MICROBIAL COMMUNITY RESPONSE DURING OIL SANDS RECLAMATION

INVESTIGATION OF MICROBIAL COMMUNITY RESPONSE DURING OIL SANDS RECLAMATION VIA LIPID AND CARBON ISOTOPE ANALYSES

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A Thesis Submitted to the School of Graduate Studies in

Partial Fulfilment of the Requirements for the Degree

Master Of Science

McMaster Unversity

MASTER OF SCIENCE (2014) McMaster University

Earth and Environmental Sciences Hamilton, Ontario

TITLE: Investigation of Microbial Community Response during Oil sands Reclamation via Lipid and Carbon Isotope Analyses

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NUMBER OF PAGES: x, 95

ABSTRACT

In this study, phospholipid fatty acids (PLFA) and carbon isotopes were used to characterize the response of *in situ* microbial communities to a pilot-scale wetland reclamation project in the Alberta oil sands, and to investigate their role in carbon cycling at the reclamation site. The Sandhill Fen reclamation project in the Athabasca oil sands region (Fort McMurray, Alberta, Canada) has created an artificial freshwater fen typical of the boreal forest region in which the oil sands occur. At this site, composite tailings (CT) residue was overlain with a thick sand cap and a freshwater fen constructed on top. Biomass in the peat material of the fen was comparable to that found in natural fens, and a comparison of PLFA profiles in peat, CT from a nearby site, and undisturbed wetlands in the area showed that microbial communities in Sandhill fen were more similar to those in the CT than those in undisturbed wetlands. Bacteria dominated the biomass, including a small percentage of sulphate reducing bacteria that are of particular interest in the reclamation project. Fungi and other eukaryotes were also present. Analyses of radiocarbon in total organic carbon (TOC) and residue from solvent extraction suggest that there was petroleum present in the peat layer of the fen. A small amount of young carbon from the fen surface has been transported into the CT layer in the form of dissolved organic carbon. Radiocarbon also showed that microbes preferentially metabolized more modern carbon within the carbon sources available to them. Biomass was more related to the age of carbon in the samples than to the TOC concentration, with younger carbon in the peat associated with higher PLFA concentration.

ACKNOWLEDGEMENTS

There are so many people whose encouragement and direction have played a big role in my journey through graduate school. First of all, I am grateful to my supervisor, Dr. Greg Slater, for his guidance, support, and optimism throughout my Master's program, especially in the face of my occasional pessimism. I am so grateful for the opportunity to do such cool science with someone who is not only an expert in his field, but also so invested in his students' success.

Thank you to the members of the Environmental Organic Geochemistry group, particularly my officemates Sam, Kelly, Mark, Margot, Danielle and Sarah, without whom my journey through this Master's degree would likely have been a bit more efficient but much less fun. All of us are deeply indebted to Jennie Kirby, without whom I am confident the entire lab would go up in flames quite quickly. Thanks to Dr. Lori Ziolkowski for training me in the laboratory and her work on this research project. Thanks to Dr. Allyson Brady for encouragement, help in the laboratory, and use of her Kingfisher CT data which appears in this thesis. A big thank you to the members of the Warren lab, who braved Fort McMurray to bring back the samples studied in this thesis, and especially to Michelle, Tara, and Katie. Thanks to Dr. Warren for all her work connecting with Syncrude, collecting samples, and providing guidance in my project and in the overarching Sandhill Fen project of which my work is a part.

My lab work and writing would never have been completed without the unwavering support of my family, who have never had a moment of doubt about my abilities and intelligence, even when I did. My boyfriend, David, has been my cheerleader throughout the past two years, and I am so grateful for his support and steady insistence that everything would work out well. Thank you to the members of my informal lunch club/trivia team for all the fun conversation and the chance to complain about grad school life without shame. The women of the WISE committee have been a great community, and I am so happy to have had the chance to work with a group that I really believe in. I'm proud of what we've accomplished together these past two years. Whatever sanity I've maintained I owe in part to my

Wednesday night violin group and the Hammer Hoppers dance club, who gave me the opportunity to think about and accomplish things outside the realm of science. Last but not at all least, thank you to my roommate Robyn, who heard about my every triumph, failure, and petty complaint over the course of this degree and without whom I would never have survived the last two years.

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1.1 Oil Sands Overview

The oil sands of northern Alberta, Canada, are one of the largest reserves of oil in the world, containing an estimated 1.7 trillion barrels of oil (Masliyah et al., 2004). This supply is composed of bituminous sands that underlie approximately 140 000km^2 of land in the Athabasca, Cold Lake, and Peace River regions of Alberta. The Canadian Association of Petroleum Producers (CAPP) report that the oil sands produced 1.9 million barrels/day (b/d) in 2013, and forecast production of 4.8 million b/d by 2030 (Canadian Association of Petroleum Producers, 2014). During surface mining, vegetation, hydrological features, and overburden are removed (Price et al., 2010) and the oil sands are scooped into large trucks to be transported to processing plants. Bitumen is extracted from the sands using the Clark Hot Water Extraction process (Masliyah et al., 2004), which requires 3m^3 of water and results in the production of 4m^3 of tailings for every 1m^3 of bitumen recovered (Holowenko et al., 2000).

1.2 Tailings Production and Management

Tailings are a slurry of water, sand, silt, residual bitumen, and residual solvents from the extraction process. Alberta's zero-discharge policy states that waste must be stored on-site, which has led to the formation of large tailings ponds in mined-out areas or above-ground containment units. Syncrude reported a total stored volume of 6.3x10^8 m^3 of tailings fluid in 2010 (Syncrude Canada Ltd., 2010). There are a number of environmental concerns associated with the tailings ponds, such as gas emissions, possible leaching of contaminants into nearby groundwater, and hazards to wildlife.

Fluid in the ponds is gradually densified by gravity into a thick slurry called mature fine tailings (MFT), releasing water which is removed for re-use in bitumen extraction (Masliyah et al., 2004). The natural rate of sedimentation and consolidation is slow, with estimates of 125-150 years for consolidation of fines (Eckert et al., 1996). Production of methane gas by methanogenic microbial consortia in the Mildred Lake

Settling Basin was found to speed up the densification rate (Fedorak et al., 2003), increasing the rate of pore water release, reducing the volume of tailings on-site, and enabling reclamation to take place sooner (Penner and Foght, 2010). However, methane is a greenhouse gas with 34 times the effect on global temperature as an equivalent mass of CO_2 over a 100 year period (Myhre and H. Zhang, 2013), so encouragement of methane production to increase densification rate is not ideal. An alternative method of tailings management currently in use is the transformation of fluid fine tailings into composite tailings by the addition of sand and gypsum ($CaSO_4 \cdot 2H_2O$) (Matthews et al., 2002). This technique was successful in field trials in 1995, and Syncrude began commercial scale use in 2000 (Matthews et al., 2002). Gypsum acts as a coagulant, causing the aggregation of colloidal particles, producing a non-segregating mixture and promoting rapid dewatering within a few hours (List and Lord, 1997). Water released is recycled back into the bitumen extraction process, and the non-segregating CT mixture is solid enough to be used as a base for land reclamation.

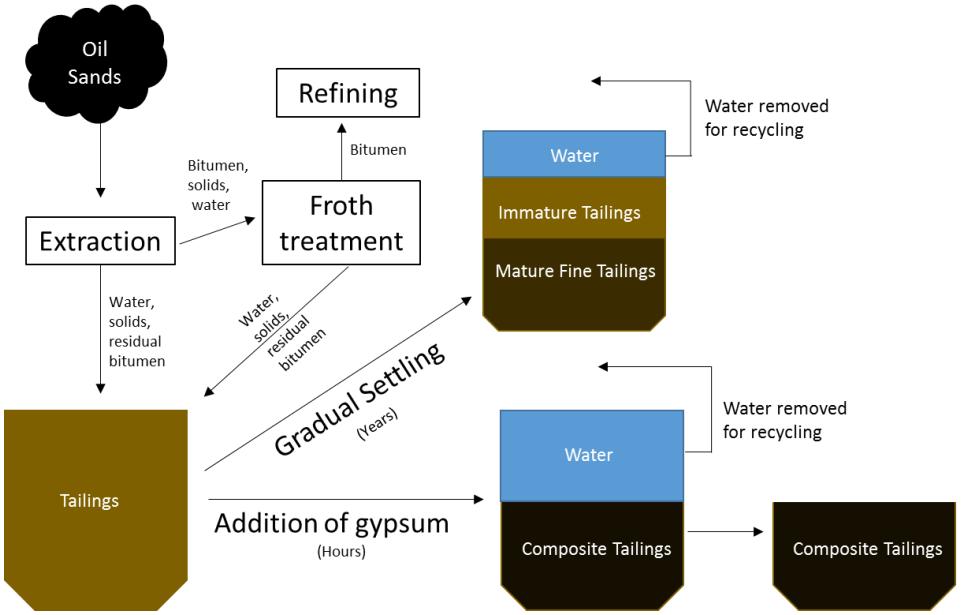

Figure 1.1: An overview of the production of tailings during bitumen extraction, and two methods of tailings densification: gradual settling and production of composite tailings by addition of gypsum and sand.

1.3 Microbes in tailings

Tailings quickly become anoxic with depth, so that the majority of microbial metabolism relies on alternate terminal electron acceptors (TEAs) with lower redox potential than oxygen. Sulphate reducing bacteria (SRB) and methanogens are common constituents of tailings (Penner and Foght, 2010). Aliphatic alkanes and some components of BTEX (benzene, toluene, ethylbenzene and xylenes) provide a labile carbon source to drive microbial metabolism (Siddique et al., 2006; Siddique et al., 2007). Methanogens are restricted to only a few substrates, specifically hydrogen, acetate, and carbon dioxide, thus they rely on syntrophic microbial communities to break down other carbon sources (Stasik and Wendt-Potthoff, 2014). Sulphate reduction provides more free energy than methanogenesis, so SRB are able to out-compete methanogens given sufficient sulphate concentrations (Holowenko et al., 2000).

1.4 Land Reclamation

In 2009, the Alberta Energy Resources Conservation Board (ERCB) issued a directive requiring that the oil sands industry find ways to shrink their tailings inventories and develop viable reclamation strategies for the land disturbed by industry activities (Energy Resources Conservation Board, 2009). About 50% of the land leased to oil sands companies is classified as wetland (Kuhry et al., 1993), mainly fens. Wetlands are defined as "areas where the land is saturated with water for long enough periods to support wet-adapted processes and plants, including hydrophytic vegetation" (Cumulative Environmental Management Association, 2007). Fens are a class of minerotrophic wetland, meaning that they are fed by mineral-rich surface or groundwater, as opposed to ombrotrophic wetlands like bogs which rely on precipitation. They are divided into rich or poor depending on water chemistry and vegetation, wherein a poor fen is more acidic and hosts a smaller variety of vegetation than a rich fen (Cumulative Environmental Management Association, 2007). Peatlands, of which fens are one class, take thousands of years to develop naturally (Price et al., 2010) and thus present a challenge for reclamation. Restoration, which is fixing wetlands

that have been damaged, is far more common than reclamation, which is required where the original wetland has been completely destroyed (Cumulative Environmental Management Association, 2007). Restoration of disturbed fens has taken place (for example, Cooper and MacDonald (2000), Cobbaert et al. (2004)), but there are no published cases of fen creation after complete destruction by industrial processes. Beginning in 2009, Syncrude began reclamation of a fen wetland atop a composite tailings deposit, called Sandhill Fen. The adjacent Kingfisher Fen area acts as an un-reclaimed control. The location of these sites can be seen in Figure 1.2.

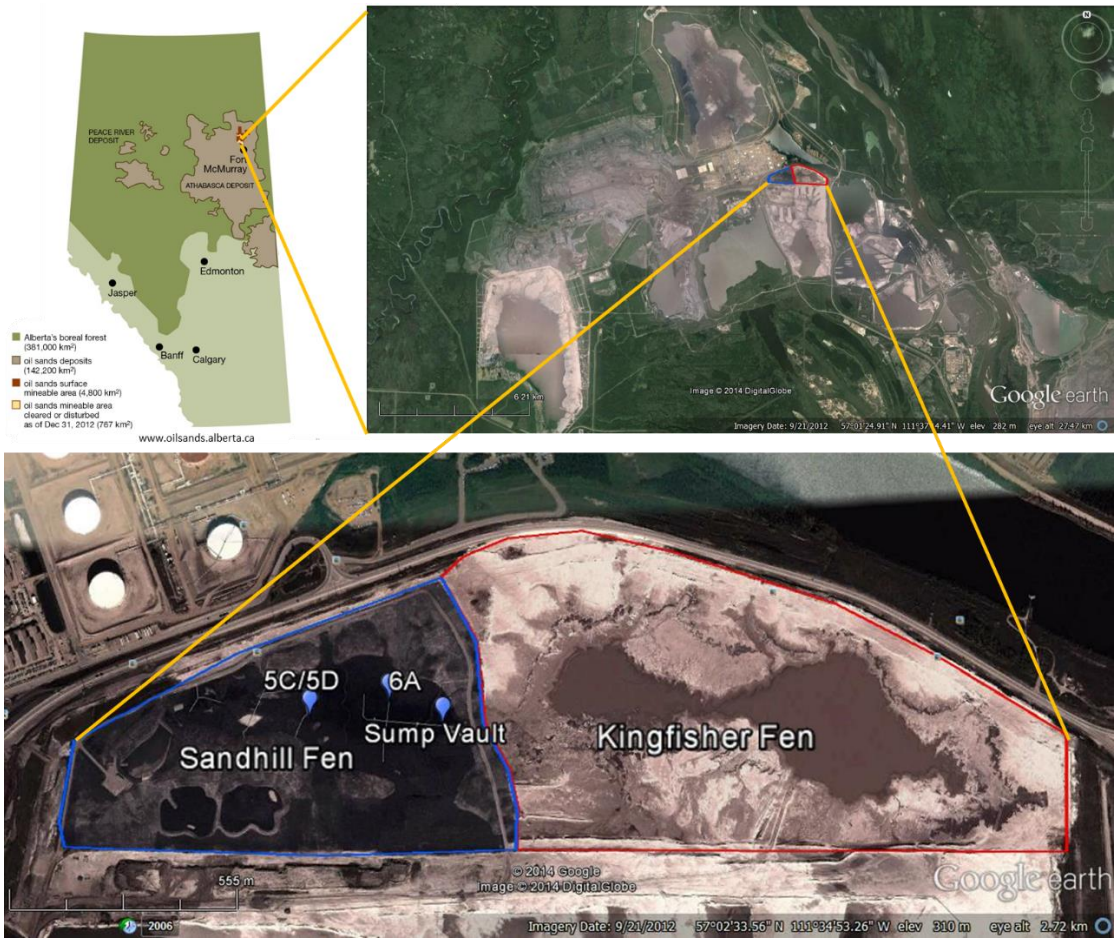

Figure 1.2: The location of the Sandhill fen reclamation site and adjacent Kingfisher Fen CT deposit. Locations of sampling sites 6A, 5C/5D, and sump vault (SV) are shown with blue markers. Images from www.oilsands.alberta.ca and Google Earth.

1.5 Sandhill Fen

Syncrude's stated goals with Sandhill Fen are to "ensure the final reclaimed landscape has capability equivalent to that existing prior to development, is integrated with the surrounding area, establishes boreal forest upland and lowland communities, yields water suitable for return to the natural environment, and is planned in direct consultation with local, directly affected stakeholders" (Syncrude Canada Ltd., 2012). Sandhill Fen is a 52ha area located in Syncrude's East In Pit, an area actively mined between 1977-1999 which has since been used as a storage pit for CT and tailings sand (Wytrykush et al., 2012). Beginning in 2009, ten metres of tailings sand was laid atop the ~35m of CT in this area, with 0.5m of clay till and 0.5m of recently salvaged peat material placed on top in 2011-2012 and seeded with fen-type vegetation (Wytrykush et al., 2012). Fresh water from Mildred Lake Reservoir was pumped into the reclamation site in the summer of 2012.

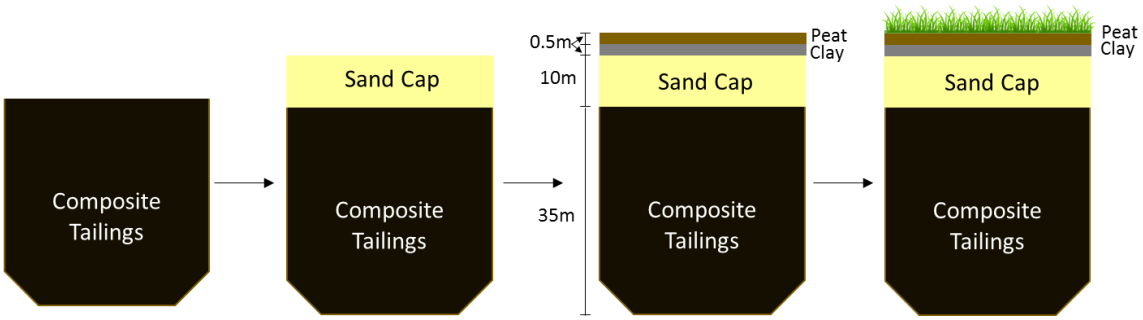

Figure 1.3: Cross-section of the reclamation site over stages of development from 2009 to 2012

Figure 1.4: Images of the development of the Sandhill Fen reclamation site

In 2009, production of hydrogen sulphide gas (H_2S), a by-product of microbial respiration that poses safety and management risks, was discovered to be occurring at the site. This lead to concerns that an influx of modern carbon from the constructed fen as it developed may stimulate metabolism of sulphate reducing bacteria (SRB) and result in increased gas production. A joint research program between Syncrude and McMaster University was developed with the goal of assessing the biogeochemistry of the Sandhill reclamation site to better understand the implications of microbial activity and geochemical cycling on this project, and to enable the development of more effective tailings management and reclamation strategies in future projects. As a subset of this overarching project, the goals of this Master's thesis were twofold:

1) To assess the microbial community at the constructed fen on the surface of the reclamation site as an indicator of ecosystem health (Mummey et al., 2002) and the progression of the constructed fen towards natural conditions, and especially to assess the presence of SRB within the microbial community in the fen

2) To investigate movement of petroleum and modern carbon at the site and their utilization by microbes in the fen and within the sand and CT layers below

To attain these goals, a combination carbon isotope and phospholipid fatty acid (PLFA) analyses were performed on samples taken from the Sandhill fen site over the course of its development between 2011 and 2013.

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Chapter 2: Introduction to PLFA and Carbon Isotopes

2.1 Phospholipid Fatty acids

Traditionally, investigation of microorganisms and their interaction with their environment has been undertaken by laboratory studies relying on isolation and culturing. These approaches are limited by the fact that only an estimated 0.001% to 1% or all organisms can be cultivated in a laboratory setting (Amann et al., 1995). Analysis of phospholipid fatty acids (PLFA) from cell membranes is one technique for studying microbial communities *in situ* without the requirement for cultivation.

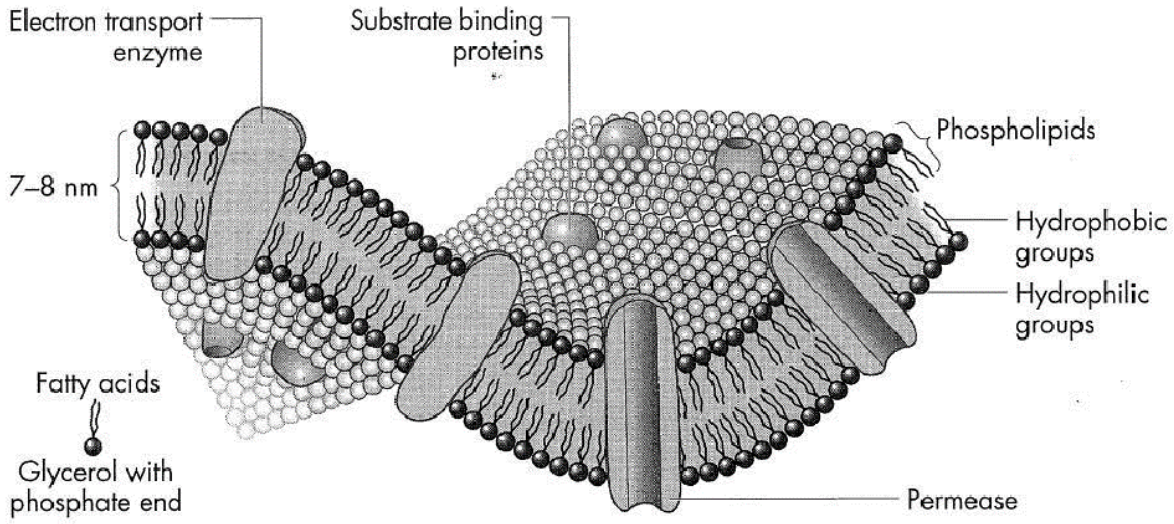

Figure 2.1: A cellular membrane, including phospholipids which make up the phospholipid bilayer (Konhauser 2009)

Phospholipids are the primary component of cell membranes in all three domains of life (Konhauser, 2009). Each phospholipid is an amphiphilic molecule consisting of a glycerol backbone bound to two hydrophobic fatty acid chains and one polar hydrophilic head group. In bacteria and eukaryotes, the fatty acid chains are ester-linked to the glycerol backbone (Denich et al., 2003), as is shown in Figure 2.2A. Intact phospholipids can be extracted from pure cultures or environmental samples using organic solvents and saponified to break the ester bonds, releasing two phospholipid fatty acids (PLFA) which can be

analyzed as indicators of the viable microbial community. PLFA can provide information on the total viable biomass of a population and the presence of specific microbial taxa through biomarker PLFA, as well as a profile or "fingerprint" of the community that can be compared between samples (Green and Scow, 2000).

2.1.1 Biomass of viable microbial communities

Because phospholipids break down by hydrolysis of the phosphodiester bond rapidly after cell death (White et al., 1979; Harvey et al., 1986), they are useful indicators of the viable microbial community. Generic conversion factors collected by Green and Scow (2000) are required to estimate cell numbers from the mass of PLFA extracted; these conversion factors range from $2x10^4$ to $6x10^4$ cells per picomole PLFA. Although there is some error inherent in conversion factors because of variation in cell size and percentage of biomass composed of phospholipids, this method has been successfully applied in many studies to demonstrate changes in total biomass; for example, Rajendran and Nagatomo (1999), Frostegård et al. (1996), Boschker et al. (2005), and Ludvigsen et al. (1997).

2.1.2 PLFA Nomenclature

By convention, fatty acids are named using the following manner: X:YωZ, where X is the number of carbon atoms in the chain, Y is the number of double bonds, and Z is the position of the first double bond counted from the aliphatic end of the molecule. Branches in *Iso*, *anteiso*, and unknown positions are denoted using the prefixes "i", "a", and "br-", respectively. If the methyl branching position is known and is neither *iso* nor *anteiso*, it is denoted with the position from the aliphatic end and "Me" as a prefix (for example, 10Me16:0). The prefix "cy" denotes cyclopropyl fatty acids.

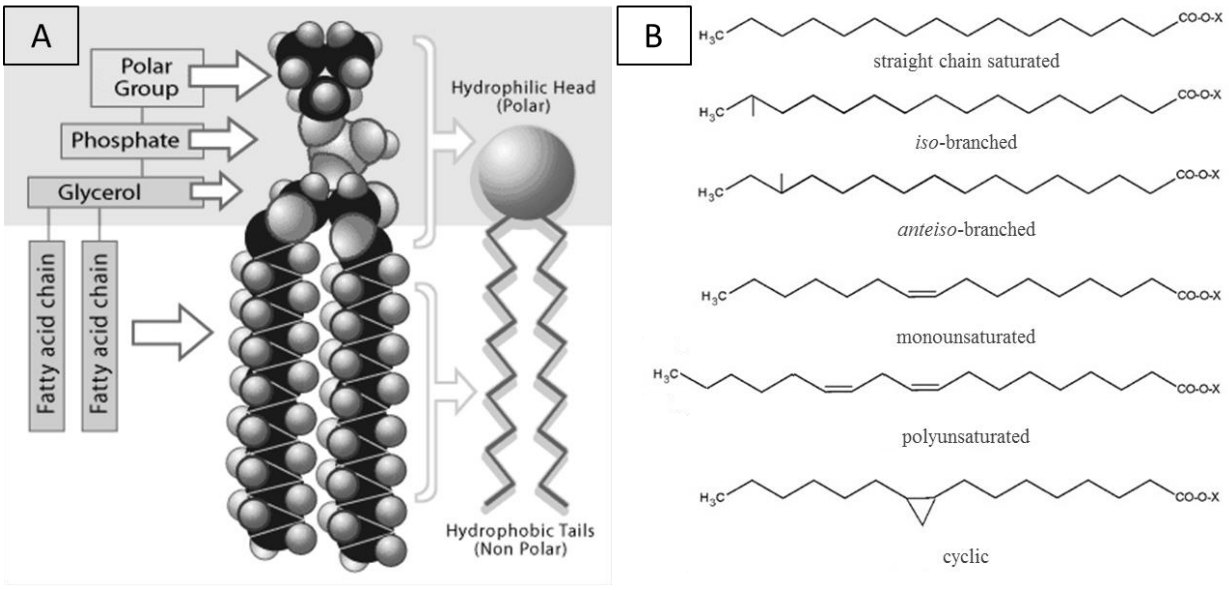

Figure 1.2 A) Structure of a phospholipid from teaching.ncl.ac.uk (Biomedical Sciences Wiki). B) Fatty acid chain structures adapted from Denich et al. (2003)

2.1.3 PLFA Biomarkers and Profiles

PLFA come in a number of conformations as presented in Figure 2.2B. Some groups of organisms are associated with particular types of PLFA, based on PLFA patterns seen in laboratory studies of pure cultures. Table 2.1 contains a brief summary of common PLFA biomarkers and the groups with which they are associated, along with selected references from literature. For example, branched PLFA are unique to bacteria (Zelles, 1999; Lechevalier and Lechevalier, 1988) while polyunsaturates are associated with eukaryotes (Zelles, 1999). Some individual PLFA are closely linked to microbial taxa, such as i17:1, which is associated with *Desulfovibrio.* Identifying PLFA biomarkers can be used to deduce the presence and prevalence of members of a microbial community.

Table 2.1: Common PLFA biomarkers and associated groups, with selected references

PLFA	Indicator for	Example References
Branched	Bacteria	Lechevalier and Lechevalier (1988)
iso-branched	Gram positives	Balasooriya et al. (2013)
Polyunsaturates (ex. 18:2)	Fungi	Frostegård et al. (1996), Zelles (1997)
20:5	Algae	Sargent et al. (1987)
Long chain saturates ≥20 C	Eukaryotes	Zelles (1999)
10Me16:0	SRB	Piotrowska-Seget and Mrozik (2003), Zelles (1999), Londry et al. (2004)
br-17:1	SRB, specifically *Desulfovibrio*	Londry et al. (2004)
Monounsaturates	Aerobes	Fang and Barcelona (1998), Mentzer et al. (2006), Guckert et al. (1985)
16:1ω8, 18:1ω8	Methanotrophs	Makula (1978)Bowman et al. (1993)
Cyclic	stationary phase stress response anaerobes	Hadwin et al. (2006), Keith-Roach et al. (2002) Balkwill et al. (1998) Guckert et al. (1985)

2.1.4 Extraction of PLFA

Phospholipids are extracted from environmental samples using a modified method first developed by Bligh and Dyer (1959) wherein a solvent solution of dichloromethane (DCM), methanol (MeOH) and phosphate buffer (PB) in a ratio of 1:2:0.8 is added to the sample, which is sonicated and left in the solvent overnight. The original method by Bligh and Dyer (1959) used chloroform, but this was modified after testing by Brinch-Iversen and King (1990) found that less hazardous DCM was equally effective. The resulting total lipid extract (TLE) is separated into neutral lipid (DCM), glycolipid (acetone) and phospholipid (MeOH) fractions using silica gel chromatography (Heinzelmann et al., 2013). Heinzelmann et al. (2013) recently tested the efficacy of this method and found that the phospholipid fraction may contain also contain small amounts of glycolipids, betaine lipids and sulfoquinovosyldiacylglycerols, while small amounts (~10%) of phosphatidylglycerides (PG), and phosphatidylethanolamines (PE) elute in the acetone rather than DCM fraction. Ester-linked phospholipids in the polar fraction are converted to fatty acid methyl esthers (FAMEs) by mild-alkaline methanolysis using potassium hydroxide in MeOH. A

different type of methanolysis is possible using HCl/MeOH, but when Chowdhury and Dick (2012) compared the two methylation procedures, they found that using HCl/MeOH resulted in higher concentrations of PLFA recovered but did not recover any branched PLFA. Since the presence of branched PLFA can significantly affect microbial community profile, the KOH/MeOH method is preferable. FAMEs are purified through a secondary silica gel, then identified and quantified using gas-chromatography mass spectroscopy (GC-MS).

2.2 Isotopes of Carbon

Carbon has three naturally occurring isotopes: ^{12}C, ^{13}C, and ^{14}C, which comprise 98.93%, 1.07%, and trace amounts of naturally occurring carbon, respectively (Rosman and Taylor, 1998). ^{12}C and ^{13}C are stable, while ^{14}C undergoes radioactive decay with a half-life of 5730±40 years (Godwin, 1962). Ratios of $^{13}C/^{12}C$ in PLFA can give insight into microbial metabolisms active in an environmental system, and ratios of $^{14}C/^{12}C$ in PLFA are valuable for distinguishing microbial carbon source(s).

2.2.1 Carbon-13

The ratio of the two stable isotopes of carbon is reported as $\delta^{13}C$ in units of parts per million (‰) and is calculated with the following equation:

$$\delta^{13}C_{sample}(‰) = \left[\frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{standard}}\right] x1000$$

wherein $(^{13}C/^{12}C)_{standard}$ is that of a known reference standard, Vienna Pee Dee Belemnite carbonate (PDB), equal to 0.0112372 (Boschker and Middelburg, 2002).

Chemical or physical processes that favour one isotope over the other can change the relative abundance of isotopes, a process known as fractionation. This is common in biological systems where reactions are enzymatically mediated, and is controlled by the Kinetic Isotope Effect (KIE), the magnitude of which

depends on the enzymes and substrates involved. Chemical bonds require more energy to break when they contain the heavier isotope (^{13}C), resulting in a preference for utilization of ^{12}C over ^{13}C in biological reactions (Galimov, 2006).

The $\delta^{13}C$ of biomolecules depends on the $\delta^{13}C$ of the substrate, the carbon assimilation pathway used by the organism, and the KIE inherent in synthesis of the biomolecule. Measuring $\delta^{13}C$ of cell components like PLFA vs. potential carbon sources can provide insight into the microbial metabolisms in use in an environmental system. Heterotrophic organisms cannot fix carbon and instead take in organic compounds from which they harvest energy and the building blocks for biosynthesis, including carbon. Organic carbon sources are typically depleted in ^{13}C compared to inorganic sources. The intake of atmospheric CO_2 and formation of organic compounds by autotrophs discriminates against ^{13}C, causing fractionation that varies depending on the type of autotroph. Among terrestrial plants there are two major isotopic categories differentiated by their photosynthetic pathways. C_3 plants incorporate CO_2 using ribulose bisphosphate carboxylase-oxygenase (Rubisco) (Ehleringer and Cerling, 2002) and produce organic compounds with $\delta^{13}C$ between -22‰ and -33‰ (Bender, 1971). C_4 plants incorporate CO_2 using phosphoenolpyruvate carboxylase (Ehleringer and Cerling, 2002), producing organic compounds with $\delta^{13}C$ in the range of -9‰ to -15‰ (Glaser, 2005). The ranges in isotope signatures are due to differences in environmental conditions during carbon fixation (Hayes, 2001). PLFA from heterotrophic bacteria that take up this carbon generally have $\delta^{13}C$ close to that of the carbon source. PLFA of heterotrophic organisms under aerobic conditions are usually within 6‰ of the carbon source (Boschker and Middelburg, 2002; Teece et al., 1999; Blair et al., 1985; Monson and Hayes, 1982; DeNiro and Epstein, 1977), while those under anaerobic conditions can be depleted by up to 14‰ relative to the carbon source (Londry et al., 2004). Figure 2.3 provides a summary of fractionation patterns in organic carbon. This information can be used to ascertain the type of microbial respiration occurring in an environment by comparing $\delta^{13}C_{PLFA}$ with $\delta^{13}C$ of the carbon source. Biogenic methane has $\delta^{13}C$ between -50‰ and -110‰

(Boschker and Middelburg, 2002), and uptake by methanotrophs causes further fractionation, so that PLFA of methanotrophic bacteria can be recognized by their considerably depleted $\delta^{13}C$. Stable carbon isotope ratios can also be used in some cases to determine the carbon source being utilized (Philp, 2007; Pancost and Sinninghe Damsté, 2003; Galimov, 2006). However, bitumen in the oil sands has a $\delta^{13}C$ of -29.6‰ (Jha et al., 1979), within the range of C_3 plants, so $\delta^{13}C$ analysis does not provide the necessary resolution to differentiate between petroleum hydrocarbons and recently photosynthesized compounds in order to determine microbial carbon source(s).

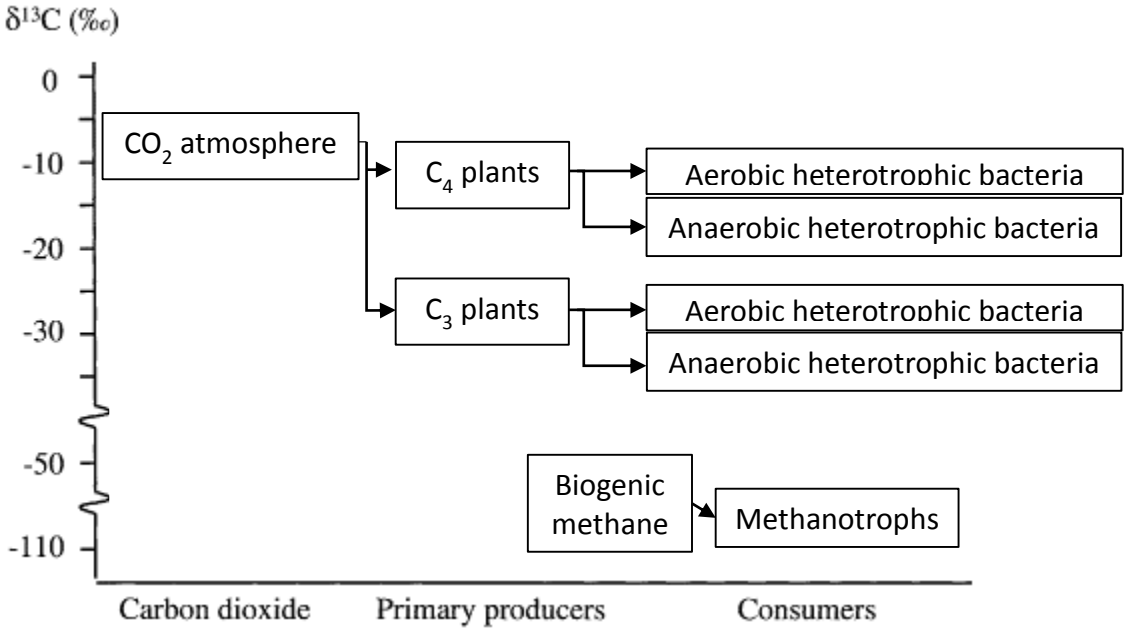

Figure 2.3: Representation of carbon isotope fractionation associated with carbon fixation and assimilation. Adapted from Boschker & Middelburg (2002)

2.2.2 Carbon-14

Radiocarbon isotope analysis is a useful tool for studying carbon cycling and distinguishing microbial carbon sources. ^{14}C is radioactive and decays over time. $\delta^{14}C$ can be calculated with an identical equation to the one used for $\delta^{13}C$:

$$\delta^{14}C_{sample}(‰) = \left[\frac{(^{14}C/^{12}C)_{sample} - (^{14}C/^{12}C)_{standard}}{(^{14}C/^{12}C)_{standard}}\right] x1000$$

where the standard is the National Bureau of Standards oxalic Acid (Stuiver & Polach 1977). But this must be corrected to account for fractionation, which is done by normalizing to a $\delta^{13}C$ of -25‰ with an equation by Stuiver and Polach (1977) that assumes that fractionation of ^{14}C is twice that of ^{13}C:

$$\Delta^{14}C(‰) = \delta^{14}C - 2(\delta^{13}C + 25)(1 + \frac{\delta^{14}C}{1000})$$

Because of this normalization, the $\Delta^{14}C$ of cellular components like PLFA is solely dependent on the $\Delta^{14}C$ of the microbial carbon source. Since ^{14}C decays over time, ancient sources will be free of ^{14}C (or "radiocarbon-dead") and have a $\Delta^{14}C$ of -1000‰. Atmospheric CO_2, on the other hand, is enriched in ^{14}C because of the testing of nuclear bombs in the mid-20th century and has $\Delta^{14}C$ >0‰ (Hua et al., 2013). Specific $\Delta^{14}C_{CO2}$ in an area is dependent on a number of environmental factors like exchange with the biosphere and burning of fossil fuels. Since plants take up atmospheric CO_2 during photosynthesis, recently photosynthesized compounds reflect the $\Delta^{14}C_{CO2}$ of >0‰. This wide range between ancient petroleum $\Delta^{14}C$ and modern $\Delta^{14}C$ of plant-produced compounds provides a convenient means of tracking transport of carbon and microbial uptake in environments where petroleum is present, such as the Sandhill Fen reclamation site.

A diagram summarizing the methods used in this thesis can be found in the Appendix.

2.3 Thesis Structure

This thesis focuses on the microbial PLFA and carbon isotope biogeochemistry at the Sandhill Fen reclamation site being constructed by Syncrude Canada Ltd. Chapter 1 is an introduction to the oil sands of northeastern Alberta and the motivation for land reclamation in the area. It discusses tailings waste produced during extraction of bitumen from the oil sands, the technology of composite tailings as a means of waste management, and a brief introduction to microbes found within tailings material. Governmental

mandates for land reclamation by oil sands companies are also discussed, as is the prevalence of wetlands in the regions leased by oil sands companies. This serves to explain the rationale for the construction of the Sandhill Fen reclamation site, which is described, along with the research questions about the presence of microbes and biogeochemistry of carbon at the site which served as impetus for this Masters project.

Chapter 2 provides an overview of the lipid and isotope analyses performed in order to address the questions presented in Chapter 1. This includes the use of phospholipid fatty acid (PLFA) analysis to determine biomass and microbial community composition, as well as stable and radiogenic carbon isotope analysis as tools to investigate carbon transport, microbial uptake of carbon, and microbial metabolism.

Chapter 3 is a manuscript in preparation for submission which focuses on PLFA analysis to determine biomass and community composition in surface material at Sandhill Fen. This analyses was done as a means of assessing the progression of the constructed fen towards natural conditions by quantifying biomass, looking at the presence of specific types of organisms using biomarker PLFA, and by comparison of PLFA profiles to those in undisturbed fens in the region using statistical methods.

Chapter 4 is also a manuscript in preparation for submission that focused on carbon biogeochemistry in the reclamation site system. Radiocarbon analysis of bulk material from the surface of the fen and from dissolved organic matter in the deeper layers of the site was used to study the transport of carbon in the system. ^{14}C analysis of PLFA showed the uptake of carbon by microbes, the trend of microbial preference for young carbon, and the relationship between age of available carbon and biomass. Chapter 5 provides a synthesis of the results and conclusions reported in Chapters 3 & 4, and suggests future work to improve upon the findings of this Masters project. The thesis ends with an Appendix containing a table of all PLFA used in cluster analysis in Chapter 3 and a method summary diagram.

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CHAPTER 3

PHOSPHOLIPID FATTY ACID (PLFA) ANALYSIS OF MICROBIAL COMMUNITIES IN A PILOT WETLAND RECLAMATION SITE IN THE ALBERTA OIL SANDS

Manuscript in Preparation for Submission

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Abstract

Microbial communities in the surface material of a pilot-scale fen wetland reclamation site in the Athabasca oil sands were characterized by phospholipid fatty acid analysis in order to assess microbial biomass development and community composition. Microbial biomass ranged from 10^7 to 10^9 cells/g, comparable to other wetland sites worldwide. Biomarkers indicate that the representation of sulphate reducing bacteria is about equal to that found in natural wetlands, despite the potential abundance of sulphate in the system due to gypsum-treated tailings present below the fen. Eukaryotes and small amounts of fungi are present, but bacteria dominate the system. Cluster analysis indicates that there are no distinct patterns of microbial community composition change over time or between material types, and that PLFA composition from materials on the surface of the reclaimed fen is more similar to that from un-reclaimed composite tailings from the adjacent site than to most undisturbed wetland PLFA profiles, indicating that the microbial community composition is influenced by that in deeper layers of the reclamation site.

3.1 Introduction

The oil sands of northern Alberta, Canada, contain an estimated 1.7 trillion barrels of oil, with production that is estimated to rise from 1.9 million barrels per day (b/d) in 2013 to 4.8 million b/d in 2030 (Canadian Association of Petroleum Producers, 2014). Extraction of bitumen requires large volumes of water and creates tailings slurry comprised of water, silt, sand, residual bitumen, and residual chemicals (Masliyah et al., 2004). Fluid fine tailings can be modified by the addition of sand and gypsum ($CaSO_4 \cdot 2H_2O$) to release water for re-use and produce composite tailings (CT) (Matthews et al., 2002), which are semi-solid and can be more easily managed. It is mandated that oil sands companies return disturbed land to the same land capability as before their industrial activity started (Energy Resources Conservation Board, 2009). The pilot-scale Sandhill Fen reclamation project is currently being undertaken by Syncrude in an

attempt to reclaim a 52 hectare area of the East In Pit, currently used as a CT storage location, to a boreal fen wetland of the type that existed in the area prior to disturbance from oil sands extraction. The reclamation site consists of layered composite tailings that have been deposited into a previously mined area to a depth of 35 metres. This CT has been topped with 10m of tailings sand, 0.5m fine-grain clay till, and ~0.5m of recently salvaged peatland material (Wytrykush et al., 2012). The adjacent site, Kingfisher Fen, is composed of CT that has undergone no reclamation activities. The goal of this reclamation project is to provide appropriate initial conditions to allow the development of a self-sustaining fen wetland, comparable to the landscape prior to mining activities, and to gain information that will guide development of future reclamation projects (Wytrykush et al., 2012). A key component of the Sandhill Fen project is to understand the biogeochemical cycling of carbon sources and nutrients within the system to understand the extent to which the activities of microbes are comparable to a naturally occurring fen and are providing effective management of CT related materials. Sulphate reducing bacteria (SRB) are of particular interest in this system, because the presence of gypsum in the CT provides abundant sulphate to act as a terminal electron acceptor during microbial metabolism that may lead to the production of hydrogen sulphide. The potential production and fate of this sulphide may have implications to the ongoing stability of the fen reclamation system. Understanding the development of the microbial community within the surface fen materials with respect to biomass and composition will indicate how this system may develop in the future and inform future applications of this reclamation strategy.

Phospholipid fatty acid (PLFA) analysis is an established method for studying *in situ* microbial communities in complex environments (Vestal and White, 1989). Phospholipids make up the membranes of bacterial and eukaryotic cells and degrade quickly after cell death (Harvey et al., 1986), so their quantification can be used to calculate an estimate of the viable biomass of a sample. Further, some lipids associated with particular groups of organisms can be used as biomarkers; for example, 10Me16:0 is associated with sulphate-reducing bacteria (Zelles, 1999), as is br-17:1 (Londry and Des Marais, 2003). Polyunsaturated

lipids are biomarkers of eukaryotes; in particular 18:2 is considered to be representative of fungi (Frostegård and Bååth, 1996), though it may also be found in some plants (Zelles, 1997).

Beyond concentration and distributions of PLFA, compound specific stable carbon isotope ($\delta^{13}C$) analysis of PLFA can be used to study carbon cycling and microbial metabolism within complex environmental systems (Boschker and Middelburg, 2002). Specifically, some isomers of monounsaturates 16:1 and 18:1 are biomarkers for methanotrophic bacteria (Bowman et al., 1993), and methanotrophy can be confirmed by the presence of markedly depleted $\delta^{13}C_{16:1}$ and $\delta^{13}C_{18:1}$ caused by lipid biosynthesis with methane carbon, which has $\delta^{13}C$ between -50‰ and -110‰ (Boschker and Middelburg, 2002). Methane production and methanotrophy are common components of carbon cycling in naturally occurring wetlands (Andersen et al., 2013).

In order to assess microbial community establishment and development during fen construction, this study used PLFA analysis to assess bacterial biomass and community composition in the surface layer of Sandhill Fen at a series of timepoints during the course of the reclamation project.

3.2 Methods and Materials

3.2.1 Sample collection and site description

Sandhill fen is a pilot-scale reclamation project in a section of Syncrude's East In Pit (57°02'23.6"N 111°35'30.0"W). Two sites within the fen were sampled for this research project: 6A and sump vault (SV), as shown in Figure 1.2. A cross-section of the Sandhill fen site across stages of development can be seen in Figure 1.3. Sampling campaigns for this study took place July 2011, August 2012, November 2012, July 2013 and September 2013. Peat placement occurred in late 2011 to spring 2012, and initial flooding took place in summer 2012. Initial surface samples collected in 2011 were collected from the sand cap overlying CT as peat had not yet been added. These samples provide a background of the microbial community and its abundance prior to installation of the peat. As fen construction progressed, surface

samples were taken of peat and exposed sand where available. Between 2012 and 2013 sampling campaigns the site was flooded with water from nearby Mildred Lake, so samples after this point were collected from beneath approximately 15-20cm of standing water. Samples were collected from a depth of 10 to 30cm below the surface of the solid material. A metal shovel and sterile silicone spatula were used to move the material into sterile Whirlpak bags or carbon-free glass jars. All tools were sterilized with 70% ethanol immediately prior to use, and glassware was pre-combusted to a temperature of 450°C. Air was expelled from the sample bags and the samples were sealed and stored in a cooler on ice until they could be moved to storage at -20°C (maximum 6 hours on ice).

As it was not possible to access the CT underlying the Sandhill fen, samples were taken from the adjacent Kingfisher Fen site, which has not yet undergone any reclamation, in order to assess the microbial community that would be expected to exist underlying the fen site. Drilling was conducted in December 2012 using an amphibious track-mounted drill platform. Samples were collected using core tubes with a diameter of 50mm and extruded in 2m increments into an Atmosbag glove bag filled with nitrogen gas. Samples were place in coolers on ice until they could be moved to storage at -20°C.

3.2.2 Lipid Extraction

For lipid quantification and composition analysis, 30-50g of material were extracted with the modified Bligh and Dyer method (Bligh and Dyer, 1959; White et al., 1979; Brinch-Iversen and King, 1990) using solvents in a ratio of 1:2:0.8 dichloromethane (DCM): methanol (MeOH): phosphate buffer (PB) added in the order DCM, MeOH, PB. Duplicate extractions on each sample were performed side-by-side. Extractions sat at room temperature overnight, then were filtered through 1.5um pore filter paper (Whatman 934-AH), which had been pre-rinsed with DCM and MeOH. Solids collected in the filter were returned to the beaker, and a second extraction was performed on these. The solvents were phase separated, the organic fraction collected and its volume reduced using a rotary evaporator (Brinkmann).

The total lipid extract from the first and second overnight organic solvent extractions were combined following phase separation. Gravity column chromatography was used to separate the organic fraction into three fractions (F1: DCM, F2: acetone and F3: methanol) using pre-combusted fully activated silica gel (Aldrich; particle size 63-200μm, pore size 0.7-0.85 cm^3/g). The methanol fraction, which contained the PLFA, was evaporated almost to dryness using a rotary evaporator as above, transferred quantitatively to glass vial and evaporated to dryness using pure N_2 (Alphagas). PLFA were converted to fatty acid methyl esters (FAMEs) via mild alkaline methanolysis (Chowdhury and Dick, 2012) using isotopically characterized methanol. A secondary silica gel chromatography (F1: 4:1 hexane:DCM, F2: DCM and F3: MeOH) was performed to isolate FAMES, which were collected in the DCM fraction. All glassware used was precombusted at 450°C overnight to remove organics.

3.2.3 FAME Identification and Quantification

FAMEs were identified and quantified using an Agilent 6890 gas chromatographer equipped with a DB-5 column of 30m length and 0.25μm film thickness (Agilent) and Agilent 5973 single quadropole mass spectrometer. The following GC oven temperature program was used: 50 °C (1 min), 20 °C/min to 130°C, 4°C/min to 160°C, 8°C/min to 300°C (5 min). FAMEs were identified by comparison of retention times with the reference standard Bacterial Acid Methyl Ester (BAME) Mix (Supelco) and mass fragmentation patterns using MSD Chemstation D.02.00.275 (Agilent). FAMEs were quantified using external FAME standards of known concentration. Relative standard deviation of duplicate extractions is recorded in Table 3.1.

3.2.4 PLFA nomenclature

Fatty acids are named using the following convention: X:YωZ, where X is the number of carbon atoms in the chain, Y is the number of double bonds, and Z is the position of the first double bond counted from the aliphatic end of the molecule. Prefixes “i”, “a”, and “br”- denote *iso*, *anteiso*, and branching in an

unknown position, respectively. If the methyl branching position is known and is neither *iso* nor *anteiso*, it is denoted with the position from the aliphatic end and "Me" as a prefix (ex. 10Me16:0). Cyclopropyl fatty acids are designated by the prefix "cy".

3.2.5 Stable carbon isotope analysis

Stable carbon isotope ratios of individual PLFA were determined using an Agilent 6890 GC equipped with a DB5-MS column of 30 m length and 0.25 µm film thickness coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. The GC program was 80°C (1 min), 4°C/min to 280°C, 10°C/min to 320°C (15 min). FAME $\delta^{13}C$ values were adjusted to account for the addition of a methyl group from isotopically characterized methanol during methanolysis using the following equation:

$$\delta^{13}C_{FA} = \frac{(n+1)\delta^{13}C_{FAME} - \delta^{13}C_{MeOH}}{n}$$

Where $\delta^{13}C_{FAME}$ and $\delta^{13}C_{MeOH}$ are the $\delta^{13}C$ values of the measured fatty acid methyl ester and the characterized methanol used during derivitization, respectively, and *n* is the number of carbon atoms in the fatty acid chain. Error is ±1.0‰ to account for analytical error and standard deviation of replicate injections.

3.2.6 Water content

Gravimetric water content was determined by drying material at 60°C overnight, and is expressed as a percentage as calculated using:

$$\frac{mass\ moist\ material - mass\ dry\ material}{mass\ moist\ material} x100 = \%\ water\ content$$

3.2.7 Statistical Analyses

As data sets were too small to assume normal distributions, non-parametric statistical analyses were used to determine significance of difference in PLFA concentration between sand and peat samples, and

significance of change in PLFA concentration within each group of samples over time. The Mann-Whitney U Test, non-parametric equivalent to Student's t-test, was used when investigating the difference between two means. The Kruskal-Wallis test, non-parametric equivalent to ANOVA, was used when comparing >2 means (ex. PLFA concentration in 6A peat in August 2012, July 2013, and September 2013). Hypotheses explored with non-parametric statistics, the test used, and results are summarized in Table 3.2. Testing was carried out using IBM SPSS 22.0 software.

Two cluster analyses were performed using Ward's method in IBM SPSS v22.0 software. Patterns of relative abundance (expressed in mole percent) of individual PLFA from Sandhill Fen sand and peat samples were compared only to each other in Figure 3.6, and with those from undisturbed wetlands in the oil sands area published by Del Rio (2004) and from samples taken during deep drilling of Kingfisher Fen CT[1] in Figure 3.7. This second analysis involved a total of 12 Sandhill Fen surface samples, 20 undisturbed wetland samples published by Del Rio (2004), and 5 CT samples. Profiles published by Del Rio (2004) include hydroxylated PLFA while the extraction method used in the present study and in the Kingfisher CT study does not, so for comparison purposes hydroxylated PLFA were removed from the profile and the mole percent of remaining PLFA adjusted accordingly. In total, 64 individual PLFA were included in each profile. See Table A1 (in Appendix) for mole percent of each fatty acid in samples from Sandhill fen and Table A2 for mole percent of each fatty acid in samples from Kingfisher fen CT used in cluster analysis.

[1] CT PLFA analysis by Dr. Allyson Brady

3.3 Results

3.3.1 Biomass

Both Table 3.1 and Figure 3.2 show the amount of PLFA extracted from fen surface material (sand or peat), in units of ng PLFA per g dry weight of material extracted, as well as the approximate biomass in cells per gram dry weight of material extracted calculated from PLFA concentration using a conversion factor of 4.0×10^4cells/pmol PLFA (averaged from conversion factors in Green and Scow (2000)). PLFA concentrations range from 3.2×10^2 to 2.8×10^3ng/g in the sand samples and 1.0×10^4 to 4.4×10^4ng/g in the peat samples. The lowest concentration of PLFA was in SV sand July 2011, while the highest was in 6A peat Aug 2012. The PLFA concentration in peat samples was consistently higher than that in sand samples, and the difference between peat and sand sample concentrations is shown to be statistically significant by the Mann-Whitney U Test (p=1.034×10^{-4}, see Table 3.2). Apparent changes in PLFA concentration within each group of samples (6A sand, 6A peat, SV sand, SV peat) are not statistically significant according to the Kruskal-Wallis Test (6A sand, 6A peat, SV peat) or Mann-Whitney U Test (SV sand). These results are summarized in Table 3.2.

3.3.2 Individual PLFA and biomarkers

Table A1 in the Appendix shows the mole percent value of each fatty acid for each sample. The PLFA 16:0 was the most prevalent fatty acid in all samples, ranging from 10% to 17% of the total moles of PLFA. All samples contained 10Me16:0 in amounts between 2% and 4%, except for SV peat in September 2013, which contained none of this PLFA. Small amounts of br-17:1 were detected in 6A sand July 2011, August 2012, and November 2012, and in 6A peat August 2012. The maximum was 2% in 6A sand in November 2012.

3.3.3 PLFA Compound Classes

Figure 3.1 shows the composition of PLFA grouped into compound classes of saturates, monounsaturates, polyunsaturates, branched, cyclopropyl, and branched+unsaturated for all samples.

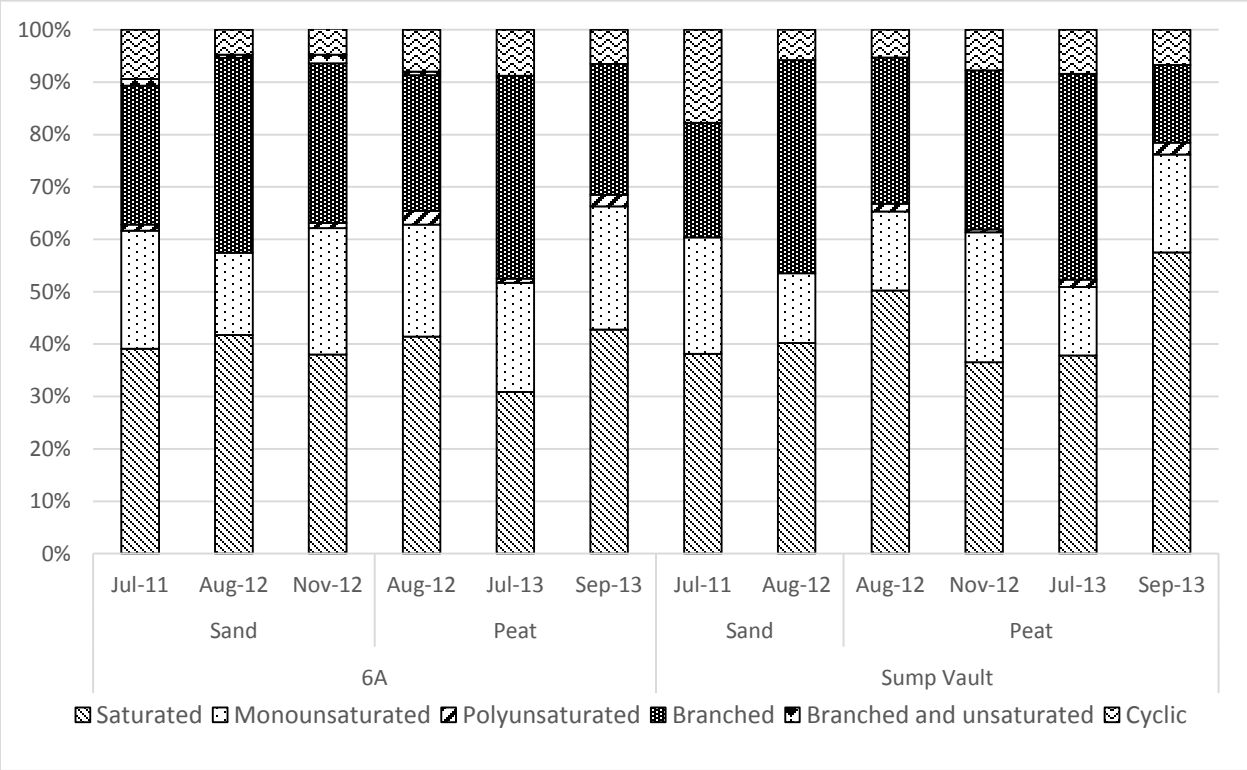

Figure 3.1: Overall mole percent PLFA composition in six categories of PLFA for each sample, averaged from duplicate extractions

Saturated PLFA were the most common type in all samples except peat from July 2013 at both sites, and SV sand August 2012, in which branched PLFA were more common. The sample with the highest percentage of saturates was SV peat Sept 2013 with 57%. Saturates with 20 or more carbons appeared in all samples, ranging from 6% to 32% of total fatty acids. Amounts in 6A sand have little variation over time, ranging from 9% to 12%, but in SV sand the amount increases from 7% to 17% from July 2011 to August 2012. In 6A peat, long chain saturates represent 15% of total fatty acids in August 2012, 6% in July 2013, and 20% in September 2013. In SV peat, they are 26% of total fatty acids in August 2012, approximately 15% in both November 2012 and July 2013, and 32% in September 2013.

The sum of branched fatty acids ranged from 15% to 41% of total PLFA. The lowest value, 15%, was in SV peat Sept 2013, a sample which had an unusually large mole percent of saturates (see above). The second lowest was 22% in SV sand July 2011. No evident difference in distributions is seen between sand vs. peat samples.

Monounsaturates ranged from 13% to 24%, with no evident pattern in sand vs. peat or moisture content (r^2*=0.0102;* Figure 3.3*).* Monounsaturates were the third most predominant category of PLFA, following saturates and branched PLFA, in all samples except SV peat September 2013, when monounsaturates were 19% of total PLFA and branched were 15%. The most common monounsaturates were 16:1 and 18:1, which combined constituted 83%-100% of total monounsaturates in each sample.

Polyunsaturates were scarce in all samples, between 0 and 3% of total FAMEs. In SV peat November 2012, 18:2 made up about half of the polyunsaturates present, while the other half was 20:5. In 6A peat September 2013, over 90% of the polyunsaturates were 18:2, and the remainder was 20:5. In 6A peat August 2012, 18:2 was 78% of the polyunsaturates, with the balance made up of 18:3 and 18:4. Other samples in which polyunsaturates were present contained only 18:2. Percent of total PLFA made up of polyunsaturates was negatively correlated with concentration of PLFA (r^2=0.33).

The percentage of cyclic fatty acids decreased between July 2011 and August 2012 in the sand at both sites. Percentages of cyclic fatty acids present increased between August 2012 and July 2013 in both 6A and SV peat, then decreased slightly in September 2013 samples. Cyclic fatty acids were 9% of the total at site 6A and 17% at the SV in July 2011. A year later, approximately 5% of total fatty acids in the sand at both sites were cyclic. Lipids from the sand sample from 6A in November 2012 were also ~5% cyclic. Peat samples from both site contained ranged from 5% to 9% between August 2012 and September 2013.

3.3.4 Stable carbon isotopes

Figure 3.5 shows $\delta^{13}C$ values of selected PLFA from all samples. Bulk $\delta^{13}C$ values of total organic carbon (TOC) fell within the range of -27.6‰ to -22.4‰. The ubiquitous saturate 16:0 has $\delta^{13}C$ values of -35.1‰ to -28.8‰. Monounsaturate biomarkers of methanotrophic bacteria had values close to that of 16:0, with $\delta^{13}C_{16:1}$ ranging from -32.6‰ to -28.7‰ and $\delta^{13}C_{18:1}$ ranging from -31.3‰ to -29.6‰. There were no distinguishable differences in $\delta^{13}C$ patterns between sand vs. peat samples or within each sample group (6A sand, 6A peat, SV sand, SV peat) as the reclamation project progressed.

3.3.5 Cluster analysis

Two cluster analyses were performed. In the first, the results of which are shown in Figure 3.6, the mole percentages of 65 fatty acids from Sandhill Fen sand and peat samples were compared to determine if patterns of similarity were present between material type, sampling site, date of sampling, water content, or some other parameter. Mole percentages of each fatty acid can be seen in Table A1. No patterns in PLFA profile were distinguishable by this method.

In the second cluster analysis, the mole percentages of 65 fatty acids from Sandhill Fen sand and peat samples, Kingfisher Fen CT samples, and undisturbed wetlands (published by Del Rio (2004)) were used to produce a cluster diagram (Figure 3.7) indicating the similarity of microbial populations in undisturbed wetlands, reclaimed fen, and tailings of the type residing below the constructed fen at the Sandhill Fen site. Mole percentages of fatty acids from four different undisturbed wetlands in the oil sands region sampled in summer months in 2000 and 2001 were taken from Del Rio (2004). Of these, three sites (H63, TR, and FM) were off-site control wetlands with low concentrations of naphthenic acids (0-3.7mg/L), and site NW was a natural wetland located near oil sands operations with higher concentrations (47.7-63.2mg/L) of naphthenic acids (Del Rio, 2004). Sandhill fen samples clustered together. KFCT samples, along with samples from two undisturbed wetlands in August 2000, formed another cluster. These two

clusters were joined in the next linkage step, before being joined to a small cluster composed of two undisturbed wetland samples and then a large cluster of 11 undisturbed wetland samples. The final cluster to join was composed of four undisturbed wetland samples from July 2000 and one from June 2000.

3.4 Discussion

3.4.1 Population size

PLFA concentration, and therefore biomass, in sand was significantly lower than in peat (p=1.034x10^{-4}; see Table 3.2), with sand biomass on the order of 10^7cells/g and peat biomass on the order of 10^8 to 10^9cells/g. Information from non-parametric statistical tests performed on PLFA concentration data, the interpretation of which can be extended to calculated biomass as well, is presented in Table 3.2. Variations within each sample group (6A sand, 6A peat, SV sand, SV peat) were not statistically significant, with p-values ranging from 0.193 to 1.000. This means that the microbial population size within each sample group remained stable despite the changes occurring at the reclamation site as peat placement, seeding of vegetation, and flooding of the fen took place, as well as over the course of changing seasons.

Table 3.1: PLFA concentrations and biomass averaged from duplicate extractions. Biomass was calculated from PLFA concentration using a conversion factor of 4.0x104cells/pmol PLFA (averaged from conversion factors in Green and Scow, (2000)).

		PLFA concentration (ng/g)	Biomass (cells/g)	Relative Standard Deviation
Jul-11	6A sand	$6.3x10^2$	$8.3x10^7$	N/A
May-12	6A sand	N/A	N/A	N/A
Aug-12	6A sand	$4.4x10^2$	$5.8x10^7$	8%
Nov-12	6A sand	$1.6x10^3$	$2.1x10^8$	28%
Aug-12	6A peat	$4.4x10^4$	$6.1x10^9$	42%
Jul-13	6A peat	$2.7Ex10^4$	$3.7x10^9$	N/A
Sep-13	6A peat	$2.6x10^4$	$1.7x10^9$	14%
Jul-11	SV sand	$3.2x10^2$	$4.6x10^7$	N/A
Aug-12	SV sand	$2.8x10^3$	$4.1x10^8$	34%
Aug-12	SV peat	$1.5x10^4$	$2.0x10^9$	31%
Nov-12	SV peat	$2.6x10^4$	$3.4x10^9$	9%
Jul-13	SV peat	$1.3x10^4$	$1.7x10^9$	N/A
Sep-13	SV peat	$1.0x10^4$	$1.4x10^9$	73%

Peat biomass is consistent with the biomass of $1.6x10^9$ to $5.7x10^9$ cells/g reported from sediment of natural wetlands in the oil sands area during summer months by Del Rio (2004), who reported biomass in nmol lipid phosphate per gram sediment, which can be converted to cells/g using conversion factors published by Findlay et al. (1989). Del Rio (2004) also studied wetlands affected by oil sands process water (OSPW) and found values in the 10^8 to 10^9 cells/g range. Similar surface peat biomass values in the 10^8 to 10^9 cells/g range are reported from studies in locations worldwide, such as early and late successional mitigated wetlands in Kentucky (D'Angelo et al., 2005) and pristine and reclaimed minerotrophic peatlands in Russia (Golovchenko et al., 2007).

Table 3.2: Hypotheses and results of non-parametric statistical tests conducted on PLFA concentration data.

Null Hypothesis	Non-parametric test	p-value, interpretation	Outcome
The distribution of PLFA concentration in sand samples is the same as that in peat samples	Independent samples Mann-Whitney U Test	1.034×10^{-4}, reject null hypothesis	PLFA concentrations found in peat samples are significantly different from those found in sand samples.
PLFA concentrations in 6A sand do not vary significantly over time	Independent samples Kruskal-Wallis Test	0.165, retain null hypothesis	PLFA concentrations in 6A sand do not vary significantly over time
PLFA concentrations in SV sand do not vary significantly over time	Independent samples Mann-Whitney U Test	1.000, retain null hypothesis	PLFA concentrations in SV sand do not vary significantly over time
PLFA concentrations in 6A peat do not vary significantly over time	Independent samples Kruskal-Wallis Test	0.223, retain null hypothesis	PLFA concentrations in 6A peat do not vary significantly over time
PLFA concentrations in SV peat do not vary significantly over time	Independent samples Kruskal-Wallis Test	0.193, retain null hypothesis	PLFA concentrations in SV peat do not vary significantly over time

There was a high degree of variation in PLFA concentration within some sample replicates, as can be seen in Table 3.1 in the form of relative standard deviation (RSD), which is equal to the standard deviation of replicates divided by the average. Some samples lack a RSD because only one extraction was performed (6A and SV sand samples from July 2011), or because an error during laboratory work resulted in the loss of one duplicate (6A and SV peat samples from July 2013). This variation demonstrates that there was small-scale spatial heterogeneity within each bag of sampled material. Multiple studies have found microbial heterogeneity at even the micro-metre scale in soil (Vos et al. (2013) and references therein). Other studies using PLFA extracted from soil or sediment have likewise found large variability within replicates (MacKenzie and Quideau, 2010; Rajendran and Nagatomo, 1999; Keith-Roach et al., 2002). Keith-Roach et al. (2002) suggests that this variation illustrates the importance of using statistical analyses to properly interpret PLFA data.

In the case of SV peat from September 2013, duplicate extractions were performed in which extraction 1 contained an abnormally high proportion (61%) of long-chain saturated PLFA, which are biomarkers of plants (Zelles, 1997). A third extraction was performed, in which the composition of PLFA was very similar to the second extraction, although the PLFA concentration was more than twice as large. It was hypothesized that an unusually large amount of plant roots were present in the first extracted subsample, which led to the high proportion of long-chain saturated PLFA. Because the focus of this study was mainly on PLFA representing microbes in the fen system and the large proportion of plant-based PLFA overwhelmed the proportions of other PLFA present, extraction 1 data was omitted from further analysis.

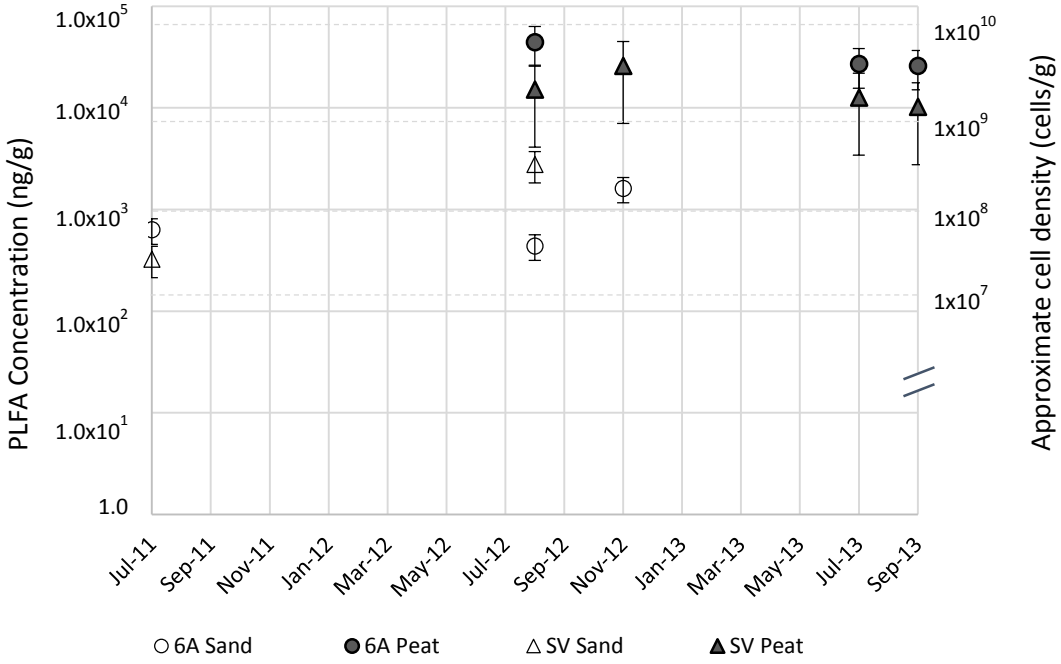

Figure 3.2: PLFA concentration and approximate biomass. Error bars represent maximum relative standard deviation from duplicate extractions for each set of samples: 6A sand 28%, SV sand 34%, 6A peat 42%, SV peat 73%.

3.4.2 Biomarkers

3.4.2.1 Sulphate-reducing bacteria

The abundance of SRB in the fen is of interest because of the large amount of sulphate available in the CT and potential production of toxic hydrogen sulphide gas as a product of SRB respiration. Sulphate reducers use sulphate (SO_4^{2-}) rather than oxygen as the terminal electron acceptor in respiration. The branched fatty acid 10Me16:0 is a putative biomarker for SRB (Fang et al., 2007; Londry et al., 2004), though it was not found in pure cultures of *Desulfovibrio* and *Desulfomaculum* studied by Londry et al. (2004). 10Me16:0 was found in small amounts in nearly all samples, with only small variations in percentage. In July and September 2013, when sampled peat was located under a small amount of surface water, dissolved sulphate concentrations in the water were found to be 43.4mg/L and 121mg/L, respectively, at site 6A and 86.1mg/L and 350mg/L, respectively, at the SV site (Michelle Reid, 2014 (thesis, unpublished)). Despite the higher concentrations of sulphate in September, SRB biomarker 10Me16:0 was more prevalent at site 6A in July 2013 than September 2013, and was present in the SV July 2013 sample and not detectable in the SV September 2013 sample. Percentages of 10Me16:0 did not differ greatly between dry and water saturated surface material, and the highest percentage of 10Me16:0 was seen in the sand sample from site 6A in August 2012. This indicates that the proportion of SRB represented by the 10Me16:0 biomarker in the community was unaltered by changes in conditions at the sample sites as peat placement and flooding took place. The branched monounsaturate br-17:1 is specific to *Desulfovibrio*, a genus of SRB (Londry et al., 2004) and was found in small amounts (<2%) in 6A sand July 2011, Aug 2012 and Nov 2012 and 6A peat Aug 2012. This suggests that SRB in sites where br-17:1 was not present or below detection limit were not *Desulfovibrio species.* Del Rio (2004) found similar amounts (1.4-2.4%) of 10Me16:0 and no br-17:1 in sediments from undisturbed wetlands in the oil sands region. Whitmire and Hamilton (2005) found measurable rates of sulphate reduction in wetland sediment 0-10cm below the water-sediment interface of two fen sites in Michigan, and concluded that in terms of total

carbon mineralization, sulphate reduction was the predominant anaerobic process in groundwater fed wetlands.

Sulphate reduction is of particular interest in the deeper layers of the reclamation site, where conditions are anaerobic and sulphate is plentiful because of the addition of gypsum to form CT. Production of hydrogen sulphide gas has been observed in Sandhill fen (Kendra and Warren, paper in prep; Holland et al., paper in prep), leading to the hypothesis that a high proportion of SRB may be seen in fen surface material as well as deeper in the sand cap and CT layers; however, biomarker fatty acids for SRB were found in amounts equivalent to those at undisturbed wetland sites in the area, indicating that the composition of the microbial population in surface material had not been appreciably impacted by the sulphur species present in the system. It is possible that there has been an impact on SRB species which do not contain the 10Me16:0 or br-17:1 lipid biomarkers, which may be elucidated using DNA analysis.

3.4.2.2 Fungi and algae

Fungal biomarkers were found in small amounts in all but one peat sample and in two of the five sand samples. Polyunsaturated PLFA represent eukaryotes (Zelles, 1999; Ringelberg et al., 2008) and 18:2 in particular has been found to correlate to ergosterol, a sterol biomarker of fungi (Frostegård and Bååth, 1996) and is therefore considered to be a fungal biomarker, though Zelles (1997) notes that it may also be present in some plant membranes. Polyunsaturates have been found in bacteria, but rarely, and only in some marine psychrophiles and cyanobacteria (Piotrowska-Seget and Mrozik, 2003) that are unlikely to be present at the study site. Only a small amount of polyunsaturated PLFA were present in samples from Sandhill fen, and 18:2 made up the majority in all samples where polyunsaturates were present. The small amounts of fungal biomarkers present is consistent with the findings of Winsborough and Basiliko (2010), that bacterial activity dominates in fens found in northern Ontario, Canada. Golovchenko et al. (2007) also found that bacteria dominate in minerotrophic peatlands (including fens) while fungi

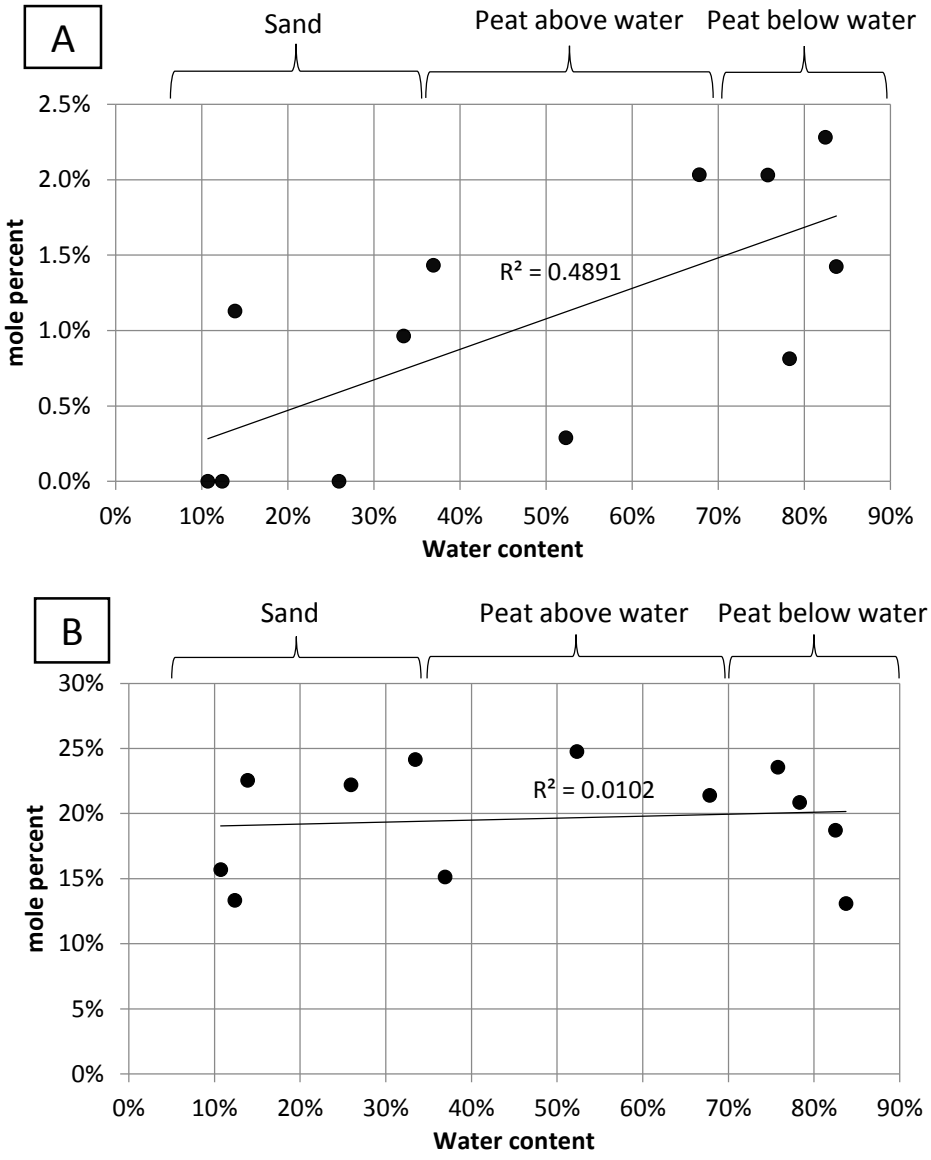

Figure 3.3: A) Mole percent of fungal biomarker 18:2 vs. water content of sample. B) Mole percent monounsaturates vs. water content of sample.

dominate in ombrotrophic peatlands. Jaatinen et al. (2008) found that the ratio of fungi to bacteria in a northern boreal fen in Finland increased under drier conditions and hypothesized that the increased recalcitrance of substrates in drier areas give fungi a competitive advantage. Conversely, the mole percent of 18:2 in Sandhill fen samples was found to be positively correlated to water content (r^2=0.489, Figure 3.3A), with the highest amount of biomarker PLFA at both sites in September 2013, when peat was sampled from below a small amount of standing water. The other polyunsaturate found in samples

indicates the presence of algae (Sargent et al., 1987). The 20:5 algal biomarker appeared in 6A peat September 2013 and SV peat November 2012, both in amounts smaller than 0.5% of total PLFA. That algae should be present in the September 2013 sample, when peat was taken from beneath water, is unsurprising, but this polyunsaturate was unexpected in the November 2012 sample, which had a water content of only 52%. Algae can be abundant in boreal wetlands (Rober et al., 2012), though this biomarker was not seen in natural wetlands in the oil sands region (Del Rio, 2004).

3.4.2.3 Eukaryotes

Long chain saturated PLFA were found in all samples, in amounts between 6% and 32%. Saturated straight-chain PLFA with ≥20 carbon atoms are considered to be long chain and are found only in eukaryotes, mosses and higher plants (Zelles, 1999). While large pieces of plant matter were removed before extraction, small roots remained in the samples, so long chain saturates from plants were expected in the PLFA profiles, especially from later samples after vegetation had been purposely planted in the fen. The origin of long chain saturates found in sand samples from both sites in July 2011, before peat and vegetation placement had occurred, is unknown. As is shown in Figure 3.4, the percentage of long chain saturates did not follow a steady pattern of increase from early samples, when vegetation had not been planted, to the most recent samples when plant life was well established in the fen. Rather, the percentage of long-chain saturated PLFA fluctuated over time, as did their concentration, showing that the fluctuating percentage is not just an artifact due to changing amounts of total PLFA.

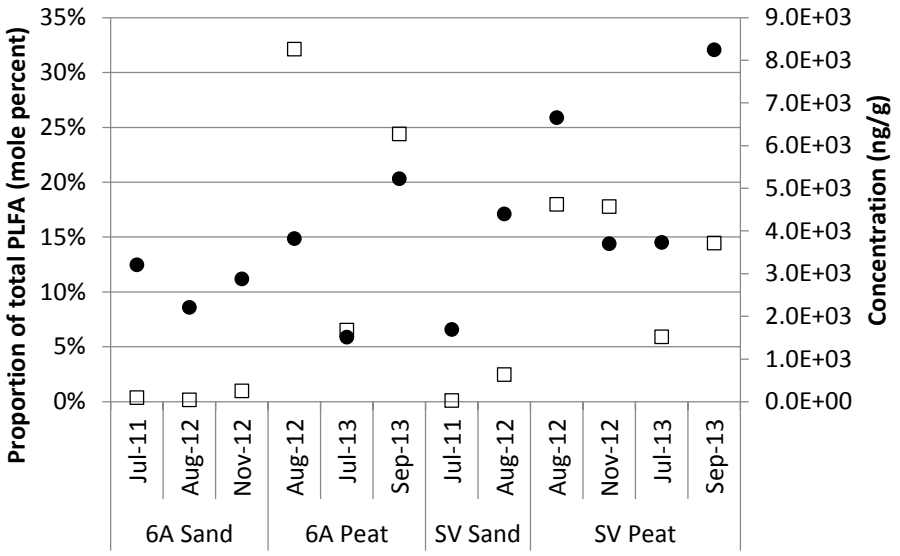

Figure 3.4: Pattern of long chain fatty acids in samples. Solid circles - proportion of total PLFA in mole percent; hollow squares – abundance in ng/g.

3.4.2.4 Methanotrophs

Monounsaturates in Sandhill Fen samples constitute 13% to 25% of total PLFA. Inglett et al. (2011) studied a Florida wetland that had undergone "extreme restoration" where soil was removed all the way down to the bedrock and replaced, and found monounsaturates made up between 43% and 52% of total PLFA, with higher proportions of monounsaturates during the wet season than dry. In Sandhill samples, there is not a strong correlation between sample water content and percent monounsaturates (r^2=*0.010*; Figure 3.3B). A high abundance of monounsaturates is characteristic of aerobic bacteria (Fang and Barcelona, 1998; Guckert et al., 1985; Mentzer et al., 2006; Rajendran and Nagatomo, 1999). Specifically, various isomers of 16:1 are characteristic of Group I methanotrophs and an isomer of 18:1 is the predominant PLFA in Group II methanotrophs (Bowman et al., 1993). While specific isomers of these monounsaturates could not be identified in samples from Sandhill fen, stable isotope analysis can aid in the distinction of biomarkers of methanotrophic microbes. Biogenic methane, the product of archaeal methanogenesis

which commonly occurs in the anoxic zones of natural wetlands (Andersen et al., 2013) produces methane depleted in ^{13}C, with $\delta^{13}C_{CH4}$ between -50‰ and -110‰ (Boschker and Middelburg, 2002). Methanotrophic metabolism and lipid biosynthesis can further fractionate against ^{13}C, causing methanotrophic bacteria to have signature highly ^{13}C depleted 16:1 and 18:1 (Boschker and Middelburg, 2002) compared to heterotrophic microbes assimilating organic carbon from sand or peat, which has a $\delta^{13}C_{TOC}$ range of -27.6‰ to -22.4‰. In Figure 3.5, the $\delta^{13}C$ of 16:1, 18:1c and 18:1t are plotted along with $\delta^{13}C$ of the ubiquitous PLFA 16:0 and of total organic carbon (TOC). There was no distinctive depletion in 16:1 or 18:1 compared to 16:0, and fractionation from $\delta^{13}C_{TOC}$ was between 3‰ and 10‰, as would be expected of heterotrophic microbes using TOC as their carbon source (Teece et al., 1999; Boschker et al., 2005).

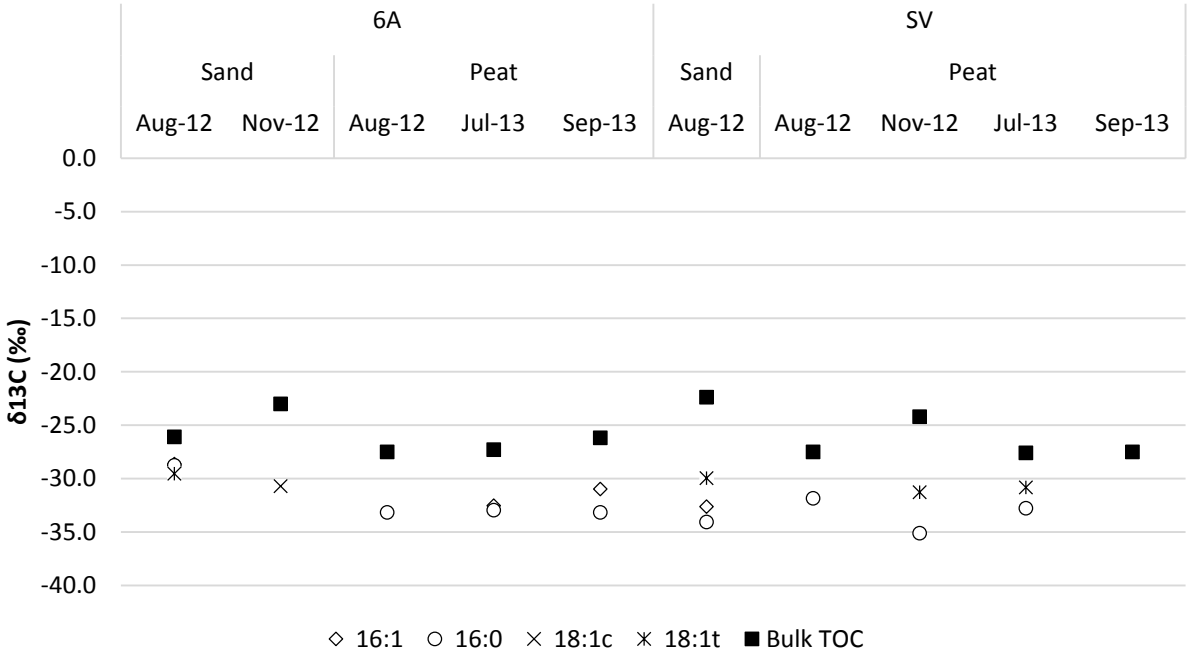

Figure 3.5: Stable carbon isotope data for selected *δ*individual PLFA. Isomers of 16:1 and 18:1 are associated with methanotrophic bacteria, and 16:0 is a common PLFA considered to be representative of the general population. Samples from July 2011 were not analyzed for *δ*13C due to low yields of PLFA, and are therefore not included in the figure.

3.4.2.5 Physiological conditions

Cyclopropyl PLFA constituted between 4% and 9% of all PLFA in all samples except SV sand July 2011, where they constitute 18%. Cyclic PLFA are common in gram negative bacteria, and are also found in anaerobic gram positives (Zelles, 1999; Ringelberg et al., 2008; Inglett et al., 2011). Guckert et al. (1985) found high proportions of cyclopropyl fatty acids in anaerobes and high proportions of monounsaturated fatty acids in aerobes during microcosm studies. However, cyclopropyl PLFA cannot be considered a biomarker for Gram negatives or anaerobes only, as other studies have correlated them with stress conditions and/or starvation (Heipieper, 2010), or communities with a high proportion of bacteria in the stationary phase of growth (Hadwin et al., 2006). Therefore, it may be that conditions at the SV site in July 2011 were more stressful to bacteria, or that a higher proportion of bacteria there were in the stationary growth phase.

3.4.3 Cluster analysis

Despite the change in surface material from sand to peat and changes taking place in the environment as the fen developed, sample PLFA profiles did not cluster by material type, date/season of sampling, water content, or other distinguishable pattern. The dendogram produced by hierarchical cluster analysis can be seen in Figure 3.6. This means that, according to the resolution available from PLFA analysis, sand and peat communities were not distinctive, despite the fact that both sand and peat would be expected to have pre-existing microbial communities before they were used as construction materials for the reclaimed fen. As discussed above, specific biomarkers did give evidence of different abundances of particular groups of organisms between samples, but the small proportions of most biomarkers means that they did not have a large influence on the overall PLFA profiles.

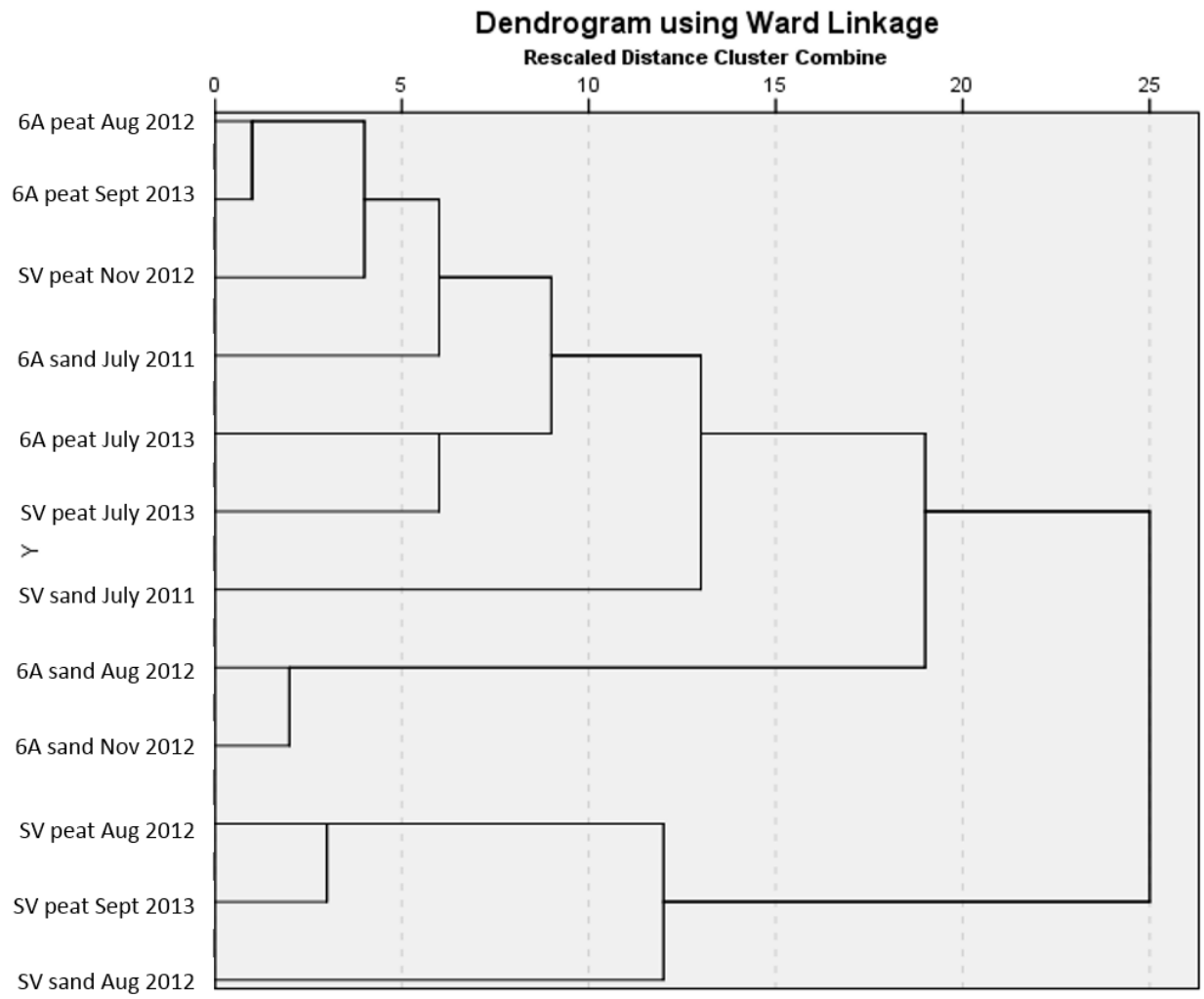

Figure 3.6: Dendogram produced using Ward's method of heirarchical cluster analysis on samples from Sandhill Fen surface material.

Hierarchical clustering was also performed using individual PLFA mole percentages from Sandhill Fen sand and peat, CT from the adjacent Kingfisher Fen site where reclamation has not taken place, and from undisturbed wetlands from the oil sands area. The resulting dendogram is shown in Figure 3.7. The clustering pattern shows that sand and peat samples from Sandhill fen were more similar to each other than to samples from the CT or undisturbed wetlands, as Sandhill Fen samples form one tight cluster. For the most part microbial communities from the surface of the reclaimed fen were more similar in PLFA

composition to those in the CT underlying the fen than they were to communities in undisturbed wetlands in the surrounding area. Had the PLFA profiles clustered by study, with Sandhill Fen and Kingfisher samples in one distinct cluster and samples studied by Del Rio (2004) in another, it would have to be concluded that slight differences in extraction and analysis between the two research groups had driven clustering rather than real differences in microbial communities. This was not the case, so it can be reasonably concluded that PLFA profiles used in the cluster analysis are comparable. The two undisturbed wetland samples that clustered with KFCT were offsite from oil sands extraction operations and contained only small amounts of naphthenic acids, a common constituent of oil sands waste (Allen, 2008), and thus were considered not impacted by oil sands industrial activity. Samples from site NW, an undisturbed wetland considered to be highly impacted because of its high naphthenic acid concentrations, clustered quite distantly from Sandhill Fen surface material samples. This suggests that if petroleum compounds had made their way into the fen from the CT below, they had not influenced the community composition, since Sandhill PLFA profiles are more similar to undisturbed wetlands than to this impacted wetland site. The closeness of Sandhill fen sample PLFA profiles to the profiles from deep within nearby CT was unexpected, and may show that the reclamation site surface was not well separated from the CT, allowing some exchange of microorganisms between CT, sand, and peat layers.

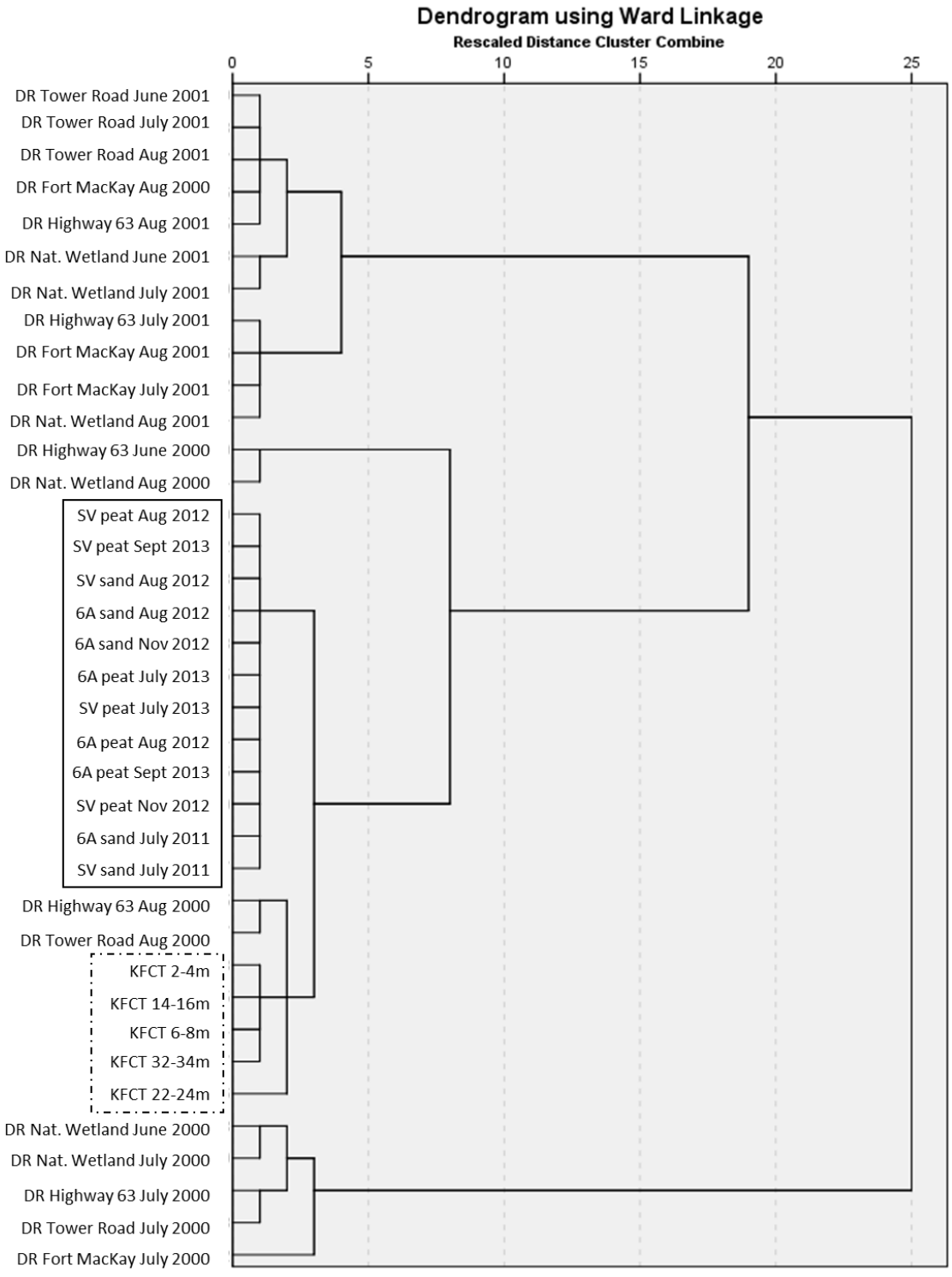

Figure 3.7: Dendogram produced using Ward's Method clustering on Sandhill Fen samples, Kingfisher Fen CT samples and undisturbed wetland samples from the oil sands area (denoted “DR”, as they came from Del Rio (2004)). The cluster of Sandhill fen samples is outlined in solid black, and Kingfisher Fen CT samples are outlined in a dashed line.

3.5 Conclusion

The microbial community at Sandhill fen had biomass in the range that has been found in natural wetlands and a diverse community composition as shown by PLFA biomarkers. Results show that SRB were a small proportion of the surface community, comparable to amounts found in naturally occurring fens. Small amounts of fungal biomarkers were present, as were varying amounts of PLFA associated with eukaryotes, likely from plant matter present in the fen. Clustering of PLFA profiles suggests that interchange of organisms may be occurring between the fen surface and CT or sand material below, indicating that the sand layer does not prevent interaction between the fen surface and CT. Metagenomic analysis could provide sharper resolution to complement work done in this study.

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CHAPTER 4

ISOTOPIC AND LIPID ANALYSES TO INVESTIGATE CARBON MOVEMENT AND MICROBIAL CARBON SOURCES IN A PILOT-SCALE WETLAND RECLAMATION SITE IN THE ATHABASCA OIL SANDS

Manuscript in Preparation for Submission

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Abstract

The transport and microbial metabolism of carbon in a wetland reclamation site in the Athabasca oil sands was analyzed using carbon isotope and lipid analyses. Sandhill fen is a pilot-scale reclamation project being undertaken by Syncrude Canada Ltd. wherein a composite tailings deposit has been overlain with sand and a fen wetland constructed on top. This study found that microbial population on the fen surface was dependent on the age of available carbon, rather than the concentration of organic carbon, and that microbes in the peat preferentially metabolized more modern carbon within organic matter available to them. Differences in the radiocarbon signatures of total organic carbon and natural organic matter indicated the presence of petroleum hydrocarbons in peat samples, however microbial lipids did not show evidence of microbes metabolizing this carbon source. There are concerns that an influx of modern carbon from the fen to the tailings layer could cause an increase in microbial population and drive production of toxic hydrogen sulphide gas. While dissolved organic carbon in water from the CT layer was found to be mainly from petroleum sources, with a small proportion of younger carbon, microbes there did show preference for more modern carbon over petroleum.

4.1 Introduction

Approximately 1.7 trillion barrels of oil are estimated to be contained in the oil sands region in Alberta, Canada (Masliyah et al., 2004). Extraction of bitumen from the sand and clay minerals to which it is attached requires $3m^3$ of water for every $1m^3$of bitumen, and produces $4m^3$of waste in the form of tailings, a slurry of water, silt, sand, solvents and residual bitumen (Holowenko et al., 2000). This waste must be kept on-site, and safe long-term storage of this material is one of the challenges faced by companies in the oil sands industry (Energy Resources Conservation Board, 2009). Addition of sand and gypsum modifies fluid fine tailings and produces composite tailings (CT) (Matthews et al., 2002), which are semi-solid and can be more easily managed. Regulations require that areas impacted by industrial activities in

the oil sands region be returned to "similar land capability" after the cessation of mining activities (Cumulative Environmental Management Association, 2007). Sandhill Fen is a pilot wetland reclamation project being undertaken by Syncrude Canada Ltd., the goal of which is to provide initial conditions to allow the development of a self-sustaining fen wetland, and to explore the feasibility and potential difficulties of future large-scale wetland reclamation projects. It is located on a 52ha section of Syncrude's East In Pit, a previously mined area which has since been used as a storage pit for CT and tailings sand (Wytrykush et al., 2012). Beginning in 2009, ten metres of tailings sand was laid atop the ~35m of CT in this area, followed by 0.5m of clay till and then 0.5m of recently salvaged peat material, which were placed on top in 2011-2012 and seeded with vegetation selected for its ability to grow in the expected conditions (Wytrykush et al., 2012). The sand cap layer was intended to act as a barrier between CT and the developing fen on top. Fresh water from Mildred Lake Reservoir was pumped into the fen in the summer of 2012. In 2009, the reclamation site was found to be producing hydrogen sulphide (H_2S) gas, a product of respiration by sulphate reducing bacteria (SRB). This led to concerns that H_2S release may be increased as a result of stimulation of SRB in the CT, sand, and fen itself by the influx of modern, labile carbon from the addition of peat and vegetation. Thus, one of the questions to be answered about the large-scale feasibility of fen reclamation atop CT is how the movement and availability of carbon affects microbial populations, especially those including SRB. This study uses carbon isotopes and microbial lipids to investigate the changes in carbon source and microbial population over the course of fen development.

Natural abundance radiocarbon (^{14}C) is a useful tool in understanding movement of carbon and its interactions with living organisms within a system. Radiocarbon degrades over time, with a half-life of 5730 years. Since the organic compounds that make up petroleum are millions of years old, they contain no detectable ^{14}C; in isotope notation, $\Delta^{14}C$=-1000‰. In contrast, recently fixed organic matter reflects the ^{14}C content of atmospheric CO_2, wherein $\Delta^{14}C$ >0‰ (Hua et al., 2013). Phospholipid fatty acids (PLFA) are cellular membrane components of bacteria and eukaryotes that degrade soon after death (Harvey et

al., 1986) and reflect the ^{14}C content of the carbon sources metabolized by the living organism (Slater et al., 2005; Wakeham et al., 2006; Ahad et al., 2010). Analysis of PLFA ^{14}C and comparison with that of available carbon sources can be used to assess the microbial uptake of petroleum carbon in a system. This method has been used in groundwater systems (Ahad et al., 2010; Cowie et al., 2010), contaminated sediments (Slater et al., 2005), and oil sands tailings ponds (Ahad and Pakdel, 2013), as well as various studies of oceanic carbon cycling (see Ingalls and Pearson (2005) and references therein). Stable carbon isotopes (^{13}C) can be used in conjunction with radiocarbon data to gain a comprehensive understanding of microbial metabolism and carbon cycling in complex environmental systems like wetlands (Chasar et al., 2000). Although petroleum and C_3 plants have ranges of $\delta^{13}C$ That overlap (Slater et al., 2005), $\delta^{13}C$ analysis can highlight the uptake of carbon from other sources like C_4 plants or algae (Ehleringer and Cerling, 2002). Fractionation of $\delta^{13}C$ between PLFA and carbon source can indicate whether microbial respiration is aerobic or anaerobic (Ahad and Pakdel, 2013).

This study used natural abundance stable ($\delta^{13}C$) and radiogenic ($\Delta^{14}C$) carbon isotopes to investigate the influence of petroleum carbon on the organic matter in the upper layers of the sand cap and peat of the Sandhill Fen reclamation site, and on the organisms, particularly bacteria, that live there. $\Delta^{14}C$ analysis of dissolved organic carbon and PLFA from sampling wells at various depths was also investigated to understand the movement of modern carbon into the deep layers of the reclamation site and its impact on microorganisms there.

4.2 Methods and Materials

4.2.1 Solid material collection and site description

Sandhill fen is a pilot-scale wetland reclamation project located in Syncrude's East In Pit (57°02'23.6"N 111°35'30.0"W). Two sites, 6A and sump vault (SV) were sampled for solid material. The location of these sites can be seen in Figure 1.2. The type of surface sample collected depended on reclamation progress

and site sampling. Initial samples in 2011 were collected from sand overlying CT, as no peat had yet been placed. As fen construction progressed in 2012, samples were taken of peat material and of exposed sand where accessible, as Syncrude policy required the peat layer not be disturbed to the degree necessary to collect sand from underneath peat. Samples were divided into four groups: 6A sand, 6A peat, SV sand, and SV peat. Sampling campaigns for sand/peat material took place July 2011, August 2012, November 2012, July 2013 and September 2013. Flooding with water from nearby Mildred Lake occurred between 2012 and 2013, raising the water table so that samples collected after this point were taken from beneath approximately 15-20cm of water. In all cases, samples were taken from 10-30cm below the surface of solid material using a metal shovel and sterile silicone spatula. All tools were sterilized with 70% ethanol immediately prior to use. Samples were sealed in sterile Whirlpak bags with air expelled and kept on ice until they could be moved to storage at -20°C (maximum ~6 hours on ice).

4.2.2 Lipid Extraction

All glassware was precombusted at 450°C overnight to remove organics. Lipid extraction was performed with the Bligh and Dyer method (Bligh and Dyer, 1959) as modified by White et al. (1979). Duplicate extractions on each sample were performed side-by-side. Between 30 and 50g dry weight of material were extracted using solvents in a ratio of 1:2:0.8 dichloromethane (DCM): methanol (MeOH): 0.1M phosphate buffer (PB) and left at room temperature overnight. Solvent was then filtered through 1.5µm pore filter paper (Whatman 934-AH) which had been pre-rinsed with DCM and MeOH. Solids caught by the filter were returned to the beaker for a second overnight extraction in the same manner as the first. After the second extraction, solids were set aside to dry, and later analyzed as Extracted Residue (Ext-res; see below). Solvents were phase separated by the addition of Milli-Q ultrapure water and the organic fraction collected. Organic fractions from both overnight extractions were combined, and the volume reduced by rotary evaporator (Brinkmann). Gravity column chromatography with fully activated silica gel (Aldrich; particle size 63-200µm, pore size 0.7-0.85 cm^3/g) was used to separate the total lipid extract into

three fractions: DCM, acetone, and MeOH. The MeOH fraction (containing PLFA) was evaporated almost to dryness by rotary evaporator and transferred quantitatively to a glass vial, where it was evaporated to dryness under pure nitrogen gas. PLFA were converted to fatty acid methyl esters (FAMEs) by mild alkaline methanolysis using MeOH characterized for ^{14}C and ^{13}C. A secondary silica gel chromatography with hexane, DCM, and MeOH was used to purify FAMEs, which eluted in the DCM fraction.

4.2.3 FAME Identification and Quantification

FAMEs were analyzed with an Agilent 6890 gas chromatographer containing a DB-5 column of 30m length and 0.25µm film thickness (Agilent) coupled to an Agilent 5973 single quadropole mass spectrometer. The GC temperature program used was: 50 °C (1 min), 20 °C/min to 130°C, 4°C/min to 160°C, 8°C/min to 300°C (5 min). FAMEs were identified using retention times compared to the Bacterial Acid Methyl Ester (BAME) Mix (Supelco) standard and mass fragmentation patterns using MSD Chemstation D.02.00.275 (Agilent). FAMEs were quantified using external FAME standards.

4.2.4 Stable carbon isotope analysis

Determination of stable carbon isotope ratios of individual PLFA was performed with an Agilent 6890 GC equipped with a DB5-MS column of 30m length and 0.25 µm film thickness coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer via a Conflo III interface. The GC program was 80°C (1 min), 4°C/min to 280°C, 10°C/min to 320°C (15 min). $\delta^{13}C$ values of FAMEs were adjusted to account for the addition of an isotopically characterized methyl group during methanolysis using the following equation:

$$\delta^{13}C_{FAME\ corrected} = \frac{(n+1)\delta^{13}C_{FAME} - \delta^{13}C_{MeOH}}{n}$$

where $\delta^{13}C_{FAME}$ and $\delta^{13}C_{MeOH}$ are the $\delta^{13}C$ values of the measured fatty acid methyl ester and the characterized methanol used during derivitization, respectively, and *n* is the number of carbon atoms in the fatty acid chain. Error is ±1.0‰, accounting for analytical error and standard deviation of replicate

injections. Stable carbon isotope ratios of total organic carbon (TOC; see below) and Ext-res were determined at Center for Applied Isotope Studies at the University of Georgia using a Finnigan MAT 251 Isotope Ratio Mass Spectrometer and expressed as $\delta^{13}C$ with an error of <0.1‰. Stable isotope ratios are expressed as $\delta^{13}C$ with respect to Vienna Pee Dee Belemite (PDB) (Stuiver and Polach, 1977).

4.2.5 Preparative capillary gas chromatography

FAMEs were purified using preparative capillary gas chromatography (PCGC). This was done using an Agilent 7890 GC equipped with two 30m DB-5 columns with 0.25µm film thickness and a flame ionization detector (FID) coupled to a Gerstel preparative fraction collector (PFC). The transfer line and PFC switching device were 320°C. The injection volume was 3µL and each sample was injected ~100 times. The same GC program as above was used. Ten percent of the effluent went to the FID, while the remaining 90% went to the PFC. Samples were cryogenically collected in glass U-traps kept at -10°C, which were then eluted into GC vials with 1mL of DCM. For most samples, all FAMEs were collected in the same U-trap. In two selected samples with a large percentage of long (≥20 carbon) FAMEs (6A peat Sept 2013 and SV peat Aug 2012), the PFC was used to separate a short FAME fraction and a long FAME fraction, which were analyzed for ^{14}C separately. Additionally, to test the necessity of PCGC purification, portions of four samples (6A peat from Aug 2012 and Sept 2013, and SV peat from Aug 2012 and Sept 2013) were held back and analyzed for ^{14}C without having gone through the PCGC step.

4.2.6 Silica gel clean up

FAMEs collected from the PCGC were run once more on the GC-MS system as described above. If siloxane contaminants were present, they were removed using silica gel chromatography with hexane, 4:1 hexane:DCM, 2:1 hexane:DCM, DCM, and MeOH. Fractions were analyzed by GC-MS as described above, which showed that all FAMEs eluted in the DCM fraction.

4.2.7 Radiocarbon analysis of PLFA

FAMEs were sent to the National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS) facility at the Woodshole Oceanographic Institute for ^{14}C analysis. FAMEs were oxidized to CO_2, which was reacted with an iron catalyst to form graphite. The ratio of $^{14}C/^{12}C$ relative to concurrently measured NBS Oxalic Acid I (NIST-SRM-4990) was measured with a 500kV Pelletron AMS, and the fraction modern (F_M) derived from this and normalized to a $\delta^{13}C$ value of -25‰ (Stuiver and Polach, 1977). F_M was converted to $\Delta^{14}C$ using the following equation:

$$\Delta^{14}C = \left[F_M * e^{(0.000121*(1950-y))} - 1\right] * 1000$$

Where F_M is the fraction modern and *y* is the year of sample collection. The accuracy of measurement is considered to be ±20‰ for $\Delta^{14}C_{PLFA}$ (Pearson et al., 1998).

4.2.8 Radiocarbon analysis of TOC and Ext-res

For TOC, a small amount of sample sand or peat was measured out and dried in an oven overnight at 60°C. Ext-res was collected during PLFA extraction, as described above. Solids were transferred to 4mL glass vials and treated with 1N $HCl_{(aq)}$ to remove inorganic carbon. $HCl_{(aq)}$ was added dropwise and the sample dried overnight at 60°C. This was repeated until no fizzing was visible on addition of acid (max. 3 times).

TOC and Ext-res samples were sent to the Center for Applied Isotope Studies at the University of Georgia. They were combusted at 900°C in evacuated/sealed ampoules in the presence of copper (II) oxide, resulting in carbon dioxide that was cryogenically purified from other reaction products and converted to graphite by the method published by Vogel et al. (1984). A CAIS 0.5 MeV accelerator mass spectrometer measured the graphite $^{14}C/^{13}C$ ratio, which was then compared to the ratio measured from the Oxalic Acid I standard (NBS SRM 4990). Error for ^{14}C analysis of bulk samples is ±10‰. A Finnigan MAT 251 Isotope

Ratio Mass Spectrometer was used to measure $^{13}C/^{12}C$ ratios, which were expressed as $\delta^{13}C$ with respect to Vienna Pee Dee Belemnite.

4.2.9 Total organic carbon concentration[2]

Solid samples were dried at 60°C and finely ground using a pre-combusted carbon-free ceramic mortar and pestle. Total carbon (TC) and total inorganic carbon (TIC) were measured with a Shimadzu TOC-L Total Organic Carbon Analyzer with a solid sampler SSM-5000A attachment (Mandel Scientific). TOC values were obtained by subtracting TIC values from TC values.

4.2.10 Biofilm units

Porous Teflon tubes packed with pre-combusted glass wool were suspended at the bottom of pre-existing monitoring wells with 1.53m screens at the bottom. Biofilm units were suspended in eight wells from July to September 2013. Of these eight, three (wells W2, 5D, and 6A) yielded sufficient PLFA for radiocarbon analysis. Well locations are shown in Figure 1.2. Well W2 was 2m deep (top of sandcap), well 6A was 8m deep (bottom of sandcap) and well 5D was 16m deep (CT). Upon retrieval, the units were wrapped in precombusted aluminum foil, packed individually in sterile Whirlpak bags, and stored in a cooler on ice until final storage at -20°C was possible. PLFA extraction and identification were performed by the same methods as described above. Because the quantity of FAMEs extracted were small and there was little concerned about co-eluted humic acids, FAMEs were sent to NOSAMS for ^{14}C analysis, as described above, without having gone through PCGC purification.

4.2.11 Dissolved organic carbon

Water samples were collected in September 2013 from monitoring wells at the Sandhill Fen site. Wells chosen for $\Delta^{14}C_{DOC}$ analysis were those in which biofilm units had sufficient growth for analysis of $\Delta^{14}C_{PLFA}$

[2] Analysis by Michelle Reid

(wells 6A, W2, and 5D), plus one additional well, 5C, which was 8m deep and located next to 5D. To sample, each well was purged by pumping approximately 3x the well volume out using dedicated polyethylene tubing and inertial lift (Waterra) pump systems. Sample water was then collected from the well into pre-combusted glass bottles using this pump system. Sample bottles were kept on ice until they could be moved to storage at -20°C (maximum ~6 hours on ice). Water was thawed at 4°C and filtered through pre-combusted glass microfiber 0.7µm filters (Whatman grade GF/F) using a vacuum filtration setup, then freeze-dried using a 4K BT XL-105 desktop model VIRTIS freeze dryer. Solids left behind by freeze-drying were collected, treated with HCl, and sent to the Center for Applied Isotope Studies at the University of Georgia, as described above.

4.3 Results

4.3.1 PLFA concentration

Average concentration of PLFA in units of g PLFA per g dry material extracted for each sample can be seen in Table 4.1, along with estimated biomass in cells per gram dry material, calculated using a conversion factor of $4.0X10^4$cells/pmol PLFA (averaged from conversion factors published in Green and Scow (2000)). Relative standard deviation were obtained by dividing the standard deviation of duplicate extractions by the average PLFA concentration of those extractions. PLFA concentrations range from $3.24x10^2$ to $2.77x10^3$ng/g in the sand and $1.02x10^4$ to $4.44\ x10^4$g/g in the peat. The Mann-Whitney U Test shows that PLFA concentrations in the sand samples are significantly different from those in the peat samples ($p=1.034x10^{-4}$; see Table 3.2 in chapter 3). Due to the high variation within extraction replicates, results from non-parametric statistical testing as summarized in Table 3.2 (chapter 3) show that variations within each group (6A sand, 6A peat, SV sand, SV peat) do not achieve statistical significance and can be attributed to natural variation and/or heterogeneity within the sites.

4.3.2 Total Organic Carbon Concentration

The concentration of TOC ranged from 7.7mg/g to 45.5mg/g in sand samples, and 18.0mg/g to 301.5mg/g in peat samples. Results are displayed in Table 4.1, along with standard error of measurements.

4.3.3 Stable carbon isotopes

Stable carbon isotope signatures of TOC, Ext-res, and individual fatty acids are presented in Table 4.3 (sand samples) and Table 4.4 (peat samples). This analysis was not performed on samples from July 2011 due to insufficient PLFA yield. The majority of $\delta^{13}C_{TOC}$ and $\delta^{13}C_{Ext\text{-}res}$ values were between -29‰ and -26‰, with a few ranging as high as -20.9‰. In 6A sand Aug 2012, $\delta^{13}C_{Ext\text{-}res}$ was 2.1‰ above $\delta^{13}C_{TOC}$, while in SV peat Aug 2012 it was 6.6‰ below. In all other samples $\delta^{13}C_{TOC}$ and $\delta^{13}C_{Ext\text{-}res}$ were equal within error. Individual FAMEs had $\delta^{13}C$ values ranging from -37.1‰ to -23.0‰. The fatty acid with the most depleted $\delta^{13}C$ was 14:0 in all cases, while the fatty acid with the least depletion varied. $\delta^{13}C$ of individual PLFA ranged from 0‰ to 14.7‰ depletion as compared to $\delta^{13}C_{TOC}$, and 0‰ to 8.1‰ depletion compared to $\delta^{13}C_{Ext\text{-}res}$.

4.3.4 TOC and Ext-res Radiocarbon

All radiocarbon results are summarized in Table 4.1 and Figure 4.1. TOC and Ext-res in sand samples were primarily petroleum derived, while TOC and Ext-res in peat samples had $\Delta^{14}C$ in the range expected for peat-derived carbon. Most sand samples had $\Delta^{14}C_{TOC}$ below -850‰ and $\Delta^{14}C_{Ext\text{-}res}$ below -715‰. The one exception to this was SV sand Aug 2012, which had a more modern $\Delta^{14}C_{TOC}$ of -423‰ and $\Delta^{14}C_{Ext\text{-}res}$ of -360‰. $\Delta^{14}C_{Ext\text{-}res}$ was higher than $\Delta^{14}C_{TOC}$ in all sand samples.

In peat, $\Delta^{14}C_{TOC}$ ranged from -504‰ to -190‰, and $\Delta^{14}C_{Ext\text{-}res}$ ranged from -389‰ to -180‰. 6A peat $\Delta^{14}C_{TOC}$ becomes increasingly modern between August 2012 and July 2013, at -411% and -190‰, respectively, dropping to -309‰ in September 2013. SV TOC shows a pattern of increase only, with $\Delta^{14}C_{TOC}$

rising from -504‰ to -280‰ over the four sequential sampling times between August 2012 and September 2013. $\Delta^{14}C_{Ext\text{-}res}$ was higher than $\Delta^{14}C_{TOC}$ in all peat samples except SV peat Sept 2013, in which $\Delta^{14}C_{Ext\text{-}res}$ was lower than $\Delta^{14}C_{TOC}$ by 76‰.

4.3.5 PLFA Radiocarbon

Radiocarbon data from PLFA also displays this division in $\Delta^{14}C$ between sand and peat. PLFA in most sand samples had $\Delta^{14}C$ below -900‰, except in SV sand Aug 2012, where $\Delta^{14}C_{PLFA}$ was -483‰, a value below this site's $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{Ext\text{-}res}$. Radiocarbon analysis could not be performed on PLFA extracted from sand at site 6A in Aug or Nov 2012, or SV July 2011, because the quantity of PLFA extracted was insufficient. Generally $\Delta^{14}C_{PLFA}$ in peat samples was higher than $\Delta^{14}C_{Ext\text{-}res}$ by values between 2‰ and 82‰. The one exception was SV peat Aug 2012, in which $\Delta^{14}C_{PLFA}$ was lower than $\Delta^{14}C_{Ext\text{-}res}$, but still higher than $\Delta^{14}C_{TOC}$.

4.3.6 PCGC testing

In two samples with high percentages of long-chain fatty acids and large quantities of total fatty acids extracted (6A peat Sept 2013 and SV peat Aug 2012), PCGC was used to separate short (<20 carbon) FAMEs from long (≥20 carbon) FAMEs in order to assess whether the source of short and long chain FAMEs could be distinguished from $\Delta^{14}C$. In these samples, $\Delta^{14}C$ of total PLFA was obtained using the following mass balance equation:

$$\Delta^{14}C_{total} = (1 - f_{long}) * \Delta^{14}C_{short} + f_{long} * \Delta^{14}C_{long}$$

where f_{long} is the fraction of total PLFA with long chains (≥20 carbon).

Results of PCGC testing, wherein a portion of the extract was purified by PCGC while another portion was sent for ^{14}C analysis without PCGC purification, can be seen in Table 4.6. For two samples, 6A peat Aug 2012 and SV peat Sept 2013, all FAMEs that went through the PCGC step were collected in a single fraction. The "PCGC" and "non-PCGC" fractions of 6A peat Aug 2012 are the same within ±20‰, the

conventional error associated with PLFA extraction and AMS measurement. For two samples, 6A peat Aug 2012 and SV peat Sept 2013, the preparative fraction collector was used to separate FAMEs into two fractions, "PCGC short" FAMEs with <20 carbons and "PCGC long" with ≥20 carbons, as discussed above, along with "non-PCGC". In both samples, short FAME fractions had higher $\Delta^{14}C$ than long. In 6A peat Sept 2013, the non-purified portion had a higher $\Delta^{14}C$ than FAMEs which had gone through PCGC purification. In SV peat Aug 2012, non-purified FAMEs had lower $\Delta^{14}C$ than short or long FAME fractions.

4.3.7 DOC and Biofilms

Radiocarbon data for DOC and biofilm PLFA is summarized in Table 4.5, and locations can be seen in Figure 1.2. Well W2 extends 2m from the surface into the sandcap layer, and DOC from this well has a $\Delta^{14}C$ of -230‰. PLFA extracted from this well's biofilm unit have a $\Delta^{14}C$ of -206‰, within error of $\Delta^{14}C_{DOC}$. Well 6A has a depth of 8m, reaching approximately the bottom of the sand layer; however, there is evidence of a leak in 6A that has allowed water from near the surface to populate the well, and thus it should not be taken as representative of the water conditions at 8m depth in the fen. Well 6A has $\Delta^{14}C_{DOC}$ of -353‰ and $\Delta^{14}C_{PLFA}$ of -97‰. Well 5C also extends 8m, and has a $\Delta^{14}C_{DOC}$ value of -904‰. Unfortunately, extraction of PLFA from the biofilm unit in 5C did not yield enough material for radiocarbon analysis. Well 5D is located next to 5C and extends 16m, reaching into the CT layer, with $\Delta^{14}C_{DOC}$ value of -878‰ and $\Delta^{14}C_{PLFA}$ of -660‰.

4.4 Discussion

4.4.1 Carbon of bulk materials

With one exception, sand samples had $\Delta^{14}C_{TOC}$ values that are close to the -1000‰ expected for petroleum hydrocarbons, as can be seen in Table 4.1 and Figure 4.1. This is consistent with the spent sand used to create the sand cap containing residual bitumen as only 88% to 95% of bitumen is recovered during hot water extraction (Masliyah et al., 2004). However, petroleum compounds may

also be making their way up from the CT, as researchers at the site report bubbles of bitumen-like material on the surface of the sand cap (L.A. Warren, personal correspondence). The one exception to this is SV sand Aug 2012, in which $\Delta^{14}C_{TOC}$ is much more modern, in the range observed for peat samples (Table 4.1, Figure 4.1). Peat and vegetation were placed atop the sand layer between sampling dates in summer 2011 and summer 2012. Based on the radiocarbon results it appears that this peat material was transported and became the dominant carbon soruce in SV sand Aug 2012. There is evidence of some input of more modern carbon at site 6A as well where the $\Delta^{14}C_{TOC}$ measured in November 2012 are shifted slightly to more modern values.

The $\Delta^{14}C_{TOC}$ of peat samples were much more modern than the $\Delta^{14}C_{TOC}$ of the sand with a mean value of -349±103‰. Peat is composed of partially decayed vegetation and other organic matter that accumulates over long periods of time, on the order of hundreds to thousands of years (Cumulative Environmental Management Association, 2007). The mean radiocarbon contents of the peat are consistent with this as they are equivalent to a radiocarbon age of ~3400 years. In natural systems, the $\Delta^{14}C$ of peat becomes depleted with depth, as the age of the material increases, so that $\Delta^{14}C$ can be used to investigate the time periods over which peat deposition occurred (Chasar et al., 2000). Peat used in the Sandhill fen reclamation was sourced from nearby areas slated to undergo mining and deposited on the reclamation site as a mixture rather than aggrading naturally. Because of this, peat $\Delta^{14}C$ cannot be assumed to have any particular pattern with depth.

Table 4.1: PLFA concentration, estimated biomass (calculated using averaged conversion factor from Green & Scow (2000)), total organic carbon concentration, and $\Delta^{14}C$ values of TOC, Ext-Res and PLFA.

			PLFA Conc. (ng/g)	Biomass (cells/g)	Relative Std. Dev.	TOC (mg/g) ±SE	$\Delta^{14}C$ (‰)		
							TOC	Ext-res	PLFA
6A	Sand	Jul-11	$6.3x10^2$	$8.3x10^7$	N/A	8.7±0.2	-920	-716	-904
		May-12	N/A	N/A	N/A	N/A	N/A	N/A	-920
		Aug-12	$4.4x10^2$	$5.8x10^7$	8%	12.7±0.6	-914	-875	N/A
	Peat	Nov-12	$1.6x10^3$	$2.1x10^8$	28%	45.5±3.5	-852	-764	N/A
		Aug-12	$4.4x10^4$	$6.1x10^9$	42%	18.0±0.9	-411	-321	-245
		Jul-13	$2.7Ex10^4$	$3.7x10^9$	N/A	63.4±2.6	-190	-180	-147
		Sep-13	$2.6x10^4$	$1.7x10^9$	14%	103.0±8.4	-309	-245	-202
SV	Sand	Jul-11	$3.2x10^2$	$4.6x10^7$	N/A	N/A	-983	N/A	N/A
		Aug-12	$2.8x10^3$	$4.1x10^8$	34%	7.7±0.0	-423	-360	-483
	Peat	Aug-12	$1.5x10^4$	$2.0x10^9$	31%	47.0±0.9	-504	-259	-277
		Nov-12	$2.6x10^4$	$3.4x10^9$	9%	230.9±1.1	-417	-389	-324
		Jul-13	$1.3x10^4$	$1.7x10^9$	N/A	262.8±29.7	-335	-333	-251
		Sep-13	$1.0x10^4$	$1.4x10^9$	73%	301.5±39.7	-280	-356	-354

Variations in the peat age due to this mixing may easily explain the observed range of $\Delta^{14}C_{TOC}$ values. However, if all the OC in a given sample is from the same source, it would be expected that the TOC and Ext-res values are the same. For all but one of the samples, the $\Delta^{14}C_{Ext\text{-}res}$values are more modern than the $\Delta^{14}C_{TOC}$ values. While this may be related to the mixing noted above, it may also be that some petroleum hydrocarbons were present in the peat material. Mass balance calculations were performed to assess the percent of organic carbon in peat samples that may be related to petroleum inputs. If it is assumed that all petroleum hydrocarbons were extracted by the Bligh and Dyer organic solvent extraction then the Ext-res is representative of natural organic matter (NOM) in peat. This assumption is based on White et al. (2005), which found that petroleum hydrocarbons were completely removed during solvent extraction from salt marsh sediment, and that $\Delta^{14}C_{Ext\text{-}res}$ reflected the residual organic carbon. This definition of Ext-res has been used in subsequent studies of tailings ponds and organic-contaminated sediments (Ahad and Pakdel, 2013; Mahmoudi et al., 2013 a; Slater et al., 2005).

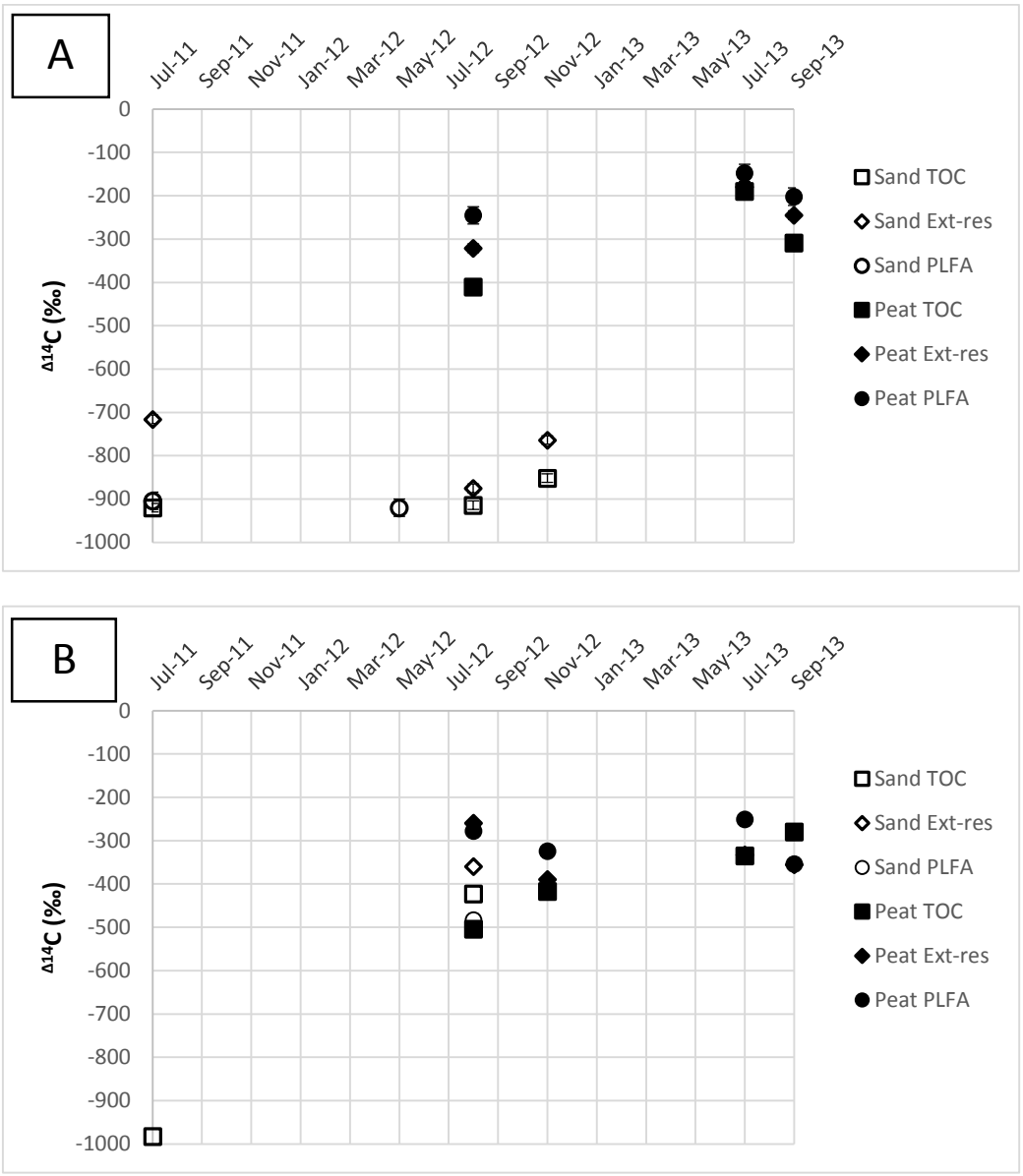

Figure 4.1: Radiocarbon signatures of total organic carbon (TOC), extracted residue (Ext-res) and PLFA at the two sites (A: site 6A, B: sump vault site) over the course of fen development. The uncertainty for $\Delta^{14}C$ was ±10‰ for TOC and Ext-res, and ±20‰ for PLFA.

Equation A below is the mass balance equation wherein *x* is the fraction of total organic carbon that is petroleum-based, and $\Delta^{14}C_{petroleum}$=-1000‰. $\Delta^{14}C_{Ext\text{-}res}$ values used are those measured for each sample. Equation B below shows the mass balance equation rearranged to solve for *x*:

A $$\Delta^{14}C_{TOC} = x\left(\Delta^{14}C_{petroleum}\right) + (1-x)(\Delta^{14}C_{Ext-res})$$

B $$x = \frac{\Delta^{14}C_{TOC} - \Delta^{14}C_{Ext-res}}{\Delta^{14}C_{petroleum} - \Delta^{14}C_{Ext-res}}$$

Results of this calculation for peat samples can be seen in Table 4.2. The sample with the highest percentage of ancient carbon was SV peat from August 2012, in which approximately one third of the TOC appears to have come from ancient carbon sources. Based on the nature of this reclamation site, the ancient carbon present is likely petroleum carbon. Whether this carbon originates from the CT and sand layers below the wetland, or was already present in the peat when it was collected from a soon-to-be mined site, is unknown.

Table 4.2: Calculated contribution of ancient carbon to total organic carbon in peat samples.
*The negative value in SV peat Sept 2013 is a result of $\Delta^{14}C_{TOC}$ being higher than $\Delta^{14}C_{Ext\text{-}res}$ in that sample; see discussion.

		% Ancient Carbon
Aug-12	6A peat	13%
Jul-13	6A peat	1%
Sep-13	6A peat	8%
Aug-12	SV peat	33%
Nov-12	SV peat	5%
Jul-13	SV peat	0%
Sep-13	SV peat	-12%*

In this system it is possible that there was an additional carbon input of recently photosythesized compounds from plant roots and their exudates. Any carbon from this source would have a $\Delta^{14}C$ higher than 0‰, since photosynthetic compounds are formed from atmospheric CO_2 which is enriched in ^{14}C due to testing of nuclear bombs in the mid-20th century (Hua et al., 2013). If this was the case, the

calculated percent petroleum carbon in TOC is an underestimation, as more radiocarbon-dead compounds would be required to offset the contribution of radiocarbon-rich plant matter. The large difference between $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{Ext\text{-}res}$ (>200‰) in 6A sand July 2011 means that some non-petroleum organic matter was present at in 6A sand before the introduction of peat or vegetation, though the source of this more modern carbon is unknown. The nonsensical calculated value of -12% petroleum carbon in SV peat from Sept 2013 is a result of the $\Delta^{14}C_{Ext\text{-}res}$ having a lower value than $\Delta^{14}C_{TOC}$. This may be explained by the presence of a large amount of recently photosynthesized carbon compounds which can be extracted by organic solvents, and therefore influence $\Delta^{14}C_{TOC}$ but not $\Delta^{14}C_{Ext\text{-}res}$. Despite these minor departures, there is strong evidence from radiocarbon data that petroleum hydrocarbons dominate the TOC in sand and peat-derived organic compounds dominate TOC in peat, with some input of petroleum carbon to the peat.

The $\delta^{13}C_{TOC}$ and $\delta^{13}C_{Ext\text{-}res}$ of most samples are -29‰ to -26.1‰ (see Tables 4.3 & 4.4), within the range expected for C_3 plants, but also close to the $\delta^{13}C$ of Athabasca oil sands bitumen, which is -29.6‰ (Jha et al., 1979). The two samples from Nov 2012, one of which is sand and the other peat, have TOC and Ext-res $\delta^{13}C$ values between -20.9‰ and -24.2‰, suggesting some contribution from carbon sources besides C_3 plant matter or bitumen. Autotrophs that use the C_4 carbon fixation pathway, including some algae and grasses (Ehleringer and Cerling, 2002) have an average $\delta^{13}C$ of -13‰ (Glaser, 2005). As described in chapter 3, no lipid biomarkers of algae were found in Nov 2012 samples. Although C_4 grasses are rare in cool climates such as would be experienced for much of the year in northeastern Alberta, they have been found in boreal fens at approximately the same latitude as the reclamation site (Kubien and Sage, 2003), so it is possible that they are present in the fen and have influenced the $\delta^{13}C$ in the two November 2012 samples. The sand sample from SV in Aug 2012 has a $\delta^{13}C_{TOC}$ value of -22.4‰, but a $\delta^{13}C_{Ext\text{-}res}$ of -29.0‰, which could be a result of the presence of carbon compounds from C_4 plants which were extracted by organic solvents and therefore not influencing the Ext-res. This sand sample

was also anomylous in that it had $\Delta^{14}C_{TOC}$ in the range of peat, as discussed above, so that carbon isotope data is unable to resolve the carbon inputs at this site.

4.4.2 Microbial Carbon Sources

In general, $\Delta^{14}C_{PLFA}$ show that microbial communities are utilizing carbon predominantly derived from the carbon source which dominates the TOC. Sand from 6A in July 2011 had a $\Delta^{14}C_{PLFA}$ of -904‰, within error of the $\Delta^{14}C_{TOC}$, showing that microbes in the sand were metabolizing the petroleum carbon rather than NOM. This could be because microbes present were types more suited to degradation of petroleum hydrocarbons and therefore preferentially metabolizing older carbon, or perhaps only a small proportion of carbon was from NOM. An additional $\Delta^{14}C_{PLFA}$ analysis was performed on a sample from May 2012, for which TOC and Ext-res data were not available, which had a $\Delta^{14}C_{PLFA}$ within error of the previous sample, showing microbes at this time point utilizing petroleum carbon. $\Delta^{14}C_{PLFA}$ in SV sand Aug 2012 is lower than both $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{Ext\text{-}res}$. Bulk carbon in this sample was unexpectedly modern, so the $\Delta^{14}C_{PLFA}$ may be explained by the microbial community being composed primarily of petroleum-metabolizing microbes, having not yet shifted towards microbes suited to metabolism of compounds in peat and recently made plant material. $\Delta^{14}C$ measurements are of bulk PLFA, so it may also be that some PLFA reflect $\Delta^{14}C$ values nearer the $\Delta^{14}C_{TOC}$, while some individual PLFA pull the bulk $\Delta^{14}C_{PLFA}$ downward. In the future, this could be explored with large-scale extractions and isolation of individual PLFA for $\Delta^{14}C$ analysis, as was done in multiple studies (Slater et al., 2005; Druffel et al., 2010; Ziolkowski et al., 2013; Cowie et al., 2009; Cowie et al., 2010), including one on microbes in oil sands tailings ponds (Ahad and Pakdel, 2013). Using a mass balance equation, as was done above for peat samples, shows that carbon in the sample was 10% petroleum-based, so these compounds were available for microbial metabolism.

The more modern carbon preferred by microbes may have been from compounds within the NOM as we have defined it above, or from recently photosynthesized matter that can be extracted from the TOC by organic solvents. Preferential metabolism of younger carbon over petroleum hydrocarbons has been reported at a contaminated salt marsh site by Slater et al. (2005), though in that case PLFA were not more enriched than Ext-res. Ahad and Pakdel (2013) reports preferential uptake of modern carbon by microbes in oil sands tailings ponds, and Mahmoudi et al. (2013 b) found evidence of microbial preference for modern carbon at a site contaminated with polycyclic aromatic hydrocarbons. Lability of carbon compounds is related to the recalcitrance of their chemical structure as well as their bioavailability. Hydrophobic organic molecules that sorb to solid particles are less available for microbial degradation than those that remain in the aqueous phase (Schwarzenbach et al., 2002), and recently photosynthesized compounds often contain functional groups that make them hydrophilic and therefor more bioavailable.

In SV peat Aug 2012, $\Delta^{14}C_{PLFA}$ was 17‰ below $\Delta^{14}C_{Ext\text{-}res}$. This is within the analytical error associated with ^{14}C analysis, so it can be interpreted as microbes metabolizing NOM without preference for modern carbon in this sample. Overall, microbes metabolize petroleum carbon in environments when it is the predominant carbon source, and metabolize younger carbon compounds within peat when peat carbon is predominant.

4.4.3 PLFA concentration

Patterns and variation in PLFA concentrations are discussed in detail in chapter 3. Table 4.1 summarizes PLFA concentration and biomass data for each sample. The relationship between $\Delta^{14}C_{TOC}$ and PLFA concentration is presented in Figure 4.2A. With the exception of sand at SV in August 2012, which had $\Delta^{14}C_{TOC}$ in the range of peat, data points clustered into two distinct groups: sand, with low PLFA concentration and radiocarbon signatures indicating mostly ancient carbon, and peat with higher PLFA

concentration and more enriched $\Delta^{14}C_{TOC}$. Figure 4.2B shows the relationship between the PLFA concentration and the amount of organic carbon available. Sand samples had low concentrations of TOC and PLFA, while peat samples had a wide range of TOC concentration, from 18.0mg/g to 301.5mg/g, which seemed to have no impact of PLFA concentration. The highest population size occurs in 6A peat Aug 2012, which has the lowest TOC concentration of any peat sample. These results show that the age of carbon available, rather than the amount, was connected to the size of the microbial population. In peat, labile compounds are degraded and the more recalcitrant compounds left behind age and become depleted in $\Delta^{14}C$. At this site, we hypothesize that the energetic advantage of metabolizing more labile carbon compounds drives the growth of microbial populations. The source of microbes present in the peat is unknown; they may have been present in the peat when it was collected from its original location, or they may have moved up from the sand layer. Cluster analysis in chapter 3 shows that sand and peat populations cannot be distinguished on the basis of PLFA profile, as no distinct sand vs. peat clusters formed when subjected to hierarchical cluster analysis (see Figure 3.7 in chapter 3).

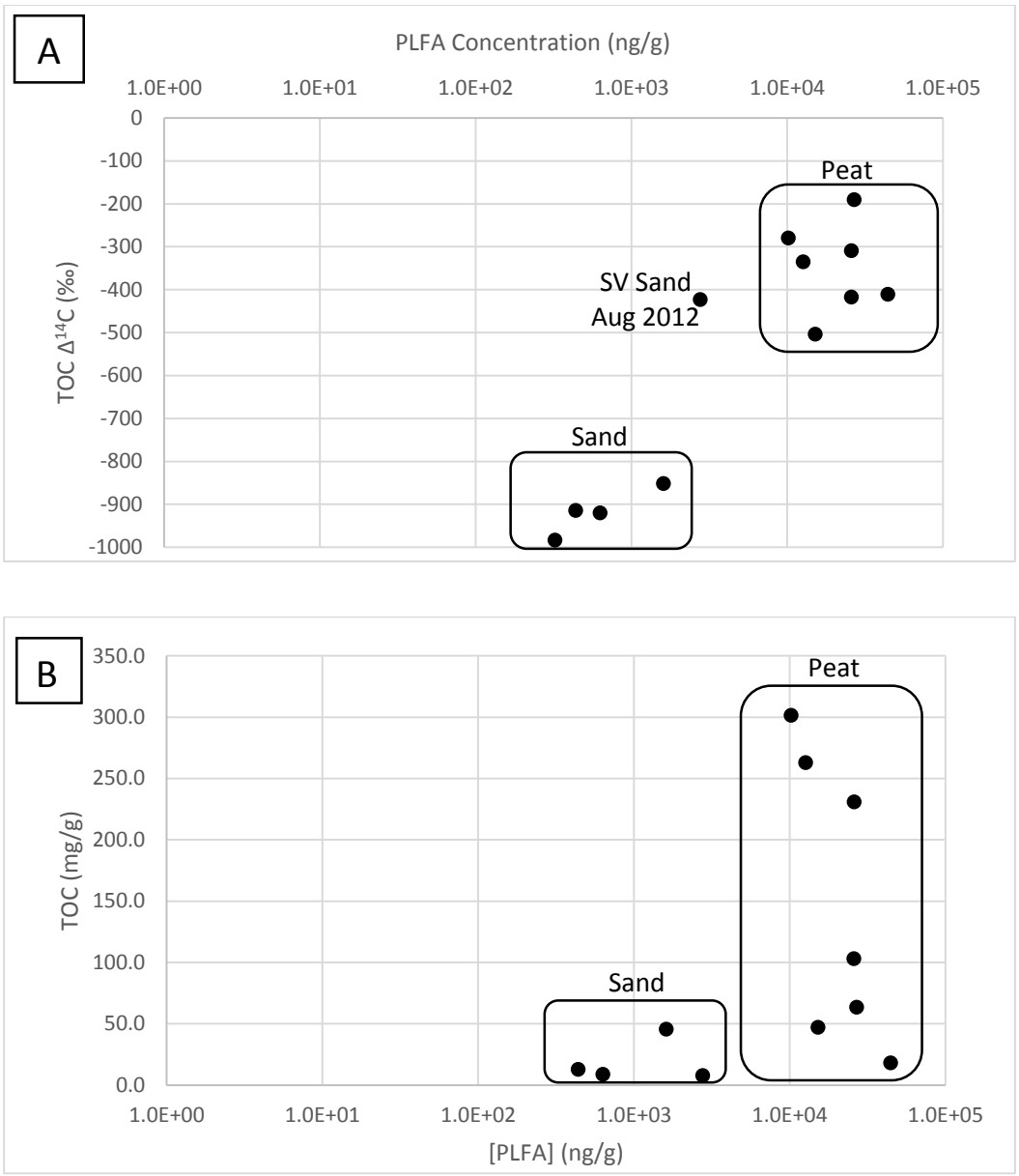

Figure 4.2: PLFA concentration plotted against (A) $\Delta^{14}C_{TOC}$ (B) TOC concentration. Note that PLFA concentration appears on the x-axis in both plots.

4.4.4 Insights into Microbial Respiration

While unable to differentiate carbon sources due to overlap in $\delta^{13}C$ between petroleum hydrocarbons and peat, depletion of $\delta^{13}C$ relative to predominant carbon source during metabolism can give insights as to

whether organisms are respiring aerobically or anaerobically. Depletion of individual PLFA compared to carbon source is due to fractionation during carbon assimilation and lipid synthesis. Stable isotope signatures of PLFA of heterotrophic organisms under aerobic conditions are usually within 6‰ of the carbon source (Boschker et al., 2005; Blair et al., 1985; Monson and Hayes, 1982; DeNiro and Epstein, 1977) while heterotrophs under anaerobic conditions have been found to have PLFA more depleted than their carbon source by up to 14‰ (Londry et al., 2004). In sand samples, radiocarbon data gives evidence that the microbial carbon source was TOC, so it can be assumed that $\delta^{13}C_{TOC}$ is that of the microbial carbon source, and the fractionation can be calculated by subtracting $\delta^{13}C_{TOC}$ from $\delta^{13}C_{PLFA}$. Results of this are shown in Table 4.3. In 6A sand Aug 2012, 10% of the PLFA for which $\delta^{13}C$ could be measured had fractionation of less than 6‰, and the remainder had fractionation of more than 6‰ compared to TOC, indicating that microbial respiration here is primarily anaerobic. In 6A sand Nov 2012, 60% of PLFA have a fractionation of >6‰. This could be a result of a mix of aerobic and anaerobic metabolism taking place, or of different amounts of fractionation occurring in the synthesis of different lipids (Hayes, 2001). In SV sand Aug 2012, all $\delta^{13}C_{PLFA}$ are depleted more than 6‰ compared to $\delta^{13}C_{TOC}$, indicating anaerobic metabolism. Interpretation of stable isotope data from the peat samples is more difficult because the radiocarbon data shows that microbes were metabolizing more modern carbon than the overall TOC or NOM in most samples, so that $\delta^{13}C_{TOC}$ or $\delta^{13}C_{Ext\text{-}res}$ cannot be assumed to represent the carbon source. Glaser (2005) summarized the range of ^{13}C signatures of C3 plants from multiple studies and recommended -27‰ as the mean value to use "for practical purposes". If this value is taken as the $\delta^{13}C$ of the microbial carbon source, all PLFA for which $\delta^{13}C$ could be measured in SV peat July 2013 had fractionation of <6‰, indicating aerobic respiration only. In all other peat samples, between 20% and 40% of PFLA in peat samples for which $\delta^{13}C$ were measured have ^{13}C fractionation of more than 6‰, as can be seen in Table 4.4, which could again be interpreted as being a result of both aerobic and anaerobic degradation taking place at the sample sites. However, $\delta^{13}C$ of C3 plants ranges from -22‰ to -33‰

(Bender, 1971; Glaser, 2005), and the specific $\delta^{13}C$ of plant matter and other organics in peat at Sandhill fen are unknown. If the carbon source is -22‰, all PLFA from peat samples have fractionation >6‰; if the carbon source is -33‰, all PLFA from peat samples have fractionation ≤6‰. A firm conclusion cannot be reached without knowing the specific carbon sources being taken up by microbes.

Table 4.3: Stable carbon isotope signatures (‰) of TOC, Ext-res and individual PLFA from sand samples. Highlighted boxes are those in which $\delta^{13}C_{PLFA}$ is more than 6‰ below $\delta^{13}C_{TOC}$. Error is ±1‰.

		6A Sand		SV Sand
		Aug-12	Nov-12	Aug-12
	TOC	-26	-23	-22
	Ext-res	-27	-21	-23
PLFA	14:0	-29	-33	-37
	i15:0	-28	-28	-28
	a15:0	-26	-27	-29
	16:1	-29	-	-33
	16:0	-29	-	-34
	10Me16:0	-29	-30	-
	cy17:0	-31	-	-33
	18:1c	-	-31	-
	18:1t	-30	-	-30
	18:0	-28	-30	-30
	cy19:0	-28	-29	-30
	20:0	-31	-	-29
	br-21:0	-33	-	-35
	22:0	-	-	-32
	24:0	-	-	-34

Table 4.4: Stable carbon isotope signatures (‰) of TOC, Ext-res, and individual PLFA from peat samples. Highlighted boxes are those in which $\delta^{13}C_{PLFA}$ is more than 6‰ below -27‰, the average $\delta^{13}C$ of C3 plants and presumed signature of microbial carbon source. Error is ±1‰.

		6A Peat			SV Peat			
		Aug-12	**Jul-13**	**Sep-13**	**Aug-12**	**Nov-12**	**Jul-13**	**Sep-13**
	Avg C3 plant	-27	-27	-27	-27	-27	-27	-27
	TOC	-28	-27	-26	-28	-24	-28	-28
	Ext-res	-27	-27	-27	-27	-23	-27	-27
PLFA	14:0	-35	-34	-36	-36	-	-	-36
	i15:0	-28	-30	-30	-27	-29	-30	-29
	a15:0	-29	-30	-31	-28	-30	-30	-30
	br-16:0	-	-30	-31	-	-	-	-
	16:1	-	-33	-31	-	-	-	-
	16:0	-33	-33	-33	-32	-35	-33	-
	10Me16:0	-	-31	-	-	-33	-32	-31
	i17:0	-29	-29	-29	-	-28	-	-
	a17:0	-29	-	-	-	-24	-	-30
	cy17:0	-33	-31	-31	-	-35	-33	-
	18:1c	-	-	-	-	-	-	-
	18:1t	-	-	-	-	-31	-31	-
	18:0	-30	-31	-29	-29	-	-33	-
	cy19:0	-31	-32	-30	-	-32	-30	-31
	20:0	-	-35	-29	-	-	-	-
	br-21:0	-35	-	-	-	-35	-	-
	22:0	-	-	-31	-	-	-	-
	24:0	-	-	-33	-	-	-	-33
	26:0	-	-	-34	-	-	-	-

4.4.5 Transport and metabolism of dissolved carbon

Table 4.5: Radiocarbon of DOC and PLFA in sampling wells. *Note that well 6A is believed to have leaked, so $\Delta^{14}C_{DOC}$ values should not be taken as representative of 8m depth.

		$\Delta^{14}C$ (‰)	
Well	**Depth (m)**	**DOC**	**PLFA**
W2	2	-230	-206
6A	8*	-353	-197
5C	8	-904	N/A
5D	16	-878	-660

The transport of modern carbon from the fen surface into sand and CT layers is of interest because of its potential to drive growth of microbial populations and increase biogenic production of $H_2S_{(g)}$ at the reclamation site. Modern compounds from the surface can be carried deeper in natural fens in the form of dissolved organic carbon (DOC) advecting with groundwater infiltrating the system (Chasar et al., 2000). $\Delta^{14}C_{DOC}$ and $\Delta^{14}C_{PLFA}$ from sampling wells and biofilm units are presented in Table 4.5. Well W2 has a depth of 2m, reaching into the top of the sand cap, and its $\Delta^{14}C_{DOC}$ is within the same range as is found in peat TOC. Well 5C reaches near the bottom of the sand cap with a depth of 8m, and 5D extends into the CT layer with a depth of 16m. $\Delta^{14}C_{DOC}$ at 8m and 16m depths shows predominantly ancient carbon. Mass balance calculations with the $\Delta^{14}C$ of modern carbon set to 0‰ show that modern carbon makes up 10% and 12% of dissolved organic carbon at 8m and 16m, respectively. Previous investigation of CT from the unreclaimed Kingfisher site adjacent to Sandhill Fen found $\Delta^{14}C_{TOC}$ values corresponding to 1% modern carbon at 4m depth and 8% modern carbon at 32m depth. The presence of modern carbon despite the lack of peat and vegetation on the surface means that some more modern carbon found the CT layers of the Sandhill site may have been present previous to wetland construction. However, downward movement of modern carbon would also be consistent with carbon transportation found in natural wetland systems (Chasar et al., 2000). It may be that modern carbon in sand and CT layers is there because of a combination of transportation from the surface wetland and other unconstrained factors.

$\Delta^{14}C_{PLFA}$ from the biofilm unit at 16m depth was -660‰, equivalent to 34% modern carbon, showing preferential metabolism of the modern carbon available in the same manner as occurred in peat samples as discussed above. It could be that rapid microbial uptake of any available more modern carbon at this depth keeps the amount of more modern carbon in the DOC low. In well W2, $\Delta^{14}C_{PLFA}$ matches $\Delta^{14}C_{DOC}$, meaning that microbes at 2m depth are metabolizing the DOC with no preference for more modern carbon. Although well 6A is considered not to be representative of conditions at 8m depth because of the leaky pipe, it does still contain evidence of microbial preference for modern carbon over more petroleum-based carbon in the DOC that was available to them. This pattern of microbial preference for modern carbon transported below the fen surface in the form of DOC is consistent with the findings in a naturally occurring fen studied by Chasar et al. (2000). Studies of microbial uptake of carbon in marine systems using naturally abundant radiocarbon have also found preferential uptake of more modern carbon within DOC (Cherrier et al., 1999; Pearson et al., 2001).

4.4.6 PCGC purification tests

Preparative capillary gas chromatography (PCGC) is usually used to separate specific compounds for further analysis – for example, isolation of a particular FAME or group of FAMEs for ^{14}C analysis was first described by Eglinton et al. (1996) and has been used in multiple studies (Ahad and Pakdel, 2013; Pearson et al., 2001; Druffel et al., 2010). In the present study, PCGC was instead used as a means of purifying the total PLFA extract of any potential contaminants that could be present. These would primarily be any potential compounds that cannot be detected or identified by GC-MS and could potentially influence the ^{14}C reading. The reason to undertake bulk analysis is that analysis of all PLFA together requires the extraction of much less sample material to get sufficient quantities for ^{14}C analysis, compared to analysis of isolated PLFA, and gives an overview of the carbon sources utilized by the whole microbial community. In order to test whether PCGC purification impacts $\Delta^{14}C$, tests were run in which samples were split, with one portion undergoing PCGC purification and the other portion not. Additionally, as an exploratory

measure, the PFC was used to separate short (<20 carbon) and long (≥20 carbon) PLFA, to determine whether they differed in $\Delta^{14}C$. Results can be seen in Table 4.6. In the two samples in which all PLFA were collected from the PCGC in one fraction, the $\Delta^{14}C_{PLFA}$ of the sample with and without PCGC purification were within analytical error of each other. In the case of 6A peat Aug 2012, values with and without PCGC were almost identical, whereas in SV peat Sept 2013 values differ by 40‰, within analytical error. In the other two samples, where the portion which went through PCGC purification was split into short vs. long fractions, the long fractions have lower $\Delta^{14}C$ than short. This was a surprising result as approximately 80% of each long fraction was composed of straight-chain saturated PLFA that are associated with eukaryotes (Zelles, 1999) like plants, which would be expected to have quite modern $\Delta^{14}C$. It may be that undegraded old plant matter from the peat was co-extracted with the PLFA of the viable organisms in the samples. Further tests should be performed to test this hypothesis. In SV peat Aug 2012, the portion which did not go through PCGC purification has a lower $\Delta^{14}C_{PLFA}$ than the short or long fractions collected from the PCGC, which could be attributed to the presence of humic acids which were unable to travel through the GC. The extract without PCGC purification was faintly yellow-brown for all samples, which has been noted by Nielsen and Petersen (2000) to possibly be a result of co-extraction of humic materials, while there was no colour visible after PCGC purification. The opposite happens in 6A peat Sept 2013, where the non-PCGC portion has higher $\Delta^{14}C_{PLFA}$ than the fractions which underwent PCGC purification. The reason for this is unknown, and requires further study.

Table 4.6: Results of PCGC tests.

			PCGC $\Delta^{14}C$ (‰)			
Sample	**Date**	**No PCGC $\Delta^{14}C$ (‰)**	**Total PLFA**	**Short PLFA**	**Long PLFA**	**Mass Balanced**
6A Peat	Aug 2012	-249	-245	N/A		
SV Peat	Sept 2013	-314	-345			
6A Peat	Sept 2013	-160	N/A	-197	-220	-202
SV Peat	Aug 2012	-360	N/A	-261	-309	-277

4.5 Conclusion

In sand samples at this site, microbes metabolized the petroleum that made up the majority of carbon available to them. In the peat, microbes preferentially utilized more modern carbon than natural organic matter present in the peat. There is no evidence of degradation of petroleum carbon by microbes in the peat, though differences between $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{Ext\text{-}res}$ do suggest that petroleum was present in the peat layer. Stable carbon isotope data suggests that a mix of aerobic and anaerobic microbial respiration was taking place, though corroborating evidence is necessary for solidify this conclusion. This study shows that the age of available carbon, rather than the total amount of organic carbon, controlled the size of the microbial population on the surface of the Sandhill Fen reclamation site, with more modern carbon associated with higher microbial populations. Only a small amount of modern carbon was transported into the sand and CT layers beneath the constructed fen, but microorganisms in these deep layers did show preference for modern carbon over the more abundant petroleum carbon. It is possible that the small influx of more modern carbon from the top layer of the site to the CT will stimulate growth of microbial populations there.

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Chapter 5: Conclusion and Future Work

5.1 Conclusion

Sandhill fen is the site of a pilot-scale wetland reclamation project currently being undertaken in the Athabasca oil sands by Syncrude Canada Ltd. It consists of a constructed fen wetland on top of a deposit of composite tailings (CT), which is a substance made by mixing gypsum and sand with tailings waste from bitumen extraction. Any waste produced by oil sands mining is required to be kept on site, so Syncrude's goal is that Sandhill fen and future projects like it will not only fulfill their land reclamation mandates but also offer a permanent means of storing bitumen extraction waste. The site consists of ~35m of CT topped with 10m of sand, then a layer of clay till topped with peat. Construction occurred between 2009 and 2012. Microorganisms play crucial roles in nutrient cycling, degradation and other important processes in both natural and reclaimed environments (Harris, 2009). One way of assessing the progression of a reclamation site towards natural conditions is to use biomarkers to look at the size and composition of microbial populations as an indicator of ecosystem health (Mummey et al., 2002). Furthermore, carbon transport and microbial carbon uptake can have implications on the function and ultimate success of the reclamation project, providing impetus to study the biogeochemistry of the Sandhill fen site. The goal of this Master's project was to 1) study the microbial biomass and community composition in Sandhill Fen using phospholipid fatty acids (PLFA) to assess the progression of the constructed fen towards natural conditions, and 2) to investigate the movement of carbon within the site and its utilization by microbes at the fen surface and in deeper layers of the site using carbon isotope and PLFA analyses. Samples were taken over the course of the reclamation site's development, from July 2011 to September 2013.

PLFA are a useful tool to determine viable biomass because they are present as a major component of the cell membrane in all bacteria and eukaryotes and degrade quickly after cell death (Harvey et al., 1986; White et al., 1979), and biomarker PLFA associated with specific groups of bacteria can be used to

determine the composition of microbial communities. Comparisons of the stable carbon isotope ratios of biomolecules vs. molecular carbon source can give insights into microbial metabolism and also work in conjunction with PLFA biomarkers to identify specific types of bacteria like methanotrophs. The radiogenic carbon isotope (^{14}C) degrades over time, so petroleum carbon is ^{14}C-free while recently photosynthesized compounds reflect the atmospheric carbon isotope composition of $\Delta^{14}C$ >0‰. This makes ^{14}C a valuable tool for studying movement of carbon within the Sandhill Fen system, and since PLFA reflect the $\Delta^{14}C$ of their carbon source it can be used to investigate microbial carbon sources at the fen surface and in wells that reach into the sand and CT layers.

Biomass in the peat layer of Sandhill Fen was comparable to that found in undisturbed wetlands in the oil sands region, as well as various other fens worldwide. Large standard deviations between duplicate extractions show a high amount of heterogeneity at the site, even within the same bag of sample material, which was also consistent with other environmental studies. PLFA concentration, and by extension biomass, in sand samples was significantly lower than in peat. Radiocarbon analysis showed that biomass was more related to the age of carbon in the samples than to the total organic carbon concentration, with younger carbon in the peat associated with higher PLFA concentration. This result supports concerns that influx of modern carbon could stimulate growth of microorganisms living in the deeper layers of the reclamation site. One sand sample had $\Delta^{14}C$ values in the range of peat and a PLFA concentration intermediate between sand and peat samples, so it is possible that there are other factors in the sand that limit microbial population size. $\Delta^{14}C$ analysis showed that carbon in all other sand samples was predominantly petroleum-derived, which could be a result of residual hydrocarbons after bitumen extraction from the sand, or movement of petroleum hydrocarbons up from the CT layer. Microbes in sand were metabolizing carbon from the petroleum, as is shown by their very low $\Delta^{14}C_{PLFA}$. Comparisons of $\Delta^{14}C$ in total organic carbon (TOC) and extracted residue (Ext-res, representing natural organic matter in the peat) suggest that up to 33% of the carbon in peat samples was ancient and potentially petroleum-

derived. If there were recently photosynthesized compounds present in TOC which can be solvent extracted and thus do not influence the $\Delta^{14}C_{Ext\text{-}res}$, the percent ancient carbon in peat may be higher than calculated. $\Delta^{14}C_{PLFA}$ values in peat were generally higher than both $\Delta^{14}C_{TOC}$ and $\Delta^{14}C_{Ext\text{-}res}$, showing microbes in peat preferentially metabolizing modern carbon, either from within the Ext-res or from recently photosynthesized compounds. This means that microbes were not degrading the ancient hydrocarbons present, which could present a hazard to the functioning of the ecosystem as some chemical constituents of oil sands petroleum have been shown to be toxic to higher organisms.

One of the concerns about the impact of fen development is the presence of sulphate reducing bacteria (SRB) and their production of hydrogen sulphide gas. Most samples had PLFA biomarkers for SRB, but in small amounts, no more than 4% of total PLFA. This is comparable to undisturbed wetlands in the region. Fungal biomarkers were present in most samples, as were long-chain PLFA associated with plants, but bacteria dominate the system in terms of biomass. Hierarchical cluster analysis of PLFA profiles from Sandhill Fen samples, samples from the adjacent unreclaimed Kingfisher Fen CT deposit, and undisturbed wetlands in the oil sands region taken from literature show that communities in Sandhill Fen were more similar to CT communities than to undisturbed wetland sediments.

Samples of water and microbes collected on biofilm units were taken from sampling wells at 2m, 8m and 16m depth. At 2m depth, at the intersection of the fen and sand layers, dissolved organic carbon (DOC) and PLFA both showed $\Delta^{14}C$ within the range of peat samples, with no microbial preference for younger carbon sources. $\Delta^{14}C_{DOC}$ at 8m and 16m depth, in the sand cap and CT layers respectively, showed a small amount of modern carbon present in the deep layers of the reclamation site. Microbes collected as a biofilm at 16m depth showed preference for the more modern carbon available. Because Syncrude policy prevents deep drilling to obtain samples of CT from beneath the fen, it is unknown what impact this small influx of modern carbon and the microbial preference have had on the size of the microbial population.

5.2 Directions for Future Research

Development of the Sandhill Fen site is ongoing, and the size and composition of the microbial community can continue to be assessed as the system moves towards more natural conditions. Archaeal lipids contain ether bonds rather than ester bonds as in PLFA (Koga and Morii, 2007), so the methods used in this thesis do not provide any analysis of archaeal populations at the study site. Archaea are common in natural wetlands, particularly in anoxic zones, so characterization of these microbes is essential to have a comprehensive understanding of the microbial life in the reclamation site. Metagenomic analysis of the communities at the fen surface and in the deeper layers would complement and expand upon the work presented in this thesis. Analysis of the chemical constituents of petroleum present in peat samples can provide information on the potential impact of these chemicals on the fen ecosystem. Sampling wells of various depth are present at a number of sites within the reclamation site, and radiocarbon analysis of DOC and biofilm PLFA from these wells will further our understanding of the movement of carbon from the surface into the sand and CT layers. Studies focused on the biogeochemistry of sulphur and iron at this site have taken place alongside this thesis work which, when combined with the findings reported in this thesis, will create a more comprehensive view of the hydrological, chemical, and microbial functions taking place in the system.

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APPENDIX

Table A1: Individual PLFA composition for each sample from Sandhill Fen, averaged from duplicate extractions, in units of mole percent of total lipids extracted. bdl=below detection limit

	6A July2011 sand	6A Aug2012 sand	6A Nov2012 sand	6A Aug2012 Peat	6A July2013 Peat	6A Sept2013 Peat	SV July2011 sand	SV Aug2012 Sand	SV Aug2012 Peat	SV Nov2012 Peat	SV July2013 Peat	SV Sept2013 Peat
11:0	bdl	bdl	1	bdl	bdl	bdl	bdl	bdl	bdl	1	bdl	bdl
12:0	2	1	<1	<1	<1	1	2	bdl	1	bdl	bdl	bdl
12:1	bdl	bdl	1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
13:0	1	bdl	bdl	bdl	bdl	bdl	bdl	1	bdl	bdl	bdl	bdl
br-13:0	1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
14:0	3	4	3	2	2	3	3	2	3	2	2	4
14:1	bdl	bdl	<1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
i14:0	bdl	bdl	<1	1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
a14:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
br-14:0	1	bdl	1	bdl	<1	1	2	bdl	1	bdl	1	<1
15:0	2	2	2	1	1	1	3	2	1	1	1	1
15:1	bdl	bdl	1	bdl	bdl	1	bdl	bdl	<1	1	bdl	bdl
i15:0	4	4	3	6	8	4	4	4	4	4	5	4
a15:0	5	5	4	4	8	4	5	4	4	4	4	3
br-15:0	1	bdl	1	<1	bdl	bdl	2	bdl	bdl	bdl	bdl	bdl
16:0	13	17	16	17	18	14	17	10	14	14	16	16
16:1	14	7	10	10	9	9	9	4	5	9	4	8
i16:0	2	1	1	1	2	2	3	3	1	2	2	1
a16:0	bdl	1	1	bdl	bdl	bdl	bdl	bdl	bdl	1	bdl	bdl
br-16:0	bdl	bdl	<1	bdl	bdl	<1	bdl	bdl	1	bdl	bdl	bdl
17:0	1	2	2	2	1	1	3	3	1	1	1	<1
17:1	bdl	bdl	bdl	bdl	bdl	1	bdl	1	1	bdl	bdl	1
i17:0	1	bdl	1	bdl	1	bdl	2	bdl	1	bdl	bdl	1

a17:0	2	2	1	1	1	bdl	2	5	2	1	1	1
br-17:0	bdl	1	<1	1	<1	4	bdl	3	bdl	2	1	1
10Me16:0	2	4	2	3	3	2	2	2	2	2	3	bdl
br-17:1	1	1	2	1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
cy17:0	7	2	5	4	5	3	11	3	3	6	4	3
18:0	3	5	4	3	2	3	4	4	4	2	3	5
18:1	8	9	10	11	12	11	13	8	8	12	9	10
18:2	1	bdl	1	2	1	2	bdl	bdl	1	<1	1	2
18:3	bdl	bdl	bdl	<1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
18:4	bdl	bdl	bdl	<1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
i18:0	bdl	bdl	<1	bdl	bdl	bdl	bdl	bdl	bdl	<1	<1	bdl
a18:0	bdl	bdl	bdl	bdl	<1	bdl	bdl	1	bdl	bdl	bdl	bdl
br-18:0	bdl	2	1	1	1	<1	bdl	2	1	<1	1	bdl
19:0	1	1	<1	<1	1	<1	bdl	bdl	1	1	<1	bdl
19:1	bdl	bdl	2	bdl	bdl	bdl	bdl	1	bdl	3	bdl	bdl
i19:0	bdl	1	1	1	<1	bdl	bdl	2	bdl	1	bdl	bdl
a19:0	bdl	1	1	bdl	bdl	bdl	bdl	bdl	bdl	<1	bdl	bdl
br-19:0	2	9	8	2	3	2	bdl	5	2	1	2	<1
cy19:0	3	2	<1	4	4	4	7	3	3	2	4	4
20:0	2	2	2	4	1	5	2	6	6	2	3	9
20:1	bdl	bdl	1	bdl	<1	1	bdl	bdl	<1	bdl	bdl	bdl
20:5	bdl	bdl	bdl	bdl	bdl	<1	bdl	bdl	bdl	<0.5	bdl	bdl
i20:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
a20:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
br-20:0	1	1	<1	<1	2	1	bdl	bdl	1	1	1	1l
21:0	bdl	1	1	1	<1	1	bdl	bdl	1	1	1	2
21:1	bdl	bdl	bdl	<1	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
i21:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
a21:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
br-21:0	bdl	2	<1	1	2	1	bdl	5	2	2	bdl	1
22:0	3	2	3	4	2	7	2	6	7	5	4	12

br-22:0	1	bdl	<1	2	2	1	bdl	1	1	1	5	bdl
23:0	1	1	1	1	1	1	bdl	1	1	1	1	1
br-23:0	2	2	<1	1	5	2	bdl	2	4	6	8	1
24:0	3	2	2	3	1	4	2	3	6	4	3	5
br-24:0	1	bdl	2	1	<1	1	bdl	2	1	2	2	1
25:0	1	bdl	<1	<1	bdl	<1	bdl	bdl	2	bdl	bdl	<1
br-25:0	bdl	1	bdl	bdl	<1	bdl	bdl	bdl	1	1	1	<1
26:0	2	1	1	1	<1	2	bdl	1	1	2	1	2
br-26:0	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	<1	bdl	bdl
27:0	bdl	bdl	bdl	bdl	bdl	<1	bdl	<1	bdl	bdl	bdl	bdl
28:0	1	bdl	1	1	bdl	1	bdl	bdl	1	1	bdl	1

Table A2: Individual PLFA composition for samples from Kingfisher CT in units of mole percent of total lipids extracted. bdl=below detection limit.

	KFCT 2-4 m	KFCT 6-8 m	KFCT 14-16 m	KFCT 22-24 m	KFCT 32-34 m
12:0	3	6	0	7	6
br-13:0	3	bdl	bdl	bdl	bdl
14:0	4	6	5	10	6
15:0	5	5	4	5	3
i15:0	6	5	7	7	6
a15:0	7	4	6	3	3
br-15:0	bdl	bdl	5	bdl	bdl
16:0	14	15	16	32	23
16:1	20	17	16	5	10
i16:0	4	bdl	5	3	3
br-16:0	4	bdl	5	bdl	2
17:0	4	5	5	3	4
br-17:0	bdl	bdl	bdl	3	bdl
10Me16:0	bdl	bdl	bdl	bdl	4
cy17:0	5	bdl	6	bdl	bdl
18:0	5	8	5	12	7
18:1	12	14	15	7	12
18:2	bdl	bdl	bdl	bdl	3
cy19:0	4	bdl	bdl	bdl	4
20:0	bdl	5	bdl	3	3
22:0	bdl	5	bdl	bdl	bdl
24:0	bdl	5	bdl	bdl	bdl

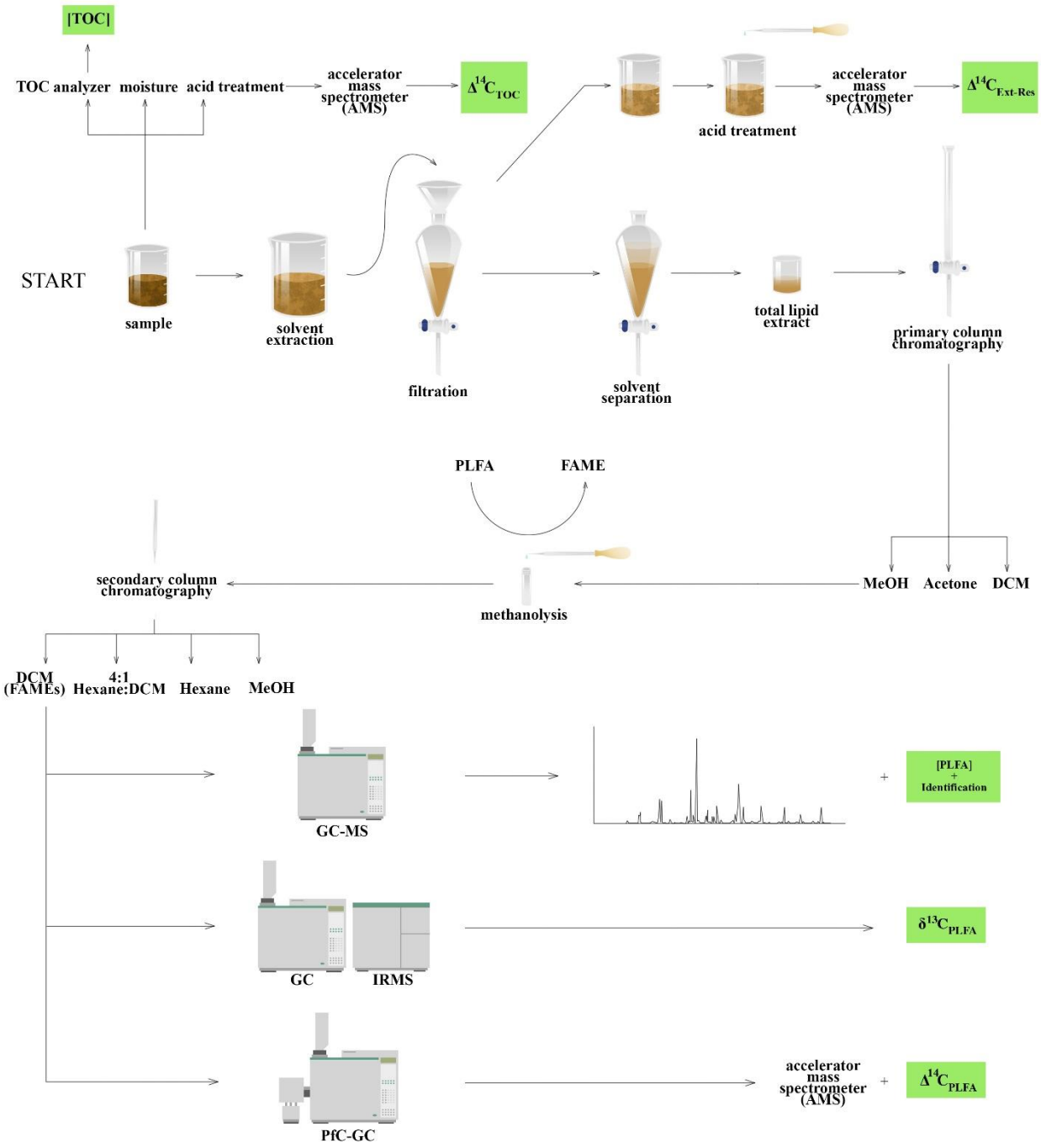

Figure A1: An overview of all methods used in this thesis. Image by Mark Belan, reproduced with permission.