.1	0.73
C28	-29.9	0.88	-30.2	0.57	-28.9	0.09	-30.5	1.67	-31.0	0.50
C29	-29.7	0.59	-30.4	0.27	-28.9	0.69	-30.1	0.77	-29.6	0.73
C30	-29.2	0.66	-30.1	0.44	-30.4	0.40	-31.0	1.55		
C31	-29.3	0.41	-30.6	0.55	-30.1	0.51	-30.5	1.10		
C32					-31.8	0.00				
C33					-28.7	0.70				

Table 2.4. Total PLFA concentrations and cell density for all five samples

	Total PLFA (mg/kg)	Cells g^{-1}*
Site 1	3.48	2.47E+08
Site 2	3.01	1.86E+08
Site 3	2.63	1.37E+08
Site 5	4.48	2.70E+08
Site 6	0.81	9.10E+07

*cells per gram estimate was estimated from PLFA concentrations

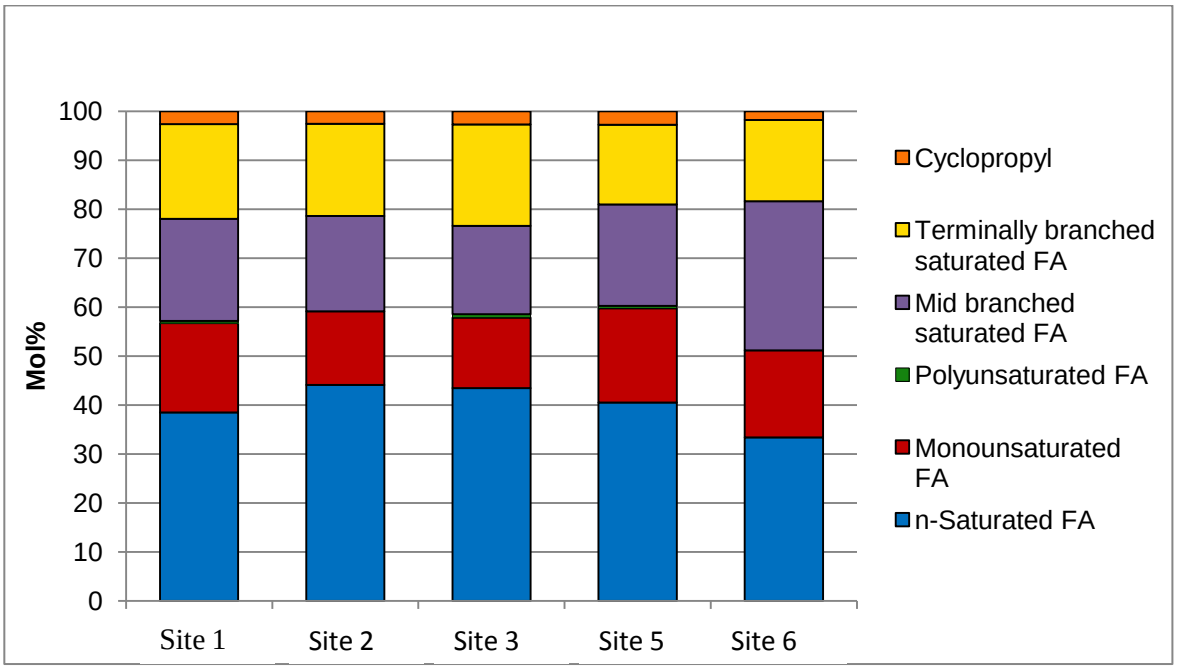

Figure 2.2. Distribution of PLFA classes expressed as mole percentage of the total.

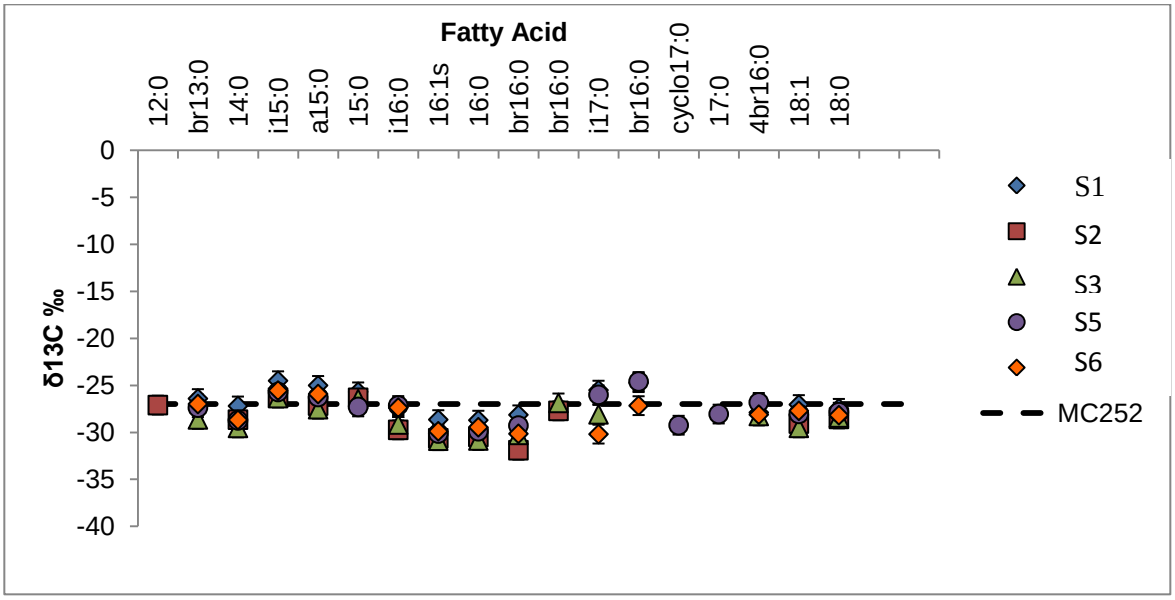

Figure 2.3. Measured $\delta^{13}C_{PLFA}$ of specific fatty acids within all 5 samples. The dashed line represents the $\delta^{13}C$ value of MC252 Crude Oil of $\delta^{13}C$= -27‰.

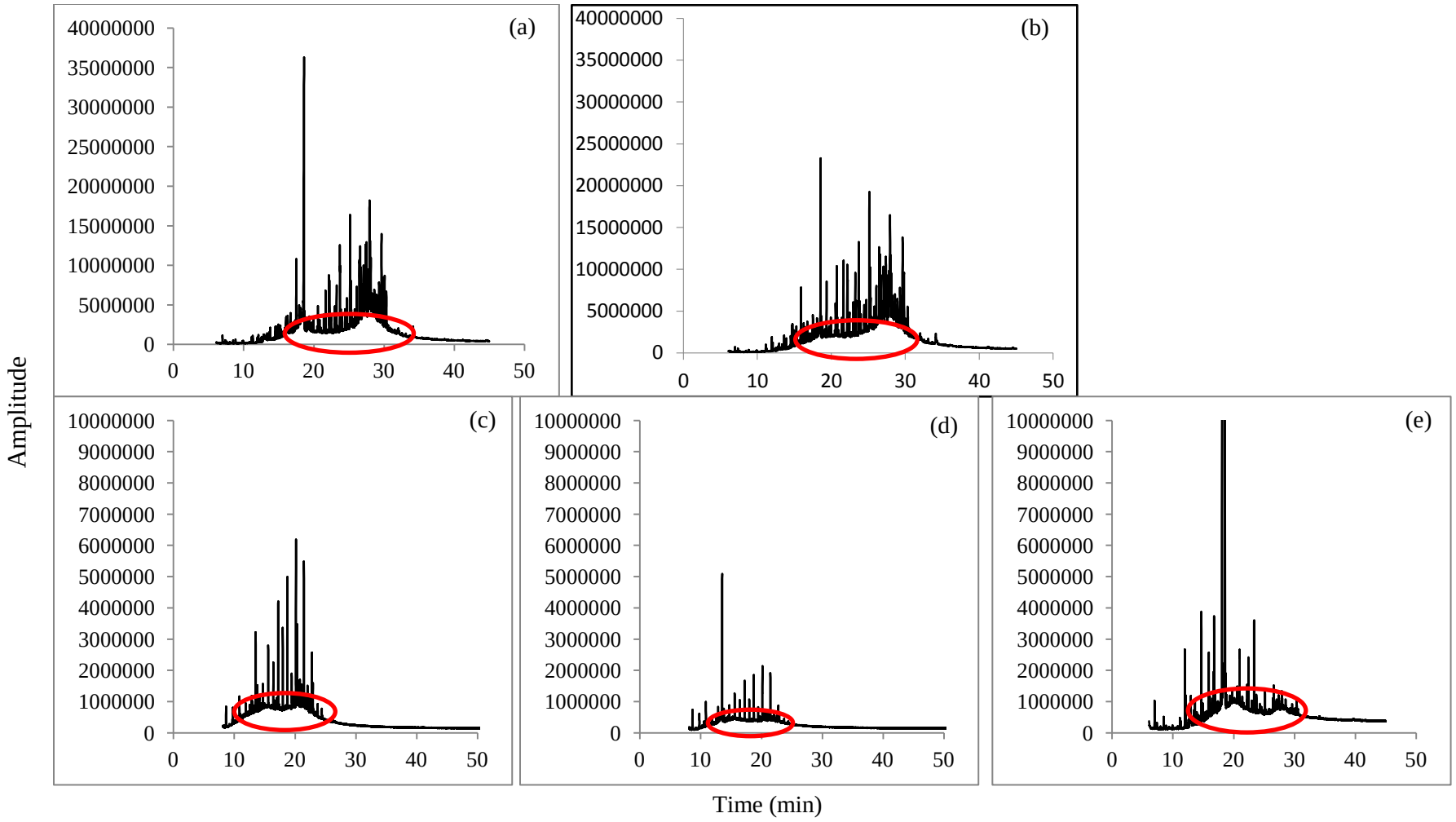

Figure 2.4. Chromatograms of extracted alkanes from Barataria Bay sediments at sites (a)S1, (b)S2, (c)S3, (d)S5, (e)S6. Red circles denote uncharacterized complex mixture (UCM). Note that S3, S5 and S6 are plotted on smaller scales than S1 and S2 due to lower concentrations.

CHAPTER 3:

IDENTIFYING MICROBIAL CARBON SOURCES DURING ETHANOL, TOLUENE, AND MTBE BIODEGRADATION IN A PILOT-SCALE EXPERIMENTAL AQUIFER SYSTEM USING ISOTOPIC ANALYSIS

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ABSTRACT

Ethanol is a non-toxic and highly labile renewable biomass-based resource which is an effective fuel oxygenate that reduces air pollution, and is commonly combined with gasoline to create more environmentally conscience transportation fuels. Recent research however, has indicated that upon accidental release into groundwater systems, the preferential microbial metabolism of ethanol can cause progressively reducing conditions leading to slower biodegradation of petroleum hydrocarbons. Therefore, the presence of ethanol can result in greater persistence of BTEX compounds and longer hydrocarbon plumes in groundwater systems. Understanding the occurrence and extent of ethanol degradation in a subsurface system can provide insight into these potential outcomes and perhaps how they might be managed Microbial biodegradation and community carbon sources coupled to aqueous geochemistry were monitored in a pilot-scale laboratory tank (80cm x 525cm x 175cm) simulating an unconfined sand aquifer. Dissolved ethanol, toluene, and methyl-tert butyl ether (MTBE) were continuously injected into the aquifer at a controlled rate over 300 days. Ethanol, toluene and MTBE all experienced significant mass loss within the aquifer. Stable carbon isotope analysis of residual hydrocarbons indicated strong evidence of biological fractionation of toluene and MTBE throughout the sampling period. Isotopic analysis of microbial phospholipid fatty acids (PLFA) indicated a strong ethanol sourced signature for almost all sampling periods with some indications of an additional carbon source such as toluene or MTBE. This study was able to

successfully identify dominant microbial carbon sources without the use of isotope labelling.

3.1 INTRODUCTION

Petroleum hydrocarbon contamination is ubiquitous in soil and groundwater systems worldwide. BTEX compounds make up approximately 15% of gasoline products and are known the be among the most persistent of compounds therein (Ulrich, 1999; Ahad, Sherwood Lollar, Edwards, Slater, & Sleep, 2000). With known toxicity and carcinogenic properties to humans and various organisms, these compounds are of great concern when released into the environment (Dean, 1985). Biodegradation, particularly intrinsic bioremediation, is of primary interest as a means to remove BTEX compounds and is known to commonly occur when gasoline hydrocarbons are released into the environment (Löffler & Edwards, 2006). Aerobically, this can occur quickly due to the favourable kinetics provided by the high oxidation potential of O_2 (Wilson, D'Adamo, Bouwer, & Hutchins, 1997). Anaerobic conditions can also facilitate degradation of harmful BTEX compounds under iron reducing conditions (Anneser et al., 2008), denitrifying conditions (Pelz, Chatzinotas, Andersen, et al., 2001;Wilson et al., 1997), sulfate reducing conditions (Anneser et al., 2008; Elsner, Zwank, Hunkeler, & Schwarzenbach, 2005; Ramos, da Silva, Chiaranda, Alvarez, & Corseuil, 2012), methanogenic conditions (Ramos et al., 2012). These various conditions often occur in an actively degrading contaminated aquifer, separating into physical zones based on which

electron acceptor is being used (Ma, Rixey, & Alvarez, 2013). Zonation will follow the redox ladder of terminal electron acceptors, moving from most energetically favourable metabolisms (oxygen) to least (methanogenesis via CO_2 reduction) as each becomes depleted in that zone (Lovley, 1997).

When a variety of additives are found in the mixture, the fate and transport of spilled gasoline can vary, as can its potential for degradation. Fuel oxygenates are commonly added to gasoline as a way to increase its combustion efficiency (Ahmed, 2001). This reduces harmful intermediates of incomplete combustion released into the atmosphere (Williams, Cushing, & Sheehan, 2003; Ulrich, 1999). A variety of oxygenates used for their anti-knocking properties are added to fuels to facilitate these altered properties (Ahmed, 2001). Tetra-ethyl lead was extensively used as a fuel additive, however adverse health effects have caused the compound to be phased out of use in gasoline in the 1970's (Thomas, 1995). Today, ethanol or Methyl tert-butyl ether (MTBE) are regularly present as fuel additives.

MTBE has a number of beneficial qualities such as cost and octane level contributing to its efficiency as an oxygenate (Deeb et al., 2003). However, an abundance of MTBE contamination in drinking water has lead to concern of health effects largely due to a noticeable odor and taste, even at low concentrations (Powers et al., 2001). When released into the environment MTBE is water soluble up to 54g/L, and is a common contaminant from leaking underground storage tanks (Deeb et al., 2003). Biodegradation of MTBE can occur both aerobically and anaerobically (Braeckevelt, Fischer, & Kästner,

2012). However, tert-butyl alcohol (TBA) is the common intermediate of MTBE degradation, created by cleaving the compound's ether bond, and has known carcinogenic properties (Schmidt, Schirmer, Weiß, & Haderlein, 2004). This is a particular concern for the release of MTBE, as its breakdown can be more harmful than the parent compound.

Due to the issues with MTBE, ethanol is now a commonly used fuel oxygenate that is considered beneficial for a variety of reasons. Like other oxygenates, its presence in gasoline is known to decreases carbon monoxide and VOCs released into the atmosphere upon combustion, however it has relatively minor health effects for humans, including no carcinogenic properties (Williams et al., 2003; Ulrich, 1999; Ma et al., 2013). As a renewable resource often derived from corn, ethanol is also a sustainable option and sometimes considered a stepping stone towards the use of biofuels (Corseuil et al., 2011; Powers et al., 2001). However, the presence of ethanol in gasoline has the potential to have significant effects on the biodegradation of BTEX and other gasoline compounds when a release to the environment occurs. Ethanol is highly labile and is miscible with water allowing it to be easily biodegraded in both aerobic and anaerobic systems much faster and more efficiently that other oxygenates such as MTBE (Schmidt et al., 2004; Corseuil, Hunt, Ferreira dos Santos, & Alvarez, 1998; Ulrich, 1999).

Studies have found that the changing properties of fuels with additives such as MTBE or ethanol can change the efficacy of microbial biodegradation (Mackay et al., 2006;Feris et al., 2008; Mackay et al., 2007; Corseuil et al., 2011; Ulrich, 1999). When ethanol is added to a gasoline mixture it's much higher oxidation potential and

degradability (due to a lack of double bonds or stable benzene rings) can alter the redox conditions in the system affecting the biodegradation patterns in an aquifer. Previous studies have found that in the presence of ethanol, BTEX contamination can be more persistent due to preferential degradation of ethanol as a more labile carbon source (D. Mackay et al., 2007; Feris et al., 2008; Ruiz-Aguilar, O'Reilly, & Alvarez, 2003; Molson, Barker, Frind, & Schirmer, 2002). As ethanol is preferentially degraded, available oxygen or other electron acceptors involved in the degradation reaction are consumed, leaving low energy reactions for the remaining BTEX contaminants (Molson et al., 2002). This was demonstrated by Nelson et al who upon constant injection of ethanol into an aquifer, observed a drastic increase in methanogens after initial degradation of ethanol took place (Nelson, LaPara, & Novak, 2010).

The methods used to analyze this altered degradation in the presence of ethanol vary. Commonly natural attenuation is monitored in a system, where concentrations of remaining hydrocarbons and degradation products are measured and can be used to assess the occurrence of any degradation (Haggblom, Youngster, Somsamak, & Richnow, 2007). By measuring concentrations in an aquifer, MacKay et al found that the presence of ethanol allowed BTEX compounds to persist in groundwater during aerobic microbial degradation, yielding longer plumes (D. M. Mackay et al., 2006). A subsequent study using similar concentration measurements found that the presence of ethanol helped to facilitate the degradation of MTBE to TBA under sulfate reduction (D. Mackay et al., 2007). Similar studies have been conducted assessing ethanol's influence on BTEX compounds under various electron acceptor conditions by measuring hydrocarbon

concentrations in groundwater, both in experimental set ups and in the field (Corseuil et al., 1998; Powers et al., 2001; Corseuil et al., 2011). Microbial indicators are also useful to assess biodegradation of a contaminant plume. By assessing/studying the microbial community and any shifts or changes, the microbial interactions with the contaminants involved can be observed. Often biodegradation occurs by means of inducible enzymes, and therefore changes in gene expression can be traced by genetic analysis indicating metabolism shifts (Ma et al., 2013)(Jechalke et al., 2011)(Kao, Chen, Chen, Chien, & Chen, 2008). Genetic analysis in an aquifer system has determined shifts in microbial communities with the addition of ethanol to fuel contamination, leading to dominant low-energy methanogenic communities slowly degrading BTEX contamination once higher energy electron acceptors were depleted through ethanol breakdown (Feris et al., 2008). Ethanol has also been found to increase the size of a microbial community in a BTEX contaminated aquifer, with genetic analysis indicating a smaller proportion of these cells to be able to degrade BTEX, suggesting that ethanol degrading organisms are dominant (Da Silva & Alvarez, 2002). In some cases however ethanol has had a positive effect on degradation potential of contaminated groundwater. The prevalence of an optimal toluene degrading gene was found in groundwater contaminated by an underground storage tank, and in many experimental settings was enhanced by the presence of ethanol (Beller, Kane, Legler, & Alvarez, 2002).

Another way to identify the occurrence of biodegradation of organic contaminants is via carbon isotopic analysis. Carbon isotopes can be used to identify individual compounds and their sources within a groundwater contaminant plume. This approach

relies on the fact that in any given pool of carbon atoms, there is a combination of different carbon isotopes, which consists of approximately 99% ^{12}C and 1% ^{13}C (Farquhar, Ehleringer, & Hubick, 1989). When an organism assimilates carbon, such as CO_2 into plants during photosynthesis, the enzymes involved in the biochemical pathway will discriminate which isotopes of carbon are taken up based on the most efficient kinetic pathway(O'Leary, 1981; Hayes, 2001). This leads to specific stable isotope ratios in the organism's tissues based on the pathway of uptake (Farquhar et al., 1989). Plants with C_3, C_4, or CAM photosynthetic pathways will therefore exhibit characteristic ratios of $^{13}C/^{12}C$, or $\delta^{13}C$ (O'Leary, 1981). Ethanol is derived from corn, a C_4 plant which has a $\delta^{13}C$ signature of around -11‰ (Freitas, Fletcher, Aravena, & Barker, 2010; Koziet, Gross, Debry, & Royer, 1991). This is distinguishable from the petroleum derived signatures of BTEX compounds and MTBE, which both tend to have isotopic compositions originally derived from C3 photosynthesis and falling in the range of -24 to -34‰ (Fang et al., 2014).

In order to more precisely trace contaminant plumes and reactions therein, compound specific isotope analysis (CSIA) has been applied in variety of groundwater contaminant studies. When breaking down a carbon source, microbes will preferentially degrade the ^{12}C isotope, based on the energy required to break the bonds of the lighter carbon atom (Meckenstock et al., 1999;Morasch, Richnow, Schink, & Meckenstock, 2001; Slater, 2003; McKelvie, Mackay, de Sieyes, Lacrampe-Couloume, & Sherwood Lollar, 2007). This causes an isotopic shift for both the remaining pool of carbon and that which is taken up into the microbial cell. After being microbially biodegraded, the

remaining contaminant pool and degradation products will be depleted in light ^{12}C and enriched in ^{13}C. This phenomenon has been used to assess the degradation of hydrocarbon plumes in groundwater, where a shift to more isotopically depleted carbon can indicate microbial degradation (McKelvie et al., 2007; Richnow, Annweiler, Michaelis, & Meckenstock, 2003; Braeckevelt et al., 2012; Freitas et al., 2010; Bouchard et al., 2007). Microbial utilization of a particular carbon source can also be identified in microbial cellular components synthesized from a particular environmental carbon source. Phospholipid fatty acids (PLFA) are an integral membrane component in bacterial and eukaryotic cells synthesised using available carbon, and rapidly degraded upon cell death (Frostegård, Tunlid, & Bååth, 1993; Slater, White, Eglinton, & Reddy, 2005). Upon uptake of a carbon source, the $\delta^{13}C$ of PLFA tends to reflect the isotopic signature of its carbon source with a depletion in ^{13}C of typically 4 to 6‰ for aerobic heterotrophy though in some cases particularly for anaerobic pathways the fractionation can be larger (Hayes, 2001; Londry, Jahnke, & Des Marais, 2004). This method can provide insight into the active microbial community at the time of sampling, and the carbon sources being used for lipid synthesis within these cells. This approach has been used to demonstrate hydrocarbon degradation in an aquifer (Pelz, Chatzinotas, Zarda-Hess, Abraham, & Zeyer, 2001), but to date has not been used to assess preferential degradation of hydrocarbons within an ethanol blended fuel.

In this study microbial interactions with ethanol, MTBE, and toluene in groundwater were assessed in an artificial sand aquifer (figure). Specifically, biomarkers and cell count estimates from phospholipid fatty acids were used to assess microbial

growth, and isotopic analysis of microbial lipids and residual hydrocarbon pools were used to assess microbial uptake. Experiments were conducted in an instrumented pilot-scale laboratory tank simulating an unconfined homogeneous sand aquifer. This unique set up allowed multi-dimensional monitoring and sampling of hydrocarbon plumes as well as corresponding microbial biofilm collection representative of the aqueous microbial community.

3.2 METHODS

3.2.1 Aquifer Design

A pilot scale laboratory tank was used for this study as per McLeod et al. (Figure 1) (McLeod, Roy, & Smith, 2014). The tank, measuring 0.8m x 5.25m x 1.75m was packed with sterilized homogeneous BARCO M49 medium silica sand (Opta Minerals Inc., Waterdown, Ontario, Canada) simulating an unconfined aquifer. While saturating the aquifer, water was inoculated with a microbial community from Base Borden that had previously been exposed to petroleum hydrocarbon contamination. The water used in the system was de-chlorinated tap water, within which a concentrated nutrient stock of NH_4NO_3 and K_2HPO_4 was dissolved. Water was gravity fed into the head tank while maintaining a constant head boundary condition, and a constant withdrawal rate from the withdrawal well 520cm down-gradient allowed an average linear porewater velocity of 20.7 ± 1.8 cm/day. This is a typical rate found in a fast flowing homogenous sand aquifer. One full pore volume therefore passed approximately every 25.4 days.

Three fully screened injection wells located 30 cm down-gradient from the head tank (HT) released a mixed solution of dissolved ethanol (Commercial Alcohols), MTBE (Sigma Aldrich) and toluene (Fisher Commercial) were continuously injected into the aquifer at a constant rate of 2.3mL/min over 330 days. Hydrocarbon solution was prepared in 20L glass bottles every other day, at concentrations of 5g toluene per 20L H_2O, 5g MTBE per 20L H_2O, and 150g ethanol per 20L ethanol. As such, ethanol was injected at a concentration 40 times greater than toluene or MTBE.

Monitoring wells (MW) were made from screened 4 inch PVC pipes located at 115cm, 250cm, and 375cm down-gradient from the head tank. These MWs were instrumented and segmented using packers to prevent vertical mixing and atmospheric diffusion, and were capped when not being sampled. These wells also housed the biofilm collection units, described below. The withdrawal well (WW) centred at the end of the lane 520cm down-gradient from the HT, was a fully screened 4-inch PVC pipe. Biofilm collection units were also located inside the WW, however it was not instrumented or segemented, allowing free mixing within.

Sampling for this experiment took place during Stage 2 of the aquifer's total duration of operation, from days 165-300. At this time, notable biological clogging occurred at shallow depths on the injection well screens due to biofilm growth and/or microbial gas production. The hydrocarbon solution was therefore primarily entering the aquifer at deeper depths, predominantly below 75cm. Samples for this experiment

focused on depths 105cm, 135cm, and 165cm below the sand surface and were not affected by any lack of hydrocarbons.

3.2.2 Dissolved hydrocarbon and solute analysis

Aquifer groundwater samples were taken using designated HDPE 30 mL syringes connected to groundwater sampler consisting of a 3-inch stainless steel, 40 µm porous cup (Chand Eisenmann Metallurgical, Burlington, Connecticut, USA) and stainless steel tubing. Samples were taken from 105cm, 135cm, and 165cm depth at 5 transects located at 70cm, 160cm, 205cm, 320cm, 445cm down-gradient.

3.2.2.1 Hydrocarbon Concentrations

Water samples were collected in 20mL glass vials with mininert valves. Samples were eradicated of any headspace and treated with 0.1g of sodium bisulphate before being stored at 4^{o}C. Samples were analysed by Purge & Trap Gas Chromatograph Mass Spectrometry (P&T GC-MS) with a detection limit of 1.10µmol/L, 0.10µmol/L and 0.10µmol/L for ethanol, MTBE, and toluene respectively. Diluted samples were loaded onto a Tekmar 3100 Purge and Trap Concentrator, followed by separation with an Agilent 6890 Gas Chromatograph equipped with a DB624 capillary column, 0.25mm diameter, 1.4µm film thickness, 60m long. The temperature profile was 45^{o}C for 3 min, ramped to 90^{o}C at 8^{o}C/min followed by a 4 min hold, then ramped at 6^{o}C/min to 200^{o}C and held for 5 min. Compounds were identified by an Agilent 5973 Mass Spectrometer by retention time compared to known standards.

3.2.2.2 Anions

Major anions, specifically NO_3^- and SO_4^{2-}, were collected in 30mL groundwater samples using HDPE bottles filtered through a 0.20 um sterile syringe filter and stored at 4^{o}C. Samples were analysed by Ion Chromatography using a Dionex 2500 IC system. The IC was equipped with a Dionex AS18 column, 4mm x 250mm, fitted with a Dionex AG18 guard column, 4mm x 50mm, operating at a flow rate of 1.0mL/min at 30^{o}C. Eluent potassium hydroxide (KOH) was held at a concentration of 22mM for 5 min, followed by an increase to 50mM over 1 min and held for 10min. Detection limits for nitrate and sulfate were 1.10uM and 0.50uM respectively.

3.2.2.3 Methane

Methane (CH_4) was measured every 60days using Passive Gas (PG) samplers installed in the aquifer upon set up. The *PG* samplers were assembled using gastight glass syringes (500 µL; VICI-Valco Instruments, Brockville, Ontario, Canada) and 8.5 cm sections of gas-permeable silicone tubing (ID 1/8”). Following a sample loop rinse, the *PG* syringes were injected directly onto 8610C SRI Gas Chromatograph (GC) using a 10 port switching valve for sequential analysis with a thermal conductivity detector (TCD), and a helium ionization detector (HID). A silica gel packed column (6-ft long) in series with a mesh column (6-ft long x 1/18” Molecular Sieve 13X 80/100) were used with helium at a carrier gas flow rate of 20 mL/min. The instrument was held for 3 minutes at 30°C, then increased at a rate of 20°C/min to 40°C for 1 minute, followed by a ramp at

8°C/min to 120°C and held for 0.1 minute and then increasing at 40°C/min to 150°C for 5 minutes.

3.2.3 Hydrocarbon ^{13}C analysis

30mL groundwater samples were inserted into 60mL glass vials prepared with mercuric chloride ($HgCl_2$) to prevent any biological growth or fractionation. Vials were sealed and left to equilibrate. Toluene and MTBE ^{13}C were analyzed from sample headspace in triplicate on a gas chromatography-isotope ratio mass spectrometry (GC-IRMS) system. This consisted of a Agilent 6890 gas-chromatograph interfaced to a Finnigan-MAT DeltaPlus via a GC Combustion III interface. The GC was equipped with a Agilent DB-5 MS column, 30m x 0.32mm with a film thickness of 0.25µm. Samples were injected manually at 45°C.

The temperature profile for hydrocarbon isotopic analaysis was 80 C held for 1 minute, ramp to 280 C at 4 C per minute, ramp to 320 C at 10 C per minute with a final hold for 15 minutes.

3.2.4 Isotope enrichment calculations

Using the Rayleigh equation, $R/R_0 = f^{(\alpha-1)}$ and the approach of Mariotti et al (Mariotti et al., 1981) and Slater (G. Slater, 2003), an isotopic fractionation factor was determined for each sample based on the stable isotope signature of the hydrocarbon plume in the aquifer as a function of its concentration as compared to the initial injected concentration and isotopic signature. The equation:

$$(\alpha - 1)lnf = ln\left(\frac{\delta^{13}C_r/1000) + 1}{\delta^{13}C_{ro}/1000) + 1}\right)$$

where $\delta^{13}C_r$=the isotopic composition of hydrocarbon the plume at a given loation in the aquifer, $\delta^{13}C_{ro}$=the initial isotopic composition of the hydrocarbon in the aquifer, f=fraction of the initial hydrocarbon concentration remaining, α=fractionation factor which can then be converted to ε in ‰ using the equation ε =1000(α-1). Calculation of the theoretical isotopic signature based on fraction remaining using established fractionation factors indicating microbial biodegradation, can be done according to $\delta^{13}C_r = \delta^{13}C_{ro} + \varepsilon lnf$ (G. Slater, 2003).

3.2.5 Microbial biofilm collection

Biofilm collection units were used to collect microbial biofilm from the aquifer's groundwater. Porous Teflon tubes 31cm x 3cm diameter with 0.6cm diameter holes, were packed with combusted glass wool and suspended in the monitoring wells at approximately 135cm depth. Units were suspended for approximately 1 month periods before being removed from the monitoring well and stored at -20 C until laboratory analysis. Biofilm measurements began at day 165 and were collected until the end of the experiment, on day 300.

3.2.6 Microbial PLFA analysis

Microbial biofilm was extracted using a modified Bligh and Dyer method (Bligh & Dyer, 1959). Biofilm collection units were extracted using 2:1 DCM:Methanol, the extract was then filtered through burned glass fiber filters to remove particles (GF/G,

Whatman). Organic extracts were separated by polarity into three fractions; non-polar, neutral and polar (F1: DCM, F2: acetone, F3: methanol) by silica gel chromatography using combusted and activated silica gel. Phospholipids from the methanol fraction were transesterified to fatty acid methyl esters (FAMEs) by mild alkaline methanolysis(White, Davis, Nickels, King, & Bobbie, 1979). The FAMEs are assumed to be from PLFA exclusively as the methanolysis hyrdrolizes and methylates only esterified fatty acids within the mixture(White et al., 1979). These were then further purified by a secondary silica gel chromatography to ensure no other carbon compounds are present in this fraction by selecting for a limited polarity range. The FAMEs were then analyzed by GC/MS. PLFA are identified based on comparison to bacterial reference standards (Bacterial Acid Methyl Esters CP, Mix, Matreya Inc, Supelco 37 Component FAME Mix), as well as retention times and mass fragmentation patterns. Designation of PLFA is according to number of carbon atoms in the chain and the number of double bonds. Prefixes are representative of structural isomers (br-methyl branching; i-iso; a-anteiso; cyc-cyclopropyl).

3.2.7 GC/MS analysis of PLFA

An Agilent 6890 GC coupled to a 5973 quadruple mass spectrometer was used for identification and quantification of PLFA FAMEs. The GC was equipped with a 30m x 0.32mm DB-5 MS column with a 0.25μm film thickness. The temperature program was 50 C for 1 minute, then ramped to 130 C at 20 C per minute, ramp to 160 C at 4 C per minute the ramped to 300 C at 8 C per minute with a final hold for 5 minutes.

3.3.5 PLFA ^{13}C analysis

$\delta^{13}C$ analysis on individual PLFAs and was performed. Specific PLFA analyzed ranged from 13 to 19 carbons long depending on the sample and available carbon mass. PLFA was obtained from the FAMEs extracted during PLFA analysis from extracted biofilm. PLFA were identified as FAMES on GC/MS prior to isotopic analysis. The $\delta^{13}C$ values were corrected for added methyl groups, a known incorporated methanol $\delta^{13}C$ value of -41.95‰ based on isotopic mass balance using a correction factor (Abraham, Hesse, & Pelz, 1998), yielding an accurate isotopic ratio of the PLFA specifically.

3.3 RESULTS

3.3.1 Nutrient concentrations

For the purposes of this study and in order to correspond with the depths of microbial sampling, species concentrations are reported for 135 cm depths. Dissolved nitrate (NO_3^-), sulfate (SO_4^{2-}), and methane (CH_4) were measured at all depths along the distance of the aquifer (Figure 2).

Nitrate was added into the aquifer's head tank at a concentration of approximately 740µmol/L, while sulfate was added at 310µmol/L. Nitrate rapidly decreased in concentration with distance down-gradient, often below the detection limit of 1µmol/L by the second sampling transect at 155cm down-gradient. Notable spikes in concentration were detection at the furthest distance down-gradient on days 165 and 265, measuring approximately 12µmol/L.

Sulfate decreased in concentration with distance down-gradient, however this decrease was much more gradual than that observed for nitrate. Sulfate did not become fully depleted at any point during the experiment, but did frequently reach low concentrations beyond 205cm sampling transect, with values as low as 1.81μmol/L at the furthest distance down-gradient.

Conversely, methane concentrations steadily increased in the aquifer with both distance down-gradient and time. With no detectable methane in the head tank or often at 115cm down-gradient, methane concentrations increased gradually, reaching up to 943μmol/L on day 300 at 445cm down-gradient.

3.3.2 Hydrocarbon concentrations

Beginning on day 165, ethanol was measured in the injection well (IW) at a concentration of 46088 μmol/L. The concentration steadily decreased with distance down gradient dropping to a final concentration between 3235 μmol/L at 445cm down gradient and 105cm depth to complete depletion and a concentration below detection on several sampling days at various depths of the 320cm and 445cm down-gradient transects (figure 3.3). These concentration drops corresponded to a range of 93% to 100% mass loss. There was no consistent trend between ethanol concentration and sampling day at any given transect, with concentrations at any given sampling point remaining fairly consistent over time.

MTBE was measured at 1153μmol/L in the IW on day 165. A general decrease in concentration over distance down-gradient was apparent, although less drastic than that

seen with ethanol. MTBE was never fully depleted in the aquifer, decreasing to a minimum of 180 μmol/L or approximately 85% mass loss (figure 3.3).

Toluene had an IW concentration of 842 μmol/L on day 165, and generally decreased in concentration with distance down-gradient. At no point did toluene become fully depleted, however reached concentrations as low as 37.23μmol/L at 445cm down-gradient, and 7.4μmol/L at 320cm down-gradient, or 95.58% and 99.12% mass loss, respectively (figure 3.3).

3.3.3 Stable isotope analysis of dissolved hydrocarbons

Water samples taken at 105cm, 135cm, and 165cm depth, corresponding to depth of collected biofilm for PLFA analysis (approximately 135cm) were analysed for $\delta^{13}C$ values of dissolved toluene and MTBE. Toluene and MTBE added to the aquifer were characterized to have an initial of $\delta^{13}C$= -28.4‰ and $\delta^{13}C$= -31.5‰ respectively. Water collected on day 185 showed some variation in toluene $\delta^{13}C$ with distance down-gradient and depth. Remaining toluene was enriched in ^{13}C at 105cm depth by as much as 2.9‰ with respect to the initial value for toluene. A slight enrichment in ^{13}C compared to the injected toluene was apparent beginning at 160cm down-gradient, measured at approximately -27‰ to -28‰ for 135cm depth and was consistent along the remaining length of the aquifer. Water taken from 165cm depth did not show enrichment outside of error of the initial toluene value. Water sampled on day 230 displayed higher consistency among sample depths and distances down-gradient, with all samples between -27.7‰ and -26.8‰. On day 265 there was a slight trend of more enriched $\delta^{13}C$ toluene values further

downgradient for all 3 depths, with the closest transect (70cm down-gradient) displaying values between -27.9‰ and -27.5‰ and the farthest between -27.4‰ and -26.5‰. Day 300, the final day of the experiment saw no trend with distance. The shallower 105cm depth however seemed to be the least enriched with generally within error of toluene's initial value. The deeper depths had values between -27.9‰ and -27.0‰.

Stable carbon isotope characterization of residual MTBE in the water samples were more variable over time compared to toluene $\delta^{13}C$ values, and showed no clear trend with depth. The initial injected MTBE $\delta^{13}C$ was -31.5‰, samples from the aquifer where enriched from this value more frequently than toluene. Samples from day 185 were consistent between 135cm and 165cm depth, with values between –31.8‰ and -29.6‰, while samples taken at 105cm depth spanned between -33.6‰ and-26.6‰. Day 230 had high similarity between samples at all three depths and all distances down-gradient, with and overall range between -31.3‰ and -29.7‰. Values displayed a slighter wider range on day 265, between -32.0‰ and -28.7‰, an enrichment in ^{13}C has large as 3.3‰. Finally day 300 saw variability among sampling locations, with a general enrichment in ^{13}C with distance down-gradient for all three depths. Samples taken here saw the largest enrichment with $\delta^{13}C$ values as high as -20.9‰ at 445cm down-gradient form 105cm deep, 10.2‰ more positive than the injected MTBE.

3.3.4 Microbial PLFA and cell abundances

The extent of microbial community growth on the biofilm units as indicated by PLFA abundances was assessed at intervals of approximately 1 month for four

consecutive months starting on day 165 and ending on day 300. Microbial cell abundances per biofilm unit were estimated using generic conversion factors of $2x10^4$cells $pmol^{-1}$ PLFA (Green & Scow, 2000). A general decreasing trend in bacterial cell abundances was seen both over time in each sampling well and with distance from source (figure 4). The opposite trend was evident in the withdrawal well (520cm down-gradient), where cell densities were consistently higher than in the monitoring wells, and tended to increase over time. Overall, biofilm collected from the head tank (0cm down-gradient) has very low PLFA abundances, the lowest measured throughout the duration of the experiment.

3.3.5 PLFA ^{13}C analysis

Stable carbon isotopic compositions ($\delta^{13}C$) of microbial PLFA extracted from biofilm units were highly similar between all sampling wells, with the exception of biofilm collected between days 185 and 230 at the furthest distance down-gradient (figure 5). The majority of PLFA $\delta^{13}C$ samples had a range of -10‰ to -18‰, while biofilm from WW between days 185 and 230 were largely within a -21‰ to -25‰ range. Samples collected between days 165 and 185 saw a high similarity between all sampling wells, with most PLFA within a $\delta^{13}C$ signature range from -10‰ to -18‰. PLFA extracted the following month, between day 185 and 230 saw a similar pattern with a high density of samples between -10‰ and -16‰. A significant depletion in samples collected from the withdrawal well (520cm down-gradient) was evident in these samples with clustering between -21‰ and -25‰, but overall samples from other locations had values as positive as -14‰. Days 230 to 265 had the majority of PLFA samples between

-10‰ and -20‰. There was no clear trend between distance down-gradient and $\delta^{13}C$ signature and no longer a prominent depletion, with samples from the withdrawal well returning to less negative values. The $\delta^{13}C$ values measured between days 265 and 300 were highly similar among all distances down-gradient, with little variation in $\delta^{13}C$ of the same FAMEs from different sampling points.

3.4 DISCUSSION

3.4.1 Dissolved hydrocarbon degradation

Concentrations of dissolved ethanol, MTBE and toluene in the groundwater demonstrate clear mass loss, which can be attributed to biodegradation. Because the system is operating at a constant flow rate and initial contaminant concentrations are stable the observed consistency in the pattern of concentrations of all 3 hydrocarbons with distance down-gradient over all sampling times indicates that the system is in a pseudo-steady state, and decreases in hydrocarbon concentrations are due to consistence and predictable processes competing at a consistent rate with hydrocarbon transport. In this case, the evidence points to microbial uptake. Despite the presence of ethanol, the more labile carbon source, all three hydrocarbons are degrading simultaneously, consistent with previous observations in groundwater systems contaminated with ethanol blended fuels (D. Mackay et al., 2007; Beller et al., 2002) .

Because groundwater flow rates in the system were constant, transport distance down-gradient can be converted into a time over which biodegradation has occurred for

any given parcel of water. Using the change in concentration at any sampling point and converting distance to a time in days enabled calculation of a pseudo-first order rate constant for biodegradation at each depth for each sampling time point based on the rate law equation of a first order reaction

$\frac{[A]}{[A_0]} = e^{-kt}$, where A = hydrocarbon concentration at a given time and location, A_0 = initial hydrocarbon concentration as measured in the injection well, t= time in days of contaminant flow calculated by $t = \frac{distance\ down{-}gradient}{aqueous\ flow\ rate}$, and k = rate constant. For each data set, the data fit the first order rate law very well, with the majority of data sets demonstrating an R^2 value of 0.60 to 0.99 (figure 3.6). Exceptions exist in the lower 165cm depths with toluene and MTBE which had depletion curves poorly fitting the first order rate equation due to largely consistent concentrations over time indicating that no degradation was occurring. These values were therefore removed from the overall rate constant data. The degradation rate constants (*k*) determined for each sampling day at a specific depth show a high degree of similarity over time throughout this phase of the experiment, between day 165 and 300 (table 1). The highly similar degradation rates over time for all three hydrocarbons dissolved in the aquifer confirm that the system is in a pseudo-steady state. This can be further demonstrated by the consistency in half-lives of the hydrocarbons, which at depth 135cm were averaged at 2.0±0.3 days, 2.2±0.2 days, and 3.7±0.7 days for ethanol, MTBE and toluene, respectively. As this phase of the experiment begins 165 days after the initial release of hydrocarbons to the aquifer, the

fact that the system has reached pseudo steady state with respect to hydrocarbon degradation is a reasonable finding.

3.4.2 Isotopic evidence of biodegradation of dissolved hydrocarbons

Isotopic enrichment in the $\delta^{13}C$ of remaining toluene and MTBE pools at various times and locations in the aquifer indicate the occurrence of biodegradation of these contaminants. It has been established that there are no significant isotope fractionation effects due to physical processes such as dissolution or evaporation, or abiotic degradation of BTEX compounds (Thullner, Centler, Richnow, & Fischer, 2012; Slater, Ahad, Sherwood Lollar, Allen-King, & Sleep, 2000; Slater, Dempster, Sherwood Lollar, & Ahad, 1999). The shifts in $\delta^{13}C$ seen in the dissolved toluene can therefore be attributed to biological fractionation. Dissolved toluene measured in groundwater was enriched in ^{13}C compared to injected toluene in almost all analyzed samples. The magnitude of this enrichment, up to 2.9‰ seen on day 185, is comparable to previous laboratory experiments measuring toluene fractionation with biodegradation (Richnow et al., 2003; Ahad et al., 2000; Vieth et al., 2005), however some studies have found much larger enrichments during toluene biodegradation, up to approximately 10‰ (Richnow et al., 2003; Meckenstock et al., 1999; Steinbach, Seifert, Annweiler, & Michaelis, 2004). The most significant enrichment is seen on day 185, when nitrate and sulfate are mostly depleted and methane is beginning to increase in concentration indicating methanogenic conditions.

Calculated isotopic enrichment factors were further used to assess the degree of biodegradation involved in the demonstrated carbon isotope fractionation, using the Rayleigh equation and initial concentrations measured in the injection well at the given time point. Measured toluene isotopic data had a mean enrichment factor of ε=-0.7 (stdev=0.22). Enrichment factors associated with toluene biodegradation reported in the literature vary, depending on factors such the substrates and metabolisms involved in the degradation, as well as the specific organisms undergoing the reaction (Richnow et al., 2003; Meckenstock et al., 1999; Ahad et al., 2000). Enrichment factors for toluene degradation outside of 0.5‰ error from the initial toluene value were compared to a theoretical value of -1.1‰, although theoretical and observed values can reach up to -2‰ (figure 7) (Steinbach et al., 2004; Richnow et al., 2003;Meckenstock et al., 1999). Calculated toluene enrichment factors falling on the line of a theoretical ε=-1.1‰ are assumed to be fractionating according to biological mechanisms with that enrichment factor. While not all data points agree, a large group of toluene fractionation factors fall on or close to this biological fractionation line. Ahad et al. (Ahad et al., 2000) found toluene biodegradation to have smaller enrichment factors of ε=-0.5 to -0.8 under anaerobic conditions. Plotting a line through the average overall toluene isotope data yields a slope confirming the enrichment factor of approximately 0.7, within the range found by Ahad et al., particularly close to the value found during sulfate reduction of MTBE, approximately -0.8. Within an error of 0.5‰ , the large majority of toluene data points fall on this line. This is strong evidence to suggest that the loss of toluene is caused biodegradation by microbial communities within the aquifer.

Biodegradation of MTBE has also been shown to involve a significant isotopic fractionation(Thullner et al., 2012; Zwank et al., 2005; Youngster, Rosell, Richnow, & Häggblom, 2010; Kuder et al., 2005; Kolhatkar, Kuder, Philp, Allen, & Wilson, 2002). Isotopic enrichments seen between days 185 and 265 reached as high as 5.4‰, however for the most part were within a 2‰ enrichment range, which is comparable to previous groundwater studies (Thornton et al., 2011; Lesser, Spinnler, Johnson, & Aravena, 2005). Isotopic fractionation of MTBE due to biodegradation can however be much greater, ^{13}C enrichments on the scale of 20‰ or more (Zwank et al., 2005; Kolhatkar et al., 2002). In this experiment large fractionation of remaining MTBE were only detected on day 300, the final sampling period. At this time enrichments were generally between approximately 4.3‰ and 11.1‰, suggesting a significant biological fractionation of residual MTBE.

MTBE's relationships between isotope enrichment and dissolved concentration in the groundwater can also be explained by biological carbon isotope fractionation, however displays a slightly different pattern. Enrichment factors calculated from MTBE isotopic signatures were highly variable, as are the reported values of MTBE biodegradation which encompass a wide range from approximately -1.5‰ to -13‰ (Hunkeler, Butler, Aravena, & Barker, 2001; Thullner et al., 2012; Kolhatkar et al., 2002; Zwank et al., 2005; Kuder et al., 2005; Youngster et al., 2010). Of note, the lowest point in this range (-1.5‰) reported by Hunkeler et al (Hunkeler et al., 2001) was generated for aquifer material originating from Base Borden, the same source as this system. Comparing the MTBE data outside of error of the initial MTBE value to a curve plotted

from an enrichment factor of -1.5, the majority of data from sampling days 165 to 265 are clustered on or near that line. This suggests that biodegradation of MTBE is occurring within the aquifer and fractionating the contaminant at a similar value to previous Base Borden communities. On day 300 however there is much more considerable fractionation of MTBE, with fractionation factors approaching the mid-range literature value of -7, especially towards the portion of the curve where a larger fraction of MTBE is remaining. This change in MTBE enrichment factors is indicative of a shift in the system, and since it affects fractionation during degradation, it likely indicates a change in the degradative pathway. However, a lack of significant change in the degradation rate (*k*) would indicate that the new pathway is working at approximately the same rate. A notable difference in data collected on day 300 is the drastic increase in methane concentration, reaching up to 943 umol/L. This generation of a large concentration of methane may be associated with a shift in metabolism used to degrade MTBE, such as methanogenic communities. Previous research, however, has found that methanogens are not efficient in MTBE degradation (Chen, Kao, Chen, Weng, & Tsai, 2006).

3.4.3 Measurements of viable biomass

The presence of PLFA on all biofilm units demonstrated the active growth of microbial communities over each month that the units were installed. However, since the mass and composition of the units are not comparable to the sand of the aquifer matrix, these cannot be converted into in situ cell abundances. Nonetheless the calculated cell abundances indicate the development of significant microbial biofilms on all collection units. It was notable that the extent of biomass growth per month decreased over the

course of the experiment. This may be related to changing redox conditions in the aquifer reflecting shifts in the microbial community.

3.4.4 Microbial metabolisms and redox zones

Nutrient concentrations indicated that microbial metabolisms within the aquifer were utilizing primarily sulphate reducing and methanogenic pathways. Nitrate was depleted to below detection prior to the first sampling point (70 cm down-gradient) indicating nitrate reduction was occurring in the region of the system closest to the HT. Microbial nitrate reduction is known to be capable of metabolizing ethanol and toluene (Zhang, Khan, Chen, & Spalding, 2006; Powers et al., 2001) and this rapid depletion of nitrate has been modelled in groundwater systems impacted by ethanol(Jin & Roden, 2011). The width of the nitrate reduction zone could only be constrained to be less than 70cm.

Sulfate was also rapidly depleted indicating the occurrence of sulfate reduction by the microbial community. Unlike nitrate however, sulfate persisted at low levels throughout much of the aquifer over time, as far as the furthest sampling point (445cm down-gradient) on all sampling dates. This suggests the potential for sulfate reduction to occur throughout the aquifer, over the entire duration of the experiment. Unfortunately, sulphide was not measured to confirm the occurrence or extent of this pathway. However, as noted above the enrichment factors calculated for toluene degradation agree quite closely to those found by Ahad et al. (Ahad et al., 2000) in sulfate reducing conditions, consistent with the concept that sulfate reduction was a consistent metabolism for

toluene degradation throughout the system supporting the decrease in sulfate but lack of complete depletion.

Methane was observed to increase with distance down-gradient and time, demonstrating increasingly methanogenic conditions in the aquifer. The production of large concentrations of methane, evident beginning on day 130, is indication of a transition to methanogenic conditions in the system. Methanogens are archaeal organisms, and therefore the increase in methanogens signals a population shift in the aquifer (Nelson et al., 2010; Feris et al., 2008). Based on the initial methane increase at the far end of the aquifer, and gradually reaching closer to the HT, it is evident that methanogenesis is most active furthest from the hydrocarbon source, while higher energy metabolisms such as denitrification are closer to the source. This is typical of an anoxic groundwater system, where microbial metabolisms can display energetic zonation, usually with that of lowest energy furthest from the source (D. R. Lovley, Chapelle, & Woodward, 1994; Bethke, Sanford, Kirk, Jin, & Flynn, 2011). As methanogens are archaea which due to a different lipid membrane structure archaea do not have PLFA and are therefore not included in this PLFA analysis (Corcelli, Chong, & Koga, 2012), an increasingly methanogenic microbial community would explain the decreasing trend in PLFA concentrations observed over the course of the experiment. An exception to this decreasing trend is evident in the withdrawal well (WW, 520cm down-gradient), where cells generally increase overtime, as well as a general increase compared to other well locations. The different structure of the WW may be contributing to this microbial

difference, as there are no packers to prevent air diffusion into the aquifer in this region or prevent mixing among the water column.

3.4.5 PLFA $\delta^{13}C$ indicating microbial carbon sources

The $\delta^{13}C$ signatures of microbial PLFA are indicative of microbial carbon sources and biodegradation. Across all sampling dates, almost all PLFA demonstrated an isotopic signature consistent with a predominant use of ethanol derived carbon, a range between -10 and -16‰ accounting for a 0 to 6‰ depletion during conversion to PLFA.

While most PLFA are in a range consistent with the utilization of ethanol, there are some specific trends within or near that range evident among specific fatty acids. The i15:0 fatty acid, associated with gram positive bacteria and anaerobic metabolisms (Fang, Hasiotis, Gupta, Brake, & Bazylinski, 2007) is consistently depleted in ^{13}C compared to much of the remaining PLFA. Considering the consistently anaerobic conditions throughout the aquifer, it is likely that anaerobic metabolisms associated with these organisms (possessing i15:0) are active. In contrast, cyc17:0 fatty acids tend to be slighty enriched in ^{13}C versus other fatty acids detected, as high as -7‰ seen in biofilm collected between days 230 and 265. As a cyclopropyl fatty acid, cyc17:0 is considered to belong to gram-negative bacteria, and often associated with sulfate reducing bacteria (Fang et al., 2007; Fang & Barcelona, 1998; Pelz, Chatzinotas, Andersen, et al., 2001). Given the evidence for sulfate reducing conditions and a major metabolism contributing to MTBE degradation in the system, when incorporated into PLFA, MTBE would show a more depleted $\delta^{13}C$ signature than ethanol use, of approximately -25 to -36‰. It is therefore

unclear why this lipid in particular tends to have a less negative $\delta^{13}C$ value, however such values have been seen before in over PLFA samples under sulfate reducing conditions (Londry et al., 2004).

Biofilm collected between days 185 and 230 displayed the only group of $\delta^{13}C$ depleted PLFA that would be indicative of microbial metabolism of toluene or MTBE. These values were as negative as -25.7‰, however were not consistently depleted for all fatty acids, suggesting multiple metabolisms occurring simultaneously within the aquifer using more than one carbon source. Some of the detected lipids were therefore synthesized from degraded ethanol and incorporating that isotopic signature, while others were from degraded toluene and MTBE causing the observation of different signatures in various lipids collected from different sampling points. All of these highly depleted fatty acids from this sampling period were found in the withdrawal well located 520cm down-gradient. Conditions here differ slightly as there are consistently higher concentrations of methane generation, as well as a non-instrumented or segmented (no packers) well, potentially allowing for mixing of water throughout the water column. Water at this location however was consistently anoxic.

Despite ethanol reaching depletion at the further distances of the aquifer, there is no strong evidence from the $\delta^{13}C_{PLFA}$ of a shift to a different carbon source, as biofilm from almost all distances along the aquifer maintain the isotopic signature characteristic of ethanol uptake, with the exception of the anomaly between days 185 and 230 as discussed above. The lack of evidence of incorporation of MTBE and toluene carbon into

the lipids may be due to several factors. Previous research tracking ^{13}C labelled carbon incorporation into PLFA in soil has found remaining signal as long as 1 year after exposure to plant derived organics (such as corn) (Tavi et al., 2013). This may be due to the recycling of ethanol derived products (Harvey, Fallon, & Patton, 1989), such as acetogenesis using CO_2 originated from the breakdown of ethanol therefore presenting a continuous ethanol isotopic range. The large difference between inputted ethanol concentrations and MTBE and toluene may simply indicate that the overwhelming process is ethanol degradation simply due to volume. Ethanol was added at a concentration 40 times greater than the contaminants, which means that it was the most readily available hydrocarbon, in addition to being highly labile and kinetically favourable to uptake. The lack of evident signal in the PLFA indicating toluene or MTBE biodegradation should not be taken to mean that these compounds were not being biodegraded by the microorganisms in the aquifer, as it is highly likely that the associated carbon isotope signature has been diluted due to the large volume of ethanol being degraded.

Overall the dominant $\delta^{13}C$ signature consistent with ethanol uptake is strong indication that ^{13}C signatures within microbial PLFA can be used to identify the uptake of dominant carbon sources. The fact that in this study, $\delta^{13}C$ of PLFA was more effective to identify ethanol metabolisms than MTBE and toluene may be a product of the drastically different ratio at which these hydrocarbons were injected. As the practical application of this study would be use in investigation of ethanol blended fuel spills, it remains to be discovered if a more realistic ratio of hydrocarbons, such as 10% by volume ethanol in an

E10 mixture, would demonstrate a more holistic picture of all carbon sources used in microbial metabolism.

Clear isotopic enrichment in remaining hydrocarbon plumes with fractionation factors agreeing with those previously observed for both toluene and MTBE biological uptake is evidence supporting the microbial biodegradation of the contaminants present. The pseudo-steady state achieved in the system, as demonstrated by largely consistent degradation rates over time, supports the conclusion loss of hydrocarbon mass is caused by biological activity as opposed to random physical processes.

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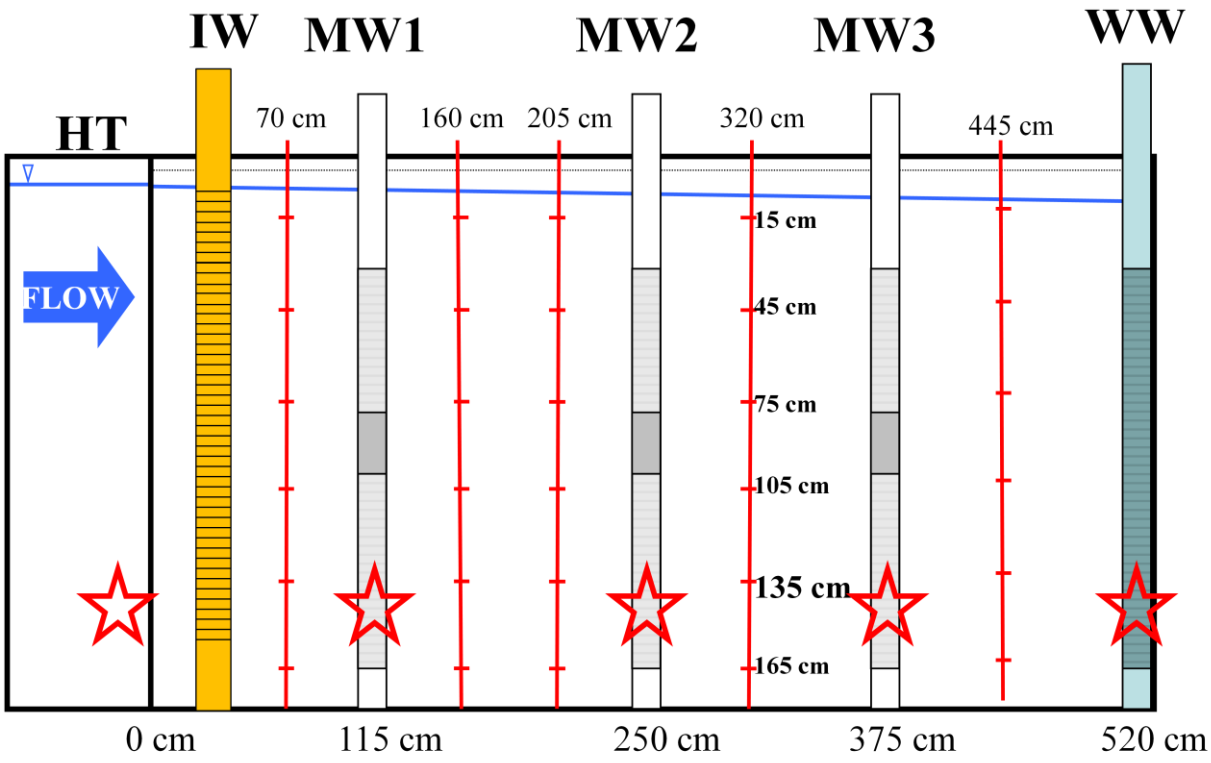

Figure 3.1. Side view diagram of aquifer set up. Stars indicate locations of biofilm collection units during the experimental period. Red lines indicate water sampling ports. HT=Head Tank, IW=Injection Well, MW=Monitoring Well, WW=Withdrawal Well.

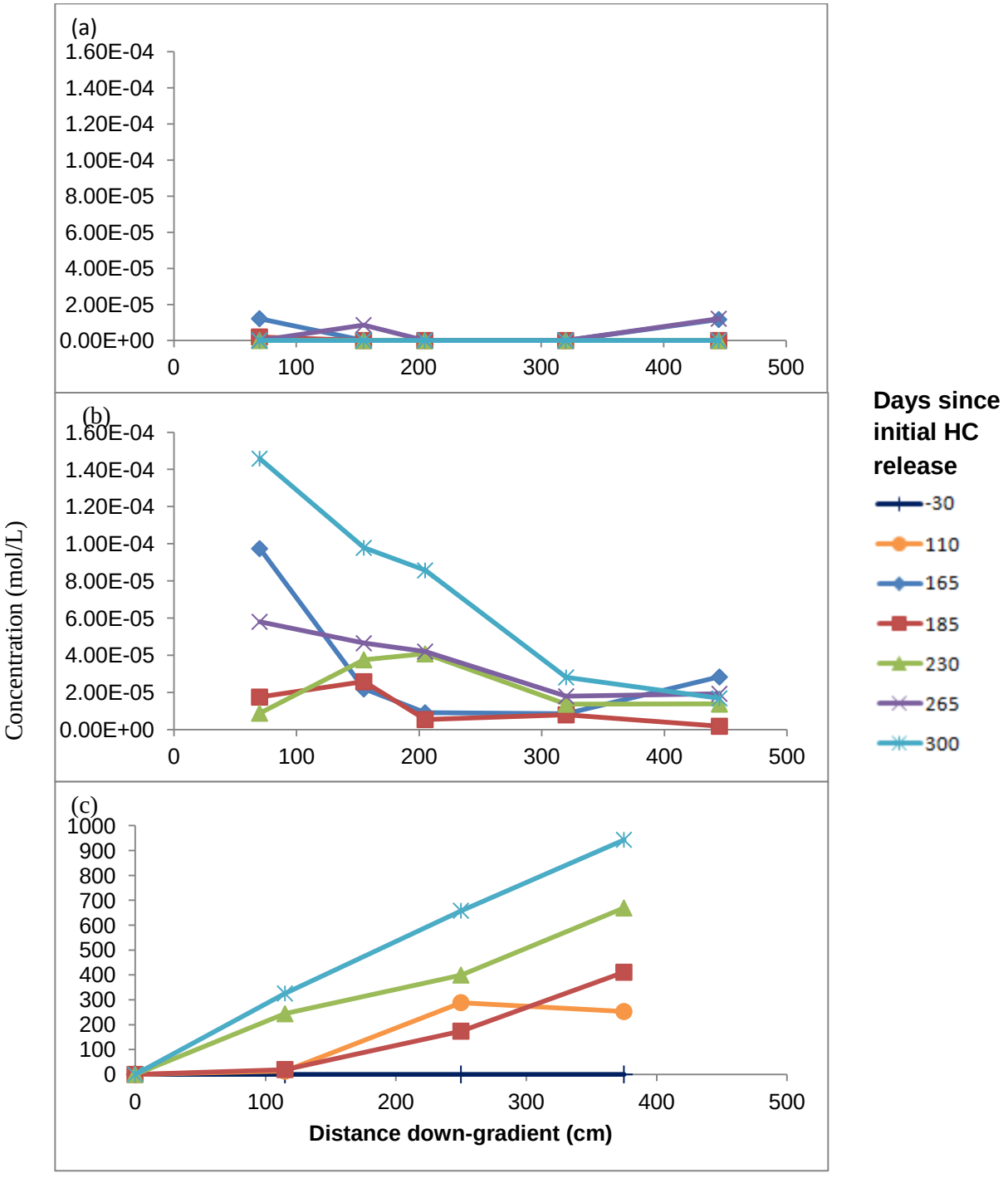

Figure 3.2. Analyte concentrations of (a) nitrate NO_3^-, (b)sulfate SO_4^{2-} and (c)methane CH_4. Groundwater samples (NO_3^- and SO_4^{2-}) were taken from 135cm belfow sand surface, and passive gas samples (CH_4) taken from 130cm below sand surface.

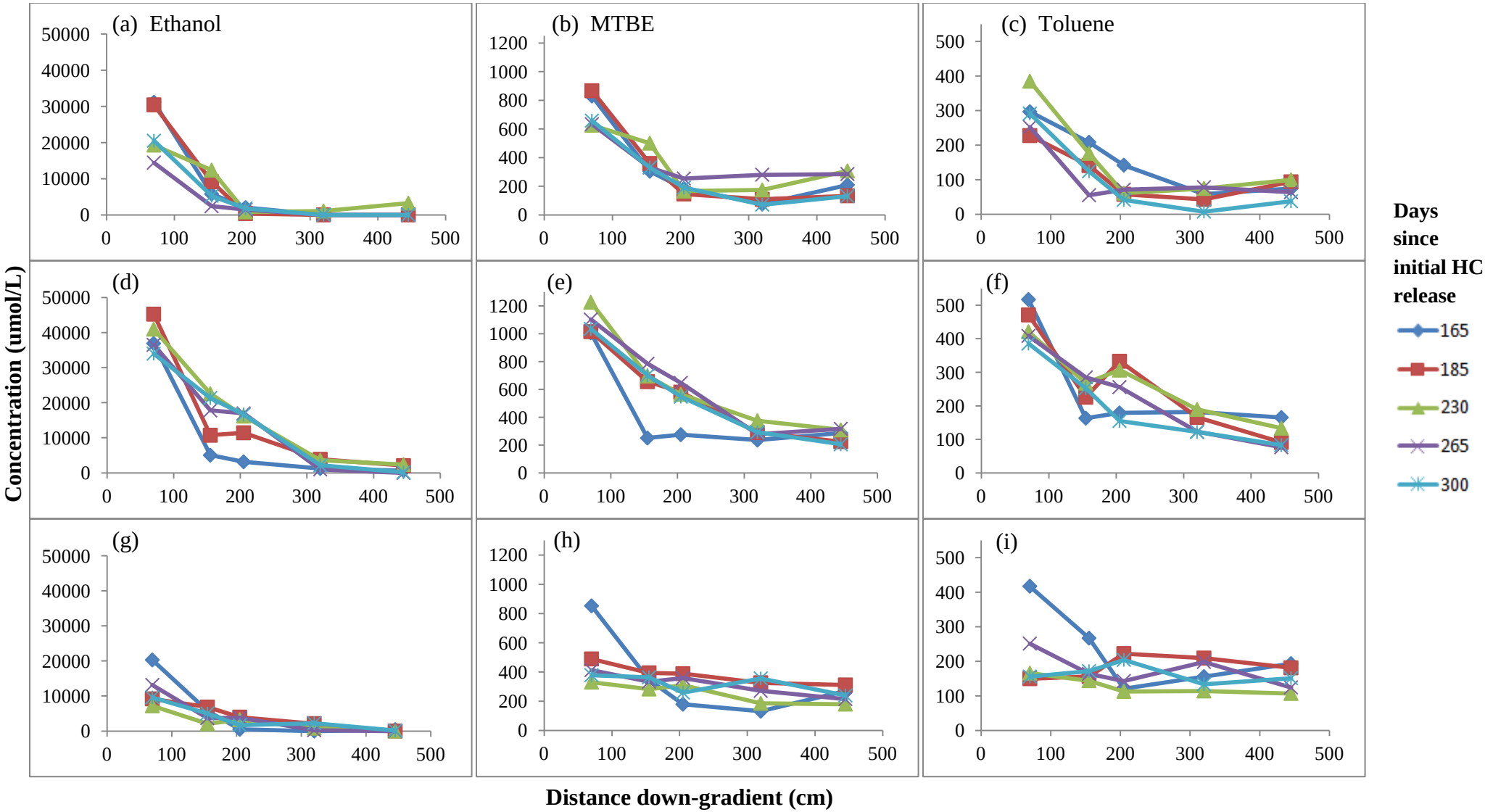

Figure 3.3. Dissolved hydrocarbon concentrations at 3 lower depths of the aquifer. Ethanol concentrations over time at depths (a)105cm, (d)135cm, and (g)165cm, MTBE concentrations at depths (b)105cm, (e)135cm, and (h)165cm, and toluene concentrations at depths (c)105cm, (f)135cm, and (i)165cm are shown. All concentrations demonstrate consistent depletion with distance and stable values over time. Note that all three hydrocarbons are on different concentration scales.

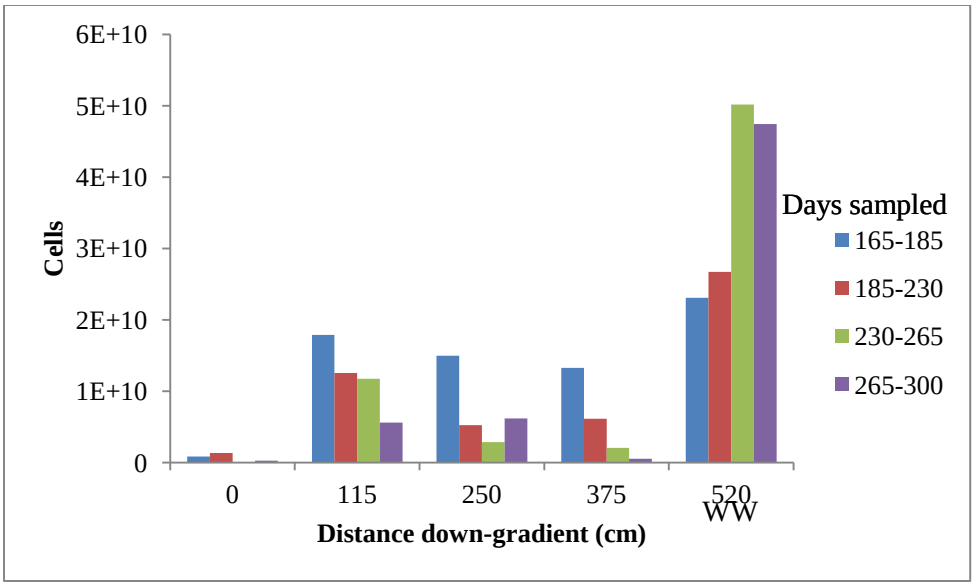

Figure 3.4. Bacterial cells per biofilm collection unit based on PLFA extraction of biofilm accumulated over approximately 1 month periods. WW = Withdrawal well.

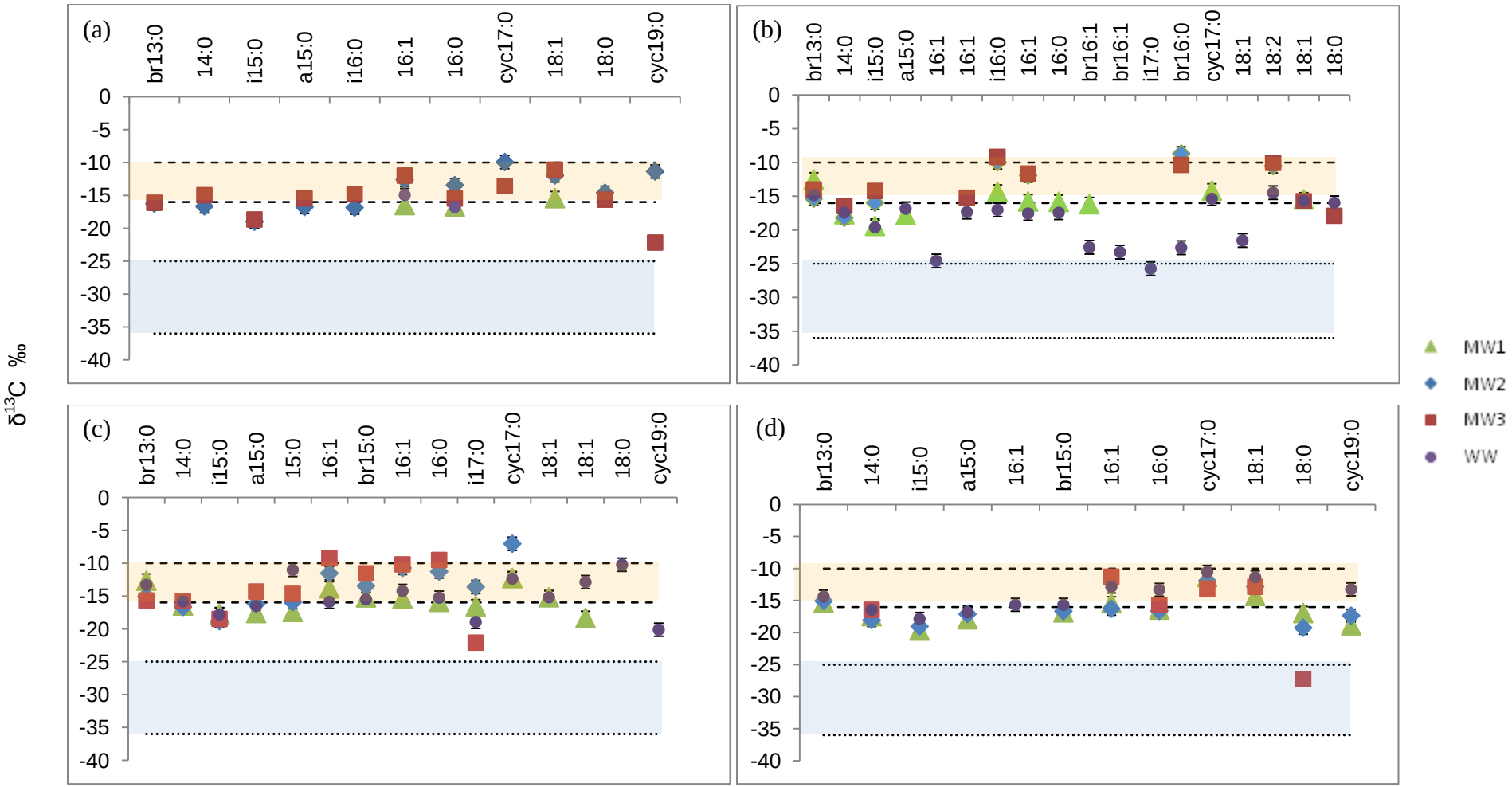

Figure 3.5 PLFA $\delta^{13}C$ signatures measured from biofilm collected from the aquifer system during (a) days 165-185 (b) days 185-230 (c)days 230-265 (d) days 265-300. Orange shaded area represents isotopic range consistent with ethanol uptake, blue shaded area indicates range consistent with toluene or MTBE uptake. Both ranges account for depletion associated with PLFA synthesis.

Table 3.1 Rate constants *k* for the degradation of ethanol, MTBE and toluene per sampling day for depths (a)105cm , (b)135cm, and (c) 165cm below the sand surface. Rate constants were determined by plotting A/Ao vs time, were the slope of the line is equal to *–k*, as outlined in the rate law equation. Note that values obtained from curves with an R^2 less than 0.6 have been removed.

(a)

Day	Ethanol	MTBE	Toluene
165	0.364	0.316	0.160
185	0.306	0.287	0.097
210	0.228	0.175	0.086
230	0.151	0.124	0.163
265	0.108	0.114	0.107
300	0.190	0.227	0.194
Mean	0.225	0.207	0.135
St dev	0.096	0.084	0.044

(b)

Day	Ethanol	MTBE	Toluene
165	0.415	0.330	0.211
185	0.414	0.307	0.215
210	0.374	0.335	0.144
230	0.334	0.281	0.150
265	0.289	0.286	0.212
300	0.329	0.340	0.218
Mean	0.359	0.313	0.192
St dev	0.051	0.026	0.035

(c)

Day	Ethanol	MTBE	Toluene
165	0.239	0.298	0.160
185	0.092	0.068	
210	0.087		
230	0.057	0.049	0.033
265	0.100	0.062	
300	0.084		
Mean	0.110	0.119	0.097
St dev	0.065	0.103	0.064

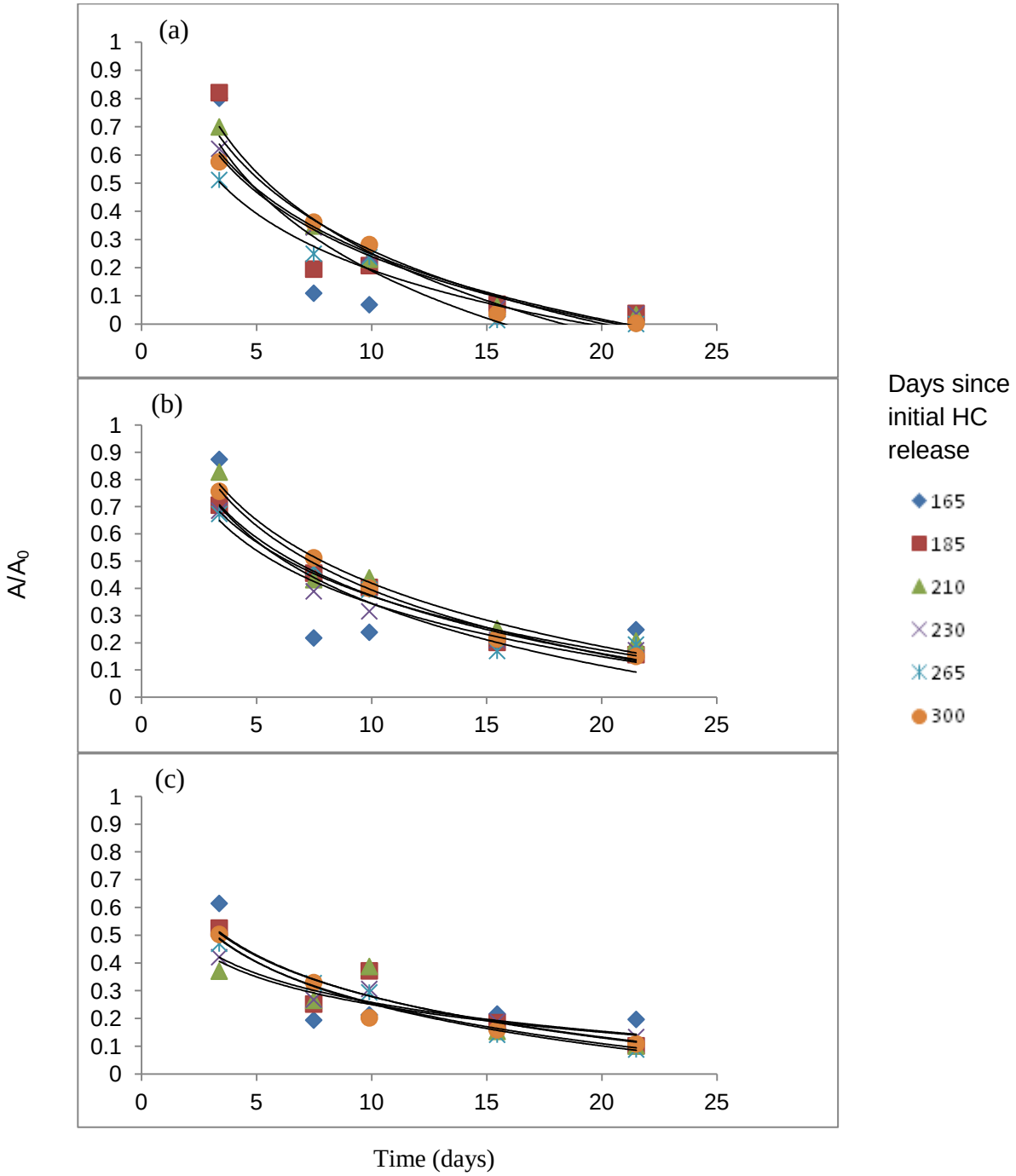

Figure 3.6. (a)Ethanol, (b)MTBE, and (c)toluene degradation rates from data collected 135cm below the sand surface, where the slope of the trendline is equal to the first order rate constant *k*. Average R^2 values of trendlines are 0.92, 0.92 and 0.84, while average half-lives are 2.0, 2.2, and 3.7 days for ethanol, MTBE, and toluene, respectively.

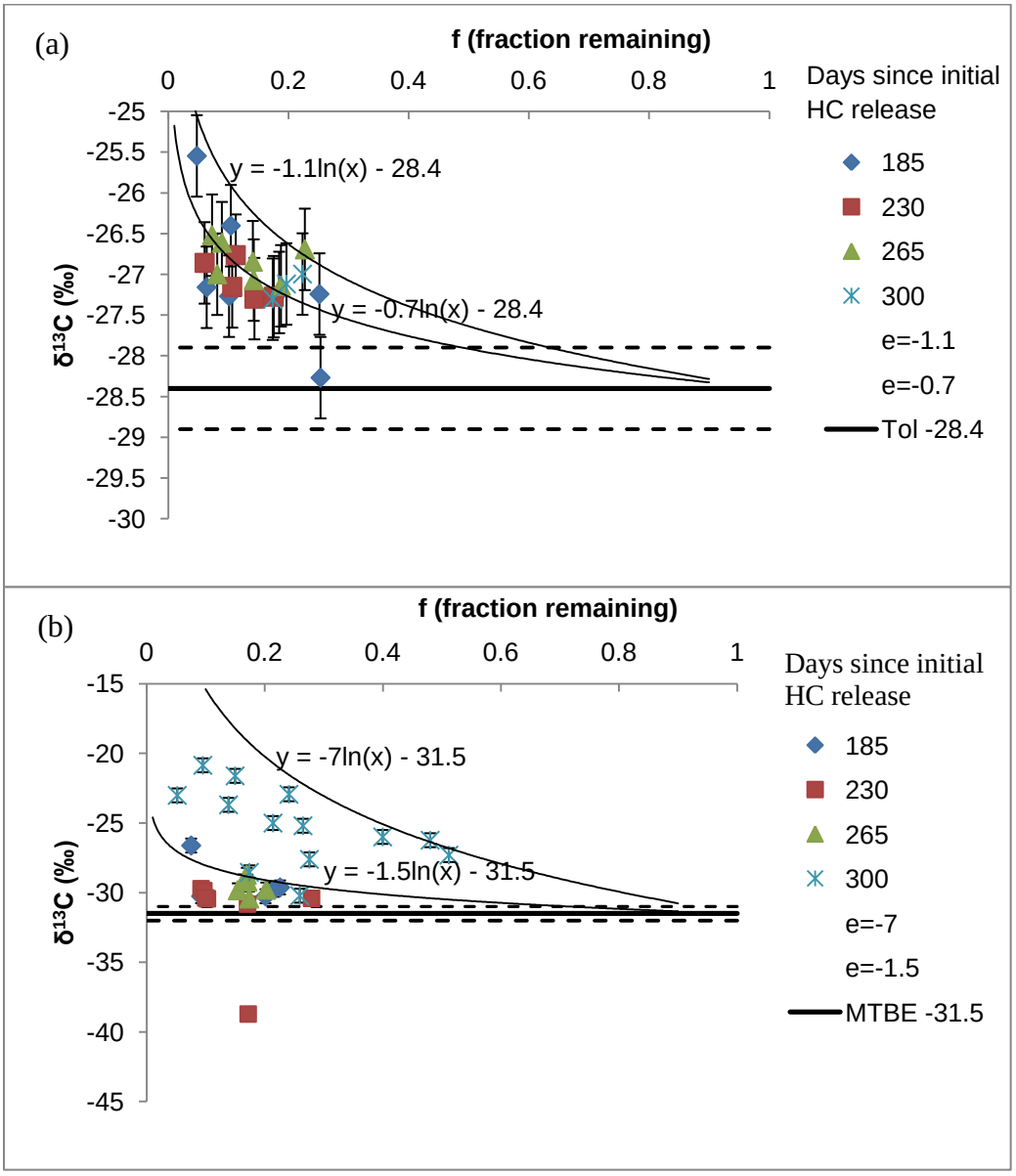

Figure 3.7. (a)Toluene and (b) MTBE ^{13}C enrichment plotted per sampling day. Literature values of ε=-1.1‰ for toluene and ε=-1.5‰ ND ε=-7.0‰ for MTBE are plotted as curves (Youngster et al., 2010)(Hunkeler et al., 2001)(Steinbach et al., 2004). Initial isotopic values for injected toluene and MTBE are marked in a solid black line, with ±0.5‰ error denoted by dashed lines. Note that values within error of these injected hydrocarbon values were removed from the graph, as they show no enrichment.

CHAPTER 4:

CONCLUSIONS

4.1 RESEARCH SUMMARY

This dissertation examined the fates of petroleum hydrocarbons in different environments when released in a spill scenario. Physical transport and behavior as well as interaction with biota and microorganisms were studied under two different environmental conditions. In addition, the effect of confounding factors, specifically the presence of additional non-petroleum compounds, was assessed for the effect on contaminant fate and microbial interaction as well as effectiveness of techniques used for this study. Overall this thesis has elucidated some notable results regarding fate and source of petroleum hydrocarbons and their behaviour under certain environmental conditions, as well as raised questions and brought forth areas of future research to better understand this important topic.

The ubiquitous release of petroleum products into the environment is a serious concern for both human and environmental health. The persistence of toxic and carcinogenic petroleum hydrocarbons creates a need to understand the fate and behaviour of these compounds in the environment in order to better comprehend and predict areas and materials of concern after chronic contaminant releases such as underground storage tanks or factory release, or acute releases from spills and environmental disasters such the Deepwater Horizon oil spill or many other large scale accidents. Contaminants can be transported and redistributed in the environment depending on a variety on chemical, physical, and environmental factors. Determining the fate of

contaminants is crucial to understanding how to effectively remediate them. An additional fate involves the interaction with microbiology. Microbial biodegradation is a naturally occurring process by which microorganisms break down contaminants into less harmful constituents, obtaining energy for their metabolism. The presence of a microbial community at a petroleum contaminated site does not directly indicate biodegradation, and specific analyses are therefore used to assess the potential and presence of this process. This dissertation sought to explore these issues of petroleum hydrocarbon fate and transport in two different sites.

In the first study, the fate of petroleum hydrocarbons in Barataria Bay sediments after the Deepwater Horizon oil spill was assessed to determine whether the erosion of coastal sediments into the bay or sinking of incoming contamination was causing hydrocarbon deposition to the surface sediments. This addressed the issue of contaminant movement and whether observed decreases in salt marsh petroleum concentrations were actually due to degradation or were a result of movement to another compartment, such as nearby sediments, where they could represent an ongoing risk. While there was a low concentration of petroleum residues such as PAHs, UCM, and alkanes in the Barataria Bay sediments investigated, the specific signatures of these compounds were not indicative of oil sourced from the spill. Stable carbon isotope signatures of n-alkanes along with alkane chain length patterns indicated terrestrially sourced alkanes from nearby plant material with some inputs from petroleum. This may include contamination from shipping traffic, factory outputs, or outflow from the nearby Mississippi River bringing with it hydrocarbons from a variety of sources, however there is no indication of petroleum from the Deepwater Horizon spill. As such, erosion was not the primary driver into these sediments. Analysis of microbial PLFA indicated that there were active microorganisms in the bay sediments at the time of sampling. Based on the various PLFA detected there was a

diverse microbial community, typical of marine sediments. Stable carbon isotope analysis was used to indicate carbon sources used by these microorganisms. The overall $\delta^{13}C$ range of sediment PLFA was not indicative of uptake of oil from the Deepwater Horizon spill, however was consistent with the incorporation of other petroleum inputs or nearby plant sourced carbon.

In the second study, petroleum hydrocarbons toluene and MTBE released in conjunction with ethanol in a pilot scale experimental aquifer were monitored from 165 to 300 days after initial release into the system. Ethanol is now a commonly used oxygenate present in most gasoline. As ethanol is a labile and highly degradable carbon source to microbial communities, the question of whether it would be degraded preferentially, and the implications of this to the fate of toluene and MTBE was the key focus of this study. Several factors indicated that biological activity was responsible for the degradation of all of these hydrocarbons in the system. A pseudo-steady state was achieved by day 165 in the aquifer, as demonstrated by consistent concentrations of all three compounds on a given time and sampling location. This was supported by highly similar degradation rate constants, and consistently high R^2 values for the rate equation curves generating these constants. The use of stable carbon isotope analysis further supported biodegradation as the sole process contributing to hydrocarbon mass loss. Dissolved hydrocarbons remaining in the groundwater were observed to be consistently enriched in ^{13}C indicating biological fractionation had taken place. Calculated enrichment factors matched previously reported values for both toluene and MTBE biodegradation, suggesting all mass loss was due to biodegradation. An evident shift in metabolism or carbon source became apparent in the MTBE fractionation factors measured on day 300. This was likely associated with the generation of high concentrations of methane at this time and may be due to a shift to a more dominant archaeal community.

Analysis of microbial biofilms growing on units installed in the aquifer was also used to assess biodegradation in the system. Using PLFA to estimate bacterial cell abundances, the presence of active bacterial communities was confirmed. Isotopic analysis of PLFA was used to assess carbon sources utilized by the bacteria to synthesize these lipids. The majority of PLFA extracted throughout the experiment were consistent with utilization of ethanol as the primary carbon source. Some data was indicative of other carbon sources such as MTBE and toluene, however the dominant signal was that of ethanol. As there is evidence of biological fractionation and degradation of both MTBE and toluene, it is likely that the lack of isotopic signal preserved in the bacterial lipids is a result of the drastically higher proportion of ethanol present in the system compared to the other hydrocarbons.

4.2 FUTURE WORK

The results of this study answered some questions regarding the fates of petroleum hydrocarbons, but raised further questions that should be addressed. In order to gain a better understanding of processes contributing to the degradation of petroleum hydrocarbons in the environment, analysis of archaea must be done. PLFA are found in bacteria and eukarya, therefore providing information about the metabolisms associated with these organisms (Green & Scow, 2000). Due to the different lipid structure in archaeal microorganisms, a different extraction and analysis must be employed to develop a more complete picture of microbial processes (Corcelli, Chong, & Koga, 2012). Archaea are known to have petroleum degrading abilities and have been found to contribute to petroleum degradation in systems similar to what was studied for this dissertation (Feris et al., 2008). The results observed in chapter 3 also point to a dominant archaeal community with the production of methane and decrease in bacterial cell abundance. In order to confirm this, the study of archaeal organisms is necessary. A similar lipid

extraction method to that used for PLFA analysis would make the use of carbon isotope analysis possible, and would allow for a more complete 3 domain assessment of microbial communities present as well as what carbon is being used for their metabolism and biodegradation.

Ethanol blended fuels are widely available and commonly used around the world. These fuels are often E5 to E10 in North America which meet requirements set by the U.S. Clean Air Act, meaning that ethanol is present as 5% by volume in E5 or 10% in E10 (Ulrich, 1999; Powers et al., 2001). In this study, ethanol was added to the aquifer system at a concentration 40 times greater than that of toluene or MTBE. This affected the results by likely masking any indication of toluene or MTBE uptake for the synthesis of PLFA, making it unclear if this method would be effective for these compounds. In order to assess whether the use of stable carbon isotope analysis in microbial PLFA would be effective to indicate biological degradation of petroleum hydrocarbons in the presence of ethanol, a ratio of these compounds which is more representative of blends used in North America must be used.

The use of carbon isotopes to identify origins of detected hydrocarbons as well as carbons sources used for lipid synthesis in microorganisms was effective throughout both of these studies. In some scenarios, such as the technique used in chapter 3 to differentiate between corn ethanol and petroleum compounds, stable carbon isotope analysis can be a clear and useful tool. However, the similarity between $\delta^{13}C$ signatures of modern plants, specifically those with a C3 photosynthetic pathway, and ancient petroleum makes distinguishing between carbon sources somewhat difficult, as was the case in chapter 2. The addition of radiocarbon analysis, $\Delta^{14}C$ can constrain ancient carbon versus modern carbon using the half-life of this unstable isotope (Hayes, 2001). Like stable carbon isotope analysis, radiocarbon can be used to identify carbon sources both in samples where petroleum may be present, or microbial lipids to identify uptake

during metabolic activity (Pearson, McNichol, Benitez-Nelson, Hayes, & Eglinton, 2001; Slater, White, Eglinton, & Reddy, 2005; Reddy et al., 2002).

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