BIOMARKERS OF MICROBIAL CELLS AND METABOLISMS IN SYSTEMS RELATED TO ENERGY INDUSTRIES

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2024)

Earth and Environmental Sciences

Collaborative Graduate Program in Astrobiology

McMaster University

Hamilton, Ontario

TITLE: BIOMARKERS OF MICROBIAL CELLS AND METABOLISMS IN SYSTEMS RELATED TO ENERGY INDUSTRIES

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NUMBER OF PAGES: ix, 98

ABSTRACT

Understanding the distribution, abundances, and metabolic activity of microbial life in terrestrial environments is fundamental to our understanding of the role that microbial life can play in many areas of interest, such as biogeochemical cycling and microbially assisted degradation, and as the foundational knowledge for the search for extraterrestrial life. Depending on location, sufficient biomass or extraction procedure can pose significant challenges to quantify and identify the microbes present in a system due to low biomass, cell resistances, or matrix effects.

This Master's Thesis project has two main aspects. The first study utilizes phospholipid fatty acids (PLFA) analysis to determine the influence of methane and other petroleum hydrocarbons on the Base Mine Lake (BML) Pit Lake (PL). This was done by extracting biofilm units that were deployed into BML at different seasons and depths. PLFA biomarkers were utilized to determine cell abundance on biofilms as well as microbial community composition. This study revealed abundant microbial growth on biofilm units. The observed microbial composition on the biofilm units was comparable to that of the water column, with notable increase of C16:1 and polyunsaturated PLFA in both the epilimnion and hypolimnion, which contains biomarkers consistent with methanotrophy (C16:1) and phototrophy (polyunsaturates). Radiocarbon analysis of PLFA from biofilm units demonstrated that the carbon source used by the microbial communities within the system was derived from petroleum carbon, with petroleum carbon contributing up to 90% of the carbon in the PLFA. Strong stable isotopic depletion of biomarker lipids ($\delta^{13}C$ = -51 ‰) for methanotrophy indicated the use of methane derived carbon by the community. In the epilimnion methanotrophy was indicated to be less important and indications of photosynthetic metabolisms were concurrent with a slightly more modern radiocarbon content consistent with inputs from autotrophy using atmospheric carbon.

The second study investigated Dipicolinic Acid (DPA), a biomarker for endospores, a highly resistant state of a variety microbial Genera. This state can survive radiation, desiccation, heat, and bacterivore digestion, and is brought on due to the environment the microbe is living on becoming harsh or unfavorable. Endospores can remain viable for extended periods of time, up to millions of years (Cano & Boruki, 1995; Vreeland, Rosenzweig, & Powers, 2000), which has made them an organism of interest for astrobiology. The presence and potential survivability of endospores within bentonite clay is also of highly applied interest to the Nuclear Waste Management Organization (NWMO) who is proposing to use bentonite within their multi-barrier spent nuclear fuel storage proposal. This study was undertaken to determine the extent of mineral matrices on the effectiveness of DPA extraction from bentonite clay, as well as other planetary analogs. This initial study demonstrated that extraction of DPA was able to effectively identify and quantify the presence of known numbers of spores added to bentonite clay. For this, plates of *Bacillus Subtilis* were grown and suspended, then cells were counted in a hemacytometer to determine concentration of the spore suspension, which was used to spike mineral matrices to learn how they influence the recovery of DPA during extraction. Further, it showed that the presence of bentonite did not have a significant effect on recovery. The only mineral matrix to see significantly lower than expected recovery was basalt. Finally, initial tests on unseeded bentonite clay showed that spores were below limit of quantification for all samples, but above the limit of detection.

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RESEARCH BACKGROUND AND FUNDAMENTAL CONCEPTS

1.01 Biomarkers and Biosignatures

Biomarkers are a substance, object, phenomenon, pattern, or temporal change that originates from a biological process or agent (Des Marais et al. 2008). Modern definitions of biosignature, due to overlapping usage of the term in a variety of fields, has been the subject of review and scrutiny of usage to avoid confusion (Malaterre et al. 2023). Environmental context can be an important factor in biosignature utilization, to avoid false-positives, and overall misunderstanding of the data. The context for biosignatures used for these papers follows the description of 'strong' biosignatures described within Des Marais et al. (2008) referring to material things such as substances and objects (Malaterre et al. 2023) that up to current understanding, is directly related to the processes and functioning of biogenic materials. Due to the thermodynamic improbability in patterns of chemical and structures composed from biologically produced material, detection of biological activity can be observed. (Des Marais et al. 2008)(Lovelock, 1965). Biosignatures and biomarkers (a term more widely used in environmental and biological sciences, defined as organic molecules recovered from sediment) (Summons et al. 2022) are fundamental tools we use to understand the activities of life on Earth, the history of life on earth and as the foundation of the search for life beyond the earth. Like fossils leaving biogenic traces within the geologic record, chemical footprints can be used to trace biogenic origins. Many biomarkers exist and encompass a wide variety of presence over time, and are useful within biomedical, food, environmental, astrobiological, and a wide variety

of other applications and can take the shape of organic molecules, isotopic ratios, mineral compositions, or morphological structures. These biomarkers are of interest in both applied research, such as understanding microbial roles in the breakdown of organic contaminants in the environment, and in basic research areas such as providing the foundation to interpret the geologic record of life and/or to inform the search for life.

This thesis focuses on the use of biomarker compounds and their isotopic compositions to understand microbial community presence and metabolisms in two systems that have both applied and astrobiological implications. The application in understanding what is happening during reclamation or storage of energy industries waste products. And astrobiological through the implications the results have to search for life in extreme environments limits and capabilities of life. The two biomarkers researched in these studies are phospholipid fatty acids (PLFA) and dipicolinic acid (DPA).

1.01.1 Phospholipid Fatty Acids (PLFA)

A useful tool for examination into the microbial communities is via extraction of phospholipid fatty acids (PLFA). Phospholipids are also referred to as intact polar lipids (IPL) and are the main building block of bacterial and eukaryotic cellular membranes. Phospholipids consist of a hydrophilic phosphate head, and a hydrophobic fatty acid tail (Figure 1.1). Phospholipid fatty acids are the fatty acid “tails” that are a component of these lipids. As such, PLFA are a representation of the intact polar lipids present. PLFA’s are strictly biogenic. Breakdown of PLFA occur within days to weeks following cell death. This allows for extant, viable microbial communities to be quantified and insights to be made. (Green & Scow, 2000; White et al. 1979).

Insight into carbon sources used by specific communities, as have been shown in many studies can also be determined (Zelles et al. 1994; Boschker & Middelburg, 2002).

Certain PLFA have been cited to be specific markers of specific taxonomic groups, described by Green & Scow (2000). However, further observations have shown that some overlap between PLFA of different groups has been noted. (Frostergard et al. 2011). Since PLFA biomass is quantifiable, the amount of PLFA per cell can be estimated via biomass conversion factors (Green & Scow, 2000).

PLFA are analyzed via transformation to fatty acid methyl esters (FAMEs) for gas chromatography-mass spectrometry analysis to indicate the presence and state of some classes of microorganisms (Zelles, 1999). PLFA can be purified and separated effectively via silica gel and gas chromatography and compared to FAME libraries to compare retention times. The fatty acid carbon content makes them also very suitable for stable carbon and radiocarbon analysis. The isotopic compositions of these PLFA can be linked to the sources of carbon being consumed by the organism and / or their biosynthetic pathways, which can lead to understanding of the community metabolism and energy sources, as described in more detail in Sections 1.4 and 1.5. The first project in this thesis applied PLFA and PLFA isotopic analysis to elucidate the carbon sources and cycling in systems such as Base Mine Lake, the first pit lake in the Alberta Oil sands region (AOSR). The goal for the work was to understand what carbon sources were being used by the microbial community, and to elucidate the extent to which petroleum hydrocarbons (PHs) were a carbon source utilized directly or indirectly via use of methane produced from the PH. The outcomes of this project will assist in the management, design, and implementation of future PLs planned for the AOSR.

1.01.2 Using $\delta^{13}C$ to constrain and determine metabolism pathways.

Stable isotope geochemistry can be used to determine the carbon sources and metabolism of the bacteria of which the lipids originated. Carbon isotope signatures within extracted lipid analysis can provide information regarding carbon source consumption by microbial communities (Jahnke et al. 1999). Stable isotopic compositions of biomarkers can be used to gain insight into the carbon sources and metabolisms occurring within a system. Stable isotope analysis involves determination of the ratio of ^{13}C to ^{12}C in a sample of organic compounds. This ratio is reported in delta notation in permille using the following equation 1:

$$\delta^{13}C\ (‰) = ((^{13}C/^{12}C_{sample}) / (^{13}C/^{12}C_{standard}) - 1) * 1000 \quad (1)$$

changes in the ratio of ^{13}C to ^{12}C in a carbon pool are referred to as fractionation effects. Fractionation effects are seen between photosynthetic pathways, crude oil, or methane, and the carbon signature of the current atmosphere. Due to differing metabolic pathways, various ranges of fractionation effects can occur, which can be used to determination of the driving metabolisms of microbial life within a system. Examples of carbon fractionation pathways in an aquatic environment can be seen in Figure 1.2 (Boschker & Middelburg, 2002). All stable carbon signatures are made in relation to a standard: Vienna Pee Dee Belemnite (VPDB). This means that all carbon signatures are made in relation to the VPDB standard, so depletion of ^{13}C is lighter than the standard, and enriched is heavier, the standardization of this technique allows for the continued comparison and widely understood natural fractionation of material to allow for global comparison. These calculated $\delta^{13}C$ values are reported in per mille (‰). Per mille is a unit

of relative difference between two sample ratios of carbon, referring to the relative difference in parts per thousand of the sample tested against an isotopic standard.

By comparing the $\delta^{13}C$ of PLFA, DIC, DOC, and methane, insight can be gained into the metabolisms that are occurring within the system. Due to fractionation within carbon metabolism pathways, stable isotopes and the ranges of the fractionation can identify carbon sources metabolized. This is because lighter isotopes, like ^{12}C, are used preferentially by microbes due to kinetic and thermodynamic favourability. Particularly, Methane from methanogenesis is very isotopically ^{13}C depleted (Boschker & Middelburg, 2002), as smaller compounds such as CO_2 and CH_4 have higher Kinetic Isotope Effects (KIEs) (Hayes, 2001). Due to the KIEs, microbial metabolism of isotopically ^{13}C depleted methane will result in a transfer of an even greater depleted signal from methane to the cell's makeup. Microbes that consume the isotopically depleted methane derived carbon will transfer this depletion into their cellular components resulting in highly ^{13}C depleted isotopic compositions (Jahnke et al. 1999; Summons et al. 1994). Biogenic methane can have values ranging from -50 to -110 ‰ (Freeman, et al., 1990; Zhang 2002). Due to this, $\delta^{13}C$ of PLFA from methanotrophs is often extremely depleted relative to $\delta^{13}C$ DIC and similar to the extreme depletion seen in methane. In contrast, autotrophically produced carbon is 10 to 30 ‰ ^{13}C depleted relative to the initial carbon source (DIC or Atmospheric CO_2) (Figure 1.2). Heterotrophic metabolisms impart a relatively small isotopic fractionation relative to their carbon source. (Boschker & Middelburg, 2002; Londry et al., 2004). Due to this, tracking isotopic signatures within the microbial communities in a system can occur, by tracing this light carbon signature (Coleman, Risatti, & Schoell, 1981).

1.01.3 Unstable carbon and Age dating using Radiocarbon analysis.

Radiocarbon is produced in the atmosphere due to solar rays and nuclear bomb testing interacting with atmospheric nitrogen via the addition of a neutron and emission of a proton to form an unstable carbon 14 atom. This unstable carbon is also incorporated into living tissues and has a half-life of 5730 years (Godwin, 1962) (Ingalls & Pearson, 2005). Most organisms will be at equilibrium with ^{14}C in their environment, with the gain and loss of ^{14}C being even. However, upon organism death, the remaining radiocarbon decays over time with no additional ^{14}C gain. Over sufficient time, generally considered 5-6 half-lives, 10 at most, all of the radiocarbon present in an organic compound will have decayed away. Such materials are referred to as radiocarbon dead and include ancient, fossil carbon such as petroleum hydrocarbons.

The radiocarbon content of organic compounds can be reported with several notations. Commonly it is reported as percent modern carbon or fraction modern, however, it can also be reported in $\Delta^{14}C$ notation, which can be used in mass balance calculations. The $\Delta^{14}C$ scale goes from -1000 ‰ for materials with no detectable radiocarbon remaining, to $\Delta^{14}C$ of circa 20 For the modern atmosphere (Basu et al, 2020), and as positive as 160 ‰ in coastal waters in the 1970s and around 700 ‰ in the atmosphere in the mid 1960s for compounds where so called “bomb spike” carbon is present. The “bomb spike” refers to the increased atmospheric radiocarbon levels that resulted from atmospheric nuclear weapons testing. In a lake environment, both pre and post war carbon can be found, with these ranges of values of

radiocarbon dead and more modern DIC, a mass balance can be used to see the impact of ancient carbon on microbial environments. The distinctions in radiocarbon content of these carbon sources can be used to differentiate the carbon sources being used by a microbial community (Slater, 2005; Graven, 2015; Turnbull et al. 2007).

^{14}C records can be determined by sediment cores, which provide a $\Delta^{14}C$ record for the period of time those sediments were formed (Hughen et al., 2004). Sediment cores can be compared to Greenland ice core CO_2 isotope record can allow to time equivalent tie points, and other records used include corals. With the method to determine the ^{14}C record, the uncertainties of each record type will influence the age dating relationship of other samples.

Due to isotopic fractionation being normalized during radiocarbon analysis microbial utilization of such radiocarbon dead carbon sources ($\Delta^{14}C$ = -1000 ‰) will result in microbial cellular components that are also devoid of radiocarbon. In contrast, recently photosynthesized carbon will have radiocarbon contents equivalent to the carbon source from which they were generated, either the atmosphere ($\Delta^{14}C$ = 15 to -20 ‰**)** in the case of plants, or the dissolved inorganic carbon in a lake **(**$\Delta^{14}C$ = -110 to -145 ‰ in some western Ontario rivers**) (**Zeidan et al 2022). This is because the influence of ancient carbon being so highly negative regarding radiocarbon, where modern sources would still have a higher ^{14}C presence (Ahad & Pakdel, 2013).

1.01.4 FAME isotopic corrections

Due to the addition of methanol in the PLFA to FAME conversion, the methanol was evaluated for $\delta^{13}C$ and $\Delta^{14}C$ to be able to provide an adjusted value for the values determined on the isotope ratio mass spectrometer (IRMS). A mass balance was used to determine the impact of the extra

carbon attached to the FAME due to derivatization during the mild alkaline methanolysis. The impact balance used is seen in equation 2:

$$D_A = ((n + 1) * D_{FAME} - D_{MeOH}) / n \quad (2)$$

Where D_A is the actual corrected PLFA $\Delta^{14}C$ value, n is the carbon chain length of the FAME, D_{FAME} is the isotopic value of the sample (pre-correction), and D_{MeOH} is the isotopic value of the methanol used in the mild alkaline methanolysis.

While the stable isotope ratios are strongly affected by metabolism in organisms, by necessity, radiocarbon analysis is normalized for fractionation so that differences in radiocarbon content can be used to determine the age of the material. This means that beyond being a dating tool, radiocarbon analysis can be used to trace carbon sources through a biogeochemical system. This approach is particularly powerful when radiocarbon "dead" carbon, such as million year old petroleum hydrocarbons, is mixing with carbon derived from the current atmosphere.

1.02 Alberta Oil sands

The Alberta Oil Sands Region ranks among the largest crude oil reserves in the world, with the Athabasca oil sands region covering an area of 142,200km^2, producing up to 3.1 million barrels of oil per day in 2018, and a proven reserve of 158 billion barrels of oil present at the start of mining (USGS.gov). Surface oil sand extraction activities result in excavation of mine pits as well as the generation of large volumes of tailings. Regulatory requirements require that these impacts on landscape be reclaimed by operators, so long-term strategies must be implemented

for reclamation of the oil sands process tailings (Allen, 2008). One such reclamation strategy is the construction of Pit Lakes. Pit Lakes reclaim land impacted by oil sands surface mining as well as storing large volumes of tailings under a water cap that isolates the tailings with the goal of preventing future release of any hazardous components (Dompierre et al. 2016). Syncrude Canada Ltd. commissioned the first full scale water-capped tailings reclamation lake through the creation of Base Mine Lake (BML) in 2012. The location of BML is north of Fort McMurray, in Alberta, Canada, and covers over 800 hectares of land with about 45 meters of fine fluid tailings (FFT) depth being covered by a water cap. Due to the settling and dewatering process, where turbidity within the lake decreases over time, the FFT present in the lake has changed from 5 meters of water cap when the lake was first established to now at a depth of 12m of water in 2019 (Syncrude, 2021). The purpose of the Pit Lake is to create a self-sustaining lake ecosystem that will be able to support life and not require active maintenance in the long term. The progress of Base Mine Lake towards establishing a self-sustaining ecosystem and maintain the isolation of FFT from the environment is the focus of a large scale, multidisciplinary research program. An important aspect of this research is understanding the role of in situ microbial communities in controlling lake biogeochemistry and for the biodegradation of oil sand by-products such as naphthenic acids or petroleum hydrocarbons (PH) (Foght, Gieg, & Siddique, 2017). To support the degradation of these potentially toxic compounds, persistent dissolved oxygen is required to allow for the metabolism of PH. This increased oxygen allows for increased aerobic oxidation of FFT by-products, and is a crucial step to develop the self-sustaining lake. This first project focuses on monitoring metabolisms of microbial communities within BML in a variety of ways to understand petroleum hydrocarbon (PH) and PH derived metabolism sources are influencing

the carbon consumed within the lake and utilization into the microbial cell makeup, which can assist in understanding oxygen consumption throughout the lake.

1.02.1 Biogeochemistry of proposed reclaimed lake

A key biogeochemical factor in the development of BML is the need for consistent dissolved oxygen within the water column is to support aerobic respiration, a major component to the viability of a self-sustaining lake. (Blumenberg, Seifert, & Michaelis, 2007). In addition, the presence of oxygen allows for microbial heterotrophy to breakdown compounds that may be released from the underlying FFT, such as methane by methanotrophy and/or residual petroleum hydrocarbons and naphthenic acids, which makes it a critical component to determine the lake's oxic boundary. In order to assess the biogeochemical cycling within BML, and therein the role of heterotrophs, including methanotrophs, in controlling oxygen levels and removal of petroleum derived contaminants, it is necessary to be able to track microbial carbon sources and metabolisms.

1.02.2 Methane utilization within BML and controls on oxic boundary

Biogeochemical cycling of methane is a key component of understanding BML development. Methane is produced within the anoxic FFT underlying BML via the activities of methanogenic microbes (Siddique et al. 2011). The methane is then released into the lake by various means of transportation. The main processes of methane transport are advective transport during FFT settling, diffusive transport across concentration gradient, and gaseous methane bubbles allowing for methane to dissolve into the water along the bubbles path to the surface. This provides methanotrophs a source of energy while removing methane from atmospheric release, but also depletes oxygen throughout the lake (Arriaga et al. 2019; Bradford et al. 2017; Chen et al. 2013).

While the oxidation of larger chemicals that are released in the dewatering of FFT such as naphthenic acids and larger petroleum hydrocarbons plays a role in the oxygen budget of the lake, previous studies have shown methanotrophic activity is a major control on oxygen supply within the water column (Slater et al. 2021; Risacher et al 2018; Arriaga et al. 2019). Nonetheless the oxidation of these larger compounds is important in preventing release of these NA and PH to the broader environment. Further, as the lake develops, it is expected that photosynthetic activity will increase in the surface waters as lake clarity increases. This provides a potential source of labile organic matter to the lake which may influence biogeochemical conditions and outcomes. Understanding the interplay between methane, petroleum and NA and recently photosynthesized carbon sources is an important component of confirming that the development of BML is on a trajectory that will meet its design goals. The goal of this research project was to assess the microbial communities composing BML, and in particular to elucidate the carbon sources driving microbial metabolism within BML, particularly the relative importance of petroleum derived versus recently photosynthesized carbon.

1.03 Microbial Metabolism

Methanotrophs consist of 3 subgroups based on metabolic pathways, which are Type I, II, and X. These 3 subgroups oxidize CH_4 via a methane monooxygenase (MMO) found either as soluble, having a Fe_2O active site, whereas particulate MMO uses copper. Methane is oxidized to and proceeds via reaction 3:

$$CH_4 + O^{2-} \rightarrow CH_3OH \rightarrow CH_2O \rightarrow HCOOH \rightarrow CO_2 \quad (3)$$

Type I Methanotrophs utilize Ribulose monophosphate (RuMP) Assimilation pathway, which uses formaldehyde, where Type II uses the Serine pathway, that converts formaldehyde to serine, and CO2 is fixed by another enzyme. Type I methanotrophy has been seen to perform better in low methane high oxygen environments (Henckel, Roslev, & Conrad, 2000). Type X methanotrophs share enzymes of both the Serine and RuMP pathways and are mostly seen growing at higher temperature environments (Jiang et al. 2010).

Heterotrophy using either PH or dissolved organic carbon may also be occurring illustrated by reaction 4:

$$CH_2O + O_2 \rightarrow CO_2 + H_2O \quad (4)$$

Photosynthesis to produce organic matter (OM) can be occurring via reaction 5:

$$6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6H_2O \quad (5)$$

The carbon sources for these reactions have two main sources. They either will derive primarily from the petroleum hydrocarbons via either direct heterotrophy or methanotrophy of methane produced from PH. Or they can be derived from heterotrophy of modern OM sources or photosynthesis within the lake or deposited in the lake.

Rates of metabolism are subject to a variety of factors that can influence consumption rates and impact the oxygen concentration. Temperature will impact bacterial growth, with conditions between 0-50 degrees Celsius allowing metabolism, but 25-30 degrees Celsius being optimal growth temperatures (Mohanty, Bodelier, & Conrad, 2007).

1.04 NWMO and the Multi-Barrier system

The Nuclear Waste Management Organization (NWMO)'s multi-barrier system, which is a robust engineered system designed to alleviate potential risks that may be associated with the long-term storage of used nuclear fuel in a deep geologic repository. In 2002, with the passing of the Nuclear Fuel Waste Act (NFWA), the NWMO was established to develop and study the most effective way to keep Canadians safe and manage the increasing number of used nuclear fuel pellets. Consistent with proposals in other countries that utilize nuclear energy, such as Switzerland, Belgium, France, Finland, Japan, Republic of Korea and Russia, the NWMO has proposed a deep geologic repository for the long-term storage of spent nuclear fuel. A key component of this proposal is the NWMO's "multi-barrier system" (MBS) (Figure 1.3) that uses natural and engineered barriers to maintain containment of used fuel until it returns to background levels of radioactivity. In the DGR MBS fuel elements, which are tubes containing the fuel pellets bundled together, are contained in a carbon steel, copper coated container that in emplaced with highly compacted bentonite clay along with clay fill within the excavated repository (NWMO.ca).

The durability of this bentonite barrier is critical as a security against any water borne transport of radionuclides should a cannister fail. But equally to prevent processes that may affect container integrity, in particular the exposure of the copper-coated container to chemicals that may lead to corrosion, such as H_2S. And while chemical reactions with local groundwater and bentonite can be constrained, it is also necessary to understand the potential impacts of metabolic activities by microbes present within the bentonite itself.

Ongoing research by the NWMO and their partners are investigating the potential for vegetal microbial growth within bentonite under conditions analogous to the DGR. The focus of this research study is to assess the potential presence of bacterial endospores (spores), which are not accounted for in traditional cell detection methods, within the bentonite that may contribute to future microbial growth.

1.05 Dipicolinic Acid

Chapter 3 focuses on another biomarker, dipicolinic acid (DPA). DPA makes up to 15% of the dry weight of both *Bacillus* and *Clostridium* endospores(spores) (Setlow, 2009; Gerhardt & Marquis, 1989). DPA assists in protecting endospores from wet heat, dry heat, and spore stability (Setlow et al. 2006). In most spores, partial dehydration of the inner core allows for increased wet-heat resistance and enzymatic dormancy (Beaman et al. 1982; Nakashio & Gerhardt, 1985). DPA is immobile within the core, and its chelation with calcium allows for extensive mineralization of the cell (Setlow 2009; Gerhardt & Marquis, 1989). Along with DPA in the core, small acid soluble proteins can also be found within the core, where they cling to and saturate DNA, morphing the structure and allowing for increased resistance to heat, UV radiation and chemicals.

Spores have increased survivability due to their extremely thick outer membranes, primarily by increasing membrane proteins, but many significant other physiological changes occur on the path to sporulation. Once complete, spores exhibit resistance to UV, heat, chemicals, digestion and are metabolically dormant while retaining very minimal water (Nicholson et al, 2000; Fox & Eder, 1969; Bloomfield & Arthur 1994). Upon asymmetric cell division, the smaller cell, the forespore, forms the spore, and is engulfed in the larger mother cell. Once the cell is engulfed,

changes to the cells physical characteristics continue. The forespore that forms is a dual-membrane structure, and further creation of membranes continue. The next protective membrane formed is the cortex, a peptidoglycan structure that sits between the two forespore membrane layers (Henriques & Moran, 2007). Next, a protein coat that encompasses the entirety of the forespore, between the outer membrane and the coat. The coat's proteins are mostly spore species specific in regards to *B.Subtilis* (Dirks, 2002). Some unique spores also create an exosporium, which forms on the outside of the spore coat. This membrane is balloon-like, and has folds and creases. The exosporium is mainly proteinous, with varying distances from the coat, and is seen to be tethered to the rest of the spore via protein-protein interaction (Stewart, 2015). Finally, innermost to the cell is the core, where DPA is located. Once the spore has 'cracked', free DPA released from the spore is degraded rapidly in aerobic and anaerobic conditions, thus detection of DPA is convincing evidence of spore presence (Seyfried & Schink, 1990). Extraction of DPA and chelation to the lanthanide metal Terbium (Tb^{3+}) for fluorescence detection paired to a High-Performance Liquid Chromatograph (HPLC) can allow for very low limits of detection of DPA (Lomstein et al 2012; Rattray et al 2021). Again, as with PLFA, cell conversion factors with a quantified biomass of DPA can lead to estimates about existing spores found within samples. (Fitchel et al 2007)

Viable but non-culturable bacteria (VNCB) as well as spore forming bacteria (SFB) are both unique and independent survival mechanisms that are difficult to see in differing, but common analysis techniques. VNCB are bacteria that do not grow on plate cultures, making them difficult to find via 16S rRNA analysis, while also breaking the distribution ratio of culturable microbes that have been allowed to grow on ideal growth medium. (Ramamurthy, Ghosh, Pazhani, & Shinoda, 2014). While these can maintain metabolic function, Sporulation is a different survival

mechanism that cuts metabolic processing to unmeasurable levels. Both are of interest to the NWMO, as both have been determined to survive the compacted bentonite clay found in the bentonite-based barrier system. (Stroes-Gascoyne, Hamon, & Maak, 2011).

1.06 Bentonite Clay usage and properties

Bentonite, depending on chemical structure, will have varying amounts of swelling capabilities, with the small particle size with large surface area, large cation exchange capabilities, and variations in exchangeable ions leading to impressive water retention and swelling these clays can possess. (Odom, 1984). Bentonites such as Wyoming's MX-80 has a major composition of monovalent Na^{+}, which allows for higher swelling capacity for MX-80's ~71% Montmorillonite content compared to divalent Ca^{+2} (Segad et al. 2010).

With the clay being a crucial component of the multi-barrier system, looking at the stability and potential issues that may arise is a major factor to consider, with a final system that should provide safety even within glaciation pressures, where 3 kilometres of ice may form on the surface. Due to the high swelling pressures of the highly compacted bentonite (HCB), the potential for metabolic activity is only expected to occur within the regions of the DGR that are not fully sealed such as interfaces between host rock or containers and the HCB, between the less dense backfill material and within fractures that may occur in the HCB.

The durability of this bentonite barrier is critical as a security against any water borne transport of radionuclides should a cannister fail. But equally to prevent processes that may affect container integrity, in particular the exposure of the container to chemicals that may lead to

corrosion, such as H_2S. And while chemical reactions with local groundwater and bentonite can be constrained, it is also necessary to understand the potential impacts of metabolic activities by microbes present within the bentonite itself.

Ongoing research by the NWMO and their partners are investigating the potential for vegetal microbial growth within bentonite under conditions analogous to the DGR. Particular to this study, the extraction of DPA from spores, which contain some sulphate reducing bacteria (SRB) species, as seen from Rattray et al (2023) and Fitchel et al (2007) was investigated to determine quantities of natural endospore counts within the natural bentonite proposed for use within the MBS.

1.06.1 Conditions within the DGR

H_2 is likely to be the dominantly produced gas in the DGR due to the radiolysis of water (Holmboe, Jonsson, & Wold, 2012; El Mendili, Abdelouas, & Bardeau, 2013). With this, and the additional presence of external pressure, clay variability, and microbial degradation, fractures within the clay barrier are possible (Graham, Harrington, & Sellin, 2016). With these factors of increasing pore/fissure space within the DGR, understanding and potential for microbial growth and clay barrier degradation. Due to the compaction of the main components of the clay barrier system, most microbes will be unable to survive, will either die, or transform into a spore state. This compaction of bentonite leads to increased dry density, decreased pore size, higher swelling pressures, and a decreased water activity. These factors limit microbial growth and nutrient cycling attempts (Stroes-Gascoyne et al. 2010). Various densities have been used in temporal studies to determine long-term effects of microbial growth in bentonite clay found that in pressure cells filled with bentonite, a minimum water activity of $\alpha_w = 0.96$ and 2 MPa of pressure

were needed, which was equivalent to a dry density of 1.6 g/cm^3 where salinity levels were at or below 50g/L (Stroes-Gascoyne et al. 2010).

1.06.2 Metabolisms of Spore Formers

Usually, Gram- Positive cells achieve sporulation state when a bacterial cell's preferred metabolising environmental carbon, phosphate, or nitrogen supply has run out (Higgins & Dworkin, 2012). This causes sporulation to occur and will cause an asymmetric cell division wherein within the cell walls, one side consumes the other. Spores are formed from a variety of genus and species of bacteria, mostly constrained to the Firmicutes phylum, which contain Clostridia, strict anaerobic bacteria, and Bacilli, mainly aerobic bacteria (Tragg et al. 2010). The *Clostridium* and *Bacillus* species are rod shaped Gram-Positive bacteria, and Clostridia species such as *Clostridium clariflavum* are not only spore-forming, but thermophilic as well (Artzi et al. 2014; Muyzer & Stams, 2008). Rattray et al. (2023) and Fitchel et al. (2007), determined that some SRB are spore formers and contain very high levels of DPA, with the fact that most SRB spores were obtained from thermophilic environments likely attributed to the increased DPA concentration.

Although the cause of sporulation is due to nutrient depletion within a microbes surroundings, whether spores are formed in situ in these environments or not has been a recurring question. Spores have been found in many areas that have historically been extremely low nutrient low biomass sites, and percolation and water transport of spores have been discussed as a potential source of transport to deep subsurface and ancient environments (Vreeland et al. 2006). This has called to question the age of viable endospores found in such environments, with the percolation

of young endospores potentially contaminating ancient environments, or whether they had grown and sporulated in situ (Kennedy, Reader, & Swierczynski, 1994; McKenney, Driks, & Eichenberger, 2013).

1.07 Spores within Bentonite clay

Over time, test bentonite plugs that have been kept for ~8 years have shown cell counts similar to pre-compacted clays (Jalique et al. 2016). The stability of the communities of microbes that survive within the clay has also been tested over time, to understand if the competitive nature of space and nutrients in the low availability environment will allow for monocultures to prevail over time in the DGR. Under different dry densities (1.5 and 1.25 g/cm^3) and buried in a 9 m deep borehole, clay microbial communities were seen to be similar in community profiles and phospholipid analysis between densities and inner/outer portions of clay over 13 months. The presence of certain bacteria like *Streptomyces* has been found within compacted and loose MX-80 bentonite likely due to spore formation. (Engel et al. 2019). In chapter 3, spores were extracted from bentonite samples in order to assess the presence of spores within the starting MBS material and thereby being able to predict and assess future potential activation and metabolic activities from spore-forming bacteria.

1.08 Astrobiology

Biosignatures are a critical component of astrobiology as understanding them on Earth provides the basis for interpreting signatures on other astrobiological targets. Analog environments are

locations on earth that share environmental or geological conditions with an extraterrestrial planet or moon, either past or present (Osinski et al. 2006). Analog studies are crucial to astrobiology as it gives insight into how early life may have formed, what that life would have looked like, and the adaptations life can take to survive and thrive in their environmental extremes and the biosignatures that life leaves behind. These close to home environments can be used to determine extraction techniques and determine what technology should be brought into the future of space exploration. Endospores in particular have been gaining increased attention within astrobiological research, as the low metabolic needs and extreme survivability of the sporulated state can allow for viability of spores after exposure to space and artificially mimicked Martian atmosphere (Nicholson & Schuerger, 2005).

Not only do spores contain some protection from radiation and UV exposure, but heat as well. With spores being exposed to UV under the cover of red sandstone or meteorite powder being found to provide radiation shielding and thus increased viability after a set timeframe. Using proxy red sand and clay, Horneck et al. (2001), found that mixing spores into meteorite proxy material or Mars analog soil could increase survival up to 100% compared to no coverage within a biopan test on a Russian earth orbiting Foton satellite. Endospores are also one of space-travels largest concerns when it comes to contamination, in part due to its ability to survive spacecraft sterilization techniques, its ability to survive in space, and also the ability to survive hypervelocity atmospheric entry (Fajardo-Cavazos et al. 2005; Tobias & Todd, 1974). Along with this, vegetative states of some spore-formers are extremophiles to begin with. Due to this, spores have been used as a model organism for studying panspermia and astrobiology due to their survivability and continued longevity in analog environments.

Along with analog environmental research, rover missions take precautions against the forward contamination of planets, referred to be NASA as Planetary Protection, in which other planets are kept uncontaminated by Earth spacecraft, and no forward contamination occurs. This is done by better restricting and monitoring endospores and other hardy organisms, which can protect against false positive detection of life on those planetary missions, and also prevent the spread of materials associated with life to be spread throughout the solar system, potentially artificially planting biosignatures (Coustenis et al 2023).

The mechanisms for endospore survival are plentiful. Along with a proteinaceous outer shell that protects chemical and enzymatic resistance. Dehydration of the core is an important factor in endospore formation, which assists in high temperature resistance. To assist in the dehydration of the core, some endospores employ Dipicolinic acid (DPA). DPA also assists in maintaining spore dormancy as well as wet heat resistance. (Nicholson et al. 2000).

Since endospores have shown to be worthy of astrobiological interest, understanding the environmental interactions endospore extractions may encounter and the differences of endospore extraction between sediment types can greatly increase the understanding of data from future Mars missions.

1.09 Research Objectives

The purpose of this thesis project was to investigate microbial communities via biomarker analysis within a range of environments related to the storage and production of energy from energy industries. These projects were undergone to better understand what

microbial communities are present and in what quantities. Along with this, better understanding of metabolic processes are occurring in these systems.

Chapter 1 includes an overview of the goals and research interests of both BML and NWMO's MBS. It also brings an introduction to PLFA biomarkers, and their relevance to understanding bacterial community distribution. Along with this, it also provides carbon isotope analysis ($\delta^{13}C$ and $\Delta^{14}C$) implications for determining and estimating microbial metabolism and metabolic activity. Similarly, with the NWMO, the purpose and importance of the MBS was expressed, and the role that bentonite plays to isolate spent nuclear fuel was explored. The introduction of spores and the usage of DPA as a biomarker to determine presence of spores was also introduced.

Chapter 2 describes a study of community structures, distribution, and metabolism within 6 biofilm units sampled from different depths and times within BML. PLFA was used to interpret distributions and microbial structures. Carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) of PLFA were compared to potential carbon sources (DIC, DOC, and PH derived methane) to determine microbial cycling and carbon source utilization.

Chapter 3 investigates the presence of spores within bentonite clay, a crucial part of the NWMO's MBS. By extracting DPA from spores, quantities of naturally occurring spores can be determined on the proposed bentonite barrier material. This study also looked into different sediment matrices to determine if adsorption of DPA may occur in particular sample types.

Chapter 4 summarizes the major conclusions of this Master's thesis project and future research directions for exploring PLFA within BML and spores within bentonite.

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1.11 Tables and Figures

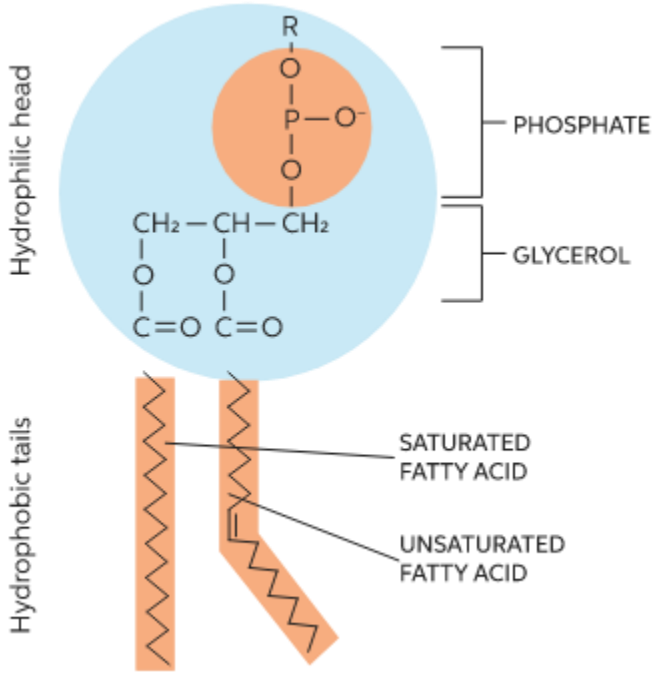

Figure 1.1 Chemical Structure of a membrane lipid.

(Image taken from https://www.chegg.com/learn/biology/anatomy-physiology-in-biology/structure-of-phospholipid)

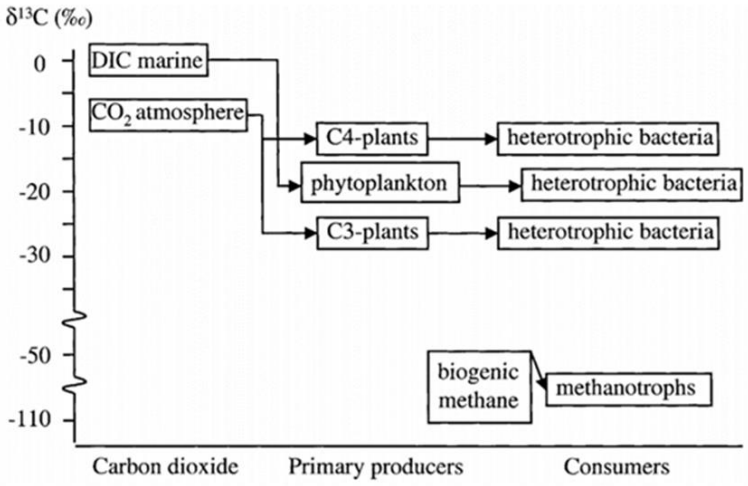

Figure 1.2 Graphic representation of metabolic stable pathway fractionation. Taken from Boschker & Middelburg, 2002

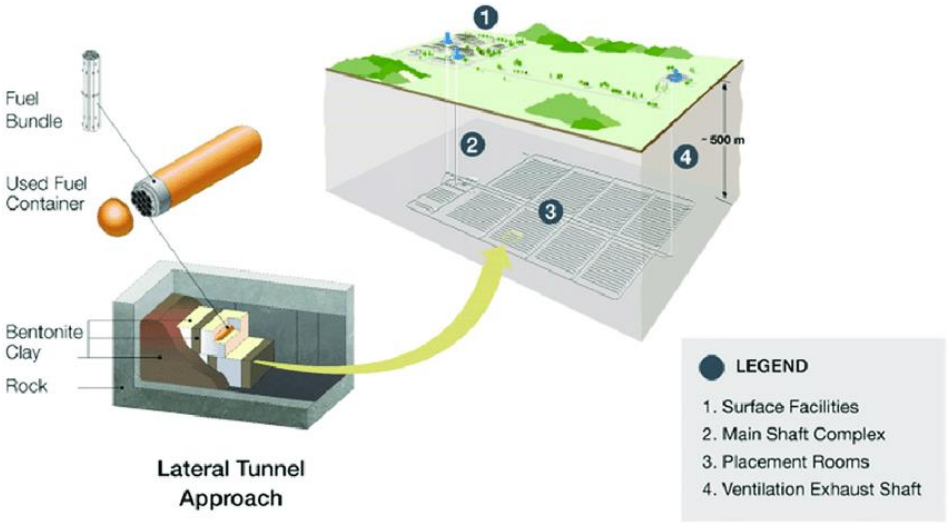

Figure 1.3 Visual representation of elements of proposed Multi-Barrier System. Taken from NWMO.ca.

CHAPTER 2:

CONSTRAINING MICROBIAL CARBON SOURCES IN BASE MINE LAKE USING RADIOCARBON AND STABLE ISOTOPIC ANALYSIS

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2.1 Abstract

Understanding the distribution, abundance, and metabolic activities of bacteria in a variety of environments is a crucial step in understanding the role that microbial life will play within many facets of the biogeochemical cycling of a system. Even within extreme environments, the ability for life to survive and the microbial distribution of these locations can provide valuable information for future analysis and exploration. Base Mine Lake (BML) is the first full scale pit lake commissioned by Syncrude Canada Ltd for the purpose of reclamation of both the oil sands extracted land, but also to manage oil sand tailings using water cap tailings technology.

In this study, we investigated the carbon sources dominantly being consumed by the microbial life found within BML, by conducting both stable carbon and radiocarbon analysis of Phospholipid Fatty acids (PLFA) extracted from the membrane's organisms growing in the water column. PLFA analysis is a widely used tool that can give insight into community structure, abundance, and metabolism through the use of biosignature lipids as well as isotopic ratios of carbon. Biofilm units were used placed at varying depths and times to sample the microbes through the water column. Microbial community abundance was observed with distributions comparable to previous water column PLFA results. Stable isotopic analysis of the PLFA showed that PLFA associated with methanotrophs were strongly ^{13}C isotopically depleted in the hypolimnion where C16:1 PLFA $\delta^{13}C$ ranged from -41 to -59 permille. In contrast, PLFA not associated with methanotrophy, such as grouped C18:1 PLFA were less ^{13}C depleted ranging from -32 to -45 permille, reflecting a lesser use of methane derived carbon. The ^{13}C depleted intermediary PLFA show that methane carbon is mixing into the general microbial communities

and likely show methane carbon being predominant throughout the microbial communities. Radiocarbon analysis of the PLFA from BML ranged from $\Delta^{14}C$ = -757 to -889 permille, demonstrating that the microbes were using petroleum derived carbon whether derived from methane or not. Notably, slightly more modern carbon was seen in the epilimnion (on average 70 ‰ more enriched), where $\Delta^{14}C$ was progressively enriched over time. The increased fraction of modern carbon in the epilimnion indicates use of more recent inputs, potentially from photosynthesis. However, mass balance estimates show that the microbial community in the lake is still dominantly using ancient carbon, with mass balance estimates indicating 10-29% modern utilization throughout the entire sampling period. Future assessment of $DI^{14}C$ within the lake can refine this estimate. Nonetheless, it is clear that the carbon cycle within the lake is dominated by ancient carbon inputs and that these are likely related to the oxidation of petroleum derived methane transported from the FFT to the water column.

2.2 Introduction

The Alberta Oil Sands Region ranks among the largest crude oil reserves in the world, with the Athabasca oil sands region covering an area of 142,200km^2, producing up to 3.1 million barrels of oil per day in 2018, and a proven reserve of 158 billion barrels of oil present at the start of mining (USGS.gov). Surface oil sand extraction activities result in excavation of mine pits as well as the generation of large volumes of tailings. Regulatory requirements require that these impacts on landscape be reclaimed by operators, so long-term strategies must be implemented for reclamation of the oil sands process tailings (Allen, 2008). One such reclamation strategy is the construction of Pit Lakes. Pit Lakes reclaim land impacted by oil sands surface mining as well as storing large volumes of tailings under a water cap that isolates the tailings with the goal

of preventing future release of any hazardous components (Dompierre et al. 2016). Syncrude Canada Ltd. commissioned the first full scale water-capped tailings reclamation lake through the creation of Base Mine Lake (BML) in 2012. The location of BML is north of Fort McMurray, in Alberta, Canada, and covers over 800 hectares of land with about 45 meters of FFT depth being covered by a water cap. Due to the settling and dewatering process, where turbidity within the lake decreases over time, the FFT present in the lake has changed from 5 meters of water cap when the lake was first established to now at a depth of 12m of water depth in 2019 (Syncrude, 2021). The purpose of the Pit Lake is to create a self-sustaining lake ecosystem that will be able to support life and not require active maintenance in the long term. The progress of Base Mine Lake towards establishing a self-sustaining ecosystem and maintain the isolation of FFT from the environment is the focus of a large scale, multidisciplinary research program. An important aspect of this research is understanding the role of in situ microbial communities in controlling lake biogeochemistry and for the biodegradation of oil sand by-products such as naphthenic acids or petroleum hydrocarbons (PH) (Foght, Gieg, & Siddique, 2017).

2.3 Biogeochemical Cycling

A key biogeochemical factor in the development of BML is the need for consistent dissolved oxygen within the water column to support aerobic respiration, a major component to the viability of a self-sustaining lake. (Blumenberg, Seifert, & Michaelis, 2007). In addition, the presence of oxygen allows for microbial heterotrophy to breakdown compounds that may be released from the underlying FFT, such as methane by methanotrophy and/or residual petroleum

hydrocarbons and naphthenic acids (NA), which makes it a critical component to determine the lake's oxic boundary. In order to assess the biogeochemical cycling within BML, and therein the role of heterotrophs in controlling oxygen levels and removal of petroleum derived contaminants, it is necessary to be able to track microbial carbon sources and metabolisms.

Biogeochemical cycling of methane is a key component of understanding BML development. Methane is produced within the anoxic FFT underlying BML via the activities of methanogenic microbes (Siddique et al. 2011). The methane is then released into the lake by various means of transportation. The main processes of methane transport are advective transport during FFT settling, diffusive transport across concentration gradient, and gaseous methane bubbles allowing for methane to dissolve into the water along the bubbles path to the surface. This provides methanotrophs a source of energy while removing methane from atmospheric release, but also depletes oxygen throughout the lake (Arriaga et al. 2019; Bradford et al. 2017; Chen et al. 2013). While the oxidation of larger chemicals that are released in the dewatering of FFT such as naphthenic acids and larger petroleum hydrocarbons plays a role in the oxygen budget of the lake, previous studies have shown methanotrophic activity is a major control on oxygen supply within the water column (Slater et al. 2021; Risacher et al 2018; Arriaga et al. 2019). Nonetheless the oxidation of these larger compounds is important in preventing release of these NA and PH to the broader environment. Further, as the lake develops, it is expected that photosynthetic activity will increase in the surface waters as lake clarity increases. This provides a potential source of labile organic matter to the lake which may influence biogeochemical conditions and outcomes. Understanding the interplay between methane, petroleum, and NA and recently photosynthesized carbon sources is an important component of confirming that the

development of BML is on a trajectory that will meet its design goals. The goal of this research project was to assess the microbial communities composing BML, and in particular to elucidate the carbon sources driving microbial metabolism within BML, in regards to the relative importance of petroleum derived versus recently photosynthesized carbon.

2.4 PLFA Usage

A useful tool for examination into the microbial communities found in BML is via extraction of the phospholipid fatty acids (PLFA). Phospholipids are the main building block of bacterial and eukaryotic cellular membranes. These are useful tools to determine community change and size and do not accumulate within the system as they are degraded quickly (Within days to weeks) (Zelles, 1999). The Phospholipid consists of a hydrophilic phosphate head, and a hydrophobic fatty acid tail (Figure 2.1). These lipids can be analyzed to gain insight into the total cellular count, community structure and variation of microbial communities, as well as food sources used by specific communities, as have been shown in many studies (Zelles et al. 1994) (Boschker & Middelburg, 2002). Total PLFA abundances can be used to estimate cell abundances based on general conversion factors (Green & Scow, 2000). In addition, biomarker FAME analysis can indicate the presence and state of some classes of microorganisms (Zelles, 1999). The fact that PLFA can be purified and separated effectively via silica gel and gas chromatography makes them also very suitable for stable carbon and radiocarbon analysis. The isotopic compositions of these PLFA can be linked to the sources of carbon being consumed by the organism and / or their biosynthetic pathways, which can lead to understanding of the community metabolism and energy sources. The goal of this project is to further constrain and determine the metabolism and age of the material being consumed within BML, and to see how alum addition and algal blooming effected the microbial life within the lake.

2.5 Using $\delta^{13}C$ to constrain and determine metabolism pathways.

By comparing the $\delta^{13}C$ of PLFA, DIC, DOC, and methane, insight can be gained into the metabolisms that are occurring within the system. Due to fractionation within carbon metabolism pathways, stable isotopes and the ranges of the fractionation can identify carbon sources metabolized. This is because lighter isotopes, like ^{12}C, are used preferentially by microbes due to kinetic and thermodynamic favourability. Particularly, methane from methanogenesis is very isotopically ^{13}C depleted (Boschker & Middelburg, 2002), as smaller compounds such as CO_2 and CH_4 have higher Kinetic Isotope Effects (KIEs) (Hayes, 2001). Due to the KIEs, microbial metabolism of isotopically depleted ^{13}C methane will result in a transfer of an even greater depleted ^{13}C signal from methane to the cell's makeup. Microbes that consume the isotopically depleted ^{13}C methane derived carbon will transfer this depletion into their cellular components resulting in highly depleted ^{13}C isotopic compositions (Jahnke et al. 1999; Summons et al. 1994). Biogenic methane can have values ranging from -50 to -110 ‰ (Freeman, et al., 1990; Zhang 2002). Due to this, $\delta^{13}C$ of PLFA from methanotrophs is often extremely ^{13}C depleted relative to $\delta^{13}C$ DIC and similar to the extreme depletion seen in methane. In contrast, autotrophically produced carbon is 10 to 30 ‰ depleted in ^{13}C relative to the initial carbon source (DIC or Atmospheric CO_2). Heterotrophic metabolisms impart a relatively small isotopic fractionation relative to their carbon source. (Boschker & Middelburg, 2002; Londry et al., 2004). Due to this, tracking isotopic signatures within the microbial communities in BML can occur, by tracing this light carbon signature (Coleman, Risatti, & Schoell, 1981).

2.6 Radiocarbon Analysis

While the stable isotope ratios are strongly affected by metabolism in organisms, by necessity, radiocarbon analysis is normalized for fractionation so that differences in radiocarbon content can be used to determine the age of the material. Radiocarbon is produced in the atmosphere due to solar rays and nuclear bomb testing interacting with atmospheric nitrogen via the addition of a neutron and emission of a proton to form an unstable carbon 14 atom. This unstable carbon is also incorporated into living tissues and has a half-life of 5730 years (Godwin, 1962; Ingalls & Pearson, 2005). Most organisms will be at equilibrium with ^{14}C in their environment, with the gain and loss of ^{14}C even. However, upon organism death, the remaining radiocarbon decays over time with no additional ^{14}C gain. Over sufficient time, generally considered 5-6 half-lives, all of the radiocarbon present in an organic compound will have decayed away. Such materials are referred to as radiocarbon dead and include ancient, fossil carbon such as petroleum hydrocarbons.

2.6.1 Age dating using Radiocarbon analysis.

The radiocarbon content of organic compounds can be reported with several notations. Commonly it is reported as percent modern carbon or fraction modern, however, it can also be reported in $\Delta^{14}C$ notation, which can be used in mass balance calculations. The $\Delta^{14}C$ scale goes from -1000 ‰ for materials with no detectable radiocarbon remaining, to $\Delta^{14}C$ of ~ 20 permille for the modern atmosphere (Basu et al, 2020), and as positive as 160 ‰ in coastal waters in the

1970s and around 700 ‰ in the atmosphere in the mid 1960s for compounds where so called "bomb spike" carbon is present. The "bomb spike" refers to the increased atmospheric radiocarbon levels that resulted from atmospheric nuclear weapons testing. The distinctions in radiocarbon content of these carbon sources can be used to differentiate the carbon sources being used by a microbial community (Slater, 2005; Graven, 2015; Turnbull et al. 2007).

In the BML environment, ancient PH carbon sources represent one potential carbon source. Due to isotopic fractionation being normalized during radiocarbon analysis microbial utilization of such radiocarbon dead carbon sources ($\Delta^{14}C$ = -1000 ‰) will result in microbial cellular components that are also devoid of radiocarbon. In contrast, recently photosynthesized carbon will have radiocarbon contents equivalent to the carbon source from which they were generated, either the atmosphere (~ 15 permille) in the case of plants, or the dissolved inorganic carbon in the lake, which can range widely depending on the source of DIC and multiple factors upstream, but in some Canadian waters has seen a range around -90 permille (Zeidan et al 2022).

2.7 Base Mine Lake During Sampling

BML was the first full scale pit lake established in 2012, with an initial water depth of 5m deepening to 12m on average due to the consolidation of underlying FFT. Previous studies have demonstrated microbial methanotrophy occurring within the water column, and the related impact of oxygen consumption throughout (Slater et al. 2021; Arriaga et al. 2019; Risacher et al. 2018).

In addition, to increase the clarity of the lake, BML had a whole lake alum addition in the Fall of 2016 (Jenssen et al. 2022). This alum addition did provide an increase in clarity and in turn increased autotrophic oxygen production in the lake. This increased algal growth phase observed in spring 2017 was followed by the observation of anoxia in the lowest 1.75m of the PL FFT-water interface during the summer of 2017 (Syncrude, 2019; Jessen et al. 2022). This bloom can be seen with an increase of PLFA biomass in 2017 in the epilimnion, but the biomass fluctuation seemed to have settled by June 2017 water samples (Slater et al. 2021). This is thought to be due to the increased epilimnion microbial growth rates allowing for more bioavailable material to settle to the deeper lake which increased aerobic heterotrophy and related oxygen consumption. This increased rate of consumption removed oxygen from the hypolimnion, where oxygen prior to the alum was already in the severely hypoxic range (~10 µM) in 2016 (Jessen et al 2022). Along with this increase of biomass accumulated in the epilimnion in 2017, the algal bloom would be expected to utilize phototrophic pathways for metabolism, consuming carbon dioxide derived carbon from sources such as DIC and DOC, that are likely significantly more modern sources of carbon than petroleum hydrocarbons. Monitoring microbial use of methane and modern carbon throughout the timeframe of the alum addition occurred and the associated impacts to assess the changes of methane utilization and how microbial community and metabolism changed.

The goals of this study were to utilize the biomass from biofilm sampling to be able to elucidate the role of petroleum derived vs photosynthetic carbon inputs within BML. By comparing biomarker PLFA and community distribution to previously studied water sampling from Slater et al 2021, the methanotrophic control of the oxic boundary can be further strengthened as a follow up the water column results. Along with this, due to the plentiful biomass presented in biofilms,

stable and radiocarbon data can be produced on the same extract, allowing assessment of the role of ancient, petroleum carbon and the extent to which this carbon is being utilized in the form of methane transported from the FFT to the water column.

2.8 Methods

2.8.1 Sampling

In order to collect sufficient microbial biomass for radiocarbon analysis, sampling for this study used Teflon biofilm units which consisted of a Teflon tube with multiple holes for areas of entry, filled with glass wool. Sampling took place from the first initial biofilm implementation in July 2016 and ended with the final biofilm removal from BML in August 2018. All biofilms were placed at the 'P3' sampling site location of BML, at a depth of 1.5 meters and 9 meters, to represent the epilimnion and hypolimnion. Dates of implementation and removal are as follows (Figure 2.2); July 2016- July 2017, Biofilm pair 'J16J17' which encompasses when the algal bloom was present in BML, having been noted at the site in May of 2017, August 2017- October 2017, biofilm pair 'A17O17', and August 2017-August 2018, biofilm pair 'A17A18'. Summer profiles of PLFA can be seen in the July – July 16/17 and August -August 17/18 biofilms, whereas August-October 2017 was chosen to represent a different season, as well as a differing biofilm sampling duration.

PLFA represent a short timeframe of weeks to days of an environment based on their degradation turnover times. Therefore, PLFA from Biofilm samplers should primarily represent the days to weeks of the lake prior to the sampler retrieval. However, due to long deployment times and potential for carbon to be cycled within the biofilm unit's microbial communities, there is a

possibility that the biofilm samples include contributions from carbon sources over a longer timeframe prior to sampler retrieval.

Teflon Biofilm units were lifted from the lake and double wrapped in pre-combusted (carbon free) aluminum foil and placed in a whirlpak and frozen until extraction. During extraction, biofilms were removed from foil with solvent rinsed tweezers to avoid contamination. Biofilms were allowed to thaw in 500 ml graduated cylinders, and once thawed proceeded to undergo the PLFA extraction method. This thawing was done to maintain the proper solution and extraction ratio.

2.8.2 PLFA extraction and FAME conversion

Extraction of PLFA from each biofilm unit was done using a modified Bligh and Dyer method. By preparing a solution of 1:2:0.8 Dichloromethane, Methanol, and Phosphate Buffer, and pouring it into the 500ml graduated cylinder. After this, a second solution of 2:1 Dichloromethane and Methanol was added to the graduated cylinder to submerge the Biofilm unit, which also assisted in maintaining a single-phase solution due to some thawing of water from the unit. Cylinders were sonicated and left in the fume hood covered overnight at room temperature. The solution was then allowed to break into the organic phase and the remaining aqueous phase in a separatory funnel, and the organic phase was collected.

The PLFA was then separated from the total lipid organic extract by silica gel chromatography with three fractions: DCM, Acetone, and finally MeOH. This final fraction is where phospholipids are found, and samples were dried down, and dissolved into characterized $\delta^{13}C$ and $\Delta^{14}C$ KOH and 1:1 toluene: MeOH and heated to 37 degrees Celsius for an hour to allow for conversion of PLFA into FAMEs for analysis on Gas Chromatography mass spectrometry. A second 3 fraction silica gel chromatography (F1= 4:1 Hexane: DCM, F2= DCM, F3= MeOH) was conducted as a cleanup step for any additional compounds that may interfere with FAME detection and analysis. The DCM fraction was brought to dryness under N_2 and hydrolyzed to form fatty acid methyl esters (FAMEs) by mild alkaline hydrolysis (White and Ringelberg, 1998). FAMEs were then analyzed via Gas-Chromatography Mass spectrometry (GC-MS) All PLFA samples were identified and quantified by mass analysis on an Agilent 6890GC (Column: DB-5MS, 0.25-μm film thickness, 30-m length, 0.32-mm ID) connected to an Agilent 5973 quadrupole mass spectrometer. The oven ramp temperature program was as follows: 50◦C for 1 min, increasing by 20◦C/min up to 130◦C, then 4◦C/min to 160◦C,and 8◦C/min to 300◦C, and a hold for 5 min. MSD Chemstation (Agilent Technologies, Santa Clara, CA, USA) was used to identify the FAMEs through the database-matching of spectra and overlaid spectra comparisons of two reference standards (Matreya PLFA mix, and Supelco 37 FAME mix). The quantification of FAMEs was based on six-point calibration curves generated for four different FAMEs (14:0, 16:0, 18:0, and 20:0; $R^2 > 0.99$), determining concentration by back-calculating using instrument areas and integration tools within MSD ChemStation.

2.8.3 Radiocarbon analysis

Samples for Radiocarbon analysis was chosen based on samples that contained adequate carbon. After FAME analysis via GC-MS and IRMS, extracted samples were suspended back into 1ml of

DCM. Each sample represented the total biofilm FAME converted sample. Samples were labeled and packed in insulated packaging with ice. Radiocarbon analysis was conducted by Woods Hole Oceanographic Institute, National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS), samples were shipped, where FAMEs were oxidized to CO_2, an iron catalyst was used to convert the sample to a graphite form, and then pressed into filaments and put into a tube that is used as the cathode to release ions that can then be tested for ^{14}C / ^{12}C(Olsen, 1970; Stuiver & Polach, 1977). By heating ionized Cesium, Graphite samples are sputtered, and ions are produced from the sample that can be used to analyze the radiocarbon content through NOSAMs Accelerator mass spectrometry system.

Due to the addition of methanol in the PLFA to FAME conversion, a mass balance was used to correct the $\delta^{13}C$ of the FAME to the value prior to the mild alkaline methanolysis. The impact balance used is seen in equation 1:

(1) $D_A = ((n + 1) * D_{FAME} – D_{MeOH}) / n$

Where D_A is the actual corrected PLFA $\Delta^{14}C$ value, n is the carbon chain length of the FAME, D_{FAME} is the isotopic value of the sample (pre-correction), and D_{MeOH} is the isotopic value of the methanol used in the mild alkaline methanolysis.

2.8.4 PLFA FAME and $\delta^{13}C$ grouping.

PLFA community analysis from Biofilm units was undergone by grouping FAMEs according to their molecular structure, which were broken down into 1) Saturated PLFA grouped together, which are PLFA produced by all microbes 2) All C16:1 unsaturated PLFA being 16

length carbon chain with a double bond anywhere along the chain, pooled together. This includes a biomarker for type I and X methanotrophs (Zhu et al. 2016). 3) All C18:1 unsaturated PLFA, being all unsaturated 18 length carbon chains grouped together. This includes a biomarker for type II methanotrophs (Zhu et al. 2016) and autotrophs (Sakamoto et al. 1994). 4) All cyclic molecules grouped together, 5) All Branched molecules grouped together, which is a general biomarker for heterotrophic organisms. 6) other monounsaturated and polyunsaturated molecules grouped together, which contains the biomarker for green algae, diatoms, and cyanobacteria (Volkman et al. 1989).

PLFA $\delta^{13}C$ analysis from Biofilm units was undergone by grouping FAMEs according to their molecular structure, which were broken down into 1) individual saturated PLFA (14:0, 16:0, 18:0) which are PLFA produced by all microbes. 2) All C16:1 unsaturated PLFA, 3) All 18:1 unsaturated PLFA, 4) Branched chain molecules grouped together. Cyclic molecules were present, but below limits of quantification for the concentration of sample used.

2.8.5 Instruments and Data Acquisition

Compound-specific stable carbon isotope analysis of PLFA was performed in triplicate on a gas chromatography–isotope ratio mass spectrometry (GC–IRMS) system consisting of an Agilent 6890 gas chromatograph (Column: Agilent: DB-5, 0.25 μm film thickness, 30m length, 0.32mm ID) attached to a Combustion III Interface, followed by a Thermo Delta Plus XP Isotope Ratio Mass Spectrometer (IRMS). Isodat NT 2.0 Software (©Thermo Electron Company) was used to analyze GC-C-IRMS results. Carbon isotope ratios were normalized to the Vienna Pee Dee Belemnite (VPDB) standard. A $\delta^{13}C$ value correction as applied to account for the methyl group addition from the isotopically characterized KOH during FAME derivatization. All

samples were injected in triplicate, with $\delta^{13}C$ standard deviation of approximately +/- 0.5 ‰ based on standard reproducibility and instrument accuracy. Due to lack of baseline separation of C16:1 and C 18:1 isomers, the $\delta^{13}C$ of these compounds were determined by integrating across the grouping of these peaks as $\delta^{13}C$ "grouped".

2.9 Results

2.9.1 PLFA biomass and Community Distribution

Figure 2.3 displays total average PLFA biomass abundances for the biofilm units were, in July 2017, 240 ug in the epilimnion and 611 ug in the hypolimnion, in October 2017 the epilimnion biofilm had 252 ug and hypolimnion had 184 ug, and in August 2018 the epilimnion biofilm contained 230 ug and hypolimnion 72 ug. This is showing plenty of growth over the period of installation.

Biofilm unit PLFA community distributions are shown in Figure 2.4. The large increase of polyunsaturates found in the epilimnion and hypolimnion of the July 2017 sample is consistent with the occurrence of the algal bloom that followed the alum addition (Slater 2021). The presence of C16:1, associated with methanotrophs are strongly represented at all time points, with a much stronger presence in the hypolimnion.

A majority of the PLFA community distribution stays consistent over the sampling period, where the 'other unsaturates' that include algal biomarkers fluctuates the most, with a decrease from 30% and 34% with depth in July to 16% and 15% with depth in October. The summer of 2018

saw an increase in other unsaturates in the hypolimnion, with 31%, but the epilimnion saw a decreasing role in this PLFA marker, with only 8% of the PLFA in this category.

General biomarker saturates stay between 19-25% over both depths and throughout the sampling period, Branched and Cyclic range from ~5-9% and ~1-6% respectively.

Grouped C16:1 saw an increase of the PLFA distribution for the first 2 sample periods, growing from 12% in the epilimnion and 20% in the hypolimnion in July to 37% and 34% in October. The population of C16:1 decreases at the next timepoint, going back to 22% in the epilimnion and 17% in the hypolimnion. Grouped C18:1 epilimnion continues growing throughout the sampling period, from July's 23% to August's 30%, with October being 27%. Hypolimnion C18:1 shares a similar trend, starting at 18% in July to August 21%. These trends are consistent and follow the same trends as previously published data from Slater et al 2021. This shows the biofilm data is representative and consistent with the water column the biofilm is surrounded by.

2.9.2 Isotope Analysis

The microbial metabolisms that dominate the biofilm microbial communities sampled by the PLFA analysis can be assessed by using $\delta^{13}C$ of PLFA as has been previously done in the water column of BML (Slater et al. 2021). Lines of best fit were not chosen to represent general trends in time due to the expectation, based on previous results, of seasonal dependent variations in the data.

2.9.2.1 Stable Isotopes in the Hypolimnion

Stable isotopic information found within the hypolimnion was determined (Figure 2.5).

Due to much lower abundances of Iso-antiso and C14 biomass, particularly in August 2018, some PLFA groupings had biomass too low to contribute accurate isotopic signatures.

The isotopic depletion of most groups of PLFA shows the microbial methane utilization throughout the hypolimnion over the sample period. These depleted values of the PLFA closely compared to the FFT source methane $\delta^{13}C$ average ($\delta^{13}C$ = -61 ‰) (Slater et al. 2021). Although PLFA biomarkers have the potential to have multiple sources contributing to the individual and grouped PLFA seen, the separation of distinct $\delta^{13}C$ sections, with the C16:1 grouped $\delta^{13}C$ ($\delta^{13}C$= -59 to -42 ‰) includes the biomarker for Type 1/X methanotrophs, along with C14:0 ($\delta^{13}C$= -53 to -43 ‰) which is also produced highly by methanotrophic organisms (Zhu et al. 2016) are consistently the most depleted of the groups analyzed.

Using the intermediary PLFA compounds, produced by most microbes, the balance of methane usage and spread throughout the microbial community can be seen. C16:0, which stays between the methanotrophic and autotrophic biomarkers through the entire sampling period in the hypolimnion, contained a $\delta^{13}C$ of -46 to -36 ‰. These intermediate PLFA still contain methane derived carbon influence, particularly in summer, where values are most depleted. In October, the general PLFA contain a $\delta^{13}C$ more representative of carbon that was not methane derived.

At the most enriched, grouped C18:1 ($\delta^{13}C$= -45 to -32 ‰) compounds that show the least incorporation of methane derived carbon. Slater et al (2021) proposed that C18:1 = -32 permille was representative of use of a carbon source derived from plants and/or PH of ~ -30 ‰ (Slater et al 2021; Ahad et al. 2013). This is supported for the samples from this study in July 2017.

However, at other timepoints the $\delta^{13}C$ of C18:1 is depleted indicating methane derived carbon may be cycling throughout the microbial community and affecting non-methanotroph organisms as well, as seen for the C16:0 results.

2.9.2.2 Stable Isotopes in the Epilimnion

In the epilimnion (Figure 2.6), the $\delta^{13}C$ results indicated less influence of methanotrophy. The C16:1 PLFA that contain the biomarker PLFA for Type I methanotrophs and the C14:0 PLFA that are produced in abundance by methanotrophs are both the most isotopically depleted PLFA in the epilimnion (δ^{13} C16:1= -47 to -38 ‰ and δ^{13} C14:0 = -44 to -39 ‰). The depletion of these values relative to the other PLFA indicates there is some methanotrophy in the epilimnion, but the influence is much less than found in the hypolimnion. This depletion is consistent with what was observed by Slater et al (2021) for the water column, displaying that methane derived carbon is still being utilized throughout the epilimnion, but to a lesser extent than the hypolimnion.

At the most enriched, grouped C18:1 ($\delta^{13}C$= -34 to -31 ‰) compounds that are not utilizing methane-derived carbon, reflecting other carbon sources aside from methane derived carbon. The isotopic compositions of these PLFA are very similar to those observed by Slater et al 2021, indicating a consistent carbon source in the epilimnion. The intermediary compounds, produced by most microbes, like C16:0 are between the methanotrophic biomarkers and autotrophic markers, but overall more enriched than what's found in the hypolimnion ($\delta^{13}C$ = -39 to -34 ‰). While most compounds analyzed became enriched in the August – October 2017 biofilm relative to the July 16 to July 17 sample, unlike the hypolimnion that returns to extremely depleted states

in the summer of 2018, the epilimnion remains more enriched, which can be seen comparing the hypolimnion C16:0 to the epilimnion from A17O17 to A17A18, (October 2017 to August 2018) where the hypolimnion returned to heavily methane influenced from -36 to -43 ‰, and the epilimnion remaining ~ -34 ‰. This enrichment throughout the epilimnion indicates that methanotrophy plays a lesser role after the alum addition, when the clarity of the lake was increased, likely due to increased autotrophic metabolism. The alum and algal trends are fairly muted within the epilimnion, where the hypolimnion saw a very large enrichment in October 2017. The epilimnion saw very little difference over the time sampled, having a slightly more enriched value over time. With the most depleted values, C16:1 and C14:0 PLFA are seen to be significantly more depleted than the other PLFA, and suggests methanotrophy is still present, albeit at lower rates than the hypolimnion. Although C14:0 PLFA is a general biomarker, the extreme depleted value of it in BML makes it reasonable to assume that methanotrophs are a major driver of this PLFA, as also found in (Slater et al. 2021).

2.9.3 PLFA radiocarbon content

The $\Delta^{14}C$ of the PLFA from BML (Figure 2.7) were dominantly derived from fossil, petroleum carbon with values that ranged from the most depleted at -889 ‰ to the most enriched (modern) value being -757 ‰. The oldest observed sample was found in the July 2017 (J16J17) hypolimnion sample, (-889 ‰) concurrent with an epilimnion value of -831 ‰. The greatest offset from epilimnion and hypolimnion was observed for the August 2018(A17A18) samples (-757 ‰ and -878 ‰) while the smallest was observed for the October(A17O17) sample (-807 ‰ and -835 ‰).

In the hypolimnion, we observe the $\Delta^{14}C$ changes from the most depleted -889 ‰ in the summer of 2017 to -835 ‰ in October 2017, and then back to -878 ‰ in the summer of 2018. With the difference between summer values being 11 ‰, between natural variation and error, the A17A18 and J16J17 values are within error of each other. In the epilimnion, the depleted radiocarbon signature continues to enrich over time, starting at -831 ‰, enriching to -807 ‰ in October and ending in August 2018 at -757 ‰.

While petroleum carbon sources dominated throughout BML, there were variations in the extent of fossil carbon present in the PLFA from the biofilm units, and between the epilimnion and hypolimnion. Overall, all the epilimnion samples contained more modern carbon than the hypolimnion samples (Figure 2.6) indicating a greater input of modern carbon within the epilimnion. This was true at each sampling point, though the extent to which the epilimnion samples contained more modern carbon than the corresponding hypolimnion samples varied. Where the epilimnion sees a more modern value that becomes more enriched over time. This is displaying an ongoing and increased input of modern carbon, which may be reflecting increased autotrophic contribution over time.

As seen in October radiocarbon data, a large enrichment of modern carbon was introduced to the microbial communities between July 2017 and Oct 2017. This follows the Alum addition in Fall of 2016 which resulted in elevated algal markers in Spring 2017 (Slater et al. 2021), and in July 2017 in this study. Concurrent with this enrichment in ^{14}C, the stable isotopic ratios of PLFA in Oct 2017 were also the most enriched, indicating the least use of methane derived carbon. These results suggest that the microbial community was composed of or utilized a greater component of photosynthetically derived carbon which was more modern and $\delta^{13}C$ enriched than the previous July 2017 timepoint and in previous data (Slater et al. 2021). This may be the result of microbial

heterotrophy of photosynthetically produced carbon resulting from the algal bloom in spring 2017, or alternatively, increased photosynthesis during the increased clarity in August (Tedford et al. 2019) of 2017. Either of these sources could have been mixed into the hypolimnion to greater extent during lake turnover in September of 2017. It may also be that with the increased clarity of the lake, more transference of bioavailable material is making its way down from the epilimnion through sinking particles. Jessen et al. 2021 saw an increase in branched and cyclic PLFA in the hypolimnion for 2018, which are formed by heterotrophic bacteria in high rates. Another potential is the addition of more water by Syncrude to maintain the water cap. This injection of new water could potentially increase the modern carbon being consumed in the hypolimnion. Whether algal bloom eutrophication, lake turnover, or water cap transference led to the enrichment, the values migrate back to a similar depleted state to the next time point of A17A18, displaying the resilience of consumption of PH derived carbon. More testing over longer time periods would be needed to better understand the cause, and as the October enrichment was only collected at one timepoint may indicate it was a short-lived outcome.

Subsequent to this, in July 2018, the microbial carbon sources in the hypolimnion had returned to being similar to previous results. Isotopic compositions of methanotroph biomarker C16:1 have returned toward more negative values and radiocarbon content has returned to more negative values. Both of these trends indicate a return to petroleum hydrocarbon derived methane playing a larger role in the hypolimnion carbon cycle. In contrast, in the epilimnion, the microbial carbon sources remained at the more enriched isotopic compositions ($\delta^{13}C$) and showed increased $\Delta^{14}C$, both consistent with an ongoing increased contribution of non-petroleum,

photosynthetically derived carbon. Though it must be noted from the $\Delta^{14}C$ that this carbon was still predominantly petroleum derived.

2.10 Discussion

In order to assess the extent of increased inputs of photosynthetically derived, more modern carbon to BML for both the epilimnion and hypolimnion samples, an isotopic mass balance was carried out between fossil petroleum carbon (-1000 ‰) and two potential modern carbon end member inputs (Figure 2.8). If the inputs were directly from the modern atmosphere, they would have a $\Delta^{14}C$ of -15 to -20 ‰. However, it is common for the $\Delta^{14}C$ of DIC within a lake to be isotopically depleted relative to the modern atmosphere (Hsueh et al. 2007; Levin, Kromer, & Hammer, 2013; Basu et al., 2020). Unfortunately, $\Delta^{14}C$ DIC values for BML were not obtained due to sample quality issues. And while it is possible that the DIC in BML has been impacted by carbonate dissolution and/or other inputs of aged carbon, given the inputs of fresh water from beaver creek reservoir and the time for air water exchange, it was assumed that the $\Delta^{14}C$ DIC of BML would approximate that of a boreal lake ($\Delta^{14}C$ = -145 ‰). In a lake dominated by petroleum derived carbon inputs, the $\Delta^{14}C$ of DIC could be heavily impacted by ancient carbon and thus quite depleted.

The results of this mass balance are shown in Figure 2.8, where ‘Ancient influence’ is the percentage of the PLFA that would be related to PH, ‘Modern influence’ is PLFA material that could be coming from atmospheric or DIC related carbon sources, and ‘Ancient influence

difference' being the difference between how much ancient and modern influence is estimated to be contributing to the lake using the two different end members in the mass balance. This shows the percentage of modern $DI^{14}C$ influence on the samples if only newly fixed carbon were being consumed as 'modern' carbon sources, the percentage of modern $DI^{14}C$ that may be contributing to the radiocarbon values within the lake given slightly more depleted values more similar to boreal lakes.

The equation for fraction of modern influence is seen below:

$$f(petroleum) = \frac{\Delta^{14}C_{measured} - \Delta^{14}C_{Source}}{\Delta^{14}C_{petroleum} - \Delta^{14}C_{Source}}$$

Where $f_{(petroleum)}$ is the fraction of sample that is petroleum derived, $\Delta^{14}C_{measured}$ is the value of the FAME analysis, $\Delta^{14}C_{petroleum}$ is the value of petroleum derived carbon, and $\Delta^{14}C_{Source}$ is the modern carbon source being compared.

By using these endmembers of $DI^{14}C$ and atmospheric values, we can see the range of impacts the modern carbon sources could be exerting on the lake.

Mass balances can be used to determine the relative impacts of two different sources, in this case the ancient petroleum derived carbon containing $\Delta^{14}C$= -1000 permille carbon, and modern carbon sources that contain significantly more radiocarbon, estimated to be around $\Delta^{14}C$= -145 permille. Since all carbon collected was put together, including methanotrophic and phototrophic PLFA, the value analyzed is an average of all the life collected. By using a mass balance, we can elucidate the impacts that each carbon source was influencing the bacterial PLFA. Firstly, the

mass balance showed that the epilimnion always contained more modern influence than the hypolimnion counterpart. This means that the hypolimnion samples contained more radiocarbon dead PLFA, and that even if the DIC values, representing modern carbon sources that are -145 permille, the hypolimnion still does not see DIC fixation contributing to more than 20% of the carbon. The potential of modern carbon usage only minorly increases in the epilimnion with the contribution of DIC fixation not reaching 30% of the carbon incorporated into microbial PLFA.

Further, the difference in the estimated DIC input via photosynthesis is not strongly influenced by the modern DIC endmember used. The difference in photosynthetic input between DIC of modern atmospheric values of ~ 15 ‰ and $DI^{14}C$ of -145 ‰ is only circa 4 %. This means that although estimates are being used, these results still strongly support that petroleum derived carbon is the primary carbon source within the lake. This is consistent, particularly in the hypolimnion, with the indication by the stable isotopic results that methane, derived from petroleum, is the primary carbon source of the microbial community.

The mass balance is not very sensitive to the scale of which modern carbon sources can be found in and around the BML environment (Table 2.1). Even if values chosen are heavily underestimated, a modern carbon source with a $DI^{14}C$ value of -400 ‰ (a value much more depleted value than the DIC observed in various other studies (Voss et al 2023; Zeidan et al 2022) was found to be impacting BML, the mass balance still remains influenced by ancient carbon more, with the most modern sample becoming ~40% modern.

Overall, the epilimnion is more influenced by modern carbon, with an average of 22% modern throughout the samples, whereas the hypolimnion sees 15% on average. The highest value of modern carbon in the hypolimnion was seen in the sample retrieved in October, with a

modern percentage of 19.2%. All other hypolimnion values were at least 5% less modern. The maximum value of modern carbon determined by biofilm was found in August of 2018 in the epilimnion, with a value of 28.4%. With the highest concentration of oxygen found within the epilimnion during summer months by Risacher et al. (2018) and Arriaga et al. (2019). This is consistent with the impact of phototropic metabolism increasing oxygen and modern carbon values. This is further supporting the suggestion that petroleum carbon is dominant and aerobic heterotrophy or methanotrophy are the major metabolisms at play within BML.

2.11 Conclusion

From this data, we can determine the epilimnion is consuming and metabolizing more modern carbon over the observed timeline. The entire lake system overall is still very much ancient carbon dominant, in both the epilimnion and hypolimnion. Dissolved methane in the lake remains a primary carbon source in the lake, particularly in the hypolimnion, as also seen by Slater et al 2021. While the influence of methane is reduced in Oct 2017, it is reestablished in the following summer. This reduction appears to be the result of influence of algal bloom carbon or epilimnion carbon being mixed during turnover of the lake.

The overall trend of decreasing methane use in the epilimnion and increasing modern carbon indicates that the lake is moving from a methane dominated carbon cycle throughout to one where more modern organic matter inputs are having influence in the epilimnion. This implies that the hypolimnion is continuing to prevent the transport of the majority of methane being input at the FFT water interface.

Further monitoring of the lake and the radiocarbon over time will give better understanding of the rates of changes the lake is undergoing. With the understanding of both the carbon 13 and radiocarbon analysis showing highly depleted values in summer and more enriched in fall, higher resolution information towards the changes of PLFA over the course of set amounts of time would greatly increase the ability to determine the causes of the changes occurring. It can be stated that the turnover of the lake clearly impacts the overall depletion of the lake's radiocarbon, and that the algal bloom that occurred in 2017 may have been a main factor in the rise of enriched carbon into the deeper sections of the lake. More testing, and testing without additional alum would be key to understanding this. With the mass balance, DIC and DOC ^{14}C numbers of the lake would be a great asset to constrain and allow for more precise information about the radiocarbon of the lake, as it stands, the maximal amount of modern carbon being metabolized into the microbial communities of the lake are less than 30%.

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2.13 Tables and Figures

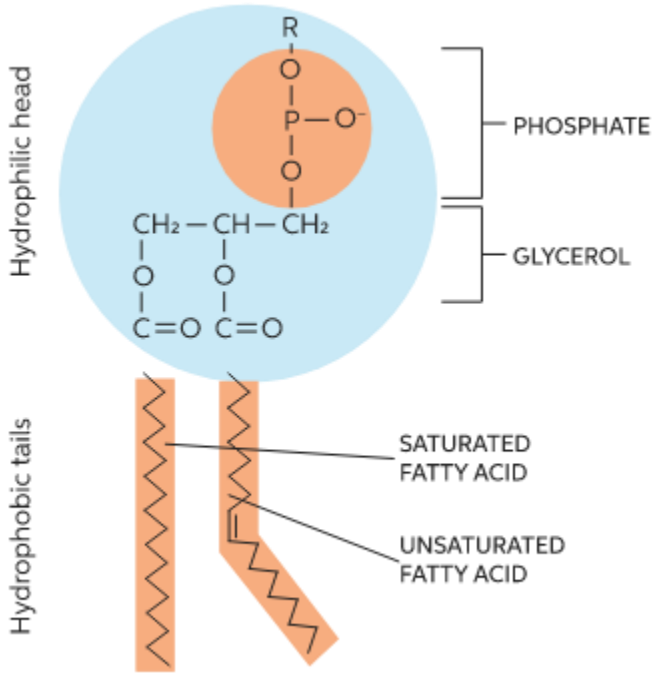

Figure 2.1 An image of an intact membrane lipid. Taken from

https://www.chegg.com/learn/biology/anatomy-physiology-in-biology/structure-of-phospholipid

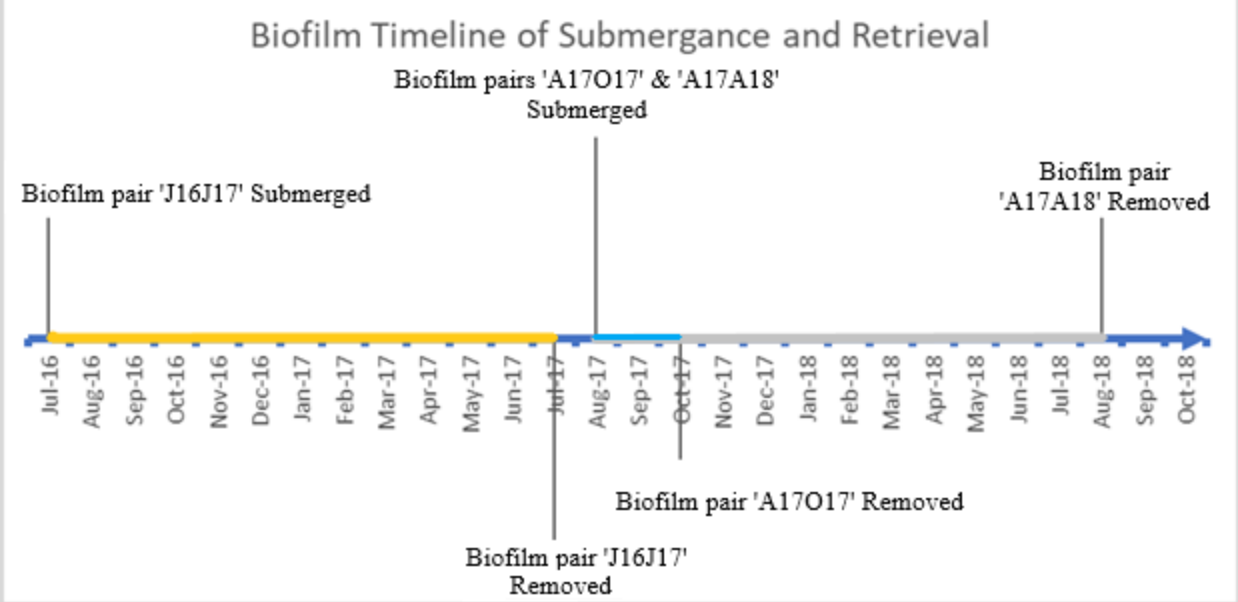

Figure 2.2 Timeline of biofilm submergence and retrieval from Base Mine Lake.

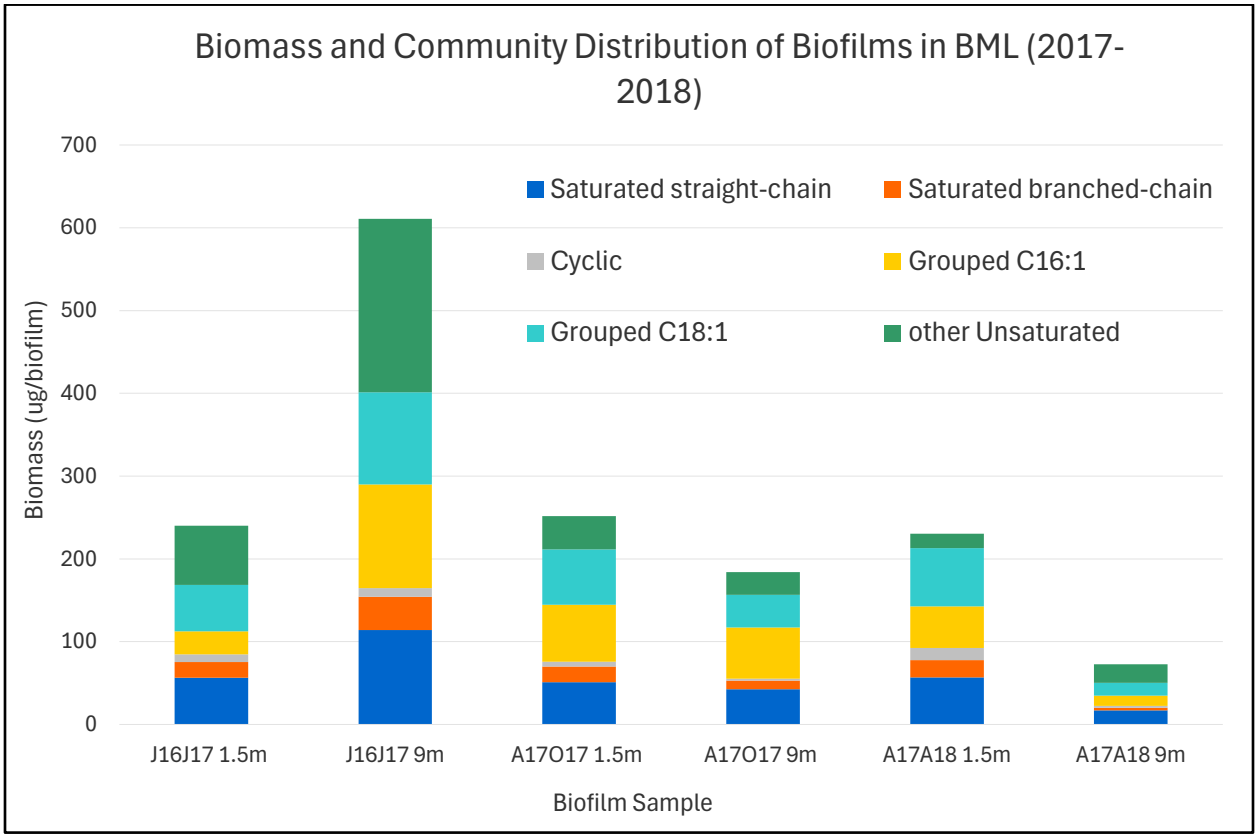

Figure 2.3 Total Biomass concentrations of Biofilms with PLFA distribution overlay.

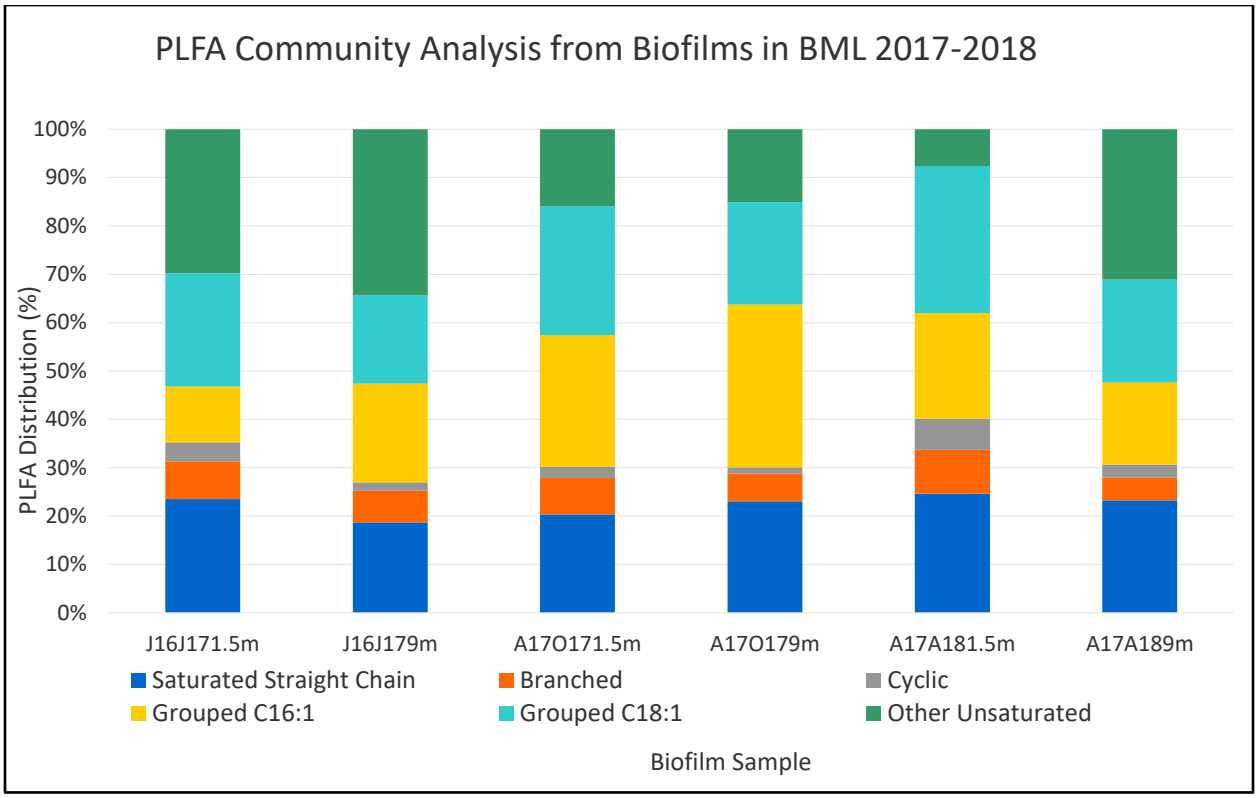

Figure 2.4 Normalized PLFA community analysis of biofilms

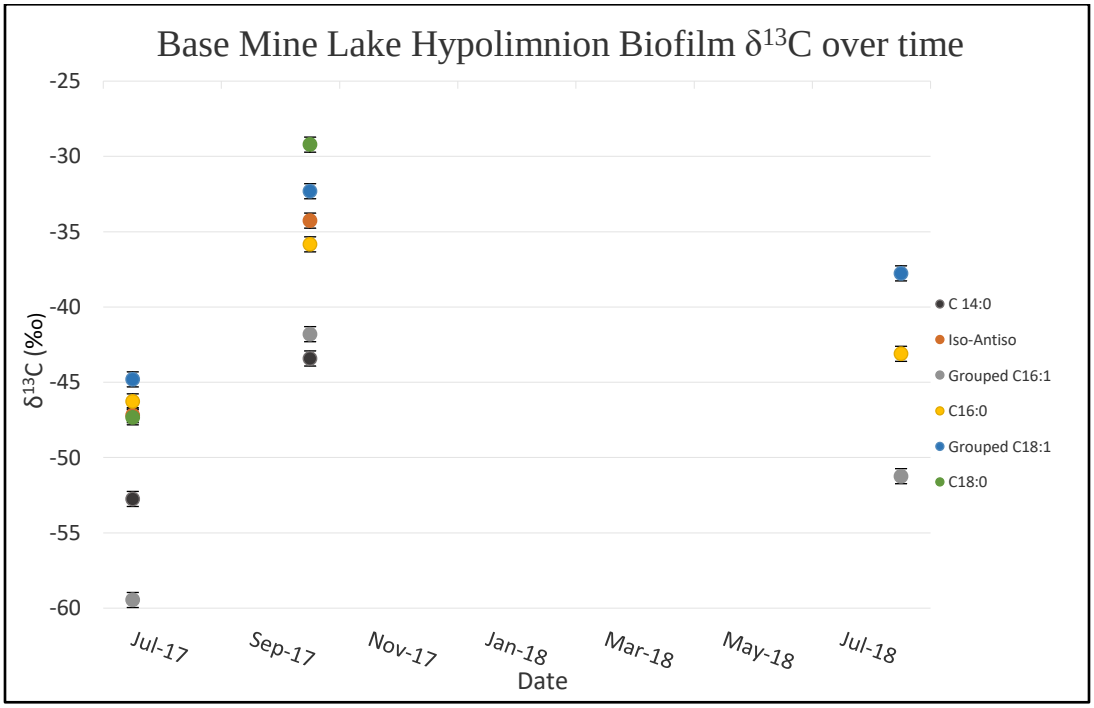

Figure 2.5 Hypolimnion PLFA stable isotopic compositions for indicator PLFA throughout biofilms sampled.

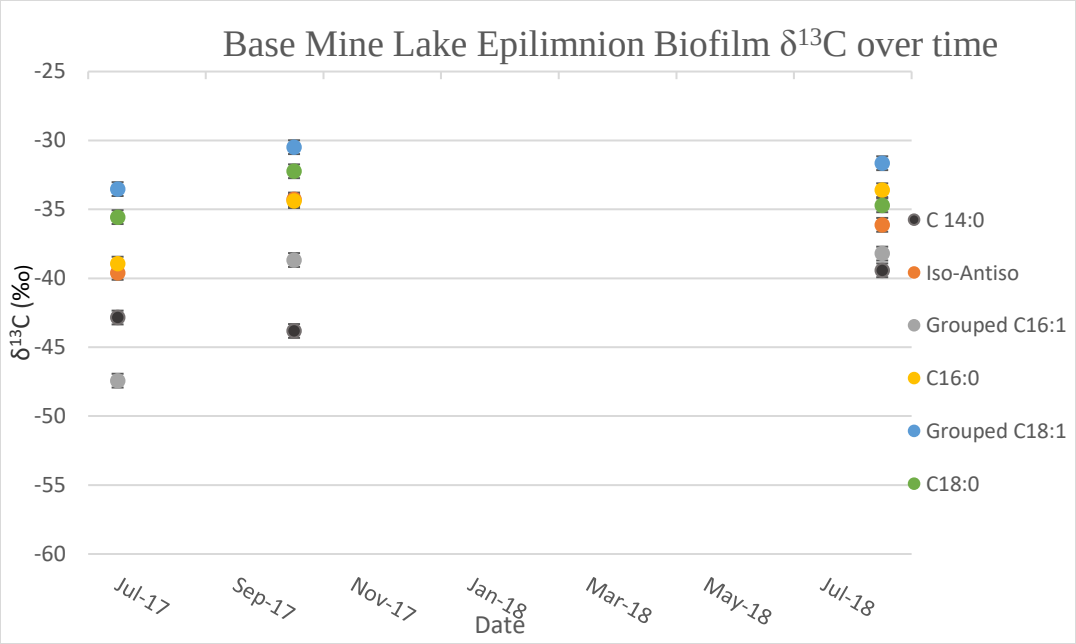

Figure 2.6 Epilimnion PLFA $\delta^{13}C$ (‰) stable isotopic compositions for indicator PLFA throughout biofilms sampled.

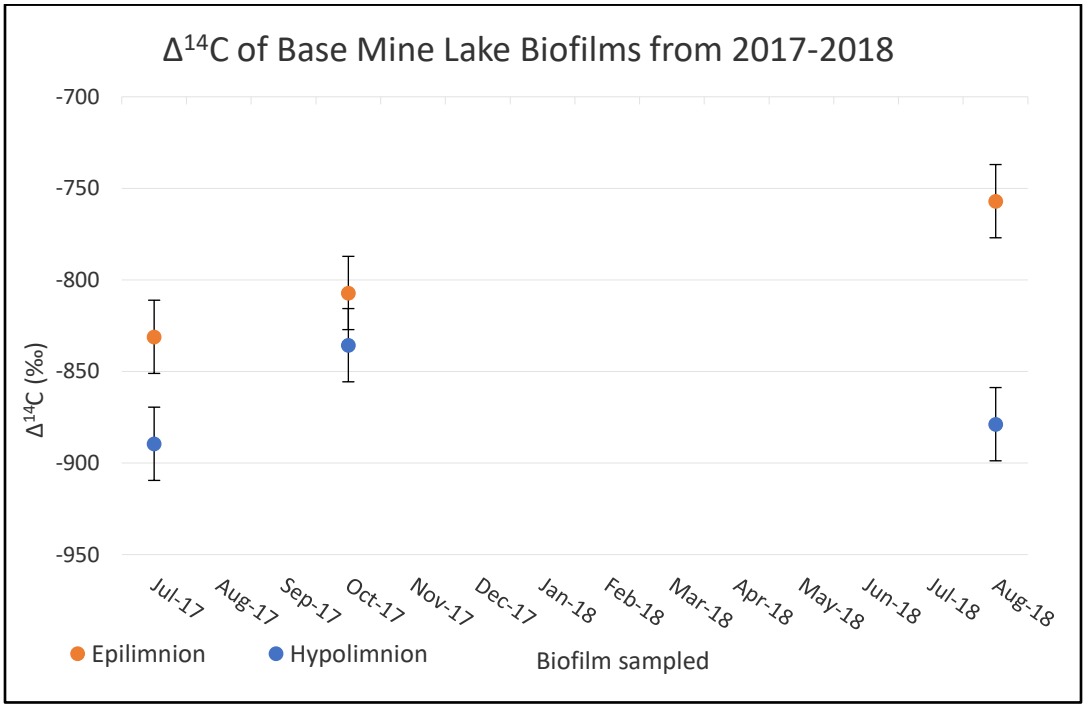

Figure 2.7 Radiocarbon analysis ($\Delta^{14}C$) of PLFA of epilimnion (orange) and hypolimnion (blue) microbial communities extracted from biofilms.

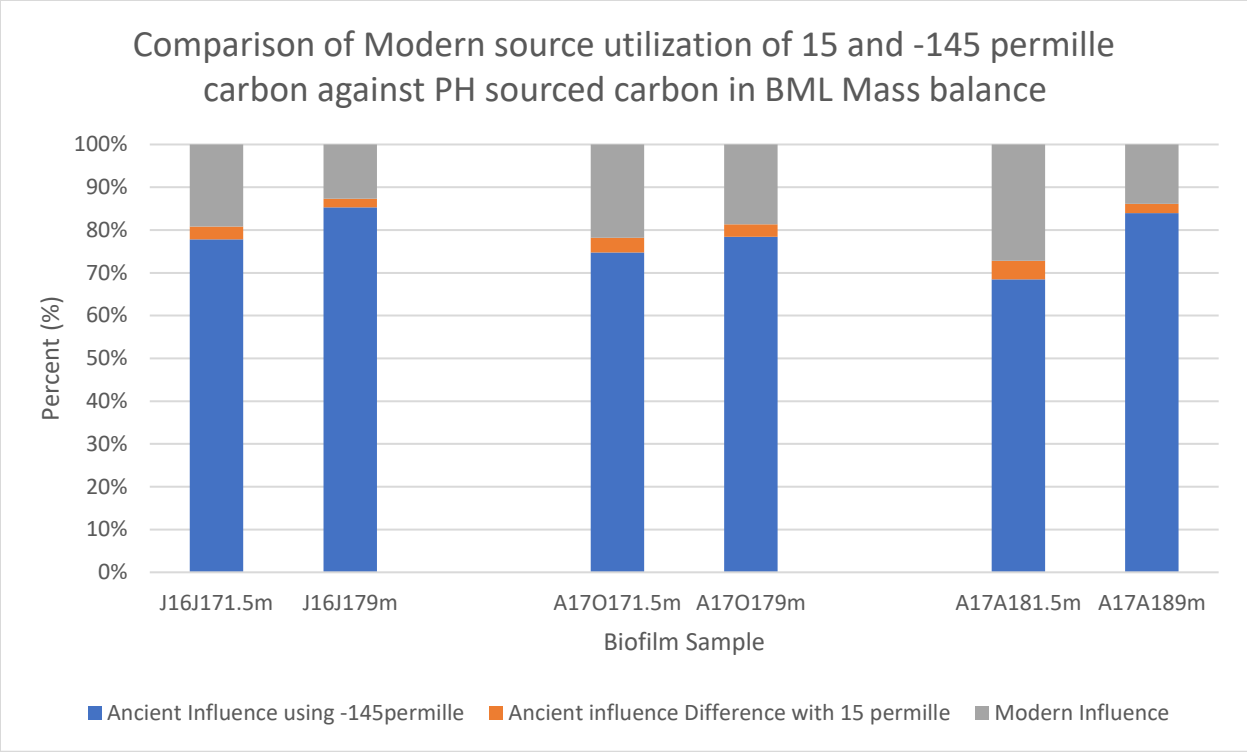

Figure 2.8 Mass balance carbon utilization of BML using atmospheric and DIC estimates. Petroleum, or ancient carbon (blue), non-petroleum sources (Grey) and the variation of carbon source percentage depending on the modern carbon source estimated value used in calculations (Orange).

Table 2.1 Values of Mass Balance equation using a variety of modern carbon source estimates.

	Modern Carbon Source (‰)	15‰	-20‰		-110‰	-145‰
Sample				**Percent Modern(%)**		
July16 - July17 1.5m		16.6	17.2		19.0	19.8
July16 - July 17 9m		10.9	11.3		12.4	12.9
August17 - October17 1.5m		19.0	19.7		21.7	22.6
August 17 to October 17 9m		16.8	16.8		18.5	19.2
August17 - August 18 1.5m		23.9	24.8		27.3	28.4
August 17 - August 18 9m		11.9	12.4		13.6	14.2

CHAPTER 3:

QUANTIFICATION OF ENDOSPORES VIA DPA ANALYSIS AND ASSESSMENT OF MATRICES EFFECTS IN BENTONITE CLAY

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3.1 Abstract

Bentonite clay is a crucial component in the Nuclear Waste Management Organization's proposal for long term storage of used nuclear fuel within a deep geological repository, in a multi-barrier system to maintain a sealing system that will survive on the scale of a million years. Culture studies have shown some bentonite clays contains sulphate reducing bacteria (SRB), which has the potential to corrode other sections of the barrier system. It is recognized that the state of the SRB and life within the DGR may be influenced by spore forming bacteria, which can survive prolonged heat, desiccation, and salinity (Masurat, Eriksson, & Pendersen, 2010). This survival is predominantly thought to be due to the sporulation of cells, in which a cell becomes dormant in harsh and unfavorable conditions. Endospores are not detected under normal cellular extraction methods, and their survivability makes them an important factor to consider into the longevity of the deep geologic repository. Endospores are not detected by most usual cell and bacterial detection methods but can be detected by Dipicolinic acid (DPA) extraction and chelation with Terbium via High Performance Liquid Chromatography – Fluorescence Detection (HPLC-FLD).

This study investigated the use of DPA analysis to detect spores in bentonite clay. DPA is a unique molecule contained within most endospores, and can be readily oxidized upon cell germination, it makes it a viable molecule for current spore count assessments. Results show that DPA recoveries from spores were consistent with expectations of $2.2x10^{-16}$ mol DPA $spore^{-1}$ as per Fitchel et al. (2007). Results showed that the DPA extraction method implemented without sediment was within error of 100% expected concentration. The presence of basalt, granite, and

bentonite clay sediments minimally impacted the recovery of expected DPA concentration within 0.5g samples, with only significant difference found with basalt extractions, being below expected recovery yield. Extraction of DPA from bentonite samples of multiple sources may not have detected DPA, indicating spores were below detection limits. Due to the interest of spores within astrobiology applications, understanding the adsorption of DPA on a variety of planetary analogs is valuable to the potential search for endospores within astrobiological significant planets.

3.2 Introduction

The Nuclear Waste Management Organization (NWMO)'s multi-barrier system is a robust system designed to alleviate potential risks that may be associated with the long-term storage of used nuclear fuel in a deep geologic repository. In 2002, with the passing of the Nuclear Fuel Waste Act (NFWA), the NWMO was established to develop and study the most effective way to keep Canadians safe and manage the increasing number of used nuclear fuel. Consistent with proposals in other countries that utilize nuclear energy, NWMO has proposed a deep geologic repository for the long-term storage of used nuclear fuel. A key component of this proposal is NWMO's "multi-barrier system" (MBS) that uses natural and engineered barriers to maintain containment of spent fuel until it returns to background levels of radioactivity. In the DGR MBS fuel elements, which are tubes containing the fuel pellets bundled together, are contained in a carbon steel, copper coated container that in emplaced with compacted bentonite clay along with clay fill within the excavated repository (NWMO.ca).

The durability of this bentonite barrier is critical as a security against any water borne transport of radionuclides should a cannister fail. But equally to prevent processes that may affect container integrity, in particular the exposure of the container to chemicals that may lead to corrosion, such as H_2S. And while chemical reactions with local groundwater and bentonite can be constrained, it is also necessary to understand the potential impacts of metabolic activities by microbes present within the bentonite itself.

Copper is a key element in the barrier system, due to the corrosion resistance to water. However, there are some reactions that can degrade the copper coating, such as reactions with sulphide, which can be produced by sulphate reducing bacteria (SRB). Confirming the presence, prevalence, and activity of sulphate reducers within bentonite clay is clearly an important aspect of demonstrating the long-term safety of the DGR.

Ongoing research by the NWMO and their partners is investigating the potential for microbial growth within bentonite under conditions analogues to the DGR. The focus of this work is to assess the potential presence of bacterial spores within the bentonite that may contribute to future microbial growth. This research project applied the extraction methods of Rattray et al 2021 and Lomstein & Jørgensen 2012 to detect and quantify dipicolinic acid (DPA), a biomarker that makes up 10-15% dry weight of some spores. Among spore formers, sulphate reducing bacteria (SRBs) are present (Fitchel et al 2007; Rattray et al 2021) and contain high amounts of DPA per cell. DPA is a molecule found in the core of a spore that assists with the dehydration of the core and thus the stability of the spore for long periods of time.

3.2.1 Storage of Used Nuclear fuel in Canada.

Used nuclear fuel is a byproduct of the nuclear energy utilization. Within the current usage of Canadian Deuterium-Uranium reactors, 3.2 million bundles (as of 2022) of spent nuclear fuel are currently in short-term storage, which are massive water filled pools, that cool the rods and allow for initial radioactivity reduction. After 7 to 10 years of water submersion, they are taken from pools and placed into dry storage vaults, that can contain the rods for ~50 years, while it awaits long term storage. An estimated 2.3 million more rods are expected to be produced within the current reactor operation timelines. Within the Waste Fuel act, the NWMO must provide a detailed approach on how to maintain safety for the surrounding area and to minimize the impact of the spent nuclear fuel until the levels of radiation would be approaching uranium ore found naturally in the earth (NWMO.ca). In order to achieve this, the NWMO has proposed construction of a DGR and multi barrier system. To get this solution approved, the NWMO must assess all potential factors that may affect DGR performance.

After a site selection search that included community awareness and indigenous community outreach, the geology and hydrogeology of two potential DGR locations are being assessed to determine their effectiveness of the location to home the DGR. Within the NWMO, extensive care and research is conducted within each element of the DGR MBS, with bentonite being a main topic of research.

Highly compacted bentonite was selected due to many of its physical properties, in part due to its swelling properties when wetted, it has very low hydraulic conductivity leading to its common use to seal groundwater wells (Stroes-Gascoyne, Hamon, & Maak, 2011). Swelling bentonite clay will hinder and halt most water, microbes and radionuclides (Stroes-Gascoyne et al. 2010;

Dohrmann, Kaufhold, & Lundqvist, 2013).This, along with host rock selection also showing low permeability, leads to very minimal water movement through the bentonite which will effectively isolate the spent fuel containers within the bentonite-based barrier system from outside influences and prevent any potential release of radionuclides in the highly unlikely event of a container failure.

Bentonite clay is used in multiple ways within the DGR, having both granulated gap fill bentonite as well as highly compacted clay boxes used to seal and fill the space between bundles. These compacted bentonite boxes are shaped for the copper-coated fuel container, and gap fill bentonite is used to fill and allow for sealing any pores or gaps within the placement room. As a DGR is drilled out a minimum of 500m deep in Canadian host rock, and have an area of $2km^2$, a lot of bentonite clay will be needed to fulfil the ideals of this engineered barrier in the MBS (Dohrmann, Kaufhold, & Lundqvist, 2013).

3.2.2 Microbial influenced corrosion

Minimising the microbial activity of the bentonite will be crucial for long term stability of the DGR. Some microbial reactions have the potential to produce H_2S, which can degrade the copper coating of the canister. Microbially influenced corrosion (MIC) is where the presence and processes of microbes allow for corrosion to be present, whether by the microbe initiating the corrosion, creating an environment that allows for corrosion, or speeds the corrosion of the metal along. Because of the low water content, limited pore space, and nutrient availability restricted by diffusion processes found in the compacted bentonite, cycling of energy will be a large component of microbial life (Stroes-Gascoyne, 2010; Pendersen et al. 2000). Spore forming SRB, such as *Desulfotomaculum* and *Desulfosporosinus*, have been found in bentonite clay.

(Campbell & Postgate, 1965), (Grigoryan et al. 2018, 2021). SRBs are able to conduct MIC due to their ability to reduce sulfate to sulfide, by using acetate in heterotrophic SRB or H_2 in autotrophic SRB like *Desulfotomaculum*.

3.2.3 Endospores

Spore states are created via stressful environmental conditions such as starvation, temperature, salt content, oxygen presence, and dryness/lack of water (Nicolson, 2002; Pinto, Santo, & Chambel, 2015). Cells create thicker membranes with increased outer-membrane protein in the pursuit of longevity, and that is thought to be the reasoning for the lack of cell division within VBNC bacteria. (Ramamurthy, Ghosh, Pazhani, & Shinoda, 2014) Endospores are the outcome of cells being stressed in their current environment, converting to an endospore to contain genetic material and remain dormant until the unfavourable environment has passed or that the spore has been transported to a favourable environment. Usually, Gram- Positive cells achieve sporulation state when a bacterial cell's preferred metabolising environmental carbon, phosphate, or nitrogen supply has run out (Higgins & Dworkin, 2012). This causes sporulation to occur and will cause an asymmetric cell division wherein within the cell walls, one side consumes the other. This spore lacks metabolic process and remains dormant in exchange for the ability to survive extreme temperatures and environments. Spores also are formed from a variety of genus and species of bacteria, mostly constrained to the Firmicutes phylum, which contain Clostridia, strict anaerobic bacteria, and Bacilli, mainly aerobic bacteria (Tragg et al. 2010). The Clostridia and *Bacillus* species are rod shaped Gram-Positive bacteria, and Clostridia species such as *Clostridium clariflavum* are not only spore-forming, but thermophilic as well (Artzi et al. 2014;

Muyzer & Stams, 2008). SRB spore formers exist as well, such as *Desulfomonas* (Kushkevych et al. 2021). The DPA concentrations from cultivated SRB spores were obtained from thermophilic environments were found to be some of the highest amount of DPA per cell (Rattray et al. 2023; Fitchel et al 2007).

Since DPA is a unique molecule contained within most endospores, and can be readily oxidized upon cell germination, it makes it a viable molecule for up-to-date spore count assessments. A terbium chelation method was developed (Hindle and Hall, 1999) but quenching of the fluorescence due to sediment matrix effects. $AlCl_3$ was found to be a suitable addition to samples to bind phosphate, found to be preferentially binding the terbium, out of the sample (Fell, Pellegrino, & Gillespie, 2001).

The purpose of this study was to look for DPA in natural bentonite to determine the spore abundance in bentonite clay of interest. The study was also undergone to discover the effectiveness of current DPA extraction methods on bentonite clays, as well as other sediment matrixes.

3.3 Methods and Material

Dipicolinic acid 99% - Sigma Aldrich, terbium (III) chloride hexahydrate 99.9%- Sigma Aldrich, HPLC Grade methanol (MeOH), acetic acid, and sodium acetate (NaOAc) were purchased from Sigma Aldrich. Polypropylene beakers, volumetric flasks were obtained from Corning and HPLC vials with Teflon caps from Agilent were also used to avoid adsorption of DPA. 50ml

Falcon tubes and 3ml Falcon Pipettes used during extraction. Bentonite clay was collected by the NWMO, basalt samples as well as granite samples were taken from previously collected samples, both having been crushed to consistent grain size.

3.3.1 Spore Suspension counting

B. subtilis was grown in the Neufeld lab in the University of Waterloo for the purpose of a control spore extraction of DPA of a known spore count. Under streak purification of bentonite clay, gap fill (granulated) bentonite was used to get pure culture *Bacillus*. This was confirmed through Sanger sequencing of the 23F-1492R 16S rRNA region and NCBI BLAST identification showing a 100% identity for *Bacillus subtilis* and *bacillus* species.

A colony was inoculated into 9 ml of liquid R2A media and incubated at 30 degrees C for 48 hours at 150 RPM and stored at 4 degrees Celsius afterwards. The purity and growth of *bacillus* can be found in supporting information

Spores were counted in a haemocytometer (Figure 3.1) to have at least 19.99 spores per square leading to an overall suspension of 7.99×10^8 spores/ml.

3.3.2 DPA Extraction

Sediment extractions were based on Lomstein & Jørgensen 2012 and Rattray et al 2021, conducted in triplicate by taking .5 grams of crushed and sieved sediment (Basalt, Bentonite, and Granite) and placing them into 50 ml polypropylene falcon tubes. 1 ml of 6M HCl and 1 mL of milli q water were poured into the falcon tube and placed into an oven heated at 95 degrees for 4

hours (Lomstein & Jørgensen 2012). Afterwards, the falcon tubes were put in an ice bath to stop hydrolysis. Solution was then transferred to a new falcon tube, and frozen. The frozen hydrolysate was then freeze dried. Once all ice was removed, falcon tubes were taken out, more milli-q water was added and frozen again. Once more the samples were freeze dried, and once taken out of freeze drier, 4 mL of 1 M NaOAc was added to resuspend sample, and 80 uL of $AlCl_3$ was added to allow the precipitation of preferential terbium binding molecules, including phosphate and other phosphorus molecules. Solution was micro filtered through a .22 um syringe filter, the first 1 mL was discarded, and the remaining solution was used in sample runs. When spore suspension samples were being extracted, the extraction method was similar but 1ml of 6 mol HCl and 1ml of the suspension was added to the falcon tube, with no sediment, to investigate the effects of bentonite on adsorption of DPA to the clay particles, some tests of burnt bentonite clay were also used to determine the DPA loss based on adsorption of free DPA within the swelling clays.

3.3.3 **Instrument HPLC-FLD Analysis and Buffer Preparation.**

Two buffers were prepared as follows: The NaOAc buffer which was prepared in Milli-Q water to 1 molar concentration, adjusted to pH of 5.6 with acetic acid, as seen in Lomstein & Jørgensen (2012). This will be further referred to as Buffer A. Buffer B was produced by diluting HPLC Grade MeOH with Milli-Q water in an 80%:20% Volume: Volume ratio. Both buffers were filtered through with 0.2 µm filters.

Samples were analysed using a modified HPLC fluorescence Tb^{3+} chelation method (Lomstein and Jørgensen, 2012; Rattray et al., 2021). An Agilent System FLD Infinity II was used for the

fluorescence detection, with a Kinetex 2.6 μm EVO C18 100 Å LC column (150 × 4.5 mm, Phenomenex, USA) column. DPA was detected using a Thermo FLD-Infinity II fluorescence detector, measuring at 270 nm emission and 545 nm excitation and compared with DPA:Tb^{3+} chelate standard curves spanning the range of DPA detected in the samples. The column was set to 32° Celsius for analysis. The NaOAc was flushed through to the purge at 3ml/min until bubbles were no longer seen, purge valve was closed, and flow was changed to .6 ml/min to maintain a pressure of around 200 bar. NaOAc vial 'blank' runs were used until the trace showed less fluctuations, and once that was reached the sample run sequence was started. Flow rate parameters are shown in Table 3.1. Endospore counts were calculated using 2.24 fmol DPA/endospore (Fichtel et al., 2007), a endospore conversion used widely in endospore conversion (Lomstein & Jørgensen, 2012; Lomstein et al., 2012).

3.4 Results

3.4.1 Standard curve for DPA concentration calculations

Standard Curves (Figure 3.2) were used to determine unknown sample concentration and spiked sample recovery concentration. Using a 5-point curve, encompassing the full range of potential values expected from the spiked recovery. This standard curve shows a R^2 of .99 and has a range of 5 nM to 1000nM of DPA. Blanks were extracted via the same method and used to verify no contamination or other peak was showing at the time of elution of the terbium and terbium-DPA complex.

3.4.2 Spore suspension recovery based on expected DPA yields.

Average recovery of Spore suspension expectations using Fitchel et al. 2007 cell conversion factor of $2.2 * 10^{-16}$ mol DPA $spore^{-1}$ with no sediment added (Figure 3.3). Error bars represent 2 Standard Deviations. With no sediment effects, recoveries were all within error of 100%. Sample 1 consisted of an expected addition of 3.5×10^3 nM of DPA, and the average recovery was 3.4×10^3 or a 97.5±2.5 % recovery. Sample 2 was an expected 7×10^3 nM of DPA with an average return of 6.8×10^3, or 96.8±12.2% recovery. Sample 3 was an expected extraction of 8.7×10^3 nM of DPA and successful extraction of 1.1×10^4, or 125±26% recovery. Sample 4 had an expected recovery of 1.3×10^4 and had 1.6×10^4 nM of DPA on average, or 122±12% recovery. Finally, Sample 5 had an expected addition of 1.8×10^4 and the average recovery was 1.9×10^4, or 108±23% recovery.

With the variability of endospore size based on species and environment, some variability between spores extracted for purpose of extraction viability between labs may occur. Having found spore-specific DPA contents vary, with different pure cultures ranging over an order of magnitude in DPA concentration (Aronson & Fitzjames, 1976; Sojka & Ludwig, 1997; He et al., 2003; Kort et al., 2005) Rattray et al. 2021 put forward a new spore conversion factor of $9.1 * 10^{-16}$ mol DPA $spore^{-1}$ using a variety of spores including thermophilic varieties seen to have higher DPA yields. Due to the *Bacillus* culture used in this study for sake of extraction yields being, the conversion factor of $2.2 * 10^{-16}$ mol DPA $spore^{-1}$ was used, as this closely matched the conditions of Fitchel et al 2007 conversion factor conditions. There is potential for spore differences to be found between species however, and underestimation of DPA per cell may have occurred using this conversion factor.

3.4.3 Recovery of spores spiked onto sediment matrices.

Using crushed basalt, bentonite, and granite as sedimentary types, recoveries from different surfaces and mineral compositions can be determined (Figure 3.4). Granite and basalt being felsic and mafic, respectively, allows for a wide variety of mineral composition to be tested. Five aliquots of each sediment type were extracted using the method previously noted. All samples were injected in triplicate, with error bars produced by using 2 standard deviations. Average overall recovery of all injections was 87%. Granite had an average recovery of 96 ± 24 %, and basalt averaged 77± 29%. Average recovery for bentonite over all samples was 86±35%, but 94±11% when removing the first sample that had an overall low recovery, likely to human error in extraction. The first bentonite extraction was the lowest recovery calculated, with a 56± 16% recovery. Human error can occur at multiple points along the extraction and preparation of samples. Improper mixing and homogenizing of the spore suspension prior to spiking samples would allow for changes in expected spore yields. Sample transfers from extraction vessels containing sediment to vessels without may not allow for transferring of all DPA, even with meticulous rinsing. Error can also arise within the bubbling up and out of extraction vessels when freeze drying, although this was not seen to occur with the samples used in this study.

With granite, a more consistent grouping of recoveries was seen, with the highest recovery of 116±10%, with the other extracts containing 97± 24%, 95± 28%, 80± 9%, and 89± 26%. Finally with basalt spiked samples, the highest recovery was 102±3.5, and the others were 83±25%, 66±6%, 61±13%, and 75±9%.

Granite and bentonite samples had no significant loss of expected yield compared to the non sediment extractions, whereas basalt saw a significant loss of expected yield in a one-way ANOVA analysis/Tukey honesty significant differences test. This may be due to the increase of

metal found in the composition of basaltic rock, with the increased ability to potentially chelate the DPA out of solution. Bentonite swell times can take a fair amount longer than extraction time to fully swell, so the physical entrapment of DPA from bentonite extractions may be a smaller factor to consider for the purpose of endospore extraction.

3.4.4 Natural DPA concentrations in Bentonite

Samples of a series of bentonite samples from several sources with no addition of spores were also extracted to determine natural endospore abundance, however while some samples and clays may have been above the limit of detection (LOD), concentration of DPA was below the limit of quantification (LOQ). At the end of testing, slight column degradation was noted as LOQ and LOD had risen above what was originally seen upon early testing. Natural bentonite extractions occurred when the LOQ converted to circa $x10^5$ spores/gram of sediment. Thus, the bentonite contained less than this amount of spores. The potential detection of DPA being present however, indicated that some spores could be present, however, given the changes in method performance, these results need to be repeated to be confirmed. Further extractions with a fresh column and standard addition analyses may be able to bring the LOQ within range of the natural spore abundance of clay of interest to NWMO. Understanding of bacterial species could also be used to better determine the cell conversion factors that best estimate the sample, as seen in Rattray et al. (2021).

3.5 Conclusion

Due to the NWMO's proposed usage of bentonite clay within their multi-barrier system, understanding factors that may influence the longevity of the barrier system must be explored.

The results determined in this study display that matrix effects minimally affect the extraction and analysis of DPA from bentonite clay, laying the foundation for further understanding of spore concentrations within the bentonite. By extracting samples with known spore amounts added, accurate understandings of expected recoveries by DPA concentration were determined. Although bentonite saw no significant loss of estimated DPA, basalt samples did show a potential to be below expected concentrations. These results have potential implications towards the search for endospores on various surfaces, including extraterrestrial biosignature exploration. These results can allow for further understanding of endospores within sediments and determination of spore counts within a variety of extreme environments, which can be used for future commercial, industrial, and astrobiological applications. Future analysis of higher mass sediment samples or standard addition techniques can lead to determination of natural spore abundances within bentonite clay. Although this study was unable to determine the quantity of spores within bentonite, this suggests minimal spore populations exist within bentonite clay of interest to NWMO. Variations within samples, species of spores present and further determination of average DPA concentration from various species of spore formers can lead to increased accuracy in cell conversion factors.

3.6 References

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3.7 Tables

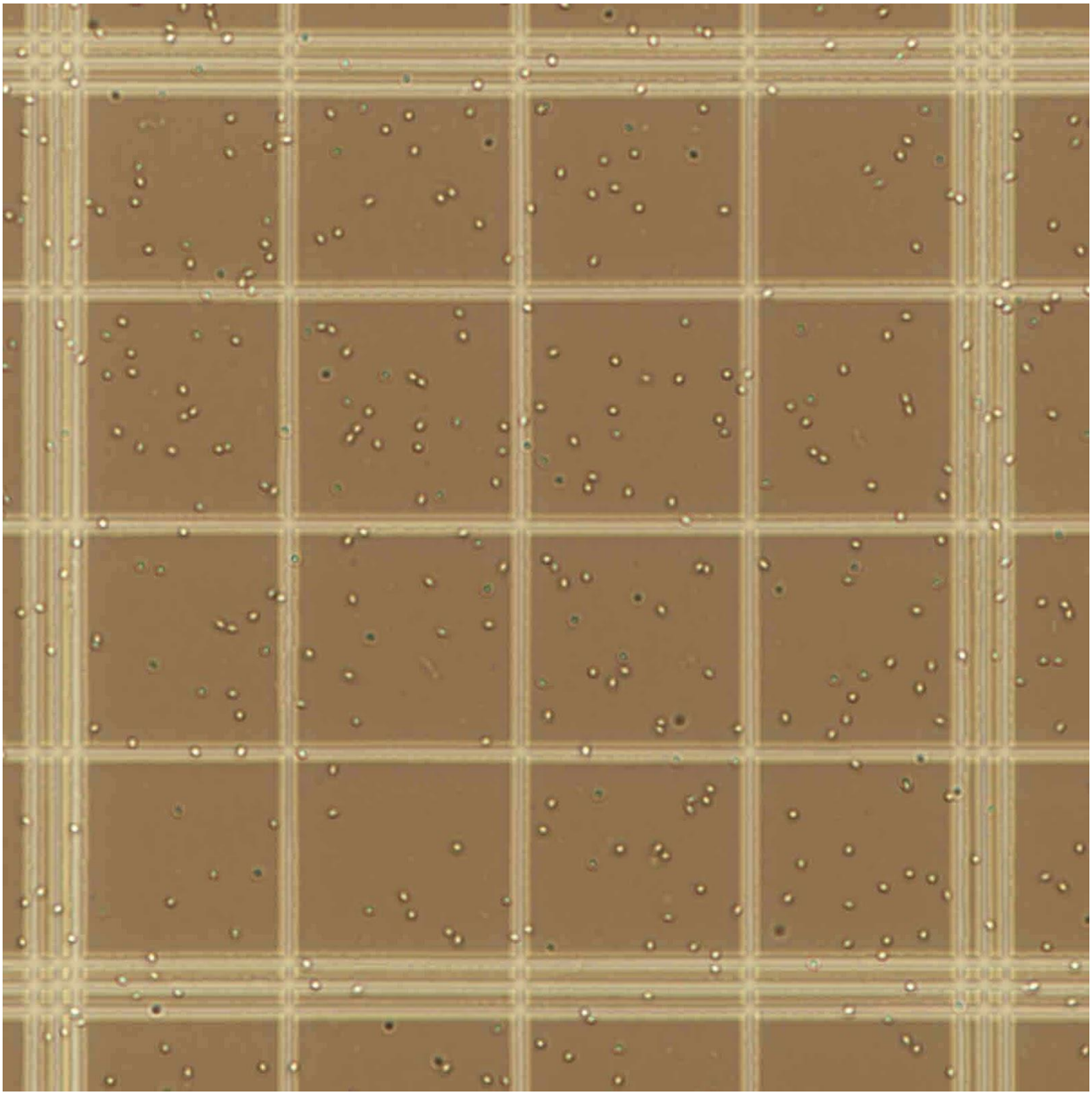

Figure 3.1 View from Microscope of Spore counting hemocytometer plate with a spore suspension, each dot representing an endospore, using the counting squares to determine a precise estimate of spore suspension.

Table 3.1 HPLC-FLD flow rate parameters

Time(min)	Flow (ml/min)	% Buffer A	% Buffer B
0	.5	100	0
1	.5	100	0
3	.5	85	15
6	.5	85	15
7	.5	100	0
10	.5	100	0

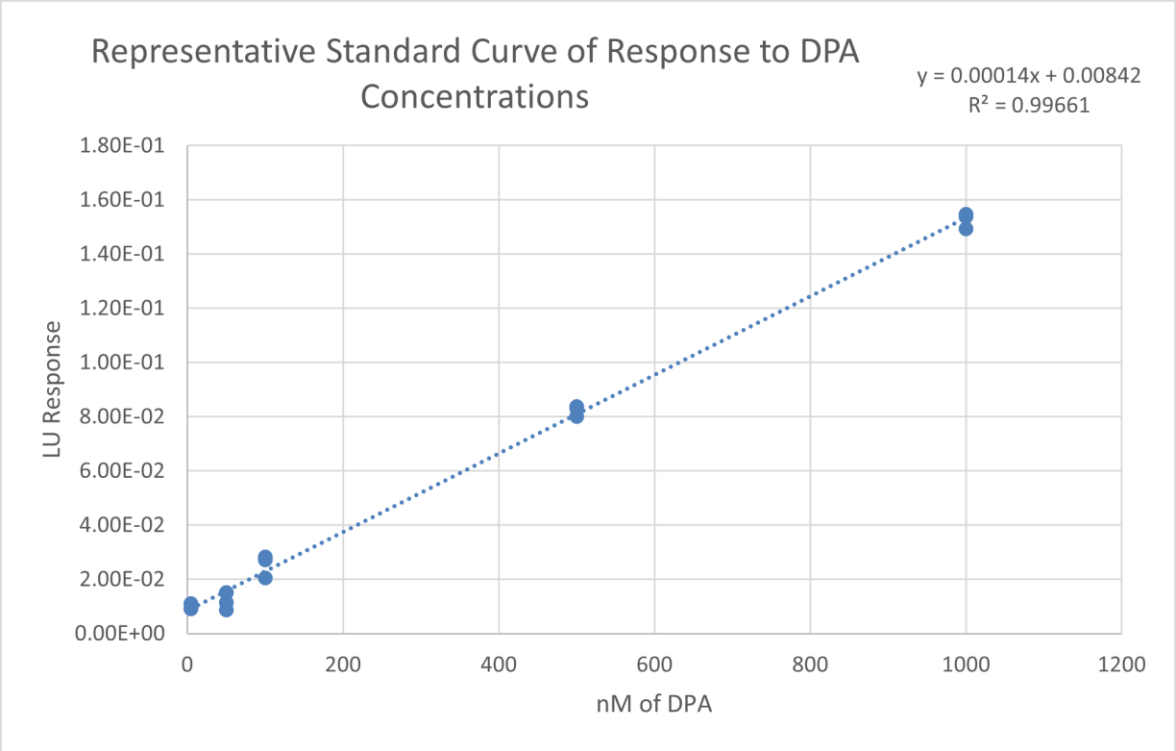

Figure 3.2 Standard Curve of HPLC-FLD DPA response. $R^2 > .99$, concentrations used were 5nM, 50nM, 100nM, 500nM, and 1000nM.

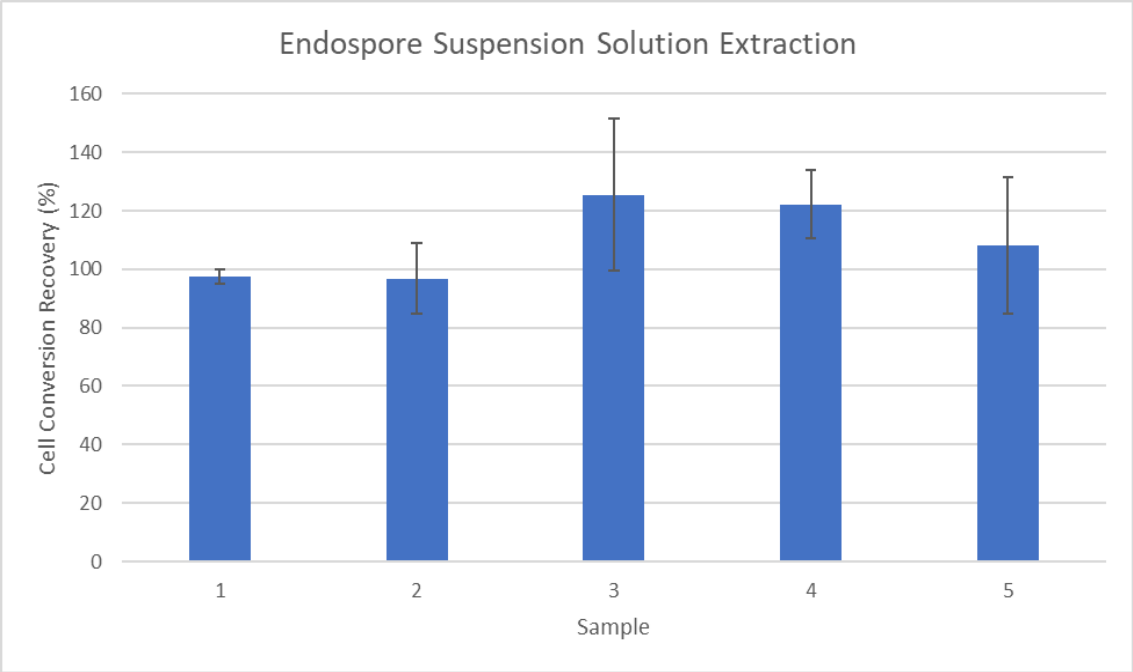

Figure 3.3 Spore suspension DPA extraction. Recovery was determined by using expected DPA concentration from cell conversion equation.

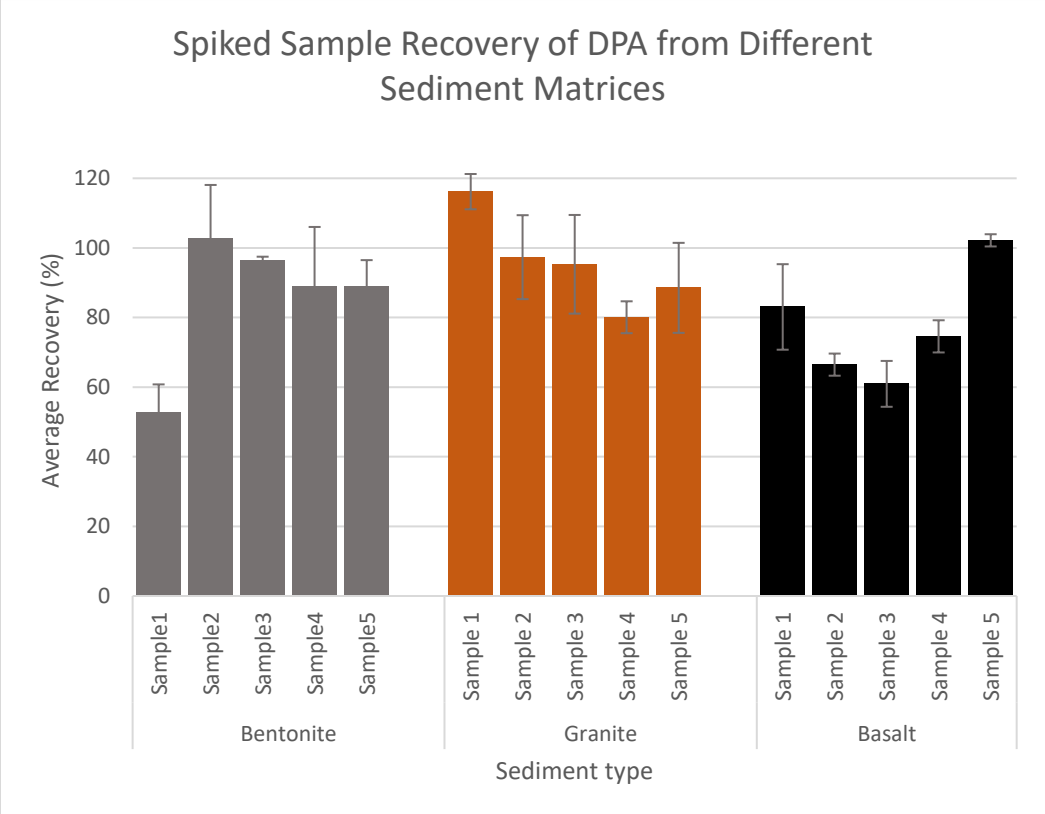

Figure 3.4 Average DPA recovery from different spiked sediment matrices of Bentonite (grey), Granite (red), and Basalt (black)

CHAPTER 4
THESIS SUMMARY AND FUTURE RESEARCH

4.1 THESIS SUMMARY

The characterization of microbial communities within BML by PLFA, $\delta^{13}C$ and $\Delta^{14}C$ analysis can contribute to the knowledge and planning of future pit lakes. Understanding the changes of metabolism and carbon sources utilized by microbial material can allow for better monitoring and understanding of the reclamation of the lake. The outcomes of this project will assist in the management, design, and implementation of future PLs planned for the AOSR.

This work allowed for the understanding of what carbon sources were being used by the microbial communities, and elucidated the extent to which PHs were a carbon source utilized directly or indirectly via use of methane produced from the PH.

4.2 The carbon source utilization of microbial life in BML

The potential carbon sources used by the microbial communities of two depths within BML was explored. The ability to determine PLFA community structure, stable isotopic values as well as radiocarbon values has allowed for deeper understanding and determination of PHs impact on the metabolism of microbial life within BML. The results displayed an extremely depleted stable isotopic signature found in methanotrophic biomarker PLFA, indicating the presence and utilization of methane as a carbon source. The radiocarbon data showed that all throughout the

lake, petroleum derived carbon is dominating the carbon being consumed. The hypolimnion showed a more depleted value across stable and radiocarbon data, showing that methane and petroleum hydrocarbons are likely more vital to the metabolism of the deeper portion of the lake, and reoccurred as a major factor for both summers sampled. Where the epilimnion saw an increase in newer material being produced/metabolized over the sampled period for radiocarbon.

4.3 The extraction of DPA from spores in different mineral matrices

The effects of Bentonite, Basalt, and Granite on DPA extraction were explored by spiking samples with known amounts of spores of *B.Subtilis.* The samples were extracted and compared against cell conversion concentrations to determine the effectiveness of the extraction within the lab, as well as determine the potential matrix effects on DPA from differing sediment compositions. The results of chapter 3 displayed no significant loss of DPA from expected values from bentonite sediment matrices. Significant loss was found to occur on basalt sediment matrix compared to spores extracted from a suspension solution by Tukey honestly significant difference test. Testing a series of natural bentonite samples from the NWMO may not have detected DPA and thereby indicating the absence of spores above the limit of detection. This implies that activation of spores is not a primary concern for the NWMO. With the limit of quantification not being reached, spores within bentonite could not be determined, but due to potential to increase the limit of detection and quantification, further analysis of bentonite sample extractions and post extraction methods may allow for spore counts to be confidently determined.

4.4 Astrobiological Implications

The results presented in chapter 2 highlight the importance of isotopic information to further determine metabolic sources, which may be able to elucidate the source of organic compounds. Biomarker analysis of organic compounds can be viable and useful tools to determine biogenic origin and habitat. Within Base Mine Lake, PLFA can be used to determine metabolism and carbon fixation sources, but other organic sources can also be used. The understanding and utilization of biomarkers and analysis of biogenic material can be used to make interpretations about systems, and applied to new systems and environments that are still being found. Applications of biomarker analysis can be applied to geologic record material and the progression of early earth. These applications can also be used to inform studies of analog sites. Chapter 3 demonstrates the capacity of spores to be found on low biomass areas such as bentonite clay, as well as the ability to extract and detect spores by using their DPA. With the discussion of panspermia needing life to be able to survive intense travel and Horneck et al, 2001 data suggesting spores can survive on mimicked mars and meteorite surfaces. Within meteoritic impact scenarios, spores are not only hardy enough to survive impact, but also, as long as they're accompanied by other small rock ejecta, can potentially successfully initiate a panspermic result. Continued testing of sporulation factors affecting DPA concentrations as well as extraction efficiencies may allow for DPA and spores to be a useful tool in learning about cell survival evolution, potentially allowing for further uncovering of biomarkers of interest towards the search for life within extraterrestrial context.

4.5 Future research directions

Further monitoring of BML and collection of biomass after 2018 can allow for further understanding of the changes made to the lake from the clearing of the turbidity by alum addition. The addition of DIC and DOC sampling for ^{14}C can allow for increased clarity on mass balance estimations. Upon determination of DIC and DOC values representative of Base Mine Lake and the river that is used to inject water, swapping the estimation values used in Chapter 2 with those determined from sampling will increase the strength of the arguments presented. While rivers see differing DIC values depending on how far downstream a sampling site is (Voss et al. 2023), source DIC sampling and $DI^{14}C$ analysis removes the estimations involved and allows for a more accurate and precise interpretation of the amount of modern and petroleum influences occurring within the lake.

Further development of lowering the limit of quantification (LOQ) for DPA using HPLC-FLD will allow for the extraction of natural spore abundance of bentonite clay to be determined. Along with bettering the LOQ, species analysis of spore formers via 16sRNA within bentonite will allow for a more precise cell conversion factor to be formed, if spore cultures are produced and extracted to determine mol DPA $spore^{-1}$. Addition of standard addition to the HPLC-FLD runs, as seen in Rattray et al 2021, can allow for further lowering of the LOQ, which could allow for concentrations below the LOQ to be determined. Larger mass extractions could also be attempted, but the additional acid needed to submerge and extract larger amounts of sediment may cause increased damage to the oven, freeze drier, and HPLC column. The potential for base to be added to minimize this may be possible, but proper acidity is needed to retain DPA's ability to chelate to Terbium.

Overall, this Master's thesis has shown biomarkers' usefulness in understanding a system. Whether it's BML or the NWMO, biomarkers can be utilized to better understand the ongoing and future developments of a system by better understanding the microbial fixation and biogeochemical cycling occurring. Biomarkers can be applied directly, but also used within an astrobiological context to build up a library that can be used to constrain and determine biogenic origins.

4.6 References

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