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MICROBIOLOGY OF LAVA TUBE SKYLIGHTS ON EARTH: IMPLICATIONS FOR ASTROBIOLOGY

M.SC. ENVIRONMENTAL TECHNOLOGY
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EIRIK G. KOMMEDAL

MARS INSTITUTE

Universitetet i Stavanger

This thesis is dedicated to the memory of my grandfather.

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ABSTRACT

Microbial life is abundant in Earth's subsurface and is extremely diverse. Subsurface habitats on extraterrestrial bodies offer microorganisms protection from hostile surface conditions. Lava tube caves are widespread on Earth; they provide access to subsurface microbial habitats and contain extensive microbial communities. In addition, they have been identified on Mars and on the Moon by their skylights. There is a lack of knowledge on how microbial communities in lava tube cave skylights differ from microbial communities in deep cave zones and overlying surface soils. This study is the first comparison of bacterial communities in lava tube cave skylights with communities in the cave deep zone and the overlying soil surface. To examine these differences, denaturing gradient gel electrophoresis (DGGE) was used to analyze and compare bacterial 16S rRNA diversity in samples from microbial mats, secondary mineral deposits, and soil samples from three lava tube caves in Lava Beds National Monument, CA, US. DGGE analysis revealed a higher bacterial diversity in the soil surface community than in the skylight and deep zone communities. The lowest diversity was found in secondary mineral deposits samples from the cave deep zone. Independent of their origin, soil samples were more diverse than secondary mineral deposits samples. Future research should focus on improving our knowledge of microbial diversity and function in lava tube caves, and how this knowledge can be used to select the locations with highest potential for detecting life on extraterrestrial bodies.

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Eirik G. Kommedal

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ABBREVIATIONS AND ACRONYMS

APS	Ammonium persulfate
B.C.	Before Christ
bp	Base pair
BSA	Bovine Serum Albumin
COSPAR	Committee on Space Research
Csp	Cold-shock protein
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
GC clamp	Guanine-cytosine clamp
GRS	Gamma-Ray Spectrometer
InSight	Interior Exploration using Seismic Investigations, Geodesy and Heat Transport
JGI	Joint Genome Institute
JPL	Jet Propulsion Lab
LABE	Lava Beds National Monument
MAST	Mean Annual Surface Temperature
MONS	Mars Odyssey Neutron Spectrometer
mRNA	Messenger RNA
NASA	National Aeronautics and Space Administration
NASA ARC	NASA Ames Research Center
NOAA	National Oceanic and Atmospheric Administration
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rrn	Ribosomal RNA operon
rRNA	Ribosomal ribonucleic acid
SLiME	Subsurface Lithoautotrophic Microbial Ecosystems
SOP	Standard Operating Procedure
TEMED	Tetramethylethylenediamine
UiS	University of Stavanger
UV	Ultraviolet

1 INTRODUCTION

1.1 Motivation

Prior to the recognition that the majority of microbial life on Earth resides in the aphotic subsurface (Whitman et al., 1998), Thomas Gold (1992) hypothesized about the widespread presence of microbial life in the Earth's subsurface where life exists in permanent darkness, independently of the photosynthetic driven surface world (Edwards et al., 2012). The knowledge that the Earth's subsurface hosts extensive microbial communities has implications for astrobiology. It is believed that the subsurface can provide habitable environments on extraterrestrial bodies where surface conditions are too hostile for life as we know it to exist, such as on Mars (Boston, 1992, Boston et al., 2001, Cockell, 2014, Cockell et al, 2016).

Lava tube caves are present wherever basaltic lava occurs on Earth and form as a results of active lava flow (Northup et al., 2011). Lava tube caves are considered extreme, oligotrophic environment due to an absence of photosynthesis in the cave deep (dark) zone. However, lava tube caves are known to host extensive microbial mats and secondary mineral deposits of biological origin of different colors (Northup et al., 2011, Lavoie et al., 2017). Skylights occur where the roof has collapsed and offer a natural way to access the shallow subsurface in lava tube caves (Lavoie et al., 2017). Series of collapsed pits, skylights, from orbiter data have enabled the detection of lava tube caves on Mars and the Moon (Leveille and Datta, 2010). The discovery of lava tube caves on Mars supports and encourages the use of lava tube caves as models in astrobiology (Lavoie et al., 2017)

The primary motivation for studying how a skylight affects the composition of bacterial communities in lava tube caves is as a terrestrial analog for Mars and other planetary bodies in the search for life in the Universe. The microbiology and mineralogy of lava tubes caves remain relatively unstudied despite their frequency (Northup et al., 2011). Lavoie et al. (2017) constitute the only study of how microbial communities within the lava tube caves related to the communities in the overlying surface. Targeting lava tube

caves in the search for life on Mars require a better understanding of the microbiology and mineralogy of lava tube caves on Earth.

1.2 Scope

This is the first study comparing lava tube bacterial communities in the skylight to bacterial communities from the overlying surface soil and the cave deep zone of each of the three caves examined in this study. We hypothesized that the bacterial communities in each cave zone were different. Denaturing gradient gel electrophoresis (DGGE) was used to analyze and compare the 16S rRNA microbial diversity found in the overlying soil, skylight region and the deep zone for each cave. Illumina MiSeq sequencing is planned for further analysis of the microbial communities, and samples have been prepared for 16S rRNA (iTag) sequencing at Joint Genome Institute. For one cave, environmental loggers were placed at the sampling sites to measure temperature, relative humidity and light levels in order to better understand these environmental variables. The environmental loggers were programmed to start recording data in the cave on October 24, 2016 and will record continuously for a one-year period, until October 24, 2017.

1.3 Outline

This master thesis on the microbiology of lava tube skylights is divided into seven chapters. Chapter 1 introduces the background and rationale for investigating the microbiology of lava tube skylights. Chapter 2 contains relevant background information and the research objectives are stated at the end. Chapter 3 describes the sampling location and the methods used to study the lava tube cave microbial communities. The results are reported in chapter 4, followed by a discussion in chapter 5, which includes an evaluation of DGGE as a method in cave microbiology. Chapter 6 summarizes the main findings and their implications, before recommendations for future research opportunities are presented in chapter 7. Appendixes are included to provide the reader with an overview of the samples from each cave.

2 BACKGROUND

During the first millennium B.C., Babylonians were the first to attempt to predict the positions of the Sun, Moon and visible planets (Mercury, Venus, Mars, Jupiter, and Saturn) (Leverington, 2007). The Greek philosophers also had a keen interest in planetary motion and tried to understand why planets move as they do. Our understanding of the Solar System and the Universe is vastly different from that of the Greek philosophers. As they studied planetary motions to understand why the planets move as they do, we search for microbial and intelligent life to understand if we are alone in the Universe.

2.1 The extent of microbial life

Earth was formed 4.5 billion years ago and has experienced physical and geological alterations throughout its history. These changes eventually led to conditions conducive to the origin of life. However, it must be noted that the conditions that allowed life to originate and appear are not necessarily the same conditions that support life once it has originated and allows it to thrive (Westall, 2012). The Earth continued to evolve and change after microbial life appeared, and life adapted to take advantage of the different environmental conditions. As a response, microbial life developed different metabolisms and physiologies to colonize and cope in a vast variety of environments. These microbial activities played an important role in the further evolution of Earth and the biosphere currently present is the result of the microbial evolution since its origin (Madigan et al., 2010).

NASA's Astrobiology Institute has defined astrobiology as "the study of the origin, evolution, distribution, and future of life in the universe" (NAI). NASA's astrobiology program seeks to answer three fundamental questions: "How does life begin and evolve? Is there life beyond Earth, and if so, how can we detect it? What is the future of life on Earth and in the universe?" (NAI). The first step is to look for life that is similar to life on Earth as this is the only form for life known. This has implications, life on Earth holds only one reference point for what life is. Additionally, there is a lack of understanding about life's physiological diversity on Earth, knowledge about alternative biochemistries, and of a definition for what life is (NRC, 2007b, Chyba and Hand, 2005, McKay, 2004).

2.1.1 Requirements for life as we know it

In their review of habitability, Cockell et al. (2016) identify five sets of requirements that must be met in order for life to prosper at a given location. These conditions are a solvent in which biochemical reactions can occur, appropriate physicochemical conditions, energy availability, main elements required for life (CHNOPS), and trace elements.

Liquid water is the required solvent for life on Earth and water is only able to exist in its liquid phase given suitable environmental conditions including temperature, pressure, and chemical composition. Microbial metabolic activity has been observed at temperatures ranges from 122°C (Kashefi and Lovley, 2003, Takai et al., 2008) to -25°C (Junge et al., 2004, Mykytczuk et al., 2013). Theoretically, water brines containing perchlorates can remain in a liquid state at temperature as far down as -65°C (Chevrier et al., 2009), and with pressures exceeding 100 bars water can remain liquid at 300°C. Even if it is possible for water to maintain a liquid phase at such extremes, it is still unknown if these conditions are able to support life (Cockell et al., 2016) and there is a lack of knowledge about how organisms adapt to multiple extremes (Harrison et al., 2013).

Microorganisms require energy for maintenance, growth, and reproduction. Depending on their method for acquiring energy, microorganisms are categorized as phototrophs or chemotrophs. Phototrophic microorganisms utilize sunlight to obtain energy, while chemotrophic microorganisms gain energy from redox process where they can use inorganic (chemolithotrophy) or organic (chemoheterotrophy) electron donors (Cockell et al., 2016).

Although life on Earth demonstrates a large diversity and complexity, it is still made up of the same atoms and molecules. C, H, N, O, P, and S are the essential elements required for cellular life on Earth and, together, these six atoms make up 97% of an *E.coli* cell (Wackett et al., 2004). These six elements form the building blocks for the nucleic acids, proteins, carbohydrates, fatty acids, and lipids which constitute the main macromolecules of life (McKay, 2004). In addition to require six main elements, microorganisms may also depend on other elements such as iron, magnesium, etc. (Wackett et al., 2004).

Regardless of where life originated on Earth, the conditions must have been such that they could sustain liquid water with temperatures that allowed organic macromolecules to be present. In addition to liquid water and organic macromolecules, bioavailable energy and essential major and trace elements had to be present for the origin of life and to sustain its evolution (Westall, 2012).

2.1.2 Life in the subsurface

A major fraction of biomass on Earth is microbial cells and microbial cells represent key reservoirs of life's essential six elements. In fact, the nitrogen and phosphorous content found in microbial cells are more than ten times higher compared to the amounts found in plant biomass (Whitman et al., 1998). The majority of our knowledge about the biospheres has come from studying life that is surface associated and dependent directly on photosynthesis or indirectly by utilizing biomass and oxygen produced by photosynthesis (Amend and Teske, 2005, Edwards et al., 2012). The general concept about life until the 1980s was that life was dependent on solar energy and photosynthesis (Gold, 1992). However, Corliss et al. reported in 1979 that they found microbial and animal communities in hydrothermal vents systems independent of surface derived energy. Numerous hydrothermal vent systems have been discovered since Corliss et al. (1979) and Russell et al. (1989), (2014) hypothesize that life could have originated in proximity of hydrothermal vents.

In 1998, Whitman et al. estimated the total number of prokaryotic cells on Earth and concluded that 55% of all prokaryotic cells were found in the marine subsurface whereas the majority of the rest was found in the terrestrial subsurface (table 2.1). This corresponds to a marine subsurface biomass on the order of 10^{17} g C and a continental subsurface biomass on the order of 10^{16} – 10^{17} g C. The distribution of cell numbers and biomass in the marine subsurface was reassessed by Kallmeyer et al. (2012) who noted that Whitman et al. (1998) had overestimated the cell numbers and biomass by, respectively 50-78% and 10-45%. McMahon and Parnell (2014) reassessed the biomass estimates provided by Whitman et al. (1998) on the terrestrial subsurface and found evidence to support a deep continental biomass of 10^{16} – 10^{17} g C, representing 2-19% of the total biomass on Earth.

Table 2.1. Prokaryotic cell numbers and biomass on Earth.

Habitat	Number of cells	Biomass (g C)	Reference
Soil	2.6×10^{29}	26×10^{15}	Whitman et al., 1998
Terrestrial subsurface	$2.5 – 25 \times 10^{29}$	$10^{16} – 10^{17}$	Whitman et al., 1998; McMahon and Parnell, 2014
Aquatic habitats	1.2×10^{29}	2.2×10^{15}	Whitman et al., 1998
Marine subsurface	2.9×10^{29}	4.1×10^{15}	Kallmeyer et al., 2012

Indications that microbial life was widespread in the deep subsurface of the Earth led Gold (1992) to hypothesize a deep, hot biosphere. In their review on intraterrestrial life on Earth, Edwards et al. (2012) defines the deep subsurface biosphere as an ecosystem that persists at least 1 m below the continental surface or seafloor and the deep, dark energy biosphere refers to ecosystems and habitats found in environments that are spatially and temporally removed from the photosynthetic world (Edwards et al., 2012).

Investigations of subsurface microorganisms have revealed that many are heterotrophic and depend on organic matter originating from photosynthetic surface life. This influx of surface derived organic matter has "contaminated" the subsurface and helps sustain microbial communities in both the continental and terrestrial subsurface (Edwards et al., 2012). Nevertheless, subsurface lithoautotrophic microbial ecosystems (SLiMEs) consuming small amounts of hydrogen and carbon dioxide have been identified where surface derived organic matter is restricted from entering and thus demonstrating that it is possible for life to exist in the absence of solar derived energy sources (Stevens and Mckinley, 1995, Stevens and McKinley, 2000, Chapelle et al., 2002).

Gold (1992) postulated an upper depth limit for life at depths of 5–10 km in the subsurface of the Earth. This was based on an upper temperature limit of 150°C for microbial life given a local pressure that would allow water to be in a liquid phase. Gold further suggested that the distribution of bacteria to these depths could be achieved by tidal pumping in less than a thousand years. This led Gold to propose a general rule: "microbial life exists in all the locations where microbes can survive" (Gold, 1992). The porosity of rock, sediment, and ice provide the necessary space for microorganisms to

live in (Parnell and McMahon, 2016). The depth limit for life remains unknown, but is believed to be governed by the availability of liquid water and an upper temperature limit (Wilkins et al., 2014).

Caves have been suggested as the link between surface and subsurface environments (Pedersen, 2000), and will be further discussed in chapter 2.2. The Lechugilla Cave in Carlsbad Caverns National Park in New Mexico, USA is a deep, extensive gypsum and sulfur-bearing hypogenic cave and provides a good example of an intraterrestrial cave environment as more than 90% of the cave lies, more than 300 m beneath the entrance (Cunningham et al., 1995).

Subsurface environments on Earth hosts a wide variety of bacteria and archaea despite the scarcity of nutrients and energy. Our lack of knowledge and understanding of their biochemistry and physiological requirements in theses stems from the absence of cultivated organisms (Hoehler and Jorgensen, 2013). Although investigations of the subsurface have identified numerous microorganisms and microbial habitats, the full potential of the deep biosphere has yet to be discovered (Edwards et al., 2012). The existence of a subsurface biosphere that is independent of solar energy has implications for our views on the likelihood of finding life beyond on Earth and how life may have originated (Pedersen, 2010, Gold, 1992).

2.1.3 Astrobiology and the search for microbial life in our Solar System

An important part of astrobiology is to improve our understanding of life on Earth, especially life in extreme environments and the limits to life on Earth. The knowledge gained from exploring the most extreme environments as potential microbial habitats gives us insights that help us understand which set of environmental conditions are capable of supporting growth of microbial life (NRC, 2007b).

The basic requirements for life as we know it are that it requires liquid water as a solvent, it draws its energy from the sun or from a chemical energy source, and nutrients. These three fundamental requirements for life form the basis for the search for extraterrestrial life and narrows it down to planets and moons where there is evidence for past or present

liquid water, where synthesis and polymerization of organic compounds can occur, and where energy sources and nutrients are present that can sustain life (NRC, 2007b).

Research on the microbiology in extreme environments and a better understanding of environments capable of supporting liquid water are two important factors for why our search for life has come to include icy worlds as well as the terrestrial planets (Marion et al., 2003).

Habitability

The aim of astrobiology has been to define the basic requirements for life that can sustain metabolic activity and reproduction in planetary environments over geological periods (Cockell et al., 2016). Cockell et al. (2016) defines habitability as the ability of an environment to support the activity of at least one known organism. Additionally, by constraining the definition of habitability to the current knowledge in biology the problem of not having a definition for life is avoided (Cleland and Chyba, 2002, Cockell et al., 2016).

The concept of habitability on Earth is intimately linked with the presence of life, but this does not necessarily have to be the case elsewhere. A habitable planetary body does not necessarily need to contain life and is important to realize when discussing habitability (Cockell et al., 2016).

A distinction is made between instantaneous and continuous habitability. The former refers to a given point in time where environmental conditions were such that the activity of at least one known microorganism was sustained, while the latter refers to a planetary body's capacity to maintain habitable conditions over geological timescales either at the surface or in the interior at local or regional scales (Cockell et al., 2016).

Another distinction is made between surface liquid worlds and interior liquid water worlds. Surface liquid worlds are planets that have liquid water both at the surface and in the interior, such as Earth, where water remains in liquid form mainly due to stellar heating, but internal heating is non-negligible. Interior liquid water worlds, such as Enceladus and Europa, do not receive enough stellar radiation for water to remain liquid

at the surface, but water is kept liquid in the interior as result of internal energy sources (McMahon et al., 2013a, Cockell et al., 2016). The lack of liquid surface water on Mars today is an example of how a planet can transition from surface liquid world to interior liquid world over geological timescales as a result of altered environmental conditions (Jakosky and Phillips, 2001).

Biosignatures

Westall and Cavalazzi (2011) define biosignatures as morphological, chemical (organic, elemental, and/or organic), and isotopic traces of organism preserved in minerals, sediments, and rocks. They represent the physical presence of the organisms as well as evidence of their metabolic activities. Biosignatures are strictly of biological origin (Marais et al., 2008). In the search for life beyond Earth it is important that we are able to discriminate between biosignatures and abiotic chemistry to avoid false positives and false negatives. These are situations where abiotic signatures resemble those of life and where real biosignatures are not detected or interpreted as origin abiotic (Horneck et al., 2016).

Strategies to look for extraterrestrial life

Our understanding of the habitability and potential of finding life in our Solar System has significantly increased during the last decade. There has been a shift and we have gone from considering Venus – Earth – Mars as habitable to seeing our whole solar system as potentially habitable. Where the icy worlds in the outer parts of our Solar System were once thought of as unhabitable, they are now considered as important targets in our search for extraterrestrial life (Westall et al., 2013, Marion et al., 2003).

The primary guiding principle in our search for life beyond Earth has been to follow the water to determine the potential for habitability. Previous astrobiology missions have primarily focused on initial exploration, but missions today are more focused astrobiology missions (McKay et al., 2014). Armed with the knowledge that liquid water has been present in the past or is present today, four suggestions for what to follow next have been published (McKay et al., 2014, Shapiro and Schulze-Makuch, 2009, Capone et al., 2006, Hoehler et al., 2007).

Shapiro and Schulze-Makuch (2009) formulated four principles to serve as guidelines for future searches for extraterrestrial life and suggested a follow-the-carbon strategy because of the chances to locate alternative carbon-based forms of life. Capone et al. (2006) argues the case for a follow-the-nitrogen approach because the presence or absence of nitrogen species can yield important clues to the potential for planetary bodies to host microbial life. The major reservoir for nitrogen on Earth is the atmosphere, while on Mars, nitrogen represents only a small fraction of a thin atmosphere (Capone et al., 2006). Shapiro and Schulze-Makuch (2009), and Capone et al. (2006) both suggested to follow one of the vital elements for life, Hoehler et al. (2007) promotes a follow-the-energy approach as energy is a key and universal requirement for life (NRC; Cockell et al., 2014). A follow-the energy approach will constrain the possible targets to bodies where bioavailable energy co-existed with liquid water, but can at the same time offer a broader basis for our search for life elsewhere (Hoehler et al., 2007). McKay et al. (2014) suggested to follow the plume of Enceladus as a specific location to investigate. The plumes of Enceladus consist of jets of vapor and icy particles that contain water, energy, carbon, and nitrogen. Thus, Enceladus represents a target that meets all the requirements McKay et al. (2014).

Rocky terrestrial planets: Mercury, Venus, and Mars

The likely presence of liquid water in the surfaces and in the subsurfaces of Mercury, Venus, and Mars all make them interesting targets where microbial life and/or biosignatures can be found (Schulze-Makuch et al., 2005). Although ice has been found close to the poles on Mercury, the conditions are considered so unfavorable for life any reasonable definition that is origin or persistence cannot be given a realistic probability (Irwin and Schulze-Makuch, 2001, Schulze-Makuch et al., 2005). Present-day surface conditions on Venus are extremely desiccating where high temperature (464°C) and absence of liquid water are the most important limiting factors for life (Cockell, 1999, Schulze-Makuch et al., 2005, Dartnell et al., 2015). While life at the surface or in the subsurface is deemed unlikely (Schulze-Makuch et al., 2005), there is potential for life in the Venusian atmosphere and the location of the habitable zone has been estimated to lie between 51 km (65°C) and 62 km (-20°C) altitude (Cockell, 1999, Schulze-Makuch et al., 2005, Dartnell et al., 2015). Mars and the Martian subsurface have long been considered as environments that are able to support chemotrophic organisms and ecosystems

(Boston et al., 1992, Gold, 1992, Cockell, 2014) and Mars is further elaborated upon in chapter 2.3.

Icy worlds: Enceladus, Europa, and Titan

Three of the most promising icy worlds targets in the search for extraterrestrial life are Enceladus, Titan, moons of Saturn, and Europa, a moon of Jupiter (Shapiro and Schulze-Makuch, 2009, McKay et al., 2014, Marion et al., 2003, Russell et al., 2014). Enceladus is another of Saturn's moons that is of astrobiological interest. The plumes being jetted out from the southern pole regions are of known composition and indicate that their area of origin is habitable to Earth-like life. Furthermore, the icy surface covers a global subsurface ocean which is likely produced by geothermal heating due to tidal forces (McKay et al., 2014). The presence of hydrothermal vents at its rocky core indicate that Enceladus could support microbial life beneath its icy surface (McKay et al., 2008, Russell et al., 2014). The Jovian moon, Europa, represents another prime target in our search for potentially habitable worlds. Marion et al. (2003) discussed potential Europan habitats for life and concluded that Europa holds sufficient energy and nutrients to support a small biomass. Based on their guidelines, Shapiro and Schulze-Makuch (2009) suggested Titan, Saturn's largest moon, as the top priority in the search for life because of the possibility of finding exotic, hydrocarbon-based life. Titan offers a rich organic chemistry which is demonstrated by an active hydrological cycle based on methane (Lunine and Lorenz, 2009).

2.1.4 Planetary protection

Definition

NASA's Office of Planetary Protection defines planetary protection as the practice of protecting solar system bodies (*i.e.,* planets, moons, comets, and asteroids) from contamination by Earth life, and protecting Earth from possible life forms that may be returned from other solar system bodies (Office of Planetary Protection).

Importance

There are good reasons for why planetary protection is such an important consideration. First, it maintains the pristine environment of the worlds to be investigated and makes sure that no contamination from Earth would interfere with our ability to detect life elsewhere if it exists. Secondly, it is to make sure that the necessary precautions are being taking to protect life on Earth. Therefore, planetary protection is generally divided into two categories: forward contamination and backward contamination. Forward contamination refers to "the biological contamination of explored solar system bodies" and backwards contamination refers to "the biological contamination of Earth as a result of returned extraterrestrial samples" (Office of Planetary Protection). Horneck et al. (2012) demonstrates that bacterial spores are able to survive both a flight and stay at Mars as long as they are protected from the solar irradiation.

Committee on Space Research (COSPAR) Panel on Planetary Protection

The 1967 United Nations *Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space, Including the Moon and Other Bodies* states that all countries party to the treaty "shall pursue studies of outer space, including the Moon and other celestial bodies, and conduct exploration of them so as to avoid their harmful contamination" and represents the first international agreement on planetary protection (Nations, 1967). Today, it is the responsibility of The Committee on Space Research (COSPAR) Panel on Planetary Protection to maintain, develop, and counsel the international community, including the United Nations, on planetary protection knowledge and policy (Kminek and Rummel, 2015). COSPAR defines five different planetary protection categories for extraterrestrial missions based on mission type and body. Target bodies with no direct interest for understanding the process of chemical evolution or the origin of life require no planetary protection (Category I), while all Earth-return missions are subject to the strictest set of planetary protection requirements (Category V). The concern for these missions is the protection of the terrestrial system with the Earth and the Moon. (Kminek and Rummel, 2015).

2.2 Life in caves

Caves offer a point of entry to Earth's subsurface and their size can vary in length from microfissures to hundreds of kilometers. Although caves might be thought of as rare by the general public, they are not. Even though most public accessible caves are limestone caves, caves formed in other calcareous rocks together with lava tubes in basaltic rocks are the most common cave types (Boston et al., 2001, Northup and Lavoie, 2001).

Chemolithotrophic microorganisms were first discovered in 1887 by Sergei Winogradsky and these microorganisms are able to influence geological formations. This is due their ability to promote redox reactions, either passively or actively, and thus, they can interact and derive energy from inorganic material (Barton, 2006, Northup and Lavoie, 2001). This is of particular importance for cave microorganisms as the presence of surface derived energy in caves is limited to the entrance zone and cave microorganisms must draw their energy from either the atmosphere or the rock (Barton et al., 2004, Chelius and Moore, 2004, Engel et al., 2003, Northup et al., 2003, Spilde et al., 2005). Our understanding of the role played by microorganisms to shape our environment will improve with an increased understanding of microbe-rock interactions (Barton, 2006)

2.2.1 Types of caves

Northup and Lavoie (2001) define the term "cave" as any natural space below the surface that extends beyond the twilight zone, and that is accessible to humans, and is the cave definition adopted here. Numerous types of cave exist and they tend to be classified according to rock type and formation method (Palmer, 1991). Caves formed in limestone and other calcareous rocks, and lava tube caves in basaltic rock are the most common type of caves (Northup and Lavoie, 2001). Other cave types such as gypsum, granite, ice, etc. also exist, but their distribution tend to be limited.

There are three primary mechanisms for forming the most common cave types, limestone and lava tube caves (Northup and Lavoie, 2001). Water passing through the soil will absorb CO_2 and the CO_2 dissolved in water leads to the formation of carbonic acid and slightly acidic water. Once the acidic water reaches the water table and stays in contact with the limestone, more calcium carbonate will be dissolved due to the presence of carbonic acid (Northup and Lavoie, 2001). Classical limestone caves are formed this way,

but limestone cave formation can also be driven by the presence sulfuric acid. Here, rising hydrogen sulfide arrives at the oxygenated zone where limestone is dissolved by the sulfuric acid that forms (Jagnow et al., 2000). Lava tube caves form as fluid lava flows out of a volcano. The lava in contact with atmosphere cools quickly and solidifies, leaving the remaining lava to flow out of the tube (Northup and Lavoie, 2001, Leveille and Datta, 2010).

2.2.2 Cave environment

Living in the subsurface offers many advantages for microbial life. They are protected from the damaging effects caused by weather conditions, desiccation, temperature fluctuations, ultraviolet radiation and they are not available as prey for higher, grazing organisms (Boston et al., 2001, Omelon, 2008).

Caves can be divided into a number of different zone. At the entrance zone, the cave will be highly influenced by the surface conditions, especially the potential for light is important. Further into the cave is the twilight zone, where light penetration is limited and the surface conditions are enhanced by cave conditions. Going even further, light will be absent and the temperature will be close to the region's mean annual surface temperature, and be very humid (Northup and Lavoie, 2001).

The cave environment is relatively stable and represents a unique subset of the deep subsurface environment on Earth (Boston et al., 2001). There is an absence of organic material produced from primary production by plants, because the lack of light. The unique physical parameters are special properties of caves, and they are usually relatively mild, predictable and constant (Northup and Lavoie, 2001). Physical characteristics of caves include stable temperatures, even though surface temperatures can have large fluctuations, high humidity, large surface area, the exchange of gases due to air circulation, often very limited in nutrients (oligotrophic), possible extreme pH values, and secondary mineral deposits can include many diverse elements (e.g., Fe, Mn, Ca, Si, S rare earth elements, etc.) (Boston et al., 2001).

Bacteria on Earth can utilize almost any energy yielding redox couple (Boston et al., 2001). Calcareous and basaltic rocks are often rich in reduced sulfur, iron, and manganese, and microorganisms at the interface between the rock and cave passage are able to utilize these reduced compounds in their metabolic processes to generate energy (Northup and Lavoie, 2001, Boston et al., 2001). Iron-, sulfur-, and manganese-oxidizing bacteria are of particular interest for cave dissolution processes because of their ability to enough acidity to dissolve cave walls (Northup and Lavoie, 2001).

Iron oxides and iron hydroxides are typically found as coatings or crust in caves. Biological iron oxide production tends to occur at low pH, while abiotic iron oxide formation occurs at pH 6 or above (Northup and Lavoie, 2001). As for iron oxides, manganese oxides can also be found as wall coatings. pH and redox reactions between Mn(II) and Mn(III, IV) control the abiotic oxidation of Mn(II), while biological manganese oxidation can occur directly or indirectly. For direct oxidation, Mn(II) can bind to intra- and extracellular Mn(II)-binding proteins or to the negatively charged compounds on cell surface (Ghiorse, 1984). Indirect oxidation is a result of altered redox conditions in the microenvironment du release of release oxidants, acids, or bases from the microbial cell (Tebo et al., 1997). Sulfur- and sulfide-oxidizing bacteria are capable of forming sulfate, which in turn can be used by sulfate-reducers as an electron acceptor. Most studies on cave sulfur-bacteria have been qualitative (Northup and Lavoie, 2001).

Continued cave exploration and survey is important to further our understanding of cave ecosystems and identify new cave biota, but in this quest it is important to remember the leave no trace conservation ethic and take care to limit human contamination in order to preserve the cave microbial habitats (Barton, 2006).

2.2.3 Cave microbiology

Because of the difficulties encountered when trying to cultivate microorganisms from an environmental sample, the general idea until the 1990s was cave life was very limited in numbers and that it was not indigenous to the cave, but rather transported into the cave by air movement or vectors (Barton, 2006, Barton and Northup, 2007). Geological features were attributed to abiotic, geochemical processes although they were difficult to explain by these processes alone. With technological advancement and the development

of new molecular techniques, researchers were able to reexamine caves and decipher their role as microbial habitats.

Our perception of cave ecosystems changed when the presence of microorganisms in geologic cave samples were demonstrated (Barton and Northup, 2006). These cave microorganisms, who have adapted to their extreme and oligotrophic environment, are potential reservoirs of important and novel discoveries similar to the discovery of Bennett and Barton, who found a microorganism capable of degrading complex aromatic compounds, benzothiazole and benzenesulfonic acid, involved in plastics manufacturing (Barton, 2006).

Although it can be difficult to observe microbial activity in caves, typical cave features that indicate microbial activity include dots on surfaces, unusual coloration, precipitates, corrosion residues, structural changes and biofilms (figure 2.1). The rule of thumb is if it cannot easily be explained by geologic phenomena, chances are that its microbiological (Barton, 2006).

The potential of caves as microbial habitats remains relatively unstudied (Boston et al., 2001) and with its active discovery, it is nearly impossible to predict where microbial activity is taking place (Barton, 2006). What is known on the other hand, is if liquid water and stable physicochemical conditions are present, then this will favor microbial growth and precipitation of secondary mineral deposits (Leveille and Datta, 2010).

The presence of biofilms in cave environment is one of the most obvious signs of microbial activity. As pointed out by Watnick and Kolter (2000), surface association in the form of a biofilm is the predominant form of lifestyle for microbes in most natural environments. An advantage of finding biofilms is that they usually form at locations where energy enters the cave. Therefore, biofilms in caves can provide valuable insights into what sources of energy can support cave life (Angert et al., 1998).

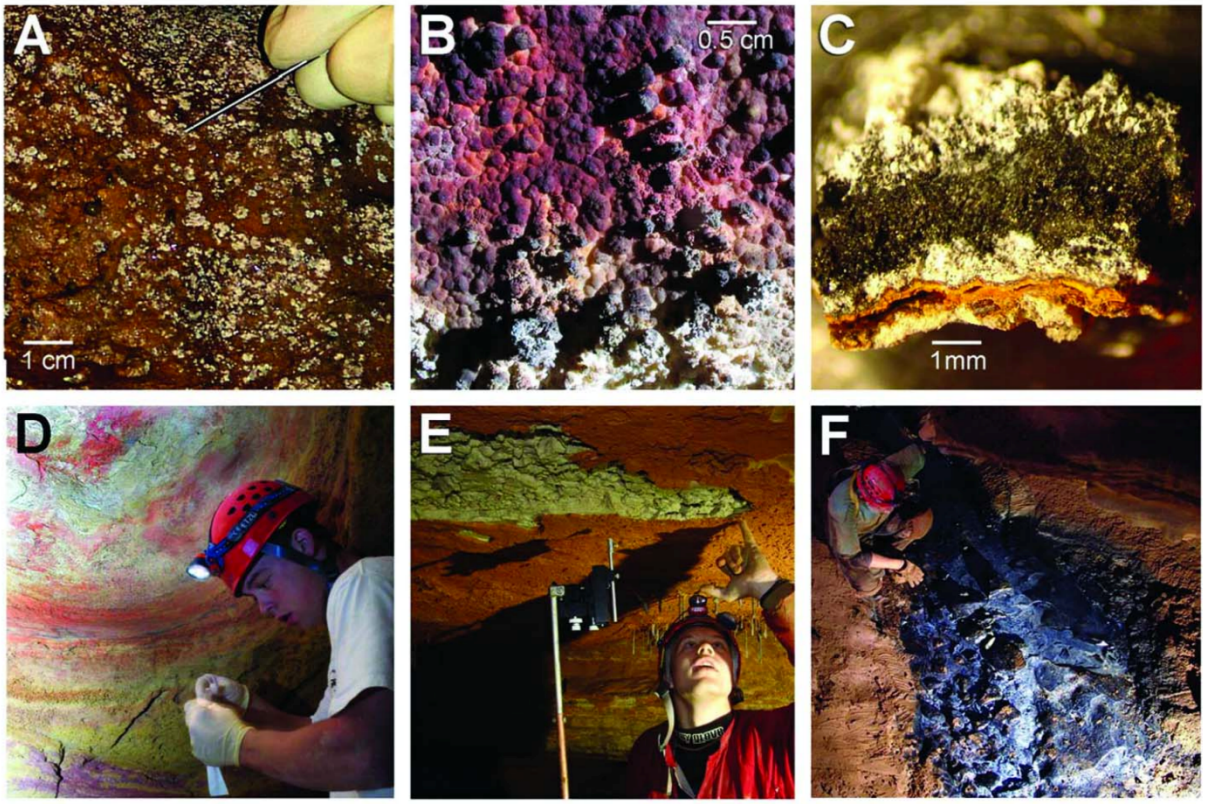

Figure 2.1. Microbiological activity in caves. A) Dots – microbial colonies on the surface of a rock being sampled using a hypodermic needle; B) Color – Microbial activity leading to the discoloration of a rock surface; C) Precipitation – Banded mineralization on a rock surface by microbial activity (the precipitate has peeled away revealing the deposit's banded formation); D) Corrosion residues – brightly-colored corrosion residue formed on a cave ceiling; e) Structural changes – using a microelectrode assembly to examine chemical gradients formed within a soft cave ceiling; F) Biofilms – a white biofilm coating in a streambed of a cave. Picture and description copied from Barton (2006).

2.2.4 Lava tubes caves

Wherever basaltic lava occurs on Earth, lava tube caves are common (Northup et al., 2011). Lava tube caves serve as point of entry to the shallow subsurface, and they are widely present on oceanic islands such as Hawai'i and Iceland, and on the continents including western United States, Spain, and South Korea (Northup and Lavoie, 2015).

The origin of lava tube caves is completely different from the origin of limestone caves. Lava tube caves are formed as a by-product of volcanic processes where molten rock rises to the surface from between 50 and 700 km depth at temperatures of 700–1200°C. Lava tube caves are the same age as the rock itself and in order to determine its age and composition, rocks from inside the cave should be used. Younger lava flows will be

located ontop of the older ones, and therefore the more recent lava flows can downcut into older lava below (Northup and Lavoie, 2015). An alternative theory for lava tube cave formation has been proposed where molten lava flows underneath the already solidified lava and lifts it up. As the molten lava is denser then the solidified lava, solid lava rocks float on the liquid lava. In the end the molten lava is drained form the lava tube cave and the lava caves seen today result from the cooling of the walls and ceiling (Northup and Lavoie, 2015).

Lava produces numerous igneous rocks that vary in chemical composition and crystal size. The most common type of lava found in lava tube caves is basaltic lava. The dark and finely crystalline basaltic lava consists of approximately 50% silicon dioxide (SiO_2), and in various amounts (in decreasing order) of aluminum (Al_2O_3), iron (FeO), calcium (CaO), magnesium (MgO), and trace amounts of other compounds (Northup and Lavoie, 2015). The environmental conditions and the chemical composition of the lava when it solidified are important parameters that determine color of the lava tube caves. The most commonly observed secondary mineral deposits are of sulfur, oxidize and hydroxide minerals, and salts formed by evaporation of infiltrating surface water rich in sulfates and chlorites (Northup and Lavoie, 2015).

Length, passage geometry, number of entrances are factors that influence the physical conditions in lava tube caves. The temperature in some lava tube caves is the mean annual surface temperature of the area and vary with latitude and elevation, while others are cold sinks where ice is present during the whole year. Five caves at the same elevation in El Malpais, New Mexico, differed from low temperature of -2°C to high temperature of 11°C, in an area of a mean annual surface temperature of 10°C. The number of passages is determined by the steepness of the terrain and is inversely related to the steepness (Northup and Lavoie, 2015).

2.2.5 Lava tube cave ecology and diversity

The first investigation into the microbial communities found in lava tube caves were culture-dependent isolation methods that studied microbial mat or slime associated microorganisms. Although there has been a revolution in culture-independent methods to identify microorganisms in the environment, the traditional cultivation and isolation

methods are still important in order to study the microbial physiology. Instead of competing against each other, these methods should be used complimentary (Donachie et al., 2007, Wackett et al., 2004). The vast microbial diversity found in lava tube caves enable us to investigate the frontiers and strategies of life in extreme, oligotrophic environments (Northup and Lavoie, 2015).

Since lava tube caves form *de novo* and their colonization is dependent on influx of microbes and organics from the surface, it is important to know which microorganisms are present at the surface, in the soil and on the volcanic rocks. The oligotrophic nature of volcanic landscapes see microorganisms taking advantage of the reduced compounds present in basalt, notably iron, sulfur and manganese (Northup and Lavoie, 2015; Barton and Northup, 2007; Lavoie and Northup, 2001). Additionally, there are indications that nitrogen is limiting for microbial growth and phosphorous even more so in carbonate and lava tube caves (Northup and Lavoie, 2015).

Gomez-Alvarez et al. (2007) investigated four recent deposits at Kilauea volcano, Hawai'i, and described their bacterial diversity. Their study found that microorganisms are able to colonize recent volcanic deposits and establish diverse communities, and that local variations are important factors that influence community composition.

Kelly et al. (2014) investigated the pioneering microbial communities colonizing the basaltic Fimmvörðuháls lava flow at Eyjafjallajökull, Iceland, formed in 2010. Both molecular and culture-dependent methods were used to reveal the primary microorganisms colonizing recent lava flows. They found that a low-diversity microbial community dominated by *Betaproteobacteria* had colonized the lava three to five months after eruption. The two main taxa were diazotrophs such as *Herbaspirillum* spp. and chemolithotrophs such as *Thiobacillus*. Cultivation and molecular analysis revealed the presence of phototrophs, although not in high abundance. This led Kelly at al. (2014) to suggest that phototrophy is not likely to be a dominant biogeochemical process in early successional basalt communities on Iceland. However, other studies have shown that for older Icelandic lava of comparable mineralogy, phototrophs make up a considerable fraction of microbial communities and that *Acidobacteria* and *Actinobacteria* dominate the non-phototrophic community fractions (Kelly et al., 2011, Kelly et al., 2010).

Northup et al. (2011) compared microbial mats and secondary mineral deposits in four caves from three different locations. The microbial mats were white and yellow in color, while the color of the selected secondary mineral deposits varied in from cave to cave. The caves represent different climate conditions including tropical and semi-arid caves in Hawai'i, temperate on the Azores, and semi-arid in New Mexico. They found that microbial mats' coverage is more extensive in areas with higher rainfall. *Actinobacteria, Alpha-, Beta-, Gamma-*, and *Deltaproteobacteria*, and *Acidobacteria* were present in all mats, while *Nitrospirae* was present in all but one cave. Phyla number per cave varied from five to eleven and the diversity at phyla level was displayed slightly higher in yellow mats than in white mats. Although the secondary mineral deposits samples share most of the 15 phyla, a higher diversity at the operational taxonomic unit (OTU) level was reported for these microbes. The most noticeable finding is that while *Actinobacteria* dominated in mats, they are only found in two of the six secondary mineral deposits samples. Additionally, table 3 in Northup et al. (2011) compared bacterial phyla found in lava caves with those in carbonate caves and found that caves appear to contain a core set of bacterial phyla.

Hathaway et al. (2014) collected white and yellow microbial mats from four lava tube caves on the Azores and Hawai'i in order to compare the bacterial diversity using 16S rRNA gene clone libraries. Hawai'i and the Azores are two widely separated archipelagos located in different oceans and experiencing different climate conditions; Hawai'i experience climatic conditions ranging from semi-arid to tropical, while the Azores experience a temperate climate. Hathaway et al. (2014) tested the Baas-Becking hypothesis "everything is everywhere, but the environment select" by investigating if local influences on lava tube cave microbial communities result in unique microbial communities or if the communities are similar despite their physical distance. They predicted that they would find novel organisms sharing <97% 16S rRNA sequence identity with known bacteria since these bacteria were subject to unique selective regimes at each location.

Hathaway et al. (2014) found that 15 bacterial phyla were found across the samples with a higher number of *Actinobacteria* clones in the Hawai'ian microbial communities and a higher number of *Acidobacteria* clones in the microbial communities from the Azores. Only 34 OTUs of the 609 OTUs defined at 97% formed with sequences from both islands. They concluded that geographical location was the major contributor to the differences observed in community composition accompanied by differences in available organic carbon, nitrogen, and copper. In addition, they found that mat color cannot predict the microbial community and that the bacterial communities in each cave are different.

The study of Lavoie et al. (2017) represents the first comprehensive comparison of bacterial communities in lava tube caves with the overlying soil community. They analyzed microbial mats from seven different lava tube caves in Lava Beds National Monument, CA, USA, and compared it with the bacterial communities from the overlying soil surface on an attempt to understand how microbial mats in lava tube caves differ from the microbial communities found in the soil surface. Tan, white, and yellow microbial mats were analyzed from each cave. The surface soils and cave microbial mats share the same phyla, but the shared OTUs overlap with only 11.2%. The contributing factors to the observed diversity difference were number of cave entrances and temperature. Although the diversity in the different mat color differed, species richness was not significantly different. *Actinobacteria* and *Proteobacteria* dominated in cave samples and soil samples, where *Actinobacteria* made up 39% of phyla in caves and 21% in surface soils, and *Proteobacteria* made 30% of all cave phyla and 36% of surface soil phyla. The next large cave phyla were *Nitrospirae* (7%), while for surface soils *Bacteroidetes* (8%) was the third largest phyla. A high degree of novelty is indicated by the lack of identification at genus level for the most abundant sequences. More OTUs and greater diversity indices were found in the surface soil than in the cave samples. Even though the microbes found in the surface soil can be transported into the underlying cave, the microbial communities inside the cave is very different because of the selective pressures from the environment.

Fungi are present in lava tube caves and they have gained increasing attention lately due to the bat infection caused by *Pseudogymnoascus destructans* called white-nose syndrome (Cryan et al., 2013). The reader is referred to Northup and Lavoie (2015) and reference therein for further information on the presence of eukaryotic microorganisms and the white-nose syndrome disease.

2.2.6 Lava tube caves as astrobiological targets

Remote-sensing and orbital imagery have identified lava tube caves (figure 2.2) and cave like features on Mars (Leveille and Datta, 2010). Their identification has led to the suggestion of lava tube caves as astrobiological targets. Stable physicochemical conditions in lava tube caves give them their unique environment and enhance secondary mineral precipitation and microbial growth (Leveille and Datta, 2010, Boston, 2010). Cave microbial communities producing secondary mineral deposits are capable of experiencing in situ preservation via mineralization. Therefore, pristine biosignatures can be found in cave environments and their physicochemical conditions can protect the biosignatures from surface weathering (Boston et al., 2001). Lava tube caves on Earth can be used to test hypotheses, improve our understanding of geomicrobiological processes and the biosignatures left behind by these processes (Boston et al., 2001, Leveille and Datta, 2010, Northup et al., 2011, Cousins and Crawford, 2011).

Popa et al. (2012) examined microorganisms living at the boundary between ice and basalt in a lava tube cave in the Oregon Cascades with perennial ice. They demonstrated the presence of neutrophilic iron-oxidizing microorganisms capable of oxidizing iron present olivine ($(Mg,Fe)_2SiO_4$), a mineral commonly found in basalts that contain around 10% Fe(II). A *Pseudomonas* sp. HerB isolate was found to use ferrous iron Fe(II) from olivine as electron donor and O_2 as electron acceptor, but growth using olivine as source of energy was favored under as low O_2 concentrations as 1.6% O_2. Since the examined environment is similar to Martian environments, the authors suggest that neutrophilic iron-oxidizing microorganisms may have thrived under past climate conditions on Mars and could still exist in Martian basalt (McMahon et al., 2013b).

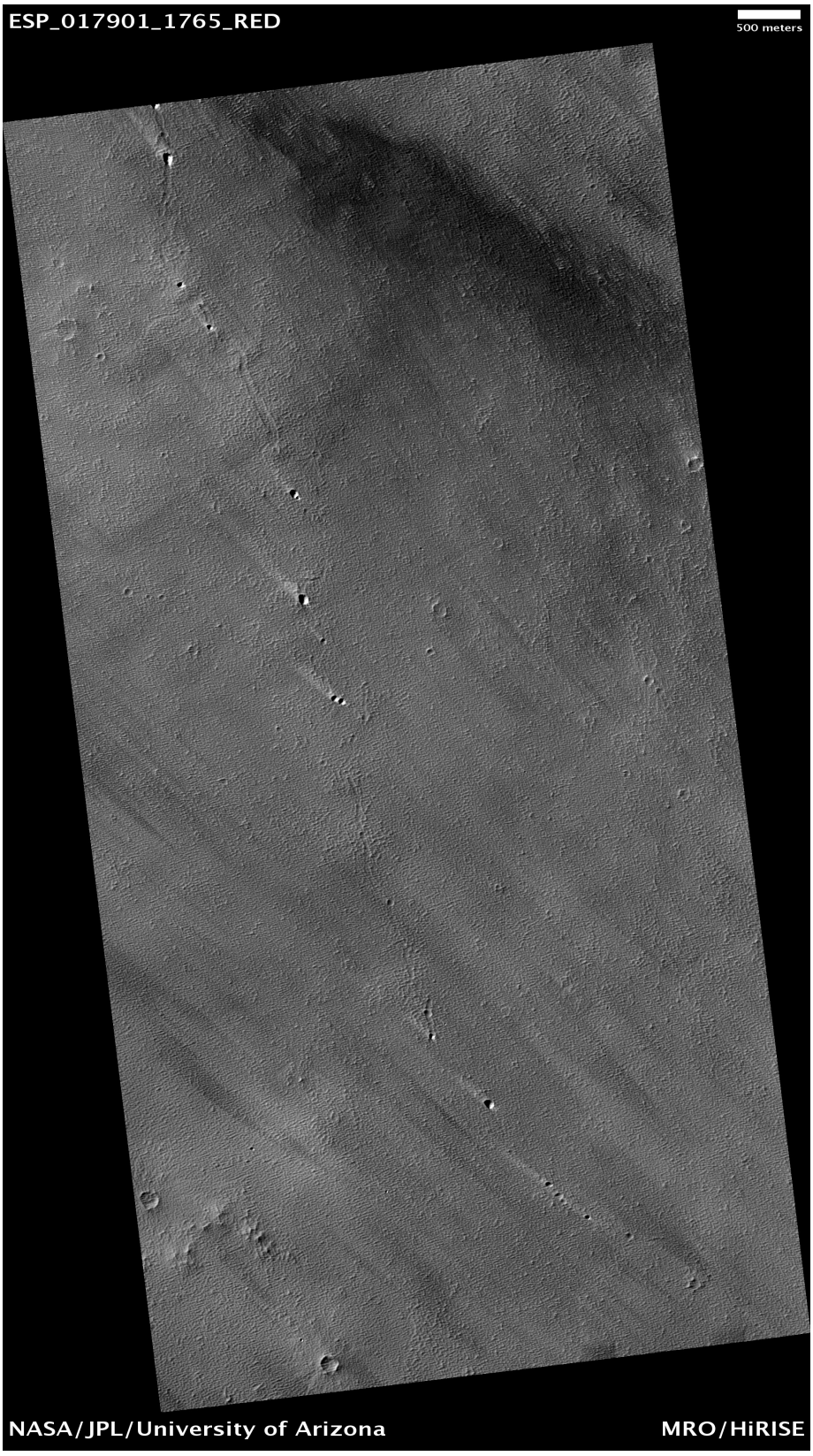

Figure 2.2. Lava tube on Arsia Mons. The lava tube is recognized by the succession of collapsed pits. Credit: NASA/JPL/University of Arizona.

Tebo et al. (2015) investigated dark oligotrophic volcanic ice cave ecosystems of Mt. Erebus, Antarctica. They collect sediment samples in three different caves, two of which are completely dark and one that receives snow-filtered sunlight seasonally. These ice caves are found in a polar alpine environment deprived of organics and with oxygenated hydrothermal circulation in highly reducing host rock. Therefore, dark oligotrophic volcanic ecosystems are rich in chemical reactants and well known for chemical exchange with Earth's surface systems, but the lack of sunlight, and thus solar derived energy and biomass, and organic matter make these ecosystems good environments to study life and explore its limits.

The likelihood of finding life in the surface of Mars is considered to be extremely low due to the extremely hostile surface conditions (NRC, 2007a, Boston, 2010, Boston et al., 2001, Boston et al., 1992, Cockell, 2014). But lava tube caves are not just of interest in our search for life beyond Earth, they offer natural protection for human exploration of Mars too (Boston, 2010).

2.3 Mars – an astrobiological target

Of all the other planets in our Solar System, Mars (figure 2.3) is the most similar to Earth. Mars is one of four terrestrial rocky planets, it is the second smallest planet in the Solar System, and it is the fourth planet from the Sun. Earth and Mars share a similar geologic history where the presence of liquid water at or near the surface since their formation has played an important role in shaping the planets as they are today. As Earth and Mars were built from the same material, carbonaceous chondrites, and they are believed to contain the same elements (NRC, 2007a). Carbonaceous chondrites formed during the early Solar System in oxygen-rich zone and consist mainly of hydrated silicates and complex hydrocarbons (Mcsween and Richardson, 1977).

As demonstrated by ESA's Schiaparelli lander, even in 2016, it is not an easy task to land on other planets. A mission to Mars or another planetary body is highly complex and very costly which is why environmental data from other planets are scarce. Mars has been and continues to be a high priority target and is the planet for which there is most information about past and present geochemical conditions (Cockell, 2014).

Earth can be used as an analog for certain environments and as our knowledge about life and environmental conditions in the subsurface increases, so does our understanding of these environments as potential habitats beyond Earth. After recognition that extreme environments on Earth were able to support microbial life, Boston et al. (1992) suggested the possibility of finding methanogenic and sulfuric based chemosynthetic ecosystems in the Martian subsurface.

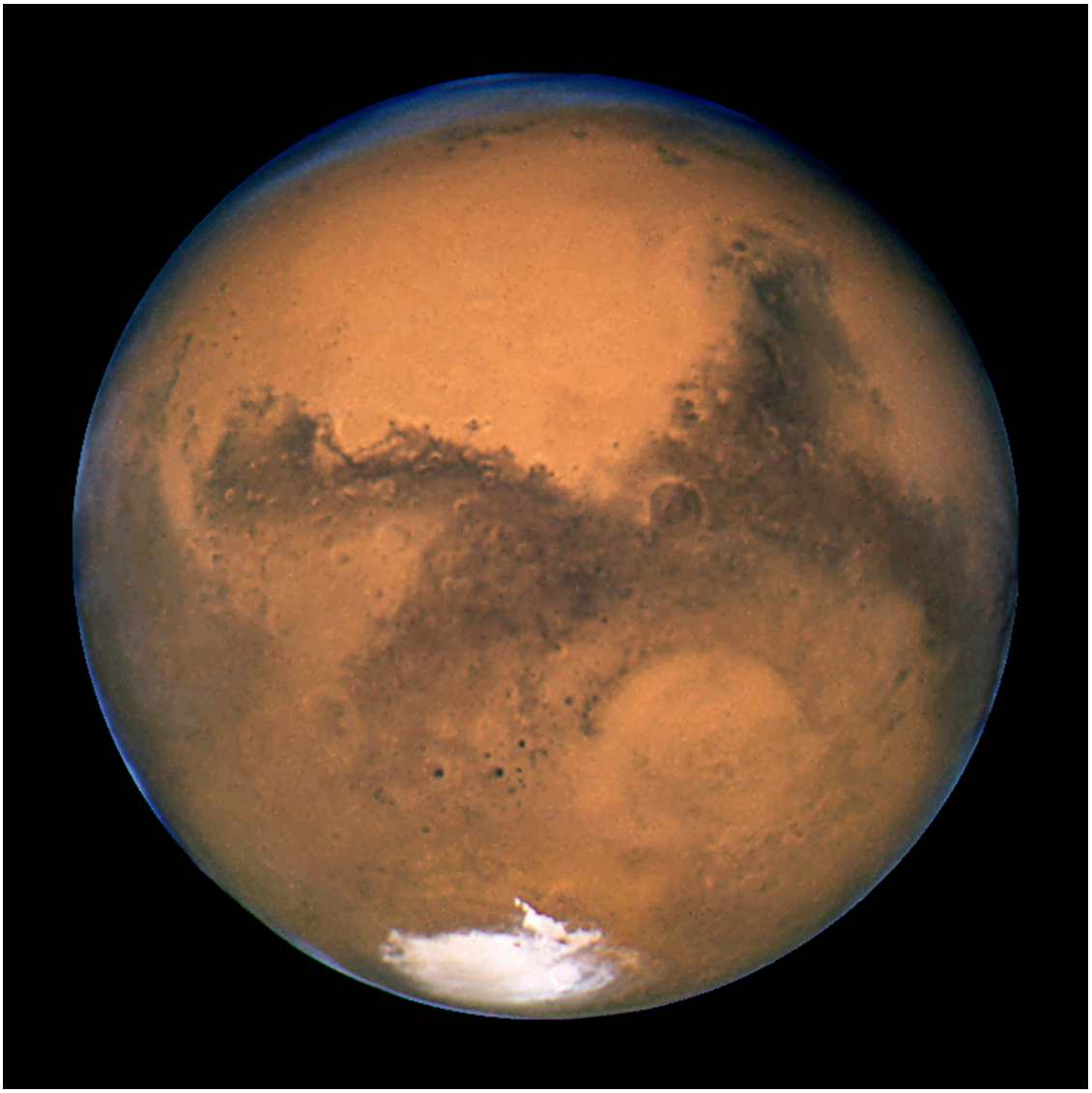

Figure 2.3. Mars Credit: NASA, ESA, and The Hubble Heritage Team (STScI/AURA)

2.3.1 Early Mars

The presence of valley networks, indications of high erosion rates, lake remnants, rivers, and other geomorphological features in terrain dating from the early Noachian (4.1 – 3.7 billion years ago) have been interpreted in favor of a wet and warm early Martian climate

that had environmental conditions that supported large bodies of standing water over time (Pollack et al., 1987, Carr, 1999, Cabrol and Grin, 2001, Clifford and Parker, 2001). However, most of this surface water was gone by the Hesperian, 3.7 – 3.1 billion years ago (Cockell, 2014). Noachian represents the best evidence that past conditions were different from today's conditions and it is often stated that the early environmental conditions on Mars were similar to those on Earth (Haberle, 1998). The geological observations linked to the Noachian suggest that it was warmer and wetter, but observations from Devon Island in the Arctic and the Dry Valleys of Antarctic (Mars analogs) have led Lee and McKay (2003) to suggest that Mars was always cold and sometimes wet. Lee and McKay (2003) state that the observed geological and geomorphological features resulting from aqueous activity may be explained by enhanced endogenic heat flow, exogenic impact and/or short-term local environmental change during an otherwise cold, low pressure (100 mbar) global climate. The greatest challenge encountered by climate models of early Mars where atmospheric conditions support a warm and wet climate is how to deal with a faint early Sun.

2.3.2 Environmental conditions on present day Mars

Although the surface of present day Mars is inhospitable to life as we know it, it is believed that conditions in the past made Mars more hospitable to life (NRC 2007a, Cockell, 2014).

Mars is considered a cold desert with a mean annual surface temperatures (MAST) of around 215 K at equator and 160 K in the polar regions. Such low temperatures help form a thick cryosphere that may extend to depths of several kilometers depending on regional variations. During summer, the temperatures above 273 K have been measured which makes for high temperature variations. Even though the temperatures may rise above 0°C for certain periods, the ground remains permanently frozen (NRC, 2007a). At such low temperatures, any water present in the cryosphere with the exception of perchlorate brines (Chevrier et al., 2009) and observations of hydrated perchlorate salts were made by Ojha et al. (2015).

Unlike Earth's 1 bar atmosphere which consists mainly of nitrogen and oxygen, the Martian atmosphere is only 5.6 mbar and consists mainly of CO_2 (NRC, 2007a). Atmospheric nitrogen makes up only 160 microbar (Capone et al., 2006). Furthermore,

this low pressure atmosphere is close to the triple point of water which makes it impossible for standing bodies of water to persist (Haberle et al., 2001, Cockell, 2014). However, due to local and regional variations in elevation, parts of the surface will experience an atmospheric pressure that is superior to the water's triple point and temporarily allow for the formation of liquid water (NRC, 2007a).

Mars also lacks a protective ozone layer, resulting in a high ultraviolet radiation surface flux. This high flux of ultraviolet radiation at the surface could lead to significant more DNA damage than on the surface of the Earth (Cockell et al., 2000), but thin layers of dust can protect against this ultraviolet radiation (Moores et al., 2007). In addition, Mars has no magnetic field and thus the surface receives much higher levels of ionizing radiation than Earth (Kminek and Bada, 2006, Dartnell et al., 2007, Pavlov et al., 2012).

2.3.2 Missions to Mars

The Viking Mission, 1975

In 1975, NASA launched two spacecrafts, Viking 1 and Viking 2, made up of a lander and an orbiter to explore the Martian surface and atmosphere. The Viking mission's three primary objectives were to obtain high resolution images of the Martian surface, characterize the atmospheric structure and composition, and search for evidence of life. Although recent missions to Mars has improved our data quality, the most complete view of Mars to date has come from the Viking experiments result. The landing sites are best described as iron-rich clay where the surface temperature varies from 150 K to 250 K (Williams, 2016).

When the Viking landers landed on Mars in 1976, Mars was considered a cold desert with a thin atmosphere. What was exciting about the Viking mission was that it was decided to investigate the biological potential of Mars, because there was a shared belief that the surface of Mars was the most promising place to look for life on another planet. The biological experiments were designed to sample Martian soil at a few centimeters depth in an attempt to discover life as we know it on Earth (McKay, 1997).

The following three biology experiments conducted by the Viking landers are : The pyrolytic release experiment (Horowitz et al., 1977) was designed to measure the ability

of Martian microorganisms to incorporate radioactively labeled carbon dioxide in the presence of sunlight, through photosynthesis (Klein, 1979, McKay, 1997). The gas exchange experiment (Oyama et al., 1977) was designed to measure if Martian microorganisms could, in the presence of water vapor in a nutrient solution, metabolize and exchange gases (Klein 1979, McKay 1997). The labeled release experiment (Levin and Straat, 1977) was designed to detect the release of radioactively labeled carbon from simple organic compounds in a nutrient solution (Klein 1979, McKay 1997). A gas chromatograph-mass spectrometer (GC-MS) was also present on the Viking landers to investigate if any organics were present in the soil (Klein, 1977, McKay, 1997). Although the GC-MS was not part of the biology experiments, it was still a key instrument, as it failed to detect organics in the soil (Biemann et al., 1977, Biemann, 1979). Even though all the biology experiments recorded activity, when the biology results are seen together with the GC-MS results, the most likely explanation is that these results are due to abiotic processes (McKay 1997).

The Phoenix lander, 2007

In 2007, NASA sent the Phoenix lander to the Martian surface to search for evidence of past or present life. The Phoenix lander was sent to the northern arctic plains where large amounts of subsurface water ice have been identified by the Mars Odyssey Orbiter. The Phoenix mission objectives were to study the water history in the arctic parts of Mars and evaluate its the biological potential and habitability JPL (2017b).

Previous missions to Mars measured the elemental composition of the Martian surface *in situ* with the use of x-ray fluorescence spectrometry. However, these measurements are unable to yield insights on the soil's aqueous chemistry, which is of vital importance for microorganisms because it is readily available for biological activity. Only the Viking experiments conducted aqueous experiments, but their focus was on specific metabolic processes (Oyama et al., 1977, Levin and Straat, 1977, Hecht et al., 2009). The Phoenix lander results from the aqueous chemical analyses of surface material and subsurface material at 5 cm dep showed no significant differences, and were determined to contain dissolved salts at 10 mM concentration with 0.4 – 0.6% perchlorate by weight (Hecht et al., 2009). Ojha et al. (2015) presented findings in favor of hydrated perchlorate salts which can contribute to contemporary liquid-mediated processes.

Mars Science Laboratory and the Curiosity rover, 2011

In 2011, NASA launched the Mars Science Laboratory and landed the Curiosity rover in Gale Crater the following year. The overall scientific goal is to explore and determine past or present habitability of the Martian surface in local area. The rover is designed to be detect complex organic molecules in rocks and soils, but it is not a life detection mission as the Viking missions were (JPL, 2017c).

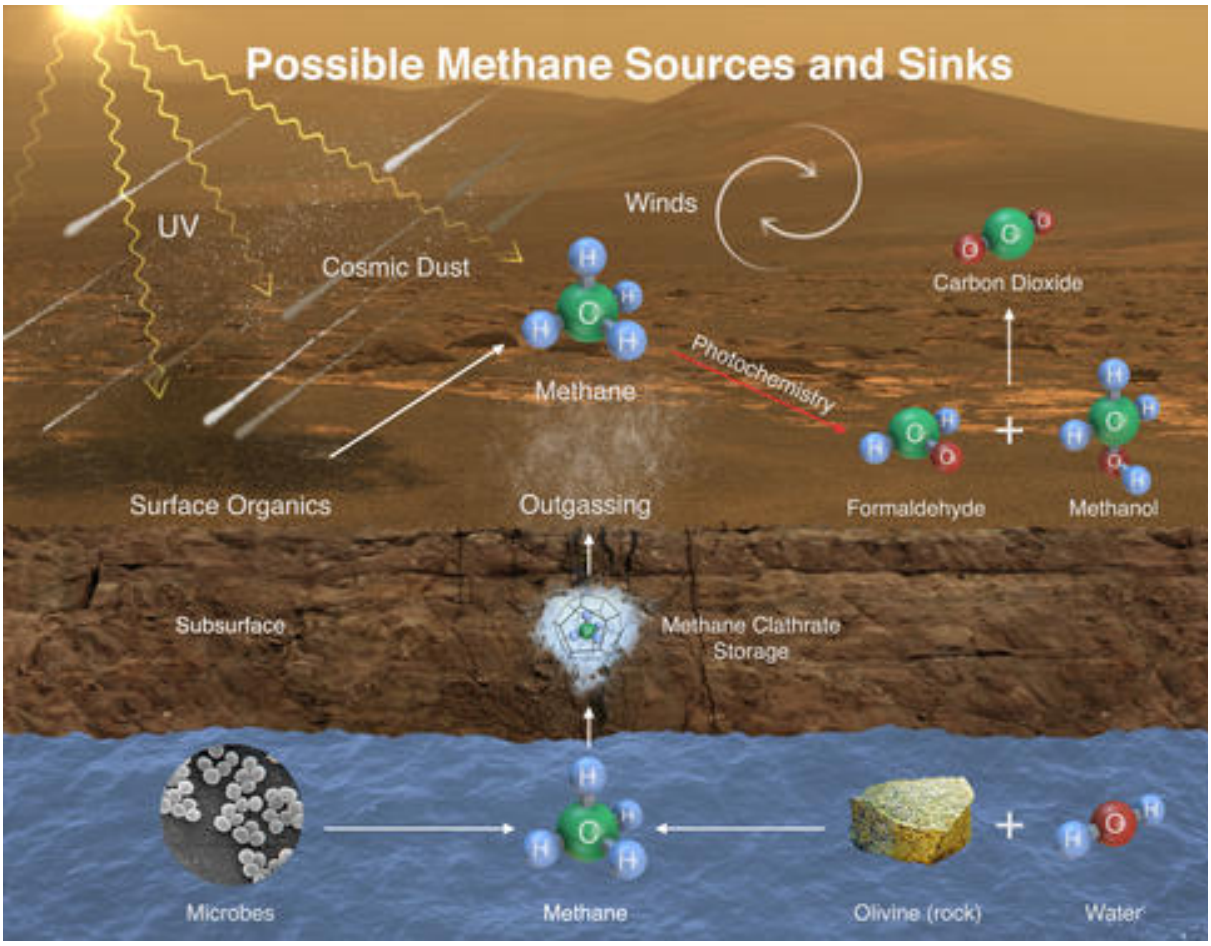

Figure 2.4. Possible methane sources and sinks.
Credit: http://mars.jpl.nasa.gov/msl/mission/science/results/

From a sample drilled in the Sheepbed mudstone in Yellowknife Bay, Curiosity demonstrated that the major elements (CHNOPS) required by life were present on Ancient Mars. This drill sample also revealed the presence of clay minerals (Grotzinger, 2014). Furthermore, Curiosity was able to uncover the presence of organic molecules from powdered rock sample obtained from the mudstone. This observation does not indicate that life is or was present on Mars, but it does show that organic molecules exist,

that they have been preserved, and they can be further studied (Williams et al., 2013). Another important observation by Webster et al. (2015) reported an elevated concentration of methane in the atmosphere for a period of two months based on *in situ* measurements. This methane spike was ten times higher than their recorded background levels and it could be caused by biotic processes as well as abiotic processes as shown in figure 2.4.

2.3.3 The possibility of finding life on Mars

Hecht et al., (2009) analyzed data from the Phoenix lander mission and found evidence for the presence of perchlorates in the soils. This finding has later been supported by data from Mars Reconnaissance Orbiter and Curiosity. The presence of perchlorates in the soil have implications for life because when they are heated, they form extremely reactive compounds and will react with any kind of carbon material present (Kerr, 2013).

Liquid water has previously been identified as a prerequisite for life as we know (NRC, 2007b, Cockell et al., 2016). The water content in the upper meter of the Martian subsurface has been mapped by the Mars Odyssey Gamma-Ray Spectrometer (GRS) and Neutron Spectrometer (MONS) and demonstrates widespread distribution (figure 2.5). The abundance of water in the form of ice and snow have been estimated to correspond to a universal water depth of 35 m (Christensen, 2006).

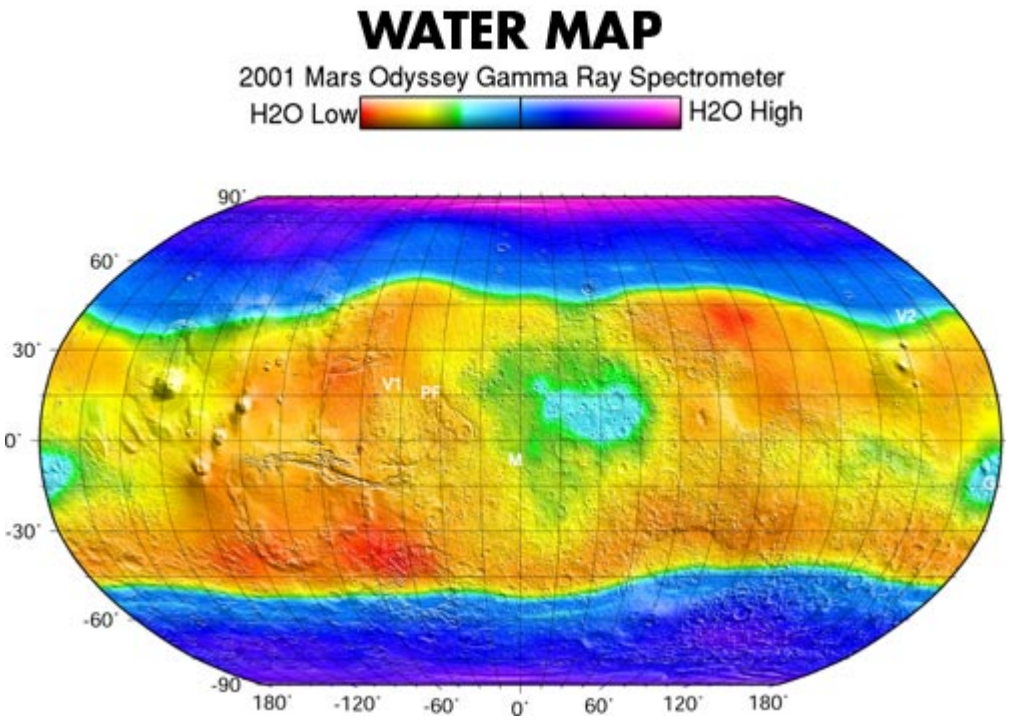

Figure 2.5. Hydrogen distribution in the upper meter of the Martian surface.
Credit: http://mars.nasa.gov/odyssey/mission/instruments/grs/

The six major elements, CHNOPS, required by all forms of life on Earth (Wackett et al., 2004) have been found on Mars (Grotzinger, 2014). 95% of the atmosphere consist of CO_2 and due to atmospheric exchange carbon is considered to have been and still be present in the near subsurface of Mars (Cockell, 2014). Additionally, the in fall of carbonaceous chondrites have contributed to the presence of organic molecules ((NRC, 2007a)). If the hydrogen distribution in figure 2.5 represents H_2O, then there is an abundance of water molecules that can be split radiolytically to yield hydrogen atoms (Lin et al., 2005). Serpentization reactions are another possible source of hydrogen on Mars (Ehlmann et al., 2010). Although, nitrogen is present in the Martian atmosphere, it is unknown whether this nitrogen can be fixed by deep subsurface life (Cockell, 2014). Lightning, impact events, and volcanic activity are abiotic processes that can fix nitrogen and thus transform nitrogen to biologically useful forms (Capone et al., 2006), but the penetration depth of these nitrogen species are uncertain (Cockell, 2014). Therefore, nitrogen may be a limiting factor for a deep subsurface biosphere on past and present day Mars (Cockell, 2014). Oxygen is bound to many of the other elements considered, CHNPS, either as CO_2, H_2O, sulfates, and even perchlorates (Cockell, 2014). The Martian surface is mainly composed of basaltic rocks and a evidence suggest that phosphate is present in

the common basaltic secondary mineral, apatite (Cockell, 2014). Sulfuric compounds and especially various forms of sulfate are widespread on Mars, as demonstrated by their widespread presence in sedimentary deposits (Gaillard et al., 2013, Cockell, 2014). *In-situ* measurements and orbital observations have shown that the Martian geosystems are strongly influenced by the sulfur cycle and results suggest that sulfur has been important in mediating aqueous processes in the Martian history (Gaillard et al., 2013).

Cockell et al. (2016) presents an extensive list of potential redox couples that can be used by microbial life on Mars. Similarly to major and trace elements, the concentration of redox couples in the deep subsurface cannot be reliably quantified (Cockell, 2014). The harsh surface conditions on Mars make photosynthetic life at the surface impossible, implying that subsurface microbial life would be limited to using organic carbon from endogenous or meteoritic sources in their chemolithotrophic pathways or for anaerobic respiration (Cockell, 2014). In their discussion of a potential microbial iron cycle on Mars, Nixon et al. (2012) conclude that there is a range of plausible electron acceptors for iron reduction in the surface and near-surface, that ferrous iron from the large reservoirs of olivine can be used as an electron donor. However, suitable electron acceptors are missing for iron oxidation.

One of the main objectives for the exploration of Mars is to search for traces of life. Westall et al. (2015) hypothesize that Martian life would have been (and may still be) chemotrophic and anaerobic. The Martian microorganisms would likely be living in similar types of environments as chemotrophic organisms on Earth, and utilize the same sources of nutrients and energy as on Earth. Therefore, fossilized traces of chemotrophic organisms on Earth can be used as proxies to understand the potential distribution of these microorganisms on Mars and their fossilized traces (Westall et al., 2015).

Mars appears to still be volcanically active and hydrothermal activity is likely in volcanic active area. The main objective of NASA's InSight (Interior Exploration using Seismic Investigations, Geodesy and Heat Transport) mission is to study Mars' deep interior in order to gain a better understanding of how the rocky terrestrial planets formed (JPL, 2017a). The Insight mission will study the geothermal flux on Mars and localized regions different heat flow regimes and possibly thinner cryosphere could be detected. The

presence of hydrothermal processes could be a driver for allowing water to reach the surface and contribute to water circulation in the Martian subsurface (NRC, 2007a). Furthermore, hydrothermal vent systems can support life independent of photosynthesis (Martin et al., 2008) and these volcanically active areas would be prime targets to search for life on Mars.

2.4 Challenges for life at low temperature

Earth is a cold planet. Large parts of the biosphere are periodically or permanently frozen and deep water with temperatures below 5°C make up the majority of the ocean. The microorganisms that are able to successfully colonize these permanently cold environments are called psychrophiles and their habitats vary from the deep sea, the polar regions and to the upper atmosphere (Feller and Gerday, 2003, Feller, 2013).

Life at low temperature has successfully overcome two important challenges, low temperature itself and its effect on viscosity of aqueous environments (D'Amico et al., 2006). Most biochemical reactions are influenced by temperature; physiological processes are slowed down, protein-protein interactions are changed, membrane fluidity is reduced, and reduced temperature leads to increased water viscosity (Georlette et al., 2004, D'Amico et al., 2006). Under sub-zero temperatures, highly saline brines containing cells, salts, minerals, and gas impurities are formed and cellular mechanisms are required to equalize the effect of physical and osmotic stress to survive during freezing (D'Amico et al., 2006). pH in biological buffers has been observed to decrease with temperature, affecting protein solubility and amino acid charge, especially histidine residues (Georlette et al., 2004).

2.4.1 Biodiversity

Large parts of our planet is exposed to low temperatures, often below 0°C. In 1887, Forster reported that microorganisms isolated from fish were able to grow and reproduce at 0°C (Georlette et al., 2004). Since then, a multitude of microorganisms that have successfully colonized cold habitats have been identified. Through evolution, these microorganisms not only survive, they thrive under these conditions (Feller and Gerday, 2003; Georlette et al., 2004; Feller, 2013)

Active bacterial metabolism has been reported for temperatures as low as -25°C in frozen permafrost microcosms (Mykytczuk et al., 2013). Gilichinsky et al. (2005) isolated and characterized a variety of anaerobic and aerobic, non-sporeforming and spore-forming, halotolerant and halophilic, pscychrophilic and psychrotolerant bacteria, mycelial fungi and yeast from 100,000 – 120,000 years old Siberian sodium-chloride brines (cryopegs) (Steven et al., 2006). Bacterial growth and reproduction have also been reported in supercooled cloud droplets (Sattler et al., 2001). Additionally, microbial studies of rocks and soils from in the Antarctic Dry Valleys have revealed unique microbial communities (Friedmann, 1982, Cary et al., 2010, Feller, 2013).

At such low temperatures, the microbial activity is confined to small amounts of unfrozen water inside the permafrost soil or the ice, and to brine channels. Liquid water flow is maintained through high concentrations of salts, exopolymeric substances and/or particulate matter and temperature gradients (Junge et al., 2004, D'Amico et al., 2006).

2.4.2 Cold temperature effect on water

Liquid water is considered to be a requirement for life and, thus, the physicochemical properties of water are of great importance to life. At cold temperatures, the physical properties of water are altered, viscosity increases as the temperatures reaches zero and solutes' diffusion rate in water is reduced. These water properties reduce biochemical reaction rates and transport rates, affecting biochemical catalysis and nutrient import and waste export (Rodrigues and Tiedje, 2008). Increased viscosity leads to higher energy expenditure as the environment is more resistant to motility which may be an important survival strategy in sea-ice brine channels (Junge et al., 2003).

A critical factor for cellular survival at freezing temperatures is the rate of cooling; it should be neither too slow nor too rapid (Mazur, 1977). Too slow cooling leads to extracellular ice formation and cellular dehydration as the cell loses water to the environment in order to maintain the chemical potential at equilibrium. Too rapid cooling leads to intracellular ice formation as the cell is not able to lose water fast enough (Rodrigues and Tiedje, 2008). To increase cellular survival at subzero temperatures, the cells should ideally experience a cooling rate which allows them to increase their intracellular solute concentration to avoid intracellular ice formation and to adapt their

physiology to the new environmental conditions (Mazur, 1977, Rodrigues and Tiedje, 2008).

2.4.3 Reaction rates

Chemical reaction rates are dependent on temperatures including chemical reactions catalyzed by enzymes. The Arrhenius equation $k=A^{(-E_a/RT)}$ is commonly used to describe how chemical reaction rates are influenced by temperature. k is the rate constant, A is the pre-exponential factor (related to steric factors and molecular collision frequency), E_a is the activation energy, R is the gas constant (8.3145 $kJ.mol^{-1}$) and T is the absolute temperature in kelvin. Any decrease in temperature will lead to an exponential decrease in the reaction rate. Typically, a temperature decrease of 10°C lowers the reaction rate by a factor 2 to 3 in biological systems. Mesophilic enzymes experiencing a downshift from 37°C to 0°C, should have their reaction rates lowered 16-80 times accordingly. Enzyme reaction rates are constrained thermodynamically by temperature, thus limiting the biochemical and physiological potential of a cellular system (Feller and Gerday, 1997, Georlette et al., 2004, Feller, 2013).

2.4.4 Protein stability

Reddy and Metpally (2009) analyzed the protein sequences in mesophilic and psychrophilic bacteria and compared the differences in amino acid composition they were able to reveal fundamental differences in proportions of amino acids. Serine, aspartic acid threonine, and alanine are overrepresented in the coil regions of the secondary structure, while glutamic acid and leucine are underrepresented in the helical regions in psychrophilic bacteria. Additionally, amino acids with aliphatic, basic, aromatic, and hydrophilic side chains are less abundant in psychrophilic proteins.

Cold-shock and cold-acclimation proteins

The cold-shock response is the beginning of cold-adaptation and involves an alteration in gene expression as microorganisms are exposed to a sudden downshift in temperature (Chaikam and Karlson, 2010). This response includes synthesizing cold-shock proteins (Csp) and may also include uptake or synthesis of compatible solutes. The combination of altered protein expression and uptake or synthesis of compatible solutes will significantly increase cold and freeze tolerance. Csps help regulate the cellular protein

synthesis, especially at a transcriptional level and initiating translation, and act as chaperones to prevent mRNA folding (Georlette et al., 2004, Phadtare and Inouye, 2008).

Cold acclimation proteins are believed to be another common and important feature in cold-adapted microorganisms. Although, their function is poorly understood, they are thought to be essential for the maintenance of both growth and cell cycle at low temperatures (Hebraud and Potier, 1999).

Cryoprotectors and antifreeze molecules

Microorganisms that experience freezing conditions produce cryoprotectors and antifreeze molecules to improve their freeze resistance and freeze tolerance. These molecules prevent ice crystals from forming and growing, which could lead to a destruction of the cellular membrane and a disruption in the osmotic balance (Feller and Gerday, 2003, Georlette et al., 2004).

2.4.5 Cold-adapted enzymes

At low temperatures, enzymes can experience cold-denaturation which leads to a loss of enzyme activity (Feller and Gerday, 1997, 2003). Hydration of polar and non-polar groups of proteins is a thermodynamically favored process at low temperatures and this is believed to be the cause for loss of enzyme activity at low temperatures. Hydrophobic forces are crucial for protein folding and stability, and it is notably the weakening of these forces that causes proteins to unfold. Thus, it is essential that cells prevent their proteins from being cold denatured in order to survive in these environments (Georlette et al., 2004, Feller, 2013).

An enzyme's catalytic cycle consists of three main phases: substrate recognition and binding, conformational changes induced by the enzyme that lead to product formation, and product release. Each of these phases involves weak interactions sensitive to temperature changes (Georlette et al., 2004, Feller, 2013).

Cold-adaption of psychrophilic enzymes is based on three key features. The main physiological adaption to cold environments is an increased specific activity that offsets low temperature inhibition on reaction rates to provide enough metabolic activity for

growth. Next, the enzyme's maximal activity is found at low temperatures, thus being more prone to inactivation and unfolding at moderate temperatures. Further, the specific activity at low temperatures is still generally lower than their mesophilic counterparts, so their adaptation is not complete (Georlette et al., 2004, Feller 2013).

For an enzyme's function to be optimized at a specific temperature, it has to balance the need for structural rigidity and flexibility so that it can keep its conformation and be able to perform its catalytic function (Jaenicke, 1991, Jaenicke, 1996). Low stability is a common feature that the majority of psychrophilic enzymes share compared to their mesophilic homologues (Georlette et al., 2004). A key aspect for enzymes adaptation to cold temperature is an increased local flexibility at the active site, while other regions may vary in stability when the enzyme is not involved in catalysis (Feller, 2013). All side chains in the active site that are involved in catalytic activities have been found to be strictly conserved (Feller 2013).

2.4.6 Membrane fluidity

Microorganisms adaptability to low temperature conditions are primarily dependent on their ability to sense to temperature changes. The membrane is the cell's interface between the internal cellular environment and the external environment. Thus, the membrane could act as one of the primary sensor of cold temperatures (Rowbury, 2003).

When bacteria are exposed to low temperature their primary response appears to a rigidifcation of the membrane(Shivaji and Prakash, 2010). The membrane fluidity can be restored by altering the proportion between of saturated and unsaturated fatty acids, by altering the fatty acid chain length, by changing from *cis* to *trans* fatty acids, and by changing from *anteiso* to *iso* fatty acids (Shivaji and Prakash, 2010). A membrane's physical properties and functions are adversely affected by decreasing temperatures, and this can typically lead to a reduction in membrane fluidity and ultimately loss of function. The membrane's physical properties are governed by its lipidic composition, which varies with the thermal habitat of the microorganism (D'Amico et al., 2006). If the membrane transitions into a gel-crystalline state, then it is no longer possible for the cell to maintain its biochemical and physiological processes such as energy generation, cell division, and transport (Shivaji and Prakash, 2010).

Fatty acid composition in the membrane is a well known adaptation at cold temperatures used to increase membrane fluidity (Los and Murata, 2004, Nichols et al., 2004). This can be achieved by introducing unsaturations in the fatty acid chains making up the lipid membrane and the expression fatty acid desaturases encoded genes is part of the well-characterized cold stress response (Russell, 2008). Another way to increase membrane fluidity is to incorporate branched-chain fatty acids (Shivaji and Prakash, 2010). As a consequence of the increased steric hindrance between the phospholipids created by the molecular adaptations to the lipid membranes, the gel-phase transition temperature for the membrane has been lowered.

Role of accessory pigments

Carotenoids, not only providing UV-radiation protection, are believed to help stabilize membranes at cold temperatures and an increased pigment production has been observed in certain bacteria at low temperatures (Shivaji and Prakash, 2010). Unsaturated and branched fatty acids contribute to destabilize the membrane and Jagannadham et al. (2000) suggested that polar carotenoid molecules may counteract this effect. Furthermore, Dieser et al. (2010) found that bacterial isolates from Antarctic environments that possessed carotenoids were more resistant to freezing compared to strains without these pigments. These findings are all in support of carotenoids having a role to play for membrane stability at low temperatures, and they may also be relevant for freeze tolerance.

2.4.7 Transcription and translation

There are fundamental challenges to transcription and translation at low temperatures that necessitate cold-adapted solutions (De Maayer et al., 2014). Enzymes involved in the protein synthesis process are expected to show the same general characteristics as cold-adapted enzymes, notably high specific activity combined with low stability (D'Amico et al., 2006)

At low temperatures, hydrogen bonding becomes stronger and so do the interactions between DNA strands. This has implications for unwinding the DNA and RNA polymerase access to the DNA. Another important aspect is the enhanced formation of stable

secondary RNA structures is at low temperatures. These secondary structures are likely to interfere unfavorably with translation (Feller and Gerday, 2003). The suggestion that a high capacity for translation and post-translation processing may be vital for growth at low temperatures (De Maayer et al., 2014) can be seen in light of this.

2.4.8 Growth and metabolism

Price and Sowers (2004) investigated the temperature dependency of metabolic rates of microbial communities at cold temperatures and found no evidence for a minimum temperature for microbial metabolism. Additionally, their work showed that the reported data on metabolism in microbial communities can be grouped in three categories: growth metabolism, maintenance metabolism, and survival metabolism. The terms maintenance and survival metabolism were introduced by Morita (1988) and refers to the energy levels required to perform osmotic regulation, maintenance of intracellular pH, macromolecular turnover, etc. without growth, and the energy levels required to repair macromolecular damage (Price and Sowers, 2004).

Studies on and our further understanding of the microbial communities that survive in cold oligotrophic environments on Earth have implications for our search for past or present microbial life on Mars, Europa, and Enceladus.

2.5 Methodology

Numerous culture-dependent and culture-independent methods are available and can be used to study cave microorganisms. Culture-dependent methods require that the microorganism in question can be grown in the lab. These methods are valuable for characterizing microbial physiology, but are limited when it comes to analyzing microbial communities and determining which microorganisms are present. Recent technological advances have improved culture-independent methods, and now, molecular analysis of cave microbial communities provides valuable insights on presence of microorganisms and structure of the microbial communities (Barton, 2006).

Molecular analysis of microbial communities is based on 16S rRNA found in the small ribosomal subunits in archaea and bacteria. The 16S rRNA is used as it is highly conserved in both bacteria and archaea and it is readily isolated. Furthermore, it allows us to see the

evolutionary distance between microorganisms (Woese and Fox, 1977). Therefore, DNA must be isolated from the community as a whole and further subject to targeted PCR amplification in order to perform molecular analysis of microbial communities.

Since the methods employed here rely on extracted DNA from environmental samples, it is important to be aware of some of the challenges related to reproducing results. One such example is that bacteria are capable of containing numerous 16S sequences (Case et al., 2007) and PCR amplification can induce biases and artifacts (Acinas et al., 2005).

2.5.1 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis is commonly used method to evaluate microbial communities. This method allows DNA fragments of similar size to be separated and can provide an overview of the differences between communities (Muyzer et al., 1993). PCR amplification of isolated DNA is carried out using GC-clamped primers where s 40-45 bp GC rich sequence is located within the last five bases from the 3'-end of the primers. The bonding between G and C bases promotes the specific binding at the 3'-end of the primers (Rettedal et al., 2010).

The amplified PCR product is then separated on a polyacrylamide gel containing the DNA denaturing agents urea and formamide in increasing amounts. The denaturing of the amplified DNA sequences depends on the sequence and number of GC base pair bindings. Once the DNA is denatured, it can no longer migrate in the gel. Therefore, sequences obtained from different microorganisms will migrate differently. Although one band should represent one population theoretically, de Araujo and Schneider (2008) found that numerous bands can represent one population, while Vallaeys et al. (1997) reported that different populations can be represented by one band. These observations can be attributed to the fact that microorganisms can contain multiple copies of the 16S rRNA (Case et al., 2007).

2.5.2 Illumina MiSeq sequencing

Illumina's MiSeq is becoming one of the most utilized sequencing platforms worldwide because of its high throughput and low sequencing costs, and its applications in i.e. metagenomics and targeted gene sequencing (Schirmer et al., 2015, Shokralla et al., 2012).

The concept of Next Generation Sequencing is based on a DNA polymerase catalyzing the incorporation of fluorescently labeled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during sequential cycles of DNA synthesis (Illumina). For each cycle, once the nucleotides are incorporated they are identified by a fluorophore excitation. Adapters are added on to the DNA fragments on both ends which in turn are loaded into a flow cell where the fragments hybridize to the surface of the flow cell. Each of these fragments are then amplified into a clonal cluster. Sequencing reagents are added next, including the fluorescently labeled nucleotides, and the first base is incorporated. The flow cell is imaged and the emission wavelength from each cluster is recorded. The incorporated base is identified based on emission wavelength and intensity. The cycle is repeated "n" times and this creates a read of "n" bases that can be aligned to a reference sequence through bioinformatics (Illumina).

In addition to bias and artifacts resulting from PCR amplification of targeted 16S regions, errors are also induced by the Illumina sequencing technology (Schirmer et al., 2015). The MiSeq is capable of producing reads lengths of 2 x 300 bp, and although substitution errors are the most common error types for Illumina sequencing, the error profiles are poorly understood (Schirmer et al., 2016, Schirmer et al., 2015).

2.5.3 Environmental monitoring

Microbial communities should not be interpreted uncoupled from their natural environment (Donachie et al., 2007), therefore HOBO® Onset® data loggers were placed in one of the caves to measure and record light levels, relative humidity, and temperature for a one-year period. Using the HOBOware® software, the loggers were programmed to record measurements every hour to capture temporal variations. The loggers are placed to capture spatial variation between the overlying soil surface, the skylight region, an intermediate zone between the skylight region and the dark zone, and the dark zone.

2.6 Research objectives

The scope of this study is to understand the microbial community in lava tube skylights and compare it with the microbial community found in the overlying surface soil and in the cave deep zone. The study is limited to three lava tube caves located in Lave Beds National Monument and it seeks to elucidate on the implications of skylights on the microbial communities in lava tube caves, a part of vulcanospeleology yet to be investigated.

We hypothesize that:

- The microbial diversity in the overlying soil surface and the lava tube skylight is different.
- The microbial diversity in the skylight region is different from the cave deep zone.

The bacterial communities were analyzed by DGGE to get an overview of and to compare the microbial diversity found in each cave zone.

3 MATERIALS AND METHODS

3.1 Project overview

To investigate the microbiology of lava tube skylights, a study proposal was submitted to the National Park Service (NPS) on September 22, 2016. This proposal gave a detailed account of the proposed work and how it aims to contribute to an enhanced knowledge of lava tube cave microbiology.

3.1.1 Field work

A reconnaissance trip was made to Lava Beds National Monument (LABE), northern California, from October 9th to 11th, 2016, during which ten lava tube caves were explored to determine if they fulfilled the criteria of having only one skylight, with at least 1 m diameter, located sufficiently far from the entrance so that entrance effects are negligible, and restricted public access. Three lava tube caves were selected based on these criteria, and field work was carried out on October 20th and October 21st, 2016, with assistance from LABE personnel.

3.1.2 Lab work

All the samples from Lava Beds National Monument were brought to the Bebout lab at NASA Ames Research Center, California, for storage and DNA extraction. Extracted DNA and rock samples were shipped on dry ice from NASA Ames to the University of Stavanger to continue the molecular work and perform microbial community analyses.

3.2 Field studies ethics statement

All sampling was done under Permit LABE-2016-SCI-0010 issued to Kommedal by the National Park Service. Lava Beds National Monument is a federally-protected area under the National Park Service, Department of Interior. No protected species were sampled.

3.3 Sampling sites

Lava Beds National Monument is located in northern California (41° 42′ 50″ N, 121° 30′ 30″ W), close to the borders of Oregon and Nevada. The monument lies northeast of the Medicine Lake Volcano and covers 190 km^2 with an almost rectangular shape. Tule Lake's old shoreline serves as the northern boundary (Larson and Larson, 1990).

3.3.1 Lava Beds National Monument

The entire monument is covered with volcanic rock that erupted over 11 000 years ago (Larson and Larson, 1990). The lava flows are largely basaltic, but do contain smaller amounts of silica-rich basaltic andesite (Lavoie et al., 2017). Most of the lava from the last eruption was distributed by lava tubes. The scenery left behind consists mainly of terrace-like borders up to 9 m high that are separated by valley-like depressions in between. Collapsed trenches mark the path of lava flow in lava tube systems. The landscape is characterized by cinder cones, spatter cones, and hornitos. The tallest cinder cones can be over 200 m above the general surface. Several craters are also present, the deepest crater being approximately 115 m deep (Larson and Larson, 1990).

More than 700 caves are found in Lava Beds National Monument which is the largest number of caves found in North America (LABE). Most of the individual caves are lava tube caves that are parts of several extensive lava tube systems. The caves vary in length from a few meters to a few kilometers. They can be both horizontally and vertically complex, having several interconnected branches or different levels (Larson and Larson, 1990)

Lava Beds National Monument is a semi-arid desert situated at a high-elevation ranging from 1737 m at the south end to 1219 m at the north end. It receives an average yearly precipitation of 390 mm, with December being the wettest month. Summers tend to be hot and dry, while it receives a monthly average of snow varying from 22.1 cm to 17.8 cm during the winter months. The temperatures range from an average low in January of -5.6°C to an average high of 28°C in July and August (LABE).

3.3.2 Description of study area

Caves L230, B220, and M760 were selected for sampling based on the criteria listed below. These three caves were formed in the Mammoth Crater lava flow between 30,000 and 40,000 years ago. Microbial sampling was done in three different zones of each lava cave. These zones were the soil surface overlying the cave in proximity to the skylight, the skylight region inside the lava tube cave, and in the deep zone of the lava tube cave.

- The lava tube must have exactly one skylight.
- The skylight must be located so that entrance effects are negligible.
- The skylight must have a diameter of at least 1 m.
- The cave cannot be open to the public.

3.3.3 Sampling microbial communities

The lava tube cave sampling took place on October 20th and 21st, 2016 in collaboration with LABE personnel. Each sample collection site was photo-documented.

The overlying soil surface was aseptically sampled using sterile 15 mL Falcon tubes. The surface layer of rocks and pebbles had been removed prior to scooping the soil into the Falcon tube. All samples from the overlying surface were taken within 1.5 m of the skylights. Triplicate samples were taken north, west, and east of each skylight. In the skylight region, soil, rock walls, and moss areas were sampled using sterile 50 mL Falcon tubes. A variety of samples were targeted in an effort to get as much diversity as possible. Skylight and deep zone soil samples were gathered the same way as the overlying soil surface. For moss samples, a flame sterilized Leatherman knife was used to cut the moss open and sample the soil underneath. Rock samples from the skylight and deep zone were collected using a flame sterilized Leatherman knife.

All microbiological sampling was done aseptically with a flame sterilized Leatherman tool. The samples were gathered in sterile 15 mL and 50 mL Falcon tubes and placed on dry ice immediately after returning from the caves. The samples were then kept on dry ice until they arrived in the Bebout lab at NASA ARC where they were stored at -80°C.

3.3.4 Monitoring environmental parameters

Onset® HOBO® environmental data loggers were placed in cave M760 to monitor temperature, relative humidity, and light levels in the deep zone, skylight region and overlying surface. Cave M760 is the largest cave and has the largest skylight, and was therefore selected for environmental monitoring because it represents the best Mars analog of the three caves.

The Onset® HOBO® data loggers were placed in cave M760 on October 21st, 2016, and were programmed to start recording on October 24th, 2016. Four HOBO® U23 (OnSet®) loggers, measuring temperature and relative humidity, and four HOBO® Pendant®, measuring temperature and light levels, were placed in a non-intrusive way, using naturally occurring hiding spots. Care was taken to place loggers in immediate proximity to the microbiological sampling sites.

Using the HOBOware® software, the loggers were programmed to record measurements every hour to capture temporal variations. The loggers are placed to capture spatial variation between the overlying soil surface, the skylight region, an intermediate zone between the skylight region and the dark zone, and the dark zone.

3.4 Lava tube cave microbial community analysis

DNA was isolated from the microbial communities and specific parts of the 16S rRNA region were amplified by PCR to analyze community profiles for each cave zone in each lava tube cave using DGGE. Two different PCR cycles were performed; one cycle to verify PCR amplification according to JGI Standard Operating Procedure (SOP) for 16S iTag sequencing, and one cycle to obtain PCR products to analyze the communities using DGGE.

3.4.1 DNA extraction

For the soil samples, DNA was extracted from approximately 0.3 g of soil using MoBio's PowerSoil® DNA Isolation Kit. DNA was extracted according to the manufacturer's instructions using 30 s of bead beating (Mini-BeadBeater-16, MIDSCI) after adding solution C1. DNA extraction from soil samples was performed in the Bebout lab, NASA ARC.

The collected rock samples were grinded into fine grain particles using a flame sterilized porcelain mortar. Between 0.5 – 0.8 g of grinded rock was then placed in the PowerBead® tubes and stored at -80°C overnight. Once thawed, DNA was extracted according to the manufacturer's instructions using 60 s bead beating at 4.5 $m.s^{-1}$ (FastPrep®-24, MP Biomedicals) after adding solution C1. Extracting DNA from the fine-grained rock samples was unsuccessful the first time, and a second, successful, extraction was performed at UiS. For DNA extraction at UiS, all spin-down steps of 30 s spins were changed to 60 s, and only 50 µL of solution C6 was used to achieve a higher concentration of DNA.

Gel electrophoresis was used to verify presence of DNA. 10 µL of extracted DNA was mixed with 2 µL 6x DNA Loading Buffer (Novagen) and loaded onto a 1% agarose gel stained with GelGreen (VWR). Extracted DNA was stored at -20°C until further processing.

3.4.2 Denaturing Gradient Gel Electrophoresis

For DGGE analysis the primers target the V3-V4 hypervariable region of the 16S rRNA gene. The extracted DNA was amplified using the universal forward primer 341F (5'-CCTACGGGAGGCAGCAG-3') with a GC-clamp attached to its 5' end (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGG-3'), and reverse primer 907R (5'-CCCCGTCAATTCCTTTGAGTT-3') (Brakstad and Bonaunet, 2006).

Table 3.1 PCR reaction components for DGGE analysis.

Component	Concentration	Reaction volume
Molecular grade H_2O, nuclease free	-	To 50.0 µL
Molecular grade Bovine Serum Albumin (BSA)	10 $mg.mL^{-1}$	2.0
5 Prime HotMasterMix	-	20.0
Forward primer	10 µM	1.0
Reverse primer	10 µM	1.0
Template DNA	-	1.0

The target sequences were amplified on 2720 Thermal Cycler (Applied Biosystems) and the thermal cycler was preheated to >90°C before placing the PCR tubes. The PCR reaction started with a denaturation step at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 50°C for 40 s, and 72°C for 60 s, and ended with an extended elongation step at 72°C for 7 min.

Amplified PCR products for DGGE analysis were run on 1% agarose gel stained with GelGreen (VWR), and loaded with 6X DNA loading dye (Novagen) to verify amplification with expected band size of approximately 550bp.

Denaturing Gradient Gel Electrophoresis was used to screen the microbial communities in the different zones in the lava tube caves. A denaturant gradient gel is a continuous gradient gel that separates DNA fragments based on their size and melting point by running them through a gradient of the denaturants urea and formamide. We chose a denaturing gradient gel ranging from 20 – 80% denaturant. A 100% denaturant solution corresponds to 7 M urea and 40% (v/v) formamide.

The solutions required to make a denaturing gel were prepared as described in table 3.3. 300 µL of a 10% ammonium persulfate solution, APS (Sigma), and 30 µL of tetramethylethylenediamine, TEMED (Sigma), was added to 30 mL of each solution immediately prior to casting the gel. The 6% polyacrylamide gel was poured on to the gel cassette in the IngenyPhorU2 system using a dispensing needle. The solutions were

connected to a peristaltic pump (Watson-Marlow Sci-Q 323), running at 8 rpm, to create an evenly dispersed gel.

Table 3.2 DGGE Solutions. All components were added to a 100-mL volumetric flask. MilliQ H_2O was added to obtain 100 mL. The solutions were filtered through a 0.2 µm filter (PALL) and stored in amber glass bottles at 4°C.

Component	20% DGGE solution	80% DGGE solution
40% Acrylamide Bis	15.0 mL	15.0 mL
50x TAE buffer	2.0 mL	2.0 mL
Formamide	8.0 mL	32.0 mL
Urea	8.4 g	33.6 g
milliQ H_2O	To 100.0 mL	To 100.0 mL

The amount of amplified DNA, loaded for each sample was determined visually from the gel electrophoresis of amplified PCR products. Volumes ranged from 10 µL to 17.5 µL of sample. Loading buffer was added to each sample in order to load 25 µL in each well. The loading buffer was contained 5 mL TAE buffer, 5 mL glycerol (100%) and 200 µL of 0.5% bromophenol blue with xylene. The polyacrylamide gel was run for 18 h in the IngenyPhorU2 system filled with 17 L 1X TAE buffer heated to 60°C at 75 V, and 45 mA. The gel was stained in 4L 1X TAE buffer to which 100 µL GelRed stain (VWR) for at least 1 hour. The gel was visualized using the BioRad Gel DOC XR Imagery System

3.4.3 16S iTag sequencing

For iTag sequencing, extracted DNA was amplified using JGI's SOP from 2015 to verify that PCR amplification will not fail in their pipeline. This SOP targets the hypervariable V4 region of the 16S rRNA using the universal forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and reverse primer 805R (5'-GGACTACHVGGGTWTCTAAT-3') (JGI, 2015).

Table 3.3 PCR reaction components for 16S iTag sequencing.

Component	Concentration	Reaction volume
Molecular grade H_2O, nuclease free	-	To 25.0 µL
Molecular grade Bovine Serum Albumin (BSA)	10 mg.mL-1	1.0
5 Prime HotMasterMix	-	10.0
Forward primer	10 µM	0.5
Reverse primer	10 µM	0.5
Template DNA	-	1.0

The target sequences were amplified on 2720 Thermal Cycler (Applied Biosystems) that was preheated to >90°C before placing the PCR tubes. The PCR reaction started with a denaturation step at 94°C for 3 min, followed by 30 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and ended with an extended elongation step at 72°C for 10 min.

Amplified PCR products for 16S iTag sequencing were run on a 1.5 % agarose gel stained with GelGreen (VWR) and loaded with 6X DNA loading dye (Novagen) to verify amplification with expected band size of approximately 250bp.

All samples have been verified to amplify according to JGI's SOP and prepared for 16S sequencing. However, due to unexpected delays these samples have not been shipped to JGI for sequencing, and therefore, 16S sequencing results have not been obtained during this thesis period and cannot be presented here.

4 RESULTS

Microbial communities from three different lava tube caves at Lava Beds National Monument were investigated using two different molecular techniques. Three different zones of each cave were sampled in triplicates and the microbial diversity was evaluated using a DGGE analysis. All samples are awaiting Illumina MiSeq sequencing at JGI to yield deeper information about the community structure for each cave.

Environmental parameters are currently being logged in LABE M760 cave. Data must be uploaded manually to a computer on site. Due to budget limitations, no data has been collected since logger placement on October 21st, 2016. Therefore, the only available results are from the DGGE analysis, and they are presented in this chapter along with field observations.

4.1 Field observations

Several caves were explored with LABE Park rangers prior to selecting which caves to investigate further. The selected three caves all had differences, but they all had one similarity a skylight that is located sufficiently far from the cave entrance to satisfy our criteria. Each lava tube cave sampled is characterized by having only one skylight that is located so that entrance effects are negligible. The skylights can be seen in figure 4.1.

All three caves varied in length, complexity and skylight size. Pictures A and B in figure 4.1 shows cave L230's skylight as seen from the surface and from inside the cave. The size of the skylight is approximately 0.75 m x 0.75 m. Moss growth is predominant in the skylight region where the sun shines, but green secondary mineral deposits are also observed on sunlight receiving rocks. Pictures C and D in figure 4.1 shows the biggest skylight, located in cave M760. Its size is about 5 m in the north-south direction and 3.5 m in the east-west direction. The skylight influence is clearly observed on the picture D (figure 4.1), where a green area, covered primarily in moss, is located directly under the skylight. The skylight region in cave B220 is seen in pictures E and F in figure 4.1. For cave B220, the skylight region inside the cave does not present the same moss covered

green area under the skylight as observed for cave L230 and M760. The skylight in cave B220 is also the smallest skylight of the three, measuring about 0.5 m x 0.5 m.

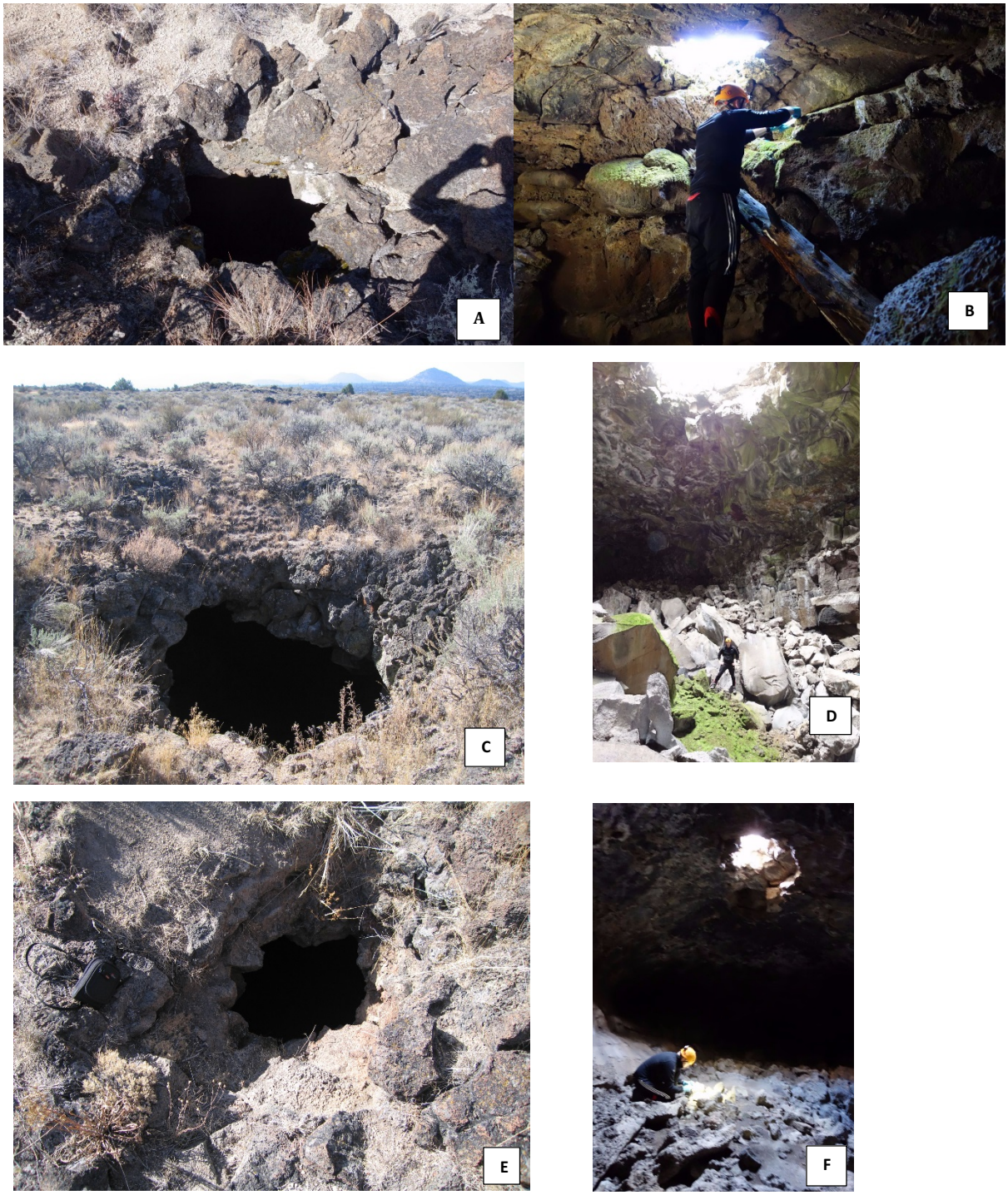

Figure 4.1. Skylights. Pictures A and B show cave L230, C and D show cave M760, and, E and F show cave B220. Each row of pictures shows each cave's skylight as seen from the surface and from inside the cave. From top to bottom, the skylight size varies from 0.75 m x 0.75 m, 5 m x 3.5 m, and 0.5 m x 0.5 m.

Figure 4.2. Sampling the various cave zones. A variety of samples were collected from three different lava tube caves and different environments were collected. Samples were taken from the overlying soil surface, the skylight region inside the cave, and the cave deep zone. A and C are secondary mineral deposits in the deep zone in cave M760. B is golden microbial mats on rough lava rock in the deep zone of cave B220. D shows golden and white microbial mats on smooth lava in the deep zone of cave L230. E shows moss and the green area in the skylight region of cave L230. F shows the overlying soil surface and vegetation for cave B220.

Figure 4.2 shows the types of environments sampled and the different types of samples gathered in each of the caves. White and golden microbial mats were sampled in the cave deep zones. Secondary mineral deposits were sampled in both the skylight region and deep zone. Sampled moss soils were only present in the skylight region. Triplicate

samples were collected for sampling locations. The overlying soil surface was sampled north, east, and west of the skylight for all three caves. The skylight regions presented internal differences, but soil samples were obtained for each region.

For cave L230, the skylight region samples included soil, moss covered soil, and green secondary mineral deposits. The deep zone samples for cave L230 included golden microbial mats from the ceiling, secondary mineral deposits present on cave walls, and wet soil on the cave floor.

The skylight region in cave B220 did not present any moss growth, but the soil present directly under the skylight was sampled. Both sides of the wall in the skylight region were sampled for secondary mineral deposits. In the cave deep zone, wet soil samples were collected along with golden microbial mats and secondary mineral deposits found in the cave deep zone.

Samples obtained from skylight region in Cave M760 were limited to the soil directly under the skylight. The upper and lower extremities of the skylight soil were sampled along with moss covered soils. The skylight floor was at an inclination, where the highest point was located towards the cave entrance and the lowest point towards the cave deep zone. The whole cave sloped downwards from the entrance to the deep zone. The inclination was such that the skylight region could not be observed from the deep zone.

4.2 DGGE

The PCR products from each individual cave were run on separate DGGE gels. Before running the DGGE, all samples belonging to one cave was amplified using PCR to target the specific region of the 16S rRNA bacterial gene. PCR products was confirmed on a 1% agarose gel using gel electrophoresis. The band intensities varied for samples varied from cave to cave, and from cave area to cave area (figure 4.3). The gel was visually analyzed to determine the amount of PCR product needed for each sample to ensure proper DGGE analysis. Each cave samples' band intensity after amplification is shown in figure 4.3, picture A shows amplification of LABE L230 samples, picture B shows LABE B220 samples, and picture C shows LABE M760 samples.

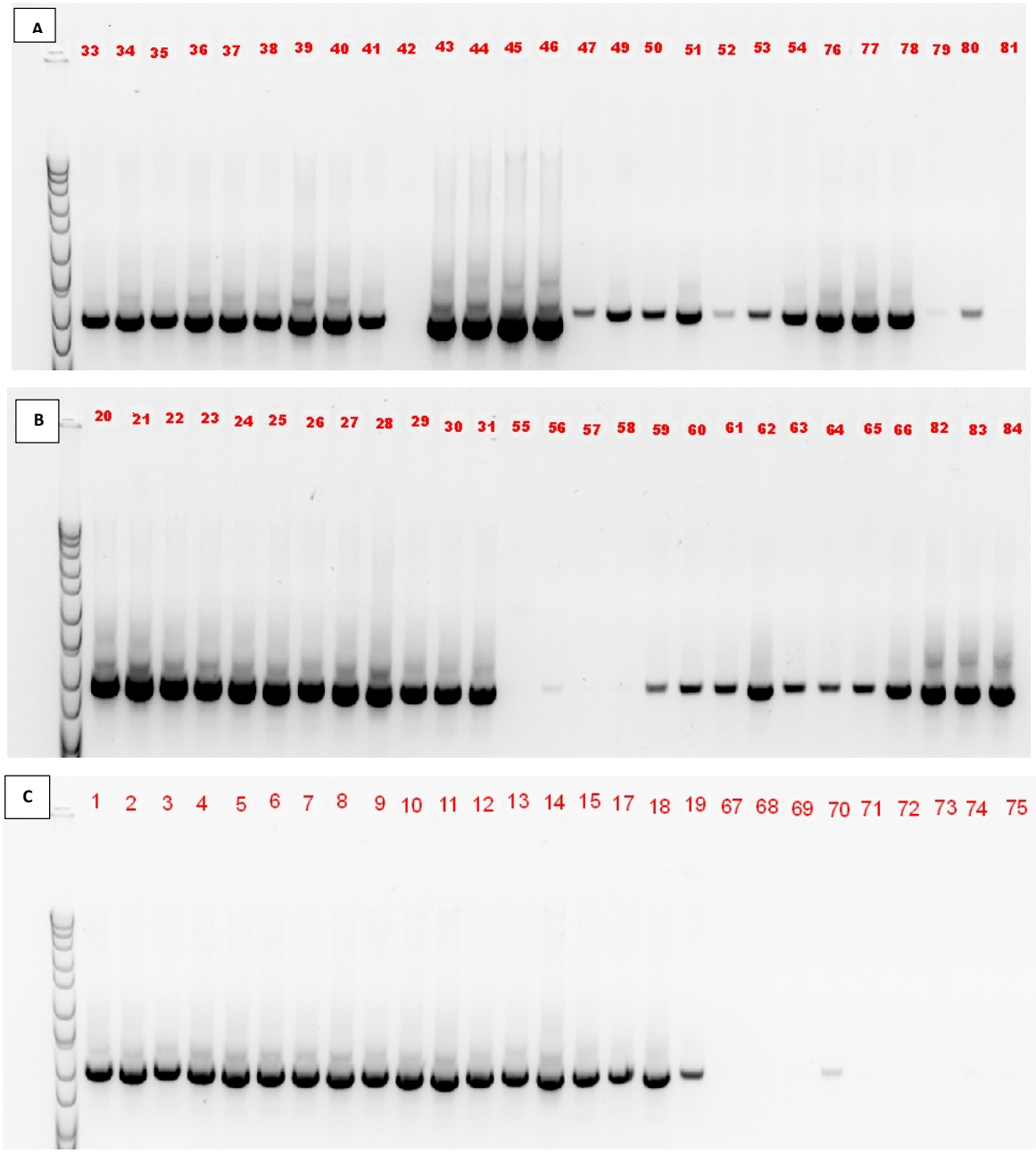

Figure 4.3. Confirmed presence of DGGE PCR products on agarose gel. A: LABE L230. B: LABE B220. C: LABE M760.

Based on visual analysis of agarose gel of amplified PCR products for DGGE analysis in figure 4.3, samples 42, 55, 57, 58, 67-75, and 81 were not used for the subsequent DGGE analysis. Although the presence of extracted DNA was confirmed by a 1% agarose gel for all samples, except sample 42, PCR amplification was unsuccessful. PCR amplification for these samples was performed multiple times without improvement, and they could therefore not be used for DGGE analysis.

The bands in each lane in the DGGE gels (figure 4.4, 4.5, 4.6) were counted to estimate the microbial diversity for each sample and compare the diversity between different cave zones. Only distinct bands were counted.

4.2.1 LABE L230 DGGE results

DGGE results from LABE L230 samples are shown in figure 4.4. From left, the first lane is a DNA marker. DNA samples 33-41 are amplified 16S rRNA PCR products from extracted DNA from the overlying soil surface, DNA samples 43-47 and 76-78 represent the amplified PCR products from the skylight region, and DNA samples 49-54 and 79-80 are from the cave deep zone.

The horizontal lines in the first nine gel lanes [33-41] represent the 16S rRNA bacterial diversity in the overlying soil surface (figure 4.4). The first three lanes [33-35], representing the northern side of the skylight, are somewhat distorted and only sample 35 is considered in the results. Of the DNA samples representing the 16S rRNA bacterial diversity in the skylight region [43-47 and 76-78], DNA sample 47 will not be taken into account because of the low band intensity. DNA samples 52 and 79-80 from the cave deep zone show too faint band intensities and will not be included in the analysis.

Overall, a higher bacterial diversity is observed in the samples from the overlying soil surface and the skylight region compared to the deep zone. Total DNA band numbers for the various cave regions ranged from 12-17, 5-11, and 3-6 for the overlying soil surface, skylight region, and deep zone respectively. Different bands indicate different microorganisms. In general, bands are shared across triplicates although their intensities vary.

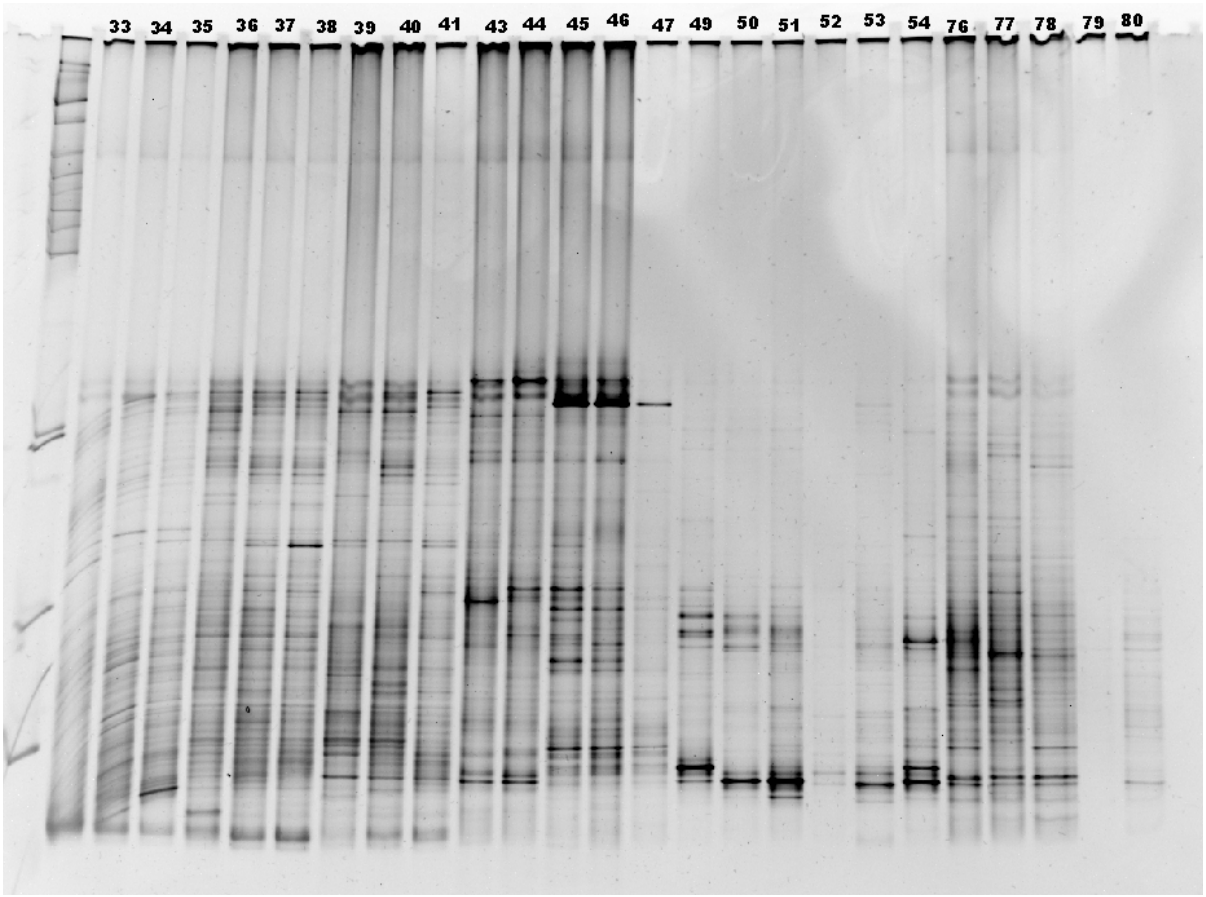

Figure 4.4. LABE L230 DGGE results. Primers targeting the 16S rRNA sequence in microbial DNA were used to amplify the extracted DNA. PCR products were subject to a DGGE gel, and the 16S rRNA for the microbial community is represented by the horizontal lines in the gel. The soil samples [33-44,76-78] show a higher diversity than the others and there is clearly less diversity in the deep zone samples [49-54, 79-80]. From left, the first lane is a DNA marker. DNA samples 33-41 are amplified 16S rRNA PCR products from DNA extracted from the overlying soil surface, DNA samples 43-47 and 76-78 represent the amplified PCR products from the skylight region, and DNA samples 49-54 and 79-80 are from the cave deep zone.

The deep zone samples show a similar gel pattern, where the most intense bands are found in the bottom half of the gel. Bands numbers indicate a higher number of microorganisms (5-6 bands) in the cave ceiling [49-51] than in the wall [53-54] (3-4 bands). These bands are found in two zones of the gel; at the middle and the bottom of the gel. Two bands are found at the bottom of the gel that are present in both ceiling [49-51] and wall [53-54] samples, although their intensities are different. The bands present at the middle of the gel differ between the ceiling [49-51] and the wall [53-54]. They do not overlap and a few more bands are present in DNA samples 49-51 than 53-54.

Three different types of samples have been analyzed from the skylight region, moss soil [43-44], soil [76-78], and secondary mineral deposits [45-47] to get as good a coverage as possible. The moss soil was found on ledges close to the skylight opening, where it receives sunlight throughout the day. The secondary mineral deposits were located on a large rock at the edge of the skylight region that receives sunlight. Only one side was covered by green secondary mineral deposits. The highest diversity was found in DNA samples from moss covered soils with 11 bands. DNA samples from the secondary mineral deposits held 9-10 bands, whereas 6-9 bands were found in the soil samples. The top two bands and the bottom two bands are present in both soil types [43-44, 76-78], but absent in the secondary mineral deposits samples. For DNA samples 45-46, the two most intense bands at the bottom of the gel are also found in two of the soil samples [76,78], but not in the samples from the moss covered soil [43-44]. DNA samples [45-46] present the most diverse community of the secondary mineral deposits samples.

The overlying soil surface samples show high diversity in all samples and there is no significant change in diversity across these samples. Although they show high diversity, they lack strong intensity bands as are identified in the other samples.

4.2.2 LABE B220 DGGE results

Figure 4.5 shows the obtained 16S rRNA bacterial diversity obtained after DGGE for LABE B220. The far right lane contains a DNA marker. Samples 20-28 are amplified PCR products from extracted DNA of the overlying soil surface samples gathered in proximity to the skylight. Samples 29-60 and 61-84 represent the diversity found in the skylight region and the deep zone, respectively.

The right side of the gel is slightly distorted, but this does not influence visual gel inspection or analysis (figure 4.5). For the skylight region, no horizontal bands are visible for sample 56 and will not be taken into account. PCR amplification did not yield sufficient levels of amplified DNA for DGGE analysis.

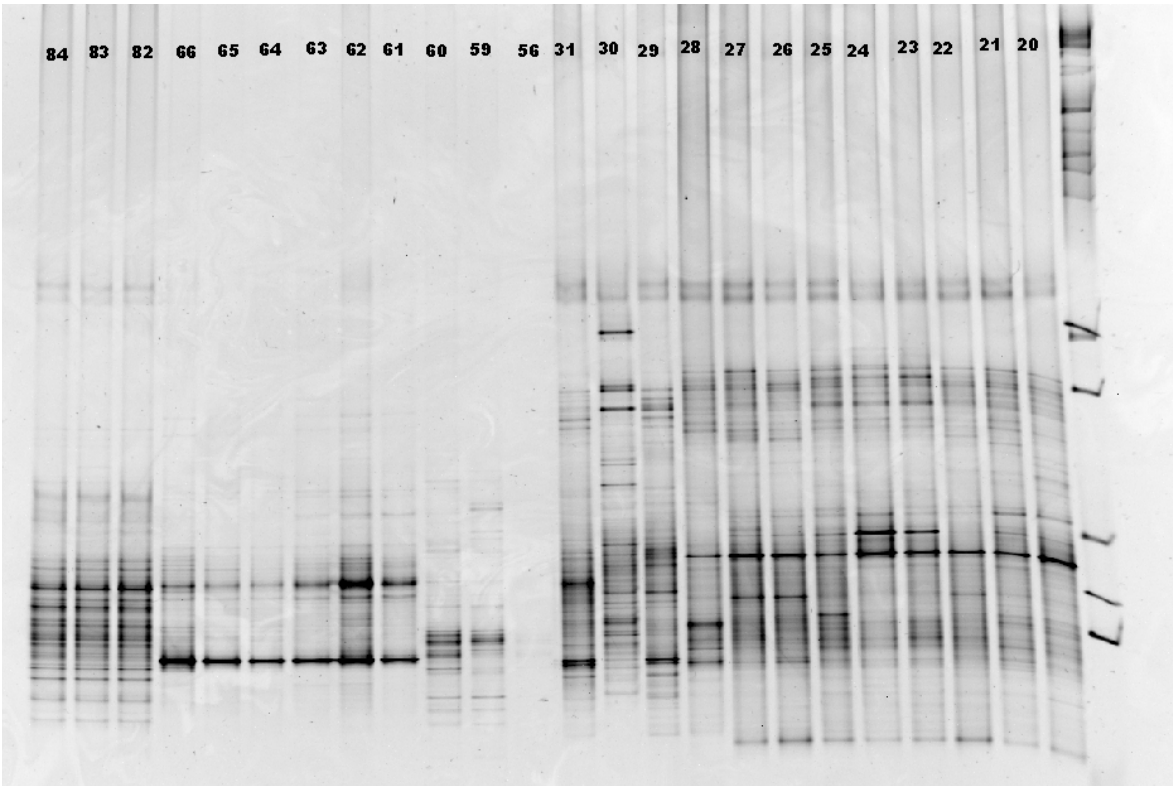

Figure 4.5. LABE B220 DGGE results. Primers targeting the 16S rRNA sequence in microbial DNA were used to amplify the extracted DNA. PCR products were subject to a DGGE gel, and the 16S rRNA for the microbial community is represented by the horizontal lines in the gel. The deep zone is clearly different from the other cave areas sampled. The gel shows a set of bands shared across all soil samples. A low diversity is obtained in the secondary mineral deposits from the deep zone and skylight region. The far right lane contains a DNA marker. Samples 20-28 are amplified PCR products from extracted DNA of the overlying soil surface samples gathered in proximity to the skylight. DNA samples 29-60 and 61-84 represent the diversity found in the skylight region and the deep zone, respectively.

A high diversity is observed in the samples from the overlying soil surface [20-28], illustrated by the number of bands ranging from 8 to 12. The samples share a similar gel pattern, but some differences are present in the lower half of the gel. DNA samples 23-25 share a band close to the middle of the gel that is absent in the others and DNA samples 26-28 share a band midway down the lower half that is absent in the others.

The two areas sampled in the skylight show very different diversity profiles. For the wall samples [59-60], band numbers vary from four to five, while for the soil sampled in the skylight [29-31] the band numbers range from 12 to 14. Bands are absent in the top half of the gel for the wall samples [59-60], while between six and eight bands are present for the skylight soil samples [29-31]. One sample from the skylight soil [30] contains an

intense band not found in any other sample. At the bottom half of the gel, skylight soil samples [29, 31] share two intense bands not present elsewhere in the gel.

The diversity profiles in the deep zone were different between the soil samples [82-84] and the secondary mineral deposits samples [61-66]. The deep zone soil samples [82-84] show a significantly higher diversity (12 bands) than the secondary mineral deposits (two or three bands). Although their diversity profiles are vastly different, the deep zone samples do share one relatively intense band halfway down the bottom half of the gel. Ceiling [61-63] and wall [64-66] samples share a common gel pattern, but more faint bands can be identified in the ceiling samples.

All soil samples [20-31, 82-84] appear to share two bands in the upper part of the gel. Further down the upper part of the gel, between two and six bands are identified in samples from the surface soil [20-28] and skylight soil [29-31] that are absent in the deep zone soil samples [82-84]. In the bottom half of the gel, a horizontally adjacent band is present in samples from the overlying soil surface [20-28], which is not present in the other soil samples [29-31, 82-84] or the secondary mineral deposits samples [56-66]. The surface soil [20-28] also share a horizontally adjacent band at the bottom of the gel that is absent in the other samples [29-84]. The highest intensity band identified in the deep zone wall [64-66] and ceiling [63-61] samples is a distinct feature of the deep zone diversity profiles.

4.2.3 LABE M760 DGGE results

16S rRNA bacterial diversity profiles obtain from DGGE for LABE M760 are shown in figure 4.6. From right to left, the first lane contains a DNA marker. Next, samples 1-9 are amplified PCR products from extracted DNA of the overlying soil surface samples gathered in proximity to the skylight. Then samples 10-19 present the diversity found in the skylight region, while sample 70 gives an impression of what can be expected from the deep zone.

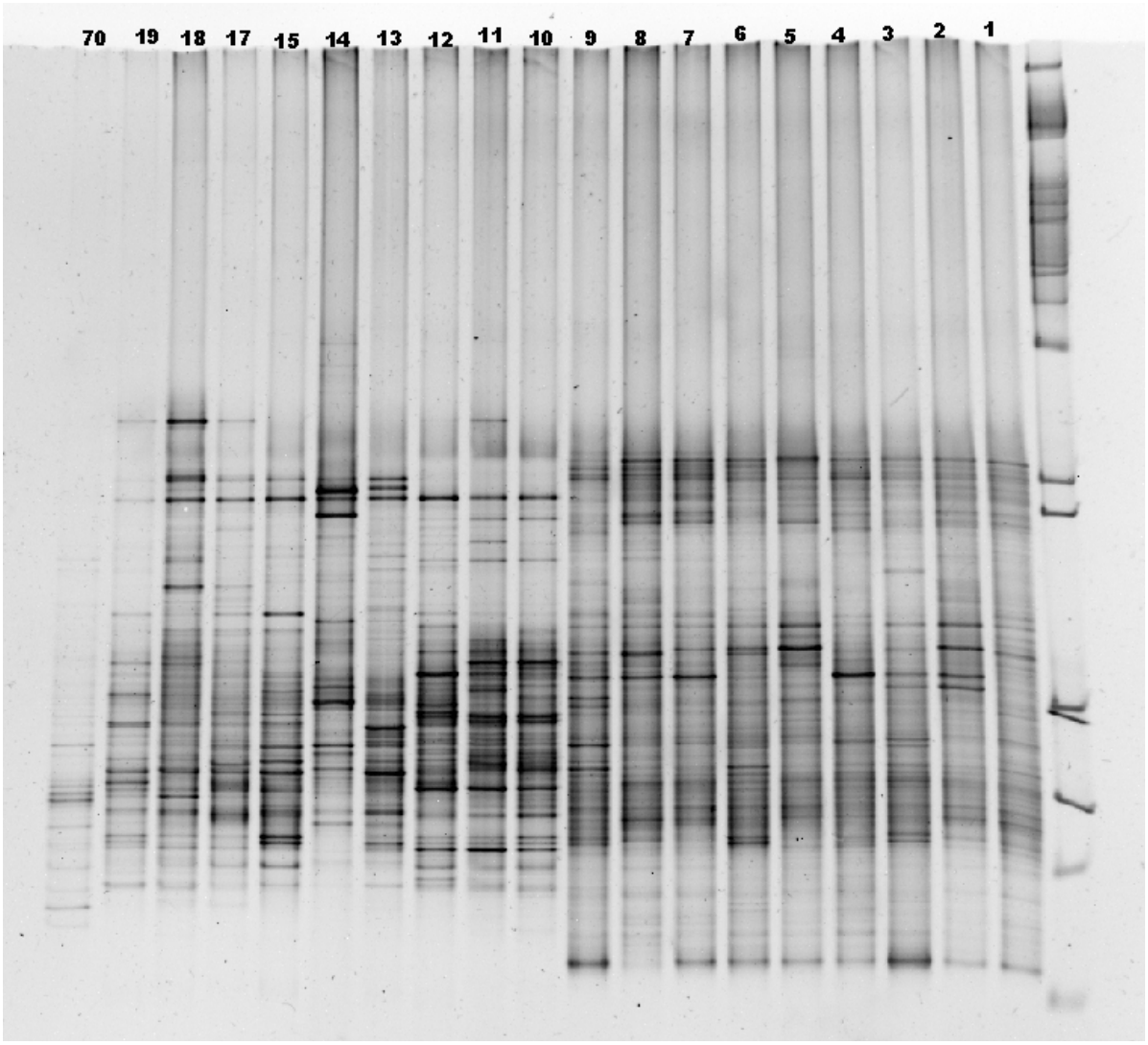

Figure 4.6. LABE M760 DGGE results. Primers targeting the 16S rRNA sequence in microbial DNA were used to amplify the extracted DNA. PCR products were subject to a DGGE gel, and the 16S rRNA for the microbial community is represented by the horizontal lines in the gel. DNA samples 1-9 are amplified PCR products from extracted DNA of the overlying soil surface samples. DNA samples 10-19 present the diversity found in the skylight region, while DNA sample 70 gives an impression of what can be expected from the deep zone. The gel displays high diversity in all soil samples [1-19], with some variations between the skylight region and overlying soil surface. One high intensity band, at the top of the gel, is shared for the skylight samples [10-19], but is absent in the overlying soil surface [1-9]. The opposite is observed at the bottom of the gel, where a band is present in samples from the overlying soil surface [1-9] and not in samples from the skylight region [10-19].

The gel is slightly distorted on the right side, but not enough to impair visual gel analysis (figure 4.6). Only one deep zone sample [70] had enough DNA quantity after PCR amplification to be subject to DGGE analysis. However, there was not enough sample material to obtain strong band intensities. Although only one sample from the deep zone is included in the figure and the bands are faint, they do indicate that there is less diversity in the deep zone compared to the skylight region and the overlying soil surface. The results presented for cave M760 are therefore limited to comparing the skylight region [10-19] and the overlying soil surface [1-9].

The soil in the skylight region in this cave was extensively sampled. The large skylight allowed the sunlight to influence a larger area in the skylight then compared to the previous two caves. Since the skylight area was at an angle with its highest point towards the cave entrance and the lowest towards the deep zone, both these sides were sampled in addition to moss covered soil. The differences in these samples are observed in DNA samples 10-19. In the upper parts of the gel, a high intensity band is shared across all the skylight samples [10-19]. Just above these adjacent lines, another horizontally adjacent band can be identified in the skylight soil samples [13-19], but not in the moss covered soil [10-12]. Furthermore, the number of bands indicate that the lowest diversity is found in the upper part of the skylight soil (10-16 bands identified), while the moss covered soil holds the highest diversity (17-19 bands identified). The number of bands found in samples from the lower parts of the skylight is intermediately located with between 15 and 17 bands identified.

High diversity is observed in the overlying soil surface samples [1-9] with band numbers ranging from 11 to 20. There are some internal variations with respect to band intensity, but the overall gel pattern is shared among all samples. A band at the bottom end of the gel is shared across the overlying surface soil samples [1-9], but is absent in the other cave zones [10-70].

4.3 Illumina MiSeq 16S rDNA sequencing

Prior to shipping samples for sequencing at JGI's facilities in Walnut Creek, the extracted DNA was subject to PCR amplification targeting the V4 region in the 16S rRNA bacterial

gene. This is a prerequisite to ensure that the samples will not fail in JGI's pipeline. Amplification was verified by gel electrophoresis on a 1.5% agarose gel (figure 4.7).

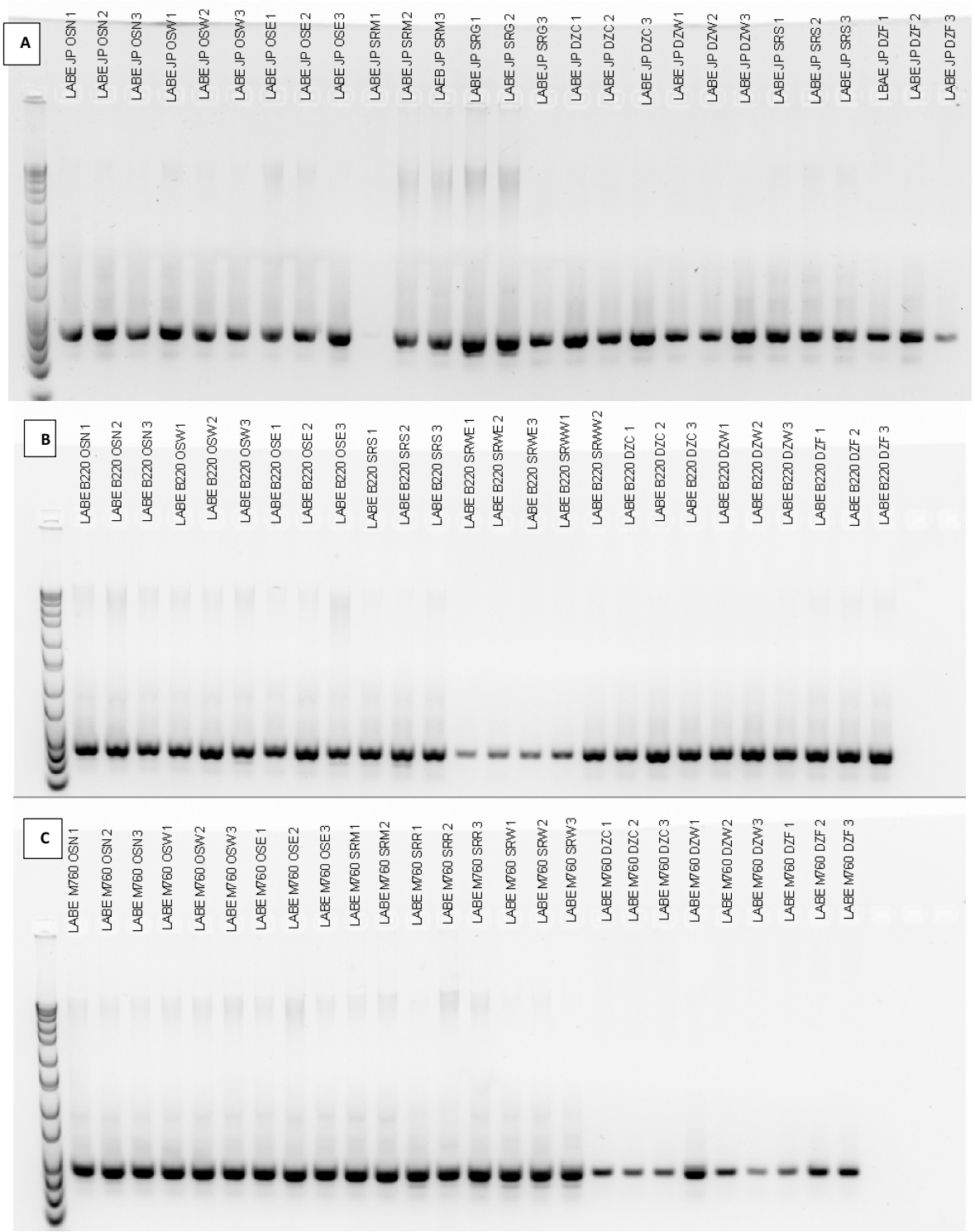

Figure 4.7. Verified amplification of 16S rRNA V4 region PCR products on agarose gel. A: LABE L230 (JP). B: LABE B220. C: LABE M760.

Amplification results targeting the V4 region of the 16S rRNA showed that not all DNA samples amplified equally well with JGI's SOP. For LABE L230, all DNA samples except LABE L230 SRM 1 were amplified (figure 4.7A). A fainter band intensity is observed for LABE L230 DZF 3 compared to the others. In general, LABE B220 DNA samples amplified well, showing strong band intensities except for LABE B220 DNA samples LABE B220 SRWE 1, 2, 3 and LABE B220 SRWW 1 (figure 4.7B). For the last cave, LABE M760, DNA samples from the overlying soil surface and skylight region amplified well, showing high intensity bands, while lower quantities of amplified PCR product were obtained from the deep zone DNA samples (figure 4.7C).

Overall, the overlying soil surface presented high intensity bands for each cave, while the less intense bands were found samples from the skylight region and the deep zone. The bands representing soil samples presented a higher intensity compared to the samples from secondary mineral deposits.

16S rRNA sequencing using JGI's Illumina MiSeq sequencing platform was planned for this thesis, but could not be obtained during this thesis period. Unexpected issues and experimental challenges caused delays in advancing this part of the project, resulting in not enough time to obtain sequencing data. The steps required to get sequencing data is highlighted in chapter 7.

5 DISCUSSION

Cave environments are characterized by stable physicochemical conditions, but there can be highly localized, small-scale variations including water and nutrient availability, and airflow induced temperature changes (Boston, 2016). This can yield fine scale differences resulting in microhabitats and with different microbial communities (Northup and Lavoie, 2001, Barton and Northup, 2007). It is important to keep these fine scale variations in mind when analyzing microbial cave communities, because the microbial community(ies) found in one or two samples do not necessarily represent the whole microbiome of the cave.

The lava tube caves selected in this study were chosen based on four criteria listed in section 3.3.2. One of these stated that the skylight diameter must be larger than 1 m. This is only valid for cave M760, not caves L230 and B220. However, these caves were chosen based on exploration and discussion with LABE personnel, and because they fulfilled the other criteria of having one skylight sufficiently far from the entrance so that entrance effects are negligible and public access to these caves is restricted.

5.1 Microbial diversity

DGGE analysis revealed differences in the microbial communities found in the various cave zones, overlying soil surface, skylight region and cave deep zone. For each gel (figure 4.4, 4.5, 4.6), the number of horizontal bands represents the total number of unique microorganisms present in a sample, and it displays the diversity across the different cave zones sampled (Muyzer et al., 1993, de Araujo and Schneider, 2008). De Araujo and Schneider (2008) constructed 27 artificial consortia of microorganisms to test whether DGGE is a reliable method to identify microorganisms present in a microbial community and their abundance. They found that DGGE is suitable to identify community members, but that band intensity does not reflect abundance. They also found that multiple bands could originate from a single organism. Triplicate samples were taken for each sampling location, and although band intensity varies, the bands are conserved across triplicates.

Samples from secondary mineral deposits presented lower band numbers compared to their soil counterparts. The number of bands for secondary mineral deposits ranged from two [64] to ten [46], with an average of 3.6 bands. The average number of bands for soil samples was 13.7, where the lowest number of bands was seven [22] and the highest was twenty [6]. DNA samples 45-46 represent the diversity found in the green secondary mineral deposits from the skylight region of cave L230. The maximum number of bands found in secondary mineral deposits from the cave deep zones was six [49]. The number of bands in samples from the overlying surface soil appear higher than the number of bands found in moss covered soils in the skylight regions of cave L230 (figure 4.4), but not for cave M760 (figure 4.6). For cave L230, the average number of bands in samples from the overlying surface is 15 [35-41], while for moss [43-44] and soil samples [76-78] from within the skylight the average number of bands are 11 and 7 respectively. Cave M760 shows the opposite. Here, the average number of bands in samples from the overlying soil [1-9] is 13.3 compared to 14.33 in the skylight soil [13-19] and 18 in moss covered skylight soil [10-12]. The highest number of bands was found in samples from the overlying soil, but distinct identification of bands from the overlying soil surface is in general made more difficult by a low relative intensity. The same observation seems to hold when comparing diversity between overlying surface soil samples [1-9] and soil samples from the skylight region [13-19] (figure 4.6). For cave B220, the soil [82-84] sampled in the deep zone display a high diversity with 12 being the average number of bands. This is significantly higher than the average number of bands, 2.5, displayed by the microbial mats [61-66]. In general, the overlying soil samples display the highest diversity, although their band intensities are lower relative to the other cave zones. This low intensity effect could be due to the number of bands present. A high diversity in samples from the overlying soil surface compared to samples from the deep zone is consistent with the findings of Lavoie et al. (2017), who described a higher species richness in samples from the overlying surface compared to samples from within caves at Lava Beds National Monument.

The cave deep zones in caves L230 and B220 are accessible through narrow crawl spaces and golden microbial mats (figure 4.2B, 4.2D) cover the ceiling and wall. A notable difference between the two cave ceilings was observed in these two pictures. The lava surface in cave L230 (figure 4.2D) is smooth with shark tooth lavacicles (a general term

for lava stalactites and stalagmites (Larson, 1991)), while the lava surface in cave B220 is rougher (figure 4.2B). Lavoie et al. (2017) found yellow mat color to be a negative predictor of diversity in caves at Lava Beds National Monument, while Hathaway et al. (2014) did not find microbial mat color to be a predictor of diversity in Azorean and Hawai'ian lava tube caves. DGGE profiles of golden microbial mats samples from the deep zone in caves L230 and B220 did not present the same diversity. For cave L230, between five and six bands were identified for the cave ceiling [49-51] compared with two to three bands identified for B220 [61-63], and a higher relative intensity is observed for B220 [61-63]. Metabolic processes and differences in metabolic processes or presence of other microorganisms have been suggested as potential causes for microbial mat coloration (Hathaway et al. 2014).

The deep zones in caves L230 (figure 4.2D) and B220 (figure 4.2B) were significantly different from the deep zone in cave M760 (figure 4.2C). In cave M760, the cave deep zone is characterized by white coatings on the walls with patches of brown and rusty color. These rusty-colored patches could be caused by enhanced local concentrations of iron where microbial cells act as nucleation sites (Ríos et al., 2011). The findings of de los Rios et al. (2011) suggest that bacterial activity is the principal determining factor for speleothem structure and formation. PCR amplification of DNA samples [67-75] from cave M760 deep zone was unsuccessful using the DGGE-primers (figure 4.3C), but PCR products were obtained using V4-primers (figure 4.7C).

The effect of the skylight on each cave is shown in figure 4.1. Sunlight can enter the cave through the skylight and provide energy for photosynthetic microorganisms, a small, green area directly under the skylight in caves L230 and M760 attest to this. The same green area was not observed in cave B220, which had the smallest skylight. Similar moss vegetation was not observed at the surface during the visits to Lava Beds National Monument in October 2016. Figure 4.6 reveal three distinct bands that are present in the moss samples [10-12], but absent in the overlying soil surface [1-9]. The two bands at lower end of the gel is proper to the moss samples, while the top band is shared with the skylight soil samples [13-19]. A similar phenomenon is observed for cave L230 (figure 4.4), where certain bands in the middle [43-47] and towards the bottom [76-78] are

unique for the skylight region. At the bottom of the gel for cave B220 (figure 4.5), DNA samples from the skylight soil [29, 31] share two intense bands not present elsewhere.

The DGGE analyses indicate that there are similarities between microbial communities for each cave zone, but also marked differences. PCR amplification of cave deep zone samples from cave M760 did not yield enough PCR product for subsequent DGGE analysis, making it impossible to compare diversity across the three cave deep zones. Further studies are required to make a comparison of the cave deep zone communities, to determine if certain microorganisms are found uniquely in the skylight soils, and to evaluate any overlap between the soil surface, skylight region, and deep zone. 16S Illumina MiSeq sequencing that could provide answers to these questions was planned, but could not be achieved during the time of this study.

Lava Beds National Monument is a semi-arid desert located at high-elevation, varying between 1291 m and 1737 m above sea level. Surface temperatures at Lava Beds National Monument ranges from an average low, -5.4°C, in January to an average high, 22.3°C, in July and August. Based on data from the National Oceanic and Atmospheric Administration (NOAA), the mean annual surface temperature, temperature values averaged over the last 20 years, for Lava Beds National Monument is approximately 8.9°C (NOAA). The temperature in the cave deep zones should therefore be close to 8.9°C (Northup and Lavoie, 2001, Barton, 2006, Barton and Northup, 2007), although the actual temperatures may vary (Northup and Lavoie, 2015). For cave B220, the deep zone temperature recorded by LABE personnel from July 2016 to mid-January 2017 shows an average temperature of 9.6°C, just above the 8.9°C estimate.

The deep zone in cave M760 was located deeper below the surface than the caves L230 and B220, and it was colder to work in cave M760's deep zone compared to L230 and B220. Due to lack of amplified 16S PCR product from the cave deep zone in cave M760 and temperature measurements for each cave deep zone, it is difficult to say whether this influenced the microbial communities. Regardless of the temperature differences between these caves, the microbial communities and microorganisms found in the deep zones are all adapted for growth at temperatures around 9°C and could potentially be psychrophilic (Moyer and Morita, 2001). Studies to determine whether these

microorganisms are psychrophilic will require examination of their optimal and maximal growth temperature, and their general physiology. If microorganisms, transported into the cave deep zone from the surface through the porous basaltic rock, adapt to their new oligotrophic environments by changing their maximal growth temperatures to 20°C or lower, then they would classify as psychrophilic. However, if they retain optimal and maximal growth temperatures above 15°C and 20°C, respectively, they would be classified as psychrotrophic (Moyer and Morita, 2007).

Lavoie et al. (2017) demonstrated that bacterial communities found in surface soils and cave microbial mats differ in composition and species richness, thus providing evidence against the belief that cave microorganisms are a subset of the microbial community found in the overlying surface. Although, the microbial communities in the surface soil and cave microbial mats differ, understanding the microbial diversity found in soil overlying caves is important as these microorganisms can potentially colonize the underlying caves, particularly for relatively shallow lava tube caves (Lavoie et al., 2017). The major cave phyla found in microbial mat samples from Lava Beds National Monument were *Actinobacteria (39%), Proteobacteria (30%),* and *Nitrospirae* (7%), while for the surface soils the major phyla were *Actinobacteria* (21%)*, Proteobacteria* (36%)*,* and *Bacteriodetes* (8%). Although there is a considerable overlap at phylum level, the overlap at OTU level was only 11.2%. The microbial diversity reported for the surface soils in Lava Beds National Monument is similar to other soils (Lavoie et al., 2017). A review of soil microbial diversity found in various environments concluded that *Proteobacteria* corresponds to 39% of all soil bacteria followed by *Acidobacteria, Actinobacteria, Verruomicrobiota, Bacteriodetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, Firmicutes,* and other/unknown (Janssen, 2006). The existence of a core set of cave microorganisms have been suggested and recent studies have identified more bacterial phyla compared to older studies, likely due to reduced sequencing costs and improved sequencing technology (Northup et al., 2011, Lavoie et al., 2017). These results indicate that some, if not all, of the major phyla identified by Lavoie et al. (2017) would also be found in the three caves examined in this study.

The surface temperature influences the temperature in the skylight region of a lava tube cave and helps circulate air in the cave. Lavoie et al. (2017) found temperature to be more

important than relative humidity in predicting species richness for lava tube caves. The skylight provides the cave with an opening to the surface through which sunlight, water, nutrients, etc. can enter (Northup et al., 2011). This provides microbial communities in the skylight with more potential sources of energy and potentially more nutrients with enhanced bioavailability than the microbial communities found in the deep zones. The high selection pressure exerted on microorganisms migrating through the soil to the cave deep zone may not be as high for microorganisms migrating to the skylight region from the surface. However, no studies regarding microbial communities in skylights have been published to date, therefore, further investigations are required to increase our knowledge about the microbiology of lava tube cave skylights.

5.2 Evaluating DGGE as a method for cave microbiology

Although, it is difficult to obtain an accurate representation of a microbial community's composition using DGGE as method, it has the advantage of being culture-independent. Using DGGE allows for detection of the most important community members and unculturable microorganisms in an environmental sample (Muyzer et al., 1993, de Araujo & Schneider, 2008). It provides a visual representation of the microbial community, making it a convenient and practical method for an initial comparison of diversity between environmental samples (Jackson et al., 2000, de Araujo and Schneider, 2008). Therefore, DGGE was chosen as primary tool to investigate the diversity of the microbial communities found in the overlying soil surface, skylight region, and deep zone.

Environmental samples contain a wide range of microorganisms whose DNA sequences may span a wide spectrum of melting temperatures. Although, a small gradient (<15-20%) may be considered to enhance band separation for DGGE analysis, Jackson et al. (2000) suggests using a large gradient because of the potential variations in DNA melting temperatures. Additionally, observations show that band clarity is reduced at smaller gradients, which can complicate the analysis of samples where several microorganisms are present (Jackson et al., 2000).

DGGE analysis provides a method to evaluate the microbial community diversity in environmental samples, but has its limitations. These limitations are important to be aware of so that interpretation does not lead to false conclusions on abundance and

diversity. The number of bands in a DGGE gel represents the microbial diversity and high intensity bands have been coupled to high abundance, but band intensity is only semi-quantitative for abundance (de Araujo and Schneider, 2008). de Araujo and Schneider (2008) found that DGGE is a suitable method to identify the numerically important community members, but is unable to determine abundance. Vallaeys et al. (1997) found that DGGE analysis is limited in its capability of estimating microbial diversity in complex environmental samples when universal primers are used to amplify small 16S rRNA fragments. They demonstrated that a DGGE gel may underestimate the total number of species present in an environmental sample and suggested that it should considered as a low limit for estimating the total diversity. Another common observation that leading to a lower apparent diversity, is when similar 16S rRNA sequences from different species co-migrate to form one band (de Araujo and Schneider, 2008). Schmalenberger et al. (2001) found that PCR amplification of the 16S rRNA region can produce multiple bands per organism. This results in a more complex band pattern that could be misinterpreted as a greater microbial diversity than is actually present. de Araujo and Schneider (2008) speculated in potential causes for observing multiple bands for one organism, including an organism's capability to contain multiple, slightly different gene copies in its genome, the presence of single-stranded DNA and amplicons with multiple denaturation domains.

PCR bias is one of the most common error sources for DGGE analysis of environmental samples. Sources of PCR bias include PCR inhibitors such as humic acids, differential amplification i.e. due to differences in primer-template hybridization, formation of PCR artifacts including the formation of chimeric molecules which are composed of parts of different sequences, and differences in the 16S rRNA sequence caused by the number of rRNA gene regions (*rrn* operons) (Wintzingerode et al., 1997).

Cell lysis is a critical step when applying molecular PCR-based methods to study microbial communities from environmental samples. A common bias affecting the observed microbial diversity is insufficient or preferential cell disruption, where not all cells are properly lysed and their DNA therefore, cannot contribute to diversity analysis (Wintzingerode et al., 1997). The secondary mineral deposits crushed into sand-sized grains in a flame-sterilized mortar prior to DNA extraction as described by Lee et al. (2015). When performing DNA extractions, bead beating was preferred over vortexing

which has been found to be more effective at releasing DNA from Gram positive cells (Lavoie et al., 2017).

5.3 Implications for astrobiology

Studies of Mars analogs on Earth contribute to an enhanced understanding of the potential of finding life on Mars. Mars analogs on Earth should in some ways resemble past or present Martian environments. First order analogs are analogous environments confirmed by direct empirical evidence (Marion et al., 2003). Lava tube caves are widespread on Earth and have been identified on Mars, making them first order analogs for Mars (Marion et al., 2003, Leveille and Datta, 2010, Northup et al., 2011). However, the lava tube caves in this study are not perfect analogs since the largest skylight in this study is 3,5 m x 5 m, while the skylights identified on Mars have diameters ranging from 100 m to 225 m (Cushing et al., 2007) and the surface conditions on Mars are significantly different. Still, an improved understanding of the microbiology of the skylight region in lava tube caves on Earth allow us to develop a better understanding for its potential in the search for life on Mars. Lava tube caves are important astrobiological targets and lava tube caves on Earth should be targeted to improve our understanding of their potential in the search for life on Mars (Leveille and Datta, 2010, Northup et al., 2011)

Careful selection of target sites in which to search for life on extraterrestrial bodies is important considering the costs and difficulties in getting to extraterrestrial bodies (Northup et al., 2011, Cockell, 2014). If Mars was ever habitable, past or present, and environmental conditions that were conducive to life existed, then biosignatures of this life are most likely to be found preserved in minerals (Banfield et al., 2001, Westall et al., 2015). The possibility of finding life in the extraterrestrial subsurface and lava tube caves has been discussed by several researchers e.x. Boston et al. (1992, 2001), Leveille and Datta (2010), Popa et al. (2012), and Cockell (2014), but little is known about the subsurface extent of lava tube caves on Mars (Cushing et al., 2007). Skylights are known geological features on Mars and Martian lava tube caves are inferred from skylight observations in volcanic regions. Although the results obtained for this thesis indicate that the skylight microbiome displays similarities and differences with the bacterial communities in the surface soil and deep zone, they are insufficient to conclude on the presence of a unique microbiome in the skylight and require further investigations.

The Martian subsurface is likely to have retained water longer than the surface (Westall et al., 2000). On Earth, water can percolate and transport organic matter through the numerous cracks and fissures present in lava tube caves (Northup et al., 2011). These cracks and fissures can also provide necessary space for microbial growth for cave microorganisms (McMahon et al., 2013). Organic matter can also fall in to caves through skylights, a common feature of lava tube caves, and air flow from the surface entering the skylights can transport and distribute fine soil particles and organic matter (Northup et al., 2011). The influx of organic matter enable heterotrophic growth as demonstrated by the presence of *Actinobacteria* in lava tube caves (Riquelme et al., 2015, Northup et al., 2011, Lavoie et al., 2017, Hathaway et al., 2014), but chemolithotrophic *Actinobacteria* have also been reported (Kanaparthi et al., 2013).

Studies of cave geomicrobiology show that cave microbial activity and interaction with minerals is found at redox interfaces (Northup and Lavoie, 2001; Barton and Northup, 2007). Basalt is a source of metabolically useful and available redox couples (McMahon et al., 2013). The generally high porosity of basaltic rocks provides a large internal surface area that can be readily colonized by microorganisms, and fluid migration through the rocks is permitted by high permeability. Jones and Bennett (2014) used laboratory biofilm reactors with both a pure culture of *Thiothrix* unzii and a mixed environmental sulfur-metabolizing community to study microbial colonization of various rock surfaces. They found that different microorganisms colonize individual mineral grains found in polymineralic rock such as basalt, and that each mineral was colonized by a unique microbial consortium expressing specific metabolic functions to take advantage of, or protect itself from, the unique aspects of that mineral. Studies on microbial colonization in partially water saturated porous media (i.e. basalt) will contribute to an enhanced comprehension of cave biofilm and microbial mat formation and development, and provide a better understanding of the potential microbial colonization of Martian lava tube caves.

The ability of microorganisms to cycle nitrogen is key to sustaining microbial communities in oligotrophic environments, where nitrogen is often a limited resource. The lack of a substantial nitrogen reservoir on Mars led Capone et al. (2006) to suggest a follow the nitrogen approach in the search for life beyond Earth. In their study of

microbial communities in Lava Beds National Monument, Lavoie et al. (2017) reported low levels of nitrite (0.31-0.98 mg/L) and nitrate (0.54-0.91 mg/L) in four of seven caves in Lava Beds National Monument. They noted the presence of nitrite oxidizers in *Nitrospirae*, nitrogen fixers in *Alphaproteobacteria*, and ammonia oxidizers in *Betaproteobacteria*, all of which are key players in the nitrogen cycle.

To explore lava tube caves and search for life on Mars, there needs to be specific criteria for selecting locations to sample and analyze. A trained eye can relatively easy identify microbial mats in caves such as those shown in figure 2 of Northup et al. (2011) and figure 4.2B and 4.2D in chapter 4. Formation of secondary mineral deposits have generally been explained by abiotic processes (Northup and Lavoie, 2001, Barton and Northup, 2007), but results from scanning electron microscopy and molecular characterization of cave secondary minerals reveal extensive microbial communities (Northup et al., 2011). The demonstration that mineral coatings contain a biological component (Boston et al., 2001, Northup et al., 2011) require further studies to determine if these communities are metabolically active. An improved understanding of the microbial communities found in secondary mineral deposits in lava tube caves on Earth will enhance our capabilities to detect biosignatures on other planets (Northup et al., 2011).

6 CONCLUSION

Microbial diversity in three lava tube caves in Lava Beds National Monument was investigated. Three different cave zones were sampled from each cave and their 16S rRNA gene was analyzed using denaturing gradient gel electrophoresis (DGGE).

Results from DGGE analysis indicate that there are similarities between microbial communities for each of the various cave zones, but also marked differences. Highest microbial diversity was observed in soil samples where the number of band ranged between 7 and 20, with an average number of 13.7 bands compared to secondary mineral deposits samples where the number of bands ranged from 2 to 10, with an average number of 3.6 bands.

The skylight region provides the lava tube cave with an opening towards the surface through which organic matter and sunlight can enter, whereas the cave deep zone is characterized as a cold and dark oligotrophic environment. The skylight's effect on the caves were directly visible in two of the three caves, where green vegetation and moss covered rocks were present. However, the results obtained in this thesis are insufficient to conclude on the presence of a unique skylight microbiome. The skylight microbiome displays similarities and differences with the bacterial communities in the surface soil and deep zone, but 16S rRNA sequencing is required to describe the various phyla present in each cave zone and evaluate any overlap in the bacterial communities.

Golden microbial mats cover the ceiling in the deep zone of caves L230 and B220, while the deep zone in cave M760 is characterized by white coatings on the walls with patches of brown and rusty cover. The bacterial diversity between the microbial mats and white coatings could not be compared because deep zone samples from cave M760 did not amplify using DGGE primers. A higher diversity was observed in the golden microbial mats found in cave L230 than in cave B220.

DGGE is suitable as a screening method in cave microbiology in order to investigate differences in community composition between samples, but 16S rRNA sequencing is required to characterize microbial community composition.

Future research is required to enhance our knowledge of microbial diversity in lava tube caves globally to test the hypothesis of a world-wide microbial biogeography. A better understanding of lava tube cave microbial communities and secondary mineral deposits is important to improve our ability to detect biosignatures in our search for life beyond Earth and evaluate the astrobiological potential of lava tube caves.

7 FUTURE RESEARCH

Several studies have been performed to characterize microbial communities found in microbial mats and secondary mineral deposits in lava tube caves. As space missions today become more astrobiology focused, it is important to improve our understanding of microbial habitats and life on Earth in order to choose areas with highest potential for finding extraterrestrial microbial life. Further investigations on the microbiology of lava tube caves are recommended.

For this thesis, denaturing gradient gel electrophoresis was used to examine the 16S rRNA microbial diversity in three different cave zones in three different caves. The obtained results indicate similarities in bacterial composition between all three cave zones, but further investigations using 16S rRNA sequencing are required to characterize the bacterial communities in each cave zone. 16S rRNA sequencing will be performed using the Illumina MiSeq platform at JGI, supported in kind by Dr. Tanja Woyke. The next steps in this project are first to receive a barcoded plate from JGI, complete the plate with sample material, ship the barcoded plate to JGI for sequencing, obtain and analyze 16S sequencing data, and, collect and analyze environmental logger data from cave M760. More studies on the microbiology of lava tube skylights should be conducted to confirm or refute the presence of a unique skylight microbiome as this cave focused on three lava tube caves in Lava Beds National Monument.

The presence and distribution of viruses in lava tube caves provides an interesting research topic that has yet to be investigated. Viruses affect geochemical cycles, and they are known to control microbial abundance and community composition (Aguirre de Carcer et al., 2015). However, our knowledge on viral biodiversity and distribution in nature in general is scarce, and absent for lava tube caves. The possibility of a cave virome is interesting in itself, but it could also provide clues to specific cave adaptation mechanisms in microorganisms.

More studies should be conducted on microbial functionality in lava tube cave environments. Nitrogen is often a limiting resource in microbial communities in caves and other extreme environments. More studies should focus on improving our understanding of the nitrogen cycle in extreme environments, particularly aiming at identifying microorganisms capable of fixing nitrogen, and oxidizing nitrite and ammonia. Long term studies of cave microbial communities is another aspect that should be further investigated to evaluate succession and potential changes in cave microbial communities over time.

The astrobiology potential of lava tube caves is widely recognized, but they remain relatively unstudied in comparison to other cave environments. This presents students and researchers with exciting research opportunities and possibilities to make an impact in the field. Therefore, it is only fitting to end this thesis with a quote by Carl Sagan: "Somewhere, something incredible is waiting to be known."

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APPENDIXES

APPENDIX 1: LABE L230 sample overview

APPENDIX 2: LABE B220 sample overview

APPENDIX 3: LABE M760 sample overview

APPENDIX 1: LABE L230 sample overview

Table A.1. Overview of LABE L230 samples.

Location	Cave	Cave Area	Sample nature	Sample name	Sample number
LABE	L230	Overlying surface	Soil	LABE L230 OSN 1, 2, 3	33, 34, 35
LABE	L230	Overlying surface	Soil	LABE L230 OSW 1, 2, 3	36, 37, 38
LABE	L230	Overlying surface	Soil	LABE L230 OSE 1, 2, 3	39, 40, 41
LABE	L230	Skylight	Moss soil	LABE L230 SRM 1, 2, 3	42, 43, 44
LABE	L230	Skylight	Secondary mineral deposits	LABE L230 SRG 1, 2, 3	45, 46, 47
LABE	L230	Skylight	Soil	LABE L230 SRS 1, 2, 3	76, 77, 78
LABE	L230	Deep zone	Secondary mineral deposits	LABE L230 DZC 1, 2, 3	49, 50, 51
LABE	L230	Deep zone	Secondary mineral deposits	LABE L230 DZW 1, 2, 3	52, 53, 54
LABE	L230	Deep zone	Soil	LABE L230 DZF 1, 2, 3	79, 80, 81

APPENDIX 2: LABE B220 sample overview

Table A.2. Overview of LABE B220 samples.

Location	Cave	Cave Area	Sample nature	Sample name	Sample number
LABE	B220	Overlying surface	Soil	LABE B220 OSN 1, 2, 3	20, 21, 22
LABE	B220	Overlying surface	Soil	LABE B220 OSW 1, 2, 3	23, 24, 25
LABE	B220	Overlying surface	Soil	LABE B220 OSE 1, 2, 3	26, 27, 28
LABE	B220	Skylight	Moss soil	LABE B220 SRS 1, 2, 3	29, 30, 31
LABE	B220	Skylight	Secondary mineral deposits	LABE B220 SRWE 1, 2, 3	55, 56, 57
LABE	B220	Skylight	Secondary mineral deposits	LABE B220 SRWW 1, 2, 3	58, 59, 60
LABE	B220	Deep zone	Secondary mineral deposits	LABE B220 DZC 1, 2, 3	61, 62, 63
LABE	B220	Deep zone	Secondary mineral deposits	LABE B220 DZW 1, 2, 3	64, 65, 66
LABE	B220	Deep zone	Soil	LABE B220 DZF 1, 2, 3	82, 83, 84

APPENDIX 3: LABE M760 sample overview

Table A.3. Overview of LABE M760 samples.

Location	Cave	Cave Area	Sample nature	Sample name	Sample number
LABE	M760	Overlying surface	Soil	LABE M760 OSN 1, 2, 3	1, 2, 3
LABE	M760	Overlying surface	Soil	LABE M760 OSW 1, 2, 3	4, 5, 6
LABE	M760	Overlying surface	Soil	LABE M760 OSE 1, 2, 3	7, 8 ,9
LABE	M760	Skylight	Moss soil	LABE M760 SRM 1, 2, 3	10, 11, 12
LABE	M760	Skylight	Soil	LABE M760 SRUP 1, 2, 3	13, 14, 15
LABE	M760	Skylight	Soil	LABE M760 SRDS 1, 2, 3	17, 18, 19
LABE	M760	Deep zone	Secondary mineral deposits	LABE M760 DZC 1, 2, 3	67, 68, 69
LABE	M760	Deep zone	Secondary mineral deposits	LABE M760 DZW 1, 2, 3	70, 71, 72
LABE	M760	Deep zone	Secondary mineral deposits	LABE M760 DZF 1, 2, 3	73, 74, 75