

MSc. Thesis – N. Ngonadi; McMaster University - School of Geography and Earth Sciences

Using PLFA to constrain microbial distribution related to S-cycling in oil-sands composite tailings during reclamation

Nwanneoma Ngonadi

MSc. Thesis – N. Ngonadi; McMaster University - School of Geography and Earth Sciences

PLFA ANALYSIS ON OIL SAND COMPOSITE TAILINGS

MSc. Thesis – N. Ngonadi; McMaster University - School of Geography and Earth Sciences

USING PLFA TO CONSTRAIN MICROBIAL DISTRIBUTION RELATED TO S-
CYCLING IN OIL-SANDS COMPOSITE TAILINGS
DURING RECLAMATION

By

NWANNEOMA NGONADI, H.B.Sc

A Thesis Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree
Master of Science

McMaster University

© Copyright by Nwanneoma Ngonadi, December 2012.

MSc. Thesis – N. Ngonadi; McMaster University - School of Geography and Earth Sciences

MASTER OF SCIENCE (2012)

(Earth and Environmental Science – Organic Geochemistry)

McMaster University

Hamilton, Ontario

TITLE: Using PLFA to constrain microbial distribution related to S-cycling in oil-sands composite tailings during reclamation

AUTHOR: Nwanneoma Ngonadi, H.B.Sc (McMaster University)

SUPERVISOR: Dr. Greg Slater

NUMBER OF PAGES: ix ,60

Abstract

Microorganisms are the most abundant living things on the planet and they drive many important environmental processes. They can do this by coupling reduction – oxidation (redox) reactions. In such reactions, the oxidation of reduced organic matter is coupled with the reduction of another compound, which serves as the electron acceptor. All microbes contain lipids in their cells; phospholipids are the main components of the cell membrane where they make up a consistent component of cell mass. Therefore, in situations where direct cell count is unrealistic, lipid analysis can be used to provide information on microbial communities. Because they hydrolyze shortly after cell death, PLFAs indicate only viable cell biomass, and PLFA analysis provides valuable insight on cell density distribution across a site. One application of PLFA analysis is within this thesis, where it was used to investigate the microbial community at Mildred Lake, Syncrude's primary tailings settling basin. At Mildred Lake, Syncrude is constructing a freshwater fen over the deposited composite tailings (CT) as part of their reclamation process. Understanding the microbial biogeochemical cycling associated with these reclamation activities is an important component for management decisions affecting the site and thus, inform future reclamation activities.

PLFA analysis on samples from the site showed variable concentrations equivalent to estimated cell densities on the order of 10^7 decreasing to 10^6 in the CT. These cell density ranges are expected for oligotrophic systems. Phospholipids can also be biomarkers if they are indicative of a specific group of microbes. The study at Mildred Lake identified biomarkers for sulfate reducing bacteria (SRBs). The presence of these

biomarkers provided a basis for the hypothesis that sulfide detected at the site was potentially from SRBs.

This thesis provides information on the fundamental concepts of lipids and the application of lipid analysis on the environmental samples from the Mildred Lake site to understand its microbial community and cycling of sulfur to prevent potential environmental issues associated with the generation of sulfide.

Acknowledgements

Dr Slater, thank you for your patience, time and effort in the last two years. Lori and Jennie, I appreciate your help especially during lab work and analyses. Nagissa, Sara and Danielle, thank you for the good times and the memories. To my family, friends and pastors, I'm grateful for the prayers, support and advice. To Ally, thank you for staying up with me while I worked on this project.

I'm thankful to Syncrude, Canada for funding this research but also for their effort to preserve the integrity of the environment. Above all, I would like to thank the Almighty God for seeing me through this. My sincere gratitude to everyone who contributed to the successful completion of my MSc degree in one-way or the other.

Table of Contents

ABSTRACT	iii
ACKNOWLEDGMENTS	v
LIST OF TABLES	vii
LIST OF FIGURES	viii

CHAPTER 1: INTRODUCTION TO MICROBIAL LIPIDS AND LIPID ANALYSIS

1.1 Microbes.....	1
1.2 Microbial Processes.....	3
1.3 Lipids.....	3
1.4 Lipid Analysis.....	4
1.5 Phospholipids	5
1.6 Phospholipid Fatty Acid (PLFA) Analysis.....	8
1.7 Conversion factors for PLFA Analysis.....	9
1.8 Phospholipids fatty acids (PLFAs) as biomarkers	9

CHAPTER 2: CRUDE OIL AND THE CANADIAN OIL SANDS

2.1 The Canadian Oil Sands.....	14
2.2 Microbial Communities at the Canadian Oil Sands.....	14
2.3 Sulfur and Sulfate Reducing Bacteria.....	17
2.4 Methane and Methanogens.....	6.....18
2.5 Syncrude Canada Limited.....	19
2.6 Thesis Structure.....	21

CHAPTER 3: USING PLFA TO CONSTRAIN MICROBIAL DISTRIBUTION RELATED TO S-CYCLING IN OIL SANDS COMPOSITE TAILINGS DURING RECLAMATION

3.1 Abstract.....	25
3.2 Introduction to Syncrude’s reclamation project.....	26
3.3 Site Description.....	30
3.4 Methods.....	33
3.5 Results.....	35
3.6 Discussions.....	43
3.7 Conclusions.....	48

CHAPTER 4: CONCLUSION

4.1 Thesis and research conclusion	50
4.2 Future Research.....	53
REFERENCES.....	55

List of Tables

CHAPTER 1

TABLE 1: Common biomarkers	10
----------------------------------	----

CHAPTER 3

TABLE 2: Sample collection timeline and methodology.....	29
TABLE 3: Mole percentages of all PLFAs.....	38
TABLE 4: Mean and standard deviation for all samples.....	38

List of Figures

CHAPTER 1	
FIGURE 1: The redox ladder	1
FIGURE 2: Bligh and Dyer method of lipid extraction.....	4
FIGURE 3: Structure of a phospholipid.....	5
FIGURE 4: Phospholipid bilayer.....	6
CHAPTER 2	
FIGURE 5: Formation of composite tailings	14
FIGURE 6: Biogeochemical cycling of sulfur.....	16
FIGURE 7: Syncrude.....	17
CHAPTER 3	
FIGURE 8: Aerial view of Mildred Lake Site.....	26
FIGURE 9a/b: Schematic and cross section of Sandhill freshwater fen.....	28
FIGURE 10: Total PLFA concentrations and cell densities.....	33
FIGURE 11: Temporal variations of total PLFA concentrations.....	34
FIGURE 12: Total PLFA concentrations of composite tailings	35
FIGURE 13: Mole Percentages of classes of PLFAs at Mildred Lake Site.....	37

Chapter 1

Introduction Part A: Microbial Lipids and Lipid Analysis

1.1 Microbes

Microbes are the most abundant living organisms on the planet (Wilson, 2003). To derive energy for their metabolism, they drive environmental reactions by recycling biologically relevant elements such as carbon and sulfur (Vestal and White, 1989), a process known as biochemical (or biogeochemical) cycling (Trudinger and Shaine, 1979, pg 1). During these reactions, microbes can couple the oxidation of organic matter to the reduction of electron acceptors (Raich, 1992). As an environmental system transitions from aerobic to anaerobic conditions, the electron acceptors used by microbes during metabolisms changes based on the redox ladder in Figure 1 below.

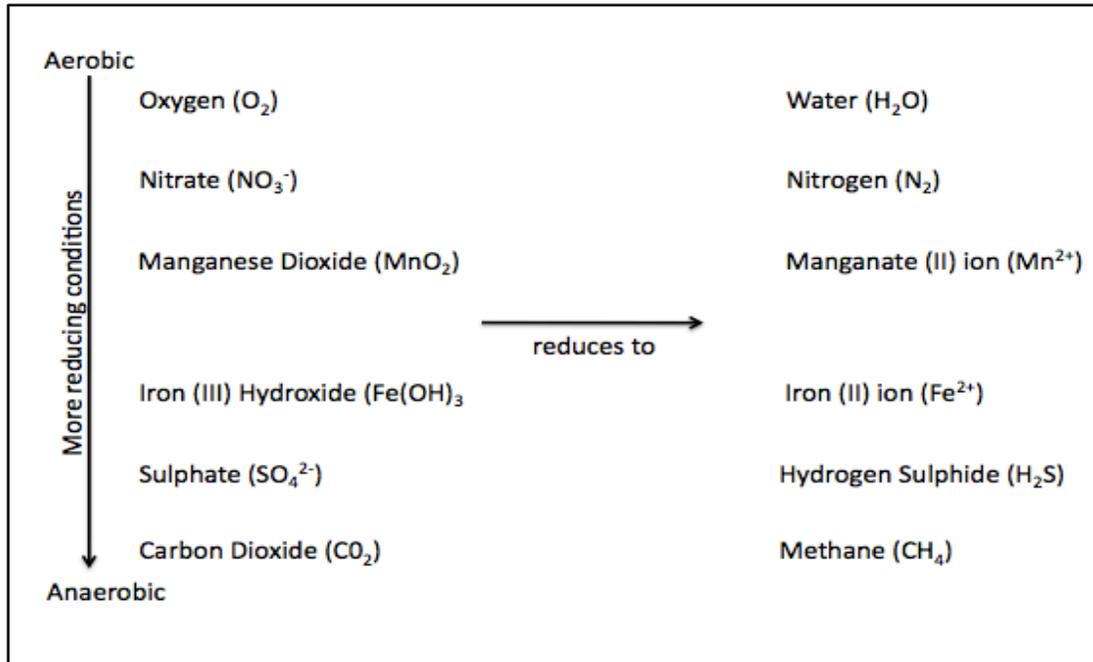


Figure 1: The reduction-oxidation (redox) ladder as the environmental conditions gradually change.

Microbes that utilize the electron acceptors at the top of the redox ladder gain more energy, allowing them to grow abundantly and outcompete other microbes (Harner et al., 2011). In the absence of electron acceptors such as oxygen and nitrate, which are at the top of the redox ladder, microbes capable of other forms of metabolism using electron acceptors lower on the redox ladder like sulfate, are able to survive and thrive. However, as in the case of sulfate reduction, the by-product sulfide, poses environmental problems due to its high toxicity, ability to easily permeate the cell membrane, and in extreme cases lead to death of the affected organisms (Reiffenstein et al.; 1992; Beauchamp et al, 1984).

1.2 Microbial Processes

Because the environment is very dynamic, it is important to know and understand the microbial biogeochemical processes occurring and to what extent, in order to predict and manage their impacts. The impacts of microbes on the environment are not always negative. For example, respiration, the process in which microorganisms convert reduced organic matter such as large complex petroleum hydrocarbons to carbon dioxide, is beneficial because it removes these pollutants from the environment. Microbial processes can also be detrimental to the environment. For example, the byproduct from the reduction of sulfate is sulfide, which is toxic (Beauchamp et al, 1984). Understanding the microbial communities provide information on their nature and functions. Since all microbes contain lipids in their cell membrane, lipid analysis is one way to study such microbial communities.

1.3 Lipids

Bacteria, archae and eukarya are the three main domains of life and they contain lipids within their cell membranes for functional and structural purposes (Dowhan and Bogdanov, 2002). Lipids can be defined as a variety of hydrocarbons more soluble in organic solvents than in water. Specifically, they are “fatty acids and their derivatives and substances related biosynthetically or functionally to these compounds” (Adibhatla et al., 2006). Some examples of lipids are phospholipids fatty acids, sterols and waxes. Because all cells are composed of lipids, analyses of such lipids can be used to investigate biomass, community structure, metabolic status and microbial activities (Vestal and

White, 1989) especially when direct cell counting and culturing is difficult. Basically, lipid analysis involves the extraction of lipids in a combination of organic solvents in which they are dissolved followed by an in depth examination of the fraction(s) of interest (Figure 2), depending on the objectives of the researcher.

1.4 Lipid Analysis

The Bligh and Dyer method is commonly used for lipid extraction. According to this method, the lipids are sonicated and dissolved overnight in three solvents; dichloromethane, methanol and phosphate buffer in the ratio 1:2:0.8 respectively. Using silica gel chromatography, the extracts are cleaned up and separated based on their polarities. The non-polar lipids are collected in the first fraction using dichloromethane; the neutral lipids are then collected in the second fraction with acetone and finally, the third fraction containing the polar lipids using methanol. Since phospholipid fatty acids (PLFAs) are polar lipids, they remain in the third fraction, which then undergoes mild methanolysis converting the PLFAs to fatty acid methyl esters (FAMES). These FAMES are identified and quantified using gas chromatography mass spectrometer (GC-MS) but reported as PLFAs. The peaks from the GC-MS are identified based on mass fragmentations and retention times compared to known standards or the lipid library.

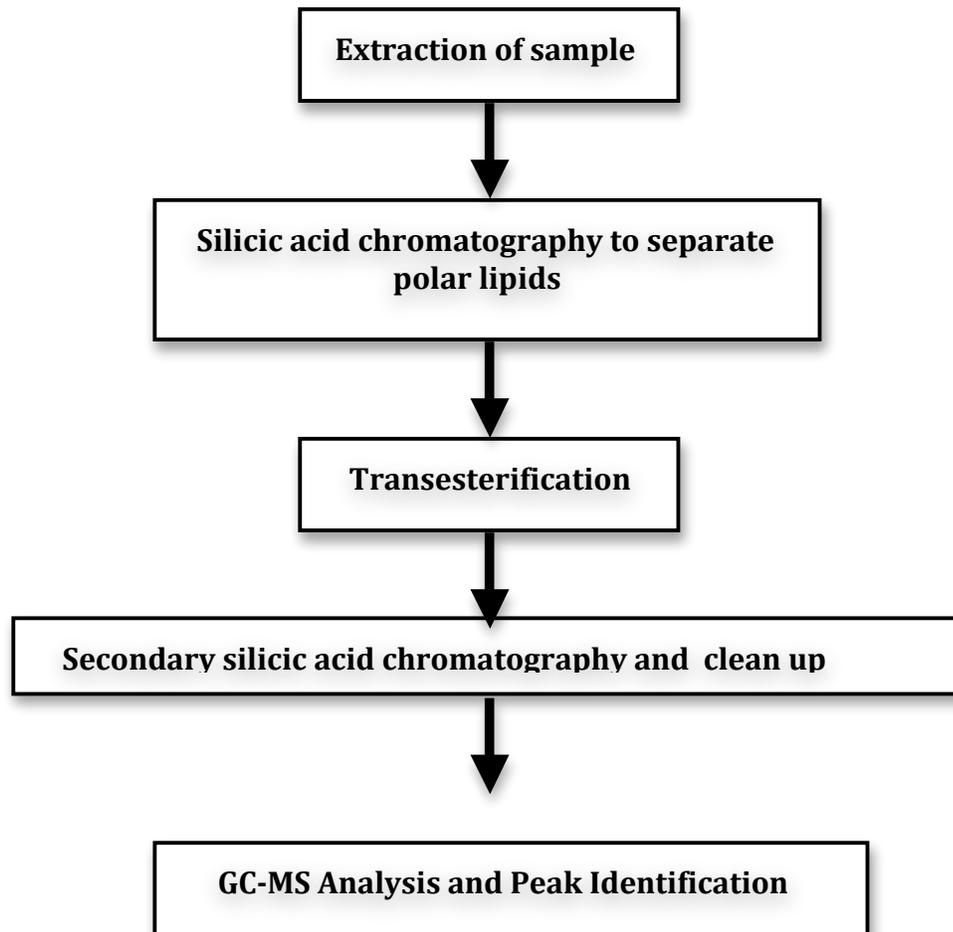


Figure 2: The Bligh and Dyer method for lipid extraction (Bligh and Dyer, 1959).

1.5 Phospholipids

A phospholipid (Figure 3) consists of one molecule of glycerol, a three-carbon alcohol, with its two hydroxyl groups bound to two fatty acid chains, and the last one bound to a hydrophilic phosphate head. In the cell membrane, a phospholipid does not exist by itself, but in close association with other phospholipids, essential carbohydrates

and proteins forming a bi-layer (Figure 4) with the hydrophilic heads oriented towards the exterior surface of the membrane and the hydrophobic (or lipophilic) ends towards the interior to repel moisture. Due to its fluid nature, the top of the bilayer is wavy enabling the easy transport of materials into the cell (Kaur et al., 2005).

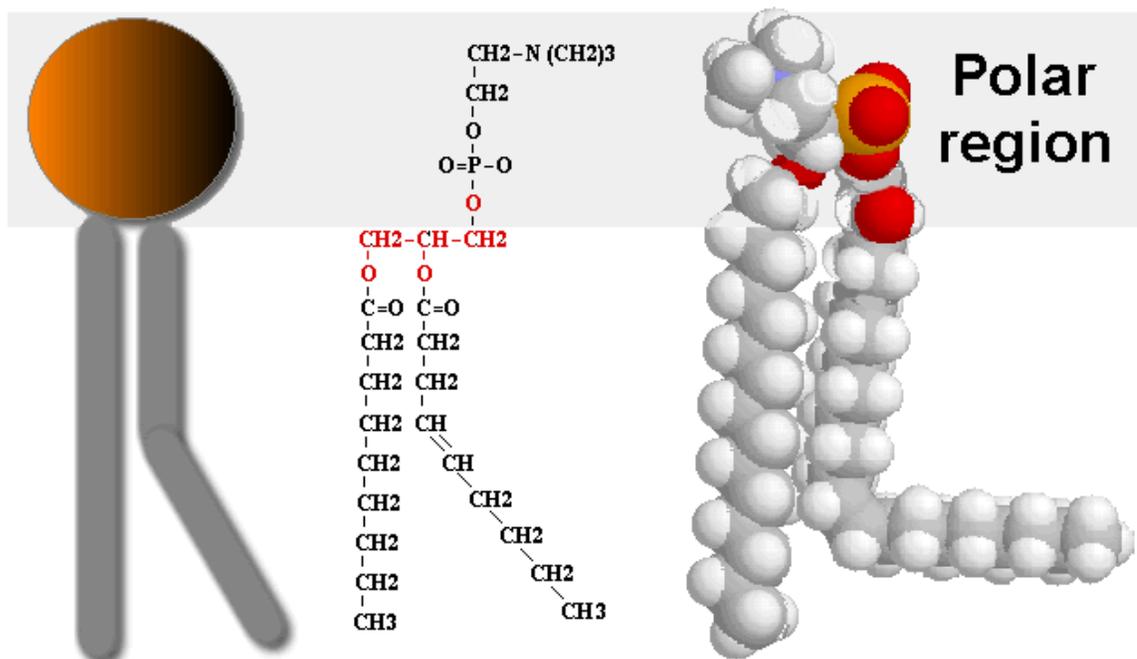


Figure 3: The chemical structure of a phospholipid. Image from Department of Biology, Western Kentucky University (2012).

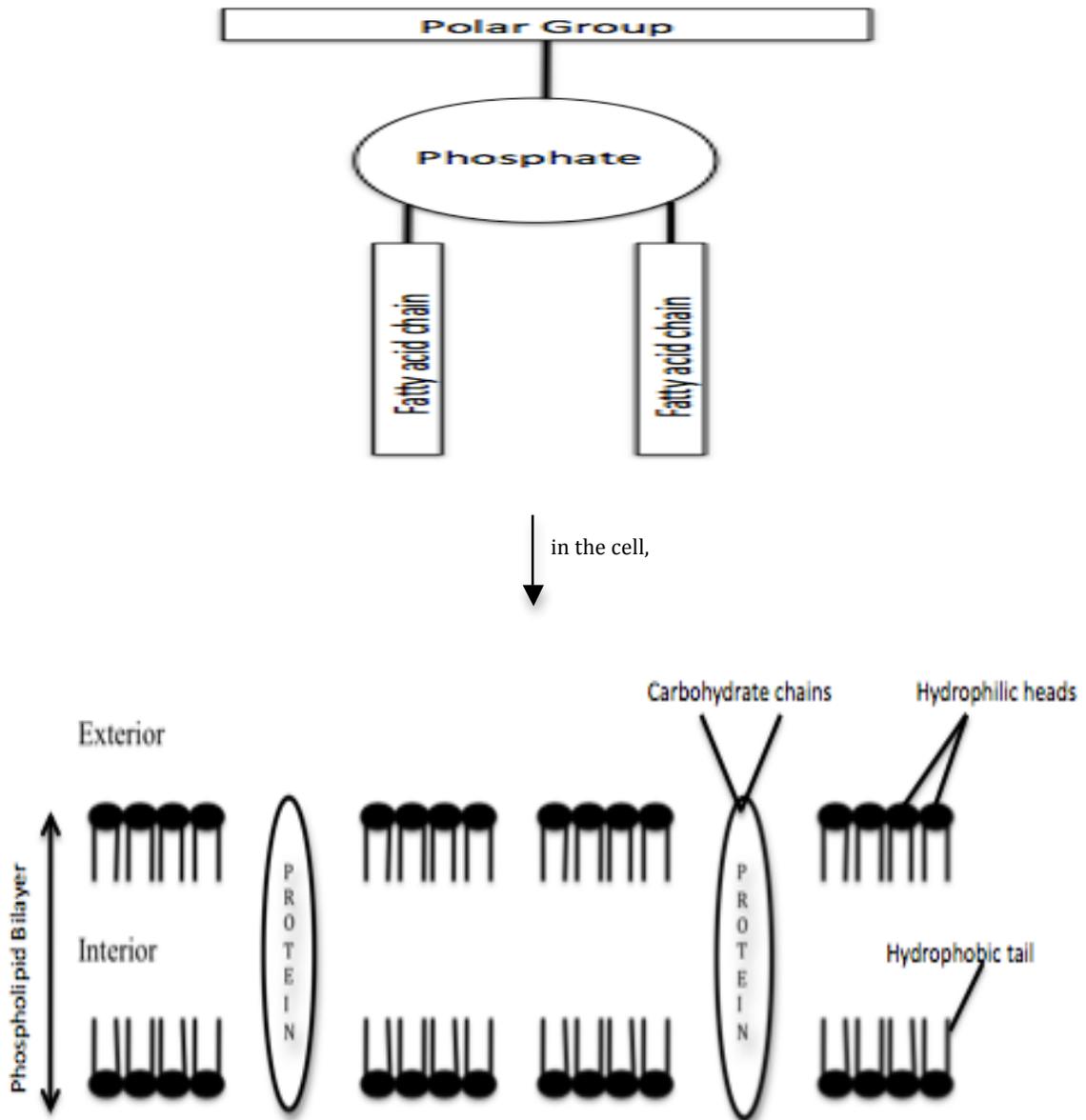


Figure 4: The top portion is a schematic of a phospholipid and the bottom portion is a schematic of the phospholipid bilayer showing how phospholipids exist in the cell membrane. Based on Dowhan and Bogdanov, 2002.

1.6 Phospholipid Fatty Acid Analysis

PLFA analysis is one way of analyzing the lipids without culturing. Compared to similar methods of lipid extraction such as total fatty acids analysis, PLFA analysis requires larger sample sizes because of its specificity and restriction to living, active cells (Vestal and White, 1989). However, this is also one of the main advantages of PLFA analysis. Because PLFAs hydrolyze rapidly after the cell death, they can be used as proxies for viable microbial biomass, in addition to providing information on the composition of the microbial community in a snapshot in time (Fang et al., 2007) indicating the metabolic activity occurring in the environment of interest (Olsson et al., 1995). The paper by Mackenzie and Quideau (2010) used PLFA analysis to investigate the total microbial biomass as an indication of microbial community on an oil sands reclaimed site using the Bligh and Dyer method for lipid extraction (Bligh and Dyer, 1959) and comparing total PLFA concentrations from different sites and those collected at different times. They found that the environmental conditions where the microbes lived reflected in their lipids, and thus, by comparing the samples collected at different times, specifically, different seasons, the difference in their lipid compositions investigated these changes in environmental conditions.

In order to be used to estimate the amount of viable biomass, the cell component measured for the lipid analysis has to be common in all cells in a fairly consistent proportion. According to Balkwill et al. (1988), phospholipids are also a good choice for lipid analysis because they can be extracted quantitatively and they are present in only in living cells in fairly uniform concentration assuming the microbes are not under

environmental stress. The high turnover of PLFAs means that they reflect changes in the environment indicating current, in situ environmental conditions (Fang et al., 2007). If the microbes live in nutrient limiting or stressed environments, this can either lead to a change in the PLFA composition or in the microbial communities, a process known as 'homeoviscous adaptation', (Sinensky, 1974), allowing the cells to still survive despite these environmental changes. For example, the ratio of cis to trans of monounsaturated fatty acids can be indicative of stress levels. Such stresses include temperatures, pH and starvation, which can alter the properties of the cell membrane such as its fluidity which then affects other factors such as transport of material in and out of the cell (Kaur et al., 2005).

1.7 Conversion factors for PLFA analysis

The extent of information provided by PLFA analysis can be broadened to include the cell densities of the analyzed samples using conversion factors to estimate cell densities from PLFA concentration. According to Green and Scow (2002) for example, the range for conversion factors for subsurface systems is 2×10^4 to 6×10^4 cells/pmol PLFA.

1.8 Phospholipid fatty acids (PLFAs) as biomarkers

Some PLFAs are biological markers or “biomarkers” for specific microbial communities. In other words, a biomarker is a lipid formed by a particular (group of) microbes, usually in the same relatively constant concentration. This level of specificity,

however, is usually rare (Boschker and Middleburg, 2002). Although most PLFAs are common to a variety of microbial groups, some are still characteristic of particular microbial groups making them potential biomarkers. For example, in the Table 1 below, the branched 15:0 PLFAs can indicate the presence of gram-positive bacteria or gram-negative sulfate reducers (Kaur et al, 2005). Fatty acids can be straight-chain saturated, or monounsaturated, branched, cyclopropyl or polyunsaturated fatty acids, but the straight-chained saturated fatty acids are not used to identify specific microbial communities since they are in all bacteria and eukaryotes (Green and Scow, 2000) unlike the branched fatty acids which are only common in 10% of eubacteria species.

Table 1: Some of the most common biomarkers (Fang et al., 2007; Green & Scow, 2000).

Phospholipid Fatty Acids	Can be a biomarker for:
16:2, 16:3, 18:3, 20:3, 20:4, 20:5, 22:5, 22:6	Cyanobacteria, protozoa, algae
i15:0, a15:0, 10Methly16:0, cyclo 17:0	Sulphate reducers
16:1, 18:1	Methanotrophs
18:2, 18:3	Fungi
cyclo 19, hydroxyl fatty acids	Aerobic gram – negative bacteria
i15:0, a 15:0, i16:0, i17:0, a18:0	Anaerobic and gram – positive bacteria

Environmental samples are composed of a variety of organic compounds, meaning that they are the habitat for a wide range of microbes. Hence, the application of biomarkers and signature lipid analysis can be a tool used to group and understand the available active microbes. An example of such complex environments is the Canadian oil sands. Therefore, identifying the PLFAs and the microbial communities represented in the samples enable a better understanding of their roles and impacts in the oil sands.

References:

- Adibhatla, R., Hatcher, J. & Dempsey, R. (2006). *Lipids and Lipidomics in Brain Injury and Diseases*. The AAPS Journal. Volume 8. Issue 2. Pages 314-321.
- Balkwill, D., Leach, F., Wilson, J., McNabb, J. & White, D. (1988). *Equivalence of Microbial Biomass measures based on membrane lipid and cell wall components, adenosine triphosphate and direct counts in subsurface aquifer sediments*. Microbial Ecology. Volume 16. Pages 73-84.
- Beauchamp, R., Bus, J., Popp, J., Boreiko, C. & Andjelkovich, D. (1984). *A critical review of the literature on hydrogen sulphide toxicity*. CRC Critical Reviews in Toxicology. Volume 13. Issue 1. Pages 25 – 97.
- BenchPrep Inc. (2012). Chemistry in AP Biology. <https://benchprep.com/>
- Bligh, and Dyer, W.J. (1959). *A rapid method for total lipid extraction and purification*. Canadian Journal of Biochemistry and Physiology. Volume 37. Pages 911-917
- Boschker, H. T. S., & Middelburg, J. J. (2002). *Stable isotopes and biomarkers in microbial ecology*. FEMS Microbiology Ecology. Volume 40. Issue. Pages 85-95.
- Dowhan, W., & Bogdanov, M. (2002). Functional roles of lipids in membranes. In D. E. Vance, & J. E. Vance (Eds.), *Biochemistry of lipids, lipoproteins and membranes* (4th Edition ed., pp. 1). Paris: Elsevier
- Fang, J., Hasiotis, S., Das Gupta, S., Brake, S. and Bazylinski, D. (2007). *Microbial biomass and community structure of a stromatolite from an acid mine drainage system as determined by lipid analysis*. Chemical Geology. Volume 243. Pages 191 – 204.
- Green, C. and Scow, K. (2000). *Analysis of phospholipids fatty acids (PLFA) to characterize microbial communities in aquifers*. Hydrogeology Journal. Volume 8. Pages 126-141.
- Harner, N., Richardson, T., Thompson, K., Best, R., Best, A., & Trevors, J. (2011). *Microbial processes in the Athabasca oil sands and their potential applications in microbial enhanced oil recovery*. Journal of Industrial Microbiology and Biotechnology. Volume 38. Pages 1761-1775.
- Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., and Kaushik, A. (2005) *Phospholipids fatty acid – A bioindicator of environment monitoring and assessment in soil ecosystem*. Current Science, Volume 89. Issue 7.

- MacKenzie, M. & Quideau, S. (2010). *Microbial community structure and nutrient availability in oil sands reclaimed boreal soils*. *Applied Soil Ecology*. Volume 44, Issue 1. Pages 32 – 41.
- Olsson, P., Baath, E., Jakobsen, I. & Soderstrom, B. (1995). *The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil*. *Mycol. Res*. Volume 99. Issue 5. Pages 623-629.
- Raich, J. (1992). *The global carbon dioxide flux in soil respiration and its relation to vegetation and climate*. *Tellus*. Volume: 44B. Pages 81-99.
- Reiffenstein, R., Hulbert, W., & Roth, S. (1992). *Toxicology of Hydrogen Sulfide*. Annual review of Pharmacology and Toxicology. Volume 32. Pages 109 – 134.
- Sinensky, M. (1974). *Homeoviscous Adaptation- A Homeostatic Process that regulates the viscosity of Membrane lipids in Escherichia coli*. *PNAS*. Volume 71. Issue 2. Pages 522-525.
- Trudinger, P & Swaine, D. (1979). *Biogeochemical cycling of Mineral Forming Elements* (3rd ed.). Amsterdam, The Netherlands: Elsevier Scientific Publishing Company.
- Vestal, J. & White, D. (1989). *Lipid Analysis in Microbial Ecology*. *Bioscience*. Volume 39. Issue 8. Pages 535 -541.
- Wilson, Edward. (2003). *The encyclopedia of life*. *Trends in Ecology and Evolution*. Volume 18. Issue 2. Pages 77 – 80.

Chapter 2

Introduction Part B: Crude oil and the Canadian Oil Sands

2.1 The Canadian Oil Sands

Globally, the largest oil sand deposits are distributed mainly within eight countries, including Canada and the United States of America (Chataturnyk et al., 2002). In Canada, crude oil is stored in oil or bituminous sands in these three main locations in Alberta; Peace River, Cold Lake and Athabasca. Oil sands are unconsolidated sandy sediments containing petroleum (Masliyah et al., 2004). The Athabasca oil sand in Fort McMurray, Alberta is approximately 21,000 square miles in area making it the largest reserve of “economically recoverable” bitumen in the country (Ramos-Padron et al., 2011). Canadian oil sands are estimated to have at least 300 billion barrels of crude oil in situ that can be economically extracted (Bellows and Bohme, 1963), and a total of about 1.3 trillion barrels (Sparks et al., 2003; Beersheba et al., 2000). Crude oil extraction dates back to as early 1920s on a smaller scale, and has since been continued today by companies such as Syncrude, which provides 15% of the nation’s petroleum requirement (Syncrude, 2012) making them the largest synthetic oil producers mining from the Athabasca oil sands.

In Canada, there are two main methods for crude oil extraction. Surface mining used for shallow reservoirs up to 75m deep and in situ pumping methods are used for sediments deeper than 75m. The mining of crude oil has its share of environmental impacts, ranging from increased greenhouse gas emissions (Charpentier et al., 2009) to

the production of tailings containing solid particulates like sands, clays and residual bitumen (Holowenko et al, 2000; Matthews et al., 2002). These tailings are very poorly consolidated and also have poor water release properties (Chataturnyk et al., 2002).

After the mining process, the responsible parties do not release their waste tailings but contain them in settling ponds where they are changed to composite tailings (CT) by adding a coagulant such as gypsum (Figure 4). The coagulant causes the CT to dewater rapidly after deposition making the CT easier to manage and providing water recycled back into the mining process. When the sediments start to coagulate, the coarser sediments settle out fastest since they are denser and then progressively, the finer tailings continue to slowly settle out, sometimes over the course of a few years, at which time they are referred to as “mature fine tailings” (Sparks et al., 2003). As the consolidation process continues, the CT continues to “dewater” and the wastewater is collected, treated and recycled. This dewatering process is also essential in making the CT manageable in the event of the site’s reclamation (Matthews et al., 2002), as in the case of Mildred Lake, since working on a dry landscape is easier than on a wet, soggy one (Masliyah et al., 2004) and recycled water provided prevents the utilization of the city’s water supplies. According to Hamza et al. (1996), shortly after the addition of the coagulant, about 40% of the process water can be recovered because the initial settling of the tailings is rapid. There are a variety of coagulants such as lime, gypsum and sulphuric acid; however, gypsum is usually the coagulant of choice since it is easily available, affordable and easy to handle (MacKinnon et al., 2001).

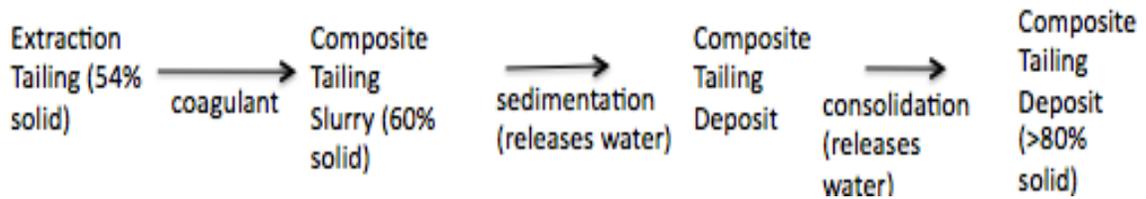
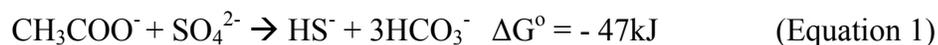


Figure 5: The formation of composite tailings (Adapted from Matthews et al., 2002).

2.2: Microbial communities at the Canadian Oil Sands

Oil sands tailings ponds become increasingly anaerobic with depth, therefore water in such systems have little or no dissolved oxygen (Harner et al., 2011). As a result, electron acceptors lower in the redox ladder are more abundant with depth causing the most common microbial communities at such depths to be anaerobic for example sulfate reducing bacteria (SRBs) and methanogens (Bordenave et al., 2010). This means that the microbial communities in tailings ponds can change with depth (Ramo-Padron et al., 2011). Carbon dioxide, hydrogen sulphide and methane gas are three of the most common products of microbial metabolism in tailings ponds. According to the redox ladder in Figure 1, methanogenesis only occurs when sulphate is limited because although, methonogens and SRBs compete for electron donors, microorganisms energetically favor sulfate reduction since they derive more energy from it compared to methanogenesis (Harner et al., 2011, Fedorak et al., 2002) (Equations 1 and 2).

For sulfate reduction:



For methanogenesis:



2.3 Sulfur and Sulfate Reducing Bacteria (SRBs)

Sulfur is one of the most versatile elements in nature. It can exist in a variety of oxidation numbers, which along with other factors such as pH will determine, if reduction or oxidation takes place (Holmer and Stockholm, 2001). Oil sands tailings contain sulfate, naturally in addition to the sulphate added by gypsum, $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, the coagulant used to consolidate the fluid fine tailings into CT at Mildred Lake. Sulphate reducing bacteria (SRBs) were the first microorganisms to be discovered from oil sands (Harner et al., 2011). One of the advantages of SRBs is that they inhibit the activities of methanogens. This is beneficial since methane is a greenhouse gas (Fedorak et al., 2002). However, sulphate reduction also poses environmental problems with the production of hydrogen sulfide, which is toxic (Figure 6) (Reiffenstein et al., 1992).

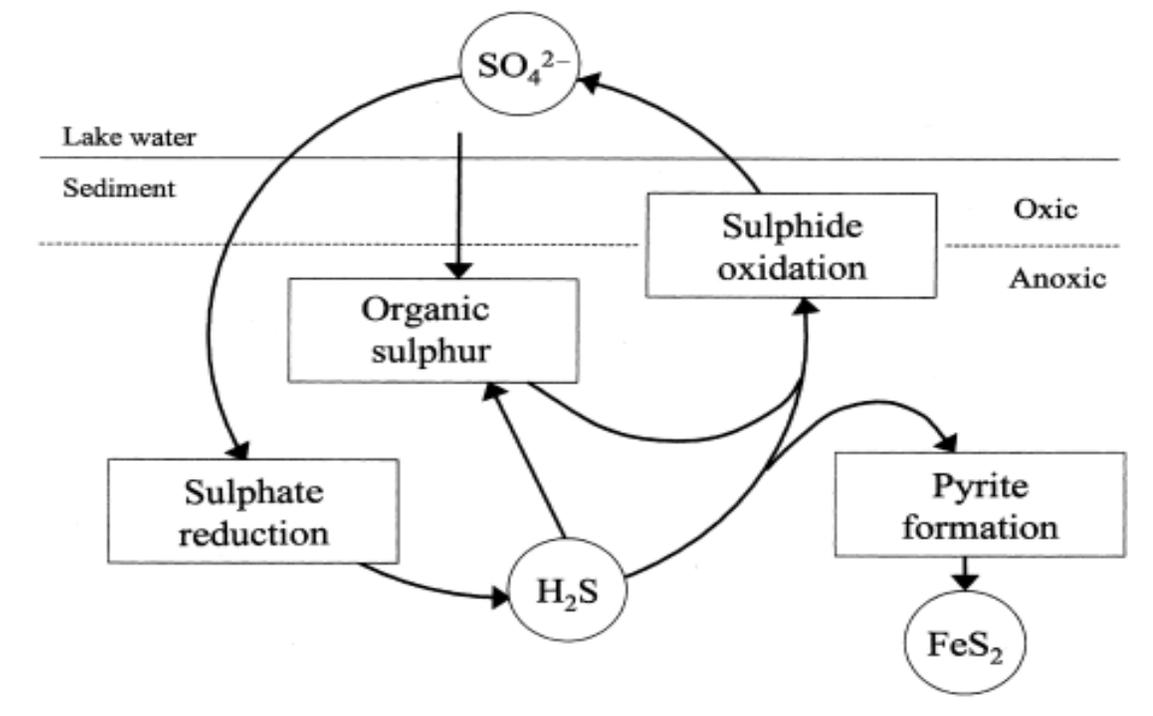


Figure 6: Biogeochemical cycling of sulfur (Holmer and Stockholm, 2001).

2.4 Methane and Methanogens

Methanogens are completely anaerobic with a variety of metabolic pathways producing methane, which accounts for over 60% of the gas flux from the oil sands. Apart from methane production and the degradation of organic matter, methanogens slow down the sedimentation and consolidation of tailings (Fedorak et al., 2002). As previously mentioned, water released by the tailings consolidation supplies 80% of water recycled in the mining process (Matthews et al., 2002). This means that methanogens also limit the amount of water available to be recycled (Harner et al., 2011). Since methanogens produce methane, which is a greenhouse gas, the amount of methane produced can be restricted by the introduction of other electron acceptors such as nitrate and sulfate, which the microbes will preferentially metabolize (Harner et al., 2011).

2.5 Syncrude Canada Limited

Syncrude Canada Ltd. (Syncrude) is located in Fort Murray, Alberta, Canada. Officially starting operations in 1978, Syncrude is a joint business venture among some of Canada's leading oil and gas companies such as Suncor Energy Oil, Imperial Oil Resources, and five others (Figure 7).

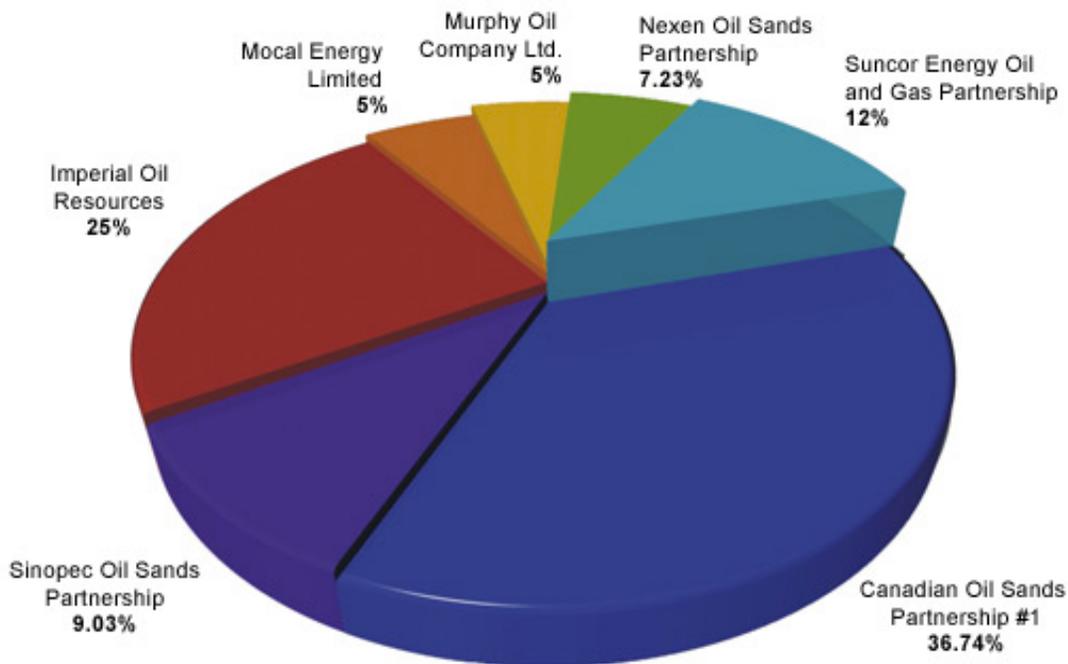


Figure 7: Syncrude as a joint venture owned by seven of Canada's top oil companies since 1978 (Syncrude Canada Limited, 2012).

At the Athabasca oil sands, two thin films of water and bitumen surround each quartz sand grain forming a matrix. This matrix contains void spaces either filled with air or water containing suspended fine particles. In the "Clark hot water extraction process" used by Syncrude, after the bitumen is separated, the coarse sediments immediately settle

while the fine sediments remain in suspension in the “process water” as tailings (Sworska et al., 2000). Syncrude is one of the first companies to have undertaken a reclamation project at its tailings settling pond by constructing a freshwater fen, to comply with the legislation in Alberta that requires that all mined land be reclaimed to the sustainable environmental standards it was prior to mining activities. Due to the detection of hydrogen sulfide at Mildred Lake as the fen is being constructed, Syncrude funded the research which is the basis for this thesis to understand the microbial community and the biogeochemical cycle of sulfur at Mildred Lake site.

2.6 Thesis Structure

Chapter 1 is an introduction to the fundamental concepts. These include lipids, lipid analysis and lipid as biomarkers. This chapter focuses on phospholipid fatty acid (PLFA) analysis as a tool to investigate the microbial communities in terms of biomass, by estimating cell densities from PLFA concentrations using conversion factors. Chapter 2 is an introduction to the Canadian oil sands and to Syncrude, the largest synthetic oil producer using the Canadian oil sands. Chapter 2 also briefly introduces the reclamation project taken up by Syncrude at Mildred Lake where they are constructing a freshwater fen above the composite tailings that have been previously deposited. Chapter 3 is written as a paper focused on understanding the microbial community and biogeochemical cycling at Mildred Lake site, Syncrude's primary settling basin for tailings. The paper used PLFA analysis to compare the sand cap and CT spatially and temporally, and the paper also identifies potential biomarkers for sulfate reduction and discusses the implications of these results in terms of cell growth and cell density distribution. Chapter 4 is the concluding chapter of this thesis. It summarizes the research done within this thesis and raises possible questions for future research.

References:

- Beersheba, F., Kotlyar, L., Sparks, B. & Chung, K. (2000). Organic coated solids in Athabasca Bitumen: Characterization and process implications. *Journal of Chemical Engineering*. Volume 78. Pages 610 – 616.
- Bellows, L. & Bohme, W (1963). Athabasca Oil Sands. *Journal of Petroleum Technology*. Volume 15. Issue 5. Pages 479-483
- Bordenave, S., Kostenko, V., Dutkoski, M., Grigoryan, A., Martinuzzi, R. (2010). *Relation between the activity of anaerobic microbial populations in oil sands tailings ponds and the sedimentation of tailings*. *Chemosphere*. Volume 81. Pages 663-668.
- Chalaturnyk, R., Scott, D. & Ozum, B. (2007). *Management of Oil Sand Tailings*. *Petroleum Science and Technology*. Volume 20. Issues 9 and 10. Pages 1025 - 1046.
- Charpentier, A., Bergerson, J. & MacLean, H. (2009). *Understanding the Canadian oil sands industry's greenhouse gas emissions*. *Environmental Research Letters*. Volume 4. Pages 1 -11.
- Fedorak, P., Coy, D., Salloum, M. & Dudas, M. (2002). *Methanogenic potential of tailing samples from oil sands extraction plants*. *Canadian Journal of Microbiology*. Volume 48. Pages 21 -33.
- Hamza, H., Stanonik, D. & Kessick, M. (1996). *Flocculation of lime-treated oil sands tailings*. *Fuel*. Volume 75. Issue 3. Pages 280 -284.
- Harner, N., Richardson, T., Thompson, K., Best, R., Best, A., & Trevors, J. (2011). *Microbial processes in the Athabasca oil sands and their potential applications in microbial enhanced oil recovery*. *Journal of Industrial Microbiology and Biotechnology*. Volume 38. Pages 1761-1775.
- Holmer, M. & Storkholm, P. (2001). *Sulphate reduction and sulphur cycling in lake sediments: a review*. *Freshwater Biology Special Review*. Volume 46. Pages 431- - 451.
- Holowenko, F., MacKinnon, M. & Fedorak, P. (2000). *Methanogens and sulphate-reducing bacteria in oil sands fine tailings waste*. *Canadian Journal of Microbiology*. Volume 46. Pages 927 – 937.

- MacKinnon, M., Matthews, J., Shaw, J. & Cuddy, R. (2001). *Water quality issues associated with composite tailings (CT) technology for managing oil sand tailing*. International Journal of Surface Mining Reclamation and Environment. Volume 15. Issue 5. Pages 235 – 256.
- Masliyah, J., Zhou, Z., Xu, Z., Czarnecki, J & Hamza, H. (2004). *Understanding water based bitumen extraction for Athabasca Oil Sands*. The Canadian Journal of chemical engineering. Volume 82. Pages 628 - 654
- Matthew, J., Shaw, W., MacKinnon, M. & Cuddy, R. (2002). *Developments of Composite Tailings Technology at Syncrude*. International Journal of Surface Mining, Reclamation and Environment. Volume 16. Issue 1. Pages 24-39.
- Ramos-Padron, E., Bordenave, S., Lin, S., Bhaskar, I., Dong, X., Sensen, C., Pournier, J., Voordouw, G & Gieg, L. (2011). *Carbon and Sulfur Cycling by Microbial Communities in a Gypsum-treated Oil Sands Tailings Pond*. Environ. Sci. Technol. Volume 45. Pages 439-446.
- Reiffenstein, R., Hulbert, W., & Roth, S. (1992). *Toxicology of Hydrogen Sulfide*. Annual review of Pharmacology and Toxicology. Volume 32. Pages 109 – 134.
- Sparks, B., Kotlyar, L., Carroll, J. & Chung, K. (2003). Athabasca oil sands: effect of organic coated solids on bitumen recovery and quality. *Journal of Petroleum Science and Engineering*. Volume 39. Pages 417-430.
- Sworska, A., Laskowski, J., & Cymerman. (2000). *Flocculation of the Syncrude fine tailings Part 1. Effect of pH, polymer dosage and Mg²⁺ and Ca²⁺ cations*. International Journal of Mineral Processing. Volume 60. Pages 143 – 152.
- Syncrude Canada Limited (2012). www.syncrude.ca

Chapter 3

Using PLFA to constrain microbial distribution related to S-cycling in oil-sands composite tailings during reclamation

Manuscript in preparation for publication

Ngonadi, N., Ziolkowski, L., Penner, T., Slater, G.

1. School of Geography and Earth Sciences, McMaster University, Hamilton, ON, Canada
2. Syncrude Canada, Limited, Fort Murray, Alberta, Canada.

3.1 Abstract

Understanding the extent and effects of microbial cycles are important to predict and mitigate their impacts on the environment. Because all microbes contain phospholipids in their cell membrane, the extraction and quantification of phospholipids can be used as a proxy to understand these microbial communities. This paper used phospholipid fatty acid (PLFA) analysis on samples from Mildred Lake, where the primary tailings pond of Syncrude, Canada is located and currently being reclaimed by the construction of a freshwater fen over the composite tailings (CT). The concentrations and distribution of PLFAs were determined for the 10m deep sand cap separating the Sandhill fen from the underlying CT, and also from within the CT. Using conversion factors, PLFA concentrations were used to estimate cell densities in the sand cap and CT. The cell densities in the sand cap were in the 10^7 range, and in the 10^6 range in the CT. These ranges, characteristic of oligotrophic systems, are between the 10^9 range of rich soils and 10^5 range of anaerobic groundwater systems. This study was also an application of PLFAs as biomarkers. The presence of branched PLFAs; iso and anteiso 15:0 and 10Me16:0 indicated the presence of sulfate reducing bacteria (SRBs) across the site. The identification of these SRBs was used to investigate cycling of sulfur, noting that the conditions in the sand cap and CT were different, with the CT possibly containing more sulfate introduced by using gypsum to convert the fluid fine tailings to composite tailing. Overall, understanding these microbial cycles helps understand the environment, and provides information, which can be used to predict the likely sources of sulfide, and help present possible mitigation steps to prevent future environmental issues at this site.

3.2 Introduction to Syncrude's Reclamation Project

Syncrude is the largest synthetic oil producer utilizing the Canadian oil sands. Using water-based extraction methods (Syncrude, 2012), they produce large volumes of fluid fine tailings composed of sands, clays and residual bitumen (Matthews et al., 2002) in quantities currently estimated at over 300 million m³. These tailings are primarily stored at Syncrude's settling pond at Mildred Lake (Figure 8) (Holowenko et al., 2000). To improve the workability and management of the fluid fine tailings, they are converted into composite tailings (CT) by the addition of a coagulant, such as gypsum, which causes the fluid fine tailings to aggregate and dewater rapidly releasing water, which is then recycled back into the mining process.

As required by the government of Alberta, any mined land has to be reclaimed after extraction to restore the "excavated land to a state of self-sustainability with no long term risks or toxicity" (Renault et al., 2003). Syncrude is one of the first companies to undertake the reclamation of oil sands tailings ponds. They are doing this by constructing a freshwater fen over the CT generated from previous mining activities. The design of this freshwater fen involved covering circa 40m depth of CT with a "sand cap", 10m in depth, above which the fen was then constructed (Figure 9b). The main purpose of the sand cap is to separate and isolate the overlying freshwater fen from the saline conditions of the underlying CT. Ultimately, these reclamation activities are planned to result in a freshwater fen that is representative of the ecological nature of the site prior to mining.

During the Sandhill freshwater fen construction, hydrogen sulfide (H₂S), a corrosive, poisonous gas with a "rotten egg smell" (Reiffenstein et al., 1992) was detected

The overarching goal of this research at the Mildred Lake was to understand the environmental processes controlling sulfide generation by assessing the presence and distribution of SRBs at this reclamation site. To achieve this goal, this study analyzed the phospholipid fatty acids (PLFAs) from sediment samples across the site; from the surface, 1m depth, and within the CT. PLFA analysis involves the extraction and purification of phospholipids from bacterial and eukaryal cells. In a reaction known as transesterification, the PLFAs were converted to fatty acid methyl esters (FAMES) (Christie, 1993; Tan et al., 2009). Since phospholipids hydrolyze shortly after cell death, within days or a few weeks, samples of intact phospholipids represent viable cell biomass capturing the in situ bacterial and eukaryal members of the microbial community being studied. Because PLFAs make up a consistent proportion of the microbial cell mass, they can be used to estimate cell densities using conversion factors (Piotrowska & Mrozik, 2003). According to Green and Scow (2000), for aquifer and deep subsurface systems, the range of applicable conversion factors is 2×10^4 to 6×10^4 cells/pmol PLFA. This approach of estimating cell densities from PLFA analysis is particularly useful when the bacteria adhere to sediments or within the soil matrix making direct cell counting difficult to achieve without a large margin of error.

The samples for this study were spatial samples collected from the sand cap and CT, in addition to the temporal samples also from the sand cap collected at the start of the fen construction and one year after. In addition to cell density estimates, PLFA analysis can provide information on specific microbial communities. Because some PLFAs are associated with specific microbial groups, they are called biomarkers. Signature lipid

analysis can be used to characterize and relate particular PLFAs to specific groups of organisms (Piotrowska and Mrozik, 2003). Although it is possible for some PLFAs to be associated with more than one group of organisms, certain PLFAs are still predominantly associated with specific microbial groups. In this study, this is particularly relevant because some PLFAs which are possible biomarkers for sulfate reduction such as iso and anteiso C15:0 and 10methylC16:0 were identified confirming the presence and activities of sulfate reducers at Mildred Lake.

3.3 Site Description:

The study site was located on Syncrude’s property, in Fort Murray, western Alberta (Figure 8, part A). The Sandhill freshwater fen (Figure 8, part C) is being constructed above a 10m sand cap separating the fen from the CT below, as part of the reclamation project of the oil sands tailing at the Mildred Lake settling basin (Figure 8, part B) used primarily to collect, consolidate and store the composite tailing generated from crude oil extraction.

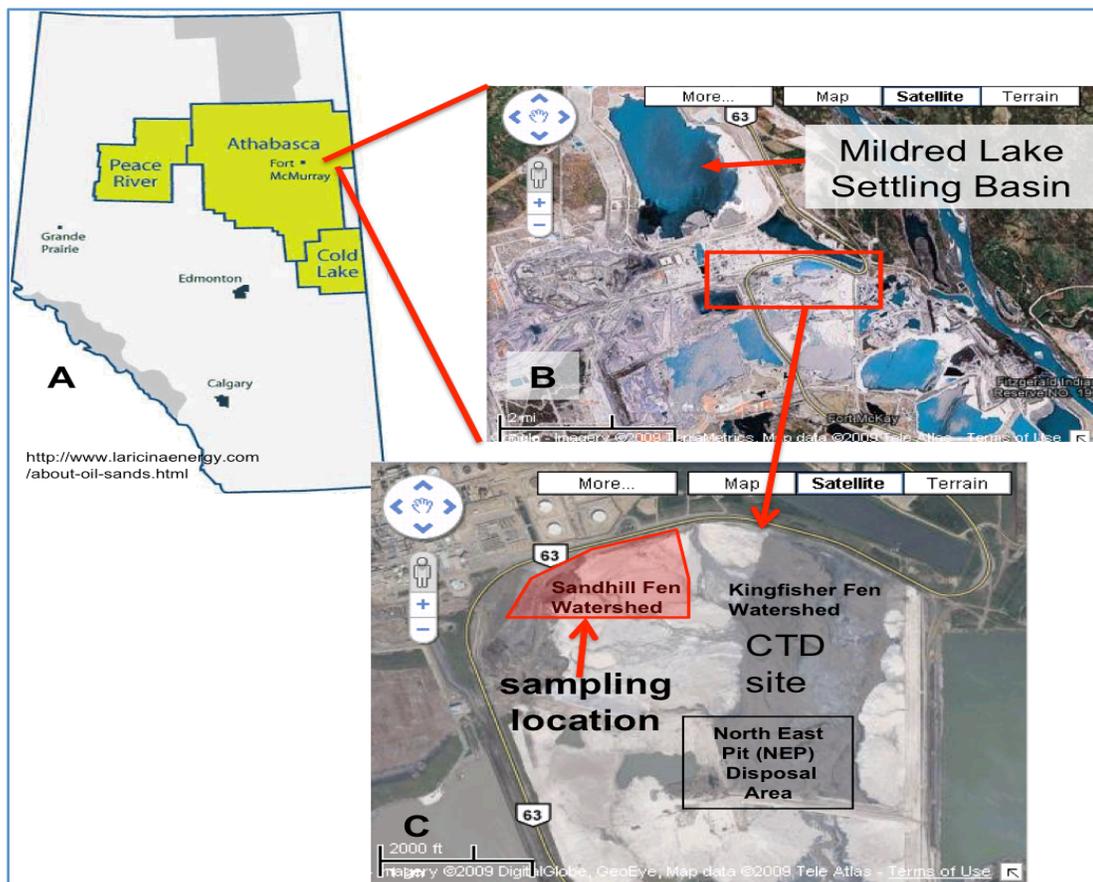


Figure 8: Aerial view of the sampling location at Mildred Lake Basin (Sandhill Fen) in Fort Murray, Alberta (Picture from Kate Stephenson, 2012).

Schematic of sampling locations at Mildred Lake:

The samples from the wells shown in the orange boxes (Figure 9a) were collected in June, 2010 (excluding the saltpan sample), and those in the blue boxes in July, 2011. The wells from which both surface and 1m deep samples were collected were 8c, 5d and 6a. The map in Figure 9a can be equally divided into three sections; upper, middle and lower fen. Figure 9b is a schematic of the site's profile with depth.

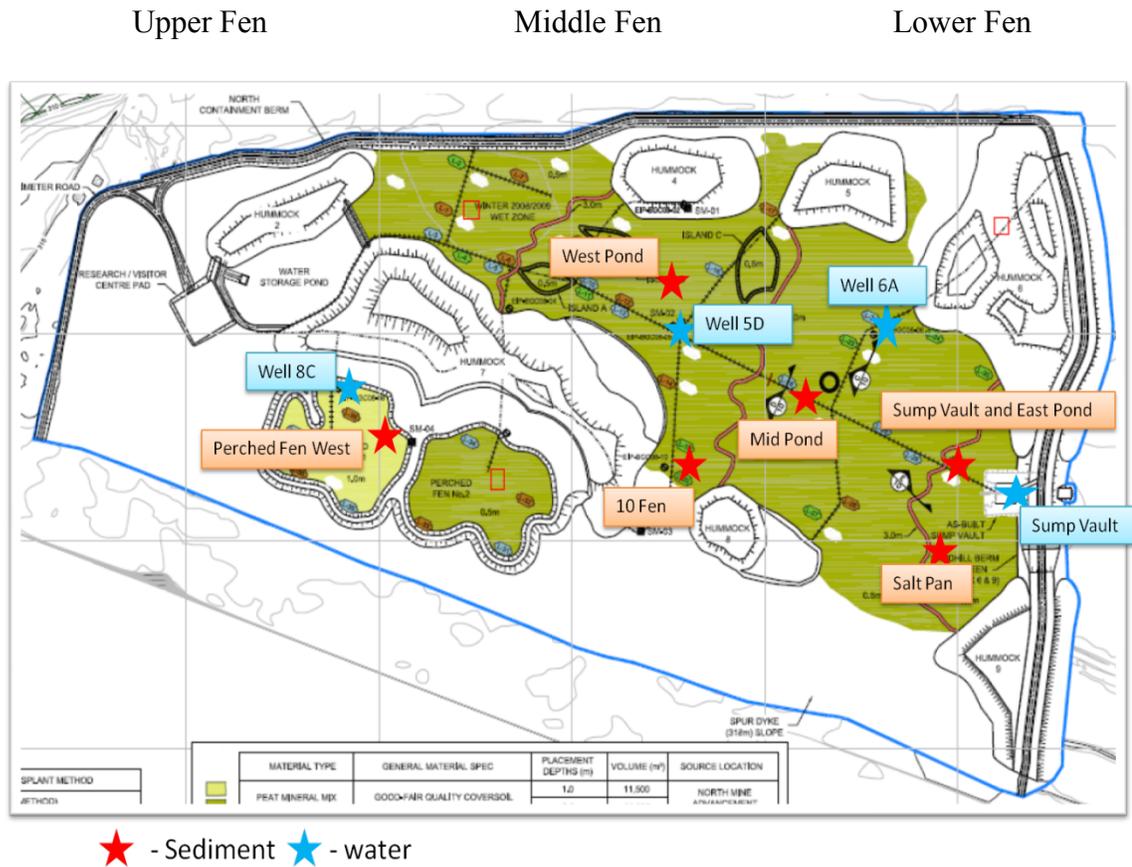


Figure 9a: Schematic of sampling locations during the construction of the Sandhill freshwater fen reclamation site.

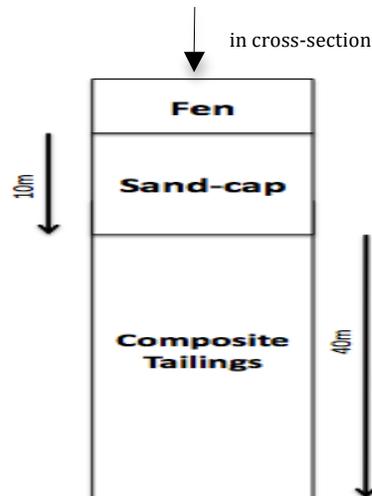


Figure 9b. Cross-section of the reclamation site with depth from the surface to the CT.

3.4 Methods:

Table 2: Sample collection timeline and methodology (locations in figure 1b):

June, 2010	Mode of collection
Samples B, C, D, E	- collected from 10-30cm depth with a sterile stainless steel shovel or polycarbonate core tubes
July, 2011	
8C, 5D, 6A (from surface and 1m depth) Saltpan	-a sterile stainless steel shovel was used to collect the surface samples, and a push core was used for the 1m depth samples..
CT samples (surface to circa 40m)	- CT samples were collected using a track-mounted amphibious drill rig with samples collected in 2m increments

Upon collection in sterile glass jars (for sand cap samples) or sterile whirl-pack bags (for CT samples), the samples were stored cold in coolers in the field, and then transferred to a freezer later that day, before they were sent to the lab, where they were stored at a temperature of -20°C until extraction. Samples were then freeze-dried and extracted using the modified Bligh and Dyer method (Bligh and Dyer, 1959), which involved sonicating the samples to be extracted overnight in methanol, dichloromethane and phosphate buffer solution (in ratios 2:1:0.8 respectively). The total lipid extracts was then separated from the remnant solids by gravity filtration. Subsequently, silica gel chromatography was used to separate the total lipid extracts, TLE, into three fractions

using the solvents dichloromethane (containing the non-polar lipids), acetone (containing the neutral lipids) and methanol (containing the polar lipids) respectively. The polar phospholipids in the methanol fraction then underwent mild methanolysis converting the PLFAs to FAMES by transesterification in the presence of potassium hydroxide catalyst. The PLFA analysis was done by gas chromatography mass spectrometry, GC-MS. The model of the GC-MS was Agilent 6890 coupled with a 5973 single quadrupole mass spectrometer. The GC column was a DB-5MS capillary column (30 m x 0.25 μ m film thickness) using a temperature program of 50°C (for 1 min.), 20°C/min to 130°C, 4°C/min to 160°C, 8°C/min to 300°C (for 5 min). The PLFAs were then identified based on their retention times and mass fragmentation patterns compared to the National Institute of Standards and Technology (NIST) library or known standards like the bacterial acid methyl esters mix by Matreya Inc., Pennsylvania, United States of America and PUFA fame mix by Supelco Analytical, Ontario, Canada.

At the onset of this study, initial testing was done to determine the quantity of each sample needed for analysis. Based on these tests, 50g of each sample was used for all extractions. The extracts from these oil sand tailings samples were organic rich and very dark in color prompting the need for more tests to investigate if the bitumen residue interfered with the results from the PLFA analysis. For this test, the asphaltene was precipitated out from the sample as suggested in Buenrostro-Gonzalez et al., (2004), and then cleaned up with silica gel chromatography first in hexane, then in a solvent made of 4:1 hexane to dcm, and finally in dichloromethane, acetone and methanol and the analysis was continued following the method already described above. There was no significant

difference in the concentrations of PLFA with or without asphaltene precipitation.

3.5 Results:

PLFA concentrations and cell density:

The mean total PLFA concentration for all samples ranged from 20-530 ug/kg as shown in Figure 10 below. The total PLFA concentrations (left axis) were converted to estimated cell densities (right axis) using the conversion factor 2×10^4 cells/pmol dry weight from Green and Scow (2000). The range of PLFAs in the sand cap was 70-530 ug/kg with no systematic variation in the PLFA concentrations of the different samples from west to east. The black diamonds, representing CT samples, were plotted such that right to left (or west to east) corresponded to top to bottom profiles from the drill core. The mean total PLFA concentration in the CT ranged from 20-80 ug/kg, which was lower than in the sand cap, excluding the sample from saltpan which has a total PLFA concentration of 80 ug/kg and the 1m depth sample from well 5d with a total PLFA concentration of 70ug/kg, both of which are comparable to the CT samples.

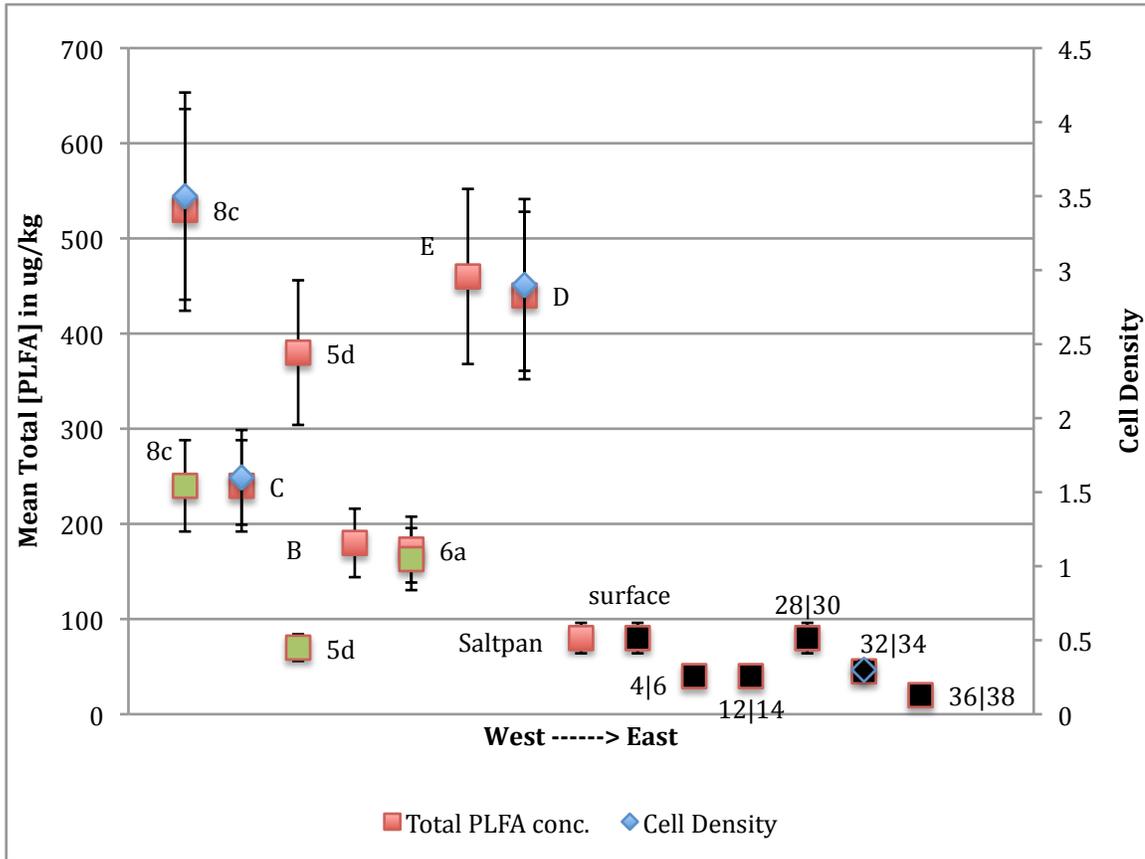


Figure 10: Graph showing mean total PLFA concentration in ug/kg and their corresponding estimated cell densities. The black data plots represent CT at depths from 0-38m and the green plots are samples from 1m depth from only wells 8c, 5d and 6a. The error bars represent $\pm 20\%$. Since this was the highest percentage of error within any of the samples from the site, therefore, plotting all samples across the site $\pm 20\%$ further highlights the differences in concentrations between the sand cap and CT.

Changes in PLFA concentration with time:

Temporal variability at the site was studied by comparing data from the 2010 and 2011 sample sets. According to the Figure 11 below, total PLFA concentrations for each of the samples were within the same order of magnitude with error bars of $\pm 20\%$.

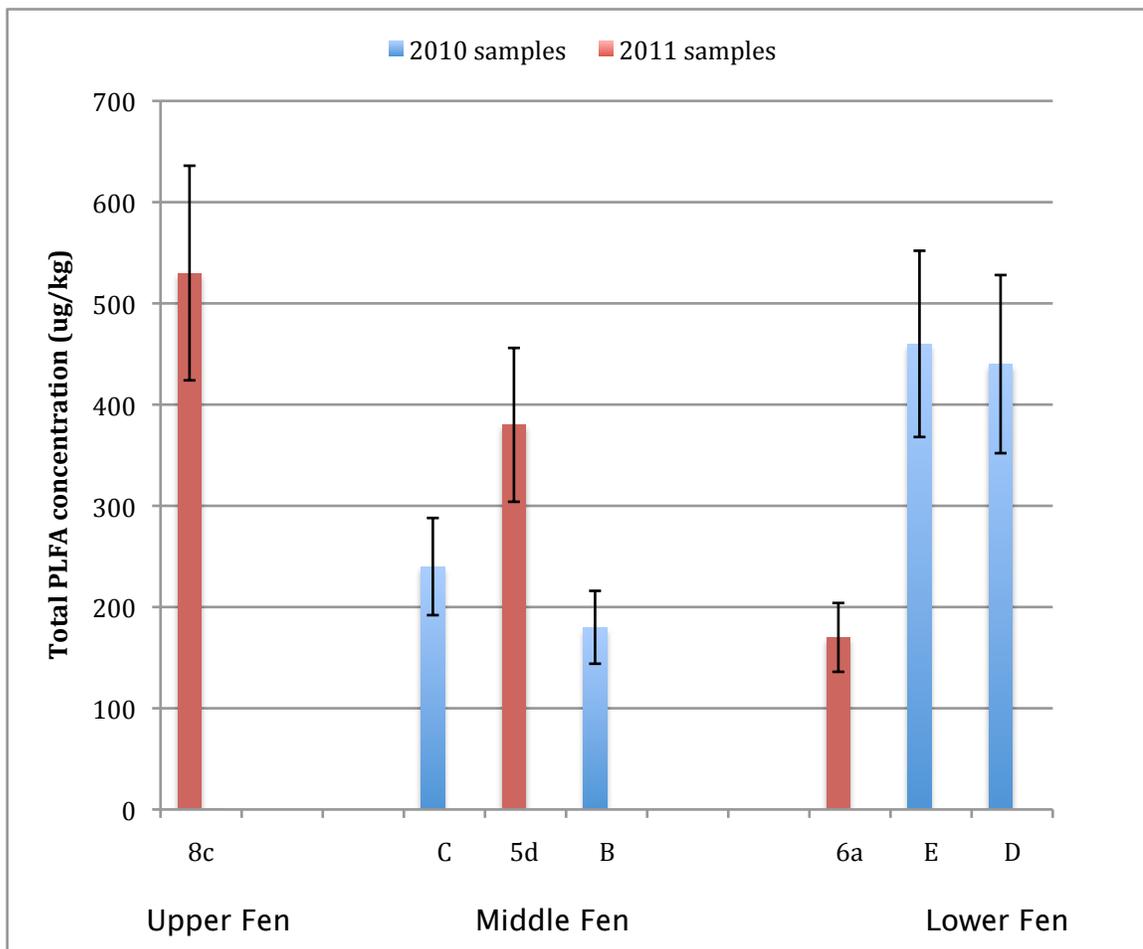


Figure 11: Temporal variation on total PLFA concentration across the fen.

Composite Tailings

Depth Profile:

Total PLFA concentrations for CT samples collected up to a depth of 38m are replotted in Figure 12. As previously noted, total PLFA concentrations were lower in the CT than in the sand cap. The variation throughout the depth profile is not systematic and thus, is likely related to heterogeneity and the varying conditions within the CT created during deposition.

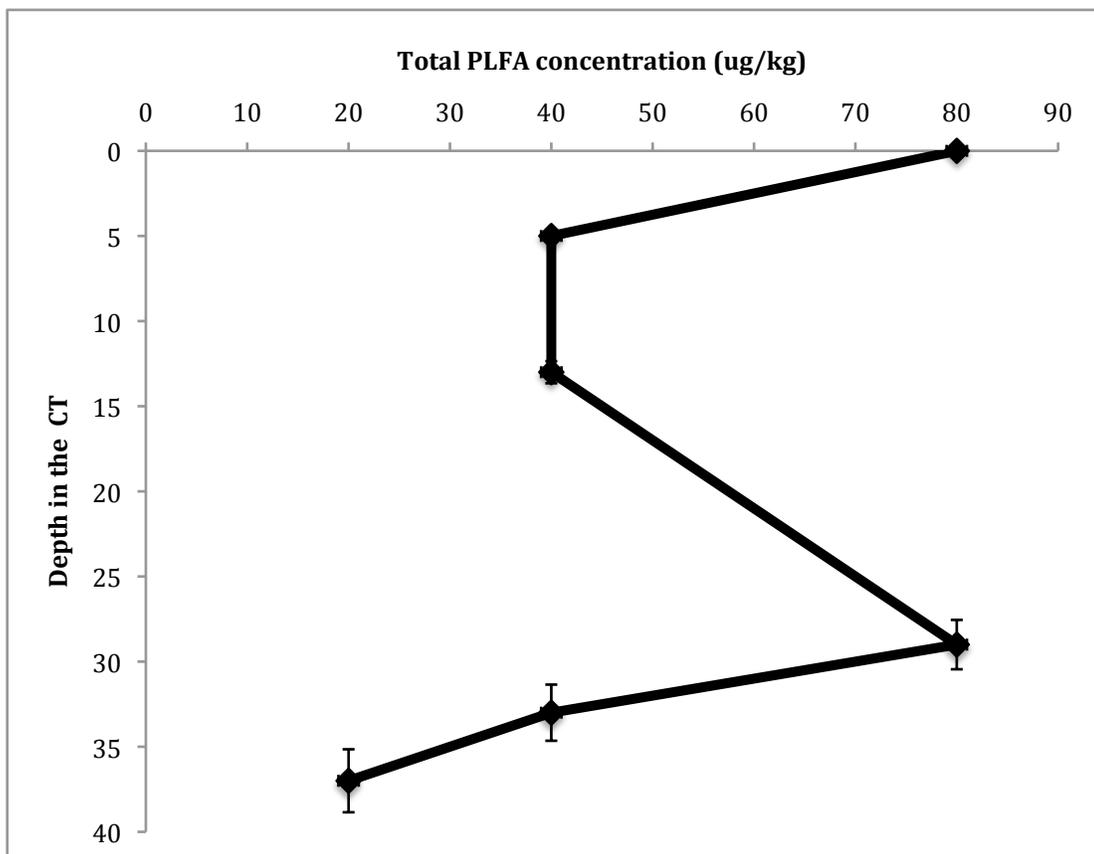


Figure 12: Graph showing total PLFA concentration of CT samples with depth from surface to circa 40m. The error bars on the x-axis are ± 1 and on the y-axis $\pm 5\%$.

Site to site comparison of classes of PLFAs:

Figure 13 shows the distributions of PLFAs for all samples grouped into four classes; saturated PLFAs, monounsaturated PLFAs, polyunsaturated PLFAs and branched PLFAs. Throughout the site, the distribution of the different classes of PLFAs was fairly uniform. However, there was a notable variation in the presence of the polyunsaturated PLFAs. In all the samples with polyunsaturated PLFAs, the fungal biomarker, C18:2 was the most abundant member of this group making up approximately 76% of the polyunsaturated PLFAs across the site (Table 3a). Some of the most common branched PLFAs are iso and anteiso C15:0, and 10MeC16:0, which together make up 53% of the total branched PLFAs across the site (Table 3b) and they are also biomarkers for sulfate reduction (Fang et al., 2007). The mole percentages of these sulfate biomarkers are comparable between the CT and the sand cap (Figure 13).

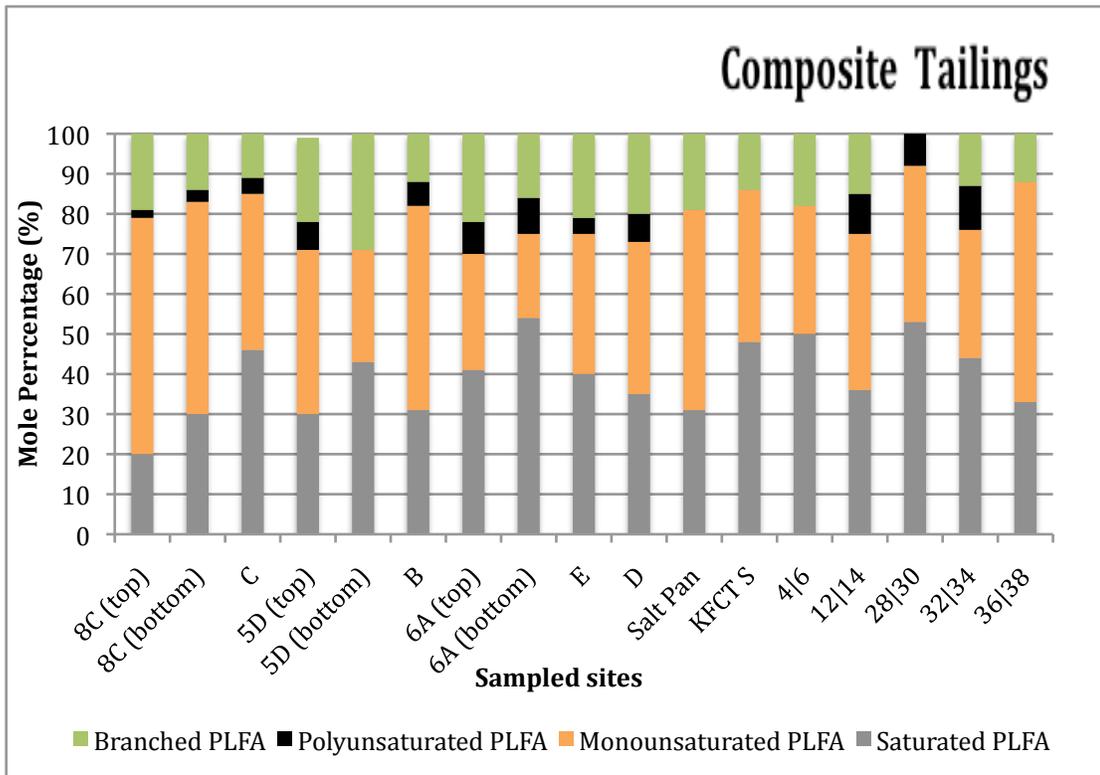


Figure 13: The graph shows the mole percentages of all samples grouped into four different classes of PLFAs. Regardless of the location, there seems to be a fairly uniform percentage of the different classes of PLFA across the site with the only notable difference being that some sites either do not have polyunsaturated PLFA or they do not have them in significant quantities above the limits of detection and quantification of the GC-MS used for this study.

Table 3a: Mole percentages for all the PLFAs in the sand cap.

SAND CAP	8C	8C(1m)	C	5D	5D (1m)	B	6A	6A(1m)	E	D	SALTPAN
C12:0	0.2	1.0	2.0	1.0	4.0	2.0	2.0	2.0	2.0	2.0	
Branched C13							2.0				
Monounsaturated C14									2.0	5.0	
C14:0	4.0	2.0	4.0	3.0	6.0	3.0	3.0	4.0	4.0	4.0	5.0
IsoC15:0	4.0	3.0	3.0	3.0	6.0	3.0	4.0	4.0	4.0	4.0	7.0
anteIsoC15:0	4.0	4.0	3.0	6.0	6.0	4.0	4.0	4.0	4.0	4.0	5.0
C15:0	4.0	2.0	3.0	1.0		2.0	2.0	2.0	4.0	2.0	4.0
Monounsaturated C16	25.0	21.0	21.0	17.0	4.0	17.0	18.0	10.0	17.0	13.0	14.0
C16:0	6.0	13.0	21.0	14.0	15.0	17.0	12.0	18.0	13.0	13.0	14.0
Branched C16	4.0	4.0		6.0	15.0		2.0	2.0	8.0	9.0	
Branched C16, 10MeC16	0.2	2.0		3.0	4.0		3.0	3.0			
Monounsaturated C17	0.2	6.0	2.0	0.3	6.0	2.0			4.0		
C17:0	0.6		3.0	3.0	4.0	2.0			4.0	2.0	
Branched C17	8.0	2.0	2.0	6.0	6.0	6.0	6.0	2.0			10.0
C18:2	2.0	3.0	4.0	2.0		6.0	3.0	4.0	4.0	7.0	
Other Polyunsaturated PLFAS -				6.0			5.0	3.0			
Monounsaturated C1	27.0	21.0	17.0	14.0	15.0	28.0	12.0	6.0	17.0	18.0	28.0
C18:0	6.0	4.0	8.0	3.0	9.0	5.0	5.0	10.0	6.0	7.0	8.0
Monounsaturated C19	6.0	4.0	3.0	3.0		3.0	4.0	6.0		2.0	8.0
Branched C19				3.0				4.0		4.0	
C20:0		3.0		3.0			4.0		4.0		
C22:0		3.0		2.0				5.0	2.0		
C23:0			3.0				4.0	6.0			
C24:0			2.0	3.0			4.0	6.0			
C26:0				2.0							

Table 3b: Approximate mole percentages for all the PLFAs in the composite tailings.

	Composite	0m	4-6m	12-14m	28-30m	32-34m	36-38m
Tailings							
Monounsaturated C14							
C14:0		9.0	9.0	8.0	6.0	8.0	5.0
isoC15:0		8.0	9.0	8.0		6.0	2.0
anteisoC15:0		8.0	9.0	8.0		6.0	2.0
C15:0		8.0	9.0	8.0	5.0	6.0	2.0
Monounsaturated C16							
C16:0		18.0	20.0	15.0	26.0	17.0	19.0
Branched C16							
C18:2				10.0	10.0	13.0	
Other Polyunsaturated PLFAS							
Monounsaturated C18							
C18:0		25.0	23.0	23.0	13.0	23.0	10.0
Monounsaturated C19							
Branched C19							35.0
							4.0

Table 4: Mean and standard deviation for all samples.

	Mean (ug/kg)	Standard Deviation	Relative Standard Deviation.
8c surface	530.0	30.0	6.0
8c (1m depth)	240.0		
C	240.0	40.0	17.0
5d surface	380.0		
5d (1m depth)	70.0		
B	180.0		
6a surface	170.0		
6a (1m depth)	160.0		
E	460.0	90.0	20.0
D	440.0	80.0	18.0
Saltpan	80.0		
CT (surface)	80.0	20.0	25.0
CT 4-6m	40.0		
CT 12-14m	40.0		
CT 28-30m	80.0		
CT 32-34m	45.0	7.0	16.0
CT 36-38m	20		

3.6 Discussions

How do the PLFA concentrations and cell densities compare across this site?

Spatial Variability

For all the samples analyzed for this study, the mean of the total PLFA concentration was 191ug/kg and the standard deviation was 270 or a relative standard deviation of 141% confirming the variability at the site. In addition, the variation within individual samples was also investigated. Using sample 8c, the mean was 530ug/kg and the standard deviation was 30 or relative standard deviation of 6% (Table 4). Variability for individual samples ranged from 6% to 25%. Thus, the variability between samples was greater than that observed for any individual sample. Therefore, the overall variation across the site is due to heterogeneity and is greater than the variation in replicates from any one sample. However, there was no systematic pattern to this variability in terms of spatial distribution across the site. It was likely that this variability was due to heterogeneity in geochemical conditions in the different samples taken. Variation was also observed between the surface and 1 m depth samples. The total PLFA concentrations were higher by up to a factor of 2 in two of the samples (8c and 5d) but not in the well 6c sample where the error within 5%. The range in variability between surface and 1m depth was the same as that observed across the site. This indicates that the heterogeneity likely responsible for these variations occurs in three dimensions. The most consistent difference in total PLFA concentrations was between the sand cap and CT samples. The student's T test was used to prove that the difference between the total PLFA concentrations from the sand cap and CT were significant. Using this formula for

“student’s T test,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s_1^2 / n_1 + s_2^2 / n_2)}}$$

*Equation from <http://www.monarchlab.org/Lab/Research/Stats/2SampleT.aspx>

where 1 denotes sand cap samples, and 2 composite tailings samples. The degree of freedom using the equation $(n_1 + n_2 - 2)$ was 15 and S was variance, n = number of samples in each group and x is mean, the calculated “t” value was approximately 26, which was higher than the critical t value of 2, hence the difference between the two samples was in fact significant. These lower concentrations in the CT may be due to its limiting conditions because it gets increasingly anaerobic with depth. However, even within the CT, the total PLFA concentrations at different depths were within the same order of magnitude, thus, slight variations may again be due to heterogeneity.

Temporal Variability

According to Figure 11, the range of total PLFA concentrations in the different sections of the fen was within the same order of magnitude. Because of the limited number of samples analyzed, it is impossible to confirm a definite trend across the site. In sample 8c for example, the relative standard deviation between the samples was 6%, thus the variation in concentrations between analysis for the same sample was not systematic

but likely due to the site's heterogeneity, and this was assumed to be the case for all the other samples. Regardless of the year the samples were from, 2010 or 2011, the variation was again still not systematic for all samples across the site, since the samples remain within the same order of magnitude and the relative standard deviations for all surface samples was between 5 % - 20%.

Implications on cell growth

The total PLFA concentrations in the sand cap ranged from 80 – 530 ug/kg and the corresponding estimated cell densities from 0.5 - 3.5 x 10⁷ cells/gram (Figure 10) using conversion factors from Green and Scow (2000). 10⁷ cells/gram is midrange between rich soils, (10⁹ cells/gram) and the 10⁵ cells/gram range of anaerobic groundwater systems, typically the lowest range reported in the literature (Fierer et al., 2002; Alfreider et al., 1997). The total PLFA concentration within the CT samples was lower than in the sand cap, by approximately an order of magnitude, and the values stayed within the same order of magnitude regardless of depth (Figure 12), ranging from 20 - 80 ug/kg ± 5% corresponding to cell density estimates of 2 x 10⁶ cells/gram – 6 x 10⁶ cells/gram. The cell densities in the sand cap and CT suggest that nutrient limitation was potentially occurring in both systems, but to a greater extent in the CT. Considering the abundance of organic matter at the site due to the presence of naphthenic acid and the abundance of sulfate both naturally and from the addition of gypsum to the CT, the apparent nutrient implication implies that either one or both of these pools were not bioavailable. This was likely because naphthenic acids are branched and cyclic hydrocarbons with large molecular weights and so can be difficult for microorganisms to

biodegrade unlike smaller chained hydrocarbons such as ethanol. Therefore, it may in fact be the case that although there seems to be an abundance of organic matter present, their nature makes them less bioavailable, resulting in a limitation of carbon sources at the site. Assuming this is the case, then the addition of more bioavailable organic matter such as by the infiltration of plant roots, which exudates from the fen, will correspond to the rise in sulfide production as the microbes oxidize this newly available carbon and reduce the already present sulfate at the site.

Implications of PLFA to microbial community distribution.

One of the main objectives of this paper was to understand the sulfur biogeochemical cycle at Mildred Lake. With regards to this, sulphate biomarkers were PLFAs of interest in this study. From Figure 13 and Tables 3a and b, it was obvious that there were significant amounts of iso and anteiso C15:0 and in some cases 10MeC16:0 which were notable biomarkers for sulphate reduction (Fang et al.; 2007). As previously discussed, this site is expected to have high concentrations of organic matter and sulphate, therefore sulphate reduction can proceed biologically via sulphate reducing bacteria, SRBs. In environmental systems like fens, the rate of sulfate reduction is 2-to 600nmol cm⁻³ day⁻¹ (McIntire & Edenborn, 1990), lower in winter when the temperature is below the optimum for the SRBs to function. For individual cells such as those from *Desulfovibrio vulgaris*, the rate of sulfate reduction can range from 0.01 – 0.05 femtomol sulfate consumed/cell/day (Villanueva et al., 2008) depending on abundance of the electron donor or acceptor. For the range of cell densities in the sand cap (assuming a

porosity of 30%, density 1800kg/m^3 (Mackay et al., 1986) for all surface samples across the site), the estimated amount of sulfide generated is estimated to be $12\text{nm day}^{-1} \text{ cm}^{-3}$ of aquifer. However, if all the voids are filled with water, this means the amount of sulfide in water will be approximately 40nm cm^{-3} or 40nm of sulfide per ml per day. The concentration of sulphate observed at the reclamation site are mostly in the range of 5-100micromoles per ml. Based on the estimated sulphate reduction rates, these concentrations could be produced in over a time span 4 months to 7 years respectively.

It was also interesting to note that the most abundant polyunsaturated PLFA is C18:2, a biomarker for fungi. Ideally, fungi were not expected to survive deep within the anaerobic conditions of the CT, but the results show otherwise. Gunde-Cimerman et al. (2009) provide evidence for “halotolerant” and “halophilic” fungi, which were able to adapt and thrive in the saline conditions such as the conditions of the CT and the study by Griffith et al., (2010) confirm that some fungi are anaerobic. The most commonly discussed metabolic pathway for such anaerobic fungi is fermentation, and Sikkema-Marvin et al., (1990) proposed that the addition of methanogens which consume hydrogen will favor the formation of acetate instead of ethanol. This is important because the presence of excessive ethanol in the system ultimately inhibits the fermentation process because the fungi cannot survive (Hoppe and Handsford, 1982).

3.7 Conclusion

The government of Alberta mandates that excavated land be restored to a “state of self-sustainability with no long term risks or toxicity” (Renault et al., 2003). In order to achieve this, Syncrude is constructing a freshwater fen over the deposited composite tailings at their primary tailings settling basin at Mildred Lake. As the fen was being constructed, hydrogen sulfide was detected at the site. Due to the toxic nature of this gas, it was important to understand its sources and biogeochemistry to prevent future potential environmental hazards. The samples analyzed for this study were collected in June, 2010 at the beginning of the fen’s construction and in July, 2011, one year later. These samples ranged from surface and 1 m depth in the sand cap to CT samples from its surface up to circa 40m. Using PLFA analysis, the total PLFA concentration for each of these samples was calculate and cell densities estimated. Across the site, the total PLFA concentration in the sand cap samples (ranged from 70-530 ug/kg), higher by up to an order of magnitude than the in the CT (which ranged from 20-80 ug/kg), except for two samples, the salt pan and the 1m depth sample from well 5d, both of which had total PLFA concentrations comparable to the CT. The temporal and spatial variability across the site were not systematic, and thus were likely due to the site’s heterogeneity.

Sulfate reduction via sulfate reducing bacteria was hypothesized to the dominant pathway for sulfide generation at Mildred Lake. The identification of sulfate biomarkers, iso and anteiso c15:0 and 10MethylC16:0 support this hypothesis. In addition to the CT and sand cap, which naturally contain sulfate, gypsum added as a coagulant to convert the fluid fine tailings to CT tailings to improve its workability, also introduced sulfate. This

study estimated that with the cells at this site being in the 10^7 range, the rate of sulfide generation is $12\text{nmol cm}^{-3}\text{ day}^{-1}$, which is approximately 40nm per ml of water per day. This will pose future problems because due to hydrogen sulfide's "rotten egg smell" and toxicity, water contaminated with hydrogen sulfide will be unsuitable for drinking. Therefore, there is a need for a sulfide sink such as converting it to pyrite, FeS_2 , which is less of an environmental issue. Also, since microbes prefer smaller, more bioavailable organic matter they can easily metabolize, limiting the amount of such bioavailable organic matter will also result in reduction on the amount of sulfide generated by SRBs.

Chapter 4

Thesis Conclusion

Microorganisms drive many environmental reactions, and understanding microbial communities helps investigate their roles and effects in environment (Wilson, 2003). Every microorganism contains lipids in their cells for structural and functional purposes. Lipids are organic compounds insoluble in water but soluble in organic solvent (Adibhatla et al., 2006). Considering that lipids are found in all microorganisms, lipid analysis can be used to investigate microbial communities when direct counting is unrealistic (Vestal and White, 1989). One type of lipid analysis uses phospholipids found in the cell membrane of all cells. A phospholipid consists of one molecule of glycerol, which is a three-carbon alcohol, with its two hydroxyl groups bound to two hydrophobic fatty acid chains, and the third hydroxyl group bound to a hydrophilic phosphate head. Since phospholipids usually serve a structural function in the cell membrane, they do not exist individually, but in groups forming a bilayer in the company of other important carbohydrates and proteins, which the cell also needs to function properly (Figure 4) (Kaur et al., 2005). Phospholipids are also only present in living cells and hydrolyze soon after cell death hence they give an indication of the viable biomass and microbial activity.

Briefly, phospholipid fatty acid (PLFA) analysis involves the extraction and identification of the lipids in the cells of the microorganism using the Bligh and Dyer method (Bligh and Dyer, 1959). The weighed samples are sonicated and extracted overnight to enable the lipid dissolve in the dichloromethane, methane and phosphate buffer in the ratio 1:2:0.8 respectively. After separating the extract from the sediments

using gravity filtration, the extracts are cleaned up using silica gel chromatography and the lipids are separated based on their polarity. The first fraction contains the non-polar lipids in the dichloromethane. The second fraction containing the neutral lipids is collected in acetone before the third fraction containing the polar lipids are contained in the methanol. Since phospholipids are polar lipids, they are contained in the methanol fraction. This fraction then goes on to undergo mild methanolysis converting the phospholipid fatty acids to fatty acid methyl esters (FAMES) by transesterification in the presence of potassium hydroxide catalyst. This step is necessary since the gas chromatography mass spectrometer used for this analysis cannot detect PLFAs. The retention time and mass fragmentation patterns are used to identify the FAME from the GC-MS and compared to known samples or to the lipid library.

The concentration of these PLFAs can be converted to cell densities using generic conversion factors such as those in Green and Scow (2000) to provide more information about the environments from which the sample was collected. In addition, since some PLFAs are characteristic of specific microbial groups, they are known as biomarkers or biological markers for the microbial communities they are related to (Fang et al., 2007). However, it should be noted that it is possible for more than one microbial community to produce a particular PLFA, therefore the application of PLFAs as biomarkers should be done with some caution (Boschker and Middleburg, 2002). The study funded by Syncrude Canada Limited, which is the primary focus of this thesis is one application of lipid analysis using PLFAs. In this study, PLFA concentrations were used to estimate cell densities at the site, and the identification of sulfate biomarkers at this site help

understand the sulfur cycle Syncrude's Mildred Lake site.

Syncrude Canada Limited is a company situated in Alberta, Canada. They are the largest synthetic oil producers in the country (Syncrude, 2012), hence they also generate large amounts of fluid fine tailings, a byproduct of the crude oil extraction process. The settling pond at Mildred Lake on Syncrude property in Alberta is their primary tailings settling pond. As mandated by the government of Alberta, lands that have been mined are required to be reclaimed to the point of long-term sustainability as it was pre mining (Renault et al., 2003). Syncrude, being one of the companies responsible for mining of crude oil, is also responsible for the reclamation of their mined land. In order to achieve this, they are constructing a freshwater fen at Mildred Lake over the composite tailings that have been previously deposited. Composite tailings (CT) are formed by the addition of a flocculants to fluid fine tailings (Hamza et al., 1996). In this instance, gypsum was the flocculant of choice because of its affordability, ease of use and availability. Sediment samples were collected across the site, from the sand-cap which separates the CT and the fen, and from within the CT itself. Using phospholipid fatty acid analysis, these samples were analyzed using the modified Bligh and Dyer method described above (Bligh and Dyer, 1959). The results from this study showed that the cell densities across this site were in the 10^7 range within the sand cap and in the 10^6 within the CT without systematic variation, temporally or spatially. These ranges were between that of rich soils which are usually in the 10^9 range and that of anaerobic groundwater systems which are typically in the 10^5 range.

One of the main objectives of the study was also to understand sulfur cycling at

Mildred Lake because of the detection of hydrogen sulfide at this site. To this effect, PLFAs that can be indicative of sulfate reduction were indentified. Oil sands are made up of a large amount of complex organic hydrocarbons such as naphthenic acids and an abundant amount of sulfate, which can occur in the CT naturally or can be introduced by the addition of gypsum, $\text{CaSO}_4 \cdot \text{H}_2\text{O}$. The microbes can therefore couple the reduction of organic carbon to the reduction of sulfate, which provides a probable source of the hydrogen sulfide detected at the site. However, these large complex hydrocarbons may not be bioavailable to the microorganism for their metabolism. It was estimated that the amount of sulfide is 40nm per ml of water in the aquifer. Biomarkers for fungi were also identified across including in the CT. Since the CT is anaerobic, the presence of fungi within it was unexpected. However, Gunde-Cimerman et al. (2009) proved that it is possible for fungi to adapt to the saline conditions of the CT and Griffith et al., (2010) showed not all fungi are aerobic, some are in fact anaerobic.

4.2 Future Research

The study raises some questions that can be addressed in future studies. This includes the source of organic carbon preferably being metabolized by the microorganisms. Understanding this becomes particularly important for the purposes of investigating bioremediation because this information provides insight on how the system can be manipulated to enhance bioremediation. For example, in a environment where methanogenesis is occurring, the addition of sulfate to such a system, will inhibit methanogenesis because the microbes will preferentially use sulfate because they gain

more energy from sulfate reduction than methanogenesis. Another aspect that can be studied further is if the introduction of more bioavailable smaller chain hydrocarbons will affect the amount of sulfide generated at the site since SRBs couple sulfate reduction to the oxidation of organic matter, thus it is expected that the more bioavailable organic carbon present, the more sulfate reduction will occur and vice versa. Finally, more studies can be done to check if the cell densities remain consistent as the fen continues to develop or if the cell densities increase or decrease providing some insight on the nutrients conditions of the site and also how the amount of sulfide at the site changes with time and ways to purify water contaminated with hydrogen sulfide so it is safe for consumption.

References:

- Adibhatla, R., Hatcher, J. & Dempsey, R. (2006). *Lipids and Lipodomies in Brain Injury and Diseases*. The AAPS Journal. Volume 8. Issue 2. Pages 314-321.
- Alfreider, A., Krossbacher, M. & Penner, R. (1997). *Groundwater samples do not reflect bacterial densities and activity in subsurface systems*. Water Reserves. Volume 31. Issue 4. Pages 832-840.
- Balkwill, D., Leach, F., Wilson, J., McNabb, J. & White, D. (1988). *Equivalence of Microbial Biomass measures based on membrane lipid and cell wall components, adenosine triphosphate and direct counts in subsurface aquifer sediments*. Microbial Ecology. Volume 16. Pages 73-84.
- Beauchamp, R., Bus, J., Popp, J., Boreiko, C. & Andjelkovich, D. (1984). *A critical review of the literature on hydrogen sulphide toxicity*. CRC Critical Reviews in Toxicology. Volume 13. Issue 1. Pages 25 – 97.
- Bellows, L. & Bohme, W (1963). Athabasca Oil Sands. *Journal of Petroleum Technology*. Volume 15. Issue 5. Pages 479-483
- Beersheba, F., Kotlyar, L., Sparks, B. & Chung, K. (2000). Organic coated solids in Athabasca Bitumen: Characterization and process implications. *Journal of Chemical Engineering*. Volume 78. Pages 610 – 616.
- BenchPrep Inc. (2012). Chemistry in AP Biology. <https://benchprep.com/>
- Bligh. and Dyer, W.J. (1959). *A rapid method for total lipid extraction and purification*. Canadian Journal of Biochemistry and Physiology. Volume 37. Pages 911-917
- Bordenave, S., Kostenko, V., Dutkoski, M., Grigoryan, A., Martinuzzi, R. (2010). *Relation between the activity of anaerobic microbial populations in oil sands tailings ponds and the sedimentation of tailings*. Chemosphere. Volume 81. Pages 663-668.
- Boschker, H. T. S., & Middelburg, J. J. (2002). *Stable isotopes and biomarkers in microbial ecology*. FEMS Microbiology Ecology. Volume 40. Issue. Pages 85-95.
- Bottcher, M.E., Thamdrup, B., Vennemann, T.W., (2001). Oxygen and sulfur isotope fractionation during anaerobic bacterial disproportionation of elemental sulfur. *Geochim Cosmochim Acta* Volume 65. Pages 1601-1609

Buenrostro-Gonzalez, E., Lira-Galeana, C., Gil-Villegas, A. & Wu, J. (2004). *Asphaltene precipitation in Crude Oils: theory and experiments*. American Institute of Chemical Engineers Journal. Volume 50. Issue 10. Pages 2552-2570

Chalaturnyk, R., Scott, D. & Ozum, B. (2007). *Management of Oil Sand Tailings*. Petroleum Science and Technology. Volume 20. Issues 9 and 10. Pages 1025 - 1046.

Charpentier, A., Bergerson, J. & MacLean, H. (2009). *Understanding the Canadian oil sands industry's greenhouse gas emissions*. Environmental Research Letters. Volume 4. Pages 1 -11.

Christie, W. (1993). *Preparation of ester derivatives of fatty acids for chromatographic analysis*. Advances in Lipid Methodology. Volume 2. Pages 69-111.

Chynoweth, D. University of Florida Agricultural and Biological Engineering.
http://faculty.abe.ufl.edu/%7Echyn/age2062/lect/lect_06/4_18.GIF

Cimerman, G., Ramos, J., & Plemenitas, A. (2009). *Halotolerant and halophilic fungi*. Mycological Research. Volume 113. Pages 1231 -1241.

Dowhan, W., & Bogdanov, M. (2002). Functional roles of lipids in membranes. In D. E. Vance, & J. E. Vance (Eds.), *Biochemistry of lipids, lipoproteins and membranes* (4th Edition ed., pp. 1). Paris: Elsevier

Ehrlich, H. (1996). *How microbes influence mineral growth and dissolution*. Chemical Geology. Volume 132. Pages 5-9.

Fang, J., Hasiotis, S., Das Gupta, S., Brake, S. and Bazylinski, D. (2007). *Microbial biomass and community structure of a stromatolite from an acid mine drainage system as determined by lipid analysis*. Chemical Geology. Volume 243. Pages 191 – 204.

Fedorak, P., Coy, D., Salloum, M. & Dudas, M. (2002). *Methanogenic potential of tailing samples from oil sands extraction plants*. Canadian Journal of Microbiology. Volume 48. Pages 21 -33.

Fierer, N., Schimel, J. & Holden, P. (2003). *Variations in microbial community composition through two soil depth profiles*. Soil Biology and Biochemistry. Volume 35. Pages 167-176.

Green, C. and Scow, K. (2000). *Analysis of phospholipids fatty acids (PLFA) to characterize microbial communities in aquifers*. Hydrogeology Journal. Volume 8. Pages 126-141.

- Griffith, G., Baker, S., Fliegerova, K., Liggenstoffer, A., van der Giezen, M., Voigt, K. & Beakes, G. (2010). *Anaerobic fungi: Neocallimastigomycota*. IMA Fungus. Volume 1. Issue 2. Pages 181 – 185.
- Hamza, H., Stanonik, D. & Kessick, M. (1996). *Flocculation of lime-treated oil sands tailings*. Fuel. Volume 75. Issue 3. Pages 280 -284.
- Handelsman, Jo. (2004). *Metagenomics: Application of genomics to Uncultured Microorganisms*. Microbiology and Molecular Biology Reviews. Volume 68. Issue 4.
- Harner, N., Richardson, T., Thompson, K., Best, R., Best, A., & Trevors, J. (2011). *Microbial processes in the Athabasca oil sands and their potential applications in microbial enhanced oil recovery*. Journal of Industrial Microbiology and Biotechnology. Volume 38. Pages 1761-1775.
- Headley, J., Peru, K., Barrow, M. & Derrick, P. (2007). *Characterization of Naphthenic Acids from Athabasca Oil Sands using Electrospray Ionization: The significant influence of solvents*. Analytical chemistry. Volume 79. Issue 16. Pages 6222-6229.
- Holmer, M. & Storkholm, P. (2001). *Sulphate reduction and sulphur cycling in lake sediments: a review*. Freshwater Biology Special Review. Volume 46. Pages 431-451.
- Holowenko, F., MacKinnon, M. & Fedorak, P. (2000). *Methanogens and sulphate-reducing bacteria in oil sands fine tailings waste*. Canadian Journal of Microbiology. Volume 46. Pages 927 – 937.
- Hoppe, G. & Hansford, G. (1982). *Ethanol Inhibition of continuous anaerobic yeast growth*. Biotechnology Letters. Volume 4. Issues 1. Pages 39-44.
- Kaneda Toshi (1977). *Fatty acids of the genus Bacillus: an Example of Branched-Chain Preference*. Bacteriological reviews. Volume 41. Issue 2. Pages 391-418.
- Kaur, A., Chaudhary, A., Kaur, A., Choudhary, R., and Kaushik, A. (2005) *Phospholipids fatty acid – A bioindicator of environment monitoring and assessment in soil ecosystem*. Current Science, Volume 89. Issue 7.
- Li, Y-L., Vali, H., Yang, J., Phelps, T. & Zhang, C. (2006). *Reduction of iron oxides enhanced by sulfate-reducing bacterium and biogenic H₂S*. Geomicrobiology Journal. Volume 23. Pages 103-117.

- Mackay, D., Freyberg, D., Roberts, P. & Cherry, J. (1986). *A natural gradient experiment on solute transport in a sand aquifer 1. Approach and overview of plume movement*. Water resources research. Volume 22. Issue 13. Pages 2017 -2029.
- MacKenzie, M.& Quideau, S. (2010). *Microbial community structure and nutrient availability in oil sands reclaimed boreal soils*. Applied Soil Ecology. Volume 44, Issue 1. Pages 32 – 41.
- MacKinnon. M., Matthews, J., Shaw. & Cuddy, R. (2001). *Water quality issues associated with composite tailings (CT) technology for managing oil sand tailing*. International Journal of Surface Mining Reclamation and Environment. Volume 15. Issue 5. Pages 235 – 256.
- Mravin-Sikkema, F., Richardson, F., Stewart, C., Gottschal, J. & Prins, R. (1990). *Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi*. Applied and Environmental Microbiology. Volume 56. Issue 12. Pages 3793-3797
- Masliyah, J., Zhou, Z., Xu, Z, Czarnecki, J & Hamza, H. (2004). *Understanding water based bitumen extraction for Athabasca Oil Sands*. The Canadian Journal of chemical engineering. Volume 82. Pages 628 - 654
- Matthew, J., Shaw,W., MacKinnon, M. & Cuddy, R. (2002). *Developments of Composite Tailings Technology at Syncrude*. International Journal of Surface Mining, Reclamation and Environment. Volume 16. Issue 1. Pages 24-39.
- McIntire, P. & Edenborn, H. (1990). *The use of bacterial sulfate reduction in the treatment of drainage from coal mines*. Mining and Reclamation Conference and Exhibition, Charleston, West Virginia, April 23-26, 1990.
- Olsson, P., Baath, E., Jakobsen, I. & Soderstrom, B. (1995). *The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil*. Mycol. Res. Volume 99. Issue 5. Pages 623-629.
- Piotrowska, Z & Mrozik, A. (2003). *Signature lipid biomarker (SLB) analysis in determining changes in community structure of soil microorganisms*. Polish Journal of Environmental Studies. Volume 12. Issue 6. Pages 669-675/.
- Raich, J. (1992). *The global carbon dioxide flux in soil respiration and its relation to vegetation and climate*. Tellus. Volume: 44B. Pages 81-99.

- Ramos-Padron, E., Bordenave, S., Lin, S., Bhaskar, I., Dong, X., Sensen, C., Pournier, J., Voordouw, G & Gieg, L. (2011). *Carbon and Sulfur Cycling by Microbial Communities in a Gypsum-treated Oil Sands Tailings Pond*. Environ. Sci. Technol. Volume 45. Pages 439-446.
- Reiffenstein, R., Hulbert, W., & Roth, S. (1992). *Toxicology of Hydrogen Sulfide*. Annual review of Pharmacology and Toxicology. Volume 32. Pages 109 – 134.
- Renault, S., MacKinnon, M. & Qualizza, C. (2003). *Barley, a potential species for initial reclamation of saline composite tailings of Oil Sands*. Journal of Environmental Quality. Volume 32. Pages 2245-2253.
- Richardson, D. (2000). *Bacterial respiration: a flexible process for a changing environment*. Microbiology. Volume 146. Pages 551-571.
- Sinensky, M. (1974). *Homeoviscous Adaptation- A Homeostatic Process that regulates the viscosity of Membrane lipids in Escherichia coli*. PNAS. Volume 71. Issue 2. Pages 522-525.
- Sparks, B., Kotlyar, L., Carroll, J. & Chung, K. (2003). Athabasca oil sands: effect of organic coated solids on bitumen recovery and quality. *Journal of Petroleum Science and Engineering*. Volume 39. Pages 417-430.
- Sworska, A., Laskowski, J., & Cymerman. (2000). *Flocculation of the Syncrude fine tailings Part 1. Effect of pH, polymer dosage and Mg²⁺ and Ca²⁺ cations*. International Journal of Mineral Processing. Volume 60. Pages 143 – 152.
- Syncrude Canada Limited (2012). www.syncrude.ca
- Tan, K., Lee, K. & Mohamed, A. (2009). *Production of Fame by palm oil tranesterification via supercritical methanol technology*. Biomass and Bioenergy. Volume 33. Pages 1096-1099.
- Trudinger, P & Swaine, D. (1979). *Biogeochemical cycling of Mineral Forming Elements* (3rd ed.). Amsterdam, The Netherlands: Elsevier Scientific Publishing Company.
- Vestal, J. & White, D. (1989). *Lipid Analysis in Microbial Ecology*. Bioscience. Volume 39. Issue 8. Pages 535 -541.
- Villanueva, L., Haveman, S., Summers, Z. & Lovley, D. (2008). *Quantification of Desulfovibrio vulgaris Dissimilatory Sulfite Reductase Gene Expression during Electron Donor- and Electron Acceptor-Limited Growth*. Applied and environmental microbiology. Volume 74. Issue 18. Pages 5850-5853.

MSc. Thesis – N. Ngonadi; McMaster University - School of Geography and Earth Sciences

Wilson, Edward. (2003). *The encyclopedia of life*. Trends in Ecology and Evolution.
Volume 18. Issue 2. Pages 77 – 80.