PHOSPHOLIPID FATTY ACIDS AS BIOMASS PROXIES AND THEIR USE IN CHARACTERIZING DEEP TERRESTRIAL SUBSURFACE MICROBIAL COMMUNITIES

By

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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 (2018)

Earth and Environmental Sciences

Collaborative Graduate Program in Astrobiology

McMaster University

Hamilton, Ontario

TITLE: PHOSPHOLIPID FATTY ACIDS AS BIOMASS PROXIES AND THEIR USE IN CHARACTERIZING DEEP TERRESTRIAL SUBSURFACE MICROBIAL COMMUNITIES

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NUMBER OF PAGES: viii, 74

ABSTRACT

Understanding the distribution, abundances and metabolic activities of microbial life in the subsurface is fundamental to our understanding of the role microbes play in many areas of inquiry such as terrestrial biogeochemical cycling and the search for extraterrestrial life. The deep terrestrial subsurface is known to harbor microbial life at depths of up to several kilometers where, in some cases, organisms live independently from the photosphere and atmosphere. Ancient fracture fluids trapped within the crystalline basement of the Canadian Precambrian Shield have been shown to be preserved on geologic timescales (millions to billions of years). Significant challenges exist when probing the deep terrestrial subsurface including the low biomass abundance, heterogeneous distribution of biomass, and the potential for matrix effects during sampling and analysis.

This Master's thesis project has two main parts. The first study utilizes phospholipid fatty acid (PLFA) analysis to determine the extent of mineral matrices on the effectiveness of PLFA extraction and analysis from deep terrestrial subsurface samples. This was done by creating a bacterial dilution series of known concentration to inoculate one of two mineral matrices, granite or bentonite. This study revealed the presence of significant influence of mineral matrices on PLFA extraction and demonstrated the unreliability of PLFA-based biomass conversion factors with respect to complex microbial communities. The second study in this thesis combine PLFA analysis with stable carbon isotope analysis to characterize microbial communities associated with fracture fluids with mean residence times of ~1.4 Ga from boreholes located ~2.4km below the surface in Kidd Creek Mine, in Timmins, Ontario. Characterizing communities in subsurface systems has large implications for the search for life on other planets and moons, acting as an analogue environment. Large volumes of water from two boreholes, 12261 and 12299, were passively filtered for 6-12 months to collect microbial biomass. Borehole adjacent biofilms were also collected along with mine service water, which served as a control. All samples had significant biomass associated with them but were distinct in PLFA fingerprint and $\delta^{13}C$ – PLFA signatures indicating the presence of three distinct microbial communities living in association with the fracture fluids and gases. These results have implications for the potential existence of ancient deep subsurface communities that have survived geologic time in isolation, in particular with relation to the subsurface of Mars, as well as give us insight into life on the early Earth.

ACKNOWLEDGEMENTS

I would like to express my utmost gratitude to my supervisor, Dr. Greg Slater, who's mentorship and guidance has helped me become a better and real scientist through this thesis project. His caring and encouraging attitude towards his graduate students, and real want for them to succeed and grow, sets Dr. Slater above and apart from many academia. It has been my absolute pleasure to work with him over these last two years and I look forward to continuing that work through my Ph.D.

To the entirety of the Environmental Geochemistry group, past and present, I thank you. Corey, Reisa, Robbyn, and Mohammad – you have all been so supportive and I cannot thank you enough for your friendship. My most sincere thanks to Allyson Brady for her mentorship and bright attitude towards science. There are very few words that can express how grateful I am for Jennie Kirby – her seemingly endless knowledge about everything in and related to the lab and the work conducted there has been invaluable. Jennie truly is an amazing scientist, mentor and friend.

To all of my collaborators at the University of Toronto, Princeton and Waterloo, and the generous funding provided by NWMO and NSERC – thank you for your continued support.

My parents, Ronelle and Tony, cannot be thanked enough for their moral, mental and financial support throughout my academic career. Their encouragement has lead me to follow my passions and dreams and has allowed me to find my place in science. To my sister, Catherine, I'd like to thank for long, sometimes late, phone calls and encouragement when things have been hard in and outside of academic life.

Finally, to all of my friends who have encouraged me, calmed me, laughed and drank with me, or offered support, empathy or a cold dose of reality when needed – thank you from the bottom of my heart. While I can't address all of you individually I'd like to specifically thank Kailee for always listening to my dramatics and being my anchor to realism. To the girl gang, Chelsi and Anya – you gals are my rock. Thanks to Maya for teaching me about statistics and giving me a crash course in R. Many thanks to all the rest: Dylan, Micaela, Dean, Devon, Kas, Luciano, Ben, David, Patrick, Rick, Polina, and so many more. I love you all.

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RESEARCH BACKGROUND AND FUNDAMENTAL CONCEPTS

1.1 THE DEEP TERRESTRIAL SUBSURFACE BIOME

1.1.1 Introduction

The presence of a microbially dominated biome within the subsurface of Earth was first reported in 1931 and has since attracted a multidisciplinary analysis of the biochemistry and ecology of this environment (Farrel & Turner, 1931; Lipman, 1931). Interest in the terrestrial subsurface has, to date, been primarily rooted in the potential of microorganisms in bioremediation of contaminated environments, such as those polluted by synthetic halogenated organic chemicals, or uranium (Finneran et al. 2002; Lovley & Chapelle, 1995; Sen & Khilar, 2006; Sims et al. 1990). The persistent nature of many contaminants makes remediation difficult and costly. Broader academic interest includes the use of Earth's subsurface for the long-term storage of industrial and nuclear waste. Sediments can act as repositories and sinks for organic pollutants where they may become immobilized or transformed and/or activated (Martínez-Jerónimo et al. 2008; Zoumis et al. 2001). Thus, identifying the impact subsurface microbes may have on such repositories is of key interest to invested parties regarding both storage and remediation (Pedersen, 1996; White & Ringelberg 1996). Microbial production of methane from shales has also been of particular interest as methane is a long-living gas responsible for the greenhouse effect in Earth's atmosphere (Kotelnikova, 2002; Martini, et al., 2003).

The discovery of the deep terrestrial biome has come to represent a substantial proportion of the global biosphere. Initially it was estimated by Whitman et al. (1998) that the total terrestrial biosphere contained more biomass than Earth's open ocean – 2.5 to 25 x 10^{29} cells. More recently these estimates have been re-evaluated and it is more likely that the true biomass of the subsurface is likely less than previously reported, though some still place the contribution of this deep biosphere as high as 50% of global biomass (Kallmeyer et al. 2012; McMahon & Parnell, 2014; Whitman et al. 1998). Deep dwelling organisms have been identified across all domains and are known to survive despite extreme environmental conditions. Determining how microbes live under these circumstances has value in determining the ultimate limits of life and reconstructing the origin of life on Earth (Nealson et al. 2005; Stevens, 1997). Given that the most likely environments for life to still exist, or be preserved, on

other planets and moons in the solar system are in the subsurface, a better understanding of subsurface life on Earth is also a key factor in our ability to search for life beyond the Earth (Rampelotto, 2010). Deep terrestrial sites such as those in the Precambrian shields of Canada and Finland offer excellent opportunities to explore potential Martian analogue environments and allow for the creation of models that may represent how life exists on other planets today (Sherwood Lollar, et al. 2007).

1.1.2 Geochemistry and Conditions for Life

The deep subsurface of Earth is considered an extreme environment where high temperature and pressure, low oxygen and nutrient availability, and variable pH and salinity of fluids act as barriers to life (Fredrockson & Fletcher, 2001; Pedersen, 2000; Rampelotto, 2010; Rothschild & Mancinelli, 2001). The upper temperature limit for life as we know it is 122˚C, a record held by the Archaean deep sea hydrothermal vent isolate *Methanopyrus kandleri*, (Takai et al., 2008). On average, geothermal gradients in the crust have temperatures increasing with depth by 25˚C per kilometer (km), suggesting that, based on temperature alone, the subsurface is habitable as deep as 5 kilometers below surface (kmbs) (Akob & Kusel, 2011; Fredrickson & Fletcher, 2001; Pedersen, 2000). Because the extent of geothermal gradients varies by location, in places where the gradient is smaller, the temperature-based habitability of the subsurface may be deeper still (Lin et al., 2006). While barophilic organisms are known to withstand pressures up to 1000 MPa, it is more likely that a thermal boundary will limit microbial growth before hydrostatic pressure (Fredrickson & Onstott, 1996; Lammer et al., 2009; Sharma et al., 2002).

Based on our general familiarity with aerobic life the absence of oxygen is often viewed as an extreme condition, however many organisms thrive in the absence of oxygen, with some exhibiting obligate anoxic lifestyles wherein cell death occurs on exposure to oxygen. Low nutrient availability and variety also limits life in the terrestrial subsurface. As depth increases, availability of organic carbon derived from photosynthesis decreases and/or becomes increasingly recalcitrant causing organisms to utilize alternate energy and carbon sources (Pedersen, 2000). As a result of low or inaccessible nutrients cellular growth is very slow with estimated doubling times as long as hundreds to thousands of years (Chivian et al, 2008; Hoehler & Jorgensen, 2013). While growth may be slow and at low density, the fractures and fluids of the deep subsurface provide a stable ambient environment with a constant, though slow, flow of chemical energy, and protection from surface UV radiation (Rampelotto, 2010).

At several kmbs, subsurface organisms are completely isolated from the products of photosynthesis and are strictly chemotrophs and gain energy from either organic chemicals (chemoorganotrophy) or inorganic chemicals (chemolithotrophy) (Stevens, 1997). Because of this, chemolithoautotrophs would act as primary producers obtaining energy from abiotically-produced inorganics (Pedersen, 1997; Stevens & McKinley, 1995). Radiolytic hydrogen (H_2) is one possible source of inorganic energy and electrons and its oxidation can be coupled to the reduction of many terminal electron acceptors such as CO_2, SO_4^{2-} and Fe(III) (Lin et al., 2006; Pedersen, 2000). Methane (CH_4) can also be produced abiotically through water-rock interactions and mantle outgassing, and together with radiolytic H_2 may provide the basis for the deep subsurface biosphere (Chapelle et al., 2002; Fang & Zhang, 2011; Sherwood Lollar et al., 2002). By utilizing available H_2 and CO_2 subsurface microbes could utilize the reductive acetyl coenzyme A (acetyl-CoA) pathway, also known as the Wood-Ljungdahl Pathway) (Wood HG 1991). This acetogenic metabolism is summarized by equation 1.1 and results in the production of acetate (CH_3COO^-) which would be easily accessible to chemoorganotrophs. CO_2 and H_2 can also be used for autotrophic methanogenesis through the reductive acetyl-Co-A pathway, summarized in equation 1.2 which provides biogenic methane for methanotrophic organisms. In the presence of acetate, methane is also produced via the reductive acetyl-Co-A pathway (equation 1.3).

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (1.1)$$

$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \quad (1.2)$$

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (1.3)$$

Methane, whether produced abiotically or biotically, can be consumed in the absence of oxygen by anaerobic oxidation of methane (AOM) which occurs via consortia with sulfate reducing bacteria (SRB) (Boetius et al. 2000; Hinrichs et al., 1999; Hoehler et al., 1994). Sulfate reduction can occur autotrophically using H_2 as the electron donor to produce hydrogen sulfide and acetate (equation 1.4), or heterotrophically using acetate (equation 1.5) or methane (equation 1.6).

$$SO_4^{2-} + 2\ HCO_3^- + 8\ H_2 + 2\ H^+ \rightarrow CH_3COO^- + HS^- + 8\ H_2O \quad (1.4)$$

$$SO_4^{2-} + CH_3COO^- \rightarrow 2\ HCO_3^- + HS^- \quad (1.5)$$

$$SO_4^{2-} + CH_4 \rightarrow HS^- + H_2O \quad (1.6)$$

These metabolisms may represent the major members of subsurface communities; however iron-reduction, nitrogen-fixation and fermentation of amino acids, purines, pyrimidines and sugars may also be present (Krumholz, 2000; Lovley, 1997; Slater et al., 2006).

1.1.3 Previous Research on Subsurface Communities

Microbial community investigations can include direct counting of microbial cells, culturing of organisms, molecular genetic approaches and biomarker analyses. All of these approaches have their strengths and weaknesses. Cell counting can estimate cell abundances but does not provide information on identity or metabolism. Culturing can be very effective to study organisms that can be cultured, however, currently it is estimated that only 5-10% of organisms can be cultured (Hallbeck & Pedersen 2012). Molecular genetic approaches are unparalleled in their ability to identify and in some cases quantify organisms present in environmental samples. However, these approaches can be impacted by biases in primer binding specificity and amplification efficiency. It is also recognized that in very low biomass environments, amplification of background genetic material present in reagents can impact the ability to interpret results (Malik et al. 2008; Spiegelman et al. 2005).

The communities within the deep terrestrial subsurface are varied in both their taxonomy and physiology. Some communities include hydrogen-consuming, methane-producing Archaea that far out-number their bacterial companions (Chapelle et al. 2002). Many of these Archaea are novel species that have been revealed as cosmopolitan in nature, representing a large proportion of the subterranean biosphere that previously was entirely unrecognized, such as *Hadesarchaea* (Baker et al. 2016). Other organisms display novel morphotypes, such as the star-shaped bacterium discovered within mine-slimes in Northam Platinum mine, South Africa, which have adapted this morphotype as a strategy to increase their surface to volume ratio (Wanger et al. 2008). Perhaps most surprisingly, was the discovery of multicellular organisms living within the deep terrestrial subsurface such as the nematode, *Halicephalobus mephisto*, discovered thriving in high temperature fracture water, aged to 3000-12000 years old in South Africa (Borgonie et al. 2011).

The majority of deep terrestrial organisms are believed to live within fracture fluid systems where sources for energy and carbon are more easily accessed (Onstott et al., 2003). Several studies have explored fracture fluid systems within deep mines to determine habitability and characterize any

communities present. Fracture fluids from 2.8 kmbs in the Mponeng gold mine, South Africa were analyzed using 16S rRNA which revealed the presence of a very low diversity community dominated by a single phylotype of Firmicutes (Lin et al. 2006). Follow-up metagenomic analysis from this site determined that >99.9% of organisms present were the thermophilic SRB *Candidatus Desulforudis audaxviator* (Chivian et al. 2008). This organism is capable of living entirely independently from the photosphere through sulfate-reduction, carbon fixation and sporulation, in these fracture fluid systems that have a residence time up to 25 million years (Lin et al., 2006).

The discovery of single-celled organisms capable of living entirely independently in a near-axenic state, as well as the presence of deep terrestrial multicellular organisms, have large implications for the search for extraterrestrial life. These findings suggest that life on other planets or moons considered habitable only by unicellular organisms, may be able to support more complex biomes that include multicellular organisms.

1.1.4 An Astrobiological Analogue

Analogue environments are locations on Earth with environmental or geological conditions that are similar to those that currently exist or may have existed in the past on Earth, an extraterrestrial planet or moon (Osinski et al. 2006). Studies conducted in analogue sites are essential to astrobiology as they help us understand how life may have arisen, adapted and/or thrived during the Archaean Eon of Earth, and/or extraterrestrially. Analogues also allow us to test and validate techniques and necessary technology for space exploration.

It has long been accepted that Mars was likely habitable during its life history, however due to harsh surface conditions only the Martian subsurface is considered potentially habitable in the present day (Boston et al. 1992; Dartnell et al., 2007). The surface of Mars does not support the presence of liquid H2O, however the present-day Martian subsurface could house saline groundwaters in deep crustal rock fractures (Sherwood Lollar et al. 2006). The deep terrestrial subsurface contains fracture fluid systems that serve as analogues of this type of potential Martian environment. These sites include the Witwatersrand Basin, South Africa, Kidd Creek Mine, Ontario, and the Lupin and Ulu Mines, Nunavut (Léveillé 2009; Dartnell et al. 2007). Fracture fluids from these sites are either habitable, or have proven inhabited, and may have the ability to support microbial communities for 25 million years to a potential 2.64 billion years (Lin et al. 2006; Sherwood Lollar et al. 2006; Holland et al. 2013). Perhaps most compelling is the discovery of a single phylotype of the sulfate-reducer *Firmicutes* living

within saline groundwater in the Archaean metabasalt at a depth of 2.8 kilometers, with no reliance on photosynthetically derived substrates for up to 25 million years (Lin et al. 2006). This discovery is promising when considering the long-term survivability of potential low-biomass microbial communities living within the Martian deep subsurface.

1.2 PLFA ANALYSIS

1.2.1 Chemical Structure and Detection

Fatty acids are polymers of acetate and are essential components in bacterial and eukaryotic lipid bi-layers. Phospholipids consist of a phosphate group and glycerol molecule that is bonded to two hydrophobic fatty acid tails and constitute the main component in biological membranes (Eigenbrode 2007). PLFAs are a major membrane component of eukaryotes and bacteria. In bacteria, the linkage between the glycerol and fatty acids is an ester bond (Figure 1.1A), and in archaea it is an ether bond (Figure 1.1B) (Green & Scow, 2000). Lipids create monolayers or bilayers of molecules due to the interaction of the polar head group and hydrophobic tail when in solution (usually water). Both the headgroup and tails of PLFAs can vary. Headgroups vary in the functional group attached to the phosphate such as choline (phosphatidyl choline) or serine (phosphatidyl serine). Fatty acid tails vary in their total chain length, and may be completed saturated, mono- or poly-unsaturated, branched, or contain cyclic groups.

Phospholipid fatty acids (PLFA) can be detected by gas chromatography – mass spectrometry (GC – MS) after conversion to volatile fatty acid methyl esters (FAMEs). To extract PLFAs from environmental samples the modified Bligh and Dyer method is used (Bligh & Dyer, 1959; White & Ringelberg, 1998). Samples are freeze-dried to remove excess water (if necessary) followed by sonication and incubation in a solvent solution containing dicholomethane, methanol and phosphate buffer in a ratio of 1:2:0.8. The resulting total lipid extraction is separated into three fractions containing non-polar, neutral and polar molecules using silica gel chromatography (Guckert et al., 1985). Intact phospholipids are recovered from the third, polar fraction in methanol, converted to FAMEs through mild-alkaline methanolysis, and finally purified by secondary silica gel chromatography (Guckert et al. 1985). The purified FAMEs are detected and separated using GC – MS and the resulting peaks are identified and quantified.

The general structure of a GC – MS is shown in Figure 1.2. Gas chromatography has two phases, a mobile phase and a stationary phase. The mobile phase, also called the carrier gas, is an inert gas

such as helium, which functions to regulate and supply pressure to the instrument, as well as carry the analyte across the column. The separation of compounds is achieved by injecting a small volume of sample into the inlet (McNair & Miller, 2009). The stationary phase is a coating on the column and determines the selectivity and ability to separate mixtures into individual components (Abraham et al. 1999). Separation occurs based on the retention time of an individual compound which is determined by polarity, boiling point and resulting vapor pressure of the compound, as well as the flow rate of the carrier gas (Zeeuw & Luong, 2002). The column is a tube made of stainless steel or glass that comes in different dimensions with different properties (Gohlke & McLafferty, 1993; Poole 2013). There are two types of columns, packed and open tubular capillary. In this work DB5 open tubular capillary columns are used which are advantageous in their reduced column bleed that leads to better signal-to-noise ratio and thus higher sensitivity. The temperature of the column is controlled by the oven and enables sequential peak elution and separation. The gas chromatograph produces a chromatogram of the sample with peaks for each compound showing area versus retention time. An example chromatogram for *Bacillus subtilis subtilis* is shown in Figure 1.3. The mass spectrometer made up of an ion source, mass analyzer and a detector (Dawson et al., 1969). The ion source creates a beam of electrons which is applied to the neutral analyte which removes an electron resulting in a cationic free radical. The resulting ions are drawn into the mass analyzer by charged plates and separated based on their mass-to-charge ratio (*m/z).* Finally, the separated ions hit the detector and the current generated is recorded. This generates a detailed mass spectrum (Figure 1.4) that includes the intensity of each ion that are used to compare against known standards or to query numerous libraries in order to identify unknown compounds.

Once identified, FAMEs are indicated using a shorthand of "pr-A:$B^{x,y,etc.}$d" where A is the total number of carbons, B is the number of double bonds, x, y, etc. are the position(s) of those double bonds, d is either *c* for *cis* or *t* for *trans*, and pr is a prefix indicating branching or the presence of a cyclic group. Branches are shown by *i* for *iso* or *a* for *antiso* or br if the branch position is unknown. Cyclic groups are indicated with the prefix cy and the suffix of Δ^{z}d where Δ indicates a cyclic group, z indicates the position of the cyclic group, and d is either *c* for *cis* or t for *trans.* For example, hexadecenoic acid methyl ester which is shown in Figure 1.4 has a shorthand of 16:0, and a theoretical FAME with a total chain length of 17, a double bond at the 6^{th} carbon in the *trans* conformation, and a cyclic group at the ninth carbon in the *cis* conformation would have a shorthand of cy17:1^{6}t Δ^{9}c.

PLFA masses are quantified using standard curves generated from FAMEs of known chain length – C_{14}, C_{16}, C_{18}, and C_{20} in this work. The concentration of the standards is known and therefore can be correlated with the area under the peak on the chromatogram. By generating a line of best fit for a set of standards, the mass of any identified compound can be determined.

1.2.2 Biomarkers and Biomass Proxies for Extant, Viable Communities

The term biomarker was first described in the context of astrobiology by Lovelock (1965) who cited the thermodynamic improbability in the patterns of chemical structures and compositions in biologically produced molecules (Eglinton & Calvin, 1967; Eglinton, *et al* 1964; Lovelock, 1965). Although they have variable definitions, in a biogeochemical context, biomarkers are defined as a substance, object, phenomenon, pattern or temporal change that originates from some biological process or agent (Des Marais, *et al*, 2008; Meadows, 2008). The biogenic origin of biomarkers is essential for the unambiguous detection of biological activity. PLFAs are strictly biogenic and degradation is known to occur within days to weeks following cell death and thus their analysis provides insight into the extant, viable microbial community (Green & Scow, 2000, White, 1993; White et al. 1979).

Some PLFA have been cited as specific markers for certain taxonomic groups summarized by Green & Scow (2000). However, the use of PLFAs as taxonomic biomarkers has come under some scrutiny citing considerable overlap in the presence of PLFA among different groups (White et al. 1997; Green & Scow, 2000). Frostergård et al. (2011) includes an excellent discussion of the caveats associated with using PLFAs to indicate taxa and/or physiological state of organisms. Due to conflicting reports and publications on the correlation of certain PLFA to specific taxa or groups, there are only a narrow few PLFA that can reliably be used in this manner (Frostergård et al. 2011; Reuss & Chamberlain, 2010). In terms of physiological state, the ratio of certain PLFA such as cis/trans or cyclo/mono-unsaturate precursor have been used to indicate stress and starvation. In either case, these ratios have been shown to be unreliable as indicators of stress. Ratios have a large range of variability, and the presence of indicator molecules, particularly the cyclic PLFA may be present in large numbers during times when stress is not expected to be occurring (Barcenas-Moreno & Bååth, 2009; Fischer et al., 2010; Frostergård et al., 2011; Kieft et al. 1994; Kieft et al. 1997). For more extensive discussion on PLFA as taxonomic and physiological indicators as it relates to this work, see Sections 3.1 and 3.4.2.

Because the mass of PLFA in a sample is quantifiable, the amount of PLFA in picomoles (pmole) per cell can be determined if cell abundance is known. Previously several biomass conversion factors have been created and summarized by Green & scow (2000) and are listed in Table 1.1. There are many pitfalls associated with using biomass conversion factors such as variation in PLFA/cell based on physiological conditions and community make-up which can create uncertainty and error in estimating biomass of environmental samples (Frostergård & Bååth, 1996; Green & Scow, 2000; Haack et al., 1994). Overall, biomass conversion factors can be acceptably used to compare related samples within a small area but are not reliable for comparing biomass at different sites (Dobbs & Findlay, 1993; Green & Scow, 2000; White et al. 1997). Biomass conversion factors are further discussed in Section 2.1 and 2.4.3.

1.3 STABLE CARBON ($\delta^{13}C$) ISOTOPE ANALYSIS

Carbon isotope analyses of PLFA and potential carbon sources, often in the form of dissolved organic carbon (DOC) or dissolved inorganic carbon (DIC), can be used to potentially determine what microbial metabolism(s) are occurring within a natural system. This is done by comparing the $\delta^{13}C$ of PLFA to DIC, DOC and/or methane. Due to the fractionation of carbon isotopes wherein lighter isotopes, such as ^{12}C, are used preferentially by microbes the ratio between the lighter and heavier stable isotopes becomes fractionated. The ratio for carbon isotopes, $^{13}C/^{12}C$, represented as $\delta^{13}C$, is defined by equation (1.7).

$$\delta^{13}\mathrm{C}(‰) = ((^{13}\mathrm{C}/^{12}\mathrm{C}_{sample})/(^{13}\mathrm{C}/^{12}\mathrm{C}_{standard}) - 1) * 1000‰ \quad (1.7)$$

The magnitude of this kinetic isotope effect is determined by kinetic isotope effects (KIEs). The $\delta^{13}C$ of PLFA depends upon the $\delta^{13}C$ of the carbon source, KIEs associated with the carbon assimilatory pathway used, and KIEs associated with biosynthesis of PLFAs (Boschker & Middelburg, 2002; Hayes, 2001). The KIEs associated with carbon fixation pathway can result in large variability in enrichment or depletion of ^{13}C relative to the associated carbon source (Boschker & Middelburg, 2002; Londry et al. 2004; Zhang, 2002). Organisms that use the reductive tricarboxylic acid (TCA) cycle generally produce lipids that are 1.8-5.5‰ depleted compared to biomass (House et al., 2002; Zhang 2002; Zhang, et al. 2003). Those that fix carbon by the 3-hydroxypropionate (3-HP) pathway have been found to have lipids with $\delta^{13}C$ values between 0.2-3.6‰ depleted relative to biomass (House, et al., 2003). However, some organisms that utilize both the 3-HP and reductive TCA cycle have lipids that are depleted by as much as 16‰ making the determination of active metabolic

pathways difficult (Zhang, 2002). Two other metabolic pathways that produce variable fractionation are the reductive pentose phosphate (PP) cycle and the acetyl-CoA (AC) pathway. Reductive PP leads to lipids that are 5.1‰ depleted relative to biomass, whereas lipids from organisms that utilize the AC pathway may have highly depleted lipids, up to 22.7‰, though the possible range is very large: 2.7-22.7‰ (House, et al., 2003). Autotrophic sulfate-reduction can produce PLFA that are up to 58‰ more depleted than their inorganic carbon source (DIC), compared to autotrophs not utilizing sulfate-reduction that produce PLFA only a few ‰ depleted compared to DIC (Boschker & Middelburg, 2002; Londry et al., 2004). $\delta^{13}C$ of PLFA from methanotrophs is often extremely depleted relative to both $\delta^{13}C$ of methane and DIC. Biogenic methane can have values ranging from -50 to -110‰ and so the $\delta^{13}C$ value for methanotroph PLFA would be further depleted still (Freeman, et al., 1990; Zhang 2002). It is important to consider than not all pathways for bacterial metabolism are known, and even for those that are known the fractionation between PLFA and DIC is not well constrained indicating wide variation in $\delta^{13}C$ values even for the same pathway (Londry et al., 2004).

1.4 RESEARCH OBJECTIVES

The purpose of this thesis was to assess the validity of techniques used to characterize deep terrestrial subsurface microbial communities and to investigate fracture fluids from the Kidd Creek Mine for their presence. Extraction, purification, identification and quantification of PLFA and subsequent stable carbon isotope analysis were used to explore answers related to this area of inquiry.

Chapter 1 provides an introductory background to the deep terrestrial subsurface as a habitat for microbial communities. Information on the geochemistry and environmental conditions of the subsurface are included, as well as an overview of recent pertinent studies of deep-dwelling organisms and how it pertains to astrobiology were also included. This section also provided information on PLFA chemical structure and its use as a biomarker. Lastly Chapter 1 introduced stable carbon isotope analysis and how $\delta^{13}C$ signatures are used to characterize microbial communities.

Chapter 2 reports an investigation into the impact of matrix effects and the validity of biomass conversion factors. Understanding the extent to which mineral matrices effect extraction and quantification of PLFA as a biomarker for extant microbial life was the first major aim of this work and was accomplished through the creation of a matrix dilution series inoculated with *Bacillus subtilis subtilis* (ATCC 6051) of known cellular concentration. Additionally, to revealing the impact of

mineral matrices, the validity of biomass conversion factors and their role in characterizing complex microbial communities is addressed.

Chapter 3 describes the investigation of ~1.4 billion-year-old fracture fluids from two boreholes (12261 and 12299) located at ~2.3kmbs in the Kidd Creek Mine, Timmins, Ontario. Fracture waters passively filtered over 6-12 months, borehole-associated biofilms, mine service water and blanks were extracted for PLFA. Purified PLFA were converted to FAMEs, identified, quantified and analyzed for $\delta^{13}C$ to determine relative biomass, and provide taxonomic and metabolic information.

Chapter 4 summarized the major conclusions of this thesis project and outlines future directions and astrobiological implications for each project.

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1.6 TABLES AND FIGURES

Table 1.1 Summary of conversion factors for calculating biomass from lipid concentration (Adapted from Green & Scow, 2000).

Conversion Factor	Source of Data
2×10^4 cells/pmole PLFA	Balkwill et al. (1988) & White et al. (1979)
2.6×10^4 *E. coli* equivalent cells/pmole PLFA	Franzmann et al (1996)
5.9×10^4 cells/pmole PLFA	Stratford (1977) & White et al.

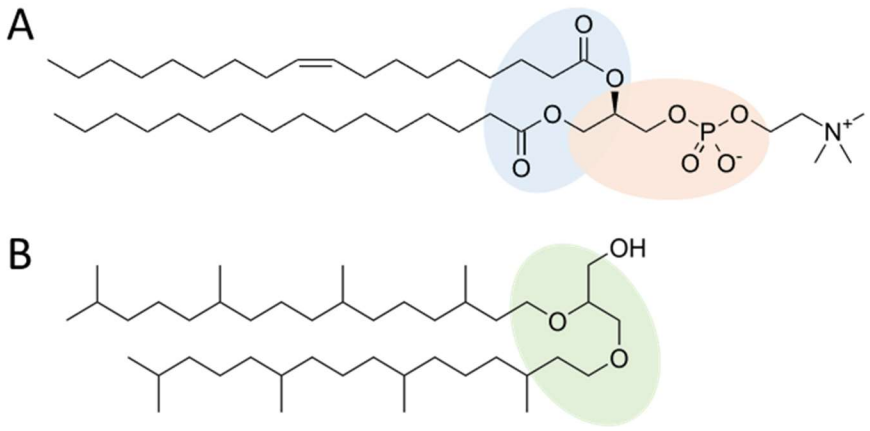

Figure 1.1 Chemical structure of membrane lipids. (A) Phospholipids such as the phosphatidyl choline (Palmitoyl-oleyl-sn-phosphatidylcholine) pictured have a phosphate-glycerol headgroup (orange) attached two fatty acid tails by ester bonds (blue). Attached to the headgroup is a functional group, in this case choline. (B) Archael lipids such as Archaeol use ether bonds (green) to connect the lipid tails to the head group. (Chemical structures taken from Wikimedia Commons https://commons.wikimedia.org/w/index.php?curid=12885201; https://commons.wikimedia.org/w/index.php?curid=9577082).

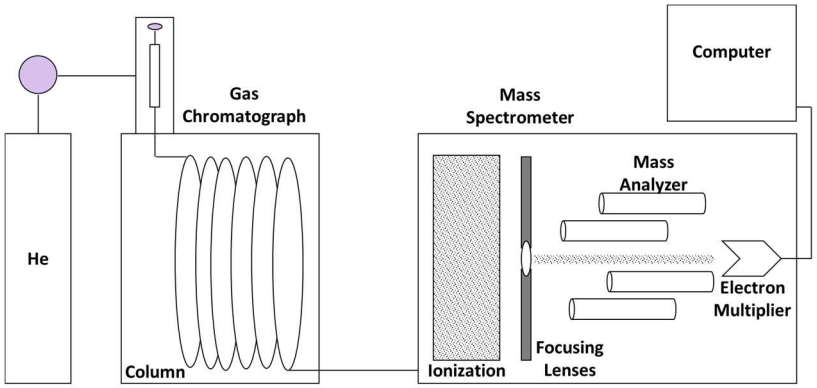

Figure 1.2 Simplified schematic of a gas chromatograph – mass spectrometer.

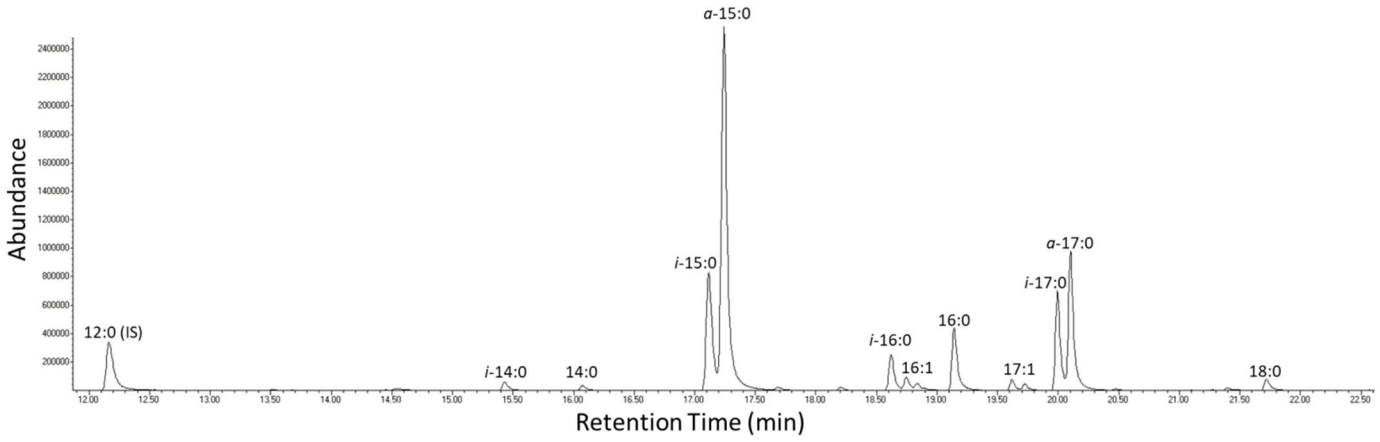

Figure 1.3 Chromatogram of *Bacillus subtilis subtilis* (ATCC 6051). Major peaks are labelled as described in the text (section 1.3.1). The peak labelled 12:0 (IS) indicates the use of an internal standard.

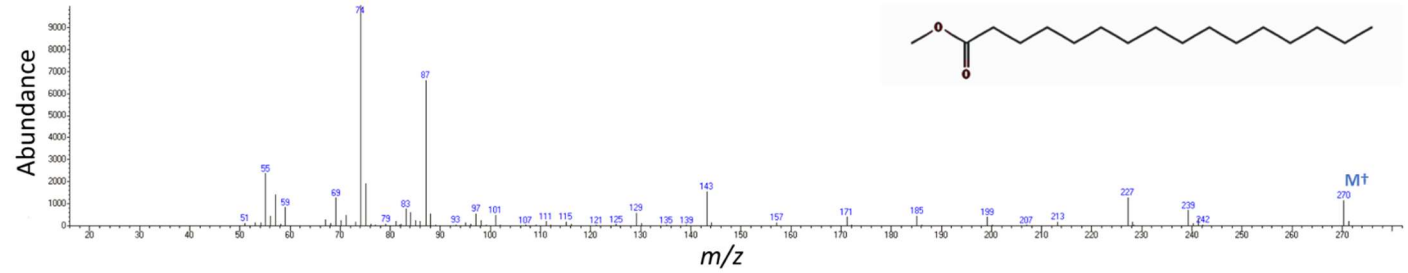

Figure 1.4 Mass spectrum of hexadecenoic acid methyl ester (16:0me). Abundance is plotted versus mass to charge ratio (*m/z)* with the total mass indicated by **M†**. The structure for 16:0me is overlaid.

CHAPTER 2

THE EFFECTS OF MINERAL MATRICES ON RECOVERY AND QUANTIFICATION OF BACTERIAL PHOSPHOLIPID FATTY ACIDS AND ASSESSMENT OF BIOMASS ABUNDANCE

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ABSTRACT

Understanding the distribution, abundances and metabolic activities of microbial life in the subsurface is fundamental to our understanding of the role microbes play in many areas of inquiry such as terrestrial biogeochemical cycling, bioremediation, and the search for extraterrestrial life. While we have made progress in investigating life in the continental subsurface in recent years, significant challenges remain. In particular, the low biomass abundance, heterogeneous distribution of biomass, and the potential for matrix effects during sampling and analysis mean that further development and optimization of methods to study subsurface life are needed.

Phospholipid fatty acids (PLFA) are a useful biosignature of extant, viable microbial communities that are applied in a wide range of environments. PLFA can provide information regarding extant biomass, temporal and/or conditional community shifts through the use of PLFA fingerprints, and metabolic activity of communities. This study had several goals. Firstly, to test the sensitivity of the modified Bligh and Dyer method of PLFA extraction with analysis by gas chromatography – mass spectrometry (GC – MS). Secondly, to determine the effects of two distinct mineral matrices, bentonite clay and crushed granite, on the extraction and quantification of PLFA. Thirdly, to analyze and comment on the validity of using biomass conversion factors derived from extracted and quantified PLFA. In order to achieve these goals Bacillus subtilis subtilis (ATCC 6051) was grown to stationary phase, and a known number of cells were extracted in pure culture or added onto either bentonite clay or crushed granite. All samples were extracted for PLFA using the mBD method and GC – MS.

All extractions yielded a linearly decreasing trend to the level of the process blank ($4.3 \times 10^6 \pm 2.5 \times 10^6$ total cells). Conversion factors generated from these experiments for the pure culture alone did not agree with previously published conversion factors, however conversion factors created from the bentonite and granite series did agree with literature values. A significant loss of extracted PLFA occurred for bentonite clay and granite samples. This loss was nearly an order of magnitude and indicates the strong presence of a mineral matrix effect which has implications with regards to detecting organisms living within such matrices.

2.1 INTRODUCTION

The biosphere of the terrestrial subsurface has gained increasing attention as its role in geochemical cycles, and potential in a variety fields and applications has become apparent. Microbial activity has been documented in many subsurface environments. The presence of microbial communities in the relatively shallow vadose-zone, and their importance in fields such as contaminant bioremediation, has been known for a long time (Brockman et al. 1992; Konopka & Turco, 1991). Much more recently, the deep continental subsurface has become an area of particular interest as these biomes and their organisms may have been isolated from the photosphere and hydrosphere for millennia (Lin et al. 2006; Onstott 2016). Some estimates place the contribution of this deep biosphere as high as 50% of global biomass (Whitman et al. 1998; McMahon & Parnell, 2014). Thus, understanding the distribution and ecology of deep-dwelling microbes is of great interest across many fields of inquiry.

Over the past three decades, terrestrial subsurface microbiology has been of considerable import in the areas of domestic and industrial contaminant remediation in groundwaters. The persistent nature of many contaminants makes remediation difficult and costly. Sediments can act as repositories and sinks for organic pollutants where they may become immobilized or transformed and/or activated (Martínez-Jerónimo et al. 2008; Zoumis et al. 2001). Microorganisms and their diverse metabolic capabilities can transform or degrade both inorganic and organic contaminants from inorganic mercury by a transgenic *Bacillus cereus* strain through volatilization and biosorption (Dash & Das, 2015), to degradation of hydrocarbons by a wide range of marine and terrestrial microbes (Ex: Himmelheber et al. 2007; a review: Perelo 2010). Additionally, the use of the deeper terrestrial subsurface as a repository for waste may also be impacted by microbial activity (White & Ringelberg 1996). How microorganisms living adjacent to repositories will impact storage has implications both in short and long-term storage of hazardous materials. By characterizing and profiling the organisms present in these systems we can better understand how microbial communities impact the areas of waste storage, and contaminant remediation and mobilization. Knowing the abundance and metabolic activities of the communities present can help predict the transformation and mobilization of contaminants, reveal new potential bioremediation techniques, and offer insight into so-called “future-proofing” storage efforts.

On a global scale, microorganisms impact key biogeochemical cycles. Their role in the production and oxidation of methane in the subsurface contributes to the global carbon cycle and greenhouse effect (Kotelnikova 2002). Calculations performed by Whitman et al. (1998) place the global carbon

content of subsurface microorganisms (5.18 x 10^{14} kg) as comparable to that of terrestrial plants (5.60 x 10^{14} kg). Subsurface microorganisms also impact the global sulfur, nitrogen, phosphorous and oxygen cycles (Anantharaman et al. 2016; Campbell et al. 2006; Hug et al. 2015; Li et al. 2016; Reinhard et al. 2017; Wankel et al. 2011).

Finally, the deep terrestrial subsurface and the characterization of the organisms therein also functions as an analogue for potential extraterrestrial biomes (Des Marais et al. 2008; Léveillé 2009). Recently near-surface organics have been detected on Mars (Eigenbrode et al. 2018), reinforcing the status of the terrestrial subsurface as an analogue for Mars, and highlighting the importance of understanding and refining how we detect, quantify and qualify these complex and cryptic microbial communities. Studies of the subsurface have significance challenges to overcome when attempting to quantify viable biomass, including: low biomass abundance, heterogeneous distribution of biomass, and sediment matrix effects.

Characterization of microbes as individuals or within a community can be accomplished through a variety of methods that can be divided into two broad types: culture-dependent and culture-independent. The former includes classical techniques such as direct and viable cell counts and culturing of environmental isolates. Culture-independent methods include molecular methods such as genetic sequencing, and biochemical methods such as characterization of intact polar lipids (IPLs) or phospholipid fatty acids (PLFA).

Culture-dependent methods rely on the ability to cultivate communities or individual isolates in a laboratory setting. Due to this reliance on culturability there is a strong bias towards organisms that can be cultured which eliminates from examination all organisms that cannot be grown in vitro. The total number of bacteria in an environmental sample outnumber those that are culturable by anywhere from one to five orders of magnitude (Amann et al. 1995; Spiegelman et al. 2005). This is especially seen in indirect cell counting methods that relies on counting colony-forming units (CFUs) which represents the number of colonies formed per unit of volume. While this technique works very well for enumerating pure cultures of bacterial isolates, it cannot give a reliable count of communities. Growth of CFUs is more often used to isolate individual organisms based on metabolism or phenotype for downstream methods such as single-colony isolate sequencing. Direct counting methods are culture-independent and advantageous as they involve the entire original environmental sample and may include all possible organisms regardless of their culturability or viability. These counting methods usually involve a fluorescent stain such as Acridine Orange (Reed et al. 2002) or DAPI (4',6-

diamidino-2-phenylindole) (Kämpfer et al. 1996; Lee et al. 2002; Sigler & Zeyer 2002; Spiegelman et al. 2005).

Molecular techniques include methods based upon the polymerase chain reaction (PCR), and direct cloning and sequencing that are independent of PCR. The use of PCR and subsequent sequencing of 16S rRNA genes is the most widely used molecular technique for characterizing environmental samples. Because PCR relies on the amplification of the 16S rRNA gene by a set of primers, primer specificity introduces bias in what organisms are amplified based on how broadly specific the chosen primer set is. Additionally, the efficiency of initial extraction and purification of the total nucleic acid content of a sample is important to ensure complete capturing of the sample. Direct sequencing, also called shotgun sequencing, involves the cloning and sequencing of the total sample without prior PCR amplification. This method avoids many of the disadvantages of other molecular methods and provides a breadth of information due to the complimentary downstream analysis that can be performed using PCR-dependent techniques or in silico analysis. For a more detailed look at the advantages and limitations of these methods see Malik et al. (2008), Spiegelman et al. (2005), and the sources therein.

Genetic analysis either by 16S rRNA or shotgun sequencing provides information regarding the identity of otherwise unculturable organisms to the species level while also potentially providing information about metabolism and other functionalities. Culture-dependent methods without downstream genetic analysis cannot provide information to the species level and offer limited knowledge on metabolic capability within a community. However, in sedimentary environments where large sample masses are needed to account for heterogeneously distributed low biomass, obtaining enough material for genetic analysis can be difficult as many molecular techniques extract nucleic acid from as little as 200 mg of sample. While small sample sizes are overcome by amplification during PCR, for shotgun sequencing the limitation of small sample size can result in non-detection. Here the complementary nature of biochemical methods such as analysis of phospholipid fatty acids (PLFA) can help overcome this limitation as well as provide complementary information (Frostegård et al. 2011). PLFA analysis can be performed on large sample masses of up to several kilograms and does provide some information on the taxa present (Green & Scow 2000).

PLFAs are biogenically produced membrane-component molecules that are extracted from environmental samples through the widely used modified Bligh & Dyer (mBD) method (Bligh & Dyer, 1959; White & Ringelberg 1998) followed by methanolysis to convert the PLFA to fatty acid

methyl esters (FAMEs) (Guckert et al. 1985). Downstream analysis of FAMEs by gas-chromatography – mass spectrometry (GC – MS) provides quantification of total FAMEs and thus PLFA, and the identification of individual FAMEs based on retention time and mass spectra relative to known standards and libraries. The presence of certain FAMEs can indicate certain classes of microbes such as the general Gram positive versus negative, to the more specific sulfate-reducing bacteria (Dowling et al. 1986; Green & Scow 2000; Kerger et al. 1988; Kohring et al. 1994; Taylor & Parkes 1983; Webster et al. 2006; White et al. 1999). By quantifying the recovered FAMEs the abundance of viable biomass can be determined using conversion factors such as those outlined and discussed in Green & Scow (2000). Quantification of biomass using PLFA conversion factors uses total environmental samples and works independently of the culturability of organisms and so has been prefered over culture-dependent methods (Tunlid & White. 1991; Wagner et al 1993, Frostegård et al. 2011). While quantification using PLFA conversion factors may eliminate the disadvantages of culture-dependent methods there are some important considerations to be made regarding the use of such conversion factors. Caution when using conversion factors has previously been advised by several groups regarding variable cellular PLFA concentration between taxa in mixed communities and in response to changing environmental conditions (Dobbs & Findley, 1993; Green & Scow, 2000; White et al., 1997; White et al. 1996). Moreover, the effects of mineral matrices on the recovery and quantification of PLFA has yet to be fully characterized.

In terms of biochemical analysis, the matrix refers to all the components present other than the analyte of interest and so the effects of this matrix on the analysis of the analyte are called matrix effects (Fitfield & Haines 2000; IUPAC 1989). The extent to which matrix effects can influence the analysis of a sample is well researched regarding the molecular analysis of nucleic acids. To minimize matrix effects on nucleic acid extraction and purification in environmental samples the evaluation and optimization of methods has been ongoing (e.g. Direito et al. 2012; Martin-Laurent et al. 2001; Miller et al. 1999; Nacham et al. 2016). Management of matrix effects has also been noticed for biological matrices such as blood, urine or saliva (e.g. Chiu et al. 2010). The extent to which mineral matrix effects influence analysis of PLFA has been characterized by only a few other studies and is largely limited to soils with a focus on methodology rather than soil or sediment type (e.g. Akondi et al. 2017; Wu et al. 2009; Petersen & Klug 1994). Currently the full extent to which mineral matrix effects impact analysis using PLFA, particularly in deep terrestrial sediments, is unknown. Due to this there exists the potential for error in biomass estimations due to reduced PLFA extraction and recovery

caused by mineral matrix effects. When evaluating and characterizing the abundance and influence of a given biome such sources of error can cause gross misestimations in final analysis.

It is the primary aim of this work to determine the effect of mineral matrices on the extraction of PLFA using the mBD method (Bligh & Dyer, 1959; White & Ringelberg 1998) and to subsequently evaluate the validity of biomass conversion factors and provide recommendation on their use and development.

2.2 METHODS

2.2.1 Dilution Series and Matrix Preparation

Bacillus subtilis subspecies subtilis (ATCC 6051, Lot# 61429838) was obtained from ATCC as a freeze-dried powder and revived according to supplier's direction. Bacteria were grown at 30˚C with shaking to maximum culture density which was determined through optical density generated growth curves. To create the dilution series 300 mL of bulk culture was grown overnight at 37˚C, then diluted step-wise. Cellular concentration of the bulk culture was determined in two ways. Firstly, plate counting for CFU was done by plating 100µL of diluted culture in triplicate for two dilutions and incubated overnight at 30˚C. Individual colonies were counted and the CFUs per millilitre of culture was determined. Secondly, because so-called non-CFU cells and dead but not degraded cells act as a source of PLFA, direct microscopic cell counts using the impermeable dye 0.5% Trypan Blue (Gibco, Thermo Fisher Scientific, Lot# 1835251) was also used, with enumeration using a Bright-Line Hemacytometer (Hausser Scientific, Horsham PA, USA) and Leica DM5000 B binocular microscope with Retiga EXi Fast 1394 camera. Finally, a combined total cell count was taken using these two methods. This single, stock culture was used to create a dilution series with the following cellular concentrations was used: 6.1×10^6, 6.1×10^5, 6.1×10^4, 6.1×10^3 cells/mL. The first dilution was created by diluting 7 mL in 693 mL of BD Difco dehydrated culture media LB media (Fisher Scientific, Thermo Fisher Scientific; Sigma-Aldrich), and the subsequent three dilution steps were made by diluting 70 mL in 630 mL of LB media, to create the concentration series shown in Table 2.1. The resulting, large volume, quantified, culture dilution series was used to inoculate the entire matrix series described below.

The mineral matrices chosen were bentonite clay (Volclay, MX80, Caldic Canada, Mississauga, ON, Canada) and granite (Grimsel, Switzerland). The granite was crushed using solvent rinsed stainless-steel mortar and pestle and sieved to 1mm; the bentonite clay was pre-crushed by the

supplier. Both minerals were extracted for PLFA using the modified Bligh & Dyer (mBD) method (Bligh & Dyer, 1959; White & Ringelberg 1998) and then combusted in a Thermolyne 30400 furnace at 450˚C for 8 hours. Using these two matrices and pure culture without a matrix, three sets of samples were created by aliquoting 50 mL of culture into precombusted glass jars, such that each glass jar for a given culture dilution received the same volume of culture from the same source, and thus the same number of cells. Three series were created in this way. The Pure Culture contained only the 50 mL aliquots of culture dilution; Bentonite contained 50 mL aliquots of culture dilution and 50.5 g (±0.58 g) of bentonite clay; Granite contained 50 mL aliquots of culture dilution and 50.5 g (±0.58 g) of granite. All samples in each series were inoculated with culture simultaneously and immediately frozen at -80˚C. Once frozen, all samples were freeze-dried using a Virtis 4KBTXL-105 for 48 hours. During freeze-drying sample jars were placed in sterilized bags and packed with crushed dry ice in order to prevent the loss of samples due to vigorous evaporation or sublimation.

2.2.2 Lipid Extraction and Quantification

All samples were extracted for PLFA by mBD with conversion to fatty acid methyl esters (FAMEs) by methanolysis (Guckert et al. 1985). The recovered FAMEs were separated using gas chromatography – mass spectrometry (GC – MS) with an Agilent GC – MS instrument (Agilent Technologies INC., Santa Clara, California, USA). FAMEs were separated on a DB5-ms+DG column (30 m, 0.25 mm, 0.25 μm) using a GC temperature program of 50˚C (1 min), 10˚C min-1 to 160˚C, 1.5˚C min-1 to 180˚C (10 min), 1.5˚C min-1 to 250˚C, 20˚C min-1 to 320˚C. FAME identity was determined using retention time and mass spectra of standards (Bacterial Acid Mehyl Esters Mix, Matreya Inc., Pleasant Gap, Pennsylvania, USA). FAMEs were named as follows – number of carbons:number of double bonds. Iso- and anteiso- are denoted by i- or a- respectively. PLFAs with branches are denoted with br- as exact position of branches was not determined as this level of resolution was not required. Double bond position was determined through retention time alone. All quantifiable peaks were summed to calculate the total quantity of FAMEs, and thus PLFA, present in each replicate of each series, and the resulting total FAMEs (pmol) was converted to cells/g of sample using conversion factors given by Green & Scow (2000).

2.3 RESULTS

2.3.1 Limit of Detection

The total picomoles of PLFA obtained for each sample in the three experimental series are shown versus total cells added to the culture (Figure 2.1A) and versus cellular abundances per gram of matrix or mL of media (Fig 1B). The detection limit determined by analysis of process blanks for this study was 765 pmole PLFA (or 7.6 pmole on column) and is shown in Figure 2.1 by the horizontal blue line. This value can be converted to find a cellular equivalent value which is $4.3 \times 10^6 \pm 2.5 \times 10^6$ total cells (Figure 2.1 A), or $8.6 \times 10^4 \pm 4.9 \times 10^4$ cells/g of sample (Figure 2.1 B).

This detection limit was used to determine which dilutions in the pure culture, bentonite and granite series could be considered reliable. Based on the intersection of the scatter plot lines for each series shown in Figure 2.1 only points that fall above this line are considered for further analysis. For all series the samples for 3.1×10^8 total cells and 3.1×10^7 total cells were used in further analysis as they fell above the limit of detection. In the granite series a third sample dilution (3.1×10^6 total cells) also fell above the detection limit threshold, however it was determined that a confounding error took place in the processing of this sample and so it is unreliable and will be excluded.

2.3.2 FAME Recovery and Conversion Factors

Because the initial concentration of cells added was known, the quantified total FAME (pmol) was used to create a set of conversion factors for comparison through equation (1).

$$\frac{\text{Initial cellular concentration } \left(\frac{\text{cells}}{\text{mL}}\right) * \text{culture volume(mL)}}{\text{Mass of PLFA (pmole)}} \quad (1)$$

Biomass conversion factors were calculated for all dilutions in all three series, however only those dilutions that were above the instrument limit of detection were considered reliable and used going forward. These conversion factors are listed in Table 2.2 as Experimental Conversion Factors. The conversion factor for the pure culture series was $5.6 \times 10^3 \pm 6.0 \times 10^2$ cells/pmol$_{PLFA}$ which is an order of magnitude smaller than the factors previously reported (2.0 to 5.9×10^4 cells/pmol$_{PLFA}$) in the literature and summarized by Green & Scow (2000). The difference in the conversion factor generated from the pure culture in this study implies that there is nearly an order of magnitude more PLFA per cell in this experiment compared to the literature values. The conversion factor for the bentonite series

is $3.6x10^4 \pm 4.8 \times 10^3$ cells/pmol$_{PLFA}$, and for the granite series is $1.7 \times 10^4 \pm 6.1 \times 10^3$cells/pmol$_{PLFA}$. These values both agree with the conversion factors previously published.

A second way to calculate biomass conversion factors from a dilution series is through equations generated from lines of best fit. These conversion factors are listed in Table 2.2 as Linear Conversion Factors. Linear conversion factors were calculated using the two dilutions above the limit of detection, and the value of the limit of detection. The equations of the line are reported in Table 2.4 with their R^2 values. All equations had R^2 values that were >0.99 indicating their reliability. The rearranged equations for the pure culture (2), bentonite (3), and granite (4), are found below where y is the mass of FAME recovered in pmole, and x is the total cells in the sample.

$$x = \frac{y}{2*10^{-4}} - 658.14 \quad (2)$$

$$x = \frac{y}{2*10^{-5}} + 482.49 \quad (3)$$

$$x = \frac{y}{4*10^{-5}} + 1055.5 \quad (4)$$

These equations give conversion factors of 4.3×10^3 cells/pmole$_{FAME}$ for pure culture, 5.1×10^4 cells/pmole$_{FAME}$ for bentonite, and 2.6×10^4 cells/pmole$_{FAME}$ for granite. These Linear Conversion Factors agree with the Experimental Conversion Factors within one standard deviation for pure culture, and two standard deviations for bentonite and granite.

2.3.3 PLFA Distribution

Previous work characterizing the PLFA of Bacillus sp. has been performed by Kaneda (1967 & 1968). In his 1967 work he lists 8 major PLFA for Bacillus subtilis ATCC 6051 (B-770 in that publication), two saturates (14:0 and 16:0) and six branched (i-14:0, i-15:0, a-15:0, i-16:0, i-17:0, a-17:0, and a-17:0) (Kaneda 1967). The distribution observed in Figure 2.2 of this work is consistent with these findings however additional PLFA were observed including two saturates (17:0 and 18:0) and two classes of mono-unsaturates (16:1 and 17:1), the bond positions of which were not determined. The detection of additional peaks is likely due to differential peak separation based on the type of capillary column and temperature program used, as well as lower detection limit and better resolution expected from a more modern GC- MS instrument.

The pattern of PLFA distribution across all samples (Figure 2.2) is generally consistent, however, variations do occur and in some cases there are losses or gains of some components that are present at

low abundances. For example, samples in the bentonite series lacked monounsaturated 16-carbon lipids (16:1) however even in the pure culture, these combined C16:1 peaks only account for 1.3% of total lipid present (0.2% for the granite series). All other lipids absent from one or both matrices are present in the pure culture series in very low abundances (>2%) and it is therefore believed that absence of lipids in the bentonite and granite series' is due solely to the loss of low abundance peaks associated with reduced PLFA recovery and not due to unique properties of the matrices themselves. The cause of the outlier distribution of the second granite point is unknown.

2.4 DISCUSSION

2.4.1 Influence of Cell Size on Conversion Factors

The discrepancy between the conversion factor calculated here and those cited in the literature can be partially explained due to the size difference in organisms used. Escherichia coli was the organism used to create the conversion factors from Franzmann et al. (1996), Stratford (1977), and White et al. (1979). B. subtilis has a surface area circa. six times larger than E. coli as calculated from the published morphology on these two organisms and summarized in Table 2.3 (Henriques et al. 1998; Jones et al 2001; Kubitschek 1990; Reshes et al. 2008; Yu et al. 2014). Cell length and width for either species are reported widely and are reliant on growth conditions and strain type, so average whole number values are used here as representative of an average bacterium. Average volume was calculated using the formula described by Volkmer & Heinemann (2011) and shown below as equation 5, where r is radius, h is length.

$$V = (\pi r^2 + h) + (\frac{4}{3}\pi r^3) \quad (5)$$

This equation was created by combining the volume of a cylinder and the volume of two half spheres, so accordingly a formula for surface area was also created in this way but combining the surface area for only the body of a cylinder and the surface area of a sphere. This equation is shown below as equation (6).

$$SA = 2\pi rh + 4\pi r^2 \quad (6)$$

These results imply that the presence of organisms of different sizes, whether due to species variation or oligotrophic conditions, can cause large errors in the estimation of cellular abundances. This is not surprising since variation in cell size between organisms is well documented, as is change in size as a response to environment.

2.4.2 Matrix Effects

When comparing the pure culture series to either bentonite or granite, a significant reduction in extracted PLFA can be seen (Table 2.2). For the bentonite series this reduction was between 79% and 88%, and between 43% and 78% for the granite series. Because all cells originated from the same culture dilution, the possibility that this is due to errors in the creation of the dilution series, or differences in culture health, is eliminated. These results suggest that matrix effects can induce large, variable errors in the estimation of cellular abundances from PLFA concentrations potentially of an order of magnitude.

The extracellular matrix (ECM) is a component of bacterial communities secreted by cells to provide biochemical and structural support, such as adhesion to surfaces. It could be speculated that the presence of significant ECM may contribute to the matrix effects seen in this study, however two points argue against this. Firstly, the methodology of culture growth and series preparation would reduce the presence of ECM. The cells added to each series were grown from the same shaken culture. Shaking cultures during incubation discourages the formation of significant ECM production. Diluting the stock culture would further reduce the ECM present. Additionally, because cultures used in this experiment were not afforded time to grow on either the granite or bentonite, no additional ECM would be produced prior to extraction. Secondly, the dichloromethane-methanol-based initial extraction used in the mBD method would be expdcted to completely break down any ECM present. This considered, only those interactions between the cells and the matrix that could happen within minutes would be accountable.

Because of the polar nature of the head groups on intact phospholipids (IPLs) such as phospholipids, it is possible that the interaction between the head groups and the mineral matrices is responsible for the reduced recovery of PLFA. This idea is based on the known adsorption of nucleic acids to mineral surfaces such as clays (e.g. Direito et al. 2012; Novinscak & Filion 2011), and perceived similarity between nucleic acids and IPLs in terms of polarity. Polarity of biomolecules can cause adsorption to complex and charges mineral surfaces and may contribute to the retention of PLFA on mineral matrices. The final potential source of mineral matrix effects is entrapment within the pore space of the sediment. Pore space could either entrap whole bacteria resulting in their exclusion from cell lysis and thus extraction, or pores could entrap total cell extract after cellular lysis. Either scenario would result in reduced PLFA recovery. Because matrix effects by their nature are highly complex, it is impossible to determine all factors, and the extent of those factors, that contribute to a total mineral

matrix effect. To reduce mineral matrix effects, optimization of PLFA methodology to reduce total effects is required. This may include variable solvent ratios, longer initial extraction times, or more vigorous agitation during initial extraction.

2.4.3 Validity of Conversion Factors as a Measure of Biomass

The validity of conversion factors has been discussed at length previously (Dobbs & Findley, 1993; Frostegård et al. 2011; Green & Scow, 2000; White et al., 1997; White et al. 1996), however their use is still widespread in the literature for estimating biomass. Green & Scow (2000) included a summary and discussion of conversion factors for counting bacterial concentration from PLFA concentration (Table 1 in that publication). However, the source of some of the values could not be easily confirmed with the published data and literature. The conversion factor 5.9 x 10^4 cells/$pmol_{PLFA}$ is reported to have been created by utilizing information from two sources. The dry weight of E. coli was apparently obtained from Stratford (1997) however this publication contains no stated dry weight for E. coli. The second paper cited, White et al. 1979, does contain a value for µmol PLFA/g dry weight however without a cellular equivalent for this dry weight, it is unclear how this conversion factor was created. The paper cited as the source of the methods (White & Frerman 1967) uses absorbance (750 mµ) versus the dry weight (mg/mL) of Staphylococcus aureus, however a subsequent publication by Bratbak & Dundas (1984) determined that the previously accepted standard value of 20% dry-matter content for bacteria was too low by about half and indicate a dry-matter content of 31 to 57%. Considering this, it calls into question the continued validity of conversion factors which were produced nearly 40 years ago and have since not been updated or revisited in depth. Additionally, it has also been demonstrated that the number of cells per gram of dry weight can vary by as much as an order of magnitude (Findlay & Dobbs 1993).

When attempting to create conversion factors it is important that all elements come from the same experiment as is the case in the conversion factor created by Franzmann et al. (1996). The conversion factor 2.6 x 10^4 cells/$pmol_{FAME}$ is stipulated as stationary phase E. coli equivalent cells (SPEE cells) indicating that the biomass converted in this way is equivalent to the cell type and stage of the organism used to create the conversion factor. This allows for the diversity of microbial species present in environmental samples to be reliably compared between samples for the same environment or study.

Poly--β-hydroxyalkanoate (PHA) is an endogenous storage lipid that is produced by many bacteria during periods of suboptimal growth and, unlike PLFA, PHA is not converted to fatty acid methyl esters during methanolysis. Because lab-cultured organisms are often grown optimally it is expected that the ratio of PLFA to PHA will be high in comparison to organisms found within limiting environments, such as the deep subsurface biosphere (Nickels et al. 1979; Findlay & White 1983; White et al. 1996; Zinn et al. 2011). It is interesting to note that in their paper Haldeman et al. (1995) create a conversion factor of 2.11 x 10^4 cells/pmole using data published by Balkwill et al. (1988) and remark that it was lower than those conversion factors calculated from surface organisms or pure cultures. The latter mentioned conversion factors, or the method by which this conversion factor was created, are not included in Haldeman et al. (1995) and so the degree of disparity between them and the Balkwill conversion factor, and therefore the validity of that claim, cannot be specifically commented on. White et al. (1996) have also remarked that the relationship between PLFA and number of cells varies by a factor of at least 4 for a wide variety of environmental samples. This work agrees with this, as indicated by the order of magnitude difference in the conversion factors reported here, and those in the previously published literature. This is important when considering using PLFA conversion factors to estimate biomass from environmental samples as growth conditions are likely to be unbalanced. The potential also exists for misestimating the total contribution of Bacteria and Eukarya to a mixed community that may also contain Archaea.

2.5 CONCLUSION

In conclusion, these results emphasize that caution is needed when converting from recovered PLFA to cellular abundance using historical conversion factors, in particular for solid samples. Variations in species present, nutrient limitations and/or matrix materials can have large potentially order of magnitude impacts on PLFA recovered per cell. Consequently, this may have large and unpredictable impacts on estimated biomass present in environmental samples and thus their contribution to local and global geochemical cycles and biospheres.

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2.7 TABLES AND FIGURES

Table 2.1 Summary of dilution series cellular concentrations

Dilution Factor	Cells/mL	Total Cells Added
1	6.1E+08	3.1E+10
10^{-2}	6.1E+06	3.1E+08
10^{-3}	6.1E+05	3.1E+07
10^{-4}	6.1E+04	3.1E+06
10^{-5}	6.1E+03	3.1E+05

Table 2.2 Data summary of recovered FAMEs and resulting conversion factors for cells/pmol$_{FAME}$

Sample	Pure Culture		Bentonite		Granite	
Total Cells Added	3.1×10^8	3.1×10^7	3.1×10^8	3.1×10^7	3.1×10^8	3.1×10^7
Total FAME (pmole)	6.2×10^4	5.0×10^3	7.5×10^3	9.8×10^2	1.3×10^4	2.8×10^3
% pmol$_{FAME}$ Loss	NA	NA	88	80	79	44
Individual Dilution Conversion Factor (cells/pmole$_{FAME}$)	5.0×10^3	6.2×10^3	4.1×10^4	3.1×10^4	2.3×10^4	1.1×10^4
Experimental Conversion Factor (cells/pmole$_{FAME}$)	5.6×10^3 ± 6.0×10^2		3.6×10^4 ± 4.8×10^3		1.7×10^4 ± 6.1×10^3	
Linear Conversion Factor (cells/pmole$_{FAME}$)	4.3×10^3		5.1×10^3		2.6×10^3	
Literature Conversion Factors (cells/pmole$_{FAME}$)						
Balwill *et al.* 1988	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4
Franzmann *et al* 1996	2.6×10^4	2.6×10^4	2.6×10^4	2.6×10^4	2.6×10^4	2.6×10^4
Stratford 1977; White *et al* 1979	5.9×10^4	5.9×10^4	5.9×10^4	5.9×10^4	5.9×10^4	5.9×10^4

Table 2.3 Cell size and morphology comparison between *Escherichia coli* and *Bacillus subtilis.*

	Escherichia coli	*Bacillus subtilis*
Length (µm)	2.0	7.0
Width (µm)	0.5	1.0
Radius (µm)	0.3	0.5
Volume (µm^3)	2.3	8.3
Surface Area (µm^2)	3.9	25.1

Table 2.4 Linear equations used to create biomass conversion factors.		
Series	Equation of the Line	R^2
Pure Culture	$y = 2*10^{-4}x-658.14$	0.9997
Bentonite	$y = 2*10^{-5}x +482.49$	0.9974
Granite	$y = 4*10^{-5}x +1055.5$	0.9947

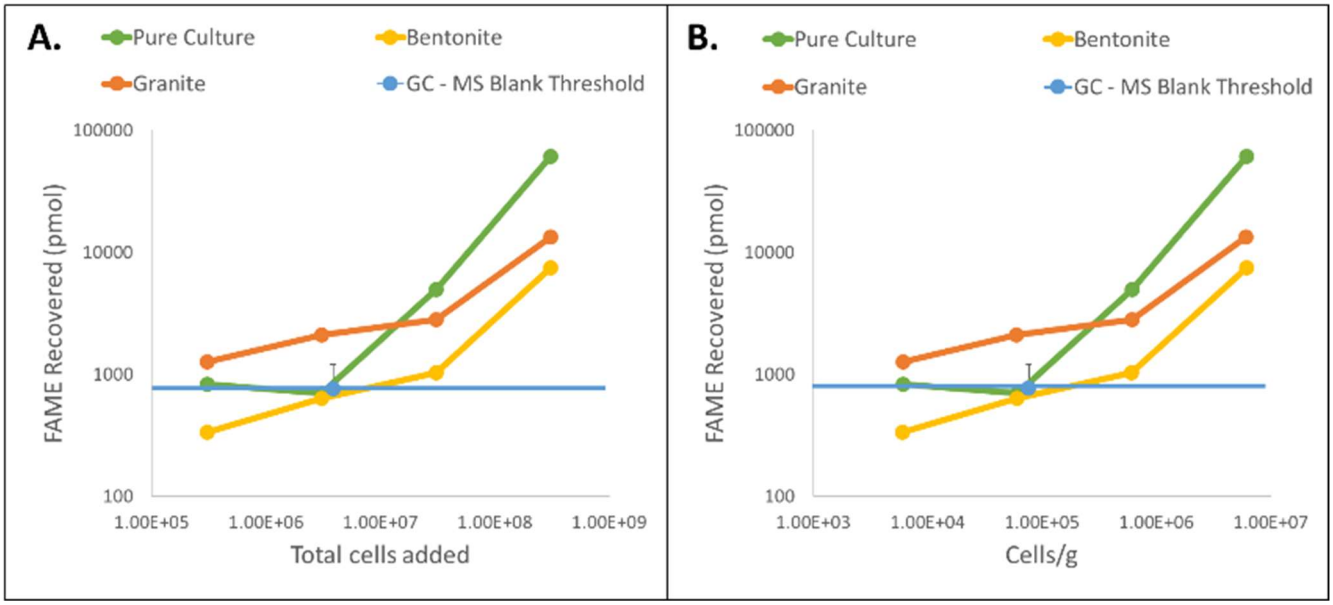

Figure 2.1 The recovered total FAMEs (pmol) from *Bascillus subtilis subtilis* per cell (A) and, per gram of doped matrix or mL of pure culture (B). Detection limits were $4.3 \times 10^6 \pm 2.5 \times 10^6$ total cells, or $8.6 \times 10^4 \pm 4.9 \times 10^4$ cells/g.

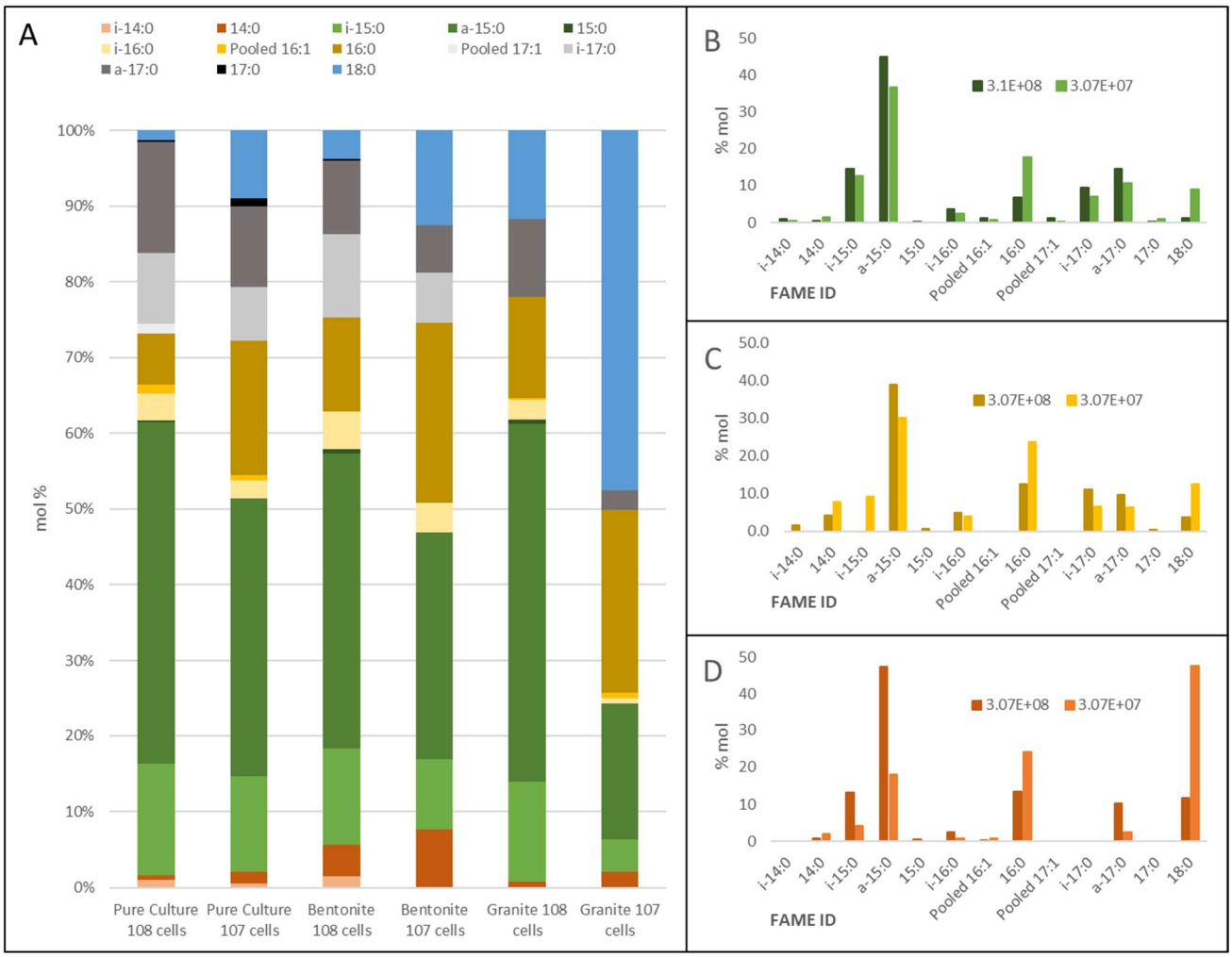

Figure 2.2 PLFA distributions (mole %) for each experimental treatment. Comparison of PLFA pattern is consistent within functional replicates of each series and between different series (A). Series One (pure culture) (B) and Series Two (bentonite clay) (C) have consistent distributions of individual PLFA. Replicate one (3.07×10^8 cells) for Series Three is consistent with Series One and Two, but replicate two (3.07×10^7 cells) appears to deviate from the normal distribution (D).

CHAPTER 3

PLFA FROM MICROBIAL COMMUNITIES ASSOCIATED WITH FRACTURE WATERS WITHIN THE PRECAMBRIAN DEEP SUBSURFACE.

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ABSTRACT

The deep terrestrial subsurface is known to harbor microbial life at depths of up to several kilometers where, in some cases, organisms live independently from the photosphere and atmosphere. Ancient fracture fluids trapped within the crystalline basement of the Canadian Precambrian Shield have been shown to be preserved on geologic timescales (millions to billions of years). To characterize the PLFA from microbial communities associated with the fracture fluids from Kidd Creek Mine, in Timmins, Ontario, large volumes of water from two boreholes, 12261 and 12299, were passively filtered for 6-12 months to collect microbial biomass.

Membrane component phospholipid fatty acids (PLFA) representative of viable microbial cells were extracted and analyzed by gas chromatography – mass spectroscopy (GC – MS) and gas chromatography-isotope ratio mass spectroscopy (GC-IRMS). Abundant PLFA were present in all samples, indicating the presence of extant microbial communities. The geochemistry of the porewaters from each borehole were similar and are thought to be sourced from the same fracture system. However, while PLFA distributions were identical between replicates of each borehole, they were distinct between boreholes suggesting differences in the microbial communities detected. Individual PLFA biomarkers indicated the presence of sulphate-reducing bacteria associated with 12261, consistent with recent MPN results for this borehole, but this marker was absent from 12299. Carbon isotope analysis of lipids for 12261 shows heavier $\delta^{13}C$ values for all PLFA, which was not observed for 12299, indicating the utilization of different metabolic pathways. Currently it is not known whether this difference is representative of microbial communities living within the fracture system, or a result of differential growth of communities within the filters over their deployment. Molecular genetic analysis is ongoing and will provide additional complementary insight into the community composition. Understanding the structure and function of microbial communities associated with fracture waters in Precambrian rock environments has the potential to provide new insights into the capabilities, limits and evolution of life.

3.1 INTRODUCTION

The deep terrestrial subsurface of Earth is known to harbor microbial life at depths of up to several kilometers (Onstott et al. 2003; Lin et al. 2006a; Stevens & McKinley 1995). Organisms that live in these isolated biomes do so independently of the photosphere and atmosphere and are often subject to extreme conditions such as high temperature, pressure, salinity, and high or low pH, and additionally they are often limited by nutrient availability. Despite this, many deep subsurface communities are thought to have survived over geological timescales (Fredrickson & Balkwill 2006.) One such example is the 25-million-year-old deep fluid systems found within a gold mine in South Africa that are host to an extant chemolithoautotrophic microbial population dominated by a single species of Firmicutes phylotype (Lin et al. 2006a; Lin et al. 2006b; Lippmann et al. 2003). In this system it is thought that the Firmicutes was introduced into the system at the time of fluid infiltration between 3 and 25 million years ago (Mya), and has been and will continue to be supported indefinitely by geological processes alone. Determining how these organisms survive in these extreme environments offers value in a variety of fields including (but not limited to) the reconstruction of early, pre-photosynthetic life, potential impact of these communities on the industrial storage of waste (White & Ringelberg 1996), and how life beyond our planet may survive in seemingly inhospitable conditions (Boston et al. 1992; Chapelle et al. 2002; Rampelotto 2010).

Studying microbial communities within the Earth's deep continental subsurface is a highly challenging undertaking. Collection of samples from the continental subsurface generally requires drilling of boreholes, either from the surface or, more cost effectively, starting at depth. Access to the continental subsurface is therefore most effectively achieved via collaboration with mining operations that are accessing deep ore deposits. However, a concern in such environments is that mining activities can have impacts on samples collected, potentially involving introduction of microorganisms via transportation or water utilization/dewatering activities within the mine. These impacts are exacerbated by the fact that biomass in the continental subsurface is generally very low. It is therefore important to bring as many complimentary perspectives as possible to bear when characterizing continental subsurface microbial communities. This can include analysis of contextual parameters such as water and rock geochemistry, as well as multiple perspectives on the microbial communities themselves.

Microbial community investigations can include direct counting of microbial cells, culturing of organisms, molecular genetic approaches and biomarker analyses. All of these approaches have their

strengths and weaknesses. Cell counting can estimate cell abundances, but does not provide information on identity or metabolism. Culturing can be very effective to study organisms that can be cultured, however, currently it is estimated that only 5-10% of organisms can be cultured (Hallbeck & Pedersen 2012). Molecular genetic approaches are unparalleled in their ability to identify and in some cases quantify organisms present in environmental samples. However, these approaches can be impacted by biases in primer binding specificity and amplification efficiency. It is also recognized that in very low biomass environments, amplification of background genetic material present in reagents can impact the ability to interpret results (Malik et al. 2008; Spiegelman et al. 2005). Membrane component phospholipid fatty acids (PLFA) are often used as a biomarker for viable, extant microbes and can provide information about total biomass, community composition and metabolic activity (Green & Scow 2000; Vestal & White 1989). PLFA analysis thus provides distinct, but highly complementary insights to that which can be obtained from molecular genetic techniques.

PLFAs are a major membrane component of eukaryotes and bacteria. Degradation of PLFAs is known to occur within days to weeks following cell death and thus their analysis provides insight into the extant, viable microbial community (Green & Scow, 2000, White, 1993; White et al. 1979). The quantification of total PLFA can be used to determine total viable biomass using previously published conversion factors (Green & Scow, 2000 and citations therein), however caution is advised when utilizing general biomass conversion factors due to the heterogeneous nature of environmental communities, including species specific cell morphology (Chapter 2). Despite this, when used to compare between samples of the same site, this type of cellular enumeration can provide insight into environmental or temporal influences on cellular abundance (Green & Scow, 2000).

The use of PLFA as a biomarker provides important information when characterizing deep subsurface microbial communities. By treating an entire distribution of PLFA as representative of a given community it acts as a fingerprint or "snapshot" of that community at the point of sampling (Bossio & Scow, 1998; Zelles & Bai 1994). By utilizing multivariate statistics, such as principle component analysis (PCA), patterns can be revealed which indicate shifts in the microbial community the fingerprint represents (Bossio et al. 1998; Green & Scow, 2000; Ludvigsen et al. 1997; Sinha & Rao 2017). The chemical structure of PLFAs can be used to determine microbial community makeup and to identify potential sources of stress. Specific PLFA structures have been found to be unique to certain microbial groups and may serve as biomarkers for specific groups (Green & Scow, 2000). For example, C18:1^{9} fatty acid was previously associated only with Eukaryotes but has been shown as an

abundant constituent of *Planctomycetales*, which include annamox genera, and is considered diagnostic when seen with 3-hydroxy-fatty acids (Kerger et al. 1988). As a response to stress some microbes produce certain PLFAs such as cyclopropyl fatty acids the ratio of which to their monoenoic precursor has been used to indicate stress (Kieft et al. 1994). However, this ratio can vary greatly and sometimes on the order of several magnitudes, with substantial cyclopropyl fatty acid content present in communities that are not under stress (Kohring et al. 1994; Green & Scow, 2000).

An additional advantage of PLFA based approaches is the ability to utilize isotopic analysis (^{13}C, ^{14}C) to elucidate the carbon sources and metabolisms supporting the microbial community. Determining the $\delta^{13}C$ of PLFA, methane (CH_4), dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) can yield additional insight into microbial carbon sources and metabolic pathways. The $\delta^{13}C$ value for PLFA varies with the carbon source that is being utilized and differs from the $\delta^{13}C$ of the carbon source due to kinetic isotope effects (KIEs) associated with biological carbon uptake and biosynthesis of PLFA (Boschker & Middelburg, 2002). If the carbon source is a small molecule, such as carbon dioxide (CO_2) or methane the associated KIEs are often large (Hayes, 2001). Methane as a carbon sources is often highly depleted relative to other carbon sources (Boschker & Middelburg, 2002). This causes the corresponding PLFA to be distinctly negative with $\delta^{13}C$ values that are 10-30‰ more depleted than the methane (Jahnke et al. 1999; Summons et al. 1994; Valentine & Reeburgh, 2000; Whiticar, 1999). This makes methanotrophy one of the easiest metabolic activities to identify in environmental samples, but the magnitude of fractionation seen in methanotrophs depends on the carbon fixation pathway involved (Londry et al. 2004). Autotrophic metabolisms have highly variable associated KIEs, up to 58‰, much like methanotrophy, due to the fixation of C1 metabolites (Brocks & Pearson, 2005; Hayes 2001). Heterotrophic pathways produce much smaller KIEs, and the general principle of "you are what you eat plus 1‰" tends to hold true, particularly for aerobic heterotrophy where the $\delta^{13}C$ values of the biomass are often no more than 4-6‰ depleted compared to the $\delta^{13}C$ of the organic carbon source utilized (Boschker & Middelburg, 2002). In anaerobic heterotrophy the difference between $\delta^{13}C$ of the biomass and the carbon source is much more variable and thus makes its detection more difficult (Boschker & Middelburg, 2002; Teece et al. 1999). Previous analysis of subsurface samples utilizing this combined analysis have demonstrated the use and cycling of microbial carbon sources in subsurface communities (Simkus et al. 2016) and have revealed the metabolic capability and interactions of microorganisms dwelling in deep subsurface fracture fluids (Onstott et al. 2007; Lau et al. 2016).

The Kidd Creek Mine is located within the Canadian Shield in Timmins, Ontario, Canada. The geology of the mine has been described previously, most recently by Li et al. (2016) and references therein. Notably, fracture fluid at some sites within the Kidd Creek Mine has a minimum mean residence time (MRT) of 1.1 to 1.7 billion years (Ga) and flows from boreholes at rates of up to a liter per minute (Holland et al. 2013; Warr et al. 2018). Accompanying the fluid is significant gas flow which includes abiogenic methane (CH_4) formed by polymerization of methane precursors (Sherwood Lollar et al. 2002, 2008), and radiolytic molecular hydrogen (H_2) (Holland et al. 2013). Recently the presence of a sulfur cycle which has prevailed within the fracture fluid over geologic time has been demonstrated by Li et al. (2016). Indirect oxidation of sulfide minerals by oxidants from radiolytic decomposition of water is the most probable source for sulfate which is the major electron acceptor in sulfate reduction - an important terrestrial biosphere metabolism (Li et al. 2016). These findings help build the case for the potential existence of isolated microbial communities that have existed for geologically relevant time within the Kidd Creek fracture fluid system (Holland et al. 2013; Li et al. 2016; Lollar et al. 2018, in review).

The goal of this study was to further investigate microbial communities associated with two boreholes in Kidd Creek mine that were part of the previous studies. Stainless steel filter housings with aluminum-coated glass wool were installed on the boreholes to allow the passive filtering of fracture fluids. Concurrently biofilm and service water samples were collected for comparison with borehole samples. Analysis of PLFA concentrations, distributions and isotopic compositions (δ13C) was used to compare the microbial communities sampled by the filters from the outflowing borehole water to the biofilms growing on the rock surfaces where water is discharged into the mine with the goal of determining whether there were differences in the carbon sources and metabolisms that the communities were using.

3.2 METHODS

3.2.1 Glass Wool Housing Preparation and Installation

Carbon-free, aluminum coated glass wool (AGW) was prepared as previously described (Mailloux et al. 2012). Cylindrical housings made of 316 stainless steel were gently packed with 21.3 g (±1.7 g) of Al-coated glass wool in the main body, with 2.25 g of uncoated glass wool placed in the housing endcaps. Complete housing units were fitted with 3/8” hose barbs and the entire unit was wrapped in aluminum foil and combusted in a Thermolyne 30400 furnace at 450˚C for 8 hours.

Stainless steel housings containing AGW were connected to the margo plugs using solvent rinsed 3/8" Tygon tubing and metal hose-clamps. Where needed, 1/4" tubing and a reducing union was used to facilitate attachment of the housings. Downstream of the housings, 3/8" tubing was used to attach other monitoring equipment and for outflow. Housings were secured to the overlying metal mesh to ensure long-term installation.

3.2.2 Sampling in Kidd Creek Mine at 7850ft from 2016-2018

The sampling site is located in Kidd Creek, a Cu-Ag-Zn mine, in Timmins, Ontario, Canada at 7850 ft (~2.4 km) below the surface. All samples were collected from one of two, ~1400 m-long uncased boreholes with identifiers 12299 and 12261. The relative position of the two boreholes and biofilm, as well as the approximate configuration of the sampling apparatus can be seen in Figure 3.1. In total, six AGW housings, two biofilm samples, and one service water sample were installed and/or collected between July 12, 2016 and January 29, 2018 (Figure 3.2). Samples for geochemistry were taken, as well as gas and water flow rates, unless otherwise specified (Table 3.1).

3.2.2.1 Sampling in 2016

On July 12, 2016 a 316 stainless steel NQ margo packer plug was solvent rinsed and semi-permanently inserted into 12261 and sealed to the inner rock walls. Sealing of the plug beyond the water level to isolate the borehole from the mine atmosphere and minimize air contamination was attempted, and water and gas was allowed to flow out of the margo plug to displace any remaining mine air, following previously described methods (Holland et al. 2013; Sherwood Lollar et al. 2002; Ward et al. 2004). The integrity of this seal appeared imperfect, however two replicate housings were connected as described in Section 2.1 and left in place with downstream flowmeters, for retrieval in 2017. At the time of installation, water was observed leaking around the margo plug. No gas flow rate was taken. Biofilm samples were aseptically collected from the collar of and below 12261.

3.2.2.2 Sampling in 2017

On January 24, 2017 the housings installed on 12261 were retrieved however the downstream flow meters did not record a change in water flowrate. On January 25, 2017 replicate housings were attached to a permanently installed margo plug in 12299 with downstream flowmeters, for retrieval in June 2017. One flowmeter was provided by Princeton University and the other by McMaster University.

On June 06, 2017 the replicate housings were retrieved from 12299. The flowmeters downstream from the housings had not recorded a change in flow, however some water was observed to be flowing out of the McMaster-provided flow meter. The margo plug in 12261 was removed for collection of other samples, then permanently resealed into the borehole. A single housing was attached to each borehole for collection in 2018. No downstream flowmeters were included. Biofilm samples were collected from the collar and below 12261.

3.2.2.3 Sampling in 2018

The housings from 12261 and 12299 installed on June 06, 2017 were collected on January 29, 2018. No biofilm samples were taken. For 12261 no water was observed flowing from the borehole at any time during retrieval, so a water flow rate could not be taken. In July 2018 approximately 20L of mine service water was obtained for comparative analysis. 17.3 L of service water was passed through 0.22µm filters under pressure; two filters were needed to filter the entire volume. The remaining 1200 mL of service water was syringe filtered through a 0.22µm sterivex filter and frozen for downstream molecular analysis.

To ensure the sterility of the apparatus used for service water filtration, 3 L of mQH_2O was filtered through the apparatus and a 0.22 µm filter. The filtrate was collected in combusted glass flasks and re-filtered through the apparatus and collected on a new 0.22 µm filter. These filters, as well as a filter blank that included two unused 0.22 µm filters, were frozen prior to processing.

3.2.3 Phospholipid Fatty Acid Extraction, Quantification and δ13C Determination

Of the total mass of Al-coated glass wool (AGW) samples 90% of each was used for analysis of PLFA. All AGW, biofilm, and 0.22 µm filter samples were extracted for PLFA by the modified Bligh & Dyer (mBD) protocol (Bligh & Dyer, 1959; White & Ringelberg, 1998) with conversion to fatty acid methyl esters (FAMEs) by methanolysis (Guckert et al. 1985). The recovered FAMEs were separated using gas chromatography – mass spectrometry (GC – MS) with an Agilent GC – MS instrument (Agilent Technologies INC., Santa Clara, California, USA). FAMEs were separated on a DB5-ms+DG capillary column (30 m, 0.25 mm, 0.25 µm) using a GC temperature program of 50˚C (1 min), 10˚C min-1 to 160˚C, 1.5˚C min-1 to 180˚C (10 min), 1.5˚C min-1 to 250˚C, 20˚C min-1 to 320˚C. PLFA identity was determined using retention time and mass spectra of standards (Bacterial Acid Methyl Esters Mix, Matreya Inc., Pleasant Gap, Pennsylvania, USA). PLFAs were named as follows – number of carbons:number of double bonds. Iso- and anteiso- are denoted by “i” or “a”

respectively. PLFAs with unknown branch location are denoted with “br”, and those containing cyclic groups are denoted with “cy”. Double bond position is indicated by a superscripted number, and where exact double bond position is unknown a superscripted letter (x or x, y) is used.

Determination of $\delta^{13}C$ for extracted PLFA was determined using an Isotope Ratio – Mass Spectrometer (IR – MS) with a GC combustion configuration (Agilent 6890N GC; GC Combustion III Interface; Delta Plus XP IRMS). FAMEs were separated using a DB5-MS capillary column (30m x 0.32 mm x 0.25 μm) with a GC temperature program of 80˚C (1 min), 4 ˚C min-1 to 280˚C, 10 ˚C min-1 to 320˚C (15 min). The injection port was 310˚C splitless, the oxidation oven was 980˚C and the reduction oven was 650˚C.

3.3. RESULTS

3.3.1 Geochemical Characteristics

A summary of all gas and water flow rates and geochemical characteristics can be found in Table 3.1. The water temperature of 12299 was 25.2˚C in January 2017, 25 ˚C in June 2017, and 23.2 ˚C in January 2018. The pH for 12299 was 6.82 in June 2017 and 6.67 in January 2018. A pH measurement was not taken for 12299 in January 2017. For 12261 the water temperature was 25.0˚C in January 2017 but was not measured during subsequent sampling trips. The pH was not recorded for borehole 12261 during any sampling trip. The service water had a temperature of 24 ˚C in January 2017, 25.1 ˚C in June 2017, and 21.6 ˚C in January 2018. The service water pH for those sampling points respectively was 6.74, 7.52, and 7.41. The $\delta^{18}O$-H_2O values for the two boreholes and service water was consistent between sampling points: -12.0‰ (±0.75) for 12299, -12.0‰ (±0.57) for 12261 and -11.2‰ (±1.0). Likewise, values for $\delta^{2}H$-H_2O also remained stable over time, but the boreholes show significant enrichment compared to the service water: -34.6‰ (±0.78) for 12299 and -33.8‰ (±0.21) for 12261 compared to -84.6‰ (±4.7) for the service water. The cations and anions recorded were Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Cl^{-}, and Br^{-}, values for which are presented in Table 3.1. Other geochemical analytes include NO_3^{-}, NO_2^{-}, HPO_4^{2-}, SO_4^{2-}, Mn, Fe, Zn, Ba, B and Sr which are found in Table 3.1.

3.3.2 Phospholipid fatty acid biomass and fingerprinting

Abundant PLFA were observed in and quantified for all AGW housings, biofilms and the service water (Table 3.2). However, since water flow was low or poorly constrained for most samples, this cannot be related to biomass per milliliter. Instead biomass per gram of AGW extracted was used for comparison with biofilm biomass. Given the long installation times, there is some potential for

microbial growth to have occurred within the filter housings, however because the AGW is not a substrate for growth this can safely be eliminated. The microbial growth observed would be using only the geochemistry of the borehole water and ideally would be considered an inoculation from the borehole.

For Borehole 12261, replicate filters collected on January 24, 2017 had biomass estimates of 3 x 10^8 cells/g_{AGW} and 2 x 10^8 cells/g_{AGW}, and 2 x 10^8 cells/g_{AGW} on January 29, 2018. For borehole 12299 replicate filters collected on June 06, 2017 had 7 x 10^7 cells/g_{AGW} and 1 x 10^8 cells/g_{AGW}, and the filter collected on January 29 2018 the biomass estimate was 2 x 10^8 cells/g_{AGW}. For the biofilms collected on July 12, 2016 and June 06, 2017, the estimated biomass was 7 x 10^9 cells/$g_{Biofilm}$ and 6 x 10^9 cells/$g_{Biofilm}$, respectively. The service water had a biomass estimate of 2 x 10^6 cells/mL.

Replicate filters collected in 2017 had identical PLFA fingerprints; this was the first collection of filters from either borehole. The major PLFA peaks from housings retrieved from 12261 on January 24 2017 were identified as cy19:0Δ^9c, 16:0, cy17:0Δ^9c, 18:1^{9}c, 18:1^{9}t, and 18:0. The 12299 AGW collected on June 06, 2017 had major PLFA peaks identified as 18:0, 16:0 and 12:0.

Temporally, PLFA fingerprints for a given borehole were different between time points. Differences included proportional loss or gain of PLFA, appearance of previously undetected PLFA, and/or disappearance of previously detected PLFA. On January 29 2018 the dominant PLFA peaks for 12261 were 18:1^{9}t, 16:0, and 18:0. The previously dominant PLFAs, cy19:0Δ^9c and cy17:0Δ^9c, decreased in relative abundance. Lipids that were previously detected as very low constituents of the sample either increased in proportional presence (ex: 12:0 0.4% to 3%) or disappeared all together (ex: all 16:1x). Three new peaks appeared: 18:2^{x,y}, 16:1^9, and 18:1^x. For 12299 the dominant PLFA on January 29 2018 were 16:0, 18:0, and 18:19c. Previously abundant PLFA saw major decreases for 12:0 and i15:0. PLFA with small proportional mass peaks in 2017 (13:0, 14:1^x, br15:0, 15:1, br16:0, i16:0, a16:0, br17:0) disappeared completely. PLFA undetected in 2017 that were detected in 2018 include 23:0 and 24:0. The only notable increase was for 18:1^{9}c. When comparing between boreholes, PLFA fingerprints are significantly different (Figure 3.3A-D).

Biofilm samples from 2016 and 2017 were statistically identical to each other. Major PLFA were identified as 16:0, 18:1^{9}c, and 18:0 (Figure 3.3E). Biofilm samples from either year are significantly different from all borehole samples. The service water sample had a PLFA fingerprint significantly

different from all borehole and biofilm samples. The major PLFA peaks were $16:1^{x}$, 16:0, and $18:1^{9}t$ (Figure 3.3F).

3.3.3 Analysis of Blanks

Three process blanks were included over three sets of extractions; however, the third process blank returned no signal above detection so is excluded from Table 3.2. Of the two process blanks that returned a signal, each contained only two PLFA peaks: The process blank from January 13, 2018 had peaks corresponding to the saturates 16:0 and 18:0 totalling 118 pmol (1.2 pmol on column); the process blank from May 14, 2018 had the saturates 14:0 and 16:0 totalling 364 pmol (3.6 pmol on column). The field blank analyzed on 13.01.18 had only the saturates 14:0 and 16:0 totalling 400 pmol (4.0 pmol on column). The minimum mass of PLFA detected in any field sample was 8 x 104 pmole, so the PLFA detected and quantified in the process blanks and field blank could account for, at most, 0.32% (±0.23%) and 0.53% of the total PLFA quantified in the field samples. The saturate PLFA found in all three blanks are considered common contaminates and this level of detection from a blank is within normal limits for the instrumentation used. All samples were significantly different from the blanks.

3.3.4 Analysis of $\delta^{13}C$

Figure 3.4 shows $\delta^{13}C$ (‰) measurements for PLFA, dissolved inorganic carbon (DIC), and methane (CH_4). DIC and CH_4 are plotted as single values and PLFA is plotted as a range from minimum to maximum. All values are compared versus the international standard PDB. $\delta^{13}C$ values for the individually detected PLFA are included in Figure 3.3 above the bar to which they correspond. The January 24, 2017 sample from 12261 had an average $\delta^{13}C$ - PLFA of -14.3‰ (±2.8‰), with a maximum of -11.9‰ (±2.3‰) and a minimum of -17.8 (±1.5‰). On January 29, 2018 borehole 12261 had an average $\delta^{13}C$ - PLFA of -24.9‰ (±4.3‰) with a maximum of -17.2‰ (±0.2‰) and a minimum of -29.2‰ (±3.1‰), indicating a depletion in ^{13}C relative to ^{12}C compared to the 2017 samples.

The borehole 12299 samples from June 06, 2017 had an average $\delta^{13}C$ - PLFA of -27.8‰ (±2.3‰) with a maximum of -24.2‰ (±0.3‰) and a minimum of -30.3‰ (±0.4‰). Samples from January 29, 2018 had an average $\delta^{13}C$ - PLFA of -29.3‰ (±1.2‰) with a maximum of -27.4‰ (±0.5‰) and a minimum of -30.3‰ (±0.4‰). Between sampling years borehole 12299 did not show significant enrichment or depletion of ^{13}C relative to ^{12}C. The $\delta^{13}C$ - PLFA values for each borehole from 2017

are significantly different from each other, with 12261 enriched relative to 12299. In 2018 the two boreholes have similar $\delta^{13}C$ – PLFA on average, however the range for 12261 is much wider.

The biofilm sample from July 12, 2016 had an average $\delta^{13}C$ – PLFA of -12.2‰ (±1.7‰), a minimum of -13.7‰ (±0.1‰) and a maximum of -10.4‰ (±0.02‰). On June 06, 2017 the biofilm sample had an average $\delta^{13}C$ – PLFA of -13.0‰ (±1.7‰), a minimum of -14.6‰ (±0.3‰) and a maximum of -11.3‰ (±0.2‰). There was no significant change in $\delta^{13}C$ – PLFA values for the biofilms between years. Relative to borehole 12261 in 2017, the values are very similar, however the biofilm remains enriched relative to the borehole in 2018. The biofilms are enriched relative to borehole 12299 during all sampling years.

The only $\delta^{13}C$ - DIC value for 12261 is from May 10, 2007 and so cannot be reported as an average but as a single value of -1.7‰. Two $\delta^{13}C$ – DIC values are available for 12299 from January 17, 2013 and April 03, 2014. Because there are only two values they are reported as a range between the two values rather than an average: -3.2 to 7.8‰. For borehole 12261 the PLFA are depleted relative to the DIC by 12.7‰ for the January 2017 sample and by 23.2‰ for the January 2018 sample. For borehole 12299 the PLFA are depleted relative to the DIC by 24.6-35.6‰ for 2017 and 26.1-37.1‰ for 2018. The biofilms are depleted relative to DIC by 10.5‰ in 2016 and 11.3‰ in 2017.

$\delta^{13}C$ – CH_4 values remain constant over time, -39.0‰ (±0.73‰) for 12261 and -39.1‰ (±0.49‰) for 12299, are not significantly different between boreholes, and are depleted relative to all $\delta^{13}C$ – DIC and $\delta^{13}C$ – PLFA values.

3.4 DISCUSSION

3.4.1 Three unique microbial communities

The distinct PLFA fingerprints for 12261, 12299 and the biofilm strongly indicate the presence of three separate and unique microbial communities living associated with the fracture fluids. Principle component analysis (PCA) shows clustering of samples into three groups that are consistent with this conclusion. The values for the three major principle components (PC) are PC1 (36.83%), PC2 (23.57%), and PC3 (20.98%). The drivers for each cluster also reinforce the importance of major PLFA peaks identified in each sample.

The $\delta^{13}C$ – PLFA values for 12299 and 12261 in 2017 indicate the utilization of different metabolic pathways in geochemically identical environments. This finding coupled with the distinct

PLFA fingerprints for these samples provides strong evidence that the two detected communities are unique from each another. The similarity between δ13C – PLFA values for the biofilms and 12661 do not indicate that the organisms present in each community are the same, rather that the organisms that are present are utilizing the same, or similar, metabolic pathways, or using metabolic pathways that result in similar PLFA fractionation. In the case of the biofilms and the 12261 filter community, the biofilm has uninterrupted access to an oxic/anoxic interface with the mine air which would promote the growth of different organisms. Due to the outflow of gases from the borehole, the microbes inside the filter housing would likely not have access to a constant oxic/anoxic interface. It is possible that the biofilm is the result of borehole organisms establishing a new community outside of the borehole. The biofilm community is also most likely to be directly impacted by the mine environment, including potential introduction of microorganisms. The different external environment may have encouraged the growth of different organisms, which would be consistent with the different PLFA fingerprints shown in Figure 3.3.

$\delta^{13}C$ values for DIC for 12261 and 12299 are only available for years in which PLFA were not sampled, and ranges of depletion of PLFA relative to DIC can vary greatly, making it difficult to determine which pathways are active in a complex community (House, et al., 2003). Despite this, some inferences can be made regarding potential metabolic pathways these communities may be utilizing. The biofilm community in both sampling years and the 12261 community in 2017 have similar DIC-PLFA values (10.5-12.7‰) that may indicate the use of any of the Acetyl-CoA (AC) pathway, reductive tricarboxylic acid (TCA) cycle, or the 3-hydroxypropionate pathway (3-HP) (House, et al., 2003; Zhang, 2002). The 2018 12261 sample and both 12299 samples have DIC-PLFA values that may indicate either the use of the reductive TCA cycle, or autotrophic sulphate reduction (Boschker & Middelburg, 2002; Londry et al., 2004).

There is some uncertainty with the grouping of the filters collected in 2018 as their spatial placement on the loadings plots is not consistent. For 12261 the 2018 sample clusters nearer to the service water sample for PC1 vs. PC3. Due to the distinct PLFA fingerprints and δ13C – PLFA values for these two samples it is unlikely that this indicates significant similarity. For the 12299 sample from 2018 it clusters with the biofilm samples for PC1 versus PC2. The uncertainty of these results can be explained by the temporal shift hypothesized to have occurred for the AGW filter housings. This shift in community composition has two possible explanations. Firstly, changes in the local environment within the stainless-steel housings containing the AGW may have selected for different organisms. For

housings collected in 2017, flow meters were connected downstream at the time of installation. This was not the case for housings collected in 2018. The configuration used for the 2017-collected housings may have provided protection from backflow of atmospheric air into the housings allowing the prevalence of a local environment containing only fluid and gas from the borehole. Without the downstream flowmeters, the 2018-collected filter set may have had an internal gas composition more similar to the external mine air. Notably, this may have created more oxic growing conditions which would have prevented the growth of anaerobic organisms, such as many sulphate reducing bacteria (SRB) that are discussed in section 4.2. Based on the differences in PLFA fingerprint, δ13C – PLFA values, as well as the real-world spatial separation of borehole 12299 and the biofilms, this clustering can be excluded as an indicator of significant similarity.

Secondly, it is possible that the temporal change in community composition is due to changes in which "compartment" of fracture fluid being sampled. Deeper fracture fluids have MRTs that are much older than shallower ones, but sampling boreholes at the same depth over time can yield changing MRTs (Warr et al. 2018). Given that subsequent determinations of MRTs for the fracture fluids sampled in Kidd Creek Mine at depth 7850 ft have changed from 1.1-1.7 Ga to 0.2-0.6 Ga (Holland et al. 2013; Warr et al. 2018), it is likely that the source of the majority of fracture fluids and gases escaping the borehole has changed. If this is the case, a community shift is a certainty as organisms from the younger fracture fluid would now make up the detected community.

3.4.2 The Case For SRB

PCA analysis indicates that the grouping of the 2017 samples for 12261 is driven by cy17:0Δ9c and cy19:0Δ9c which are associated with anaerobic sulfate reducing bacteria (SRB) (Dowling et al. 1986; Green & Scow 2000; Taylor & Parkes 1983; White et al. 1999). Sulfate reduction can utilize several potential electron donors including organic acids (formate, acetate, etc.), alcohols (methanol and ethanol), methane, molecular hydrogen (Liamleam & Annachhatre, 2007) and metallic iron (Fe(0)) (Kato, 2016). Sulfate is the major electron acceptor for SRB which is present in the Kidd Creek fracture fluids in quantities that have previously been determined to be adequate to support a community based on this metabolism (Li et al. 2016). The gas flowing from the boreholes is rich in H2 (Holland et al 2013) which is the electron donor for many SRBs (Liamleam & Annachhatre, 2007). Its abiotic production in crystalline rock fracture fluids has previously been shown to support subsurface SRBs when ample sulfate is present (Lin et al. 2005). Competition for H2 as an electron donor exists between SRBs, methanogens and homoacetogens (Widdel, 1988; Liamleam & Annachhatre, 2007).

Sulfate reduction has a much more favorable free energy than that of methanogensis and thus may outcompete methanogenic organisms for available H2, and the lack of evidence for biotic methane production in the Kidd Creek boreholes again supports a community free of methanogenic microorganisms. The presence of homoacetogens cannot be confirmed or denied based on PLFA information alone. In the greater geochemical context of the Kidd Creek system, the presence of SRB utilizing the fluids and gas from boreholes is not surprising and widely supported by the previous analysis of the established sulfur cycle (Li et al. 2016) and demonstration of similar deep-dwelling sulfate-reducing organisms (Lin et al. 2006).

An important consideration when using PLFA as a biomarker for SRBs is the context in which their existence is being suggested. The majority of PLFA biomarkers for SRBs previously identified have been done in marine sediments, or enrichments thereof, where sulphate-reduction is associated with anaerobic oxidation of methane (AOM) (Elvert et al. 2001; Elvert et al. 2003; Hinrichs et al. 2000; Pancost et al. 2000; Thiel et al. 1999; Zhang et al. 2002). Thus, what is known regarding SRB-indicating PLFA is largely restricted to those species involved with AOM which is not applicable to the organisms detected and profiled here based on the absence of AOM. The terminally branched iso-15:0 and antiso-15:0 are often cited as indicators for SRBs, including acetate-oxidizers, such as *Desulfobacter, Desulfococcus and Desulfosarcina* species, however the organisms from these genera that have been examined are AOM-associated SRBs (Dowling et al. 1986; Webster et al. 2006).

While the use of individual PLFA, or proportions between PLFA, have been used as biomarkers for specific genera, caution has been advised regarding this practice especially in regard to complex and unique communities such as those associated with the fracture fluids of Kidd Creek Mine (Frostergård et al. 1993; Frostergård et al. 2011; Pombo et al. 2002; Willers, C., Jansen van Rensburg, P.J., Claassens, S. 2011). One of the strengths of PLFA fingerprinting is its complimentary nature to geochemical information, and genetic techniques such as sequencing and subsequent phylogenetic and bioinformatic analysis. Geochemical analysis of Kidd Creek indicates sulphate is present in the fluids of both boreholes at levels between 10 and 25mg/L. In order to concretely confirm the presence of SRBs to the genus or species level, genetic analysis will be performed in the future.

The reduction of sulphate by SRBs often mediates anaerobic oxidation of methane by methanotrophic Archaea (ANME) (Knittel & Boetius, 2009). SRBs and ANME have been shown to create consortia in many environments including marine, freshwater and terrestrial sediments (Knittel & Boetius, 2009; Lloyd et al., 2006; Takeuchi et al., 2011). The presence of methanotrophic bacteria is

characterized by highly depleted δ13C – PLFA relative to δ13C – CH4 (Valentine & Reeburgh 2000). Because the δ13C – PLFA is enriched relative to δ13C- CH4 no methanotrophy is thought to occur due to bacteria in the Kidd Creek system, however methanotrophy by archaea cannot be eliminated.

3.4.2.1 Exclusion of cyclo PLFA as stress indicators

Variation in PLFA fingerprint profiles in Figure 3.3 indicate changes in the microbial communities they represent. Some changes in PLFA expression have been attributed to stress-response in the organisms (Green & Scow 2000). The ratios between certain PLFA have been used previously to indicate stress, such as cis- versus ¬trans¬- fatty acids (Heipieper et al. 1992; Hedrick et al. 1991; Ludvigsen et al. 1999), and cyclopropyl versus monoenoic precursor fatty acids (Green & Scow 2000; Kieft et al. 1994; Kieft et al. 1997).

For samples taken from 12261 in 2017 the ratio of cyclopropyl 17:0 PLFA to monoenoic 16:1 and the ratio of cyclopropyl 19:0 to monoenoic 18:1 may indicate that the population is under stress based on short-term starvation and desiccation experiments conducted by Kieft et al. in 1994. These studies were conducted on laboratory isolates and produced ratios ranging from 0-1.5 for cy17 to 16:1 and 0-0.4 for cy19 to 18:1, however comparative works looking at aquifer sediments, vadose sediments, and deep cores produce ratios ranging from 0-8 (Albrechtsen et al. 1995; Balkwill et al. 1998; Lehman et al. 1995; Ringelberg et al. 1997). Subsequent long-term studies by Kieft and colleagues in 1997 indicate that changes in the ratio of cyclopropyl fatty acids to their monoenoic precursors as a response to stress are temporally short lived, lasting only days. Additionally, the natural ratio of cyclopropyl to monoenoic fatty acids for laboratory grown sulfate-reducing isolates can range from 0-29.8 and has been recorded as high as 5.5 in deep subsurface isolates (Green & Scow, 2000; Kohring et al. 1994). The ratios seen in the samples from 12261 in 2017 are well within these ranges at 2.1 (cy17 to 16:1) and 1.1 (cy19 to 18:1), with comparative biofilms showing ratios of 0.12 and 0.1, respectively. The sample from 12261 in 2018 had ratios of 0.25 for cy17 to 16:1 and 0.02 for cy19 to 18:1, but because physiological indicators such as these ratios appear to respond strongly to community composition, the presence of cyclopropyl fatty acids in the 12261 samples from 2017 and their absence in the 12261 sample from 2018 is hypothesized to represent a change in community composition rather than an indicator of total community stress. The ratio of cis to trans unsaturated fatty acids such as 18:19 has similar shortfalls when used to indicate stress with respect to microbial communities instead of laboratory isolates (Green & Scow 2000). 18:19 has been used to indicate Eukarya and methanotrophs

(Green & Scow 2000), but also as a general marker for gram negative bacteria (Wilkinson 1988). The presence of eukaryotes in either the fracture fluid or in the biofilm cannot be excluded.

3.4.3 Implications for Ancient, Isolated Communities and Extraterrestrial Biomes

The validity of the isolation of truly ancient organisms is a topic of much debate. The ability to prove exterior sterility, and continued sterility throughout laboratory procedures and analysis is considered one of the most important factors and cannot be overstated (Vreeland & Powers, 1998; Vreeland & Rosenzweig, 2002). The possibility that the organisms observed in this study were introduced during borehole drilling and have subsequently established communities associated with the boreholes and fracture fluids can be eliminated based on the significantly different profiles of each sample. If organisms had been introduced to the system from the exterior mine environment or the adjacent biofilm, it would be expected that the PLFA fingerprints and $\delta^{13}C$ – PLFA values between all samples would be statistically identical because the geochemistry between the boreholes is identical. Because this is not the case, cross-contamination can be eliminated. Enrichment or selection for the growth of different organisms deep within the boreholes can also be eliminated based on the available geochemistry to support microbial growth between 12299 and 12261.

Whether the biofilm around 12261 is the result of borehole organisms colonizing the exterior environment, or vice versa, is less certain. However, the case that the community detected in 12299 is derived from organisms living in the deep fracture fluid can be made more confidently. Inoculation of the boreholes by adjacent biofilms can be confidently eliminated for 12299 as there is no biofilm growing around the collar of the borehole, and the margo plug in this borehole has been permanently in place for many years.

Understanding the origins, metabolic activities and biosignatures of communities living in deep subsurface systems, and in particular determining whether there are indigenous communities in these systems, provides perspective for interpretation of potential subsurface communities elsewhere, such as in the subsurface of Mars. As an analogue environment, terrestrial subsurface biomes exist independently of the photosphere and atmosphere and are self-reliant in organics and energy (Stevens et al. 1995; Rampelotto, 2010). Kidd Creek Mine has previously been cited as a prime candidate for analogue studies (Léveillé 2009; Dartnell et al. 2007), and the demonstration of organisms subsisting off fracture fluids in this system contributes to this classification. Although the presence of liquid water on the surface of Mars is debated, the present-day subsurface of Mars could house saline

groundwaters in similar deep crustal fractures (Sherwood Lollar et al. 2006). With the recent detection of the near-surface presence of organics (Eigenbrode et al. 2018), interest in the potential habitability of the Martian subsurface has been reinforced. In this context the fracture fluid system of Kidd Creek represents a potential analogue environment for potential Martian fracture fluid biomes and while the ancient nature of the organisms detected in association with the Kidd Creek fluids is debatable, these findings do indicate that life can utilize the geochemistry of such systems to thrive. Potential exists for systems similar to the ~1.4 Ga fracture fluids to exist on extraterrestrial bodies where they may support life for geologically relevant time scales where surface conditions are no longer conducive to life as we know it.

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3.6 TABLES AND FIGURES

Table 3.1. Summary of Geochemistry

Date	May 10 2007		January 17 2013		April 03 2014		July 12 2016			January 24-25 2017			June 06-07 2017			January 28-29 2018			July 2018		
Sample*	12261	12299	12261	12299	12261	12299	Service Water	12261	12299	Service Water	12261	12299	Service Water	12261	12299	Service Water	12261	12299	Service Water	12261	12299
Gas Flow Rate (mL/min)							na			na	3082	12333	na	2729	5938	na	46	4398	na		
Water Flow Rate (mL/min)							na	284		na	280	146	na	134	127	na	nm	115	na		
Temperature (± 0.1 °C)								26		24	25	25.2	25.1	nm	25	21.6	nm	23.2			
pH (± 0.02 pH)								6.32		6.74	nm	25.2	7.52	nm	6.82	7.41	nm	6.67			
δ^{13}C-CH_4 (‰)	-39.6					-39.7	na			na	-39.6	-38.5	na	-38.2	-39.1	na	-38.5	-39.0	na		
δ^{13}C-DIC (‰)	-1.71			-3.2		7.8															
δ^{18}O-H_2O (‰)										-10.6	-12.4	nm	-12.4	-11.6	-11.1	-10.7	nm	-12.4			
δ^{2}H-H_2O (‰)										-80.8	-33.6	-12.4	-89.8	-33.9	-34.1	-83.1	nm	-35.5			
Li^+ (ug/l)										<500	<500	-34.2	<500	<500	636	< 500	nm	< 5000			
Na^+ (mg/l)										377	17300	616	194	17900	17300	261	nm	14900			
K^+ (mg/l)										53	108	15300	25	118	123	54	nm	195			
Mg^{2+} (mg/l)										106	2020	100	76	2120	2460	52	nm	2110			
Ca^{2+} (mg/l)										422	65500	2220	350	68300	58200	322	nm	50800			
Cl^- (mg/l)										252	136000	49700	201	120000	110000	232	nm	125000			
Br^- (mg/l)										3	2000	115000	2	1910	1760	3	nm	1890			
NO^{3-} (mg/l)										18	< 2	1730	15	< 5	< 5	6	nm	< 20			
NO^{2-} (mg/l)										2	< 2	< 2	< 0.1	< 5	< 5	< 0.2	nm	< 20			
HPO_4^{2-} (mg/l)										< 0.2	< 4	< 2	< 0.2	< 10	< 10	< 0.4	nm	< 30			
SO_4^{2-} (mg/l)										1930	20	< 4	1300	10	10	1300	nm	< 40			
Mn (µg/L)										3370	33400	25	2500	33900	28700	1100	nm	22100			
Fe (mg/l)										<5	26	24900	<5	17	16	1	nm	10			
Zn (µg/L)										36900	<250	35	29500	<250	<250	7400	nm	< 500			
Ba (µg/L)										<50	43800	<250	<50	43300	42900	< 200	nm	32700			
B (µg/L)										<1500	3140	41700	<1500	3450	3540	nm	nm	nm			
Sr (mg/l)										4	1200	2880	3	1100	997	3	nm	971			

*nm = not measured; na = not applicable

Table 3.2. Characterization of phospholipid fatty acid content of biomass and biofilms.

Sample*	13.01.18_ McMaster _PB†	14.05.18_ McMaster _PB†	13.01.18_ KC7850_ FB_AGW‡	xx.07.18_ KC7850 _SW	24.01.17_ KC7850_ 12261_ AGW_ PLFA1	24.01.17_ KC7850_ 12261_ AGW_ PLFA2	29.01.18_ KC7850_ 12261_ AGW	06.06.17_ KC7850_ 12299_ AGW_ McMaster	06.06.17_ KC7850_ 12299_ AGW_ Princeton	29.01.18_ KC7850_ 12299_ AGW	12.07.16_ KC7850_ 12261_BF	06.06.17_ KC7850_ 12261_BF
Borehole	NA	NA	NA	NA	12261	12261	12261	12299	12299	12299	12261	12261
Sample Type	Process Blank	Process Blank	Al-Coated Glass Wool	Service Water	Al-Coated Glass Wool	Al-Coated Glass Wool	Al-Coated Glass Wool	Al-Coated Glass Wool	Al-Coated Glass Wool	Al-Coated Glass Wool	Biofilm	Biofilm
Installation Date	NA	NA	NA	NA	12-07-16	12-07-16	06-06-17	24-01-17	24-01-17	06-06-17	NA	NA
Retrieval Date	NA	NA	NA	NA	24-01-17	24-01-17	29-01-18	06-06-17	06-06-17	29-01-18	12-07-16	06-06-17
Mass of sample (g)§	NA	NA	19.45	NA	21.3	21.3	21.3	21.3	21.3	21.3	6.3	9.8
Volume of sample (L)	NA	NA	NA	17.3 L	NA	NA	NA	NA	NA	NA	NA	NA
Number of PLFA peaks	2	2	2	21	29	23	20	26	23	17	17	24
Total pmole PLFA	118	364	400	1.4E+6	3.1E+5	1.7E+5	2.0E+5	7.6E+4	1.4E+5	1.8E+5	2.3E+6	2.8E+6
pmole PLFA on column	1.2	3.6	4.0	1.4E+4	3.1E+3	1.7E+3	2.0E+3	7.6E+2	1.4E+3	1.8E+3	2.3E+4	2.8E+4
pmole PLFA/g	NA	NA	21	NA	1.4E+4	8.0E+3	9.2E+3	3.6E+3	6.6E+3	8.4E+3	3.7E+5	2.9E+5
pmole PLFA/mL	NA	NA	NA	83	NA	NA	NA	NA	NA	NA	NA	NA
Total Cells	2E+6	7E+6	8E+6	3E+10	6E+9	3E+9	4E+9	2E+9	3E+9	4E+9	5E+10	6E+10
Cells/g	NA	NA	4E+5	NA	3E+8	2E+8	2E+8	7E+7	1E+8	2E+8	7E+9	6E+9
Cells/mL	NA	NA	NA	2E+6	NA	NA	NA	NA	NA	NA	NA	NA
δ^{13}C PLFA (‰) Average	NA	NA	NA	-34.6	-14.6	-14.3	-24.9	-27.9	-27.9	-29.3	-12.2	-13.0
δ^{13}C PLFA (‰) Maximum	NA	NA	NA	-30.9	-10.2	-11.3	-17.2	-24.0	-24.4	-27.4	-10.4	-11.3
δ^{13}C PLFA (‰) Minimum	NA	NA	NA	-39.0	-18.9	-16.7	-29.2	-29.9	-30.6	-30.3	-13.7	-14.6

*Naming Convention: Date Retrieved_Level_Borehole_Sample Type_Replicate Indicator. Date Convention: DD.MM.YY. Sample Type: PB, Process Blank; FB, Field Blank; AGW, Al-coated Glass Wool; BF, Biofilm. Replicate Indicator: Only included for samples that had replicate housings attached to the same borehole

†Process Blanks (PB) are date-marked for the day the extraction was completed and have "McMaster" for Level.
‡The Field Blank (FB) is date-marked for the day extraction was completed as it was carried into the mine on several trips.
§Sample mass for filters is the total average across all filters, except for the Field Blank which is known.

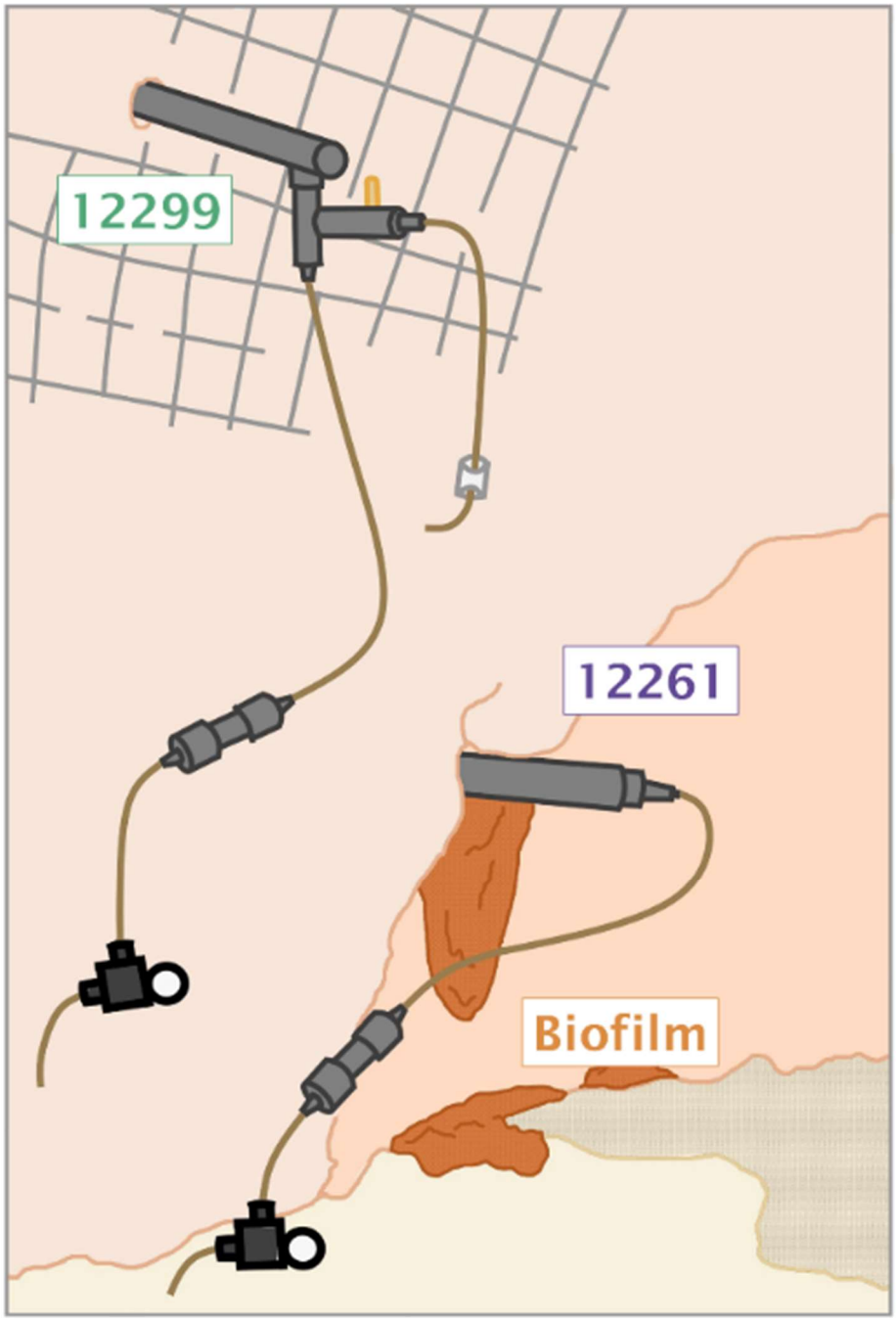

Figure 3.1. Schematic of the Kidd Creek sampling site at 7850 ft below the surface. Two boreholes are pictures (12299 and 12261) as well as the biofilm growing around and below 12261. The schematic shows the approximate configuration of the McMaster sampling apparatus.

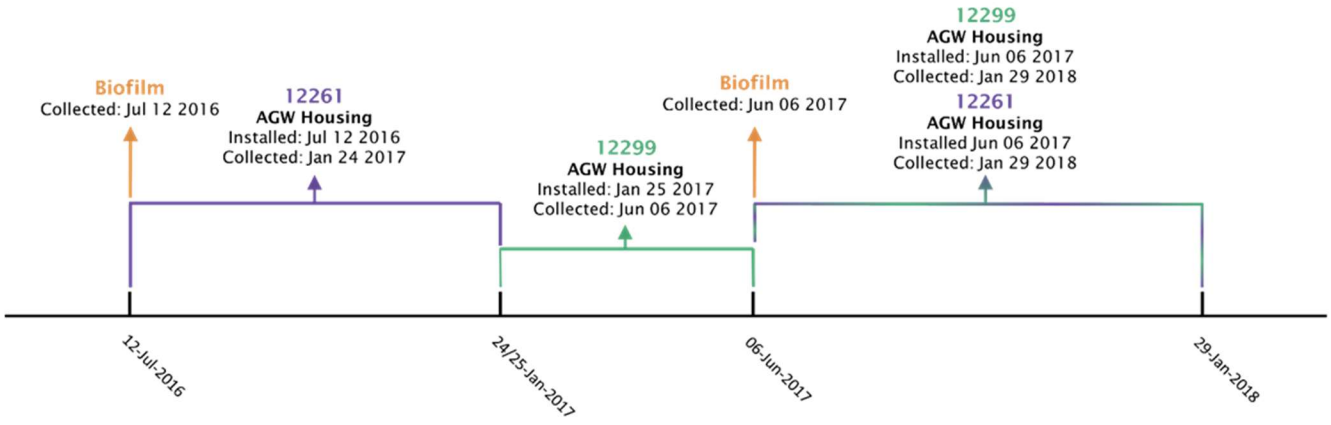

Figure 3.2. Timeline of sampling with installation and collection dates noted.

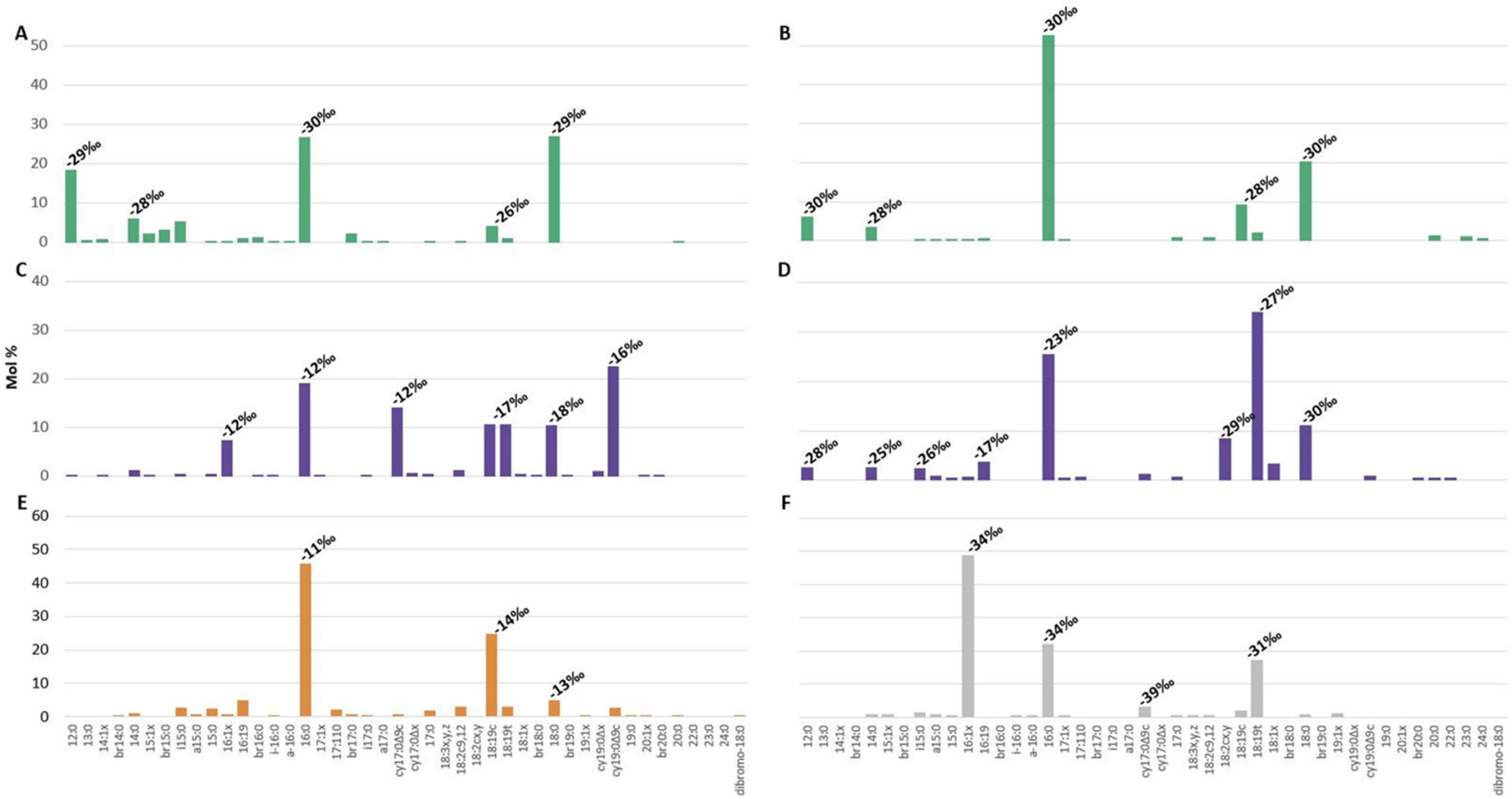

Figure 3.3. Mol% distributions of PLFA for each sample with δ13C values (‰) overlaid. (A) Average of replicates for 12299 in 2017 and (B) single filter from 2018. (C) Average of replicates for 12261 in 2017 and (D) single filter from 2018. (E) Average of biofilm samples from 2017 and 2018. (F) Service water from 2018.

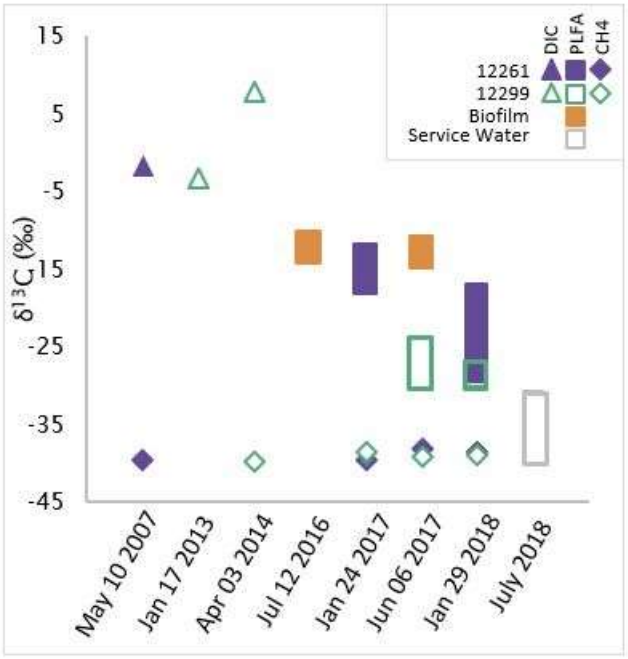

Figure 3.4. δ13C (‰) values for dissolved inorganic carbon (DIC) (triangles), PLFA (rectangles) and methane (CH4) (diamonds). Samples taken from borehole 12261 are in solid purple, from borehole 12299 are in green outline, biofilms are solid orange, and service water is in grey outline. DIC and CH4 are presented as single values whereas PLFA is presented as a range from minimum to maximum averaged over all PLFA peaks.

CHAPTER 4

THESIS SUMMARY AND FUTURE RESEARCH

4.1 THESIS SUMMARY

The characterization of microbial communities within the Earth's deep terrestrial subsurface contributes to our understanding of the limits of life on Earth including the potential for communities to remain viable and isolated for geologically relevant periods of time. Investigating these biomes can act as analogues providing insight into the potential presence of similar communities on extraterrestrial planetary bodies, such as Mars. Difficulties associated with sampling in the deep subsurface can include heterogeneous distribution of organisms, mineral matrix effects, and limited access to sampling sites, and have resulted in this area of inquiry remaining largely unexplored. In this Master's thesis inquiry was made in two main areas of interest: (1) the extent to which mineral matrix effects reduce the recovery of phospholipid fatty acids, and (2) the detection and subsequent characterization of potential microbial communities associated with ancient fracture fluids at ~2.4 km depth within the Kidd Creek Mine, Timmins, Ontario. Mineral matrix effects were characterized by adding known total *Bacillus subtilis subtilis* (ATCC 6051) to either bentonite clay or crushed granite and subsequently extracting phospholipid fatty acids (PLFAs) from these samples. This study showed significant *circa* order of magnitude loss in PLFA recovered due to mineral matrix effects and allowed for the discussion of currently published biomass conversion factors. To detect microbial communities associated with ancient Kidd Creek fracture fluids, aluminum-coated glass wool (AGW) was packed into stainless steel housings and left to passively filter fracture fluids and gases. Additionally, borehole-associated biofilms and mine service water were also sampled. PLFA were extracted and detected in all fracture fluid-associated samples and indicated significant biomass present. Comparison of PLFA fingerprints and stable carbon isotope analysis ($\delta^{13}C$) confirm the presence of three distinct microbial communities utilizing identical geochemistry through different metabolic pathways. This study demonstrated that ancient isolated fracture fluids can support diverse microbial communities that are, potentially, indigenous to the formation from the time of burial.

4.1.1 The impact of mineral matrix effects on extraction and quantification of phospholipid fatty acids, and subsequent biomass estimates

The effects of mineral matrices on extraction of PLFA was explored by using a dilution series of *Bacillus subtilis subtilis* (ATCC 6051) as a PLFA source and adding a known abundance of cells to one of two mineral matrices, bentonite clay or crushed granite. The results of this study indicated significant mineral matrix effects that resulted in a *circa* order of magnitude loss in PLFA recovered when compared to pure culture extracts, and that this loss effected all detected individual PLFA equally. Relative to the pure culture, the bentonite clay caused an 80-88% loss in PLFA recovered and the granite caused a loss of 44-79%. Because the number of cells added was known, two sets of conversion factors were generated from this study. The first set, called the Experimental Conversion Factors, was created by using the known cellular abundance and the quantified PLFA mass, and the second set, the Linear Conversion Factors, was created using the line of best fit from the dilution series for the pure culture and each mineral matrix. The Experimental Conversion Factor for the pure culture (5.6×10^3 cells/pmole$_{FAME}$) was an order of magnitude smaller than all previously published conversion factors ($2\text{-}5.9 \times 10^4$ cells/pmole$_{FAME}$). This was not the case for the bentonite (3.6×10^4 cells/pmole$_{FAME}$) or granite (1.7×10^4 cells/pmole$_{FAME}$). All Linear Conversion Factors ($2.6\text{-}5.1 \times 10^3$ cells/pmole$_{FAME}$ were an order of magnitude smaller than published conversion factors. The disagreement between the conversion factors created in this study and those previously published was due to the use of different organisms to create the factors. Because bacteria can vary largely in size and shape, so to does their surface area to volume ratio which effects the amount of PLFA per individual cell. Additionally, organisms used to create conversion factors are often grown optimally and so their physiological state may not accurately represent that of microbes extracted from the environment. Overall this study advises caution when using conversion factors to estimate biomass in environmental samples, especially those extracted from complex matrices, as biomass can be greatly misestimated due to multiple confounding factors.

4.1.2 Three distinct microbial communities living associated with Precambrian fracture fluids in the crystalline basement of the Canadian Shield

To detect viable microorganisms associated with fracture fluid systems with a mean residence time of ~1.4 Ga in the deep terrestrial subsurface, long-term passive filtration of waters and gases escaping two boreholes (12261and 12299) 7850 ft below land surface was performed. Biofilms growing adjacent to 12261, as well as mine service water, were also sampled. PLFAs were extracted

from all samples and used to estimate biomass, and to characterize and compare the detected communities. Analysis of carbon isotopes ($\delta^{13}C$) of PLFA, methane and DIC was used to characterize potential metabolic activity, and by combining $\delta^{13}C$ of PLFA with fingerprinting differences in the detected communities could be further supported and confirmed. This combined analysis indicated three unique communities growing associated with the fracture fluids; each borehole filter had a distinct PLFA fingerprint and $\delta^{13}C$-PLFA signal, and so did the biofilm community. Estimated microbial biomass for the filters ranged from 7×10^7 to 3×10^8 cells/g_{AGW}, and for the biofilm the range was $6 - 7 \times 10^9$ cells/$g_{biofilm}$. Replicate filters showed statistically identical PLFA fingerprints and $\delta^{13}C$ values. Biofilm samples taken in separate years also had statistically identical PLFA fingerprints and $\delta^{13}C$ values indicating no temporal change for this community. A temporal change in PLFA fingerprint but not in $\delta^{13}C$ was seen for the community detected in 12299 filters. Both PLFA fingerprint and $\delta^{13}C$ changed temporally for the 12261 community. Temporal changes can be attributed to several factors. First, a change in sampling apparatus configuration may have caused accidental oxygenation of filtration units in 2018, but not 2017, thus changing the growth environment causing selection for aerotolerant organisms. Secondly, it is possible that as different, younger fracture fluids begin escaping the fracture system, they bring different organisms that colonize the AGW inside the filter housing.

The presence of cyclopropyl PLFAs in the 2017 12261 filters strongly indicates the presence of SRBs, a finding that is supported by previously published work on the sulfur-cycle within the Kidd Creek fracture system, and a most-probably number (MPN) study currently being conducted at the University of Toronto. The $\delta^{13}C$ value for PLFA is enriched compared to the methane present indicating that no methanotrophy is occurring in the fracture system. The $\delta^{13}C$ for DIC 12261 and 12299 was compared to $\delta^{13}C$ for PLFA from all samples in attempt to determine what metabolic pathways were being used. Due to variance in ranges of fractionation associated with metabolisms, no one pathway could be correlated to a given community.

This study demonstrates the presence of three microbial communities that are distinct from each other. The individual PLFA, fingerprints and $\delta^{13}C$ of PLFA, DIC and methane indicate that these organisms live independently from surface processes and are likely self-sufficient. Furthermore, the case can be made for the indigenous nature of the communities detected in the filtration units.

4.1.3 Astrobiological Implications

The results from the study presented in Chapter 2 highlight the importance of collecting sufficient sample mass when working in environments that have heterogeneously distributed and low biomass. Should extraterrestrial samples be analyzed for PLFA either *in situ* or back on Earth, mineral matrix effects may result in non-detection if the obtained sample mass is too low. Chapter 3 demonstrates the capability of microbial communities to live isolated from surface processes, and thus self-sufficiently, in the deep terrestrial subsurface. Further, it also raises the possibility of these communities being indigenous to the fracture fluid system which would place the maximum length of time the community has existed at 1.7 Ga. These findings have implications in the search for extraterrestrial life, particularly in the potential for life to exist in the subsurface of other rocky bodies, such as Mars. The presence of liquid water on the surface of Mars is still debated (Dundas et al., 2017; Malin & Edgett, 2000; Martin-Torres, et al., 2015), and the potential for subsurface waters has long been discussed, citing terrestrial subsurface rock and fluid systems as analogue sites (Boston et al., 1992; Chapelle et al., 2002; Dartnell et al 2007, Pfiffner et al., 2008; Sherwood Lollar et al., 2006). Most recently the presence of a Martian subglacial lake has recently been confirmed (Orosei et al. 2018), adding support for the Martian subsurface as a potentially inhabited biome.

4.2 FUTURE RESEARCH DIRECTIONS

To expand confidence in the conversion factors and mineral matrix effects presented in Chapter 2, additional replicates in each series should be run. In order to minimize mineral matrix effects, the method by which lipids are extracted from samples should be optimized. Optimization could include comparison of different initial extraction protocols such as mBD versus microwave assisted extraction, or optimization could be adjusting the parameters of the initial extraction of mBD. Creation of additional dilution series on different matrices, such as basalt, shale or various soils, will help reveal the extent of variable lipid recovery caused by matrix effects. Finally, the mineral matrix study could be performed for biosignatures other than PLFA, such as archaeal lipids or the spore-indicating dipicolinic acid (DPA).

To gain more insight into the metabolic pathways being used by the microbial communities, further analysis of carbon isotopes ($\delta^{13}C$) of DIC should be performed. The $\delta^{13}C$ for DIC that is included in this thesis is sufficient to comment on possible metabolisms only, and not to concretely confirm the use of any given metabolic pathway. By determining $\delta^{13}C$ of DIC from samples taken

between 2016 and 2018, the accuracy of conclusions regarding metabolic pathways will be more concrete. In order to determine what species of microorganisms are present and what metabolic pathways they may be utilizing, 16S rRNA and metagenomic sequencing will be performed. Previously the power of combined PLFA and nucleic acid analysis has been demonstrated for other deep subsurface systems (ex. Magnabosco et al. 2016). Whole genome shotgun sequencing will compliment the results presented in Chapter 3, allowing for extensive bioinformatics and examination of metabolic potential of organisms detected.

Analysis of PLFA only provides information on the bacteria present, so analysis of archaeal cells and their lipids would provide information on the role of Archaea in these deep terrestrial fracture systems. This analysis would include detection and quantification of archaeal lipids by High Performance Liquid Chromatography (HPLC), and stable carbon analysis ($\delta^{13}C$) of the extracted archaeal lipids. Molecular genetic analyses targeting Archaea would provide the same information described above for bacteria.

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