CULTURE-INDEPENDENT MOLECULAR PHYLOGENETIC ANALYSIS OF MICROBIAL COMMUNITIES LIVING AT SATURATING LEVELS OF MAGNESIUM SULFATE

A Thesis by

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CULTURE-INDEPENDENT MOLECULAR PHYLOGENETIC ANALYSIS OF MICROBIAL COMMUNITIES LIVING AT SATURATING LEVELS OF MAGNESIUM SULFATE

The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science with a major in Biological Sciences.

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DEDICATION

This thesis work is dedicated to my wife, Lisa, who has been a source of unconditional encouragement and constant support throughout graduate school and life. I am truly blessed to have her in my life. To my children, Brayden Leif and Amelia Mae, I give my deepest expression of love and gratitude for your encouragement and the sacrifices you have made so that I may complete my research. This work is also dedicated to my parents, Richard and Vickie Kilmer, who have always supported me no matter what path I chose to take.
“Research is what I’m doing when I don’t know what I’m doing.”

- Wernher von Braun
ABSTRACT

Athalassohaline epsomite lakes in the northwestern plains of North America contain high concentrations of magnesium sulfate (MgSO$_4$). Hot Lake located outside of Oroville, WA is one such lake in which the MgSO$_4$ precipitates as epsomite (MgSO$_4$$\cdot$7H$_2$O). Natural environments containing high concentrations of MgSO$_4$ are rare and there has been little previous microbiological analysis of the communities that dwell in Hot Lake or of any analogous epsomite-rich environment. It was unclear whether the microbial communities inhabiting this distinctive environment were archaea, novel bacteria endemic to epsomite environments, bacteria commonly associated with hyperhaline environments, or typical forest soil bacteria which have adapted strategies for survival under conditions of osmotic-stress. This study utilized culture-independent and culture-dependent techniques to analyze the microbial diversity of Hot Lake.

The culture-dependent campaign resulted in nearly 100 isolates which were isolated on 10% NaCl or 2 M MgSO$_4$ agar plates. The clone library was dominated by Gram-positive clades, specifically sequences related to uncultured actinobacteria. Sequences related to sulfur-reducing Deltaproteobacteria were also abundant in the culture-independent library. Conversely, the isolate collection produced a near even split between Gram-negative and Gram-positive clades. Halomonas, Idiomarina, Marinobacter, Marinococcus, Nesterenkonia, Nocardiopsis and Planococcus were the most abundant genera found. This research was supported by NASA ROSES Planetary Protection (PPR), Kansas NASA EPSCoR, NIH NCRR NIGMS KINBRE, and NSF GK-12.
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CHAPTER 1

INTRODUCTION

1.1 Background

Athalassohaline lakes are saline lakes that are non-marine in origin and have chemical compositions high in sulfate, magnesium or carbonate. The makeup of their waters is distinctly influenced by the geology of the area where they develop (Grant, 2004). This can occur in the form of ions leaching from the surrounding geology into fresh waters during artesian flow or via the resolution of salt deposits from an earlier evaporative event (Grant, 2004). In the northwestern plains of North America and Canada there is an abundance of athalassohaline lakes that are at, or near, saturating levels of sodium sulfate, sodium chloride, magnesium chloride, and/or magnesium sulfate (Hammer, 1986; Last and Slezak, 1988; Last and Ginn, 2005).

There are a few athalassohaline lakes that are of particular interest because they are both sulfate rich and contain high levels of magnesium which, at saturating levels, precipitates as epsomite ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$). Despite a considerable interest in the microbiology and biogeochemistry of extreme environments there is a wide gap in our knowledge due to the lack of studies that have been conducted on microbial life at high concentrations of magnesium sulfate and other salts beyond NaCl. Furthermore, most microbial studies have focused on the water columns and there have been very few investigations looking at the sediments or marginal soils of hypersaline lakes (Mesbah et al., 2007).
These epsomite-dominated hypersaline lakes represent unique habitats which may hold vastly different microbial communities from those found in other hypersaline environments. The study of the microbial life that thrives in such harsh environments provides a high likelihood of discovering novel organisms with novel capabilities. These particular microorganisms are of great interest for their prospective use in industrial applications as well as for their relevance to finding life on Mars and Jupiter’s moon Europa (Grant et al., 1998; Litchfield, 1998a; Landis, 2001; Oren, 2002; Tehei et al., 2002; Prieto-Ballesteros et al., 2003). It has been shown that halophilic microorganisms obtained from hypersaline environments can produce polymers, enzymes, and compatible solutes that are of interest to the biotechnology industry (Ventosa and Nieto, 1995). Additionally, halophilic/halotolerant bacteria have been of great interest in studying the ecology of haloviruses (Bath and Dyall-Smith, 1998; Dyall-Smith et al., 2003; Porter et al., 2005; Bath et al., 2006).

1.2 Mars

In addition to the inherent value of discovery, the investigation into the microbial diversity found in hypersaline environments rich in hydrated magnesium sulfates also has a direct relevance to forward contamination by robotic landers on Mars. In an effort to reduce the risks of forward contamination NASA’s Office of Planetary Protection and the Committee on Space Research (COSPAR) have set planetary protection guidelines on the level of microbial bioload that can be present on any spacecraft that is intended to land on planetary bodies deemed to be potentially capable of supporting life (Chyba et al., 2006). While these guidelines have considerably reduced the spacecraft’s microbial bioload, terrestrial microbial communities representative of those communities
found in the air and surfaces of class 100K clean rooms of the spacecraft assembly facilities persist at the time of launch (La Duc et al., 2003, 2004; Chyba et al., 2006; Moissl et al., 2007, 2008; Stieglmeier et al., 2009). Many of these microorganisms have the ability to tolerate radiation, peroxides, and high salinities (La Duc et al., 2003, 2004; Link et al., 2003; Kempf et al., 2005, Venkateswaran et al., 2001, 2003). These studies make the potential for forward contamination of Mars abundantly clear; however, it is uncertain if Earth’s terrestrial microbes would be able to survive and propagate in the Martian soils.

Mars’ surface challenges microbial life with many different environmental extremes such as low temperatures, high radiation, low water activities, and oxidizing minerals (Clark, 1998). Studies on the composition of cements found in Mars’ regolith have indicated that the initial primordial solutions were salt-rich and have shown that there are areas of Mars surface that are rich in sulfate salts (King et al., 2004; Clark et al., 2005). Data produced using the visible to near-infrared imaging spectrometers of Mars Express OMEGA and CRISM (Compact Reconnaissance Imaging Spectrometer for Mars), as well as, data from the lander missions of the Mars Exploration Rover (MER), Viking, Pathfinder, and Phoenix have given evidence that a large portion of these areas are dominated by sulfur compounds like the sulfates of magnesium, calcium, and iron (Clark, 1979 and 1993; Wänke et al., 2001; Roach et al., 2007; Bishop et al., 2008; Wiseman et al., 2009; Foster et al., 2010; McKay et al., 2013). Additionally, at the Viking 2 lander site, Utopia Planitia, evidence was found of water in patches of ephemeral frost which formed in a thin, almost continuous layer or in much thicker, patchy layers (Svitek et al., 1990). Due to the dry nature of the regolith any melt water
from the frost should rapidly dissipate via capillary action into the pore spaces of soil grains and those pore spaces could then provide thin films of biologically available water (Jakosky et al., 2003). The presence of sulfate salts along with this limited availability of subsurface water on Mars implies that any liquid water on Mars should occur as (magnesium) sulfate-rich brines (Foster et al., 2010; McEwen et al., 2011). The intermittent availability of these conditions could provide the means for sporadic microbial growth.

### 1.3 Bacterial Biodiversity

The greatest abundance and diversity of all of Earth’s living organisms are represented in Bacteria and Archaea (Whitman et al., 1998; Torsvik et al., 2002). It has been estimated that there are more microorganisms per ton of soil than there are stars in our galaxy ($10^{16}$ and $10^{11}$ respectively) (Curtis and Sloan, 2005). Many of these microorganisms possess metabolic capabilities which are responsible for catalyzing Earth’s natural, life sustaining processes from organic decomposition to the cycling of inorganic elements. Furthermore, these microorganisms work together in a symbiotic relationship within a community to accomplish difficult environmental processes such as methanogenesis, sulfate reduction, and metal reduction reactions just to name a few (Paerl and Pickney, 1996). Most, if not all, of life on Earth is reliant on its close interaction with microorganisms or the environmental processes performed by microbial communities (Berman-Frank et al., 2003). Life’s dependence on these microorganisms provides the niches needed for microbes to flourish in most habitats, but it is their broad array of metabolic plasticity which creates their ability to thrive in extreme environments (Head et al., 1998).
There is a relatively broad range of microorganisms that can tolerate life in extreme environmental conditions; however, there are a few microorganisms which require the presence of that extreme condition in order to survive (Madigan and Oren, 1999). These microorganisms are referred to as extremophiles and they can be found in such harsh conditions as high radiation levels, extreme high or low temperatures or pH, high pressures, desiccation, oxygen tension, chemical extremes, and high concentrations of salts (Stetter, 1999; Rothschild, 2001; Johnson & Hallberg, 2003). It is the microorganisms living in the extreme environment of saturating levels of MgSO$_4$ in which the efforts of this study are focused.

1.4 Culture-dependent vs. culture-independent practices

For many years, microbial community analyses were limited to the use of culture-dependent techniques which required pure live cultures or known co-cultures (Amann et al., 1995; Pace, 1997). Early studies on bacterial diversity simply involved the culturing and isolation of various microbial community members. Once isolated to a pure culture the organisms were subjected to a variety of phenetic tests which allowed researchers to categorize the microorganisms into operational taxonomic units (OTU). These OTUs could then be used to determine what types, how many types, and the relative abundance of each type of microorganism present within the sampled community. However, early researchers found that the standard practices of quantifying viable bacteria by counting OTUs on a nutrient agar did not correlate with the number of cells observed by direct microscopic examination (Amann et al., 1995). This irregularity between counts was great, usually by several factors of 10, and became known as the "great plate count anomaly" (Jones, 1977; Ferguson et al., 1984; Staley and Konopka,
1985). This lead to the realization that the selective culture conditions being used had inherent biases toward the microorganisms that could be cultured and that culture-dependent work alone does not provide a good representation of a microbial community and leads to an underestimation of diversity. It has been estimated that up to 99.9% of bacteria are currently unculturable (Hill et al., 2000; Chatterji et al., 2008).

Some biases caused by the inability to culture bacteria might be because we cannot reproduce the specific conditions required by these bacteria or the fact that some bacteria enter a state referred to as viable but not culturable (VBNC) (Janssen, 2002). The VBNC state is thought to be used as a survival mechanism during periods of environmental stress or the bacteria may enter this state just prior to death (McDougald et al., 1998). Resuscitation of bacteria in the VBNC state has not been conclusively shown to occur making unculturable cells or dead cells indistinguishable from those cells that are simply waiting for the appropriate growth conditions (Barer et al., 1998; Kell et al., 2004). While there have been some advances that have improved our ability to isolate new bacterial cultures, such as individual cell isolation via microcolony cultivation, culture-dependent work continues to be limited (Ferrari et al., 2005).

Understanding the diversity, distribution, and relative abundance of microbial populations was hindered for many years due to the difficulty of accessing true diversity by culture-dependent methodologies. Since a vast majority of microbes defied cultivation by common practices a culture-independent approach of identifying microbial phylotypes via the 16S rRNA gene was suggested (Olsen et al., 1986; Pace et al., 1986). With an average nucleotide length of 1.5 kb (kilobases), a highly conserved
sequence domain along with nine variable regions, and ubiquitous distribution in high copy numbers among prokaryotes the 16S rRNA gene allows for greater analysis and identification of signature sequences over a wide taxonomic range (Van de Peer et al., 1996). In addition, the 16S rRNA genes can be obtained from environmental samples without the use of cultivation making it the most commonly used tool in the determination of evolutionary relationships and diversity of bacterial communities in a natural environment solely based on molecular phylogeny (Giovannoni et al., 1990; Amann et al., 1995; Pace, 1997; Santos and Ochman, 2004). The 16S rRNA genes are obtained via the extraction of DNA, whether it is from a pure culture or an environmental sample, and the amplification of the target genes by polymerase chain reaction (PCR). These amplified gene fragments, commonly referred to as amplicons, are then used to construct a clone library that can then be subjected to gene sequencing (Head et al., 1998). A majority of sequences found in the environment can be detected via PCR resulting in a remarkably significant difference in the estimation of bacterial diversity between culture-dependent and culture-independent practices.

The molecular techniques used in the culture-independent investigations are not without their own limitations. Much bias can be created in the first step of molecular analysis during the extraction of DNA. The efficiency of DNA extraction is dependent on the methods used, the cell type, and the physical matrix (Whyte and Greer, 2005). Milder extraction methods will exclude DNA retrieval from organisms possessing tougher cell walls, while more harsh extraction methods will shear the DNA and limit the detection of organisms with weaker cell walls. The method of PCR has also been accused of excluding or creating biases by discriminating against high G+C DNA
templates due to their low efficiency of strand separation and against unknown taxa because primers are designed to target only known organisms (Reysenbach et al., 1992; Rainey et al., 1994). Another PCR derived problem that can skew phylogenetic analyses is the formation of chimeras (Maidak et al., 2000). A chimera forms when there is an interruption in the sequence synthesis of one template strand, usually due to template damage or degradation, and is continued on another template strand (Pääbo et al., 1990). Chimeric sequences are the most frequently displayed 16S rRNA artifact by new submissions to public databases like GenBank and can difficult to identify, especially when there is more than 85% homology (Theron and Cloete, 2000; Ashelford, 2005).

1.5 Microbial life in high salt concentrations

1.5.1 Sodium chloride

Many studies have been conducted to investigate the microbial communities that inhabit environments with high concentrations of NaCl. The microorganisms that live in these environments are commonly referred to as either halophilic or halotolerant. The name halophilic is restricted to microorganisms having an explicit requirement for NaCl. Halophilic organisms are unable to grow in the absence of NaCl concentrations that are not above 1.0 – 1.5 M. By contrast, microorganisms termed halotolerant have the ability to grow in the presence of high NaCl concentrations despite having no specific NaCl requirements (Grant, 2004).

Numerous environments have been found that contain higher than normal levels of NaCl and they can be aquatic, whether marine or non-marine in origin, or terrestrial
such as the barren salt flats found at the Great Salt Plains of Oklahoma. Seawater is probably the best known NaCl environment and it has been and still is being studied to understand the diversity and structuring of its rich microbial communities. Nevertheless, seawater, with a NaCl concentration of approximately 3.5%, does not represent a harsh environmental condition for many microorganisms. There are, however, numerous hypersaline environments that have a greater NaCl concentration than that found in seawater. Hypersaline environments can also be aquatic or terrestrial and include crystallizer ponds from solar salterns, natural lakes such as the Great Salt Lake, salt flats, and some deep-sea basins (Javor, 1989).

Both cultivation and molecular studies of halophiles conducted on solar saltern crystallizer ponds at saturating NaCl salinity levels, 30% to 35%, have shown that the bacterial and archaeal diversity is fairly limited (Benlloch et al., 1995, 2001, 2002; Litchfield et al., 1998b; Mouné et al., 2003; Pašić et al., 2007). Another study found that at NaCl concentrations of 30% all species that were found at lower salinities disappeared, no cyanobacteria or anoxygenic phototrophic bacteria were present, and halobacteria were a key species present (20% or 64 of 327 strains) (Rodriguez-Valera, 1985).

1.5.2 Magnesium chloride

Many studies have been conducted on the microbial communities living at high concentrations of magnesium chloride. These studies have resulted in conflicting data on the presence of microbial life. While researching the bitterns of the ESSA saltern at Guerrero Negro, Baja Javor (1984) made the claim that these "bitterns were apparently devoid of life." However, Javor discovered this saltern to have many culturable
halophiles. Later culture and molecular based studies of the same site conducted by Spear et al., (2003) and Sabet et al., (2009) expanded on Javor's findings. Sabet isolated 35 halophilic strains with some of those isolates being produced from dilutions as high as $10^{-6}$ suggesting that these organisms are present in relatively high abundance. The Dead Sea is another study site that is rich in magnesium and chlorides and has been the subject of many microbial samplings that have found those waters to be rich with life, particularly haloarchaeal species (Oren, 1983 and 1988). In addition, studies have suggested the presence of microbial life in other magnesium chloride rich waters such as the Discovery Brine pool found at the benthic layer of the Mediterranean Sea where concentrations can be greater than 5 M (van de Wielen et al., 2005; Hallsworth et al., 2007). Whether these organisms are viable at these concentrations of MgCl$_2$ is unknown.

### 1.5.3 Magnesium Sulfate (Epsomite)

There has been a relatively small amount of data reported on microbial life found at high concentrations of magnesium sulfate. A culture-based study using epsomite at high concentrations in microbial growth media was conducted using garden soil isolates. This study did find isolates that had the ability to grow at 0.42 M MgSO$_4$ (Markovitz, 1961; Markovitz and Sylvan, 1962). In other studies microbial isolates have also been produced from epsomite formed in the efflorescences of degrading stone (Laiz et al., 2000; Mandrioli and Saiz-Jimenez, 2002). Laiz et al. (2000) reported that they were able to obtain isolates from efflorescences with epsomite concentrations as high as 25%, which is slightly higher than 1M. Javor (1984) reported that some isolates from the Guerrero Negro salterns were able to grow at 0.9 M MgSO$_4$. The greatest
reported MgSO$_4$ tolerance has been for isolates from the Great Salt Plains in Oklahoma, growing at 2 M MgSO$_4$ (Crisler et al., 2009). In fact, this study found that bacterial growth was significantly greater in 2 M magnesium sulfate than in 1 M magnesium chloride.

With regards to epsomite lakes, the most extensive microbiological study was conducted on Hot Lake by Anderson (1958) and was limited to some descriptive work. Anderson stated that the lake had a brown stagnant layer which smelled of hydrogen sulfide. Below this brown layer he describes a green layer which he postulates to be Chlorobium-dominated. In addition to this layering, Anderson reports finding cyanobacterial mats consisting of Plectonema, Oscillatoria, and Anacystis at the bottom of Hot Lake. A more recent study by Lindemann et al. (2013) reports deep sequencing data on the microbes inhabiting these phototrophic microbial mats. Neither of these reports mentions any sampling from the 15-ft solid epsomite layer found at the bottom of the lake.

In British Columbia, Canada, a series of sulfate-dominated, magnesium-rich playas, collectively known as Basque Lake, have been the subject of a couple studies to detect the signatures of life using IR spectroscopy (Hyde et al., 2007; Foster et al., 2010). These studies reported enrichment cultures (containing approximately 1.5 M MgSO$_4$ • 7H$_2$O) that displayed positive biphasic growth. These studies also make mention of a green, pink, or red coloration in some of the enrichment cultures and epsomite crystals. These colors are consistent with organisms that are known to be halotolerant such as cyanobacteria and/or eukaryotic algae (green) and halotolerant bacteria and/or haloarchaea (pink – red). Despite this persuasive evidence for
epsotolerant microbial life provided by these studies there have been no microbial isolations, measures of microbial abundance or metabolic activities, or molecular ecology studies reported for any of the epsomite lakes.

1.6 Hot Lake

Hot Lake is a natural, sulfate-dominated brine that has been exposed to few surveys of its microbial communities. Besides a recent study reporting isolate and deep sequencing data on the microorganisms inhabiting the lake’s phototrophic microbial mats (Lindemann et al., 2013); there have been no studies, to our knowledge, into the molecular ecology of this lake’s marginal soils nor has there been any effort to produce microbial isolates from these soils or the lake’s water. This lake is a shallow, meromictic athalassohaline lake that has a low concentration of chloride and is dominated by sulfate along with possessing a high level of magnesium. In this lake sulfate is the dominant anion and magnesium represents the dominant cation. The evaporation of water from Hot Lake causes the concentration of magnesium sulfate to reach a level of saturation, resulting in the precipitation of the crystalline mineral epsomite. The focus of this study is centered around this lake in the Okanogan forest in the state of Washington based on its accessibility along with it being one of the lakes with the highest recorded concentrations of magnesium sulfate. An image of Hot Lake can be seen in Figure 1.
Hot Lake is maintained by the Bureau of Land and Mines and is located in the Washington’s Okanogan forest (48° 58’N, 119° 29’W) at an elevation of approximately 2000 feet (Figure 2). This lake received its name because freezing is rare due to the fact that it retains solar heat year round. Hot Lake is saturated with its dominant mineral epsomite and has a benthic layer that consists of a thick deposit of solid precipitates.
and in the early 1900’s this lake was used to mine epsom salt. Hot Lake covers an area of about 1.27 hectares and is characteristically shallow with a maximum depth of 3.25 meters and mean depth of only 1.06 meters (Anderson, 1958). Due to evaporation, Hot Lake mostly exists as a series of smaller pots (playas) which surround a main body of water. The playas of Hot Lake can be seen in the image found in Figure 1. However, throughout the year, depending on rainfall or snow melt, these playas do become congruent with the main section of the lake. In the marginal areas where there is complete surface water evaporation a solid mineral layer forms a powdery deposit. A schematic illustrating the diurnal and annual cycles characteristic of playas can be seen in Figure 3.

Figure 2. Location of Hot Lake

Hot Lake is not the only epsomite rich lake to be found in the northwestern plains of North America. In fact, this area is home to several small magnesium rich, sulfate-dominated lakes with brines that are derived from the chemical weathering of mafic minerals, glass, and/or sulfides by groundwater (Foster et al., 2010). Only a few miles
from Hot Lake there is a privately owned epsom lake named Poison Lake. Poison Lake was subjected to heavier mining than Hot Lake and while this lake does precipitate epsomite there have been no published data on its mineral composition. In addition to the epsomite lakes found in the United States the Canadian Plains are rich with sulfate-dominated athalassohaline lakes (Hammer, 1986; Last and Slezak, 1988; Last and Ginn, 2005). The Kamloops and Basque regions of British Columbia, as well as, the Saskatchewan area also possess many magnesium-rich, sulfate-dominated lakes.

![Annual and diurnal sedimentary cycles of cold saline playa lakes](image)

Figure 3: Annual and diurnal sedimentary cycles of cold saline playa lakes; modified from Last and Ginn 2005.

1.7 Chaotropy, Kosmotropy and Water Activity

Natural brine lakes, like Hot Lake, are distinctive because they are not dominated by chloride (such as you would find in marine or marine derived environments) but are sulfate dominated. Magnesium and sulfate exist as bivalent ions which create different ionic ratios compared to the monovalent ions, sodium and chloride, found in sea water. These ionic ratios are biologically essential due to the fact that the osmotic strength of a
solution changes with the valence of the dominant ions within that solution depending on the level of total dissolved solids. The gap between the solubility of evaporite salts can vary, sometimes, widely depending on temperature. This variation in solubility can be seen in Figure 4. The interaction between these ions and therefore their effect on water structure is described by chaotropicity and kosmotropicity (Last and Ginn 2005). Chaotropic ions have the tendency to destabilize and destroy complex molecules such as nucleic acids or proteins because they have low charge densities and interact weakly with water. Kosmotropic ions generally have high charge densities and strong interactions with water, resulting in more hydrogen bonding between water molecules which contributes to the stability and structure of water-water interactions (Hribar et al., 2002; Van Rantwijk and Sheldon, 2007).

Figure 4. Temperatures impacts on solubility of commonly occurring evaporite lake minerals (Taken from Last and Ginn, 2005).
The chaotropicity of magnesium chloride is much greater than that of magnesium sulfate and it has been suggested that it is the chaotropicity of these hypersaline waters that determines their suitability for microbial growth (Hallsworth et al., 2007). Furthermore, when compared to magnesium chloride, magnesium sulfate has a higher affinity for retaining its ionic bond at high concentrations. Crisler et al., (2012) demonstrated the chaotropic effects of different salt solutions (magnesium sulfate, magnesium chloride, lithium sulfate and sodium sulfate) on the modification of gel-point temperature of agar solutions over a range of salt concentrations and can be seen in Figure 5. This resistance of magnesium sulfate to disassociate in saturating conditions results in higher water activities than those seen in magnesium chloride-dominated waters at the same salinities (Hallsworth et al., 2003; Marion et al., 2003; Grant, 2004). Water activity is the ratio of the vapor pressure of water in a material to the vapor pressure of pure water at a given temperature (pure water has a water activity value of 1). The concept of water activity has been widely accepted as a way of defining an environment’s water availability (Grant, 2004). Water activity levels are known to influence microorganism behaviors such as growth, sporulation, and the production and stability of toxins, therefore, environments with higher water activities have a propensity to be more favorable for microbial life (Beuchat, 1987). In order to survive most bacteria require a minimum water activity value of 0.90, survival at water activity levels below this are largely occupied by xerophilic/xerotolerant or halophilic/halotolerant organisms (Grant, 2004).
Figure 5: The chaotropicities of different salt solutions were determined by their effects on the gelation temperature of agar (Taken from Crisler et al., 2012).
CHAPTER 2

AIM

2.1 Hypotheses

Making predictions on the outcomes from this research was difficult since the parameters shaping an environment’s microbial diversity are poorly understood (Ward \textit{et al.}, 1998; Horner-Devine \textit{et al.}, 2003) in addition to the fact that there had been no previous analysis of the microbial communities that dwell in Hot Lake or any analogous epsomite-rich environments. Furthermore, forecasting the presence of a particular organism, or group of organisms, based on its specific metabolic abilities is complicated by the fact that there has been no reported effort by other studies to identify any of the isolates produced from epsomite enrichment cultures. However, based on the data of some similar microbial studies of extreme hyperhaline environments hypotheses were developed suggesting the outcomes of this research.

1. Epsomite lakes, including Hot Lake, represent an extreme environment with saturating salinities making survival a difficult task for any organism. Due to the unique extremes of this environment it is likely this research will find the microbial communities occupying it will have low diversity.

2. The microbial community found in the margin soil of Hot Lake is expected to be dominated by organisms that are known to be halophilic or halotolerant even though these environments are lacking in significant concentrations of sodium chloride.
The first hypothesis was selected based on data collected from other studies which indicate a direct correlation between low community diversity and an increase in the extremes of an environment (i.e. high/low temperatures, pH, or salinity) (Frontier, 1985; Atlas and Bartha, 1997).

The second hypothesis was chosen because we felt that the key parameter allowing these particular microorganisms to function at saturating concentrations of epsomite will be tolerance to high osmolarity rather than specifically high salinity. In order to prevent death by dehydration during droughts or rupturing during excessive rainfall, microbes in many environments have developed the ability to tolerate a range of osmolarities (Bremer, 2002). This osmotolerance capability may be mediated by the same DNA repair mechanisms often associated with UV light or chemically induced DNA damage (Wilson et al., 2004). Furthermore, we expected to find that the most osmophilic/osmotolerant of these organisms would include gram-positive cocci, *Halobacteria*, *Vibrio* and representatives of the *Pseudomonas-Alteromonas-Alcaligenes* group (Rodriquez-Valera et al., 1985).

### 2.2 Objectives

The primary objective of this study was the assessment of microbial taxonomic diversity and structure in the lake margin soils of Hot Lake, an epsomite rich lake, through the use of culture-independent molecular and phylogenetic analyses. The phylogenetic data is also supplemented with data from culture-dependent work conducted on a bacterial isolate collection obtained using fresh samples from the same site. The specific stepwise aims of this research were to;
1. Obtain lake margin soil samples from Hot Lake.
2. Produce representative DNA extracts from the Hot Lake margin soil samples.
3. PCR amplify bacterial 16S rRNA gene sequences from those extracts.
4. Clone the bacterial amplicons into a competent cell line.
5. Produce a bacterial clone library.
6. Sequence 200 to 300 clones from the library.
7. Produce phylogenetic trees representing the bacterial community of the Hot Lake margin soil.
8. Use the data gathered to make determinations of microbial community structure (i.e. species richness, diversity and abundance) for the Hot Lake margin soil.
CHAPTER 3

EXPERIMENTAL PROCEDURES

3.1 Sample Collection and Transportation

In order to obtain fresh samples arrangements were made with a collaborator that had access to the Hot Lake location. Brent Cunderla from the United States Department of Interior - Bureau of Land Management based in Wenatchee, WA agreed to personally collect samples from a variety of locations around Hot Lake. Sterile 50-ml plastic centrifuge tubes, sterile spatulas, and a shipping container were packaged in our lab at Wichita State University and sent to Mr. Cunderla in order to aid him in the collection of the samples and to reduce any financial burden he may incur in the process of said collection. The sampling of random sites around Hot Lake (T. 40 N., R. 27 E., W.M. Section 7, SE¼SE¼, Section 18, NE¼NE¼, Okanogan County, WA) took place on November 12, 2009 between the hours of 11:15 am and 1:15 pm Pacific Standard Time. Random water and lake margin soil samples were aseptically collected into sterile plastic 50-ml centrifuge tubes from 7 sites around Hot Lake. A topographic map of the Hot Lake Basin labeled with sampling sights can be seen in Table 1 and a description and image of each of the sampling sites can be seen in Table 2. At each sampling site two 50-ml plastic centrifuge tubes were filled with an appropriate sample from that site. Upon the collection of the two samples from each site one sample was allowed to remain at an ambient temperature and one sample was immediately frozen in the field by being placed in dry ice. This separation of the samples was done
because the samples kept at an ambient temperature were to be used for cultivation work while the field frozen samples were reserved for molecular analysis. The samples were then shipped overnight to our lab via FedEx. Upon the arrival of the samples at Wichita State University the still-frozen samples were placed into a freezer at -80°C until they could be processed for molecular analysis (see “DNA Extraction”) and the “fresh” cultivation samples were immediately used to inoculate a variety of growth media (see “Isolation of Culture Collection”).

**TABLE 1**

TOPOGRAPHIC VIEW OF HOT LAKE BASIN SAMPLING LOCATIONS

<table>
<thead>
<tr>
<th>Topographic Map Marking Sampling Sites</th>
<th>Public Land Survey System Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. 40 N., R. 27 E., W.M. Section 7, SE¼SE¼, Section 18, NE¼NE¼, Okanogan County, WA</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2
HOT LAKE BASIN SAMPLING LOCATIONS

<table>
<thead>
<tr>
<th>Sample Number*</th>
<th>Sample Type</th>
<th>Sample Site Description</th>
<th>Sample Site Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &amp; 1-F</td>
<td>Water</td>
<td>Samples were collected from a small pond in the northwest corner of the Hot Lake basin (approximately 50’ southeast of vegetation line in northwest corner of the lake basin).</td>
<td>![Sample Site Image]</td>
</tr>
<tr>
<td>2 &amp; 2-F</td>
<td>Lake Margin Soil</td>
<td>Epsomite crystals at the north end of the Hot Lake basin. Sample location is approximately 100’ south and in the center of the northern lake basin.</td>
<td>![Sample Site Image]</td>
</tr>
<tr>
<td>3 &amp; 3-F</td>
<td>Water</td>
<td>Sample was collected from the north end of Hot Lake (NW corner) from the northern most area of the lake. A small channel connects this deeper pond with Hot Lake. Sample site is about 75’ south of Sample Site 1.</td>
<td>![Sample Site Image]</td>
</tr>
<tr>
<td>4 &amp; 4-F</td>
<td>Lake Margin Soil</td>
<td>Sample location is from a small divide between sections of the main Hot Lake (1/3 from north end of lake). Epsomite crystal crust (1/4 inch) underlain by a greenish and reddish layer with black organic material below.</td>
<td>![Sample Site Image]</td>
</tr>
<tr>
<td>5 &amp; 5-F</td>
<td>Water</td>
<td>Sample site is from the mid-portion of Hot Lake along west side of lake.</td>
<td>![Sample Site Image]</td>
</tr>
</tbody>
</table>
**TABLE 2 (continued)**

<table>
<thead>
<tr>
<th>Sample Number*</th>
<th>Sample Type</th>
<th>Sample Site Description</th>
<th>Sample Site Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 &amp; 6-F</td>
<td>Water</td>
<td>Sample site is form a small pond just south (20’) of the main Hot Lake. Pond is about one foot deep and red in color.</td>
<td><img src="image" alt="Sample Site Image" /></td>
</tr>
<tr>
<td>7 &amp; 7-F</td>
<td>Lake Margin Soil</td>
<td>Sample site is at the south end of the Hot Lake basin about 75’ from southern end of vegetation line. Sample site is located 25’ south of Sample 6 site. Sample consistent color tan-white with dark colored rock chips mixed within lake margin soil sample.</td>
<td><img src="image" alt="Sample Site Image" /></td>
</tr>
</tbody>
</table>

* Designation of "F" means frozen duplicate sample

### 3.2 Isolation of Culture Collection

Due to the lack of previous efforts to isolate microorganisms from MgSO₄-rich environments there were no known defined enrichment medium recipes. Since a community analysis was the focus of this study nutrient-rich enrichment mediums were necessary to help prevent the exclusion of some microorganisms due to a lack of specific nutrient requirements. In the absence of an experimentally proven enrichment medium study specific modifications were made to the “SP” and “MH” mediums described by Caton et al. (2004). The SP and MH are nutrient-rich, high-salinity (NaCl) mediums that were designed for the isolation of halophilic and halotolerant bacteria and archaea, respectively, from soils at the Great Salt Plains in Oklahoma.
The base SP medium contained (per liter): 2.0 g KCl, 10.0 g yeast extract, 5.0 g Bacto-tryptone, 1.0 g dextrose, 0.36 g CaCl$_2$•2H$_2$O, 0.06 g NaHCO$_3$, 0.23 g NaBr, 1.0 mg FeCl$_3$•6H$_2$O, and 0.5 ml trace minerals. In addition to the base each liter of these different SP mediums contained either: 100.0 g (10%) MgSO$_4$•7H$_2$O and 1.0 g NaCl, 100.0 g (10%) NaCl and 1.0 g MgSO$_4$•7H$_2$O, or 493.0 g (2M) MgSO$_4$•7H$_2$O and 1.0 g NaCl. The 10% MgSO$_4$•7H$_2$O and the 10% NaCl SP mediums were made in both liquid and solid form but the 2M MgSO$_4$•7H$_2$O SP could only be made as a liquid. The solid SP media used for plates also contained 15.0 g/L Bacto-agar. The MH medium contained (per liter): 220.0 g NaCl (20%), 10.0 g MgSO$_4$•7H$_2$O, 5.0 g KCl, 3.0 g sodium citrate, 1.0 g KNO$_3$, 5.0 g Bacto-tryptone, 1.0 g yeast extract, 0.20 g CaCl$_2$•2H$_2$O, 1.0 mg FeCl$_3$•6H$_2$O, and 0.5 ml trace minerals. As with the SP medium, the MH medium was prepared as a liquid and a solid with the solid form containing 15.0 g/L Bacto-agar.

The effort to isolate microorganisms began immediately upon the arrival the “fresh” Hot Lake samples and was completed by Dr. Schneegurt and made available to me. Aliquots from each sample were used to inoculate the previously mentioned mediums by direct plating, dilution plating and liquid enrichment. The direct plating of was done by spreading approximately 0.5 g of each lake margin soil sample or 500 µl of each water sample onto the surface of the different solid mediums. Dilution plating was only done using the lake margin soil samples. This was done by making a 1:10 dilution of the lake margin soil using a 0.1% sodium pyrophosphate (NaPP) solution. This slurry was vigorously agitated for 30 minutes on a shaker before being allowed to settle for approximately 10 minutes. Once the particulates had settled 500 µl of the dilution was spread plated onto the different solid mediums. All plates were wrapped in parafilm and
stored in a humid chamber while incubating to help prevent the drying of the agar surface.

Each lake margin soil and water sample was used in the inoculation of the different liquid enrichment mediums. This was done by inoculating 250 ml Erlenmeyer flasks containing 100 ml of the different liquid mediums with aliquots of 0.5 g of lake margin soil, 1.0 ml of water sample, or 1.0 ml of the lake margin soil dilutions. All plating and liquid enrichment cultures were done in duplicate and incubated at 7, 25 and 37°C. The liquid enrichment cultures were incubated on shakers at approximately 150 rpm. Upon microbial growth in the liquid cultures 100 µl of that culture was spread onto agar plates with medium corresponding to that of the liquid culture. However, since 2M MgSO₄•7H₂O solid medium plates will not gel the positive growth liquid cultures of this medium were plated onto 10% NaCl and/or 10% MgSO₄•7H₂O plates. These plates were then wrapped in parafilm and incubated at the same temperature as the liquid culture they came from.

Colonies were collected from the plates within a couple days of manifestation and plates were returned to incubation as to not exclude slower growing microorganisms. Selection of the colonies to be used for isolation was done on basis of gross morphological and physiological features such as differences in pigment, shape, size, or growth rate (Caton et al., 2004). In an attempt to limit the size of the culture collection apparent duplicate colonies derived from the same Hot Lake sample were excluded. Isolates were produced by transferring the selected colonies onto fresh agar plates, of medium corresponding to that of the plates they originally grew on, and streak plating them. To ensure the purity of isolate colonies each isolate underwent a
minimum of five consecutive streak platings. Once a pure culture isolate was obtained it was grown (in duplicate) on agar slants, of appropriate medium, and stored in humid containers at room temperature. To prevent contamination of the isolate collection one set of these pure cultures was kept sealed until they were used to make additional duplicate sets while the other set was used for the phenetic characterization. In addition, a set of isolates were made in 50% glycerol stocks and stored at -80°C in case something were to happen to the extant collection.

3.3 DNA Extraction

Two different methods were used for the extraction of DNA. This was done because the mechanical action used to extract DNA from the Hot Lake margin soil samples was reasonably harsh and would most likely have resulted in excessive DNA shearing if used in the extraction of DNA from the pure culture isolates. DNA from the lake margin soil sample was extracted and purified using an UltraClean® Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer’s protocol for maximum yields. This protocol involved a combination of bead-beating and detergent for cell breakage, an inhibitor solution to reduce humic and protein contamination, and a silica gel spin filter for binding the DNA.

In order to extract DNA from the pure-culture isolates obtained from Hot Lake a freeze-thaw method modified from the one described by Caton et al., (2004) was used. This protocol involved centrifugation to harvest cells from 2 – 3 ml of overnight culture for each isolate. The cells were then resuspended in 0.3 ml of 10 mM Tris-Cl (pH 8) and alternately frozen in liquid nitrogen (1 minute) and thawed in a 90°C water bath (1 minute) for a total of six cycles. The suspensions where then clarified via centrifugation
and the supernatant was placed back into the 90°C water bath for 10 minutes. This is a crude method of DNA extraction that lacks steps for the removal of humic material and organics; however, this was not a concern in these pure-culture samples.

3.4 PCR Amplification

The bacterial 16S rRNA sequences from both the Hot Lake margin soil sample and isolate DNA extracts were amplified using the forward primer pA which corresponds to the *E. coli* nucleotide positions 8 through 28 and the reverse primer pH that corresponds to the complement nucleotide positions 1542 through 1522 (Edwards et al., 1989). These primers are broadly applicable for phylogenetically distinct bacteria because they span the conservative DNA fragment consisting of nearly the entire 16S rRNA gene (Edwards et al., 1989). The sequences of both PCR primers used in this study for amplification of extracted DNA are listed in Table 3.

**TABLE 3**

 Universal Bacterial Oligonucleotide Primers Used in PCR Amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Specificity</th>
<th>Sequence (5′ to 3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Bacterial 16S rRNA</td>
<td>AAG GAG GTG ATC CAG CCG CA</td>
<td>Edwards et al., (1989)</td>
</tr>
</tbody>
</table>

PCR reactions for the amplification of the 16S rRNA genes were performed using an Eppendorf Mastercycler® Personal thermal cycler. Each PCR reaction was set up in
a 0.2 ml thin-walled PCR tube (Eppendorf) with a total reaction volume of 25 μL which included a genomic DNA template of approximately 5 μL, 0.2 μM of each primer (pA and pH), 200 μM of each dNTP, 5 μL 10X PCR buffer and 1 U Takara Ex Taq® (Takara Bio, Shiga, Japan). In order to test for possible reagent contamination a negative control reaction (without DNA template) was run with each set of PCR reactions. In addition, a positive control containing a DNA template from a known organism (*Bacillus subtilis*) was also run with each set of reactions to verify the success of the procedure. The PCR reaction profile included the following thermal cycling parameters: an initial denaturation at 95°C for 4 minutes followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C, and extension for 1 minute at 72°C. These 40 cycles were followed by a final extension for 5 minutes at 72°C. To visually confirm successful amplification of the desired 16S rRNA gene fragments (~1.5 kb) 5 μl from each reaction was run through a 1.0% agarose gel via electrophoresis and stained with ethidium bromide. The amplicons produced from the DNA extracted from the Hot Lake isolate collection were then cleaned using Wizard® SV Gel and PCR Clean-Up System (Promega) and stored at -20°C until they were shipped for sequencing.

3.5 16S rRNA Gene Clone Library Construction

The heterogeneous nature of environmental samples affects the spatial distribution of microbial communities which, in turn, produces a nugget effect that results in analytical biases of microbial community structure (Young and Ritz, 1998). To help alleviate this issue I ran 10 simultaneous PCR reactions from the same Hot Lake margin soil extract. Following electrophoresis through a 2% agarose gel and band
excision the lake margin soil amplicon consortium was then spin-purified to remove reactants using Wizard® SV Gel and PCR Clean-Up System (Promega). Once the amplicons had been purified, they were again visualized via electrophoresis in a 1% agarose gel and the DNA concentration was estimated using the spectrophotometer reading at a wavelength of 260 nm.

To produce the lake margin soil clone library a ligation reaction was set up at a ratio of 1:3 (ng vector: ng insert) followed by transformation into JM109 competent cells using pGEM®-T Easy Vector Systems (Promega) according to the manufactures instructions. The transformed cells were spread plated onto LB (Lysogeny Broth) agar plates containing 50 µg/ml ampicillin that had been prepared with 40 µl of 25 mg/ml X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 100 µl of 100 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The addition of X-GAL and the inducer IPTG allow for blue/white colony selection to help determine whether a transformed CFU (colony forming unit) is the product of a successful ligation reaction. These plates were incubated at 37°C for approximately 24 hours before a total of 342 of the white CFUs were then randomly plucked and transferred to a new LB with ampicillin plates in an ordered fashion before again being incubated at 37°C for approximately 24 hours before being stored at 4°C.

To further ensure that a great majority of the clone library contained the inserted amplicon sequence of interest and were not the products of self-ligation a subset of 50 CFUs were haphazardly chosen to have their plasmid DNA extracted using Wizard® Plus Minipreps DNA Purification System (Promega). The extracted plasmid DNA was digested using the restriction enzyme EcoRI before being run on a 1.0% agarose gel via
electrophoresis and stained with ethidium bromide in order to visually confirm the presence of the desired fragment. Upon confirmation of a high percentage of desired ligations the clone library was prepared for sequencing by randomly collecting and inoculating 96-well plates (containing 200 µl LB/ampicillin/10% glycerol liquid media) with approximately 200 random bacterial 16S rRNA clones. The 96-well plates were then incubated at 37°C for 12 hours before they were sealed using adhesive foil and stored at -80°C until being placed on dry ice and shipped.

3.6 Sequencing and Phylogenetic Analysis

For the subsequent phylogenetic analyses the Hot Lake margin soil clone library was sequenced via single-pass high-throughput sequencing using 3 µM of the same forward primers used in the amplification of the 16S rRNA (pA) by Beckman Coulter Genomics (formerly Agencourt Biosciences, Danvers, MA). In preparation of sequencing the bacterial isolates, derived from the culture collection, a 50X dilution was prepared using 1 µl from each isolates purified amplicons. Using a spectrophotometer, the absorbance reading at a wavelength of 260 nm was used to estimate each samples DNA concentration. This data along with the remainder of the purified amplicons and a 10 µM of the forward primer (pA) were shipped on dry ice to Dr. Mike Grose at the University of Kansas Biodiversity Institute for single-pass sequencing using the EUBPA primer. Nucleotide sequences of between 900 – 1,200 bases were determined using either a Li-Cor 4000L or 4200 IR2 automated DNA sequencer (Li-Cor, Lincoln, NE).

Sequences were automatically aligned using Clustal-W (Thompson et al., 1994) and then manually examined and trimmed in MacClade v4.08 (Sinauer Associates). Contextual 16S rRNA gene sequences were identified in GenBank using BLAST (Basic
Local Alignment Search Tool) (Altschul et al., 1990) or by comparison to relevant literature. PAUP* 4.0 b10 (Phylogenetic Analysis Using Parsimony) (Swofford, 1998) generated phylogenetic trees using distance analysis with Jukes-Cantor rules and the neighbor-joining algorithm. Sequences were trimmed to equal lengths, with sequences less than 500 bp removed, and positions with gaps and ambiguous bases ignored, giving 500-600 positions for analysis. Bootstrap analysis was used to assess the relative support for each branch with a total of 100 replicates conducted heuristically using the distance-based neighbor-joining algorithm and the nearest-neighbor-interchange algorithm in PAUP*. The trees were rooted using *Methanospirillum hungatei* as the functional outgroup. Putative chimeras (approximately 15% of the sequences) were identified through iterative analyses using Pintail within MOTHUR (Schloss et al., 2009), manually examined, and removed if necessary. Distance files were further analyzed using the MOTHUR statistical package to determine Chao 1 estimators, Simpson indexes, non-parametric Shannon indexes, rarefaction curves, and OTUs at various levels of sequence similarity. Library comparisons were made using BLAST.
CHAPTER 4

RESULTS

The main objective of this research was to provide an assessment of the microbial taxonomic diversity and structure in the margin soils of Hot Lake. Several diversity parameters and analyses were used to meet this objective and to answer the questions presented in the hypotheses.

4.1 Collection and Identification of Bacterial Isolates

Bacterial isolates were obtained from all seven of the “fresh” Hot Lake water and margin soil samples. These halotolerant and epsotolerant isolates were obtained via direct plating, liquid enrichment and dilution plating of the samples on modified SP medium. These methods produced approximately 100 aerobic heterotrophic bacterial isolates which were chosen based on unique phenotypic colony morphology within each sample. Despite the success of the bacterial isolation campaign, no archaea were isolated from Hot Lake even though the samples were from near-saturated salinities and there were enrichment attempts in high-salt media. Nearly 25% of this isolate collection was derived from each of the sampling Sites 2, 4, and 7.

With 44 isolates the Gram-negative bacteria *Halomonas* were the most abundant culturable genera isolated from the seven water and soil samples. In addition to the
Halomonas, Gram-negative isolates were also obtained from the genera Marinobacter and Idiomarina. Another abundant genera isolated in the culture-dependant collection was the low G+C Gram-positive Marinococcus with a total of 17 isolates. Other low G + C Gram-positive genera isolated included Planococcus and two actinomycetes Nocardiopsis and Nesterenkonia. Gram-positive Bacillus species accounted for nine of the isolates in the collection. Other, less-abundant, isolates produced by the culture campaign included Corynebacterium, Exiguobacterium, Kocuria, Staphylococcus, and Virgibacillus.

4.2 Culture-Independent Clone Library

A bacterial 16S rRNA gene clone library was prepared from direct DNA extracts from a Hot Lake margin soil sample (7-F) collected at 1:03 pm on November 12, 2009 and frozen in the field using dry ice. This sample was chosen due to the large number of bacterial isolates that were produced from its fresh (never frozen) counterpart. Repeated attempts to extract and amplify archaeal DNA only provided weak banding indicating low abundance therefore a archaeal clone library was not pursued. In an effort to reduce biases in the library, ten PCR reactions were set up and run simultaneously from the same 7-F DNA extract and the resulting amplicons were then combined for use in the cloning reaction. In total, 192 bacterial 16S rRNA gene clones were sent out (to Beckman Coulter Genomics, Danvers, MA) to be sequenced. After an initial analysis of the 192 sequenced clones 13 (6.8%) were rejected for being too short or having too many ambiguous bases and were removed from any further analysis. The remaining 179 clone library sequences were then subjected to evaluation using the Pintail algorithm, built into the program Mothur (Schloss et al., 2009), to screen for
quality and the presence of chimeric sequences. From these, 3 (1.7%) sequences were flagged as being potential chimeras and therefore they were removed (Huber et al., 2004) from our data analysis leaving us with 176 non-chimeric sequences to be used in the analysis of community composition.

### 4.3 Analysis of Community Composition

In order to characterize the community composition of lake-margin soil the sequences obtained from the 16S rRNA library were compared to the databases of known sequences provided by GenBank and the Ribosomal Database Project (RDP). Using RDP’s Classifier program a pie chart was produced (Figure 6) depicting the breakdown of phyla the 176 clone sequences fell into.

![Figure 6. Phyla Classification of OTUs from the Hot Lake clone library using the RDP Classifier.](image)

Figure 6. Phyla Classification of OTUs from the Hot Lake clone library using the RDP Classifier.
Out of the 176 clone sequences analyzed by the RDP classifier, 99.4% (all but one) were found to fall into the bacterial domain. Within the domain Bacteria the RDP classifier identified sequences belonging to 4 distinct phyla and the remainders were categorized as Unclassified Bacteria. The one sequence representing the leftover 0.6% was categorized as an Unclassified Root. The Unclassified Root category is for sequences that did not pass the 80% threshold required by the classifier (de Bruijn, 2011). The largest portion of the sequences (41%) were categorized as Unclassified Bacteria, meaning some random subsets of these query sequences did not always obtain an 80% or greater match with sequences within the RDP database. Phylum richness was low with only representation from 4 different phyla. Of the sequences assigned to a distinct phylum, Actinobacteria made up the largest group with 26% of the OTUs and was followed closely by Proteobacteria at 24%. Phylum abundance was not very even with the remaining represented phyla of Firmicutes, 5%, and Chloroflexi, 4%.

4.4 Estimation of Diversity and Coverage

Statistical estimates of the Hot Lake margin soil microbial community’s richness and evenness, as well as, the efficiency of sampling were all conducted using the program Mothur (Schloss et al., 2009). To evaluate the sampling efforts rarefaction curves (Figure 7) were produced to reflect the commonly used thresholds of taxonomic relatedness of strain (99%), species (97%), genus (94%), and division (88%) between the OTUs (Caton et al., 2009). As expected the number of OTUs increased with an increase in the level of sequence identity. In order to project the total number of OTUs expected to be found at the different levels of sequence identity Chao1 estimations were made (Table 4) (Chao, 1984). There were 84 OTUs identified within the bacterial
sequences at the 99% (strain) level of relatedness. The Good’s coverage values (Table 4) were relatively high and consistent at all levels of taxonomic relatedness (Schloss et al., 2009).

![Rarefaction curves of different levels of sequence identity](image)

**Figure 7.** Rarefaction curves of different levels of sequence identity based on 16S rRNA gene sequences obtained from the bacterial clone library as analyzed using MOTHUR.

At a sequence identity of 97%, 65 OTUs were identified compared to 47 OTUs being identified at 88% similarity within the bacterial library. Coverage was calculated using the equation $C = 1 - \left(\frac{n1}{N}\right)$, where $n1$ is defined as the number of phylotypes (OTUs) with only one representative and $N$ is the total number of clones (Good, 1953). The coverage values ranged from 79% at 99% sequence identity to 77% at a sequence identity of 88%. Coverage data shows that the 16S rRNA library likely contains a
majority of diversity present at the phylum level and higher. However, 77% coverage at 97%, 94% and 88% identity levels, in addition to a lack of leveling off of the rarefaction curves (Fig. 7) indicate more sampling is needed to completely portray the diversity of this community. A frequency distribution further illustrated this showing that 32% of the sequences appeared only once at a sequence identity of 97% while only 11% appeared only one time at a sequence identity of 80%. Both the Shannon index (H) and Simpson index (D) imply that there is an increase in bacterial diversity at higher levels of sequence identity. The H/H\textsubscript{max}, a measurement of evenness, is relatively high across all levels of sequence identity indicating the library has a relatively even distribution of bacterial taxa. The diversity estimates of the bacterial community at Hot Lake are comparable to those frequently observed in bacterial diversity studies of environments rich in NaCl (Caton et al., 2004; Wani et al., 2006; Mesbah et al., 2007; Valenzuela-Encinas et al., 2009); however, there has been no previously described equivalent to this epsomite environment for a direct comparison. A more expansive community depiction could be drawn through deep sequencing; however, deep coverage of this bacterial community is beyond the scope of this study.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level of sequence identity</th>
<th>Level of sequence identity</th>
<th>Level of sequence identity</th>
<th>Level of sequence identity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>99%</td>
<td>97%</td>
<td>94%</td>
<td>88%</td>
</tr>
<tr>
<td>OTUs</td>
<td>84</td>
<td>65</td>
<td>55</td>
<td>47</td>
</tr>
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<td>0.77</td>
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<td>0.77</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Average</td>
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<td>108</td>
<td>84</td>
<td>68</td>
</tr>
<tr>
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<td>130-290</td>
<td>83-165</td>
<td>66-131</td>
<td>54-106</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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<td>3.71</td>
<td>3.42</td>
<td>3.19</td>
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<td>0.69</td>
<td>0.63</td>
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<tr>
<td>Simpson index ($D$)</td>
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<td>0.062</td>
<td>0.080</td>
<td>0.092</td>
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</table>

### 4.5 Phylogenetic Groups Recovered

The 16S rRNA clone library sequences were used to produce phylogenetic trees which also included contextual sequences from both cultured and uncultured bacteria found in the GenBank database. These phylogenetic trees were divided up into Gram-negative bacteria (Figure 8), Gram-positive bacteria (Figure 9), and Chloroflexi and unclassified bacteria (Figure 10). The contextual sequences were obtained by searching through rRNA gene sequences which a BLAST analyses indicated were closely related to those from Hot Lake. Overall, the clustering of major monophyletic groups in these trees followed expected patterns.
The clone library was dominated by Gram-positive bacteria. A majority of these Gram-positive bacteria (>85%) were closely related to cultured and uncultured Actinobacteria. Other Gram-positive clones of interest included the sulfate-reducing bacteria Desulfonispora and Thermodesulfobium. The BLAST analysis also identified clones closely related to the Gram-positive bacteria Bacillus, Clostridia, Lactococcus, Staphylococcus, and Virgibacillus. Staphylococcus and Virgibacillus are two of the few genera found in both the culture-dependent and culture-independent collections.

Clones related to Legionella and uncultured bacteria from the Gemmatimonadetes phylum were the most abundant of the clones representing Gram-negative bacteria. Coxiella, a close relative of Legionella, was also found to have a strong Gram-negative presence in the library. Not surprisingly, this sulfate-rich environment provided an abundance of Gram-negative sulfate-reducing bacteria related to Deltaproteobacteria. Some of the sulfate-reducing genera related to these clones, from this division, included Desulfobacterim, Desulfobulbus and Geobacter (sulfurreducens). Other clones were shown to be related the Gram-negative genera Acidovorax, Erythrobacter, Halothiobacillus and Lysobacter.
Figure 8. Phylogenetic tree for Gram-negative bacteria from Hot Lake based on 16S rRNA gene sequences. Bootstrap values greater than 50% are shown.
Figure 9. Phylogenetic tree for Gram-positive bacteria from Hot Lake based on 16S rRNA gene sequences. Bootstrap values greater than 50% are shown.
Figure 10: Phylogenetic tree for Chloroflexi and unclassified bacteria from Hot Lake based on 16S rRNA gene sequences. Bootstrap values greater than 50% are shown.
CHAPTER 5
DISCUSSION

5.1 Overview

The main focus of this research was the characterization of the taxonomic diversity and community composition of microorganisms inhabiting the epsomite-rich margin soil of Hot Lake. Natural environments containing high concentrations of MgSO$_4$ are rare and there has been no previous microbiological analysis of the communities that dwell in Hot Lake’s waters, lake margin soils, or of any analogous epsomite-rich environment. Prohibitively high epsomite concentrations have been suggested to potentially be the most extreme of the near-surface conditions on Mars. The implication that high concentrations of epsomite may be prohibitive to microbial survival and the lack of previous comparable studies to support a hypothesis made the prediction of what organisms made up the microbial communities in Hot Lake a difficult one. These communities could have been dominated by unique bacteria or archaea endemic to epsomite-rich environments or commonly known halotolerant and halophilic organisms found in other hypersaline environments. Based on studies of other environments with wide ranging osmolarities (Rodriquez-Valera et al., 1985; Bremer, 2002) it was suggested that the microbial community would be dominated by organisms known to be halophilic or halotolerant. Furthermore, the microbial community inhabiting Hot Lake was anticipated to have a low diversity and be made up of organisms like gram-positive cocci, Halobacteria, Vibrio and representatives of the Pseudomonas-Alcaligenes group.
The fresh margin soil sample from Hot Lake’s site 7 provided a large number of bacterial isolates via the culture-dependent campaign and therefore made a seemingly good site choice for the culture-independent work. In order to obtain a representation of a microbial community residing in Hot Lake DNA was extracted from the frozen margin soil sample acquired from site 7. Bacterial 16S rRNA gene clone libraries were prepared from those direct DNA extracts which were sent away to be sequenced. There were 192 bacterial 16S rRNA gene clone sequences obtained from the Hot Lake site 7 clone library. Out of the original 192 sequences there were 176 sequences that survived an initial analysis. The sequences that did not move forward for further analysis were discarded because they were either too short or had too many ambiguous bases (13 sequences) or were flagged as potentially being chimeric (3 sequences).

From the 176 bacterial clone sequences used in the RDP phyla classification a significant portion (41%) were assigned as unclassified bacteria. The designation of “unclassified” is given to sequences when some random sequence subsets fail to consistently obtain a ≥ 80% match with sequences within the RDP database. These unclassified bacteria, like an estimated 99.9% of all bacteria, are currently considered unculturable and isolates have not been reported.

At the phylum level, richness was found to be relatively low with only 4 different phyla being represented in the bacterial 16S rRNA clone library. *Actinobacteria* was the most commonly observed phylum in the library with approximately 26% of the OTUs identified. Many of the isolates found in the culture-dependent collection also fell into this phylum. This is an interesting find because *Actinobacteria* are Gram-positive bacteria most commonly found in the soil fulfilling the function of decomposition and
humus formation and are not widely reported from hyperhaline environments. Bacteria in the phylum *Proteobacteria* were almost as abundant as the *Actinobacteria* making up nearly 24% of the sequenced OTUs identified. *Proteobacteria* are Gram-negative bacteria that are considered to be the biggest and most diverse group of known bacteria. The abundance of *Proteobacteria* in the Hot Lake microbial community is not completely surprising since this phylum is known for its extreme range of metabolic capabilities. The remaining two phyla identified from the library only made up approximately 9% of the sequenced OTUs. *Firmicutes*, which made up about 5% of the sequenced library, is another highly diverse phylum. This phylum consists of Gram-positive bacteria with many species well known for their ability to survive in a broad range of osmolarities by the formation of desiccation-resistant endospores. Making up the final 4% of the sequenced library OTUs is the phylum *Chloroflexi*. Chloroflexi, also known as green non-sulfur bacteria, are common in marine and freshwater sediments; however, very little is known about this phylum and its metabolic characteristics (Hug et al., 2013).

5.2 **Hypothesis #1: Due to the unique extremes of this environment it is likely this research will find the microbial communities occupying it will have low diversity.**

This hypothesis was postulated based upon previous studies conducted on the microbial communities in other extreme environments. These studies have shown a direct correlation between decreasing community diversity and the increase in the extremes of an environment (i.e. high/low temperatures, pH, or salinity) (Frontier, 1985; Atlas and Bartha, 1997).
While it was expected that we would find low diversity in this harsh environment this study has shown that a diverse microbial community can survive and thrive in natural environments with saturating concentrations of MgSO$_4$. The Shannon index ($H'$) values were relatively high indicating that bacterial diversity was high. Furthermore, these values increased with an increase in sequence identity implying higher diversity at higher levels. The Simpson index ($D$) values also indicated the same trends in diversity as these values were all relatively low and decreased with an increase in sequence identity. As mentioned earlier, a direct comparison with other similar epsomite environments could not be made; however, Hot Lake’s bacterial community diversity estimates are similar to those found in NaCl rich environments (Caton et al., 2004; Wani et al., 2006; Mesbah et al., 2007; Valenzuela-Encinas et al., 2009).

Despite the statistical evidence indicating a diverse bacterial community being represented in the 16S rRNA clone library there is other evidence that we are only seeing part of the picture. The Good’s coverage values were reasonably high throughout all levels of identity; however, these values also indicate that an estimated 21% to 23% of the total diversity is not represented in the clone library. In addition, the relatively steep rarefaction curves point to the conclusion that the total diversity of the Hot Lake margin soil bacterial community was not completely identified.

5.3 **Hypothesis #2:** The microbial community found in the margin soil of Hot Lake is expected to be dominated by organisms that are known to be halophilic or halotolerant even though these environments are lacking in significant concentrations of sodium chloride.
Microbes in many environments have developed the ability to tolerate a range of osmolarities in order to prevent death by dehydration during droughts or rupturing during excessive rainfall (Bremer, 2002). This idea lead us to postulate that the key parameter allowing the mentioned organisms to survive and function in saturating concentrations of epsomite is the ability to tolerate high fluctuations in osmolarity rather than a specific tolerance to high salinities. Since we felt it was not specifically the epsomite that was the limiting factor it was anticipated that the organisms found at Hot Lake would be similar to those found in other extreme salty environments.

Most of the culture-dependent collection and many of the culture-independent’s clones met our expectations and were shown to be from taxa commonly found in hyperhaline environments. A majority of the isolates (44 total isolates) were identified as belonging to the genus *Halomonas*. *Halomonas* are well known to possess a wide range of salt-tolerances (Vreeland et al., 1980), including some of the widest ranges of salt-tolerance found in bacteria (Vreeland, 1992). *Halomonas* have been found in a vast array of habitats encompassing a wide range of temperatures, pH, and salinities (Ventosa et al., 1998; Reddy et al., 2003; Kaye et al., 2004). Another interesting genera found in abundance in the isolate collection were the *Planococcus* which contain some species known to be halotolerant (Russell, 1993; Romano et al., 2003). In addition to exhibiting halotolerance, members of this group have been reported to be able to not only survive, but grow in sub-zero temperatures (Mykytczuk et al., 2012). Other abundant genera, commonly known to be halotolerant, produced by the culturing campaign included *Bacillus*, *Idiomarina*, *Marinobacter* and *Marinococcus*. Several of the isolates were assigned to the actinomycetes *Nesterenkonia* and *Nocardiopsis*. 
Nesterenkonia have been found, through metagenomic analysis, to be present in the sub-glacial Lake Vostok 4,000 M beneath the Antarctic ice sheet (Rogers et al., 2013). Interestingly, Lake Vostok has been shown to contain soluble Mg\(^{2+}\), Na\(^{+}\), Cl\(^{-}\) and SO\(_4^{2-}\) (Siegert et al., 2003). While these solutes are not at precipitating concentrations, as they are at Hot Lake, they are available for utilization. The other actinomycete, Nocardiopsis, is frequently isolated from environments such as soils, marine sediments and salterns which contain moderate to high salt concentrations (Sabry et al., 2004). In addition to the above genera, which were isolated in abundance, isolates were also obtained from Corynebacterium, Exiguobacterium, Kocuria, Staphylococcus and Virgibacillus. All of these genera contain species which have been identified as being halotolerant or possessing some other adaptation for dealing with osmotic stress (Yasuhiro et al., 1972; Komeratat and Kates, 1975; Cordero and Zumalacárregui, 2000; Vreeland et al., 2000; Rodrigues et al., 2008; Fränzel et al., 2010; Yun et al., 2011).

While the isolate collection was dominated by Halomonas, these bacteria were not detected in the clone library. At first glance the culture-independent clone library appears to be more representative of a common soil microbial community than that of an extreme hyperhaline environment, like what was found in the culture-dependent’s isolate collection. A vast majority of the sequences produced by the clone library were assigned to taxa closely related to cultured and uncultured Actinobacteria. Gemmatimonadetes, a bacterium estimated to make up approximately 2% of all soil bacteria (Janssen, 2006), were also abundant in the clone library. The clone sequences also showed the presence of the common soil bacteria Acidovorax, Bacillus, Clostridium and Legionella. While these genera are associated with commonly being
present in ordinary garden soil, many of them have also been reported from soils and sediments of some extreme environments indicating the adaptation of osmotic-stress strategies for survival. \textit{Actinobacteria}, the dominant clone sequence reported from the library, are known to be abundant in marine sediments (Stach et al., 2003). \textit{Gemmatimonadetes}, while recognized for being abundant in soil, have been suggested to have adapted strategies to survive in low soil moisture (DeBruyn et al., 2011). The genus \textit{Acidovorax} is also not commonly thought of when you look at extreme environments; however, \textit{Acidovorax} species have been found in the harsh soils of Antarctica (Shivaji et al., 2004) and discovered to be abundant in the high-alkaline saline soil of a former soda lake bed Texcoco (Mexico) (Valenzuela-Encinas et al., 2009).

Sequences from the clone library were also assigned to genera that would be expected to be present in an environment saturated in sulfate salts. Of these, \textit{Halothiobacillus}, a purple sulfur bacterium in the \textit{Gammaproteobacteria} division, seems the most obvious. \textit{Halothiobacillus} literally means “salt-loving sulfur rodlet” and their distribution is ubiquitous across marine, freshwater and soil environments, especially sulfur springs, sulfur deposits, or in sulfide minerals where oxidizable sulfur is abundant (Kelly and Wood, 2000). Lysobacter was another Gammaproteobacteria detected in the clone library. Lysobacter were found to be abundant in hyperalkaline and hypersaline industrial residues (Brito et al., 2013) and have been isolated from hypersaline Lakes like Urmia Lake in NW Iran (Asem et al., 2014). \textit{Deltaproteobacteria}, a division which is rich in sulfate-reducing bacteria, were also shown to have an abundant presence in the Hot Lake clone library. Within this division clone sequences were assigned to genera
like *Desulfobacterium*, *Desulfobulbus* and *Geobacter*. Other sulfate-reducing bacteria identified in the clone library included the Gram-positive genera *Desulfonispora* and *Thermodesulfobium*.

### 5.4 Study Significance

The research reported here was unique because it is the first study to take a microbiological look at an epsomite lake’s margin soils. Having been no reports on microbial communities from a comparable environment it was unknown whether the organisms found would be archaea, novel bacteria endemic to epsomite environments, bacteria commonly associated with hyperhaline environments, or typical forest soil bacteria which have adapted strategies for survival under conditions of osmotic-stress. This study has revealed the first conclusive evidence that a natural environment with saturating concentrations of MgSO$_4$ can support a diverse microbial community.

Through culture-dependent and culture-independent methods this study found Hot Lake to be home to many bacteria, like *Bacillus*, *Halomonas*, *Planococcus* and *Marinococcus*, that come from taxa commonly observed in hyperhaline environments. As expected, the culture-dependent isolate collection was dominated by bacteria identified as known hyperhaline dwellers, especially *Halomonas*. In contrast to the isolate collection, the clone library did not produce any sequences which were reported as *Halomonas* and instead was dominated by the common soil bacteria *Actinobacteria*. While *Actinobacteria*, and many of the other clone library bacteria typically associated with soils, are not widely described as being found in hyperhaline environments there are known osmo-tolerant relatives (Stach et al., 2003; Sabry et al., 2004; Shivaji et al., 2004; Valenzuela-Encinas et al., 2009; DeBruyn et al., 2011; Brito et al., 2013; Rogers
et al., 2013; Asem et al., 2014). The clone library does indicate, however, that sulfate-reducing and anaerobic bacteria are abundantly present in Hot Lake. While Hot Lake was shown to be home to many bacteria commonly associated with hyperhaline environments, both the culture-dependent and culture-independent portions of this study found that archaea was virtually absent. Some of the Hot Lake samples did produce weak banding of archaeal 16S rRNA gene sequences when amplified via PCR; nevertheless, abundance appeared to be too low to necessitate a clone library and no archaeal isolates were present in the culture-dependant collection. Archaea are frequently reported to dominate in environments with near saturating salinities; however, many of these organisms are known to be extreme halophiles requiring a minimum of 1.5 M NaCl for growth (Mohr and Larsen, 1963; Schneegurt, 2012). High concentrations of NaCl are not present in Hot Lake and it appears that haloarchaea’s need for it cannot be substituted with other salts (Mullakhanbhai and Larsen, 1975; Onishi et al., 1980; Vreeland and Martin, 1980). The lack of NaCl levels high enough to meet haloarchaea’s requirements suggests that perhaps the few archaea present are sulfur-dependent archaebacteria or salinotolerant methanogens (Kilmer et al., 2014).

5.5 Additional Data

5.5.1 Epsotolerance and Halotolerance of Isolate Collection

While not included in this study, additional data has been collected from the isolates obtained by the culture-dependent work and via comparisons with the clone sequences produced from the 16S rRNA clone library. The isolate collection was subjected to tolerance testing to reveal each isolate’s ability to grow in high concentrations of NaCl and MgSO$_4$. A vast majority (82%) of the isolates was capable
of growth at 50% MgSO$_4$ and an impressive 58% of them grew at 60% MgSO$_4$, which is at near saturating levels. Interestingly, none of the isolates were found to require high concentrations of MgSO$_4$ for survival. The osmoadaptation of wide ranging epsotolerance seen in the Hot Lake isolates is likely a result of fluctuating concentrations of the salt due to the seasonal cycling of summertime evaporation and springtime snowmelt. When tested for their tolerance to high concentrations of NaCl all of the isolates grew at 10% salinity and approximately 75% of these showed the ability of growth at 20% salinity. Furthermore, despite being from an environment without significant chloride concentrations, 16% of the isolate collection was capable of growth at near saturating concentrations (30%) of NaCl. As with MgSO4, none of the isolates appeared to have a requirement of high NaCl concentrations for growth. While the majority of the isolates had broad epsotolerance and/or halotolerance there were no epsophilic or halophilic bacteria present in the collection (Kilmer et al., 2014).

### 5.5.2 Hot Lake vs. NASA’s JPL and ESA’s SAFs

NASA’s Jet Propulsion Laboratory (JPL) and the European Space Agency (ESA) provided us with their DNA sequence libraries produced from samples obtained from their Spacecraft Assembly Facilities (SAF). Using a BLAST analysis these sequences were compared to the Hot Lake sequences obtained from the isolate collection and the clone library. Nearly 13% of the Hot Lake sequences matched JPL SAF library sequences at the species level (≥97% identity). At the genus level of sequence identity (≥94%), the JPL SAF library matched approximately one third of the Hot Lake sequences. The Hot Lake isolate collection provided the best matches at these levels, particularly *Bacillus*, *Halomonas*, *Kocuria* and *Nocardiopsis*. Like the Hot Lake clone
library, the JPL SAF library was dominated by bacteria frequently identified in soils. In contrast, ESA SAF sequence library mostly consisted of bacteria commonly associated with humans and therefore showed little overlap with the Hot Lake sequences (Kilmer et al. 2014).

The high number of sequences found to be in common between the JPL SAF library and those obtained from Hot Lake demonstrate the potential for forward contamination of celestial bodies by robotic landers. Many studies conducted on the regolith of Mars have indicated that these areas are rich in sulfate salts and are dominated by sulfur compounds including magnesium sulfate (Clark, 1979; Clark, 1993; Wänke et al., 2001; King et al., 2004; Clark et al., 2005; Roach et al., 2007; Bishop et al., 2008; Wiseman et al., 2009; Foster, 2010; McKay et al., 2013). In addition, evidence of water in the form of ephemeral frost has been reported on Mars (Svitek et al., 1990). The presence of sulfate salts along with this limited availability of water on Mars implies that any liquid water on Mars should occur as (magnesium) sulfate-rich brines (Foster et al., 2010; McEwen et al., 2011). The intermittent availability of these conditions could provide epsotolerant organisms the means for growth increasing the likelihood of contamination by spacecraft.

5.6 Further Research

As the first reported study to investigate the microbial life in an epsomite lake this research has provided a baseline for which many further investigations can begin. This study represents only a small glance into the true diversity of epsotolerant microorganisms present in Hot Lake. The rarefaction curves and frequency distribution produced in this study indicate more sampling is needed to completely portray the
diversity of this community. Deep sequencing of samples from multiple locations at Hot Lake may reveal rare organisms and in turn unveil a broader picture of microbial diversity (Lindemann et al., 2013). Furthermore, sampling from the same locations at different times of the year would provide a deeper understanding of these microbial community’s responses to Hot Lake’s seasonal cycling. A more extensive characterization of the microorganisms inhabiting saturated MgSO$_4$ conditions will help build a deeper understanding of those organism’s adaptations to osmotic-stress which could result in technology beneficial to industrial applications or may lead to enhancements of the life detection parameters for future missions of planetary exploration.
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