MICROBIAL COMMUNITIES IN THE DEEP TERRESTRIAL SUBSURFACE

EXPLORING MICROBIAL COMMUNITIES AND CARBON CYCLING WITHIN THE EARTH'S DEEP TERRESTRIAL SUBSURFACE

By

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ABSTRACT

Investigating the presence of microbial communities in the Earth's deep terrestrial subsurface and the metabolic processes taking place in these environments provide insight into the some of the ultimate limits for life on Earth, as well as the potential for microbial life to exist within the subsurface of other planetary bodies. This Master's thesis project utilized phospholipid fatty acid (PLFA) analysis, in combination with carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$), to explore the presence and activity of microbial communities living within deep terrestrial subsurface fracture water systems and low permeability deep sedimentary rocks.

Deep fracture water systems, ranging from 0.9 to 3.2 km below land surface, were sampled for microbial communities via deep mine boreholes in the Witwatersrand Basin of South Africa. PLFA concentrations revealed low biomass microbial communities, ranging from $2x10^1$ to $5x10^4$ cells per mL and the PLFA profiles contained indicators for environmental stressors, including high temperatures and nutrient deprivation. $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFAs and potential carbon sources (dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and methane) identified microbial utilization of methane in some systems and utilization of DIC in others. Evidence for microbial oxidation of methane and chemoautotrophy in these systems is consistent with a self-sustaining deep terrestrial subsurface biosphere that is capable of surviving independent of the photosphere. Viable microbial communities were also identified within deep (334 to 694 m depth) sedimentary rock cores sampled from the Michigan Basin, Canada. PLFA analyses identified microbial cell densities ranging from 1-3 x 10^5 cells/mL and identified PLFA indicators for environmental stressors. These results demonstrated the ubiquity of microbial life in the deep terrestrial subsurface and provided insight into microbial carbon sources and cycling in subsurface systems that may survive in isolation over geologic timescales.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 1:

INTRODUCTION TO RESEARCH AND FUNDAMENTAL CONCEPTS

1.1. LIFE IN THE DEEP TERRESTRIAL SUBSURFACE

1.1.1. Introduction

Microbial life in Earth's deep terrestrial subsurface is currently known to reach depths of several kilometers within the continental crust (Chivian et al., 2008; Lin et al., 2006a; Moser et al., 2003; Onstott et al., 2003; Pfiffner et al., 2006; Ward et al., 2004). The deepest of these microbial communities are believed to live entirely independent of the photosphere, in high temperature and pressure conditions, are often limited by the availability of nutrients and, in some cases, are thought to have survived over geological timescales (Fredrickson and Balkwill, 2006; Lippmann et al., 2003; Pedersen, 2000). Their ability to survive despite these extreme environmental conditions places the deep subsurface biosphere as a priority site for exploring the ultimate limits for life on Earth. Understanding the mechanisms by which these microorganisms survive in isolation from the photosphere is also valuable for reconstructing early life on Earth, before the rise of photosynthesis (Nealson et al., 2005; Stevens, 1997). Applying this knowledge to other planetary bodies, where surface conditions may be inhospitable to life, provides insight into the possibilities for microbial life to exist in subsurface environments beyond Earth. The discovery of Earth's deep terrestrial subsurface biosphere has provoked a strong interest to search for life in the subsurface of other planets and moons in our solar system, including the subsurface of Mars (Chapelle et al., 2002). For these reasons, investigating the biogeochemical

processes occurring in Earth's deep continental biosphere is of particular interest to astrobiologists and is a major focus for understanding the origin of life on Earth.

Thirty years ago, life was generally considered to be a surface phenomenon; the conditions below Earth's surface were believed to be too extreme for life to survive. However, by the end of the 1980s, following the discovery of life within deep ocean hydrothermal vents, evidence began to arise that an extensive biosphere existed in the Earth's terrestrial subsurface (Corliss et al., 1979; Gold, 1992). Thomas Gold (1992) postulated that microbial life in Earth's deep terrestrial subsurface was widespread and that the extent of this biosphere may be comparable to microbial life above Earth's surface. He noted that some microbial communities in the deep terrestrial subsurface may be completely isolated from the products of photosynthetic activity and, as a result, may be entirely independent of the photosphere (Gold, 1992). A few years later, Whitman et al. (1998) proposed that the total microbial cell abundance in the deep terrestrial subsurface falls between $2.5x10^{29}$ and $25x10^{29}$ cells, exceeding the total microbial cell abundance in the Earth's open ocean. Recently, Kallmeyer et al. (2012) re-evaluated these estimates, focusing primarily on marine subsurface sediments, and concluded that Earth's subsurface biomass is likely lower than previously proposed. Nevertheless, low biomass microbial communities in Earth's deep terrestrial subsurface are generally accepted to be ubiquitous systems wherever temperatures, pressures and nutrient availability permit their growth (Fredrickson and Fletcher, 2001; Pedersen, 2000). As a result, questions arise as to what particular microbial groups are living in the Earth's deep terrestrial subsurface, what microbial metabolisms are active in these environments, and what environmental stressors ultimately limit the growth of these microbial communities.

1.1.2. Physical and geochemical limitations

Microbial communities in the deep terrestrial subsurface require the ability to survive a range of environmental stressors, including elevated temperatures and pressures, low oxygen levels and low nutrient availability (Fredrickson and Fletcher, 2001; Pedersen, 2000). Geothermal gradients in the continental crust vary by location, largely depending on the ability of the geological material to conduct heat; however, on average, temperatures increase with depth by approximately 25°C per km (Akob and Kusel, 2011; Fredrickson and Fletcher, 2001; Pedersen, 2000). The upper temperature limit for life as we know it is currently believed to be ~121°C (Kashefi and Lovley, 2003). This suggests that, based on temperature alone, the deep terrestrial subsurface biosphere should be capable of extending as deep as 5 kmbls (Akob and Kusel, 2011). In locations where the geothermal gradient is lower (e.g. 10°C per km in the Witwatersrand Basin of South Africa), microbial communities may be capable of reaching depths of up to 12 kmbls (Lin et al., 2006a; Omar et al., 2003). Microorganisms are generally tolerable to hydrostatic pressures of 10 to 100 MPa, and some barophilic organisms have been shown to withstand pressures of up to 1000 MPa (Fredrickson and Onstott, 1996; Lammer et al., 2009; Sharma et al., 2002). The depth at which hydrostatic pressure in the continental crust begins to exert an influence on a microbial community depends on the depth to groundwater within the system. However, for most locations, a thermal boundary is more likely to limit microbial growth before elevated hydrostatic pressures (Fredrickson and Onstott, 1996). Another potential limitation for subsurface communities in the continental crust is low nutrient availability. With an increase in depth, photosynthetically-derived organic carbon becomes limited and/or highly recalcitrant (Pedersen, 2000). Many deep subsurface microbial communities are forced to utilize alternative energy and carbon sources produced abiotically in

the subsurface. These mechanisms are outlined in more detail in Section 1.1.3. Severe nutrient deprivation in the deep subsurface results in very slow cell growth, with cell doubling times that are believed to reach hundreds to thousands of years (Chivian et al., 2008; Hoehler and Jorgensen, 2013). With long cell doubling times, microorganisms in the deep subsurface may tend to live in a semi-inactive state, waiting for conditions that are favorable for growth and reproduction (Chivian et al., 2008; Lomstein et al., 2012).

The geochemistry and habitability of deep terrestrial subsurface environments largely depends on the geology of the system. Most sedimentary rocks are porous in nature, which allows for a continuous flux of nutrients by groundwater flow. These rock units contain large amounts of buried organic carbon and have not been subjected to extreme temperatures or pressures (Stevens, 1997). For this reason, any microbial communities present at the time of deposition have the potential to survive for extended periods in these environments. In contrast, igneous and metamorphic rocks have been subjected to high temperatures and/or pressures, generally have low contents of organic matter, and lack sufficient pore space (Stevens, 1997). Microbial growth is typically restricted to the surfaces of fractures within crystalline igneous and metamorphic rocks. Due to the lack of organic material present, microbial communities within these fractures are likely based on in situ primary production, utilizing inorganic gases that are produced by water-rock interactions (Nealson et al., 2005).

1.1.3. Energy and carbon sources for subsurface communities

All microbial life on Earth requires an energy source for survival and reproduction, whether it be from sunlight (phototrophy) or chemical compounds (chemotrophy). In the absence of sunlight, subsurface microbial communities must derive their energy from chemical sources. Chemotrophs can either utilize organic chemicals (chemoorganotrophy) or inorganic chemicals

(chemolithotrophy) as sources of energy. In the shallow subsurface, microbial communities are ultimately linked to the photosphere through metabolism, as they utilize electron donors and acceptors, such as organic carbon and molecular oxygen, that are derived from surface photosynthetic activity (Lin et al., 2006b). Evidence suggests, however, that microbial communities living deep within crystalline rocks, at several kmbls, are completely isolated from the products of photosynthesis (Stevens, 1997). In the absence of solar energy and photosynthetically-derived organic carbon and oxygen, deep subsurface lithoautotrophic microbial ecosystems could act as primary producers, obtaining energy from abiotically-produced inorganic chemicals (Pedersen, 1997; Stevens and McKinley, 1995). As illustrated in Figure 1.1, a proposed inorganic source of energy and electrons for these communities is radiolytic hydrogen (H_2), which can be produced by several abiotic geological processes, including out-gassing, water-rock interactions, and radiolytic decomposition of water (D'Hondt, 2013; Itavaara et al., 2011; Lin et al., 2006b; Pedersen, 2000). H_2 oxidation can be coupled to the reduction of various terminal electron acceptors, including CO_2, SO_4^{2-}, Fe(III), NO_3^-, and HCO_3^-, and potentially provide the energetic basis for the deep subsurface biosphere (Amy and Haldeman, 1997; Chapelle et al., 2002; Fang and Zhang, 2011).

High concentrations of H_2, methane and higher hydrocarbon gases are detected in deep, saline fracture waters from mines in Canada, South Africa and the Fennoscandian Shield (Lollar et al., 2008). Hydrocarbon gases, including methane, are commonly produced autotrophically by CO_2-reduction (i.e. methanogenesis). Methanogens and acetogens can couple CO_2 reduction to H_2 oxidation to produce methane and acetate, respectively, and acetoclastic methanogens can convert this acetate to methane gas (Fang and Zhang, 2011; Pedersen, 2000). Methane and higher hydrocarbons are also produced thermogenically by the breakdown of organic matter

(Ward et al., 2004). Abiotic sources of methane and higher hydrocarbons in the deep terrestrial subsurface include mantle out-gassing and water-rock interactions (Lollar et al., 2002). Isotopic signatures and concentration patterns of $\delta^{13}C$ and $\delta^{2}H$ are analyzed to distinguish between the various abiotic and biotic origins of hydrocarbon gases in the continental subsurface. Distinguishing between hydrocarbon biosignatures and abiosignatures not only provides insight into microbial metabolisms beneath the Earth's surface, but may also be applied to interpreting hydrocarbon gases on other planetary bodies, including Mars (Onstott et al., 2006b).

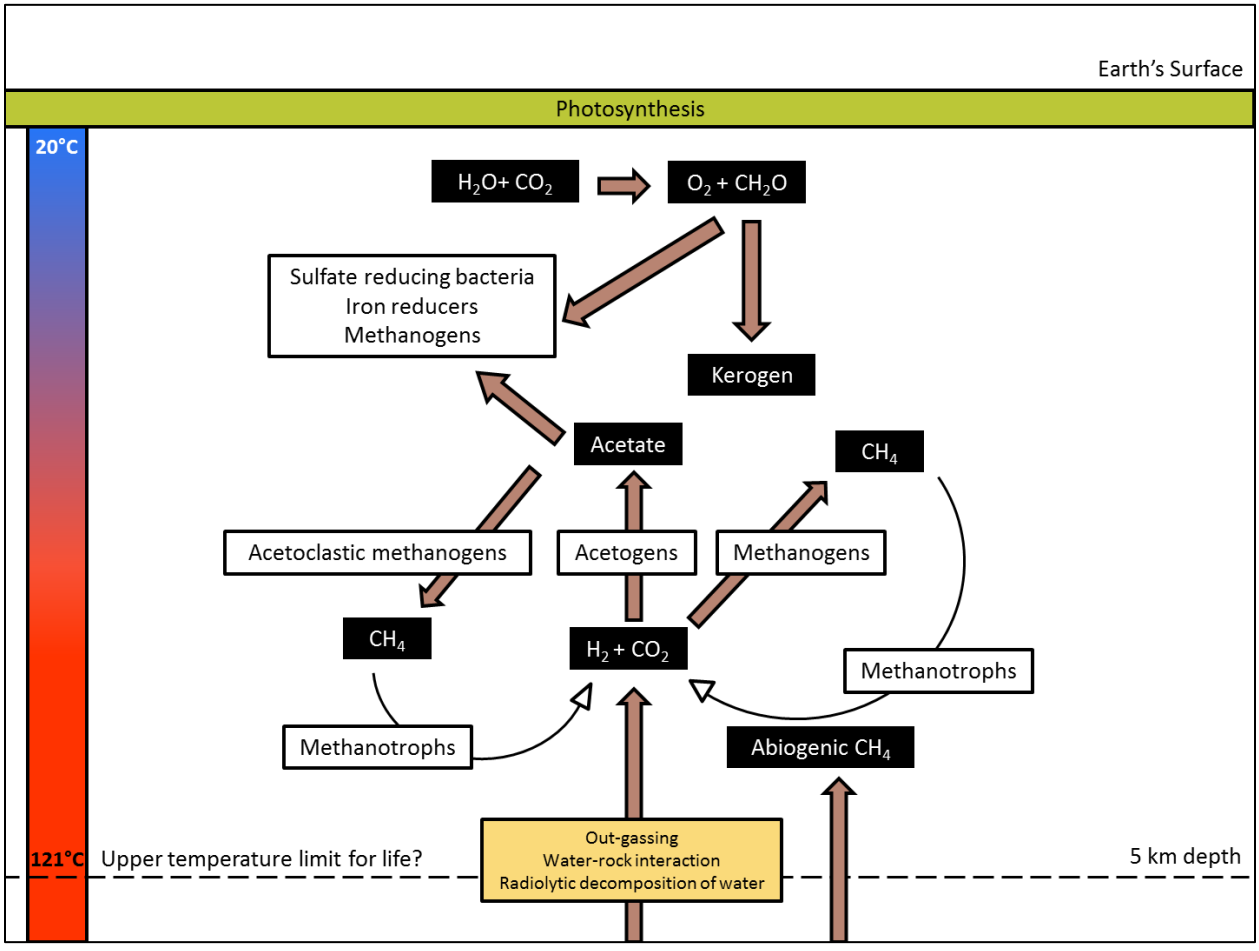

Figure 1.1. Microbial carbon cycling in a deep terrestrial subsurface hydrogen-driven microbial ecosystem (Pedersen, 2000; Stevens and McKinley, 1995).

Methane is a key potential carbon and energy source in the deep terrestrial subsurface, as it is produced by various biotic and abiotic reactions. Microorganisms have been shown to oxidize methane in both aerobic and anaerobic environments via various mechanisms (Boetius et al., 2000; Hanson and Hanson, 1996; Hinrichs et al., 1999; Hoehler et al., 1994; Joye, 2012). In aerobic environments, type I and type II methanotrophic bacteria are capable of utilizing methane as their sole carbon and energy source via the enzyme methane monooxygenase (MMO) (Hanson and Hanson, 1996). Anaerobic oxidation of methane (AOM) commonly occurs in marine subsurface sediments via a consortium of sulphate reducing bacteria and archaea (Boetius et al., 2000; Hinrichs et al., 1999; Hoehler et al., 1994). Recently, AOM has also been shown to occur by individual microbial groups: the denitrifying bacterium *Methoxymirabilis oxyfera* (Ettwig et al., 2008) and the ANME-2 clade of archaea (Milucka et al., 2012). Isotopic evidence for AOM by type I and type II methanotrophic bacteria in subsurface environments suggests that these bacteria may be capable of oxidizing methane in anaerobic conditions, using an alternative electron acceptor, such as sulphate, manganese or iron (Beal et al., 2009; Ettwig et al., 2008; Mills et al., 2010).

1.1.4. Sampling sites

Sampling from the deep terrestrial subsurface biosphere presents many challenges, due to the inaccessibility of these microbial systems. A common approach used to sample deep subsurface microbial communities is drilling and coring (Fredrickson and Fletcher, 2001; Fredrickson and Onstott, 1996). In addition to the high costs associated with drilling, it is difficult to eliminate the introduction of external contaminants to subsurface samples. The equipment used for drilling requires a lubricant which, in many cases, consists of petroleum products (Hallmann et al., 2011). Pure water can be used as a drilling fluid to minimize sample

contamination; however, this can lead to equipment failure and is often found to contain low levels of contamination (Fredrickson and Onstott, 1996; Sherman et al., 2007). As a result, it is difficult to unequivocally state that any biosignatures observed in a sample are derived from the subsurface environment, rather than introduced during sampling or sample handling. For this reason, sensitive tracer techniques, such as dyes or fluorescent microspheres, can be applied to the drilling water to assess the potential impact of contamination during sampling (Fredrickson and Fletcher, 2001; Onstott et al., 2003). Furthermore, the geochemistry or microbiology of the subsurface samples can be compared to the drilling fluid and other sampling materials to distinguish between indigenous microbial communities and contamination (Fredrickson and Onstott, 1996).

Deep mining sites, reaching depths of several kilometers, can also provide exceptional access to deep terrestrial subsurface systems via exploratory boreholes (Davidson et al., 2011; Lin et al., 2006a; Onstott et al., 2006a; Pfiffner et al., 2006; Wanger et al., 2006). These boreholes often tap into large reservoirs of subsurface fracture water that can be sampled and analyzed for microbial communities. Extremely low microbial cell densities typically observed in deep terrestrial subsurface environments require large sample sizes in order to detect microbial cells or associated biosignatures. Via deep mine boreholes, thousands of liters of water can be filtered and concentrated, and extremely low levels of microbial biomass can be detected as a result. Nevertheless, there is potential for the introduction of contamination during the process of borehole drilling. As a precaution, the natural fracture water should be allowed to flow from the borehole for a sufficient amount of time prior to sampling, in order to flush out any potential contaminants introduced during drilling.

1.1.5. Previous investigations of deep subsurface microbial communities

Microorganisms in the deep subsurface are identified and quantified using a combination of phospholipid fatty acid (PLFA) analyses, genetic analyses and culturing experiments. A wide array of studies across various field sites have identified a range of microbial ecosystems, consisting of heterotrophic organisms, including sulfate-reducing bacteria, iron-reducing bacteria and nitrogen-fixing bacteria, and chemolithoautotrophs, including methanogens, acetogens, sulphate reducers and iron reducers (Chivian et al., 2008; Davidson et al., 2011; Itavaara et al., 2011; Slater et al., 2006). The microbial abundances vary considerably from site to site; however, cell densities are found to range from 10^3 to 10^6 cells per mL of groundwater or gram of sediment, and generally decrease with depth (Fang and Zhang, 2011; Itavaara et al., 2011; Onstott et al., 1998; Pedersen, 2000; Whitman et al., 1998). Most microbial diversity studies are conducted by analyzing fracture water samples and their associated biofilms. The bulk of the deep terrestrial subsurface biosphere is believed to concentrate within these fracture systems (Onstott et al., 2003). Rock core samples have also been analyzed, although to a lesser extent, to assess the abundance and diversity of deep subsurface microorganisms living within natural pore spaces in crystalline rocks (Colwell et al., 1997; Onstott et al., 2003; Onstott et al., 1998). Onstott et al. (2003) combined PLFA analysis with 16S rDNA sequencing to assess the microbial diversity and approximate viable biomass within deep South African mine rock samples. Their results revealed a community dominated by thermophilic sulfate reducing bacteria, and a cell density of approximately 10^3 cells per gram (Onstott et al., 2003). The overall heterogeneity observed between studies and sample types suggests for a site-specific deep subsurface biosphere.

Fracture water samples from deep mines have been explored in several studies to assess the presence and diversity of microbial communities. Lin et al. (2006b) analyzed fracture water sampled from 2.8 kmbls in the Mponeng mine in South Africa. The results from their 16S rRNA analysis revealed a very low microbial diversity, dominated by one single phylotype of the Firmicutes phylum (Lin et al., 2006b). Chivian et al. (2008) performed a follow-up metagenomic analysis of filtered fracture water from the same site in the Mponeng mine in South Africa. Their results were striking: *Candidatus Desulforudis audaxviator*, a thermophilic sulfate-reducing bacterium from the Firmicutes phylum, comprised >99.9% of the microorganisms identified. This bacterium is abundant in almost all fracture fluids that have been sampled from the Witwatersrand Basin in South Africa (Chivian et al., 2008). Capable of sulfate reduction, carbon fixation and sporulation, this bacterium is capable of an entirely independent lifestyle, completely isolated from the Earth's photosphere. Fracture fluids analyzed from other sites in the Witwatersrand Basin have been found to be much more diverse (Davidson et al., 2011). In addition to bacteria, a range of archaeal species, including methanogens, have also been identified in deep terrestrial fracture waters in South African mines (Takai et al., 2001; Ward et al., 2004).

Until recently, life in the deep subsurface was believed to consist solely of single-celled microorganisms. A combination of high temperatures, low oxygen, low nutrient availability and lack of physical space were assumed to preclude the evolution of multicellular organisms in the deep subsurface. However, Borgonie et al. (2011) discovered multicellular eukaryotic species, *Halicephalobus mephisto*, from the phylum Nematoda in 3,000 to 12,000 year-old fracture water from deep mines in South Africa. These organisms, found at 3.6 kmbls, reproduce asexually, graze on subsurface biofilms, and are capable of tolerating temperatures of up to 41°C (Borgonie

et al., 2011). This discovery holds large implications for the search for extraterrestrial life, suggesting that multicellular life in the deep subsurface of other planetary bodies is, in fact, a possibility.

1.1.6. Astrobiological implications

Investigating Earth's deep terrestrial subsurface biosphere provides insight and support for the theory that life may exist beneath the surface of other planetary bodies. Life on the surface of Earth is ubiquitous for several reasons. Most importantly, liquid water, believed to be a requirement for life to evolve, is plentiful at the Earth's surface, largely due to ambient surface temperatures maintained by greenhouse gases in a thick atmosphere. Secondly, Earth's ozone layer currently protects surface life from bombarding ultraviolet radiation (UV). Many rocky planets and moons lack shielding atmospheres and, as a consequence, their surfaces are continually exposed to damaging levels of UV radiation (Lammer et al., 2009). In addition, liquid water on the surfaces of other planetary bodies is believed to be a relatively uncommon feature (Chapelle et al., 2002; Lammer et al., 2009). Mars is a major focus in astrobiological research, largely due to its close proximity to Earth, but also because its geochemical and physical properties are potentially suitable for life. The surface of Mars is generally believed to be uninhabitable, due to the planet’s thin atmosphere and lack of liquid water at the surface; however, evidence suggests that Mars held liquid water on its surface in the past, and that its subsurface may contain liquid water today (Pfiffner et al., 2008). If life on Mars exists, it is likely isolated in the subsurface of the planet. For this reason, chemolithoautotrophic self-sustaining systems in the subsurface of Mars are particularly intriguing for understanding the mechanisms by which microbial communities could survive in the subsurface of other planetary bodies.

Exploring microbial metabolisms active in Earth's deep terrestrial subsurface can also be used as a method to further understand early life on Earth, prior to the rise of photosynthesis. The ability of microbial communities to survive without input from surface photosynthetic activity suggests that some of the earliest forms of life on Earth could have been deep terrestrial subsurface chemolithoautotrophs. This also implies that microorganisms could have survived deep within Earth's continental crust as major meteorite impacts bombarded the early Earth. Potential sterilization of the Earth's surface due to cosmological events such as the late heavy bombardment would have had little to no impact on the survival of the deep terrestrial subsurface biosphere. This holds large implications for the potential origins of life on other planetary bodies as well, where similar meteorite impacts would have occurred.

Finally, Earth's deep subsurface biosphere provides an opportunity to explore the ultimate physical and geochemical limitations for terrestrial life. The "critical zone" is defined as the outer zone on a planetary body where all of the chemical, physical and biological processes required for life are occurring and interacting (Akob and Kusel, 2011). Earth's critical zone is currently hypothesized to extend down to depths greater than 5 kmbls, based on estimations of temperature, porosity and nutrient availability (Akob and Kusel, 2011). Currently, life on Earth is believed to require a set of criteria, including tolerable temperatures (< 0-113°C), pressures (< 100 Mpa), pH (< 12), UV radiation and sufficient water activity (Lammer et al., 2009; Sharma et al., 2002). Deep terrestrial research has not yet fully reached these extremes; however, the investigation of these systems has only recently begun and there are certainly many more discoveries to be made.

1.2. RESEARCH APPROACHES

For this Master's thesis project, two deep terrestrial subsurface environments were investigated for microbial communities: (1) water-filled fracture networks accessed through deep mines in the Witwatersrand Basin of South Africa and (2) natural pore spaces in deep sedimentary rock cores sampled from the Michigan Basin, Canada. A combination of phospholipid fatty acid (PLFA) analysis and carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) were used to investigate microbial community structures and active microbial carbon cycling within six water-filled fracture networks. The sedimentary rock cores were analyzed for PLFAs to assess the presence and abundance of microbial communities living within the natural pore spaces of a set of six sedimentary rock samples from various depths.

1.3. PHOSPHOLIPID FATTY ACID ANALYSIS

All microbial cells, including bacteria, eukarya and archaea, contain cellular membranes, and analyzing the microbial lipids that compose these membranes can provide information about total microbial biomass, community structure, and metabolic activity of microbial communities (Vestal and White, 1989). Microbial cell membranes are composed of a wide variety of lipids and the types of lipids composing these membranes are dependent on the microorganism, as well as environment factors. The various classes of lipids include phospholipids, waxes, sterols, and hopanoids. Ester-linked phospholipid fatty acids (PLFAs), in particular, are useful for assessing the total abundance and compositions of viable bacterial and eukaryotic microbial communities. In addition, carbon isotope signatures ($\delta^{13}C$ and $\Delta^{14}C$) of the PLFAs in an environment can be diagnostic for specific microbial metabolisms. As a result, combining PLFA analysis with carbon isotope analyses can be used to elucidate active microbial processes, without the use of culturing experiments or genetic analyses.

1.3.1. PLFA chemical structure and identification

Phospholipids are the primary building blocks of bacterial and eukaryotic microbial cell membranes (Dowhan and Bogdanov, 2002). The arrangement of phospholipids in the cell membrane forms a semi-permeable phospholipid bilayer that controls the passage of ions and molecules into and out of the cell (Figure 1.2). The phospholipid chemical structure consists of a polar hydrophilic phosphate group and a glycerol molecule bonded to two hydrophobic fatty acid chains. In bacteria and eukarya, the linkage between the fatty acid chains and the glycerol group is an ester bond; whereas, in archaea, the linkage is an ether bond (Green and Scow, 2000). Variations in PLFA structures observed among bacteria and eukarya include saturated, mono-unsaturated, poly-unsaturated, branched and cyclopropyl PLFAs with varying fatty acid chains lengths.

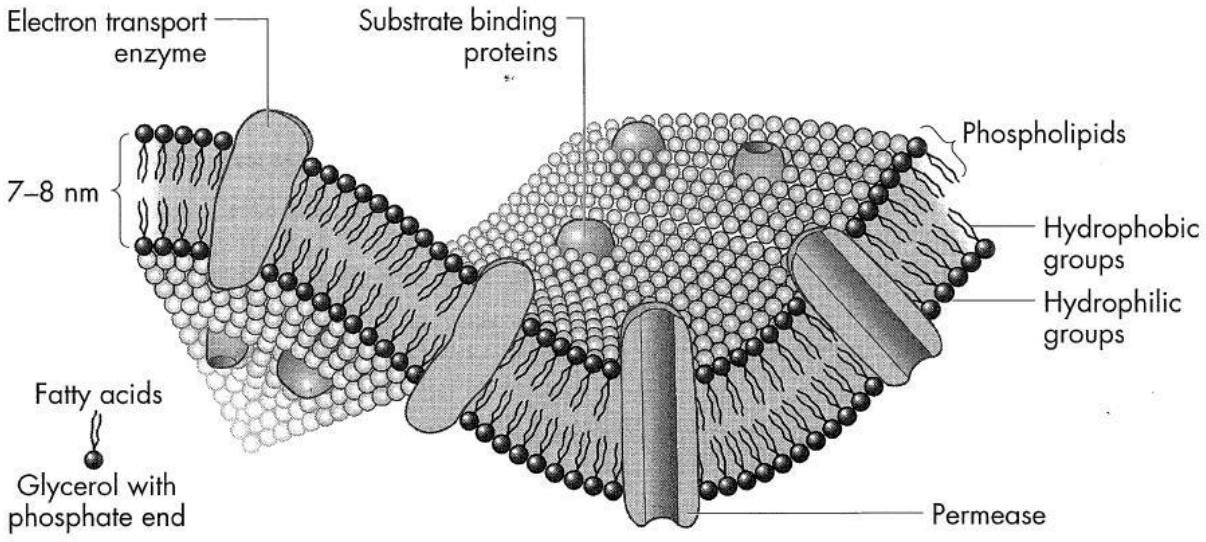

Figure 1.2. Microbial cell membrane composed of a phospholipid bilayer (Konhauser, 2007)

PLFAs can be extracted and analyzed from environmental samples using the Bligh and Dyer (1959) method, whereby a sample is extracted and sonicated in a solvent solution of dichloromethane, methanol and phosphate buffer, at a ratio of 1:2:0.8. The resulting PLFA

extracts are separated into non-polar, neutral, and polar fractions using silica gel chromatography (Guckert et al., 1985). Ester-linked phospholipids recovered from the polar fractions are converted to fatty acid methyl esters (FAMEs) via mild-alkaline methanolysis and subsequently purified by a secondary silica gel chromatography (Guckert et al., 1985). Individual microbial FAMEs are identified and quantified using gas chromatography-mass spectrometry (GC-MS).

1.3.2. PLFAs as indicators for viable microbial communities

Phospholipids are known to degrade rapidly following cell death via the hydrolysis of the phospholipid polar head group and fatty acid chains (Harvey et al., 1986; Logemann et al., 2011; White et al., 1979). In surface environments, this process is known to occur within days to weeks following cell death (White et al., 1979). As a result, intact phospholipids are considered to be indicative of viable microbial communities in an environment. In deep terrestrial subsurface systems, where oxygen levels are expected to be low, rates of phospholipid degradation are potentially slowed by up to 40% (Logemann et al., 2011). Furthermore, phospholipid degradation is primarily a biotic process and, in the absence of an active microbial community, phospholipid degradation is expected to occur very slowly (Logemann et al., 2011). Despite potential variations in phospholipid degradation rates in subsurface environments, PLFAs can provide a range of information about the viable bacterial and eukaryotic communities, including total viable cell abundances, viable community structures and active microbial metabolisms (see Sections 1.3.3, 1.3.4, 1.4.0).

1.3.3. PLFA-based cell density estimates

The total concentrations of PLFAs extracted from an environmental sample can be used to estimate the microbial cell density in a system. Based on assumptions regarding the average size of a microbial cell and the number of PLFAs composing a microbial cell membrane,

conversion factors can be used to convert PLFA concentrations to microbial cell densities. For deep subsurface environments, conversion factors commonly used to convert PLFA concentrations to microbial cell densities range from $2x10^4$ to $6x10^4$ cells per picomole PLFA (Green and Scow, 2000). Based on these PLFA conversion factors and other methods for cell enumeration, typical microbial cell densities within deep subsurface environments have been found to range from 10^3 to 10^6 cells per mL/gram of rock (Kallmeyer et al., 2012; Parkes et al., 1994; Pedersen, 1993; Whitman et al., 1998).

1.3.4. PLFA biomarkers

Microbial cell membranes can contain a range of PLFA compositions and, in some cases, particular PLFA structures are diagnostic for specific microbial groups. The presence of these biomarker PLFAs are considered as indicators for specific microorganisms in an environment. Table 1.1 lists commonly identified PLFA biomarkers and the corresponding microbial groups represented by these biomarkers. For example, type I and type II methanotrophs can be identified by the presence of the monounsaturated PLFAs 16:1ω8c and 18:1ω8c, respectively (Hanson and Hanson, 1996). Sulphate reducing bacteria can be identified by the branched PLFA 10Me16:0 (Dowling et al., 1986). In some cases, particular PLFA structures reflect microbial responses to environmental stressors. For example, microbial communities subjected to severe nutrient deprivation have been shown to produce a greater mole percentage of cyclopropyl PLFAs and a greater proportion of *trans* versus *cis* monoenoic PLFA configurations (Kieft et al., 1994; Petersen and Klug, 1994). The production of *iso* over *anteiso* fatty acids has also been shown to relate to environmental stress factors, such as high temperatures (Kieft et al., 1994; Petersen and Klug, 1994). Identifying PLFA biomarkers and PLFA stress indicators can be used to assess the

composition of a microbial community and the impact of environmental conditions on the microbial community.

Table 1.1. Common PLFA biomarkers and associated microbial groups (Green and Scow, 2000)

PLFA Biomarker	Interpretation	Reference
*i*15:0, *a*15:0, 15:0, 16:1 *i*17:0, 17:0, 18:1	Common in most bacteria	Tunlid and White (1992)
Branched PLFAs	Gram-positive bacteria	Hardwood and Russell (1984)
Monoenoic PLFAs	Gram-negative bacteria	Wilkinson (1988)
10Me16:0	Sulphate-reducing bacteria (*Desulfobacter*)	Dowling et al. (1986)
16:1ω8c	Type I methanotrophs	Makula (1978)
18:1ω8c	Type II methanotrophs	Makula (1978)

1.4. CARBON ISOTOPE ANALYSES

Carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) of microbial cellular components (PLFAs) and potential carbon sources can be used to investigate active microbial carbon cycling within the natural environment. $\delta^{13}C$ analysis provides insight into the active microbial metabolisms in a system, whereas $\Delta^{14}C$ analysis provides information about microbial carbon sources. The combination of these two techniques provides information about *in situ* microbial carbon sources and cycling in the deep terrestrial subsurface.

1.4.1. Principles of $\delta^{13}C$ analysis

There are three naturally-occurring isotopes of carbon (^{12}C, ^{13}C and ^{14}C), the most abundant of which is ^{12}C, composing 98.9% of carbon in the environment (Brocks and Pearson, 2005). ^{12}C and ^{13}C are stable carbon isotopes and ^{14}C is an unstable, radioactive isotope. $\delta^{13}C$ is a measurement of the ratio of the two stable isotopes of carbon (^{13}C and ^{12}C), compared to an internationally established reference standard, the Vienna Pee Dee Belemnite (VPDB) carbonate ($^{13}C/^{12}C$ ratio of 0.0112372) (Boschker and Middelburg, 2002). The relative $\delta^{13}C$ value of a given sample is reported in parts per million (‰) and calculated using the following equation:

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

The $\delta^{13}C$ values of carbon sources and microbial biomass are largely controlled by kinetic isotope effects (KIE) involved in biological reactions. Chemical bonds containing the heavier stable isotope (^{13}C) require more energy to break, relative to bonds that contain the lighter stable isotope (^{12}C) (Brocks and Pearson, 2005). As a result, there is a preferential utilization of ^{12}C over ^{13}C during biological reactions. The magnitude of this KIE is dependent on the metabolic pathway and enzymes involved. Measuring the $\delta^{13}C$ value of microbial cellular components (such as PLFAs) and potential microbial carbon sources (such as inorganic carbon, organic carbon and methane) can provide insight into the active microbial metabolisms taking place in an environment.

1.4.2. Controls on PLFA $\delta^{13}C$ signatures

The $\delta^{13}C$ value of PLFAs is determined by three factors: (1) the $\delta^{13}C$ value of the source of substrate carbon, (2) the carbon assimilation pathway involved in the biological uptake of

carbon, and (3) KIEs involved in the biological synthesis of PLFAs. The KIEs involved in the biological uptake of carbon, due to the preferential utilization of the lighter isotope (^{12}C), is the primary control on the $\delta^{13}C$ values of carbon sources and microbial cell biomass (Boschker and Middelburg, 2002). The magnitude of the KIE involved in the uptake of carbon depends on the carbon assimilating pathway.

1.4.2.1. Microbial carbon sources

Microorganisms are capable of utilizing a range of inorganic and organic carbon sources to incorporate carbon into their cell biomass. Autotrophic organisms are the "primary producers" of organic material; they utilize inorganic carbon sources (CO_2 and dissolved inorganic carbon (DIC)) to produce complex organic compounds. Heterotrophic organisms are the "consumers" of organic material, utilizing only organic carbon sources to build their cell biomass. Methanotrophic bacteria are capable of using methane as their sole carbon and energy source (Hanson and Hanson, 1996). The carbon isotopic signature of the substrate carbon greatly influences the carbon isotope signature of microbial cell biomass, and the carbon assimilation pathway (see Section 1.4.2.2) determines the magnitude of the fractionation involved in the uptake of a particular carbon source (Figure 1.3). Typically, inorganic carbon sources are isotopically enriched in ^{13}C, relative to organic carbon sources. This is due to the KIEs associated with the biological production of organic carbon from inorganic carbon sources (Hayes, 2001). Methane is generally highly depleted in ^{13}C, with $\delta^{13}C$ values typically ranging from -50 to -110‰, depending on the methane source (i.e. abiogenic methane vs. biogenic methane) (Boschker and Middelburg, 2002).

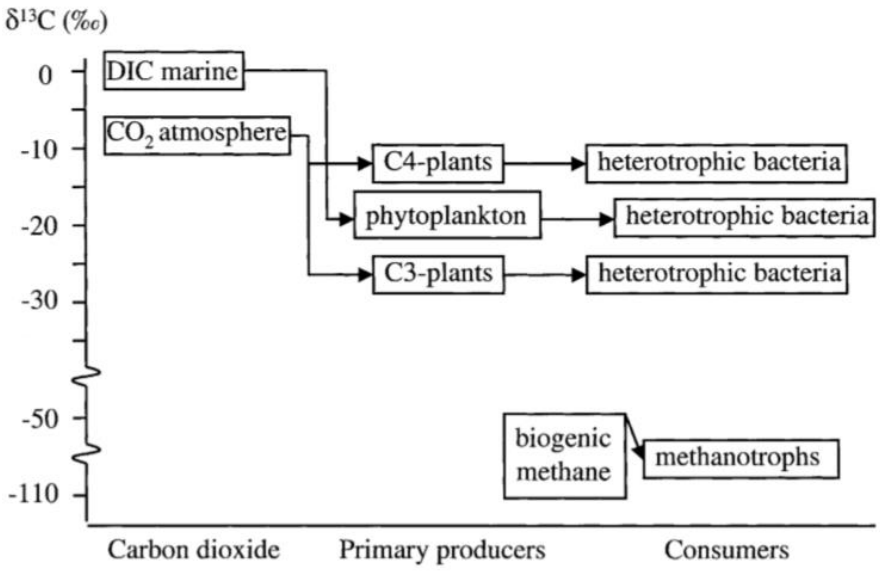

Figure 1. 3. Representation of general carbon isotopic signatures of microbial carbon sources and carbon isotope fractionations involved in carbon fixation/assimilation pathways (Boschker and Middelburg, 2002).

1.4.2.2. Carbon assimilation pathways

The mechanism by which an organism assimilates carbon largely influences the carbon isotope value ($\delta^{13}C$) of the cellular components. If the carbon source is a small molecule, such as CO_2 or methane, large KIEs are associated with the fixation or assimilation of this carbon (Hayes, 2001). In contrast, if the carbon source is a large molecule, such as carbohydrates or organic acids, KIEs involved in the assimilation of this carbon source will generally be small (Hayes, 2001).

Autotrophic microorganisms convert inorganic carbon sources (CO_2, H_2CO_3, HCO_3^-, CO_3^{2-}) to complex organic molecules via a series of carbon fixation pathways (Brocks and Pearson, 2005). Carbon isotope fractionations associated with microbial autotrophy are widely variable and can reach up to 58‰; however, the magnitude of the KIE depends on the carbon fixation pathway involved (Londry et al., 2004). The largest KIEs are associated with the Acetyl-CoA pathway, intermediate KIEs are associated with the Calvin-Benson cycle, and the smallest

KIEs are associated with the rTCA and 3-HP pathways (Brocks and Pearson, 2005). The speciation of the inorganic carbon source also influences the $\delta^{13}C$ value of the microbial biomass, as atmospheric CO_2 tends to be isotopically lighter (-6 to -8‰), dissolved HCO_3^- and CO_3^{2-} tend to be slightly isotopically heavier (-2 to +2‰), and dissolved CO_2 tends to vary, depending on whether it is derived from respiration of organic carbon or residual from methanogenesis (Brocks and Pearson, 2005).

In contrast to large KIEs involved in autotrophic metabolisms, heterotrophic metabolisms generally exhibit small KIEs (Boschker and Middelburg, 2002). The principle of "you are what you eat, plus 1‰" is used to describe the small KIEs involved in aerobic heterotrophy; the $\delta^{13}C$ values of heterotrophic biomass are generally similar to the $\delta^{13}C$ values of the organic carbon sources utilized by these organisms (Boschker and Middelburg, 2002). In anaerobic environments, the negative offset between the $\delta^{13}C$ value of the substrate organic carbon and the $\delta^{13}C$ value of the microbial cell biomass generally ranges from 1-2 ‰ (Boschker and Middelburg, 2002). In anaerobic environments, however, heterotrophic metabolisms can result in much larger KIEs (Teece et al., 1999).

Methanotrophy exhibits large KIEs, similar to autotrophy, as both of these carbon assimilation pathways involve the fixation of small C_1 metabolites (Hayes, 2001). Methanotrophic bacteria are often easily identified using $\delta^{13}C$ analysis, as their methane carbon sources are generally highly depleted relative to other carbon substrates (Boschker and Middelburg, 2002). When an organism consumes methane and incorporates this carbon into its cellular biomass, the $\delta^{13}C$ value of the cellular components is often distinctly negative. Carbon isotope fractionations involved in methanotrophy typically result in $\delta^{13}C_{PLFA}$ values that are 10-

30‰ more depleted that the methane carbon source (Jahnke et al., 1999; Summons et al., 1994; Valentine and Reeburgh, 2000; Whiticar, 1999).

1.4.2.3. PLFA synthesis

KIEs are also involved in the biological synthesis of PLFAs. PLFAs are essentially polymers of acetate derived from acetyl-CoA (Hayes, 2001). The formation of acetyl-CoA involves the production of pyruvate, and the subsequent decarboxylation of pyruvate to acetyl-CoA via the enzyme pyruvate dehydrogenase (Hayes, 2001). This reaction is believed to be responsible for the carbon isotope fractionations observed between the $\delta^{13}C$ values of microbial cell biomass and $\delta^{13}C$ values of PLFAs for most organisms (Brocks and Pearson, 2005; Hayes, 2001). In general, microbial PLFA $\delta^{13}C$ values are depleted by 3-6‰ relative to the $\delta^{13}C$ value of the total microbial biomass (Boschker and Middelburg, 2002; Hayes, 2001). In anaerobic environments, however, this carbon isotope fractionation can range from 5-10‰, due to KIEs involved in alternative mechanisms of acetyl-CoA production (Hayes, 2001; Scott and Nealson, 1994; Teece et al., 1999).

1.4.4. Principles of $\Delta^{14}C$ analysis

Variations in KIEs associated with the various potential microbial metabolisms, as well as variations in $\delta^{13}C$ values of potential carbon sources, can complicate the interpretation of $\delta^{13}C$ signatures in the environment. In cases where the $\delta^{13}C$ values do not provide a definitive interpretation of the active microbial metabolisms, radiocarbon ($\Delta^{14}C$) analysis can be used as an additional tool to elucidate microbial carbon sources and cycling. $\Delta^{14}C$ analysis is based on the fact that, unlike the lighter isotopes of carbon (^{12}C and ^{13}C), ^{14}C is radioactive and decays over time. The equation for $\Delta^{14}C$ is:

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

where

$$\delta^{14}C = \frac{^{14}C/^{12}C_{sample} - ^{14}C/^{12}C_{standard}}{^{14}C/^{12}C_{standard}} \times 1000‰$$

and the definition for $\delta^{13}C$ is analogous to that of $\delta^{14}C$ (Stuiver and Polach, 1977). $\Delta^{14}C$ is a measure of the ratio of ^{14}C to ^{12}C, relative to an oxalic acid standard. As ^{14}C decays over time, modern atmospheric carbon will have a $\Delta^{14}C$ value of 55 ± 50‰ (Turnbull et al., 2007) and an ancient carbon source (≥ 60 000 years) that contains no significant ^{14}C will have a $\Delta^{14}C$ value of -1000‰ (Stuiver and Polach, 1977). Kinetic isotope effects involved in the microbial uptake of ^{14}C are corrected for by a $\delta^{13}C$-based normalization: $2(\delta^{13}C + 25)$ (Stuiver and Polach, 1977). This term corrects for KIEs, assuming that the carbon isotope fractionation of ^{14}C is twice that of ^{13}C, with a normalization to $\delta^{13}C$ = 25‰. As such, the $\Delta^{14}C$ value of microbial cellular components, such as PLFAs, is solely dependent on the $\Delta^{14}C$ value of the microbial carbon source. The large range of $\Delta^{14}C$ values observed in nature allows the incorporation of fossil carbon into microbial biomass to be traced directly by comparing the $\Delta^{14}C$ values of PLFAs to the $\Delta^{14}C$ values of potential carbon sources (DIC, DOC and methane). Provided that there are significant levels of ^{14}C in these carbon pools, observing equivalent $\Delta^{14}C$ values for PLFA and a carbon source (DIC, DOC or methane) would indicate recent microbial incorporation of carbon from this source into the cellular components.

1.5. RESEARCH OBJECTIVES

The purpose of this thesis project was to investigate microbial communities living within a range of environments in the deep terrestrial subsurface with an aim to better understand what

microbial communities are present, what are the approximate cell abundances in these systems, and what are the range of metabolisms involved in the deep terrestrial subsurface carbon cycle. PLFA analysis and carbon isotope analyses were used to gain a big picture view of the microbial communities present and the metabolisms involved.

Chapter 1 provided a background overview of microbial life in the deep terrestrial subsurface, the implications of these microbial systems and the research performed to-date in these environments. It also provided an introduction to PLFA analysis and carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) and the applications of these techniques for investigating microbial community structure and metabolisms.

Chapter 2 describes a study of the microbial community structures, abundances and metabolisms within six deep subsurface fracture networks sampled from several deep mines in the Witwatersrand Basin of South Africa. PLFA concentrations and PLFA structures were used to assess microbial cell densities and community structures across the six subsurface systems. Carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) of the PLFAs and potential carbon sources (DIC, DOC and methane) were utilized to investigate active microbial carbon cycling across the range of geochemical conditions present in these sites. The purpose of this study was to determine what microbial community groups were present and how methane, DIC and DOC tie in with the deep subsurface carbon cycle.

Chapter 3 investigates the presence and abundance of microbial communities living within the natural pore spaces of sedimentary rock core samples from the Michigan Basin in Canada. PLFAs were extracted and analyzed from the interior material of six rock core samples, ranging from depths of 334 to 695 mbls, to assess whether viable microbial communities were

present within the rock core interiors. Comparisons between PLFA concentrations/distributions in the rock core interiors and the contaminated outer surfaces were analyzed to assess whether the PLFAs were indigenous to the rock core interiors, while addressing the question of whether PLFAs can be used as indicators for viable communities in the deep terrestrial subsurface.

Chapter 4 summarizes the major conclusions of this Master's thesis project and future research directions for exploring deep terrestrial subsurface microbial systems.

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CHAPTER 2:

EXPLORING VARIATIONS IN MICROBIAL CARBON SOURCES AND CYCLING IN THE DEEP TERRESTRIAL SUBSURFACE USING PHOSPHOLIPID FATTY ACID (PLFA) AND CARBON ISOTOPE ANALYSES

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ABSTRACT

Microbial communities and carbon cycling within six anaerobic deep terrestrial subsurface fracture water sites, sampled from the Beatrix, Driefontein, Tau Tona and Kloof gold mines in the Witwatersrand Basin of South Africa, were investigated using a combination of phospholipid fatty acid (PLFA) analysis and carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$). Sample depths ranged from 0.9 to 3.2 kilometers below land surface (kmbls). PLFA concentrations revealed very low microbial cell densities, ranging from $2x10^1$ to $5x10^4$ cells per mL of deep subsurface fracture water, which decreased as fracture water temperature increased. The extremely low bacterial cell density ($2x10^1$ cells/mL) measured for one of the deepest subsurface samples (KL445) raises the question of whether this site is an abiological system or potentially a community solely comprised of archaea. PLFA profiles of the various sites contained indicators for environmental stressors, including an increased ratio of *iso*- over *anteiso*- fatty acids in response to higher temperatures, and the presence of cyclopropyl PLFAs, potentially representing microbial responses to nutrient deprivation and/or other stress conditions. $\delta^{13}C_{PLFA}$ values, which ranged from -72.9‰ to -11.0‰, were compared to $\delta^{13}C$ values of potential carbon sources (dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), and methane) to elucidate microbial carbon sources and cycling. The most depleted $\delta^{13}C_{PLFA}$ values indicated active microbial oxidation of methane at two anaerobic sites (Dr5IPC and Be326 Bh2), potentially representing anaerobic methanotrophy by bacteria or sulphate-dependent anaerobic oxidation of methane by bacteria and archaea. There was also evidence of autotrophy in Be326 Bh2, based on comparisons between $\delta^{13}C_{PLFA}$ values and $\delta^{13}C_{DIC}$ at this site. The results from $\Delta^{14}C$ analyses of PLFAs and potential carbon sources for Dr5IPC and Be326 Bh2 were consistent with the active cycling of methane in both of these systems as well as the contribution

of autotrophy in Be326 Bh2. $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFAs and potential carbon sources from the two Tau Tona mine samples (TT109 Bh2 and TT107) indicated microbial utilization of DIC, providing evidence for autotrophically-supported ecosystems in these sites. The combination of $\delta^{13}C$ and $\Delta^{14}C$ analyses in this study was able to resolve patterns of active microbial utilization of distinct carbon sources, with methane as a major carbon source in some systems and DIC as a major carbon source in others. Overall, this evidence for predominantly methanotrophic and autotrophic communities present at depths of up to 3.2 kmbls is consistent with a self-sustaining deep terrestrial subsurface biosphere.

2.1. INTRODUCTION

2.1.1. The deep terrestrial subsurface biosphere

In crystalline rock of the Earth's deep terrestrial subsurface, water-filled fracture networks represent potential microbial habitats (Onstott et al., 2006; Onstott et al., 1998; Wanger et al., 2006). The range of metabolic processes taking place in these environments is still largely unknown, despite the potential ubiquity and global significance of these microbial systems (Onstott et al., 1998; Whitman et al., 1998). Understanding the metabolic processes involved in sustaining microbial systems in the deep terrestrial subsurface is important for developing an understanding of the limits of habitability on Earth, as well as the potential for microbial life to exist in the deep subsurface of other planetary bodies. Photosynthetically-derived organic carbon buried within Earth's subsurface can be utilized as a carbon source by subsurface microbial communities; however, in many deep terrestrial subsurface systems, this organic carbon source is limited and/or highly recalcitrant (Pedersen, 2000). Microorganisms in the deep terrestrial subsurface must, therefore, often rely on alternative carbon sources to build their cellular components. Potential alternative microbial metabolisms include autotrophic utilization of

dissolved inorganic carbon (DIC) by methanogens, acetogens, sulfate reducers or iron reducers, or microbial oxidation of methane derived from abiotic or biotic sources (Lollar et al., 2006; Pedersen, 2000; Stevens and McKinley, 1995). Methane is a key carbon pool in the deep terrestrial subsurface as it is produced autotrophically by CO_2-reducing microorganisms (methanogenesis), thermogenically by the breakdown of organic matter (thermogenesis), as well as abiotically by reactions analogous to Fischer-Tropsch synthesis (Lollar et al., 2006). Microbial oxidation of methane has been shown to occur both aerobically and anaerobically by methanotrophic bacteria, as well as anaerobically via a consortium of sulphate reducing bacteria and archaea (Beal et al., 2009; Ettwig et al., 2008; Hinrichs et al., 1999; Hoehler et al., 1994; Mills et al., 2010; Mills et al., 2013). The production and cycling of methane in the deep terrestrial subsurface could be a major component of a biosphere that is ultimately independent of the photosphere.

2.1.2. Deep subsurface chemolithotrophic microbial communities

Primary production in the deep terrestrial subsurface is believed to be based on chemolithotrophy, by which microorganisms derive their energy and electrons from inorganic chemicals. Molecular hydrogen (H_2) has been proposed as the energetic basis for these chemolithotrophic systems, as it can be produced by several abiotic geological processes, including out-gassing, water-rock interactions, and radiolytic decomposition of water (Lin et al., 2005; Pedersen, 1997; Stevens and McKinley, 1995). A range of chemolithotrophs, including methanogens, acetogens, sulfate reducers and iron reducers, are capable of utilizing H_2 directly (Fang and Zhang, 2011). Methanogens and acetogens, coupling CO_2 reduction to H_2 oxidation, can produce methane and acetate, respectively. Acetoclastic methanogens contribute to the methane pool by converting acetate to methane gas. This methane gas can be used as both a

carbon source and energy source by microbial communities via both aerobic and anaerobic oxidation reactions (Beal et al., 2009; Ettwig et al., 2008; Hinrichs et al., 1999; Hoehler et al., 1994; Mills et al., 2010; Mills et al., 2013). In addition to fueling autotrophic microbial communities, H_2 is also required for Fischer-Tropsch synthesis reactions, whereby CO/CO_2 and H_2 react to produce hydrocarbons of various molecular weights, including methane (Lin et al., 2005; Lollar et al., 2002). This mechanism of abiogenic hydrocarbon production provides another source of methane and higher hydrocarbons, to subsurface microbial communities, independent of photosynthetically-derived organic matter.

2.1.3. Mechanisms of microbial oxidation of methane

Microbial utilization of methane as a carbon source requires the ability to oxidize methane either aerobically or anaerobically, depending on the oxygen levels within the environment. There are currently several mechanisms by which various microbial groups, including bacteria and archaea, are known to oxidize methane (Joye, 2012). Type I and Type II methanotrophic bacteria are capable of oxidizing methane in aerobic conditions via the enzyme methane monooxygenase (MMO) (Hanson and Hanson, 1996). Anaerobic oxidation of methane (AOM) is thought to most commonly occur as a cooperative process between methanotrophic archaea (termed "ANME" for "anaerobic methanotrophs") and sulphate reducing bacteria (Boetius et al., 2000; Hinrichs et al., 1999; Hoehler et al., 1994). This association between ANME and sulphate reducing bacteria is thought to oxidize methane to CO_2 while reducing sulphate to hydrogen sulphide in anoxic sediments (Boetius et al., 2000). AOM has also been shown to occur without the presence of a microbial consortium. For example, the denitrifying bacterium *Methoxymirabilis oxyfera* is capable of producing its own oxygen in order to fuel AOM without a syntrophic partner (Ettwig et al., 2008). The ANME-2 clade of archaea has also

been shown to perform AOM on its own, by single-handedly mediating both methane oxidation and sulphate reduction (Milucka et al., 2012). AOM by type I and type II methanotrophs has not yet been confirmed; however, phospholipid fatty acid biomarkers for type II methanotrophs have been identified in several anaerobic subsurface systems, combined with evidence for microbial cycling of methane. These observations could be an indication that type II methanotrophs are capable of methanotrophy in the absence of oxygen, using an alternative terminal electron acceptor such as sulphate, manganese or iron (Beal et al., 2009; Ettwig et al., 2008; Mills et al., 2010).

2.1.4. Assessing the presence, structure and metabolic state of subsurface microbial communities

Phospholipid fatty acid (PLFA) analysis provides a "snapshot" of the viable microbial community in an environment and an estimate of the total bacterial and eukaryotic microbial cell biomass. Phospholipids are a major component of bacterial and eukaryotic microbial cell membranes and PLFA degradation is known to occur within days to weeks following cell death (Green and Scow, 2000; White, 1993; White et al., 1979). As a result, analyzing the fatty acid structures derived from intact phospholipids provides information about the viable microbial community. Firstly, PLFA concentrations can be used to estimate microbial cell densities in an environment, based on known conversion factors (Green and Scow, 2000). Secondly, the chemical structures of the PLFAs can be used to characterize microbial communities and potential environmental stressors. Certain PLFA structures are unique to particular microbial groups and serve as biomarkers for these organisms (e.g. 10Me16:0 for sulphate-reducing bacteria, or 16:1ω8c and 18:1 ω8c for methanotrophs) (Green and Scow, 2000; Hanson and Hanson, 1996). Microorganisms are also known to produce certain PLFAs in response to

environmental stressors (e.g. increased production of cyclopropyl PLFAs in response to stressors such as nutrient deprivation, or an increased ratio of *iso*- over *anteiso*- PLFAs in response to high temperatures). The presence of these PLFA structures can provide insight into the impact of subsurface environmental conditions (Kieft et al., 1994; Petersen and Klug, 1994).

2.1.5. Investigating microbial carbon cycling using $\delta^{13}C$ and $\Delta^{14}C$ analysis

Stable carbon isotope analysis ($\delta^{13}C$) of PLFAs and potential microbial carbon sources provides insight into active microbial carbon cycling in an environment. $\delta^{13}C$ signatures within microbial systems are largely influenced by biological carbon isotope fractionations, whereby microorganisms preferentially consume the lighter isotope of carbon (^{12}C). This preferential utilization of the ^{12}C over ^{13}C is caused by a faster rate of reaction associated with ^{12}C-containing molecules. The magnitude of this kinetic isotope effect (KIE) depends on the pathway involved, and comparing the $\delta^{13}C$ value of PLFAs to potential carbon sources can provide insight into which metabolic pathways are active. The $\delta^{13}C$ value of microbial PLFAs depends on the following: (1) the $\delta^{13}C$ value of the carbon source; (2) kinetic isotope effects (KIEs) associated with the carbon assimilation pathway (autotrophy vs. heterotrophy vs. methane oxidation); and (3) KIEs involved in the microbial synthesis of PLFAs (Boschker and Middelburg, 2002; Hayes, 2001). Comparing the $\delta^{13}C$ values of PLFAs to the $\delta^{13}C$ values of dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and methane can potentially distinguish between microbial autotrophy, heterotrophy and methane oxidation. Carbon fixation pathways involved in autotrophic metabolisms generally produce organic matter that is highly depleted in ^{13}C relative to the inorganic carbon source (DIC) and the extent of this carbon isotope fractionation is highly variable (Boschker and Middelburg, 2002). For example, autotrophic sulfate reducing bacteria, have been found to produce PLFAs that are up to 58‰ more depleted in ^{13}C than their carbon

source (DIC); whereas, some autotrophic bacteria produce PLFAs that are only several ‰ more depleted than DIC (Boschker and Middelburg, 2002; Londry et al., 2004). The products of heterotrophic metabolisms in aerobic environments generally show little carbon isotope fractionation, producing $\delta^{13}C_{PLFA}$ values that are typically 4-8‰ more depleted than the organic carbon source; however, in anaerobic environments, heterotrophic bacteria have been shown to produce PLFAs that are up to 21‰ more depleted than their organic carbon substrate (Boschker and Middelburg, 2002; Teece et al., 1999). Microbial oxidation of methane generally results in very depleted $\delta^{13}C_{PLFA}$ values, as $\delta^{13}C_{Methane}$ values are typically very negative and carbon isotope fractionations ($\Delta\delta^{13}C_{Methane\text{-}PLFA}$) can range from 10-30‰, depending on the mechanism involved (Jahnke et al., 1999; Valentine and Reeburgh, 2000; Whiticar, 1999).

The carbon isotope separations between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{methane}$ may provide some insight into the microbial production and utilization of methane in a system; however, a combination of carbon and hydrogen isotope analyses ($\delta^{13}C$ and $\delta^{2}H$) of methane and higher hydrocarbons are required in order to fully distinguish between the various sources of methane in the deep terrestrial subsurface (Lollar et al., 2002). During the production of methane via methanogenesis in a closed system, KIEs cause the methane pool to become isotopically lighter ($\delta^{13}C_{Methane}$ becomes more negative) and the residual DIC pool to become isotopically heavier ($\delta^{13}C_{DIC}$ becomes more positive). As a result, the KIEs associated with methanogenesis can produce carbon isotope separations between DIC and methane ranging from 55‰ to 95‰ (Whiticar, 1999). Microbial oxidation of methane tends to counteract this fractionation effect, reducing the carbon isotope separation between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$. Through the same principle as outlined above, the lighter carbon isotope of methane is preferentially consumed, the residual methane pool becomes isotopically heavier ($\delta^{13}C_{Methane}$ becomes more positive) and the DIC pool

becomes isotopically lighter ($\delta^{13}C_{DIC}$ becomes more negative). Overall, the mass balance between carbon pools during microbial oxidation of methane tends to reduce the carbon isotope separation between DIC and methane. Interpreting the carbon isotopic compositions of $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$ to gain insight into microbial cycling of methane requires taking into consideration both of these effects.

Variations in kinetic isotope effects associated with the various potential metabolisms and variations in the $\delta^{13}C$ values of potential carbon sources may complicate the interpretation of $\delta^{13}C$ signatures. In systems where the measured $\delta^{13}C$ values do not provide a definitive interpretation of the microbial metabolisms involved, radiocarbon ($\Delta^{14}C$) analysis can be used as an additional tool to differentiate microbial carbon sources. Using a combination of $\delta^{13}C$ and $\Delta^{14}C$ approaches provides a greater level of certainty for the interpretation of microbial carbon cycling in a system. $\Delta^{14}C$ analysis has been used to trace the flow of microbial carbon sources in a range of environments (Brady et al., 2009; Mills et al., 2010; Mills et al., 2013; Slater et al., 2006; Slater et al., 2005). $\Delta^{14}C$ is a measure of the ratio of ^{14}C to ^{12}C, relative to an oxalic acid standard. As ^{14}C decays over time, modern atmospheric carbon will have a $\Delta^{14}C$ value of 55 ± 50‰ (Turnbull et al., 2007) and an ancient carbon source (≥ 60 000 years) that contains no significant ^{14}C will have a $\Delta^{14}C$ value of -1000‰ (Stuiver and Polach, 1977). Kinetic isotope effects involved in the microbial uptake of ^{14}C are corrected for by a $\delta^{13}C$-based normalization. As such, the $\Delta^{14}C$ value of microbial cellular components, such as PLFA, is solely dependent on the $\Delta^{14}C$ value of the microbial carbon source. The large range of $\Delta^{14}C$ values observed in nature allows the incorporation of fossil carbon into microbial biomass to be traced directly by comparing the $\Delta^{14}C$ values of PLFAs to the $\Delta^{14}C$ values of potential carbon sources (DIC, DOC and methane). Provided that there are significant levels of ^{14}C in these carbon pools, observing

equivalent $\Delta^{14}C$ values for PLFA and a carbon source (DIC, DOC or methane) would indicate recent microbial incorporation of carbon from this source into the cellular components. In addition, comparing the $\Delta^{14}C$ values of DIC and methane can provide further insight into active methane cycling (Slater et al., 2006). The microbial production of methane from DIC will tend to shift the $\Delta^{14}C_{Methane}$ value towards $\Delta^{14}C_{DIC}$. Likewise, the microbial oxidation of methane to DIC will produce $\Delta^{14}C_{DIC}$ values that are shifted towards $\Delta^{14}C_{Methane}$.

2.1.7. Exploring deep subsurface communities via deep mine boreholes

Deep terrestrial subsurface microbial communities are largely inaccessible due to the challenges and high costs involved in sampling from several kilometers depth. However, deep mining sites and their network of tunnels and crosscuts allow exceptional access to the deep terrestrial subsurface. The Witwatersrand Basin of South Africa hosts the world's deepest mines, some of which exceed 4 kilometers below land surface (kmbls). During the course of normal mining operations, the advancing tunnels and exploratory boreholes facilitate sampling for microbial communities by intersecting natural water-bearing fractures. An advantage of sampling from water-bearing fractures is that a very low microbial biomass system can be concentrated via filtering. Extremely low microbial cell densities typically observed in these environments, often reaching cell densities as low as 10^3 cells per mL, requires filtering thousands of liters of deep subsurface fracture water from the borehole in order to collect sufficient biomass for PLFA analysis and carbon isotope analyses. Other challenges involved in subsurface sampling include capturing representative samples of indigenous subsurface microbial communities, avoiding the introduction of contaminants during borehole drilling and sampling, and demonstrating that any observed biosignatures are derived from the subsurface system.

In this study, PLFAs were sampled and analyzed from six deep mine sites in South Africa (Dr5IPC, Be326 Bh2, Be326 Bh1, TT109 Bh2, TT107 and KL445) to investigate the presence and activity of subsurface microbial communities. The concentrations of PLFAs in each fracture water system were measured to assess microbial cell densities. The PLFA profiles from each site were used to interpret the microbial community compositions and identify potential indicators for environmental stressors. Carbon isotopic compositions ($\delta^{13}C$ and $\Delta^{14}C$) of the PLFAs and potential microbial carbon sources (DIC, DOC and methane) were measured and compared to elucidate microbial carbon sources and cycling within the six deep subsurface systems.

2.2. SITE DESCRIPTIONS

Fracture water samples for this study were collected from four gold mines (Beatrix, Driefontein, Tau Tona and Kloof) in the Witwatersrand Basin of South Africa, representing different geographical locations, geologies and depths. The sampling sites from Beatrix and Driefontein have been previously described by Borgonie et al. (2011). The geology of the region has been previously described by Lin et al. (2005) and Onstott et al. (2006).

In brief, the Witwatersrand Basin of South Africa is a large Archean, intracratonic basin composed of the 2.9 Ga Witwatersrand Supergroup (quartzite, shale and volcanic strata), overlain by the 2.7 Ga Ventersdorp Supergroup (mafic volcanic sequence), and the 2.5 Ga Transvaal Supergroup (dolomite). Strata in the eastern and southern portions of the Witwatersrand Basin are overlain by the Permo-Carboniferous Karoo sandstone and shales. Beatrix mine (Gold Fields Ltd.) is located near the towns of Welkom and Virginia, approximately 240 kilometers southwest of Johannesburg, in the Free State province of South Africa. The mine is located along the Southern rim of the Witwatersrand Basin. The two boreholes (Be326 Bh1 and Be326 Bh2) are located on the level 26 of the #3 shaft at 1.3 kmbls.

They are located in the Witwatersrand Supergroup which, at this location, is overlain by 400-800m of the Carboniferous Karoo sediments. Driefontein mine (Gold Fields Ltd.) is situated 70 kilometers west of Johannesburg near Carletonville in the Gauteng Province of South Africa. The mine is located on the North Western rim of the Witwatersrand Basin. The borehole sampled (Dr5IPC) was located in the intermediate pumping chamber (IPC) at 0.9 kmbls of #5 shaft and intersects the regional Transvaal dolomite aquifer. Tau Tona mine (Anglo Gold Ashanti Ltd.) is located in close vicinity to Driefontein mine, near Carletonville. The sampling sites (TT107 and TT109 Bh2) were nearly horizontal boreholes located on levels 107 and 109 at depths of 3.1 and 3.2 kmbls, respectively. Kloof mine (Gold Field Ltd.) is located 60 kilometers west of Johannesburg, on the North Western rim of the Witwatersrand Basin. The fracture zone sampled, known as Danies fault zone, is parallel to other faults and dykes of the Ventersdorp Supergroup (2.7 Ga). The borehole sampled in this study (KL445) is on level 45 at the #4 Shaft of the Kloof mine.

2.3. MATERIALS AND METHODS

2.3.1. Sampling methods

All water and gas samples were collected from borehole collars after the methods of Lollar et al. (2002) and Ward et al. (2004). Prior to sampling, formation-derived water and gas was allowed to flow from the sampling borehole, in order to displace any potential contaminants introduced during borehole drilling. The flow of gas and water was subsequently directed into a sampling bucket. The water flow rate was determined by the average time required to fill the graduated sampling bucket with fracture water. The gas flow rate was determined by filling the sampling bucket with water, and displacing a known volume of water from an inverted graduated beaker. A packer was subsequently placed inside the opening of the borehole and securely sealed

to minimize contamination from the mine air. A stainless steel manifold with control valves was connected to the packer, and PTFE tubing was attached to the ball valves on the sides of the manifold. Temperature, pH, conductivity and reduction potential (Eh) were measured from the water with probes, and dissolved O_2, H_20_2, Fe(II), total Fe and sulfide were measured using Chemetrics test kits (Chemetrics Inc., Calverton, VA). All vials for gas and water sampling were pre-fixed with saturated aqueous $HgCl_2$ to ensure that no post-sampling microbial activity would alter the gas composition or isotopic signatures. Water for $\delta^{13}C_{DIC}$ analysis was collected through a 0.2µm filter into 40 mL glass amber vials, which were rinsed in 10% HCl, combusted at 450°C for 8 hours, and treated with 50µL of saturated $HgCl_2$ solution prior to sampling. Water for $\Delta^{14}C_{DIC}$ analysis was collected through a 0.2µm filter into 500 mL glass bottles, which were pre-treated with 1 mL of saturated $HgCl_2$ solution. Water for $\delta^{13}C_{DOC}$ and $\Delta^{14}C_{DOC}$ analysis was collected through a 0.2µm filter into 250 mL glass bottles sealed with PTFE lined caps. For gas sampling, water and gas were directed to an inverted graduated funnel via gas-impermeable plastic tubing. Gas samples for compositional analysis, $\delta^{13}C$ analysis and $\Delta^{14}C$ analysis were collected from the top of the inverted funnel using a needle and transferred to pre-evacuated borosilicate vials, which were previously combusted at 450°C for 8 hours, pre-treated with 100µL of saturated $HgCl_2$ solution and sealed with blue butyl rubber stoppers. Samples for PLFA analysis were collected using carbon-free, aluminum coated glass wool filters that capture microbial cells through electrostatic interactions (Mailloux et al., 2012). The lipid cartridges were packed with aluminum-coated glass wool and combusted for 24 hours at 450°C prior to sampling (Mailloux et al., 2012). The lipid filter cartridges were connected to the boreholes via the stainless steel manifold and water was allowed to flow through the aluminum coated-glass wool lipid filters for up to several weeks. Total volumes of filtered water were recorded for each

site (Table 2.5). Lipid filters were subsequently removed from the boreholes and stored at sub-zero temperatures until analysis.

2.3.2. Phospholipid fatty acid extraction and analysis

Phospholipid fatty acids (PLFAs) were extracted from the aluminum-coated glass wool filters at McMaster University. All lipid filters were freeze-dried prior to extraction. PLFAs were extracted twice from each lipid filter using a modified Bligh and Dyer method (Bligh and Dyer, 1959). The resulting extracts were separated into non-polar (F1 and F2), neutral (F3) and polar (F4) fractions using silica gel chromatography, using hexane (F1), DCM (F2), acetone (F3) and methanol (F4) (Guckert et al., 1985). Phospholipids recovered from the polar fractions were converted to fatty acid methyl esters (FAMEs) via mild-alkaline methanolysis and subsequently purified by a secondary silica gel chromatography, using 4:1 hexane:DCM (F1), DCM (F2) and methanol (F3) (Guckert et al., 1985). Microbial FAMEs were identified and quantified using gas chromatography-mass spectrometry (GC-MS) on an Agilent 6890 GC-MS (Agilent Technologies Inc., Santa Clara, CA, USA). Samples Be326 Bh1 and Be326 Bh2 were run on the GC-MS in 2011 on an HP-88 column with a temperature program of 80°C (for 1 min.), 10°C/min to 175°C (for 12 min.), 2°C/min to 190°C (for 10 min.), and 10°C/min to 230°C (for 10 min.). Samples Dr5IPC, TT109 Bh2, TT107 and KL445 were run in 2012 on a DB-5MS capillary column (30m x 0.25µm film thickness) with a temperature program of 50°C (for 1 min.), 20°C/min to 130°C, 4°C/min to 160°C, and 8°C/min to 300°C (for 5 min.). The microbial FAMEs were identified based on their retention times and mass fragmentation patterns, compared to known standards (Bacterial Acid Methyl Esters Mix, Matreya Inc., Pleasant Gap, Pennsylvania, USA). 10Me16:0 could not be identified in Be326 Bh2 and Be326 Bh1 as these samples were not run with the 10Me16:0 standard.

Fatty acids identities are listed with the following nomenclature: total number of carbon atoms followed by the total number of double bonds (ex. 16:1 represents a 16 carbon monounsaturated fatty acid). Terminal-branching fatty acids are indicated by the prefixes *i (iso)* and *a (anteiso)*. Mid-branching positions are represented by the number of carbon atoms from the carboxyl group to the methyl group (ex.10Me16:0). Cyclopropyl fatty acids are represented by the prefix *cy*.

2.3.3. Direct cell counts

Microbial cell densities based on direct cell counts were calculated for Be326 Bh2 and Be326 Bh1 at the University of Delaware, using epifluorescence microscopy. These cell density estimates were used as a comparison for the PLFA-based cell density estimates in this study. Bacteria were isolated and stained using SYBR Gold nucleic acid gel stain and subsequently enumerated using epifluorescence microscopy. Direct cell counts were performed for 15 fields of view, and an average was taken of these values.

2.3.4. $\delta^{13}C$ analyses

The $\delta^{13}C$ compositions of the PLFAs and potential carbon sources (DIC, DOC and methane) are referenced to the internationally accepted standard carbonate rock Vienna Pee Dee Belemnite (PDB) and reported in the standard $\delta^{13}C$ notation (Sessions, 2006):

$$\delta^{13}C_{sample} = [(R_{sample} - R_{std}) / R_{std}] \times 1000‰$$

where R_{sample} and R_{std} are the $^{13}C/^{12}C$ isotope ratios of the sample and standard, respectively. PLFA $\delta^{13}C$ values were measured using gas chromatography-isotope ratio mass spectrometry (GC-IRMS) at McMaster University. Aliquots of microbial FAMEs were injected into a split/splitless injector set to splitless mode at 300°C prior to separation using GC-MS on an

Agilent 6980 GC-MS. For samples Be326 Bh2 and Be326 Bh1, an HP-88 column with a temperature program of 80°C (for 1 min.), 10°C/min to 175°C (for 12 min.), 2°C/min to 190°C (for 10 min.), and 10°C/min to 230°C (for 10 min.) was used. For samples Dr5IPC, TT109 Bh2, and TT107, a DB-5MS capillary column (30m x 0.25µm film thickness) and a temperature program of 50°C (for 1 min.), 20°C/min to 130°C, 4°C/min to 160°C, and 8°C/min to 300°C (for 5 min.) was used. Individual FAMEs were combusted to CO_2 as they eluted from the column via a combustion oven set a 960°C. Evolved CO_2 was analyzed using a Delta^{Plus} XP continuous flow IRMS. Only PLFAs with sufficient mass of carbon were measured for $\delta^{13}C$ analysis. Isotopically-characterized methanol was used for mild alkaline methanolysis. To account for the addition of one methyl group per fatty acid, $\delta^{13}C_{PLFA}$ values were corrected using the following equation:

$$\delta^{13}C_{PLFA} = [(N+1) \times \delta^{13}C_{measured} - \delta^{13}C_{MeOH}]/N$$

where N is the number of carbon atoms. DIC and DOC concentrations and $\delta^{13}C$ compositions were measured using cavity ring-down spectroscopy (CR-DS) in the laboratory of Dr. T.C. Onstott at Princeton University. Gas compositions and methane $\delta^{13}C$ compositions were measured using GC-C-IRMS in the laboratory of Dr. Barbara Sherwood Lollar, University of Toronto. The GC-C-IRMS system was composed of a Varian 3400 capillary gas chromatogram and an oxidation oven at 980°C interfaced directly to a Finnigan 252 gas source mass spectrometer, and a temperature program of 35°C (for 6 min.), 30°C/min. to 110°C, and 5°C/min. to 220°C (for 5 min.) was used. The accuracy and reproducibility for $\delta^{13}C$ analysis was ± 0.5‰ for DIC, DOC, methane and PLFA.

2.3.5. $\Delta^{14}C$ analysis

Methane for $\Delta^{14}C$ analysis was separated on a Varian 3300 GC equipped with a 6 m packed molecular sieve (60Å) and combusted to CO_2 via the method described in Slater et al. (2006). Methane $\Delta^{14}C$ analysis was performed via accelerator mass spectrometry (AMS) at the National Ocean Sciences Accelerator Mass Spectrometer (NOSAMS) facility at Woods Hole Oceanographic Institution as per methods described by McNichol et al. (1994). DIC $\Delta^{14}C$ analysis was performed via AMS at NOSAMS by acidification and collection of the CO_2 generated as per methods described by McNichol et al. (1994).

Due to the very low abundances of PLFAs detected in these samples, the masses of carbon comprising the individual PLFAs were below the minimum mass required for $\Delta^{14}C$ analysis by AMS (< 20µg of carbon). In order to achieve this minimum mass of carbon, the PLFAs extracted from each site were sent as bulk fractions to NOSAMS for $\Delta^{14}C$ analysis. Bulk $\Delta^{14}C_{PLFA}$ values, therefore, represent the full set of PLFAs identified at a site. PLFA samples were combusted to CO_2 and converted to graphite for $\Delta^{14}C$ analysis via AMS at NOSAMS. The accuracy and reproducibility for $\Delta^{14}C$ analysis was ±10‰ for DIC, DOC and methane and ±20‰ for PLFA.

2.4. RESULTS

2.4.1. Fracture water geochemistry

The geochemical parameters of the fracture waters sampled from the six deep subsurface sites are listed in Tables 2.1 and 2.2. Gas and water flow rates and methane concentrations are listed in Table 2.3. A wide range of water temperatures were measured, from 26.8°C in Dr5IPC to 54.5°C in KL445. The pH values varied from 7.4 to 9.4. Conductivity measurements were

highest for Be326 Bh1, Be326 Bh2 and KL445 (8.98 to 14.43 mS/cm), and lowest for TT109 Bh2, TT107 and Dr5IPC (0.47 to 0.52 mS/cm). All of the measured Eh values were negative, indicating reduced environments. DIC concentrations were highest in Dr5IPC and TT109 Bh2, at 28.44 ppm and 12.37 ppm, respectively. Be326 Bh2, Be326 Bh1 and TT107 yielded lower DIC concentrations, ranging from 4.68 to 6.84 ppm. KL445 contained the lowest DIC concentration at 1.08 ppm. All measured DOC concentrations were very low (< 0.7 ppm), as well as oxygen levels (< 2.5 ppm), H_2O_2 concentrations (<0.1 ppm), soluble Fe (II) (< 0.5 ppm), total Fe (< 0.6 ppm) and H_2S (< 0.6 ppm). Dr5IPC contained the lowest methane concentration at 0.002mM, followed by Be326 Bh2 at 0.4 mM. Methane concentrations from TT109 Bh2, KL445 and TT107 ranged from 1.4 to 7.5 mM.

2.4.2. $\delta^{13}C$ measurements of DIC, DOC and methane

The $\delta^{13}C$ values measured for DIC, DOC and methane from the six deep subsurface sites are reported in Table 2.4. Figure 2.1 illustrates the $\delta^{13}C$ values of DIC, DOC and methane in comparison to the $\delta^{13}C_{PLFA}$ values. Low gas levels in Be326 Bh1 precluded the $\delta^{13}C$ analysis of methane. Low levels of DOC in Be326 Bh1, TT107 and KL445 precluded the $\delta^{13}C$ analysis of DOC for these samples. Two groups of $\delta^{13}C_{DIC}$ values were observed: highly ^{13}C-depleted values (-41.9‰ to -25.0‰) from Be326 Bh2, Be326 Bh1 and KL445 and less ^{13}C-depleted values (-9.3‰ to -5.0‰) from Dr5IPC, TT109 Bh2 and TT107,. All of the $\delta^{13}C_{Methane}$ values, ranging from -57.2‰ to -36.7‰, were isotopically depleted relative to the corresponding $\delta^{13}C_{DIC}$ and $\delta^{13}C_{DOC}$ values. The $\delta^{13}C_{DOC}$ value for Be326 Bh2 (-28‰) was more isotopically enriched than both $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$ at this site. The $\delta^{13}C_{DOC}$ values for Dr5IPC (-43‰) and TT109 Bh2 (-33‰) fell in between their respective $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$ values.

2.4.3. $\Delta^{14}C$ measurements of DIC, DOC and methane

The $\Delta^{14}C$ values measured for DIC, DOC and methane from the six deep subsurface sites are reported in Table 2.4. Figure 2.2 illustrates the $\Delta^{14}C$ values of DIC, DOC and methane in comparison to the $\Delta^{14}C_{PLFA}$ values. $\Delta^{14}C_{DOC}$ was only measured for Be326 Bh2. The $\Delta^{14}C_{Methane}$ values measured from TT109 Bh2, TT107 and KL445 ranged from -990‰ to -991‰. These values were within ±10‰ error of -1000‰, indicating insignificant or very small amounts of ^{14}C in these samples. $\Delta^{14}C_{Methane}$ values from Dr5IPC (-968‰) and Be326 Bh2 (-974‰) were more positive, indicating slightly higher levels of ^{14}C in these methane pools, relative to the other samples. The $\Delta^{14}C_{DIC}$ values from Dr5IPC, Be326 Bh2 and KL445 were very negative (-984‰ to -930‰), whereas DIC from Be326 Bh1, TT109 Bh2 and TT107 contained higher levels of ^{14}C (-863‰ to -497‰). For Be326 Bh2 and KL445, the $\Delta^{14}C_{Methane}$ and $\Delta^{14}C_{DIC}$ values fell within ±10‰ error of one another. As was observed for the $\delta^{13}C$ measurement, the $\Delta^{14}C_{DOC}$ value measured for Be326 Bh2 (-494‰) was significantly enriched relative to all other carbon pools at this site. Notably, DIC from Be326 Bh2 was re-sampled 7 months following the first measurement and yielded a $\Delta^{14}C_{DIC}$ value of -922‰, 60‰ more enriched than the original value.

2.4.4. PLFA concentrations and cell density estimates

The total masses of PLFA (µg) recovered from each site and total PLFA concentrations (picograms of PLFA/mL of filtered water) are listed in Table 2.5. These concentrations were used to estimate microbial cell densities for each of the six subsurface sites. Conversion factors used to estimate microbial cell densities in deep subsurface environments based on PLFA concentrations typically range from $4x10^4$ to $6x10^4$ cells/picomole PLFA (Green and Scow, 2000). In this study, PLFA-based cell density estimates for each site were calculated using the conversion factor of $6x10^4$ cells/picomole PLFA in order to calculate the upper limits for

microbial cell densities in these low biomass systems. Overall, the microbial cell densities estimated for the six subsurface sites were very low, with the highest cell densities on the order of 10^4 cells per mL of filtered fracture water (Figure 2.3). KL445 yielded an extremely low cell density, estimated as $2x10^1$ cells per mL of water. Direct cell counts via epifluorescence microscopy for Be326 Bh2 and Be326 Bh1 were found to be relatively consistent with the PLFA-based cell density estimates, although at slightly lower values (Figure 2.3). These direct cell counts for Be326 Bh2 and Be326 Bh1 produced microbial cell density estimates of $8x10^3$ cells/mL and $3x10^4$ cells/mL, respectively, compared to PLFA-based cell density estimates of $2x10^4$ cells/mL and $5x10^4$ cells/mL, respectively.

2.4.5. Phospholipid fatty acid profiles

The individual PLFAs identified in each of the six subsurface samples are listed in Table 2.6. Relative abundances as mole percentages of the individual PLFAs are illustrated in Figure 2.4 and the relative abundances of PLFA classes (cyclic, branched, unsaturated and saturated) are illustrated in Figure 2.5. Dr5IPC was composed primarily of branched PLFAs (79.1 mol%), with *a*-15:0 and *i*-16:0, constituting 30.3 mol% and 26.3 mol%, respectively. 10Me16:0, a PLFA biomarker for sulfate reducing bacteria, was observed in Dr5IPC, constituting 2.7 mol%. No unsaturated or cyclic PLFAs were observed in Dr5IPC, although they were observed in all other samples. Be326 Bh2 and Be326 Bh1 yielded very similar PLFA profiles, both dominated by 16:0, with relatively high proportions of the unsaturated PLFAs 16:1 and 18:1, similar proportions of the short-chain PLFAs, and the presence of *cy*17:0 and *cy*19:0. In TT109 Bh2, two PLFAs comprised 77.8 mol% of the sample: 16:0 (57.8 mol%) and *cy*17:0 (20.0 mol%). In addition to *cy*17:0, TT109 Bh2 also contained the cyclopropyl PLFA *cy*19:0 (3.2 mol%). The PLFA profile from TT107 was comprised predominantly of branched (45.0 mol%) and saturated

(40.7 mol%) PLFAs, and contained the cyclopropyl PLFA *cy*17:0. The PLFA profile from KL445 consisted entirely of three PLFAs: 16:0 (42.5 mol%), 18:0 (29.5 mol %), and 18:1 (28.0 mol %); however, this observation may be an artifact of the low total abundance of PLFA detected in KL445. The detection limit for these samples was essentially 1 cell per mL of fracture water, which was achieved by filtering very large volumes of water (> 1000 liters) during sampling. In samples that have very low cell abundances, a very low detection limit allows a very small input of contamination to be detected. For KL445, where the cell density estimate is as low as $2x10^1$ cells per mL, the PLFA profile may in fact be indicative of a low level of contamination, rather than of an indigenous microbial community. Due to the very low concentrations of the monounsaturated 16:1 and 18:1 PLFAs and minimal sample mass available for analysis, the double bond positions of 16:1 and 18:1 could not be identified. This limitation precluded the identification of the common biomarkers for methanotrophic bacteria (16:1ω8*c* and 18:1ω8*c*) in these samples (Hanson and Hanson, 1996).

2.4.6. δ^{13}C Phospholipid fatty acids

The $\delta^{13}C$ values measured for PLFAs are listed in Tables 2.4 and 2.7. Only those PLFAs with sufficient mass of carbon were measured for $\delta^{13}C$. Containing only a total of 0.4 µg of PLFA, KL445 did not contain sufficient mass of carbon to measure $\delta^{13}C_{PLFA}$. The $\delta^{13}C_{PLFA}$ values varied widely across the five subsurface sites, as well as among the individual PLFAs at each site (Table 2.7 and Figure 2.1). The most depleted $\delta^{13}C_{PLFA}$ values were observed at Dr5IPC, ranging from -72.9‰ to -70.3‰. Be326 Bh2 PLFAs were slightly more enriched in ^{13}C, with $\delta^{13}C$ values ranging from -64.6‰ to -55.8‰. Out of the five subsurface sites measured for $\delta^{13}C_{PLFA}$, the TT107 $\delta^{13}C_{PLFA}$ values were the most enriched in ^{13}C and were also the most variable among individual PLFAs, ranging from -25.5‰ to -11.0‰. $\delta^{13}C_{PLFA}$ values for TT109

Bh2 and Be326 Bh1 were less variable, ranging from -36.3‰ to -30.6‰ and -51.6‰ to -45.1‰, respectively.

2.4.7. $\Delta^{14}C$ Phospholipid fatty acids

Bulk PLFA $\Delta^{14}C$ values are listed in Table 2.4 and illustrated in relation to $\Delta^{14}C_{DOC}$, $\Delta^{14}C_{DIC}$ and $\Delta^{14}C_{Methane}$ in Figure 2.2. Low abundances of PLFAs in Be326 Bh1 and KL445 precluded the $\Delta^{14}C$ analysis of PLFAs for these two sites. Out of the four samples measured for $\Delta^{14}C_{PLFA}$, the $\Delta^{14}C_{PLFA}$ value from Be326 Bh2 contained the lowest levels of ^{14}C (-940‰). The $\Delta^{14}C_{PLFA}$ values from Dr5IPC (-893‰) and TT109 Bh2 (-848‰) were slightly more positive, and the $\Delta^{14}C_{PLFA}$ value from TT107 (-640‰) was the most highly enriched in ^{14}C.

2.5. DISCUSSION

2.5.1 Deep subsurface environmental conditions

The geochemistry of the deep subsurface fracture water systems indicated varying environmental conditions across the six sites (Tables 2.1, 2.2 and 2.3). Several similarities were observed between Be326 Bh2 and Be326 Bh1, including ambient temperatures (36.8°C and 31.6°C), relatively high pH values (8.8 and 9.4), slightly higher conductivities (9.04 and 8.98 mS/cm), low DIC concentrations (6.12 and 4.68 ppm) and low oxygen levels (<0.01 ppm). TT109 Bh2 and TT107 were similar in terms of their high temperatures (48.7°C and 52.1°C) and low conductivities (0.47 and 0.50 mS/cm). Water temperatures across the six sites were found to increase with depth by approximately 10°C per km, ranging from 26.8°C at 0.9 kmbls (Dr5IPC) to 54.5°C at 3.2 kmbls (KL445). Fracture water pH values did not appear to correlate with depth and were relatively neutral for Dr5IPC, TT109 Bh2 and KL445 (7.4 to 8.0) and more alkaline for TT107, Be326 Bh2 and Be326 Bh1 (8.6 to 9.4). Conductivity measurements appeared to

increase with depth, with the exception of TT109Bh2 and TT107, which were two of the deepest sites in the study and yet yielded the lowest salinities. All DOC and oxygen measurements were low, as is expected for deep terrestrial subsurface environments (Pedersen, 2000). DIC and methane levels were more variable across the six sites and, in general, did not correlate with one another. Notably, Dr5IPC contained the highest levels of DIC (28.44 ppm) and the lowest levels of methane (0.002 ppm). The wide range of environmental conditions observed across the different deep subsurface sites suggests that the microbial community structures and metabolisms will vary concurrently.

2.5.2 Microbial biomass distributions

The six deep subsurface samples yielded very low concentrations of PLFAs, ranging from 0.1 to 208.2 picograms of PLFA per mL of filtered water (Table 2.5). Based on these PLFA concentrations, microbial cell densities were very low, ranging on the order of 10^1 to 10^5 cells per mL of filtered water (Table 2.5). Microbial cell densities commonly reported for deep terrestrial subsurface pelagic systems range from 10^3 to 10^7 cells per mL of water (Fredrickson and Fletcher, 2001; Mills et al., 2010; Moser et al., 2003; Pedersen, 1997; Pfiffner et al., 2006). The cell densities calculated in this study fall within this range of expected values, with the exception of TT107 and KL445, which yielded extremely low cell densities of $2x10^2$ cells/mL and $2x10^1$ cells/mL, respectively. Direct cell counts for Be326 Bh2 and Be326 Bh1 via epifluorescence microscopy yielded microbial cell densities of $8x10^3$ and $3x10^4$ cells per mL, which were similar, but slightly lower than, the PLFA-based cell density estimates of $2x10^4$ and $5x10^4$ cells per mL, respectively (Figure 2.3). This small discrepancy may indicate average cell sizes that differ from those used to calculate PLFA conversion factors (Green and Scow, 2000). Nevertheless, these values confirm the presence of very low biomass in these systems. With the

exception of DR5IPC, the estimated microbial cell densities appeared to decrease as water temperatures increased (Tables 2.1 and 2.5). The deepest of the sites (KL445), with the highest water temperature and the highest salinity yielded the lowest cell density estimate. This trend may indicate that high water temperatures are a limitation to the bacterial community in these systems.

When interpreting microbial cell density estimates in subsurface fracture water systems, the ability to collect a representative sample of the microbial community must be taken into consideration. Our sampling method is believed to collect primarily pelagic cells that are mobile within the fracture water itself, which may potentially exclude those microbial cells that are attached to rock surfaces within the fracture water systems. It is possible that a proportion of the microbial community in these systems is living as biofilms on rock surfaces, rather than as pelagic cells within the fracture water, and that, as a result, these microorganisms are not sampled during the filtering process (Fredrickson and Fletcher, 2001; Pfiffner et al., 2006; Wanger et al., 2006). As such, the cell densities derived from our sampling method could result in an underrepresentation of the indigenous microbial community, due to a lack of sampling from rock surfaces.

2.5.3. Microbial community structures

Differences among the individual PLFA profiles of the six deep subsurface sites indicate varying community structures and/or microbial responses to environmental stressors (Figures 2.4 and 2.5). All six PLFA profiles contained relatively high abundances of 16:0, a common fatty acid in all bacteria (Boschker and Middelburg, 2002). Unlike the other five sites, Dr5IPC contained a high relative abundance of branched PLFAs, which may indicate a high proportion of gram-positive bacteria in this system (Hardwood and Russell, 1984; Kaneda, 1991). The

presence of the PLFA biomarker 10Me16:0 in Dr5IPC is an indicator for sulphate-reducing bacteria at this site (Green and Scow, 2000). Be326 Bh2 and Be326 Bh1 appear to contain similar microbial communities based on their PLFA profiles, as the majority of the PLFAs identified in Be326 Bh2 and Be326 Bh1 are common to both systems, including the short chain PLFAs 12:0, 13:0 and *i*-14:0, which were not identified in any other system. Both Be326 Bh2 and Be326 Bh1 are dominated by 16:0, followed by the monounsaturated PLFAs 16:1 and 18:1, which are common in gram-negative bacteria (Green and Scow, 2000). Be326 Bh2 contains a slightly higher relative proportion of the monounsaturated PLFAs 16:1 and 18:1, in comparison to Be326 Bh1, potentially indicating a relatively higher abundance of gram-negative bacteria in this system. All of the PLFA profiles, except for those of Dr5IPC and KL445, contained some cyclopropyl PLFAs. While cyclopropyl fatty acids are potential indicators for anaerobic bacteria (Fang and Barcelona, 1998; Green and Scow, 2000), they have also been shown to be produced in response to environmental stressors, such as severe nutrient deprivation (Guckert et al., 1986; Kieft et al., 1994). Nutrient deprivation, as well as other environmental stressors, such as high temperatures and pressures, are expected for deep terrestrial microbial systems. The observation of cyclic PLFAs in Be326 Bh2, Be326 Bh1, TT109 Bh2 and TT107 may thus be an indicator for microbial responses to environmental stressors. This is consistent with the fact that Dr5IPC is the shallowest system with the lowest water temperatures observed across the sample set and does not contain cyclopropyl PLFAs. TT109 Bh2, in particular, contains a relatively high proportion of *cy*17:0 (20.0 mol%). Aside from KL445, TT109 Bh2 is the deepest environment in this study and contains one of the highest water temperatures, indicating potentially higher stress conditions in this site.

A relative increase in the production of *iso*- versus *anteiso*- fatty acids has been shown to occur in response to increasing temperatures, due to differing melting temperatures of *iso*- versus *anteiso*- fatty acids (Petersen and Klug, 1994). The melting point of the fatty acid *i*-15:0 is 51.7°C, whereas the melting point of *a*-15:0 is only 23.0°C (Kaneda, 1991). This relative increase of *iso*- over *anteiso*- fatty acids has been shown to increase once temperatures reach approximately 25°C (Petersen and Klug, 1994). In Dr5IPC, the ratio of *i*-15:0 over *a*-15:0 is very low (= 0.1) which is consistent with the lower water temperature (26.8°C) measured for this system. In TT107, the ratio of *i*-15:0 over *a*-15:0 is relatively high (= 2.4), which is consistent with TT107's high water temperature (52.1°C). TT109 Bh2, Be326 Bh1 and Be326 Bh2 all contain higher proportions of *i*-15:0 than of *a*-15:0, with ratios of 1.8, 1.6, and 1.8, respectively. These values are consistent with the warm water temperatures observed at these sites, ranging from 31.6°C to 48.7°C (Table 2.1). Overall, these observations illustrate a clear trend of microbial responses to high water temperatures in these sites. The varying environmental conditions, in combination with variations in microbial community structures, suggest that the active microbial metabolisms in these systems will also vary widely.

As noted above, due to very low PLFA concentrations observed in KL445, any interpretation of the indigenous microbial community in this system must be approached with caution. The KL445 PLFA profile consisted only of three PLFAs (16:0, 18:0 and 18:1). 16:0 and 18:0 are very common and non-diagnostic PLFAs in bacteria; however, monounsaturated PLFAs such as 18:1 are indicators for gram-negative bacteria (Wilkinson, 1988).

2.5.4. Evidence for microbial cycling of methane in Dr5IPC

Based on $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFAs and potential carbon sources, methanotrophy appears to be a predominant metabolism in Dr5IPC (Figures 2.1 and 2.2). The $\delta^{13}C$ values of the

PLFAs are highly depleted (-72.9‰ to -70.3‰) and negatively offset from $\delta^{13}C_{Methane}$ (-55.4‰) which is consistent with the preferential uptake of isotopically lighter methane by methanotrophs, in combination with KIEs associated with the synthesis of PLFAs (Boschker and Middelburg, 2002; Hayes, 2001; Summons et al., 1994). The carbon isotope separation between $\delta^{13}C_{PLFA}$ and $\delta^{13}C_{Methane}$ ($\Delta\delta^{13}C_{Methane\text{-}PLFA}$ = 14.9‰ to 17.5‰) is consistent with the KIEs typically involved in microbial uptake of methane (i.e. $\Delta\delta^{13}C_{Methane\text{-}PLFA}$ ranging from 10 to 30‰), providing further evidence for methanotrophy in this system (Hayes, 2001; Jahnke et al., 1999). Microbial utilization of DIC as the main carbon source in Dr5IPC can be ruled out based on the $\delta^{13}C$ analysis of DIC and PLFAs. The isotopically heavy $\delta^{13}C_{DIC}$ value (-7.4‰), compared to the isotopically light $\delta^{13}C_{PLFA}$ values (-72.9‰ to -70.3‰), translates to a very large carbon isotope separation ($\Delta\delta^{13}C_{DIC\text{-}PLFA}$ = 62.9‰ to 65.5‰). The magnitude of this separation is greater than the largest autotrophic carbon isotope fractionation observed to date ($\Delta\delta^{13}C_{DIC\text{-}PLFA}$ = 58‰), indicating that microbial autotrophy in Dr5IPC is likely not a dominant metabolism (Londry et al., 2004).

The $\Delta^{14}C$ from Dr5IPC is consistent with methanotrophy as a major metabolism at this site. The very negative $\Delta^{14}C_{PLFA}$ value (-893‰) is consistent with microbial uptake of an ancient carbon source, such as methane (-968‰) or DIC (-930‰). The $\Delta^{14}C_{PLFA}$ and $\Delta^{14}C_{DIC}$ values are very similar (7‰ outside of error), indicating that the microbial community in Dr5IPC is likely assimilating a portion of its carbon from DIC. This scenario is consistent with active methanotrophy, as type II methanotrophs are known to assimilate carbon from methane and DIC, in a ratio that is between 1:1 and 2:1 (Hanson and Hanson, 1996; Jahnke et al., 1999; Mills et al., 2010). As the $\delta^{13}C_{DIC}$ and $\delta^{13}C_{PLFA}$ values from Dr5IPC do not indicate microbial utilization of DIC as a major carbon source, the methanotrophic bacteria are likely assimilating the majority of

their carbon from methane (i.e. a ratio of methane to DIC that is closer to 2:1). In addition to providing evidence for methanotrophy, $\Delta^{14}C$ and $\delta^{13}C$ analyses of methane and DIC also provide isotopic evidence for methanogenesis by archaea in Dr5IPC. The $\Delta^{14}C_{Methane}$ value (-968‰) indicates that, in contrast to the majority of the sites in this study, the methane pool in Dr5IPC contains some ^{14}C. This low level of ^{14}C is most likely derived from DIC (-930‰) via methanogenesis. Consistent with this observation, the large carbon isotope separation of 49.8‰ between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$ in Dr5IPC is characteristic of autotrophic methanogenesis by archaea (Londry et al., 2008; Whiticar, 1999). Active methanotrophy is expected to counteract this effect by reducing the carbon isotope separation between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$; however, the relative sizes of the methane and DIC pools can greatly influence the magnitude of this effect. The relatively small methane pool and relatively large DIC pool in Dr5IPC indicates that this carbon isotope separation effect may not be very apparent (Tables 2.2 and 2.3). Furthermore, without information about the relative rates of methanotrophy and methanogenesis in this system, the influence of active methanotrophy on the carbon isotope separation between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$ cannot be easily constrained.

If microbial utilization of methane as a carbon source in Dr5IPC is an active process, it must be occurring via anaerobic reactions, as Dr5IPC contains very low levels oxygen (Table 2.2). One possible mechanism by which the community may be capable of oxidizing methane is via bacterial methanotrophy using an alternative electron acceptor such as sulphate, manganese or iron (Beal et al., 2009; Ettwig et al., 2008; Mills et al., 2010). The water temperature (26.8ºC) and pH (7.4) measured from Dr5IPC fall within the range of optimum conditions for methanotrophy by type I and type II methanotrophs (25ºC to 35ºC and pH values of 7.0 to 7.6) (Hanson and Hanson, 1996). Anaerobic methane oxidation by type I and type II bacterial

methanotrophs has not yet been confirmed; however, the presence of biomarkers for type II methanotrophs in anaerobic environments, combined with evidence for active microbial cycling of methane in these systems, suggests for the presence of such a mechanism (Mills et al., 2010). The PLFA biomarkers for type I and type II methanotrophs could not be identified in Dr5IPC; therefore, the presence of these methanotrophs cannot be confirmed. However, $\Delta^{14}C$ evidence for microbial utilization of DIC by a methanotrophic community in Dr5IPC is consistent with the metabolisms of type II methanotrophs (Hanson and Hanson, 1996; Jahnke et al., 1999; Mills et al., 2010). Anaerobic oxidation of methane can also occur as a consortium between sulphate reducing bacteria and the ANME group of archaea (Boetius et al., 2000; Hinrichs et al., 1999; Hoehler et al., 1994). Via this mechanism, $\delta^{13}C$ analysis of the archaeal lipids tend to yield extremely depleted $\delta^{13}C$ values, reaching as negative as -130‰ with $\Delta\delta^{13}C_{Methane\text{-}lipids} \approx 60‰$. The $\delta^{13}C_{PLFA}$ values of the sulfate reducing bacteria tend to fall intermediate between the $\delta^{13}C$ of the methane source and the $\delta^{13}C$ value of the archaeal lipids (Hinrichs et al., 2000; Valentine and Reeburgh, 2000). Based on the $\delta^{13}C$ values of the archaeal lipids and bacterial PLFAs in these consortiums, archaea are thought to be the primary drivers of methane oxidation via reverse methanogenesis, and the sulphate reducing bacteria are thought to feed off an organic intermediate produced by the archaea (Boetius et al., 2000; Valentine, 2011; Valentine and Reeburgh, 2000). The PLFA biomarker for sulphate reducing bacteria (10Me16:0) was identified in Dr5IPC at 2.7 mol%, and although the abundance of this biomarker (~2.0 µg) precluded the $\delta^{13}C$ analysis of this particular PLFA, the presence of 10Me16:0 at a site where $\delta^{13}C_{PLFA}$ values are extremely depleted raises the question of whether sulphate reducing bacteria are involved in methane cycling in this system. $\delta^{13}C$ analysis of archaeal lipids, which was beyond the scope of

this study, would potentially confirm the presence or absence of sulphate-dependent methane oxidation in Dr5IPC.

2.5.5. Evidence for methanotrophy and autotrophy in Be326 Bh2

In comparison to Dr5IPC, Be326 Bh2 yielded a wider set of $\delta^{13}C_{PLFA}$ values, ranging from -64.6‰ to -55.8‰. Taking into consideration the various potential carbon sources and the KIEs associated with different metabolisms, the wide range of $\delta^{13}C_{PLFA}$ values in Be326 Bh2 potentially represents more than one active metabolism in Be326 Bh2. The most highly ^{13}C-depleted $\delta^{13}C_{PLFA}$ values, representing 14:0, 16:1, 16:0, *cy*17:0, and 18:1, range from -62.7‰ to -64.6‰ and are negatively offset from $\delta^{13}C_{Methane}$ by 11.3‰ to 13.2‰. These negative offsets fall within the range of carbon isotope fractionations typically observed for methanotrophy ($\Delta\delta^{13}C_{Methane\text{-}PLFA}$ ranging from 10‰ to 30‰) and are, therefore, likely indicative of microbial utilization of methane as a carbon source (Hayes, 2001; Jahnke et al., 1999). Consistent with this interpretation, the set of PLFAs represented by these highly ^{13}C-depleted values, particularly the unsaturated PLFAs 16:1 and 18:1, are commonly observed in methanotrophic communities (Bodelier et al., 2009; Gebert et al., 2004; Mills et al., 2010). The two remaining PLFAs that were measured for $\delta^{13}C$ from Be326 Bh2 (*i*-15:0 and *a*-15:0) yielded $\delta^{13}C_{PLFA}$ values of -55.8‰ and -60.1‰, with negative offsets from $\delta^{13}C_{Methane}$ ($\Delta\delta^{13}C_{Methane\text{-}PLFA}$ = 4.4‰ and 8.7‰) that are likely too small to be a result of methanotrophy (Hayes, 2001; Jahnke et al., 1999). As such, *i*-15:0 and *a*-15:0 are likely derived from microbes using an alternative carbon source, such as DIC (Hayes, 2001; Jahnke et al., 1999). The $\delta^{13}C$ values of *i*-15:0 and *a*-15:0 are negatively offset from $\delta^{13}C_{DIC}$ by 13.9‰ and 18.2‰, respectively, falling within the range of possible carbon isotope fractionations associated with microbial utilization of DIC, and may indicate acetogenesis, sulfate reduction and/or iron reduction (Blaser et al., 2013; Boschker and

Middelburg, 2002; Londry et al., 2004; Ruby et al., 1987). Consistent with this observation, *i*-15:0 and *a*-15:0 are generally uncommon in methanotrophic bacteria (Bodelier et al., 2009; Gebert et al., 2004).

$\Delta^{14}C$ analyses of PLFAs and potential carbon sources from Be326 Bh2 provide further isotopic evidence for the presence of both active methanotrophy and autotrophy in this system (Figure 2.2). The $\Delta^{14}C_{PLFA}$ value measured for this site is very negative (-940‰,), indicating very low ^{14}C content. $\Delta^{14}C_{DIC}$ (-982‰) and $\Delta^{14}C_{Methane}$ (-974‰) fall within ±10‰ error of one another and contain very little ^{14}C. These very similar $\Delta^{14}C$ values, in close proximity to $\Delta^{14}C_{PLFA}$, indicate that DIC and methane are equally likely carbon sources for the microbial community in Be326 Bh2. The $\Delta^{14}C_{PLFA}$ value is slightly more positive than both DIC (12‰ outside of error) and methane (4‰ outside of error); however, this difference may simply be due to the heterogeneity of the fracture water geochemistry. Notably, a replicate $\Delta^{14}C_{DIC}$ measurement from Be326 Bh2, sampled 7 months later, yielded a more positive $\Delta^{14}C_{DIC}$ (-922‰) that falls within error of the $\Delta^{14}C_{PLFA}$ value. This difference in $\Delta^{14}C_{DIC}$ over time represents variability in the fracture water geochemistry over space and/or time and indicates that small differences between $\Delta^{14}C$ values should be interpreted with caution. The $\Delta^{14}C_{DOC}$ value (-494‰) measured from Be326 Bh2 is more positive than $\Delta^{14}C_{PLFA}$ by 446‰, ruling out DOC as a major carbon source in this system.

Overall, the $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFA and potential carbon sources from Be326 Bh2 provide evidence for methanotrophy as an important process in this system, but also indicate that it is not the sole active metabolism, due to isotopic evidence for autotrophy for a subset of the PLFAs. As such, two distinct components of the microbial community, utilizing different carbon sources (methane and DIC), have been identified via carbon isotope analyses. The

specific mechanism by which microbial oxidation of methane is occurring in Be326 Bh2 is speculative, as was the case with Dr5IPC. The PLFA biomarkers for type I and type II methanotrophs (16:1ω8c and 18:1ω8c), as well as the biomarker for sulphate reducing bacteria (10Me16:0) could not be identified in Be326 Bh2; therefore, the presence and activity of these microbial groups cannot be confirmed. Nevertheless, methanotrophy in Be326 Bh2 is occurring via a mechanism of anaerobic oxidation of methane.

2.5.6. Autotrophic microbial communities in TT109 Bh2 and TT107

Microbial communities in TT109 Bh2 and TT107 are primarily using DIC as a carbon source, either via autotrophic acetogenesis, or sulphate or iron reduction, based on $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFAs and potential carbon sources in these systems (Figures 2.1 and 2.2). Carbon isotope fractionation effects resulting from autotrophic metabolisms tend to be large and variable depending on the pathway involved (Boschker and Middelburg, 2002). At both TT109 Bh2 and TT107, the $\delta^{13}C_{PLFA}$ values are depleted in ^{13}C in relation to DIC, with negative offsets ($\Delta\delta^{13}C_{DIC\text{-}PLFA}$ = 21.3‰ to 27.0‰ for TT109Bh2, and $\Delta\delta^{13}C_{DIC\text{-}PLFA}$ = 6.0‰ to 20.5‰ for TT107) that are consistent with the preferential uptake of the lighter isotope of carbon from DIC by the microbial communities (Boschker and Middelburg, 2002; Londry et al., 2004). Methane as a major carbon source can be ruled out in both of these systems, as the $\delta^{13}C_{PLFA}$ values are enriched in ^{13}C in relation to methane, which is inconsistent with kinetic isotope effects associated with methanotrophy (Jahnke et al., 1999; Valentine and Reeburgh, 2000; Whiticar, 1999). However, the $\delta^{13}C_{PLFA}$ values from TT107 are highly variable and shifted towards the $\delta^{13}C_{Methane}$ value, indicating that a minor influence from methane to the $\delta^{13}C_{PLFA}$ values in TT107 is a possibility.

$\Delta^{14}C$ analysis of PLFAs and potential carbon sources from TT109 Bh2 confirm the presence of a microbial community that is primarily using DIC as a carbon source, as the $\Delta^{14}C$ values of the PLFAs (-848‰) and DIC (-863‰) fall within error of one another (Figure 2.2). The $\Delta^{14}C_{Methane}$ value is very negative (-990‰) in comparison to these values, indicating that methane in TT109 Bh2 is not a significant carbon source. The presence of ^{14}C in both the PLFAs and the DIC pool, and the lack of ^{14}C in the methane pool, indicate that the carbon composing the PLFAs in TT109 Bh2 is largely derived from DIC.

The $\delta^{13}C_{DOC}$ value (-33.1‰) measured for TT109 Bh2 falls directly within the range of $\delta^{13}C_{PLFA}$ values and may indicate that the DOC collected from TT109 Bh2 is largely composed of organic carbon derived from microbial biomass (Figure 2.1). PLFAs are generally 3-6‰ more depleted than the bulk microbial biomass; therefore, the $\delta^{13}C$ value of the bulk microbial biomass in TT109 Bh2 could be as enriched in ^{13}C as -24.6 to -30.3‰. As the $\delta^{13}C_{DOC}$ value is slightly more depleted in ^{13}C than these values, microbial biomass is likely not the only source of carbon to the DOC pool in TT109 Bh2.

For TT107, $\Delta^{14}C$ analysis of PLFAs and potential carbon sources provides support for microbial utilization of DIC as a major carbon source, but also indicates a potential influence from methane. Specifically, the $\Delta^{14}C_{PLFA}$ value measured for TT107 contains a lower level of ^{14}C than the $\Delta^{14}C_{DIC}$ from this site. The difference between $\Delta^{14}C_{DIC}$ and $\Delta^{14}C_{PLFA}$ of 143‰ indicates that an alternative carbon source with relatively low levels of ^{14}C, such as methane, has significantly influenced the $\Delta^{14}C_{PLFA}$ value at this site. Consistent with this observation, the most ^{13}C-depleted PLFAs (16:0 and 18:0, with $\delta^{13}C_{PLFA}$ values of -21.1‰ and -25.5‰, respectively) are shifted negatively towards $\delta^{13}C_{Methane}$ by up to 14.5‰ relative to the other PLFAs. 16:0 and

18:0 appear to be influenced by both $\delta^{13}C_{DIC}$ and $\delta^{13}C_{Methane}$ and, therefore, likely represent both microbial utilization of DIC and methane as carbon sources.

Although $\delta^{13}C$ and $\Delta^{14}C$ analyses from TT107 and TT109 Bh2 clearly indicate microbial utilization of DIC, the mechanisms of autotrophy taking place in these systems is speculative and may consist of acetogenesis, sulphate reduction and/or iron reduction (Blaser et al., 2013; Boschker and Middelburg, 2002; Londry et al., 2004; Ruby et al., 1987). The lack of the PLFA biomarker for sulphate reducing bacteria in these two systems suggests for the absence of this bacterial group and metabolism. The PLFA profile from TT107 largely consists of branched PLFAs, which are indicative of gram-positive bacteria and also consistent with the potential presence of acetogenic bacteria (Blaser et al., 2013). The PLFA profile from TT109 Bh2 is mainly comprised of saturated PLFAs, which are non-diagnostic for specific microbial groups. As such, the PLFA profiles cannot conclusively identify the mechanisms of autotrophy in these systems. $\delta^{13}C$ and $\Delta^{14}C$ measurements of acetate in TT107 and TT109 Bh2, compared to $\delta^{13}C_{PLFA}$ and $\Delta^{14}C_{PLFA}$ values, could potentially confirm the presence or absence of autotrophic acetogenesis in these systems.

Low gas levels in Be326 Bh1 precluded the analysis of $\delta^{13}C$ of methane in this system. Based on $\delta^{13}C$ values of PLFA and DIC alone, it appears that the microbial community is actively utilizing DIC as a carbon source. The negative offset from $\delta^{13}C_{DIC}$ to the $\delta^{13}C_{PLFA}$ values ($\Delta\delta^{13}C_{DIC\text{-}PLFA}$ = 15.1‰ to 21.6‰) in Be326 Bh1 is similar to, but slightly smaller than, the offset from $\delta^{13}C_{DIC}$ to $\delta^{13}C_{PLFA}$ in TT109 Bh2, a microbial community that is likely predominantly autotrophic. However, without $\delta^{13}C$ measurements for methane or DOC, this is only speculative. The geochemical conditions in Be326 Bh1 and Be326 Bh2 were very similar, as well as the PLFA profiles derived from these sites, indicating that the microbial communities

in these two systems are potentially very similar. If this is the case, then Be326 Bh1 may be utilizing methane as a carbon source, in addition to DIC.

2.5.7. The possibility of KL445 as an abiotic system

The extremely low cell density estimate calculated for KL445 ($2x10^1$ cells/mL) based on the total PLFA concentrations from this site raises the question of whether KL445 contains any viable microbial biomass or whether the microbial community is composed solely of archaeal cells, as archaea cannot be identified via PLFA analysis. In total, 5,121 liters of fracture water were filtered from KL445 and only 0.4 µg of PLFA were detected. By filtering several thousand litres of water, the detection limit for KL445 was lowered to essentially 1 cell per mL of water. Due to this high sensitivity of analysis, extremely low levels of contamination, either introduced during sampling or during sample handling, may be detected via PLFA analysis. If the PLFAs detected in KL445 are derived from contamination, this suggests that KL445 does not contain any viable bacteria or eukarya. Notably, KL445 is one of the deepest systems investigated in this study, at 3.2 kmbls, and contained the highest water temperatures and highest salinity observed among the six sites. This could potentially indicate an inhospitable environment or, alternatively, a microbial community that is composed entirely of thermophilic archaea. PLFA analysis of microbial communities only provides information about bacterial and eukaryotic microbial cells in an environment, and excludes information about the archaeal community structure and abundance. In contrast to ester-linked phospholipids, archaeal lipids contain ether linkages between the glycerol molecule and the fatty acid chains (Green and Scow, 2000). Due to this difference in chemical structure, identifying archaeal lipids requires a separate method of analysis. In cases where an environment appears to be devoid of life, it is important to consider the possibility that archaea, which are left undetected using the PLFA method, are actually

dominant in the system. In order to confirm the presence or absence of viable microbial life in this system, analyses of archaeal lipids and/or genetic analyses would certainly provide further insight.

2.6. CONCLUSIONS

The results of this study revealed varying microbial community structures and microbial metabolisms in the deep terrestrial subsurface via PLFA analysis and carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$). The observation of very low PLFA concentrations across the six subsurface sites indicated very low microbial cell densities overall, two of which (TT107 and KL445) were lower than typically observed for subsurface systems (Fredrickson and Fletcher, 2001; Mills et al., 2010; Moser et al., 2003; Pedersen, 1997; Pfiffner et al., 2006). Extremely low PLFA concentrations in one sample (KL445) may indicate a microbial community that is dominated entirely by archaea, or potentially an abiological system. The observation of very depleted $\delta^{13}C_{PLFA}$ values, negatively offset from $\delta^{13}C_{Methane}$ values, indicated microbial utilization of methane as a major carbon source in two subsurface systems (Dr5IPC and Be326 Bh2). $\delta^{13}C_{PLFA}$ values, negatively offset from $\delta^{13}C_{DIC}$ in Be326 Bh2 also provided evidence for microbial utilization of DIC via chemoautotrophy. $\Delta^{14}C$ data for PLFA and potential carbon sources was consistent with these observations. The combination of $\delta^{13}C$ and $\Delta^{14}C$ analysis in TT109 Bh2 and TT107 indicated microbial communities dominated by autotrophic metabolisms in these sites. Overall, this study was able to identify distinct ecosystems utilizing carbon sources that are known to be produced in the deep terrestrial subsurface. In some communities, methane played a key role as carbon source, whereas other systems were dominated by autotrophic processes. These results indicate a potentially self-sustaining deep terrestrial subsurface biosphere that may be capable of surviving over geologic timescales without interaction with surface processes.

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2.8. TABLES AND FIGURES

Table 2.1. Sample mine information, depth, and geochemical parameters for deep subsurface fracture water samples

Sample	Mine	Depth (kmbls)	Temperature (°C)	pH	Conductivity (mS/cm)	eH (mV)
Dr5IPC	Driefontein	0.9	26.8	7.4	0.52	n.d.
Be326 Bh2	Beatrix	1.3	36.9	8.8	9.04	-297
Be326 Bh1	Beatrix	1.3	31.6	9.4	8.98	-223
TT109 Bh2	Tau Tona	3.2	48.7	7.6	0.47	-250
TT107	Tau Tona	3.1	52.1	8.6	0.50	-318
KL445	Kloof	3.2	54.5	8.0	14.43	-235

n.d. - not determined

Table 2.2. Geochemical parameters measured from six deep subsurface fracture water samples

Sample	DIC (ppm)	DOC (ppm)	O_2 (ppm)	H_2O_2 (ppm)	Fe(II) (ppm)	Total Fe (ppm)	H_2S (ppm)
Dr5IPC	28.44	0.25	0.06	n.d.	<0.05	0.25	<0.1
Be326 Bh2	6.12	0.13	<0.01	n.d.	n.d.	n.d.	n.d.
Be326 Bh1	4.68	n.d.	<0.01	n.d.	n.d.	n.d.	n.d.
TT109 Bh2	12.37	0.69	0.1	<0.1	<0.1	0.6	0.6
TT107	6.84	n.d.	0.2	<0.1	<0.1	0.15	0.2
KL445	1.08	n.d.	2.5	<0.1	0.5	1.0	<0.1

n.d. - not determined

Table 2.3. Gas and water flow rate data and calculated methane concentrations

Sample	Methane in gas (%)	Gas flow rate (mL/min.)	Methane flow rate (mmol/min.)	Water flow rate (L/min.)	Methane concentration (mM)
Dr5IPC	1.9	30.5	0.02	13.1	0.002
Be326 Bh2	60.0	2.0	0.05	0.1	0.4
Be326 Bh1	56.2	n.d.	n.d.	n.d.	n.d.
TT109 Bh2	62.5	160.0	4.1	3.0	1.4
TT107	55.0	1000.0	22.6	3.0	7.5
KL445	52.3	882.0	19.0	4.3	4.4

n.d. - not determined

Table 2.4. Carbon isotope ($\delta^{13}C$ and $\Delta^{14}C$) measurements for PLFA, DIC, DOC and methane

Sample	$\delta^{13}C$ (‰)				$\Delta^{14}C$ (‰)			
	PLFA[a] ± 0.5‰	DIC ± 0.5‰	DOC ± 0.5‰	Methane ± 0.5‰	PLFA ± 20‰	DIC ± 10‰	DOC ± 10‰	Methane ± 10‰
Dr5IPC	-70.3 to -72.9	-7.4	-43.0	-55.4	-893	-930	n.d.	-968
Be326 Bh2	-55.8 to -64.6	-41.9	-28.0	-51.4	-940	-982	-494	-974
Be326 Bh1	-45.1 to -51.6	-30.0	n.d.[b]	n.d.	n.d.	-717	n.d.	n.d.
TT109 Bh2	-30.6 to -36.3	-9.3	-33.1	-42.3	-848	-863	n.d.	-990
TT107	-11.0 to -25.5	-5.0	n.d.	-44.1	-640	-497	n.d.	-996
KL445	n.d.	-31.5	n.d.	-36.5	n.d.	-984	n.d.	-991

[a]$\delta^{13}C_{PLFA}$ values only include PLFAs of sufficient mass for $\delta^{13}C$ analysis (see Table 2.7).
[b]n.d. - not determined

Table 2.5. Volumes of filtered subsurface water (L), total mass of PLFA detected (µg) and microbial cell density estimates based on PLFA concentrations

Sample	Volume of filtered water (L)	Total mass of PLFA detected (µg)	PLFA concentration (pg/mL)	Cell density estimate[1] (cells/mL)
Dr5IPC	16,444	78.3	4.8	$1x10^3$
Be326 Bh2	5,854	469.1	80.1	$2x10^4$
Be326 Bh1	1,086	226.1	208.2	$5x10^4$
TT109 Bh2	25,828	3,785.4	146.6	$3x10^4$
TT107	46,599	41.2	0.9	$2x10^2$
KL445	5,121	0.4	0.1	$2x10^1$

[1]Cell density estimates are based on a conversion factor of $6x10^4$ cells/picomole PLFA (Green and Scow, 2000).

Table 2.6. Total mass of individual phospholipid fatty acids (µg) and their relative abundances (mol%) from DR5IPC, TT107, TT109 Bh2, Be326 Bh1, Be326 Bh2, and KL445

	Dr5IPC		Be326 Bh2		Be326 Bh1		TT109 Bh2		TT107		KL445	
PLFA	µg	mol%	µg	mol%	µg	mol%	µg	mol%	µg	mol%	µg	mol%
12:0	0	0	1.2	0.3	1.2	0.7	0	0	0	0	0	0
13:0	0	0	1.5	0.4	1.4	0.7	0	0	0	0	0	0
i-14:0	6.9	10.7	1.5	0.4	1.8	0.9	0	0	0	0	0	0
14:0	1.9	2.9	6.8	1.6	4.9	2.4	0	0	3.2	9.5	0	0
i-15:0	1.9	2.8	8.4	1.9	11.6	5.4	6.3	1.8	8.2	23.2	0	0
a-15:0	20.8	30.3	4.6	1.1	7.2	3.4	3.4	1.0	3.4	9.5	0	0
15:0	1.7	2.5	4.0	0.9	3.8	1.8	3.1	0.9	2.5	7.2	0	0
i-16:0	19.1	26.3	3.6	0.8	4.6	2.0	0	0	2.0	5.4	0	0
16:1	0	0	129.7	28.3	44.9	20.1	10.6	7.4	0	0	0	0
16 unsat.	0	0	3.7	0.8	8.3	3.7	0	0	0	0	0	0
Me16:0	1.6	2.2	0	0	0	0	0	0	0	0	0	0
10Me16:0	2.1	2.7	0	0	0	0	0	0	0	0	0	0
16:0	9.1	12.6	167.7	36.3	91.2	40.4	215	57.8	6.1	16.2	0.09	42.5
i-17:0	1.1	1.5	3.7	0.8	2.4	1.0	10.1	2.6	1.1	3.5	0	0
a-17:0	1.9	2.6	4.2	0.9	3.3	1.4	0	0	1.4	3.5	0	0
Me17:0	0	0	0	0	0	0	0	0	0	0	0	0
Δ17:0	0	0	29.1	6.0	9.2	3.9	77.5	20.0	1.6	4.1	0	0
17:1	0	0	0	0	0	0	0	0	0	0	0	0
17 unsat.	0	0	7.0	1.5	1.8	0.8	0	0	0	0	0	0
17:0	1.2	1.6	4.0	0.8	2.6	1.1	4.9	1.3	1.2	3.1	0	0
Me18:0	0	0	2.1	0.4	2.6	1.0	0	0	0	0	0	0
18:1	0	0	65.3	12.9	14.9	6	10.9	2.7	3.0	7.2	0.06	28
18 unsat.	0	0	2.0	0.4	0	0	0	0	0	0	0	0
18:0	1.1	1.4	5.4	1.1	3.6	1.5	3.8	0.9	2.0	4.7	0.07	29.5
19:0	0	0	0	0	0	0	0	0	0	0	0	0
Δ19:0	0	0	13.4	2.5	4.7	1.8	13.8	3.2	0	0	0	0
20:1	0	0	0	0	0	0	2.4	0.5	0	0	0	0

Table 2.7. Carbon isotope values ($\delta^{13}C$) measured for individual PLFAs from six deep subsurface fracture water systems

PLFA	$\delta^{13}C$ (± 0.5) ‰				
	Dr5IPC	Be326 Bh2	Be326 Bh1	TT109 Bh2	TT107
i-14:0	-72.9	n.d.	n.d.	n.d.	n.d.
14:0	n.d.	-62.7	-49.8	n.d.	-12.3
i-15:0	n.d.	-55.8	-47.3	-30.6	-11.0
a-15:0	-71.6	-60.1	-47.7	-31.3	n.d.
15:0	n.d.	n.d.	-46.0	-31.4	-17.8
i-16:0	-72.6	n.d.	-45.1	n.d.	-12.4
16:1	n.d.	-64.5	-50.6	-33.2	n.d.
16:0	-70.3	-63.5	-50.0	-34.7	-21.1
i-17:0	n.d.	n.d.	n.d.	-32.0	n.d.
Δ17:0	n.d.	-64.6	-48.8	-33.5	n.d.
18:1	n.d.	-63.9	-51.6	-33.9	n.d.
18:0	n.d.	n.d.	n.d.	-32.7	-25.5
Δ19:0	n.d.	n.d.	n.d.	-36.3	n.d.

n.d. - not determined

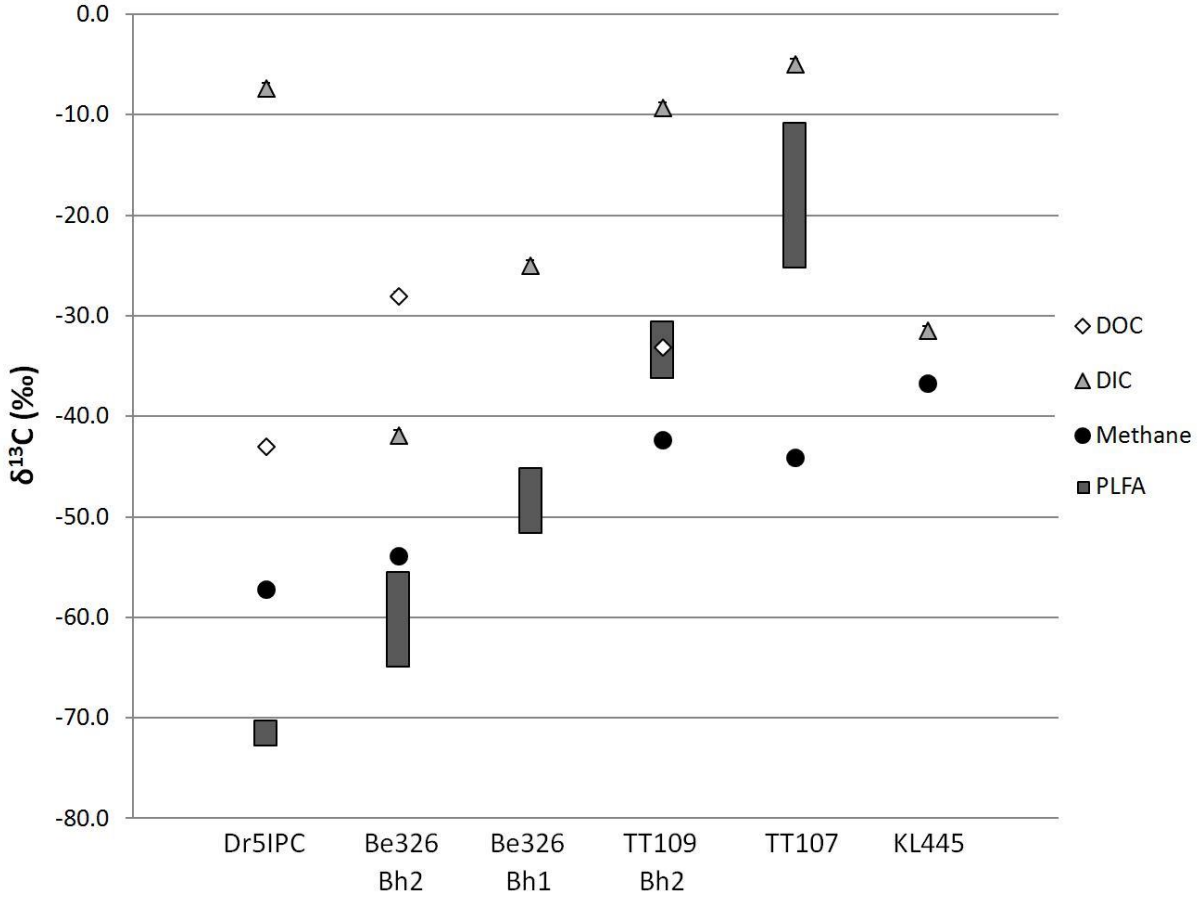

Figure 2.1. $\delta^{13}C$ values for DIC, DOC, methane and PLFA from six deep subsurface fracture water sites. PLFA $\delta^{13}C$ values are ranges measured for the total set of fatty acids identified at each site. KL445 did not contain sufficient carbon from PLFA to measure $\delta^{13}C$. Low DOC levels precluded the $\delta^{13}C$ analysis of DOC for Be326 Bh1, TT107 and TT109 Bh2. Low gas levels precluded the $\delta^{13}C$ analysis of methane at Be326 Bh1.

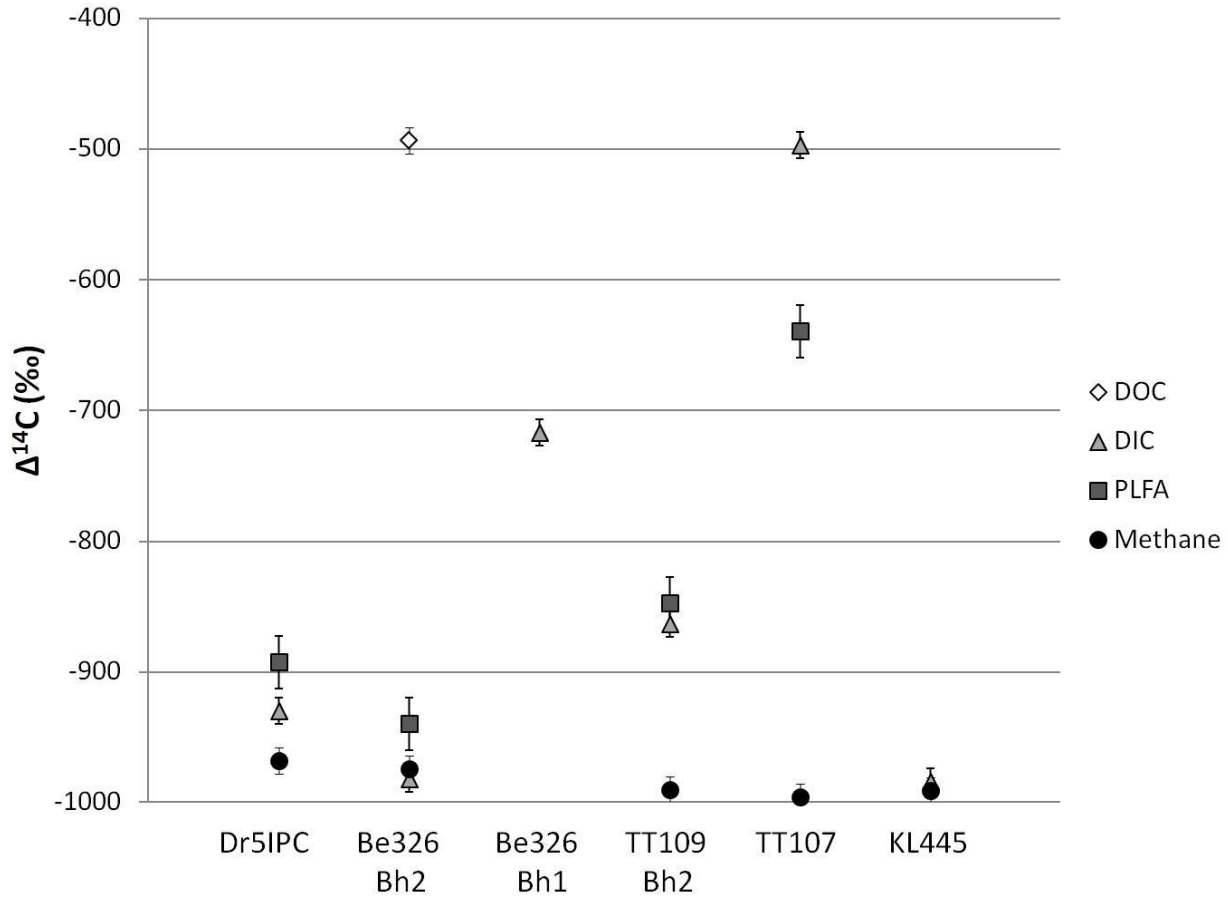

Figure 2.2. $\Delta^{14}C$ values for DOC, DIC, PLFA and methane from six deep subsurface fracture water sites.

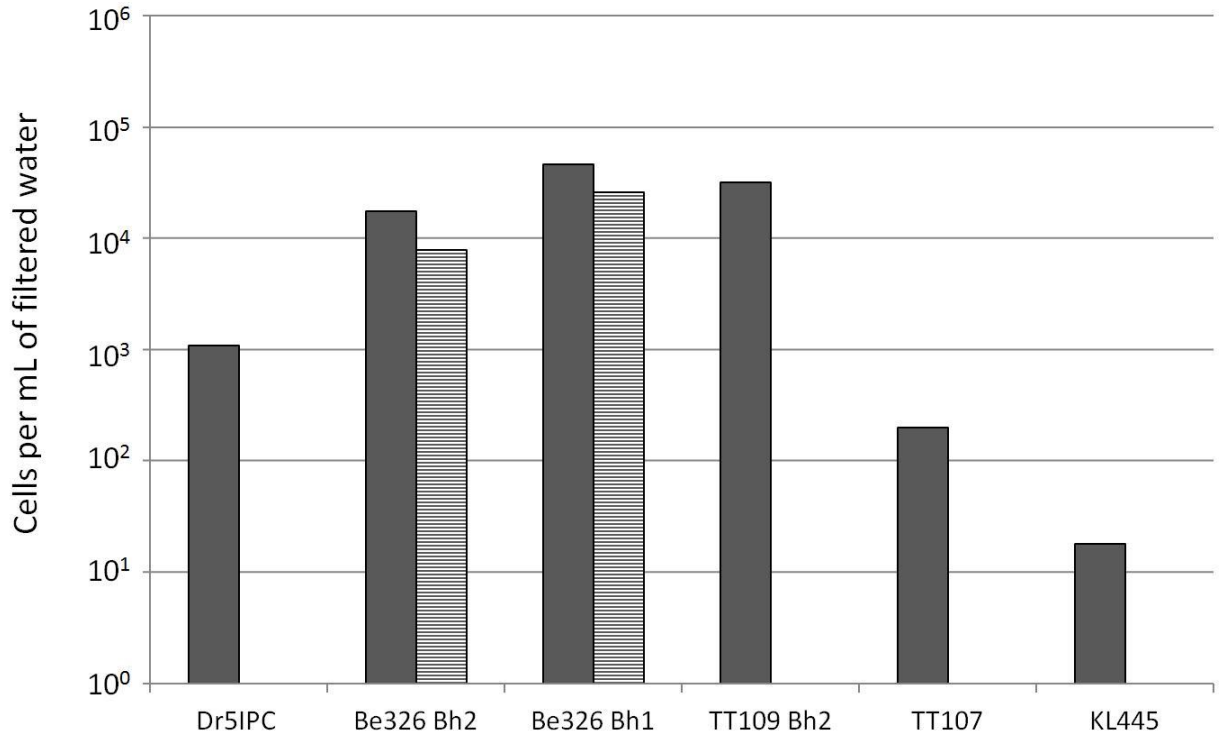

Figure 2.3. Estimates for number of cells per mL of deep terrestrial subsurface water. Cell estimates displayed as dark grey bars are based on PLFA concentrations and a conversion factor of $6x10^4$ cells per picomole of PLFA (Green and Scow, 2000). Cell density estimates displayed as light grey bars are based on direct cell counts via epifluorescence microscopy.

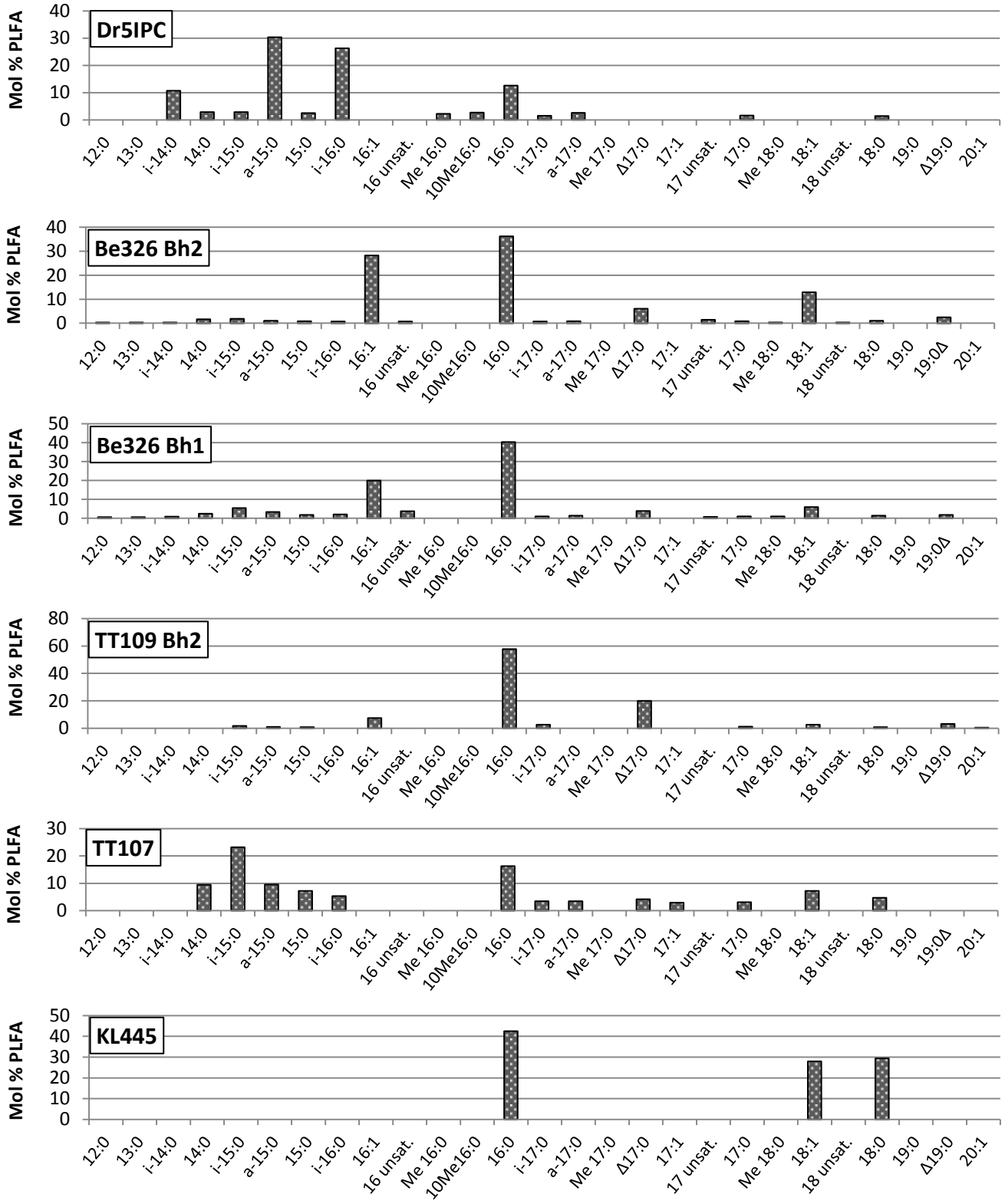

Figure 2.4. Relative abundances (mol %) of cyclic, branched, unsaturated and saturated PLFAs detected in filtered water samples from six deep subsurface sites.

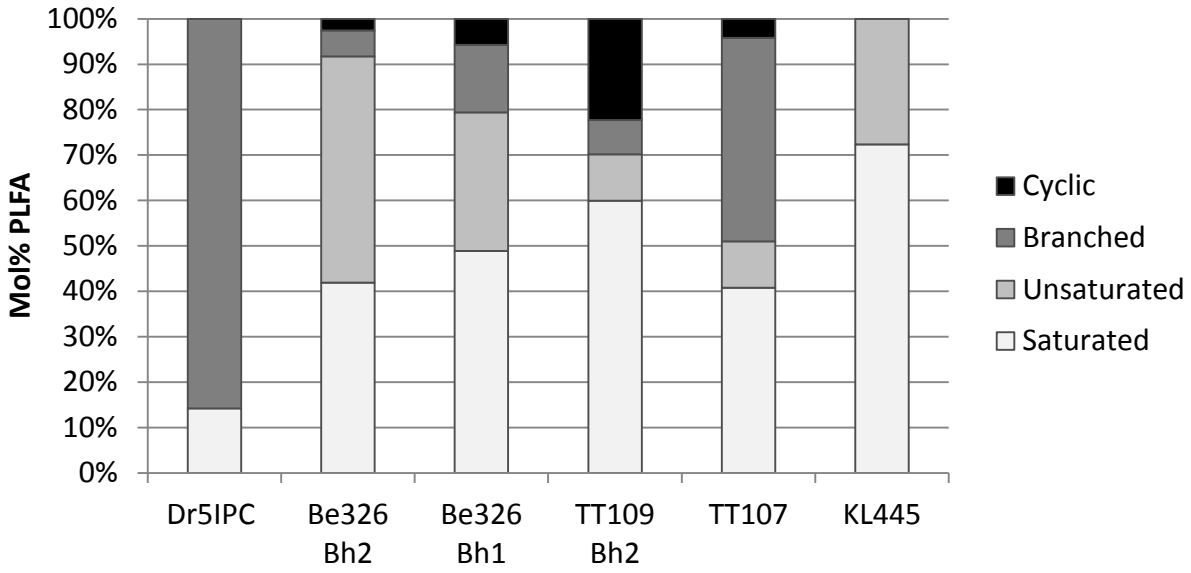

Figure 2.5. Relative abundances (mol %) of PLFA detected in filtered water samples from six deep subsurface sites.

CHAPTER 3:

INVESTIGATING THE PRESENCE OF INDIGENOUS MICROBIAL COMMUNITIES IN LOW PERMEABILITY DEEP SEDIMENTARY ROCKS BASED ON PHOSPHOLIPID FATTY ACID (PLFA) ANALYSIS

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ABSTRACT

Phospholipid fatty acid (PLFA) analysis was used to assess the presence of viable microbial communities living within the natural pores spaces of low permeability deep subsurface sedimentary rocks sampled from the Michigan Basin, Canada. Cores were collected from depths of 334 to 694 meters, including Middle Ordovician to Upper Silurian argillaceous dolostone, argillaceous limestone and shale lithologies. In order to determine whether the PLFAs detected were indigenous to the rock core interiors or introduced during sampling and/or sample handling, the PLFA concentrations and distributions within the rock core interiors, the outer rock core surfaces, and the drilling water were compared. The PLFA concentrations measured for the rock core interiors were consistent across the sample set, representing cell densities of 1-3 x 10^5 cells per gram of rock. All samples were dominated by the PLFAs 16:0, 18:0 and 18:1, with variations in the presence and relative abundances of minor PLFAs across different depths. For all samples, the exterior rock core surfaces were found to contain PLFA concentrations that were equal to or greater than the PLFA concentrations within the rock core interiors. Two of the rock core samples contained relatively high PLFA concentrations on their outer surfaces, representing cell densities that were one and two orders of magnitude higher than the corresponding rock core interiors. This observation, in combination with differences between the PLFA profiles of the interior and exterior materials, indicated a distinct source of PLFAs that was introduced onto the outer surfaces of the rock cores during sampling and/or sample handling without influencing the interior PLFA content. One sample of drilling water was found to contain PLFAs at a very low concentration (0.4 ng of PLFA per mL), representing a cell density of less than $8x10^4$ cells per mL, while other drilling water samples contained no detectable PLFAs. The lack of observation of PLFAs in the drilling water samples is hypothesized to be due to the high salt content that was

added to Lake Huron surface water, hydrolyzing the cells that were originally present. Thus, while the observation of PLFAs in the drilling water may indicate a potential source of contaminants to the rock core surfaces, alternatively, this may indicate some contribution from the subsurface microbial community to the drilling fluid as a result of the re-circulation of the water during the sampling process. The compilation of results from this study presents evidence for the presence of a deep subsurface microbial community living within the pore spaces of sedimentary rock units several hundred meters below land surface.

3.1. INTRODUCTION

3.1.1. Investigating the presence of microbial communities in deep subsurface rock

Assessing the presence and/or activity of indigenous microbial communities within low permeability rocks is extremely challenging and has only been undertaken in a handful of systems (Colwell et al., 1997; Fredrickson et al., 1997; Onstott et al., 2003; Onstott et al., 1998). Exploring microbial communities in subsurface environments presents a number of specific challenges including: sample access and the ability to demonstrate that any observed biosignatures are not related to sampling artifacts; sensitivity of analysis, which can be limited by sample sizes available; and uncertainties as to whether information generated via surface community studies can be transferred to subsurface systems. All of these factors are made more challenging by the fact that these oligotrophic deep terrestrial subsurface systems are generally expected to have very low microbial biomass, due to limited nutrient sources, often in combination with anaerobic conditions, high salinity, and elevated temperatures (Colwell et al., 1997; Kallmeyer et al., 2012; Krumholz, 2000; Krumholz et al., 1997; Parkes et al., 1994; Pedersen, 1997). Microbial studies of deep terrestrial subsurface environments have yielded microbial cell density estimates on the order of 10^3 to 10^7 cells per mL in most systems

(Fredrickson and Fletcher, 2001; Fredrickson et al., 1997; Mills et al., 2010; Moser et al., 2003; Pedersen, 1997; Pfiffner et al., 2006). The majority of these samples are collected from artesian aquifers or pumped water collected from geologic units of interest. A relatively small number of studies have investigated microbial communities living within subsurface rock material, and very few of the samples have been derived from low permeability rocks (Colwell et al., 1997; Fredrickson et al., 1997; Onstott et al., 2003; Onstott et al., 1998). Studies that investigate deep subsurface water for microbial communities primarily sample pelagic microbial cells and are expected to miss any cells that are adhered to rock surfaces. As such, a limitation of sampling from subsurface water is that microbial cell densities estimated from these samples are potentially an underrepresentation of the total microbial community, due to the exclusion of cells that are adhered to surfaces. Regardless of these factors, little water can be obtained from low permeability systems. Investigating indigenous microbial communities in low permeability sedimentary rocks thus requires pulverizing and analyzing rock material to access matrix surfaces in natural pore spaces.

3.1.2. Quantifying microbial biomass from consolidated matrix surfaces

Sampling matrix surfaces to assess subsurface biomass abundance has been undertaken, for the most part, in unconsolidated marine sediments. Original work by Parkes et al. (1994) estimated microbial cell abundances in deep sediments to be on the order of 10^5 cells per cm^3 via cell counting approaches. Recently, Kallmeyer et al. (2012) used new approaches to demonstrate that highly oligotrophic marine sediments can reach cell abundances as low as 10^3 cells per gram. However, this type of direct cell counting cannot be applied to consolidated rock material. Applying direct cell count methods to consolidated material would require physical separation of the cells from the rock surfaces. The preparation of consolidated materials, which requires

pulverizing the rock, would be expected to damage microbial cell membranes. Any damaged cells would be excluded from direct cell counts, ultimately resulting in an underrepresentation of the total cell abundance. As such, ideal cell enumeration methods for consolidated materials involve quantifying molecular biomarkers (such as phospholipid fatty acids) or genetic material, as these compounds are more likely to remain intact throughout sample processing.

3.1.3. Analytical techniques for detecting subsurface life

Key approaches for detecting subsurface microorganisms fall into three classes: characterization of biomarkers such as phospholipid fatty acids (PLFAs), characterization via molecular genetics, and culturing of microorganisms sampled from subsurface substrates. In the latter case, if an organism can be cultured, this is the strongest possible evidence for indigenous viable cells. However, this approach is limited because it is generally felt that less than 1% of microorganisms in environmental systems are culturable (Amann et al., 1995; Pace et al., 1986). Furthermore, as is the challenge involved in direct cell counting, pulverization steps involved in sampling from consolidated rock material may damage intact cells and preclude the ability to culture. This means that biomarker approaches, such as PLFA analysis, and genetic analyses are the most appropriate methods with which to assess microbial populations in low permeability rocks.

A fundamental challenge that arises due to the low microbial biomass expected for subsurface systems is sensitivity of analysis. Methods that rely on direct detection of microbial cells or biomarker compounds can be limited in their ability to detect very low concentrations of microbial cells. Molecular genetics approaches often use amplification steps to overcome this limitation. These approaches are extremely powerful and, in principle, can detect one cell present in a sample. However, the amplification steps must be undertaken with care in order to avoid

contamination at such extremely low levels. Furthermore, matrix effects from mineral and/or organic components of samples can cause interferences and make amplification challenging. These effects are most typically observed in environmental conditions where genetic analyses have not been commonly applied.

An alternative method for overcoming sensitivity limitations is to extract and concentrate relatively large sample sizes. While this can be a logistical challenge, the advantage of such an approach is that the presence of microbial biosignatures can be assessed directly, without the use of an amplification step. This allows techniques, such as phospholipid fatty acid (PLFA) analysis, to not only detect the presence of subsurface microbial communities, but also provide an estimate for the total cell abundances in these systems. Phospholipids are a major component of bacterial and eukaryotic microbial cell membranes and have been shown to hydrolyze within days to weeks upon cell death under ambient surface conditions (White et al., 1979). Therefore, PLFAs collected from environmental samples represent a snapshot of the viable microbial community within a particular system (White et al., 1979). PLFA analysis of environmental samples can provide two primary types of information about the microbial community at a site. Firstly, PLFA concentrations provide estimates for the total amount of viable microbial biomass present within a system. Cell density estimates are based on generic conversion factors relating PLFA concentrations to microbial cell densities (Green and Scow, 2000). The total amount of PLFAs composing a microbial cell membrane is determined by the size of the microbial cell. Cell densities estimates based on PLFA concentrations clearly involve assumptions regarding average cell size and shape (White, 1993); however, this approach remains highly valuable as a means to estimate microbial cell mass in environments where cells cannot be quantified by direct counting techniques. PLFAs can also serve as indicators for particular microbial groups or for

responses of microorganisms to environmental conditions or stresses. Using literature records of PLFAs produced by specific bacteria and eukarya, PLFA biomarkers can be used to identify particular microbial groups (Fang et al., 2007). Alternatively, the presence or ratio of certain PLFAs can indicate physiological conditions of the cells (e.g. stress responses). For instance, in response to stress or resource depletion, microbial communities produce greater mole percentages of cyclopropyl PLFAs and a greater proportion of *trans* versus *cis* monoenoic PLFA configurations (Guckert et al., 1986; Kieft et al., 1994; Petersen and Klug, 1994).

3.1.4. Assessing the potential for contamination

In order to confirm the presence and abundance of microbial biomass in low permeability rocks, it must be demonstrated that any biosignatures observed in the sample are, in fact, derived from indigenous organisms, and not derived from contamination during sampling. The low microbial biomass expected for subsurface systems and the relative ease with which some techniques, such as molecular genetics, can be affected by sampling-related factors are key concerns during the development of methods to assess indigenous microbial communities. Previous approaches have compared outer rock core surfaces, where impacts are expected to be maximized, to internal rock core material to demonstrate that genetic or geochemical indicators were indigenous to the core samples (Fredrickson et al., 1997; Fredrickson and Onstott, 1996; Sherman et al., 2007). Tracers such as dyes or fluorescent microspheres, as well as assessments of extractable hydrocarbons outside and inside the core, have shown that penetration into low permeability rocks is low, as expected, on the order of 1-5 mm (Kallmeyer et al., 2006; Onstott et al., 2003; Sherman et al., 2007). Comparing the outer rock core surfaces to internal rock core material can be used to confirm that removing the outer > 5 mm of surface rock material will, in fact, remove any sampling influences. Confirming that sampling influences have been removed

is important for demonstrating that the interior rock core material is pristine and representative of the indigenous subsurface microbial community.

3.1.5. Research approach

The goal of this study was to identify and quantify PLFAs from rock core samples to determine whether there was evidence for indigenous microbial communities and at what total cell abundances. Comparisons between exterior and interior rock core material were used to distinguish between indigenous microbial cells and potential sampling influences. PLFA analysis of the drilling water was used to assess a potential source of contamination. The PLFA profiles derived from the rock core interiors were used to assess the presence of particular microbial groups and microbial responses to environmental stressors in the deep terrestrial subsurface.

3.2. METHODS

3.2.1. Overview

Six sedimentary rock cores, sampled from a range of depths from the Michigan Basin, Ontario, were analyzed for phospholipid fatty acids (PLFAs) to assess the presence of viable microbial communities within the rock core interiors (Table 3.1). Using a flamed hammer and chisel, rock cores of 9 cm diameter were broken to lengths of 20-30 cm. All cores were handled with sterile nitrile glove, stored in Ziploc bags, frozen onsite and subsequently stored in a -20°C freezer at McMaster University in order to minimize contamination and prevent further microbial growth. High concentrations of NaCl were added to the Lake Huron drilling water to approximate the chemistry of the subsurface pore water. 60mL samples of filtered drilling water and 1L samples of bottled drilling water were collected at time points, corresponding to the sampling of individual cores and were analyzed to determine whether the drilling water used

during sampling contained any detectable PLFAs. Tracer approaches, such as adding fluorescent microspheres or chemical tracers to the drilling water, can be used to assess whether contaminants have been introduced to sample interiors during drilling processes (Fredrickson and Onstott, 1996). However, in this study, these approaches were not available to us. As a result, in order to assess whether PLFAs were introduced to the rock cores during the drilling process and/or during sample handling, the abundances and patterns of PLFAs within the rock core interiors and on the outer rock core surfaces were compared. In order to sample from the outer rock core surfaces, each core sample was rinsed with Bligh and Dyer (1959) extraction solvents prior to cutting or crushing, and these solvents were analyzed for PLFAs. Based on the work of Sherman et al (2007), in combination with observations of PLFA extraction solvent penetration several mm into the rock core interiors, it was assumed that penetration of contaminants would be limited to the outer 5 mm of the rock. Analysis of the rock core interiors to assess the presence of indigenous microbial populations based on PLFA involved the removal of the outer surfaces of the core (> 5mm) to avoid any surface influences. All rock core samples analyzed in this study are identified using the following nomenclature: "Depth(i/o)_StratigraphicUnit", where 'i' denotes the "inside" (interior) rock material of a core and 'o' denotes the "outside" (exterior surface) of a core.

3.2.2 PLFA extraction and analysis

PLFAs were extracted from all samples using a modified Bligh and Dyer PLFA extraction method (Bligh and Dyer, 1959). The resulting total lipid extracts (TLE) were separated into non-polar (F1), neutral (F2) and polar (F3) fractions using silica gel chromatography, using DCM (F1), acetone (F2) and methanol (F3) (Guckert et al., 1985). Phospholipids recovered from the polar fractions were converted to fatty acid methyl esters

(FAMEs) via mild-alkaline methanolysis and subsequently purified by a secondary silica gel chromatography, using 4:1 hexane:DCM (F1), DCM (F2) and methanol (F3) (Guckert et al., 1985). Microbial FAMEs were identified and quantified using gas chromatography-mass spectrometry (GC-MS) on an Agilent GC-MS (Agilent Technologies Inc., Santa Clara, CA, USA) with a DB-5MS capillary column (30m x 0.25µm film thickness) and a temperature program of 50°C (for 1 min.), 20°C/min to 130°C, 4°C/min to 160°C, and 8°C/min to 300°C (for 5 min.). In order to optimize sensitivity and allow the maximum sample mass to be injected via the auto-sampler system on the GC-MS, samples were run in 50 µL or 100 µL volumes with 2 µL injections. The lower limit of quantification for this system was determined to be the lowest concentration standard that can be reliably analyzed, which is 2 ng/µL for a 1 µL injection on the GC-MS. Sample peaks with area responses lower than this standard could be detected, but not quantified. All reported concentrations consider only quantifiable peaks.

3.2.3. Drilling water

The PLFA content of the drilling water was analyzed to assess a potential external source of contamination during sampling. 60 mL samples of drilling water were filtered using Millipore Sterivex 0.2 µm filters and the filters were extracted using the PLFA method. No PLFAs were detected from these extracts; however, this is likely a result of the microbial cell densities being below the detection limit of 10^5 cells/mL. To address this, four 1L samples of drilling water, collected during drilling at depths of 662, 670, 682, and 695 meters, were filtered in the lab using the same syringe filter system, lowering the detection limit to approximately $3x10^4$ cells per mL (Table 3.2). The filters were subsequently extracted and analyzed for PLFAs.

3.2.4. Rock core rinses

Rinses of the outer core material were analyzed for PLFAs to assess the presence and abundance of potential contaminants on the outer surfaces of the rock cores. The cores were individually rinsed with Bligh and Dyer (1959) extraction solvents (45 mL DCM, 90 mL methanol and 36 mL phosphate buffer) prior to cutting and crushing. These solvents were analyzed for PLFAs via the method outlined in Section 3.2.2.

PLFA concentrations and cell density estimates calculated for the rock core rinses were calculated based on the assumption that the outer 3 mm of rock material are extracted during rinsing with extraction solvents. This assumption is based on the observation of a 3 mm outer rim of solvent-saturated rock material after rinsing. In support of this observation, Sherman et al. (2007) reported the presence of external contaminants within the outer 3-5 mm rim of rock core material. The PLFA concentrations and cell density estimates calculated for the rock core rinses will change depending on this assumption. In order to assess the extent of this change, PLFA concentrations and cell density estimates were re-calculated using alternative assumptions (i.e. that the outer 1 mm or the outer 5 mm of rock material are extracted during rinsing) (Table 3.7). The total mass of rock material extracted during rinsing was calculated as follows: The total volume of rock material composing the interior of the rock core (excluding the outer 1, 3 or 5 mm of material) was first calculated, using $V = L\pi r^2$, where L is the length of the rock core minus the outer 1, 3, or 5 mm and r is the radius of the rock core minus the outer 1, 3, or 5 mm. This volume was subsequently subtracted from the total volume of the entire rock core sample, to calculate the total volume of rock material within the outer 1, 3, or 5 mm of the rock core. This volume was multiplied by the density of the rock core sample to determine the mass of rock material within the outer 1, 3 or 5 mm.

3.2.5. Rock core interiors

The outer surfaces of the six cores were removed using a hydraulic jack equipped with stainless steel chisels (Figure 3.1). The purpose of this step was to remove a minimum of the outer 5 mm of rock material that may have been impacted by external contaminants during drilling and to achieve a pristine interior sample of rock core material for analysis. The stainless steel chisels were rinsed with dichloromethane, methanol, hexane in between each step. The interior pieces of the rock cores were crushed using a stainless steel mortar and pestle, and then powdered using a stainless steel puck mill on a SPEX machine, both modeled after Sherman et al. (2007) (Figure 3.1). The stainless steel mortar and pestle and puck mill were washed and rinsed with distilled water, and solvent rinsed with dichloromethane, methanol, hexane and acetone in between each sample preparation. The powdered interior material was subsequently extracted for PLFAs. Blank analysis of pre-combusted sand crushed in the stainless steel mortar and pestle and puck mill were found to contain no detectable PLFAs.

3.3. RESULTS

3.3.1. Drilling water

The results of the PLFA analyses of filtered drilling water are shown in Table 3.2. No PLFAs were detected in any of the four 60 mL filtered drilling water samples. The detection limit for these 60 mL water samples was $5x10^5$ cells per mL. Increasing the sample volume to 1L increased the sensitivity by lowering the detection limit to $3x10^4$ cells per mL. One of the 1L filtered drilling water samples (DWR DGR8 - 013) did not contain any indication of PLFAs. Two of the 1L filtered drilling water samples (DWR DGR8 - 012 and DWR DGR8 - 015) appeared to contain very low levels of PLFAs, but their abundances were below quantification. The fourth 1L drilling water sample (DWR DGR8-014) contained a total of 0.4 µg of PLFAs.

Based on a conversion factor of $6x10^4$ cells per picomole of PLFA, this PLFA abundance represents a microbial cell density of $8x10^4$ cells per mL of drilling water, which is just above the detection limit of $3x10^4$ cells per mL (Green and Scow, 2000). The PLFAs present in the DWR DGR8-014 sample were 16:0, 17:0, 18:1 and 18:0. The relative abundances of the specific PLFAs present in DWR DR8-014 are illustrated in Figure 3.2.

3.3.2. Rock core interiors and rinses

3.3.2.1. PLFA Concentrations

All six rock core interiors and their exterior rinses were found to contain PLFAs. The masses of PLFAs detected and the PLFA concentrations for each sample are listed in Tables 3.3 and 3.4. The total concentrations of PLFAs in the rock core interiors and the rock core rinses are illustrated in Figure 3.3. In all six cases, the total concentrations of PLFAs in the rock core interiors were equal to or lower than in the rock core rinses. Interior PLFA concentrations across the six rock core samples were similar, ranging from 0.4 to 1.5 ng of PLFA per gram of rock. PLFA concentrations in four of the exterior rinses were comparable to these values ranging from 1.0 to 3.4 ng of PLFA per gram of rock. However, the rinses of 464o_Queenston and 529o_Georgian Bay yielded very high concentrations of PLFAs (17.8 and 76.2 ng of PLFA per gram of rock, respectively) relative to the other four rock core rinses.

PLFA concentrations for the rock core rinses were calculated based on the assumption that the outer 3 mm of rock core material was extracted during rinsing with extraction solvents. In order to assess the effect of this assumption, cell densities were calculated for the rock core rinses based on alternative depths of extraction (i.e. 1 mm vs. 3 mm vs. 5 mm). The results from these calculations are listed in Table 3.7, and indicate that the effect of changing this assumption

to 1 mm or 5 mm was negligible. Even if the solvents extract up to 5 mm into the core, the concentrations of PLFAs on the outer rock core surfaces are greater than or equal to the concentrations in the interiors. There were no cases where the PLFA concentrations in the rock core interiors were higher than the concentrations on the rock core surfaces.

3.3.2.2. Cell Density Estimates

Cell density estimates based on PLFA concentrations for the rock core interiors and the rock core rinses are listed in Tables 3.3 and 3.4, respectively. Cell density estimates are based on a conversion factor of $6x10^4$ cells per picomole of PLFA (Green and Scow, 2000). For the rock core interiors, cell density estimates were relatively consistent, within the range of $1x10^5$ to $3x10^5$ cells per gram of rock. Cell density estimates calculated for the four exterior rinses that contained lower PLFA concentrations were relatively consistent, ranging from $2x10^5$ to $8x10^5$ cells per gram of rock. Cell density estimates for the two exterior rinses (464o_Queenston and 529o_Georgian Bay) that contained relatively high PLFA concentrations were one and two orders of magnitude higher than the other four rinses, respectively.

3.3.2.3. PLFA Distributions

The relative abundances (in mole percentage) of individual PLFAs detected in the extracts of the rock core interiors and rock core rinses are listed in Tables 3.5 and 3.6 and illustrated in Figure 3.4. The three most abundant PLFAs were 16:0, 18:0 and 18:1 in all six rock core rinses and all six rock core interiors. Generally, the samples contained relatively low abundances of other PLFAs, which largely consisted of of branched and cyclopropyl PLFAs. Two exceptions were 334o_SalinaA1 and 464i_Queenston, both of which only contained the three PLFAs 16:0, 18:0 and 18:1.

3.3.2.4. Rock Core Appearances

Cores 334_SalinaA1 and 661_Coburg were found to contain very thin natural fractures, along which the cores broke once the pressure of the hydraulic jack was applied (Figure 3.5). The material along these fractures was very dark and oily in nature. For 334_SalinaA1, the darker material was initially analyzed separately from the rest of the core. The PLFA composition and concentrations did not differ between the dark material and the rest of the 334_SalinaA1 sample. For 661_Coburg, the fracture ran along the entire length of the core; therefore, the darker material was analyzed in combination with the rest of the interior material.

3.4. DISCUSSION

3.4.1 Evidence for viable indigenous microbial communities

3.4.1.1. Comparing interior and exterior PLFA concentrations

Total PLFA concentrations for the rock samples were very low and indicative of a very low biomass subsurface community. PLFAs were identified in the rock core interiors at concentrations of 0.4 to 1.5 ng PLFA/g of rock, representing relatively consistent microbial cell density estimates of $1x10^5$ to $3x10^5$ cells/g of rock. These cell density estimates are comparable to some of the lowest estimated cell densities reported for solid subsurface samples (Colwell et al., 1997; Fredrickson et al., 1997; Parkes et al., 1994). PLFA concentrations calculated for the rock core exteriors (1.0 to 76.2 ng PLFA/g of rock) were either greater than or equal to the PLFA concentrations observed for the rock core interiors (Figure 3.3), representing microbial cell densities of $2x10^5$ to $2x10^7$ cells/g of rock. Consistencies between several of the exterior and interior PLFA concentrations either implies that the exterior rock core material sampled the same microbial community as the rock core interiors (i.e. that there was no effect from sampling), or

that any effects during sampling were communicated throughout the entire rock core sample (i.e. the interior values are also a result of contamination).

The exterior surfaces of the samples 464_Queenston and 529_GeorgianBay contained exceptionally high concentrations of PLFAs, representing cell densities that were one and two orders of magnitude higher than the rest of the samples, respectively (Figure 3.3). PLFA concentrations within the rock core interiors of samples 464_Queenston and 529_GeorgianBay were no higher than the other rock core interior PLFA concentrations. These differences are attributed to the presence of contaminants on the outer rock core surfaces of these two samples and the absence of these contaminants in the rock core interiors. Tests of various assumptions regarding the depth of solvent penetration during extraction confirmed that the observation of higher PLFA concentrations on the outer rock core surfaces was not an artifact of underestimating the total mass of extracted rock material during rinsing (Table 3.7). The observation of differences for these two samples illustrates that contamination can be identified and likely occurred on the outer surfaces of these two cores. The absence of an increase in cell biomass on the surfaces of the other rock cores suggests that the observed cell densities are indigenous rather than due to contamination. This argument can be further tested via examination of PLFA distributions.

3.4.1.2. Comparing interior and exterior PLFA distributions

Differences in the distributions of individual PLFA structures within the interior and exterior rock core materials supported the argument that the PLFAs observed in the rock core interiors were from indigenous microbial communities rather than contaminants during sampling. Hypothetically, if the PLFAs identified within the rock core interiors were derived from contamination during sampling, the range of PLFAs identified and their relative

abundances would be consistent between rock core interiors and the outer rock core surfaces where contamination effects would be expected to be greatest. Any observation of PLFAs, or patterns in PLFA distributions, that are unique to the outer rock core surfaces could thus be considered indicators or "tracers" for contamination.

All of the rock core interiors and rock core rinses contained the PLFAs 16:0, 18:0 and 18:1; however, in some cases, the relative proportions of these individual PLFAs greatly differed between the rock core interiors and the corresponding rock core rinses (Figure 3.4). For example, a relatively high proportion of 18:1 was observed in 334o_SalinaA1 (70.8 mol%), relative to the proportion of 18:1 in the corresponding interior sample 334i_SalinaA1 (17.7 mol%). The same observation was made for the two samples that contained relatively high exterior PLFA concentrations (464_Queenston and 529_GeorgianBay). The interiors of 464_Queenston and 529_GeorgianBay contained low abundances of 18:1 (32.1 mol% and 18:0 mol%, respectively) relative to the exterior surfaces (67.9 mol% and 39.4 mol%, respectively). These distinct differences in the PLFA distributions between the interior and exterior materials indicates a distinct source of PLFAs introduced onto the outer rock core surfaces, containing a high proportion of 18:1. As this source does not appear to be the inner cores, it suggests that it is contamination, which did not appear to affect the interior PLFA content. The distinct high relative abundance of 18:1 identified on the outer surfaces of 334_SalinaA1, 464_Queenston and 529_GeorgianBay may represent a similar source of contamination to these surfaces. This is consistent with the fact that 334_SalinaA1, 464_Queenston and 529_GeorgianBay were sampled from relatively similar depths and may have been influenced by the same sampling materials.

Further evidence for distinct sources of contamination was revealed by similarities in the PLFA profiles of the 661_Coburg and 682_Coburg rock core rinses. The outer surfaces of these

cores (661o_Coburg and 682o_Coburg) contained relatively high abundances of the PLFA 12:0, at 12.9 mol% and 23.8 mol%, respectively. Yet, 12:0 was not observed in either of the corresponding rock cores interiors or any of the other rock core samples in this study. This observation indicates that a distinct source of PLFAs containing 12:0 was introduced to the surfaces of these two cores and did not influence the PLFA content of the rock core interiors. Again, this is consistent with the fact that 661_Coburg and 682_Coburg were sampled from relatively similar depths. Overall, these differences in the PLFA distributions between the interior and exterior rock core material indicate distinct microbial communities and provide evidence for indigenous microbial communities that were not affected by sampling influences.

3.4.1.3. Is drilling water the source of the contamination?

The PLFA content of the drilling water was compared to the concentrations and distributions of PLFAs in the rock core samples to assess whether the drilling water was a potential source of contamination during sampling. The results indicate that the drilling water was likely a minor source of contamination to the outer rock core surfaces and an unlikely source of PLFAs to the rock core interiors. All but one of the 1L filtered drilling water samples lacked quantifiable PLFAs, indicating that the microbial cell densities within these samples were below the detection limit of 0.2 ng of PLFA per mL, or $3x10^4$ cells per mL. One of the 1L filtered drilling water samples, DWR DGR8-014, was found to contain 0.4 ng of PLFA per mL which represents $8x10^4$ cells per mL, based on a conversion factor of $6x10^4$ cells per picomole of PLFA. This cell density estimate was an order of magnitude lower than the cell density estimates calculated for the rock core interiors ($1x10^5$ to $3x10^5$ cells/g) and one to three orders of magnitude lower than those of the rock core rinses ($2x10^5$ to $2x10^7$ cells/mL). A relatively low cell density estimate for one drilling water sample, in combination with a lack of quantifiable

PLFAs in the three other drilling water samples, suggests that the drilling water is not a major source of microbial cells, and thus PLFAs, to the rock core samples.

The low microbial cell density estimated for the drilling water can likely be attributed to its high added NaCl content. The source of the water was originally fresh surface water from Lake Huron, which likely contained relatively high abundances of bacterial and eukaryotic cells, based on average lake water microbial cell densities of 10^6 cells per mL (Whitman et al., 1998). However, the addition of high levels of NaCl created salinities of 140-170 ms/cm, approximately three times the salinity of sea water, and comparable to the salinities of the groundwater in the geologic units being sampled. The high levels of NaCl may have acted as a bactericidal to a large portion of the cells via plasmolysis and ultimately resulted in a very low microbial cell density in these samples. A microbial cell density that is equal to, or lower than, $8x10^4$ cells per mL of drilling water indicates that the drilling water was likely a very minor external source of PLFAs to the rock core surfaces and an unlikely source of PLFAs to the rock core interiors.

Another possible explanation for the PLFAs observed in the drilling water is that they are the result of the drilling water "sampling" the rock units during drilling. The drilling water samples were collected following the recirculation of the water along the core. As such, it must be considered that, although the drilling water may have introduced PLFAs to the rock core samples, the converse situation is also possible (i.e. rock material may have introduced PLFAs to the drilling water). The PLFA profile from the DWR DGR8-014 drilling water sample consisted of four PLFAs: 16:0, 17:0, 18:1 and 18:0. The corresponding rock interior sample at this depth (682i_Coburg) did not contain 17:0. However, 661i_Coburg, which was sampled from a similar depth, was the only rock core to contain 17:0. This could suggest that the drilling water was sampling the indigenous microbial communities as it was re-circulating through the subsurface.

While difficult to conclusively assess, the results from this study suggest that a combination of both effects was occurring; the drilling fluid may have been sampling from the subsurface microbial community while also introducing low levels of microbial contaminants to the outer rock core surfaces.

3.4.2. PLFA biomarkers and stress indicators

Differences in the PLFA profiles among the interior samples may indicate distinct shifts in microbial community structure with depth. Furthermore, the detection of certain PLFA structures in the rock core interiors provided information about the microbial community composition and environmental stressors in these deep subsurface environments. The three most abundant PLFAs in all of the samples were 16:0, 18:0 and 18:1. Monounsaturated PLFAs such as 18:1 are commonly attributed to gram-negative bacteria, while 16:0 and 18:0 are common PLFAs in most bacteria and are not considered to be indicative of specific microbial groups or environmental conditions (Green and Scow, 2000). Beyond these three PLFAs, several of the rock core interiors contained branched PLFAs, which are common in gram-positive bacteria (Fang and Barcelona, 1998; Green and Scow, 2000). The PLFA profiles of 661i_Coburg and 682i_Coburg both contained the PLFAs *i*-17:0 and *a*-17:0, which were not observed in the interiors of any other samples. This observation may indicate some similarity in the microbial community structures of these two sites, which is consistent with the similar depths of these two samples. 661i_Coburg contained the cyclopropyl PLFA *cy*17:0 at 6.5 mol% and 694i_ShermanFalls contained the cyclopropyl PLFA *cy*19:0 at 11.3 mol%, which are potential indicators for microbial responses to environmental stressors. Microorganisms have been shown to produce higher proportions of cyclopropyl PLFAs in response to stress conditions, such as severe nutrient deprivation during cell growth (Kieft et al., 1994; Petersen and Klug, 1994).

Alternatively, cyclopropyl PLFAs are potential indicators for anaerobic bacteria (Fang and Barcelona, 1998). Notably, these cyclopropyl PLFAs were present within two of the deepest samples, at 661m and 694 m depth, which is consistent with greater stress conditions, including higher pressures and lower nutrient availibity. While it is difficult to assign the PLFAs observed to specific microbial groups or environmental responses at these low abundances, indicators for anaerobic bacteria or environmental stressors are both consistent with low oxygen levels and low nutrient availability commonly observed for deep terrestrial subsurface systems (Colwell et al., 1997).

3.4.3. Assessing the viability of subsurface communities

Using PLFAs as indicators for viable microbial communities is based on the assumption that phospholipids hydrolyze within days to weeks after cell death (White et al., 1979). This assumption is based on studies that were performed under surface conditions with high microbial cell abundances, which would be expected to contribute high levels of phospholipases (i.e. enzymes that accelerate phospholipid hydrolysis). One question that arises is whether hydrolysis of phospholipids, and thus the loss of PLFAs, would occur at the same rate in subsurface systems or whether the observed PLFAs may be relics that are preserved for long timescales. The implication of the latter case is that PLFA studies of subsurface systems could appear to reveal viable microbial communities in environments that are, in reality, devoid of viable organisms. This topic is important for this study as it addresses the validity of our results, as we demonstrated the presence of viable microbial communities in low permeability, deep terrestrial subsurface environments.

Harvey et al. (1986) examined the degradation rates of phospholipids in the presence of various levels of organic matter and found that phospholipids were degraded more slowly in the

presence of high levels of labile organic matter. In low organic content conditions, phospholipids degraded fairly rapidly; approximately 50% of the phospholipids had degraded within the first 12 hours, and that 70% had degraded by the end of the 4 day period. Their results also revealed that degradation rates were slowed by up to 40% in anaerobic conditions. Logemann et al. (2011) investigated the degradation of rates of phospholipids in marine sediments and demonstrated that phospholipid degradation is primarily a biotic process. The abiotic controls showed no sign of microbial activity and, concurrently, only a slight decrease in the concentration of phospholipids over the course of the 100-day experiment. This suggests that phospholipids may be preserved over long periods of time in systems where the microbial community has recently died off, or is metabolically inactive, and there is an absence of phospholipases. These results are directly applicable to deep terrestrial subsurface environments, where low organic contents, low oxygen levels and potentially abiotic systems are expected (Pedersen, 2000). In order for PLFAs to be preserved in the subsurface over geologic timescales, reaction rates must be slowed by many orders of magnitude. Such an extreme reduction in reaction rates may be unrealistic. Ideally, reaction rates would be determined under conditions more relevant to subsurface systems, including a combination of high pressures, low oxygen levels and low nutrient availability, in order to test whether assumptions regarding phospholipid degradation rates are causing estimates of viable cells to be higher than they should be, due to preservation.

3.4.4. Subsurface lithologies and implications for indigenous microbial communities

Based on the results of this study, viable microbial communities appear to exist at low abundances (on the order of 10^5 cells per gram) within the small natural pore spaces of low permeability sedimentary rock units. These stratigraphic units, composed of argillaceous dolostone, argillaceous limestone and shale lithologies, are located at depths ranging from 334 to

694 meters below land surface (mbls). Similar microbial cell densities have been observed previously for other sedimentary systems, including sandstone and shale units, up to depths of several kilometers below land surface (kmbls) (Colwell et al., 1997; Fredrickson et al., 1997; Krumholz et al., 1997; Lehman et al., 1995; Onstott et al., 2003). These studies have identified deep subsurface microbial communities largely consisting of iron reducers, sulfate reducers, anaerobic heterotrophic bacteria and methanogens, via combinations of PLFA analyses, genetic analyses, and culturing experiments.

The sediments that compose the stratigraphic units analyzed in this study were deposited between the Middle Ordovician and the Upper Silurian time periods (between 470 and 400 mya). Microbial communities currently living within these sedimentary units may have been present at the time of deposition, in which case they would have persisted over 400 myr in isolation from the Earth's surface environments. Groundwater flow likely introduced microbial communities into this system until the more recent past; however, diagenesis and compaction of sediments would have greatly reduced the permeability of the sediments over time. As such, at several hundred mbls, low porosity limits the flux of nutrients from the Earth's surface and microbial communities must rely on endogenous electron acceptors, such as sulfate, iron and organic matter (Colwell et al., 1997; Fredrickson and Balkwill, 2006). Organic-rich sedimentary units can potentially fuel heterotrophic microbial communities as both an energy and carbon source. However, in many systems, ancient organic carbon is highly recalcitrant or inaccessible to microbial communities due to small pore sizes and low permeability, in which case microbial communities must rely on alternative metabolisms, including H_2-based chemolithoautotrophy (Fredrickson and Balkwill, 2006; Stevens, 1997). In the presence of low nutrient availability, deep subsurface microbial communities are likely adapted to slow growth over long periods of

time (Krumholz, 2000). Microorganisms have been found to concentrate within the larger interconnected pore spaces of sedimentary rocks, as larger porosity allows for the diffusion of nutrients and migration of microbial cells (Fredrickson et al., 1997; Krumholz, 2000). In this study, PLFA concentrations along the natural fractures of the rock cores were no higher than the PLFA concentrations within the rest of the matrix material. Limestone and dolostone lithologies are generally more porous in nature than shale and would potentially host more abundant microbial communities. However, the microorganisms identified in our study did not appear to concentrate within particular lithologies, as the microbial cell densities were relatively consistent with depth.

3.5. CONCLUSIONS

The results from this study indicate the presence of a low biomass indigenous microbial community in these geologic units and are consistent with previous observations of microbial communities in deep terrestrial subsurface rock (Colwell et al., 1997; Fredrickson et al., 1997; Krumholz et al., 1997; Lehman et al., 1995; Onstott et al., 2003). Differences in PLFA concentrations and distributions between the rock core interiors and the outer rock core surfaces indicate a minor input of external contaminants onto the exterior surfaces of the rock cores. This influence of external sources did not appear to affect the interior material of the cores, based on consistent PLFA concentrations within the rock core interiors. A low abundance of PLFAs in one drilling water sample, in combination with a lack of quantifiable PLFAs in all other drilling water samples, indicates a very low microbial cell density in the drilling water used for sampling. The drilling water may have acted as a very minor source of contaminants to the outer rock core surfaces; however, it is unlikely to have impacted the interior rock core material. As such, the PLFAs identified within the rock core interiors are likely indigenous to the samples, representing

viable microbial communities at low abundances within deep sedimentary rocks. The observation of indigenous microbial communities in low porosity sedimentary rocks demonstrates the ubiquity of microbial systems in the deep terrestrial subsurface, and the ability of subsurface communities to persist over geologic timescales, despite a range of environmental stressors.

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3.7. TABLES AND FIGURES

Table 3.1. Rock core sample depths, corresponding stratigraphic units and lithologies.

Core	Depth (m)	Stratigraphic Unit	Lithology	Geologic Period
334_SalinaA1	334.4	Salina A1	Argillaceous dolostone	Upper Silurian
464_Queenston	464.9	Queenston	Shale	Upper Ordovician
529_GeorgianBay	531.6	Georgian Bay	Inter-bedded shale and limestone	Upper Ordovician
661_Coburg	661.6	Coburg	Argillaceous limestone	Middle Ordovician
682_Coburg	682.9	Coburg	Argillaceous limestone	Middle Ordovician
694_ShermanFalls	694.6	Sherman Falls	Argillaceous limestone	Middle Ordovician

Table 3.2. Filtered drilling water sample names, volumes, and depths, total PLFA detected and cell density estimates based on PLFA concentrations.

Sample	Volume of filtered water (mL)	Depth (m)	Total mass of PLFA detected (μg)	Cell density estimate (cells/mL water)
DWR DGR8 001 –Blank	60	n/a	n.d.	n.d.
DWR DGR8 – 001	60	n/a	n.d.	n.d.
DWR DGR8 – 003	60	334.42	n.d.	n.d.
DWR DGR8 - 007	60	531.57	n.d.	n.d.
DWR DGR8 - 012	1000	661.63	n.q.	n.q.
DWR DGR8 - 013	1000	670.10	n.d.	n.d.
DWR DGR8 - 014	1000	682.86	0.4	$8x10^4$
DWR DGR8 - 015	1000	694.57	n.q.	n.q.

n.d. - No PLFA Detected
n.q. - Non-Quantifiable

Table 3.3. Rock core interior sample masses, total PLFA detected, PLFA concentrations, and cell density estimates.

Core	Mass of interior material extracted for PLFA (g)	Total mass of PLFA detected (µg)	PLFA concentration (ng PLFA/ g of rock)	Cell density estimates (cells/ g of rock)
334i_SalinaA1	1108.4	1.3	0.8	$2x10^5$
464i_Queenston	875.0	1.1	1.2	$3x10^5$
529i_GeorgianBay	1257.8	0.6	0.4	$1x10^5$
661i_Coburg	972.3	1.3	1.4	$3x10^5$
682i_Coburg	1008.3	1.5	1.5	$3x10^5$
694i_ShermanFalls	859.4	0.8	1.0	$2x10^5$

Table 3.4. Total mass of rock material extracted during rinsing, total PLFA detected, PLFA concentrations, and cell density estimates for rock core rinses.

Core Rinse	Mass of rock material extracted during rinsing (g)	Total mass of PLFA detected (µg)	PLFA concentration (ng PLFA/g of rock)	Cell density estimates (cells/g of rock)
334o_SalinaA1	525.5	1.4	2.7	$6x10^5$
464o_Queenston	523.3	9.3	17.8	$4x10^6$
529o_GeorgianBay	445.2	33.9	76.2	$2x10^7$
661o_Coburg	456.8	1.5	3.4	$8x10^5$
682o_Coburg	459.2	0.8	1.8	$4x10^5$
694o_ShermanFalls	429.4	0.4	1.0	$2x10^5$

Table 3.5. Total masses of the individual phospholipid fatty acids (µg) and their relative abundances (mol%) identified within the rock core interiors of 334_SalinaA1, 464_Queenston, 529_GeorgianBay, 661_Coburg, 682_Coburg and 694_ShermanFalls

	334i SalinaA1		464i Queenston		529i GeorgianBay		661i Coburg		682i Coburg		694i ShermanFalls	
PLFA	µg	mol%	µg	mol%	µg	mol%	µg	mol%	µg	mol%	µg	mol%
12:0	0	0	0	0	0	0	0	0	0	0	0	0
14:0	0	0	0	0	0.1	21.6	0	0	0.1	9.1	0	0
i-15:0	0	0	0	0	0	0	0	0	0.1	7.4	0	0
a-15:0	0.1	10.5	0	0	0	0	0	0	0.1	7.7	0	0
15:0	0	0	0	0	0	0	0	0	0	0	0	0
16:1	0	0	0	0	0	0	0	0	0	0	0	0
16:0	0.4	30.8	0.4	35.8	0.2	34.1	0.4	33.1	0.4	30.0	0.1	19.2
Me17:0	0	0	0	0	0	0	0	0	0	0	0	0
i-17:0	0.2	16.1	0	0	0	0	0.1	6.6	0.1	5.9	0	0
a-17:0	0	0	0	0	0	0	0.1	6.4	0.1	5.9	0	0
cy-17:0	0	0	0	0	0	0	0.1	6.5	0	0	0	0
17:1	0	0	0	0	0	0	0	0	0	0	0	0
17:0	0	0	0	0	0	0	0.1	6.7	0	0	0	0
18:2	0	0	0	0	0	0	0	0	0	0	0	0
18:1	0.2	17.7	0.4	32.1	0.1	18.0	0.1	9.5	0.2	10.3	0.4	52.0
18:0	0.3	24.9	0.4	32.1	0.2	26.2	0.4	31.1	0.3	18.5	0.1	17.4
Me-19:0	0	0	0	0	0	0	0	0	0	0	0	0
cy-19:0	0	0	0	0	0	0	0	0	0	0	0.1	11.3
19:0	0	0	0	0	0	0	0	0	0	0	0	0
20:1	0	0	0	0	0	0	0	0	0	0	0	0
20:0	0	0	0	0	0	0	0	0	0.1	5.1	0	0

Table 3.6. Total masses of the individual phospholipid fatty acids (µg) and their relative abundances (mol%) identified in the rinses of 334_SalinaA1, 464_Queenston, 529_GeorgianBay, 661_Coburg, 682_Coburg and 694_ShermanFalls

	334o SalinaA1		464o Queenston		529o GeorgianBay		661o Coburg		682o Coburg		694o ShermanFalls	
PLFA	µg	mol%	µg	mol%	µg	mol%	µg	mol%	µg	mol%	µg	mol%
12:0	0	0	0	0	0	0	0.2	12.9	0.4	23.8	0	0
14:0	0	0	0.1	1.6	0.7	2.4	0.1	8.4	0.2	13.6	0	0
i-15:0	0	0	0	0	0.1	0.4	0	0	0	0	0	0
a-15:0	0	0	0.1	1.3	0.1	0.5	0	0	0	0	0	0
15:0	0	0	0.1	1.3	0.2	0.7	0	0	0	0	0	0
16:1	0	0	0.1	1.4	0.8	2.5	0	0	0	0	0	0
16:0	0.2	12.8	0.8	8.8	7.9	24.7	0.6	36.2	0.4	22.4	0.3	29.8
Me17:0	0	0	0.1	1.0	0	0	0	0	0	0	0	0
i-17:0	0	0	0.1	1.0	0.2	0.5	0.1	5.4	0	0	0.2	21.9
a-17:0	0	0	0.1	1.1	0.3	0.8	0.1	5.5	0.2	9.6	0	0
cy-17:0	0	0	0	0	0	0	0	0	0	0	0	0
17:1	0	0	0	0	0.3	0.9	0	0	0	0	0	0
17:0	0	0	0.1	1.1	0.5	1.6	0	0	0	0	0	0
18:2	0	0	0.2	2.5	1.2	3.3	0	0	0	0	0	0
18:1	1.0	70.8	6.4	67.9	13.8	39.4	0.2	10.5	0.3	14.3	0.2	23.1
18:0	0.2	16.4	0.8	8.0	7.4	21.1	0.4	21.1	0.3	16.3	0.3	25.2
Me-19:0	0	0	0.2	2.0	0	0	0	0	0	0	0	0
cy-19:0	0	0	0	0	0	0	0	0	0	0	0	0
19:0	0	0	0	0	0.1	0.3	0	0	0	0	0	0
20:1	0	0	0	0	0.3	0.7	0	0	0	0	0	0
20:0	0	0	0.1	1.0	0.2	0.4	0	0	0	0	0	0

Table 3.7. Comparison of PLFA concentrations and cell density estimates for rock core rinses, assuming that the outer 1, 3 or 5 mm of rock material are extracted during rinsing

Core Rinse	PLFA concentration (ng/g of rock)			Cell density estimate (cells/g of rock)		
	1 mm	3 mm	5 mm	1 mm	3 mm	5 mm
334o_SalinaA1	7.8	2.7	1.7	$2x10^6$	$5x10^5$	$3x10^5$
464o_Queenston	51.7	17.8	11.0	$1x10^7$	$4x10^6$	$2x10^6$
529o_GeorgianBay	220.7	76.2	47.4	$5x10^7$	$2x10^7$	$1x10^7$
661o_Coburg	9.8	3.4	2.1	$2x10^6$	$8x10^5$	$5x10^5$
682o_Coburg	5.3	1.8	1.1	$1x10^6$	$4x10^5$	$3x10^5$
694o_ShermanFalls	2.8	1.0	0.6	$6x10^5$	$2x10^5$	$1x10^5$

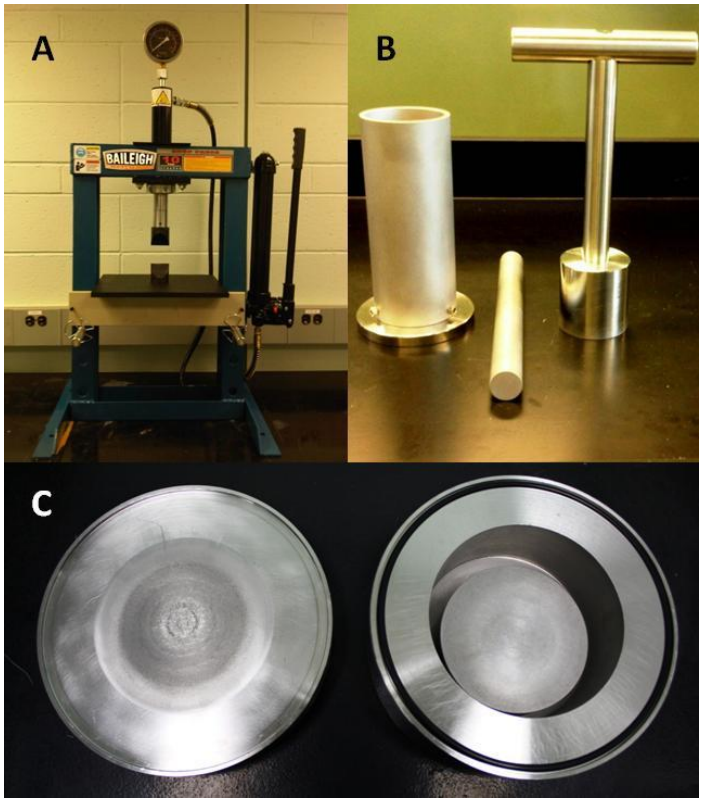

Figure 3.1. Rock core sample processing equipment. (A) Hydraulic jack rock cutter equipped with stainless steel chisels (B) Stainless steel mortar and pestle (C) Stainless steel puck mill used for powdering interior rock material.

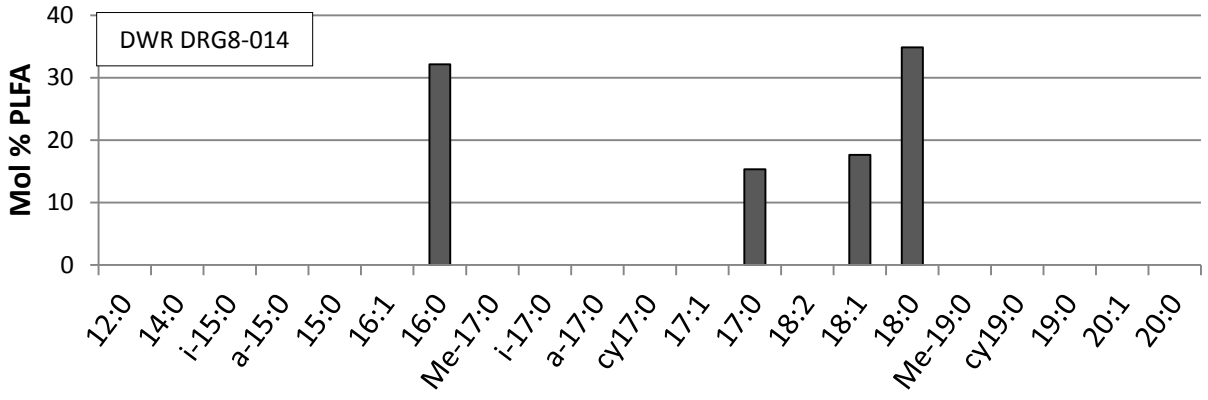

Figure 3.2. Relative abundances (mol %) of PLFA detected in a 1 liter drilling water sample (DWR DGR8-014).

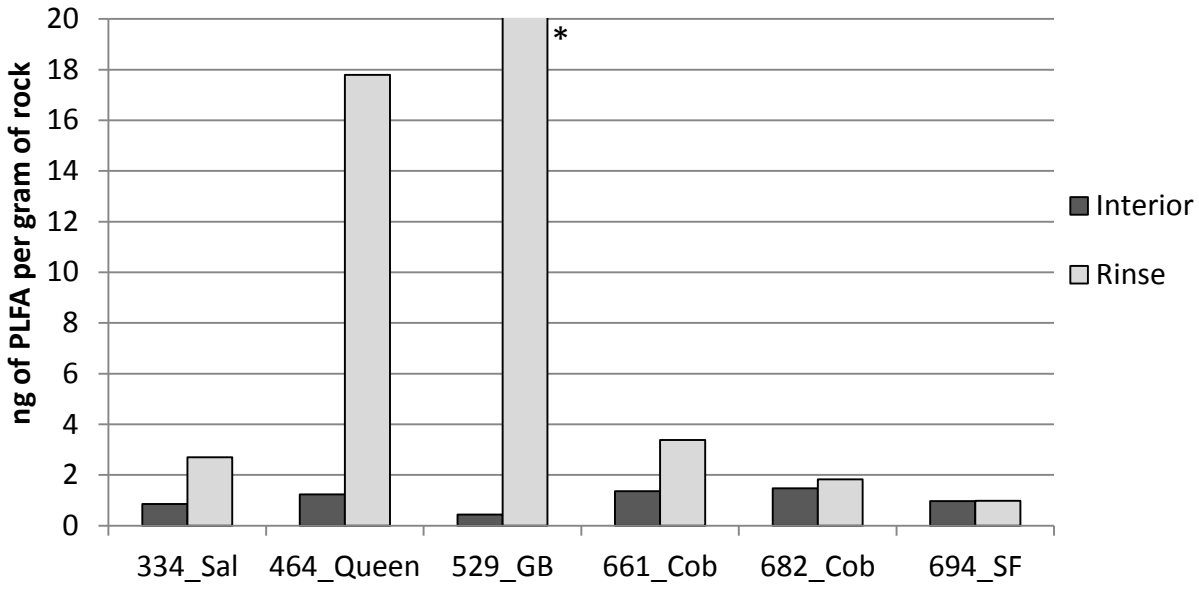

Figure 3.3. Total concentrations of PLFA (ng of PLFA per gram of rock) detected from core rinses and core interiors. (*) Actual 529o_GeorgianBay concentration is off the scale of this graph at 76.2 ng/g.

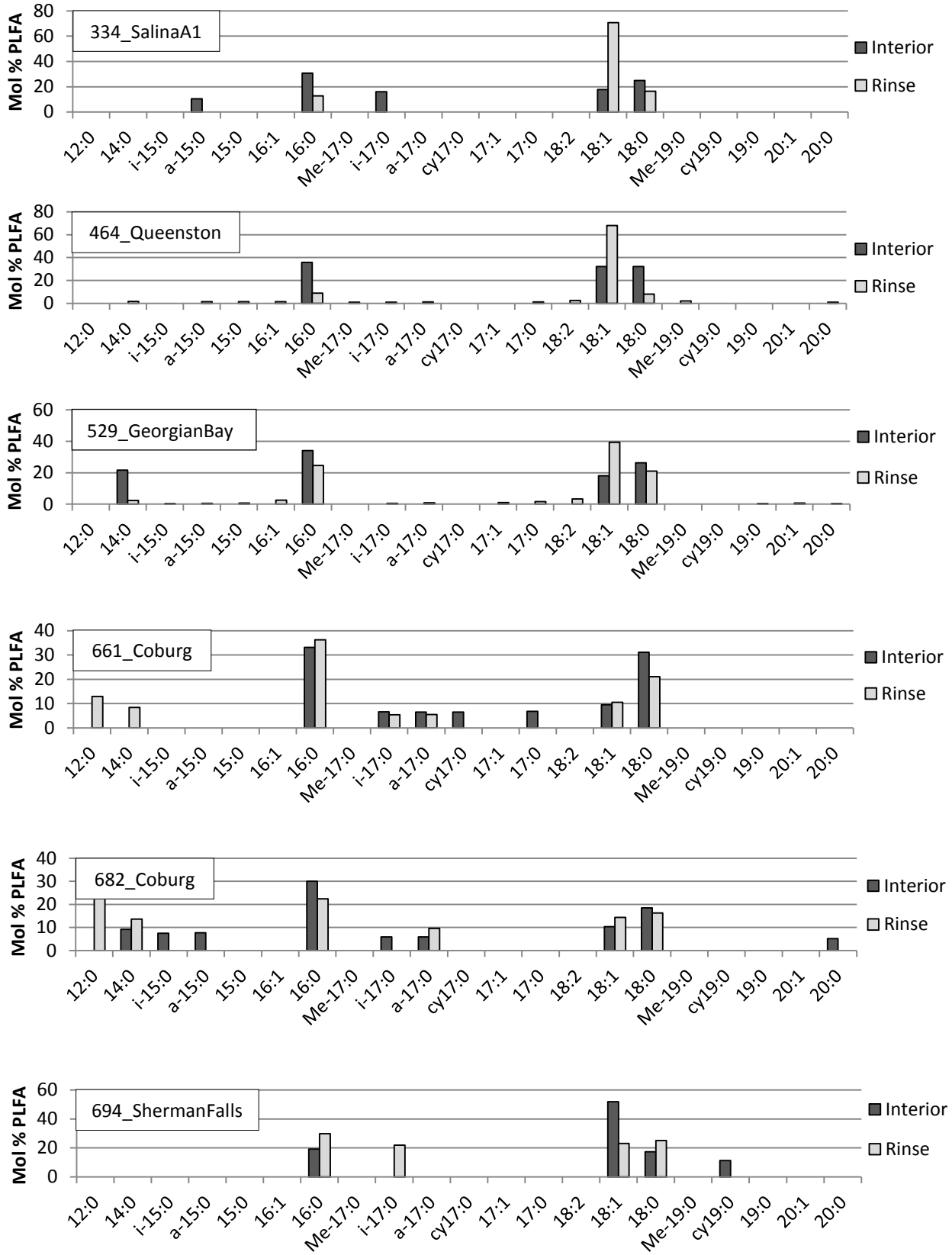

Figure 3.4. Relative abundances (mol %) of PLFA detected in rock core interiors and rock core rinses.

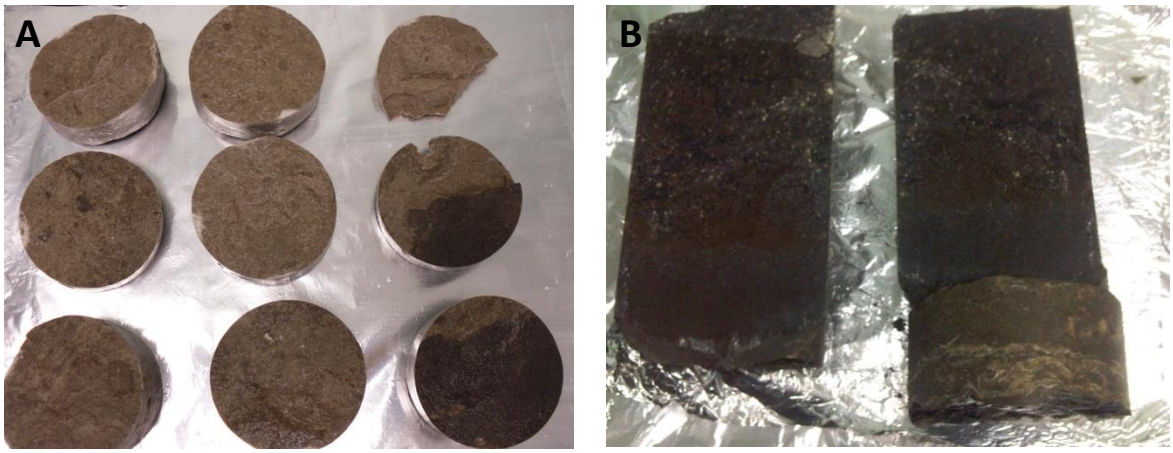

Figure 3.5. (A) Individual slices of 334_SalinaA1. The two darker pieces in the bottom right-hand corner resulted from a break along a natural fracture in the core. (B) Interior of 661_Coburg, illustrating the dark material covering the surfaces along a natural fracture in the rock.

CHAPTER 4:

CONCLUSIONS: THESIS SUMMARY AND FUTURE RESEARCH

4.1. THESIS SUMMARY

Investigating microbial life in the Earth's deep terrestrial subsurface provides insight into the ultimate limits for life on Earth, as well as the potential for microbial life to exist in the subsurface of other planetary bodies. Due to challenges involved in sampling from the Earth's deep terrestrial subsurface, the biogeochemical processes taking place in these systems and the range of environmental conditions tolerated by subsurface microbial communities remain largely unexplored. Despite these limitations, microbial communities have previously been identified at depths of up to several kilometers within the continental crust, some of which are believed to have been isolated from surface microbial processes over geologic timescales (Chivian et al., 2008; Lin et al., 2006; Moser et al., 2003; Onstott et al., 2003; Pfiffner et al., 2006; Ward et al., 2004). For this Master's thesis project, two subsurface environments were investigated to further explore the presence and activity of microbial communities living in the Earth's deep terrestrial subsurface: (1) water-filled fracture networks accessed through deep gold mines in the Witwatersrand Basin of South Africa and (2) natural pore spaces in deep sedimentary rock cores sampled from the Michigan Basin, Canada. Phospholipid fatty acids (PLFAs) were extracted and analyzed as biosignatures for viable microorganisms in the deep subsurface samples. In addition, carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) of the PLFAs and potential carbon sources were used to elucidate *in situ* microbial carbon sources and cycling. These studies revealed the presence of viable microorganisms across a range of extreme subsurface conditions and identified active microbial metabolisms including anaerobic oxidation of methane and chemoautotrophic

processes. These findings provided insight into the range of conditions tolerated by microbial communities in the deep continental crust and the biogeochemical processes occurring in these systems.

4.1.1. Microbial carbon sources and cycling within deep subsurface fracture water systems of the Witwatersrand Basin, South Africa

Microbial carbon sources and cycling within deep terrestrial subsurface fracture water systems were explored via deep mine boreholes, 0.9 to 3.2 kilometers below land surface, in the Witwatersrand Basin of South Africa. PLFAs were extracted from filtered fracture water samples to identify, quantify and characterize the indigenous microbial communities. Carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) of the PLFAs and potential carbon sources (dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and methane) were used to elucidate microbial carbon sources and cycling in these systems. The results from this study revealed microbial communities with very low biomass and variations in microbial metabolisms across the range of subsurface sites, including anaerobic oxidation of methane and microbial utilization of DIC. Microbial cell densities, based on PLFA concentrations, ranged from $2x10^1$ to $5x10^4$ cells per mL and decreased as fracture water temperatures increased. An extremely low bacterial cell density ($2x10^1$ cells/mL) observed in one of the systems (KL445) may represent a subsurface environment that is devoid of viable biomass or, alternatively, a system composed entirely of archaea. Various PLFA indicators for environmental stressors were observed, including an increased ratio of *iso*- over *anteiso*- fatty acids in response to higher temperatures, and the presence of cyclopropyl PLFAs, representing microbial responses to nutrient deprivation and/or other stress conditions. $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFAs and potential carbon sources indicated active microbial oxidation of methane in two anaerobic sites (Dr5IPC and Be326 Bh2). Be326 Bh2 also revealed

evidence for active microbial utilization of DIC, based on comparisons between $\delta^{13}C_{PLFA}$ and $\delta^{13}C_{DIC}$. The microbial communities in the two Tau Tona sites (TT09 Bh2 and TT107) were found to be predominantly chemoautotrophic, based on $\delta^{13}C$ and $\Delta^{14}C$ analyses of PLFAs and potential carbon sources, possibly indicating active acetogenesis, or sulphate or iron reduction. The distinct carbon isotope patterns observed across the range of subsurface fracture water systems in this study revealed that the microbial communities were utilizing methane and DIC as carbon sources, consistent with a self-sustaining deep terrestrial subsurface biosphere that functions independently of the Earth's surface metabolic processes.

4.1.2. Indigenous microbial communities living within low permeability deep sedimentary rocks in the Michigan Basin, Canada

The presence of viable microbial communities living within the natural pore spaces of deep subsurface sedimentary rocks were investigated by extracting PLFAs from rock core samples collected from the Michigan Basin, Canada. The PLFA distributions and abundances within the rock core interiors, compared to the PLFA contents of the outer rock core surfaces and the drilling water, indicated the presence of low biomass microbial communities living within deep sedimentary rock. The PLFA concentrations were consistent among the rock core interiors (0.4 to 1.5 ng of PLFA per gram), representing low microbial cell densities of 1-3 x 10^5 cells per gram of rock. Relatively high PLFA concentrations (17.8 and 76.2 ng of PLFA per gram) measured on the outer rock core surface of two samples (464o_Queenston and 529o_Georgian Bay) indicated a distinct source of PLFAs that may have been introduced during sampling and/or sample handling, and did not appear to affect the interior rock core material. This difference allowed the indigenous microbial communities in the rock core interiors to be distinguished from the outer rock core contaminants. Furthermore, differences between the PLFA profiles of the

interior and exterior rock core materials indicated a distinct source of PLFAs on the outer rock core surfaces, providing further support for indigenous microbial communities in the rock core interiors. One drilling water sample was found to contain a very low abundance of PLFAs, representing less than $8x10^4$ cells per mL, while all of the other drilling water samples lacked quantifiable PLFAs. This low abundance of PLFAs was attributed to the high salt content of the drilling water, which may have acted as a bactericidal to the subsurface community. These observations indicated that the drilling water was likely a minor source of contamination to the outer rock core surfaces and an unlikely source of contamination to the rock core interiors. Consistent with the presence of indigenous subsurface microbial communities, the PLFA profiles derived from the interior rock core material contained cyclopropyl PLFAs, indicating microbial responses to environmental stressors in the deep subsurface.

4.1.3. Implications for microbial life in the deep terrestrial subsurface of Earth and other planetary bodies

The results from these studies demonstrate the ubiquity of microbial life in Earth's deep terrestrial subsurface. Detecting viable microbial communities across a range of extreme environmental conditions, including high water temperatures (up to 54.5°C), low oxygen levels, low levels of organic carbon, and limited porosity within deep sedimentary rocks, demonstrates the resilience of microbial life to environmental stressors. These findings hold large implications for understanding the ultimate limits for life on Earth and the potential for life to exist in the subsurface of other planets, such as Mars. The surface conditions on Mars today are believed to be inhospitable for microbial life, due to the planet's thin atmosphere and a lack of liquid water on the planet's surface (Lammer et al., 2009). The subsurface of Mars, however, may contain liquid water today and could potentially host viable microbial communities that are supported by

H_2-based chemolithotrophy (Chapelle et al., 2002). Furthermore, if methane is produced abiotically in the subsurface of Mars, methanotrophic communities may be capable of utilizing methane as a carbon source via anaerobic methane oxidation reactions. Overall, the findings from this Master's thesis project demonstrate the capabilities of microbial communities to survive within the Earth's deep terrestrial subsurface and provide further support for the possibility of microbial life to exist in the deep subsurface of Mars and other planetary bodies.

4.2. FUTURE RESEARCH DIRECTIONS

In order to gain a better understanding of the total microbial cell abundances and microbial biogeochemical processes occurring in the Earth's deep terrestrial subsurface, archaeal populations should be identified and quantified in addition to bacteria and eukarya. PLFA analysis only provides information about the presence and activity of bacterial and eukaryotic cells and excludes information about archaeal cells, as their unique phospholipid structure require a separate method of analysis (Green and Scow, 2000). Investigating the presence of viable archaeal cells within these deep terrestrial subsurface environments is a key next step for several reasons. Firstly, searching for all three domains of life in the subsurface will provide a more accurate estimate for microbial cell abundances. In Chapter 2, for example, KL445 contained extremely low PLFA concentrations, potentially representing a system that is devoid of viable bacteria and composed solely of archaea. A method of analysis that includes archaeal cells is required in order to confirm this hypothesis. Secondly, applying carbon isotope analyses ($\delta^{13}C$ and $\Delta^{14}C$) to archaeal cells would provide insight into the role of archaea in the deep terrestrial subsurface carbon cycle. For example, identifying extremely ^{13}C-depleted $\delta^{13}C$ signatures of archaeal lipids may indicate archaeal utilization of methane as a carbon source via reverse methanogenesis reactions (Valentine and Reeburgh, 2000). Anaerobic oxidation of

methane in the deep terrestrial subsurface is currently poorly understood and exploring the role of archaeal cells in this process would potentially identify the mechanisms involved.

Further exploration of microbial communities in the deep terrestrial subsurface could also include investigations of bacterial endospores. In the absence of solar energy, chemolithotrophic microbial communities depend on chemical energy sources such as H_2, and in many subsurface systems, energy fluxes are expected to be low (Chapelle et al., 2002; Chivian et al., 2008). It is unclear whether microbial communities in the deep terrestrial subsurface are adapted to extremely low energy fluxes or whether they are in a dormant, spore-like state, waiting for conditions that are favorable for growth and reproduction (Lomstein et al., 2012). Concentrations of dipicolinic acid, a marker for bacterial endospores, can provide an estimate of the total endospore abundance in an environment (Lomstein et al., 2012). Identifying endospores in the deep terrestrial subsurface would provide information about the tolerance of subsurface microbial communities and their methods of survival in extreme conditions.

Phospholipid degradation rates in the environmental conditions of the deep terrestrial subsurface should also be investigated in order to fully understand the applicability of PLFA analysis to deep subsurface environments. Several studies have examined the rates of phospholipid degradation in anaerobic conditions with low organic contents, and have found that degradation rates are slowed by up to 40% in these settings (Harvey et al., 1986; Logemann et al., 2011). Logemann et al. (2011) found that phospholipid degradation in abiotic conditions occurred at extremely slow rates, which raises the question of whether phospholipids would remain intact for long periods of time in the deep terrestrial subsurface following the death of the microbial community, or in a system of very low microbial activity. Preservation of

phospholipids over geologic timescales in the deep terrestrial subsurface may be unrealistic; however, fully investigating these degradation rates should be a key consideration.

4.3. REFERENCES

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