MICROBIAL ADAPTATION AND DIVERSITY IN THE HIGH-ELEVATION EXTREME CRYOSPHERE: IMPLICATIONS FOR ASTROBIOLOGY

by

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Microbial adaptation and diversity in the high-elevation extreme cryosphere: implications for astrobiology

Thesis directed by Professor Steven K. Schmidt

ABSTRACT

Life on Earth has been found in every extreme environment explored to date where liquid water is at least occasionally available. The discovery of extreme environments and organisms inhabiting them has provided valuable insights into the search for life beyond our own planet. Terrestrial analogues are environments on Earth exhibiting a subset of conditions similar to those found on other planetary surfaces such as Mars, the best candidate for harboring life in our solar system. A number of environments of the Earth's cryosphere are analogues of the three main climatic stages of Mars and may help in assessing the habitability of past and present Mars. Here I describe previously unexplored analogue environments and show how they support thriving microbial communities, as well as discuss potential strategies microbes use to survive and grow in these extreme systems. In chapter 2, I present the first study of microbial diversity in ice and soils from the top of Mt. Kilimanjaro, a terrestrial analogue of the middle age of Mars, and demonstrate that this site supports both cosmopolitan and endemic microbial communities. In chapter 3, I demonstrate that the dominant microeukaryote (*Naganishia friedmannii*) at high elevations (5100 to 6300 m.a.s.l.) on several Atacama volcanoes (analogues of the third epoch of Mars), is able to grow rapidly during extreme diurnal freeze-thaw cycles and is metabolically versatile. In chapter 4, I show that high-elevation transient ice structures known as “nieves penitentes” are oases that support thriving communities of snow algae and other microbes on Volcán Llullaillaco, one of the Atacama volcanoes and the second tallest volcano on Earth.

In chapter 5, I present work from microcosm and field experiments showing that extremophilic communities can respond to water and nutrient amendments in soils from Volcán Llullaillaco. In chapter 6, I describe the draft genome of *Naganishia friedmannii* isolated from tephra soils at an elevation of 6030 m.a.s.l. on Volcán Llullaillaco. I show it has a streamlined genome compared to closely related fungi and possesses genes known to play a role in coping with environmental stressors prevalent at extreme elevations.

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CHAPTER I

INTRODUCTION AND OVERVIEW

Introduction

The quest for life beyond Earth has sparked the imagination of humankind since the beginning of history. However, only in the past few decades have we come to a better understanding about the extremes of life on Earth and its potential to be found beyond our planet (Merino et al. 2019, Rothschild and Mancinelli 2001). What are the limits of life still remains one of the greatest unanswered questions in biology. In the last several decades, environments once thought to be devoid of life have revealed to be populated by varying levels of biodiversity, supporting the almost inescapable notion that life, at least on Earth, always finds a way (Solon et al. 2018).

Life on Earth is found everywhere where it is possible to find a favorable combination of physical and chemical parameters allowing metabolic reactions to occur. These parameters delimit specific ranges where life can exist. What were previously thought to be insurmountable chemical and physical barriers to life, we now consider as another niche for organisms that we call extremophiles (Rothschild and Mancinelli 2001). Life on Earth has been found anywhere where liquid water is at least occasionally available and where there is an energy source to power its metabolism. Life has been uncovered from a number of extreme environments that present physical and geochemical extremes. Physical extremes include extremely high or low temperatures, such as hydrothermal pools (Brock and Freeze 1969) or polar and high elevation environments (Siebert and Hirsh 1988, Lynch et al. 2012), and high pressure such as deep-sea vents (Lonsdale 1977). On the other hand, geochemical extremes comprise environments that expose life to desiccation, such as deserts (Azua-Bustos et al. 2012), high salinity (Oren 2008), or extremely high or low pH, like in soda (Jones at el. 1998) or acidic lakes (Gyure et al. 1990).

In addition, many extreme environments combine multiple environmental stressors and are home to what are known as polyextremophiles (Capece et al. 2013, Seckbach et al. 2013), such as microbes living on mountain summits where they are exposed to cold temperatures, high UV radiation, low nutrient and water availability. The environments inhabited by polyextremophiles, which thrive at multiple extremes, can provide excellent models for extraterrestrial habitats (Seckbach et al. 2013) and may give us insight on what life beyond Earth would be like. Such habitats are known as terrestrial analogues and have environmental, mineralogical, geomorphological, or geochemical conditions similar to those observed on extraterrestrial locations (Preston and Dartnell 2014), such as the Martian surface. These sites offer insight for astrobiological studies on the habitability potential of other worlds.

Mars is considered one of the best candidates for harboring extraterrestrial life in our solar system. It has undergone three main climatic stages throughout its history (Fairén et al. 2010). During the first epoch (> 3.95 - 3.7 billion years ago) it was potentially habitable because basic requisites for life as we know it were present, such as liquid water on its surface (Fairén et al. 2009) and energy sources in the form of hydrothermal systems (Schwenzer and Kring 2009). Some of Earth analogues that best represent this epoch are the Canadian Arctic, the Pilbara region in Australia and the Rio Tinto in Spain (Preston and Dartnell 2014). During its middle epoch (3.7 – 3.1 billion years ago) Mars became increasingly drier and colder, becoming the so-called "snowball Mars" (Fairén et al. 2010). Despite the increasing aridity and lowering of temperatures during this epoch, Mars still presented active volcanic areas. A number of places on Earth are analogues of this stage, the high elevation volcanic areas in Kamchatka, sub-glacial volcanoes in Iceland, or the top of Mt. Kilimanjaro (Fairén et al. 2010). Mars then transitioned into a third and present-day climatic stage (3.1 billion years ago to present) that

turned the planet to a hyper arid and extremely cold world that lost its surface habitability. During this stage it lost most of its atmosphere and free water, and its surface was bathed by a full spectrum of solar radiation. The best places on Earth that simulate these conditions are some of the harshest deserts known, such as the Mojave and Namib deserts, the Antarctic Dry Valleys and the barren expanses of high elevations sites in the Atacama region (Solon et al. 2018).

Today the Martian surface is very cold with an average temperature of -60°C (Haberle et al. 2001). However, surface temperatures can occasionally go above freezing near the equator. But even at the equator, Mars is subject to extreme thermal fluctuations and is bathed by a full spectrum of solar radiation, including the UV B and C radiation. Absence of liquid water is probably the most serious constraint for life anywhere on Mars today (Fairén et al. 2010). This was not always the case, as Mars Orbiters and Landers have obtained evidence that liquid water existed on the surface of Mars. Despite the presence of water far back in the past, it is likely that any existent - and past - microbial life on Mars would have to adapt to the conditions currently found on the surface or near subsurface, likely using strategies similar to organisms that inhabit extreme arid regions of the Earth cryosphere (Fairén et al. 2005). One of the main questions about Mars today is if there are any habitable niches on its the surface or subsurface.

There are no sites on Earth that present the entire suite of extremely harsh environmental conditions found on the Martian surface through time; however, in this dissertation I discuss how high-elevation, cold desert soils are possibly some of the best earthly analogues for conditions on Mars today. These deserts occur on several mountain ranges and near both poles between the upper limit of plant distribution and the lower boundary of permanently ice-covered regions (Rhodes et al. 2013). Among these types of ecosystems, some of harshest are found at high elevations on volcanoes in the Andes, especially in areas bordering the Atacama

Desert. Conditions faced by life there are in many ways even more extreme than those of the lower elevation Atacama Desert (Lynch et al. 2012). In this seemingly lifeless environment microbial life has to cope with a number of environmental stressors such as extreme UV flux, extreme freeze-thaw cycles, low pH values, low atmosphere pressure and extremely low nutrient and water content (Schmidt et al. 2018). High elevations in the Atacama are therefore a model ecosystem to profile microbial diversity and adaptation in soils at the hyperarid margin of the cryosphere.

Even in the most extreme environments on Earth, we can find oases for microbial life that can mitigate some of the stressors of the surrounding environment. These oases can provide a source of water, heat, or nutrients in an otherwise harsh landscape, which allows for the development of more complex microbial communities than those in the environment surrounding them. The notion that some niches within extreme environments could sustain "oasis-like" ecosystems is an attractive one to astrobiology as they represent new model systems for environments where it is more likely to find extant biota on other planets. Niches that alleviate at least one environmental limitation (e.g. fumaroles and ice penitentes) have been described in the last decades on Volcán Socompa and Volcán Llullaillaco (Costello et al. 2009, Solon et al. 2018, Vimercati et al. 2019b).

The characterization of microbial life in terrestrial analogues of Mars is of fundamental importance to address questions about the potential of life beyond Earth, according to the guidelines of the NASA Astrobiology Roadmap (DesMarais et al. 2008). Among the objectives of the NASA Astrobiology Roadmap are to determine the physiological state of microbial cells found in extremely dry and temperature fluctuating environments, to characterize the dynamics of organism survival in conditions currently present on the surface of Mars, and to explore molecular adaptations of polyextremophilic microorganisms (DesMarais et al. 2008).

Thesis Overview

To study how microbial life adapts to some of the harshest environments of the cryosphere, I explored the microbial communities of a number of terrestrial analogue environments and investigated strategies used to cope with the different environmental stressors. I used a combination of 16S and 18S rDNA gene surveys, pure-culture physiological experiments, and microcosm manipulations to examine the distribution and potential activity of microbes in extreme high-elevation environments. In addition, I examined the genome of one of the main microorganisms found in my study systems.

In Chapter II, I discuss how the top of Mt. Kilimanjaro (5893 m.a.s.l.) represents an analogue of the middle epoch of Mars and describe the microbial life that is found on the top of this mountain and how related it is to other extreme environments of the cryosphere (published as Vimercati et al. 2019a).

In Chapter III, I describe the dominant microeukaryote found at high elevation on multiple Atacama volcanoes (analogues of the third epoch of Mars), *Naganishia friedmannii* (ex. *Cryptococcus friedmannii*), its ability to grow under extreme freeze-thaw cycles, and its physiology and its metabolic potential (published as Vimercati et al. 2016).

In Chapter IV, I show that high-elevation ice pinnacles known as "nieves penitentes" are oases that mitigate environmental challenges in harsh landscapes and harbor the only known phototrophic communities on Volcán Llullaillaco (Vimercati et al. 2019b).

In Chapter V, I manipulated extremophilic communities of a high elevation Atacama volcano to investigate how they respond when some of the environmental limitations such as water and nutrients are alleviated (Manuscript in prep).

Lastly, in Chapter VI, I present the draft genome of *Naganishia friedmannii* and examine the potential strategies and adaptations that this polyextremophilic microeukaryote uses to survive in its hostile environment (Manuscript in prep).

CHAPTER II

THE DISAPPEARING PERIGLACIAL ECOSYSTEM ATOP MT. KILIMANJARO SUPPORTS BOTH COSMOPOLITAN AND ENDEMIC MICROBIAL COMMUNITIES

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Abstract

Microbial communities have not been studied using molecular approaches at high elevations on the African continent. Here we describe the diversity of microbial communities from ice and periglacial soils from near the summit of Mt. Kilimanjaro by using both Illumina and Sanger sequencing of 16S and 18S rRNA genes. Ice and periglacial soils contain unexpectedly diverse and rich assemblages of Bacteria and Eukarya indicating that there may be high rates of dispersal to the top of this tropical mountain and/or that the habitat is more conducive to microbial life than was previously thought. Most bacterial OTUs are cosmopolitan and an analysis of isolation by geographic distance patterns of the genus *Polaromonas* emphasized the importance of global Aeolian transport in the assembly of bacterial communities on Kilimanjaro. The eukaryotic communities were less diverse than the bacterial communities and showed more evidence of dispersal limitations and apparent endemism. Cercozoa dominated the 18S communities, including a high abundance of testate amoebae and a high diversity of endemic OTUs within the Vampyrellida. These results argue for more intense study of this unique high-elevation "island of the cryosphere" before the glaciers of Kilimanjaro disappear forever.

Introduction

High-elevation microbial diversity has been studied in several of the highest mountain ranges on Earth (Costello et al. 2009, Lynch et al. 2012, Schmidt et al. 2011), however microbial communities have not been studied at high elevation in the African continent and in particular, on the climate sensitive glaciers and periglacial soils on the top of Mt. Kilimanjaro. Kilimanjaro, Africa's highest mountain and the tallest free standing mountain on Earth, is located on the Kenya-Tanzania border, about 370 km south of the equator and approximately the same distance from the Indian ocean (3 04'S, 37 21' E). This massive stratovolcano (about 80 by 50 km) consists of 3 main peaks, of which Kibo (5893 m) is the highest and the only one still retaining glaciers. Its summit has collapsed to form a caldera, enclosing the Reusch crater, which is about 800 m across and still presents a continuous geothermal heat flux. In recent years Kilimanjaro and its dramatic ice loss have become an "icon" of climate change, attracting broad interest in its fate (Kaser et al. 2004). The three remaining ice fields on the plateau and slopes are both shrinking laterally and rapidly thinning (Thompson et al. 2009) leaving behind just the ragged fringe of an ice cap, which is believed to have once covered the entire summit of the mountain (Humphries 1959). Of the ice cover recorded in 1912, 85% has disappeared, with a peak reduction rate registered from 1989 to 2007 of about 2.5% per year (Thompson et al. 2009). A drastic drop in atmospheric moisture starting at the end of the 19th century and an increase of shortwave incoming radiation due to decreased cloudiness are currently believed to be the main drivers of glacier retreat (through sublimation) on Kilimanjaro, as well as other equatorial east African glaciers (Mölg et al. 2003). It is believed that under present conditions, rapid glacier shrinking at the top of Kilimanjaro will continue unabated and the entire summit of the mountain is expected to be devoid of ice for the first time in 11000 years by mid-century (Kaser et al. 2004).

As the glaciers on top of Kilimanjaro continue to recede, unique microbial communities and paleodiversity archives will be gradually compromised and lost. Glaciers are known to function as reservoirs of airborne microorganisms that can preserve information about their relationship with climatic and environmental changes through time (Xiang et al. 2009). To date there have been no investigations published on the microbiology of the habitats at the top of Kilimanjaro and the expected dramatic change of these environments in the next decades makes it important to study the biological communities dwelling within the ice and soil. Its high elevation and its considerable isolation from any other mountain range also makes it an ideal site to further advance our knowledge of microbial endemicity and biogeographic patterns. Aerial deposition and post-depositional selection are the main drivers of microbial community composition at high elevations (Xiang et al. 2009) but relatively little is known about their specific roles in the establishment of microbial communities.

The top of Mt. Kilimanjaro is also of interest to the field of astrobiology. Kilimanjaro's periglacial soils may be some of the most extreme environments on Earth and are therefore considered as potential analogues for habitable zones on Mars (Ponce et al. 2011). Microbial life at the top of this mountain has to cope with a complex interplay of parameters that are similar to those that could be found on Mars or other planetary bodies, such as a high UV flux, extreme diurnal freeze-thaw cycles, low atmospheric pressure and an extremely low nutrient content and water activity (Ponce et al. 2011). For this reason, these oligotrophic soils and glaciers have received some attention as Martian analogues prior to the launch of the Mars Science Laboratory (MSL) Curiosity Rover and as a field test for a planned Mars 2018 mission (Ponce et al. 2011). Preliminary data from Ponce et al. 2011 showed that Kilimanjaro soils in proximity to glacier walls may be nearly sterile with a total organic carbon (TOC) content of less than 1000 μg/g. TOC has been

traditionally correlated with viable microbial biomass and the value observed for Kilimanjaro soils are slightly higher than TOC levels of oligotrophic mineral soils found in extreme environments in Antarctica, high elevation sites in the Andes and the Atacama Desert (Parsons et al 2004, Costello et al. 2009, Drees et al. 2006). In the present study we follow up on the preliminary work of Ponce et al. 2011 to provide the first culture-independent analysis of the microbial communities in ice and soil on top of Mt. Kilimanjaro.

Materials and methods

Site Location and Sample Collection

The study area at the top of Mt. Kilimanjaro was accessed from the Machame trail on June 4th, 2012, at the beginning of the dry season. Prevailing weather conditions at the top were clear, windy and cold, with most of the surface covered in fresh snow. Samples were collected at the border of the tabular-shaped plateau glacier in the Southern Ice Field (SIF) at 5772 m elevation (S 03 04. 839' E 037 21. 628') (Figure 2.1). The collection site was far off the main trail with no apparent foot traffic. A total of 8 samples were collected along a transect from the base of the glacier wall: 2 from the ice wall (N3 and N8) and 6 from soils at increasing distance from the base of the glacier wall at distances of 0.4 m (N1), 2.5 m (N2), 5 m (N4), 7 m (N6), 7.5 m (N5) and 8 m (N8) (Table 2.1). Latitude, longitude and elevation data were collected using a handheld GPS device. The original plan to collect soil samples along a longer perpendicular transect was made impossible by deep snow on the ground, thus samples were collected from areas close to the glacier that were devoid of snow. Ice samples were collected by scraping the ice wall with a sterile spatula, while soil samples (50g each) were collected from the top 0-4 cm of soil. Both types of sample were placed in sterile 50 ml centrifuge tubes (Fisher Brand, Hampton, NH, USA) and kept on ice for 1 day during the descent from the

mountain. They were subsequently stored at -20°C for a week and then shipped to the University of Colorado at Boulder. Upon arrival they were stored at -80°C for later use in molecular and chemical analysis. A subset of collected samples has been archived at -80°C for long-term storage to be available for potential future studies.

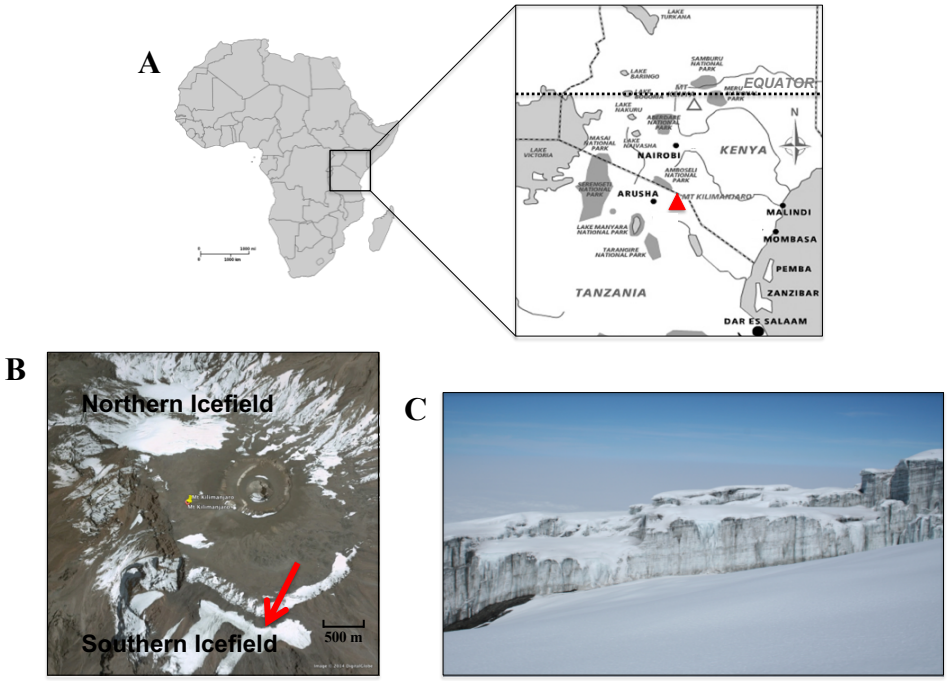

Figure 2.1. Location of Kilimanjaro sampling sites. (A) A map of Eastern Africa showing the location of Mt. Kilimanjaro on the Tanzania-Kenya border (red triangle). Mt. Kilimanjaro is located 370 km south of the Equator. (B) Google Earth imagery captured after field collections were made (Map data: Google, DigitalGlobe). Red arrow indicates sampling location at the border of the tabular-shaped plateau glacier in the Southern Ice Field at 5772 m elevation (S 03 04. 839' E 037 21. 628'). (C) This panel features the tabular-shaped plateau glaciers of the Southern Icefield facing the sampling location and shows the large amount of snow cover that was present on the sampling day.

Table 2.1. Kilimanjaro sample sites and soil characteristics, sampled June 2012.

Sample	Location	Elevation	Distance from Glacier	Type	pH	DOC[a] (µg/g soil)	TDN[b] (µg/g soil)	% of H_2O
N3	S 03° 04.839′ E 037° 21. 628	5772 m	on glacier wall	Ice				
N8	S 03° 04.837′ E 037° 21. 638′	5772 m	on glacier wall	Ice				
N1	S 03° 04.839′ E 037° 21. 628′	5771 m	0.4 m	Soil	7.5	44.8	25.3	28.1
N2	S 03° 04.839′ E 037° 21. 637′	5770 m	2.5 m	Soil	7.61	174.8	14.1	19.8
N4	S 03° 04.839′ E 037° 21. 636′	5772 m	5 m	Soil	7.49	22.3	9.5	13.6
N5	S 03° 04.839′ E 037° 21. 635′	5772 m	7.5 m	Soil	7.69	11.7	1.5	7.2
N6	S 03° 04.836′ E 037° 21. 634′	5772 m	7 m	Soil	7.93	7	3.1	7.7
N7	S 03° 04.835′ E 037° 21. 632′	5771 m	8 m	Soil	7.6	8.7	2.4	6.9

[a]DOC, Dissolved organic carbon.

[b]TDN, Total dissolved nitrogen.

DNA extraction, PCR and 16S/18S rRNA gene clone library construction

Total environmental genomic DNA from soil and ice samples was extracted using PowerSoil® and PowerWater® DNA Isolation Kits respectively (MO BIO, Carlsbad, CA USA), according to the manufacturer's instructions. PCR of 3 soil (N1, N5 and N7) and ice samples was performed using 1-3 µl of template DNA, 12.5 µl of AmpliTaq Gold® 360 MasterMix 5 Units/µL (Applied Biosystems, Foster City, CA, USA) and 1 µl of Forward and Reverse primers (10 µM) in a G-Storm GS2 thermal cycler (GRI Ltd., Essex, UK). Bacterial 16S rRNA genes were amplified using the bacterial domain-specific primer 8F (5' - AGA GTT TGA TCC TGG CTC AG - 3') and universal primer 1391R (5' - GAC GGG CGG TGW GTR CA - 3'). Eukaryotic 18S rRNA and archaeal 16S rRNA genes were amplified using the universal primer pair 4 Fa-short (5' –ATT CCG GTT GAT CCT GC- 3') and 1492r (5' –GGT TAC CTT GTT ACG ACT T- 3'). PCR amplification for 16S rRNA genes was carried out using a program of 95°C for 10 min followed by 35 cycles of 95°C, 1 min; 53°C, 30 sec; 72°C, 2 min and 30 sec; with a final elongation step of 72°C for 10 min. 18S eukaryotic and 16S archaeal rRNA genes were amplified by a program of 95°C for 10 min followed by 35 cycles of 95°C, 1 min; 49°C, 30 sec; 72°C, 2 min and 30 sec; and a final elongation step of 72°C for 10 min. PCR products of the appropriate length were excised from agarose gels and purified following the protocol of the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA USA), with Hyperladder IITM as a reference. PCR products were ligated into TOPO TA ® cloning vectors and transformed into OneShot™ *E. coli* cells (Invitrogen, Carlsbad, CA, USA). Transformed cells were grown on Ampicillin 50 µg/mL agar plates overnight at 37°C. Single colonies containing the inserts were pelleted and randomly arrayed on 96-well plates. Functional Biosciences (Madison, WI, USA) performed Sanger sequencing bi-directionally using vector-targeted T7 (5' - AAT ACG ACT CAC TAT AG - 3') and M13R-9 (5' - GCT ATG ACC ATG ATT ACG - 3') primers.

Sanger Sequencing and phylogenetic analysis

Geneious (Biomatters, Auckland, New Zealand) was used to edit sequences, trim primers and assemble contigs. Edited sequences were aligned using the SINA aligner tool (Pruesse et al. 2007) and imported into ARB (Ludwig et al. 2004) where they were manually fine-tuned. Putative chimeras were identified using a combination of Bellerophon (Huber et al. 2004) and the Mallard Program (Ashelford et al. 2006). Once putative chimeras were removed, remaining sequences were used to generate a phylip-formatted distance matrix and cluster analysis was performed with MOTHUR (Schloss et al. 2009) using the average neighbor algorithm implementation to define OTUs at the minimum threshold of 97% sequence identity. Phylogenetic affiliation of OTUs and related sequences were found using both the basic local alignment search tool (BLAST) and ARB through the parsimony insertion function. Phylogenetic trees for both bacterial and eukaryotic OTUs were inferred using maximum likelihood (ML). The ML analysis was conducted using a general time reversible (GTR) model of evolution with a gamma distribution (g) and a proportion of invariant sites (I) with MEGA6.06. (Tamura et al. 2013). Node support was estimated using 300 bootstrap replicates.

PCR and MiSeq Illumina sequencing

Amplification of the bacterial V4-V5 16S rRNA gene region and eukaryotic 18S rRNA gene for all soil and ice samples was performed using the oligonucleotide primers sets 515F/806R and Euk_1391f/EukBr respectively (Earth Microbiome Project, accessible at http://www.earthmicrobiome.org/emp-standard-protocols/16s-18s/). All forward and reverse primers were modified to include a unique 12 nucleotide barcode. PCR reaction mixtures contained 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 1 µL of template and 12.5 µL of MM Gotaq Hot start Colorless Master Mix (Promega Corporation, Madison, WI, USA). The reaction volume was adjusted to a total of 25 µL with ultrapure DNase/RNase free

water. Thermal cycles for 16S rRNA gene amplification consisted of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 1 min; 50°C for 1 min; and 72°C for 105 sec; with a final elongation step of 72°C for 10 min. The program for 18S rRNA gene amplification consisted of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec; 57°C for 1 min; and 72°C for 1.5 min; with a final elongation step of 72°C for 10 min. To prepare amplicons for sequencing, amplicon purification and normalization was done with Invitrogen SequalPrep Normalization Kit (Invitrogen Inc., CA, USA). Amplicons were combined into a single pool and sequenced using the Illumina MiSeq platform (BioFrontiers Institute, Boulder, CO) using pair-end 2x150 bp chemistry.

Forward-oriented sequences were demultiplexed, quality filtered and processed using the QIIME pipeline (Caporaso et al. 2010b). Paired-end sequences did not work for eukaryotic reads and only the read corresponding to the 1391F primer was used because it overlaps more with sequences in the NCBI and SILVA databases (Darcy and Schmidt 2016). Singletons were excluded from further analysis and sequences with > 97% SSU rRNA gene sequence identity were clustered into an OTU via UCLUST. Representative sequences for each OTU were chosen for classification and the Greengenes and Silva 104 reference database for 16S and 18S rRNA gene sequences respectively were employed to assign taxonomy identification to each OTU. Sequences were aligned with PyNAST (Caporaso et al. 2010a) and a phylogeny was built with the FastTree algorithm (Price et al. 2009). OTU tables were rarified to the level of the lowest number of sequences in the lowest populous sample and were used to assess alpha diversity and relative abundance of all taxa.

Diversity Measures and Statistical Analysis

Diversity within individual communities (alpha diversity) was evaluated by measures of phylogenetic dominance, evenness and richness with MOTHUR

(Schloss et al. 2009). Dominance was estimated through the Berger and Parker index (Berger and Parker 1970), which calculates the ratio between the abundance of the most represented OTU and the total OTU abundance. The Shannon Index was used to estimate community diversity taking into account OTUs richness and evenness. Phylotype richness was determined through the S_{chao1} estimator and an estimate of how well sampling had covered true community diversity was obtained with the non-parametric estimator Good's coverage.

Weighted UniFrac Analysis, which quantifies shared evolutionary history through shared branch length (Lozupone et al. 2006) was used to construct beta diversity matrices for both the ice and soil datasets (both 16S rRNA and 18S rRNA genes) and a one-way Analysis of Similarity test (ANOSIM) from the vegan R package (Oksanen et al. 2013) was used to test significance of phylogenetic difference in beta diversity between ice and soil samples. Rarefaction curves were plotted for both bacterial and eukaryotic sequences with a sequence similarity cut-off value of 3% to evaluate if sampling effort had revealed true phylogenetic diversity within the site. Additionally, Principal Coordinate Analysis (PCoA) ordination was constructed based on weighted Unifrac distance matrices in order to visualize differences among community compositions of soil and ice samples.

Environmental classification of OTUs

Environmental classification of long-read bacterial OTUs containing 2 or more sequences was done following a modified version of the method of Herbold et al. (Herbold et al 2014). Each OTU representative sequence was queried against the NCBI nucleotide database using BLAST. OTUs were classified as "non-endemic" if closest matches to OTUs were > 97% identical, "endemic" if closest matching sequences were < 97% identical (Table A2.1). Within the "non-endemic" group, reads were classified as "cryophilic" if all database entries that matched the representative unique sequence with > 97% identity had been previously observed

in perennially cold environments, as "non-cryophilic" if all matches were observed in temperate environments and as "polythermal" if the matches were observed in both perennially cold and temperate environments. Reads for the endemic OTUs were classified as "subnovel" or "novel" if the nearest match was between 97% and 95.5% or 95.5-88.5% respectively (Herbold et al. 2014).

Biogeographic analysis

In order to examine the biogeographic distribution within the cryosphere of the most abundant phylotypes retrieved on top of Mt. Kilimanjaro, a genetic isolation by geographic distance analysis was performed on the 2 most abundant long-read OTUs for which geographic coordinates of closely matching sequences were available in Genbank: a *Polaromonas* clade within the Bacteria (Figure A2.1) and a *Chlamydomonas* clade within the Eukarya. Other long-read sequences from glacial and periglacial environments (Figure A2.2 and A2.3) closely matching the sequences of the clades were downloaded from GenBank (Table A2.2 and A2.3). Geographic distances between sample sites were computed in R (R Development Core Team, 2012) using the Fields package (http://CRAN.R-project.org/package=fields) and an uncorrected genetic distance matrix was created using the Ape package (http://CRAN.R-project.org/package=ape). To test for a correlation between the matrices, Mantel tests were performed in R using 1000 randomized permutations per test. A Mantel correlogram was constructed and the application of Sturge's rule resulted in data being partitioned in distance classes. Further Mantel tests were carried out on each distance class with a Bonferroni correction.

Soil Analyses

Soil pH was determined according to the method of King et al. 2010. Specifically, 2 g of soil and 2 mL of DI water were placed into 15 mL centrifuge tubes and shaken horizontally for 1 hr at 175 rpm. Soil pH was then measured

with an Oakton benchtop pH meter (OAKTON Instruments, Vernon Hill, IL, USA). Total water content of soil samples was measured by placing 1g of soil of each sample in 15 mL conical sterile tubes left open to dry at 60°C in an oven for 24 hrs. Water content was determined as the percentage of the ratio between the water loss and dry weight of the sample.

Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were measured using a modification of the method described in Weintraub et al. 2007. 2g of soil of each sample was shaken with 25 mL of 0.5M K2SO4 for 1 hour. Solutions were then filtered using a 0.2 μm IsoporeTM membrane filter (Millipore, Darmstadt, Germany). DOC and TDN were measured using a Shimadzu TOC-V CSN Total Organic Carbon Analyzer with TNM-1 module.

Nucleotide sequence accession numbers

The SSU rRNA gene sequences from Sanger libraries from this study were deposited in the GenBank database under accession numbers KX771236 to KX772158 while the SSU rRNA gene sequences from Illumina MiSeq libraries were deposited in the SRA (Short Read Archive) database under Bioproject ID PRJNA340181 and PRJNA340027.

Results

Alpha and Beta Diversity

Bacterial richness (Chao 1) and Alpha Diversity (PD) were comparable for soil and ice samples (Table 2.2), whereas the eukaryotic community showed more diversity in ice compared to soils. The Shannon Index implied than evenness was similar in soils and ice for both bacterial and eukaryotic communities. The Berger Parker Index revealed relatively low dominance in both ice and soil bacterial communities, meaning that the most abundant OTUs in the samples are only represented by a small number of sequences. Eukaryotic communities in soil and

ice displayed a higher Berger Parker Index than that seen for bacterial communities showing that the most abundant OTUs constitute 40-50% of total abundance.

Table 2.2. Diversity and dominance indices, coverage, number of observed OTUs and phylogenetic diversity index (PD) for bacterial and eukaryotic communities in soil and ice samples.

Sample	Chao1	Shannon	Berger-Parker	Good's Coverage	Observed OTUs	PD
Bacteria						
Soil	603.37	6.37	0.12	0.97	418	20.74
Ice	646.83	6.32	0.09	0.97	400	19.89
Eukarya						
Soil	184.36	2.65	0.47	0.99	113.3	38.48
Ice	284.85	2.73	0.39	0.98	143.5	45.59

Rarefaction curves (Figure A2.4) yielded similar patterns as those estimated by Chao1 index and were close to saturation at a 97% clustering level. The number of OTUs observed was ~ 3.5 times higher for Bacteria compared to Eukarya. Within the Eukarya, sampling coverage for ice communities was about twice as high as soil communities. Good's coverage index demonstrated that the estimated proportion of phylotypes represented in our libraries was very high (> 0.97), however, "species-level" sampling coverage was not complete as the observed number of OTUs was less than that estimated with the Chao1 richness.

ANOSIM analysis revealed that there was a significant difference in composition (beta diversity) between ice and the soil samples for Bacteria ($R = 0.8$, $P = 0.03$), but there was not a significant difference between ice and soil eukaryotic assemblages, perhaps indicating that the soil Eukarya came from the ice. Interestingly both the bacterial and eukaryotic composition of the soil sample closest to the ice wall (N1) was more similar to that of ice samples compared to soil (Figure A2.5). Ice assemblages showed a lower weighted UniFrac dissimilarity

value to the soil sample closest to the glacier wall than the other soil samples for both Bacteria (0.13 vs 0.202) and Eukarya (0.212 vs 1.49).

Community composition and phylogenetic analysis

Bacterial OTUs from soil and ice were distributed among 18 phyla, including 4 candidate divisions and were dominated in both ice and soil by Proteobacteria, Bacteroidetes, Actinobacteria and Acidobacteria (Figure 2.2). Betaproteobacteria represented the dominant fraction of the community in both ice (25%) and soil (22%) and most of these were in the Comamonadaceae (*Polaromonas*, *Variovorax*, *Rhodoferax*, *Curvibacter*, *Methylibium*) and Oxalobacteraceae in the soil and Rhodocyclales in the ice. Bacteroidetes accounted for 9 and 22% in ice and soil, respectively, with the majority in the Chitinophagaceae family (*Ferruginibacter*, *Segetibacter* and *Sediminibacterium*). Actinobacteria accounted for 5 and 8% of the total sequences in ice and soil, respectively, with most being in the Actinomycetales (*Frigobacterium*, *Nocardioides* and *Pseudonocardia*). Cyanobacteria only represented 4% of sequences in the soil, whereas they were more abundant in the ice (10%), with most sequences in the Oscillatoriales and Chroococcales. Overall soil communities harbored higher abundances of Actinobacteria, Bacteroidetes and Acidobacteria than ice, while sequences related to Betaproteobacteria, Alphaproteobacteria and Cyanobacteria displayed higher abundance within ice communities (Figure 2.2).

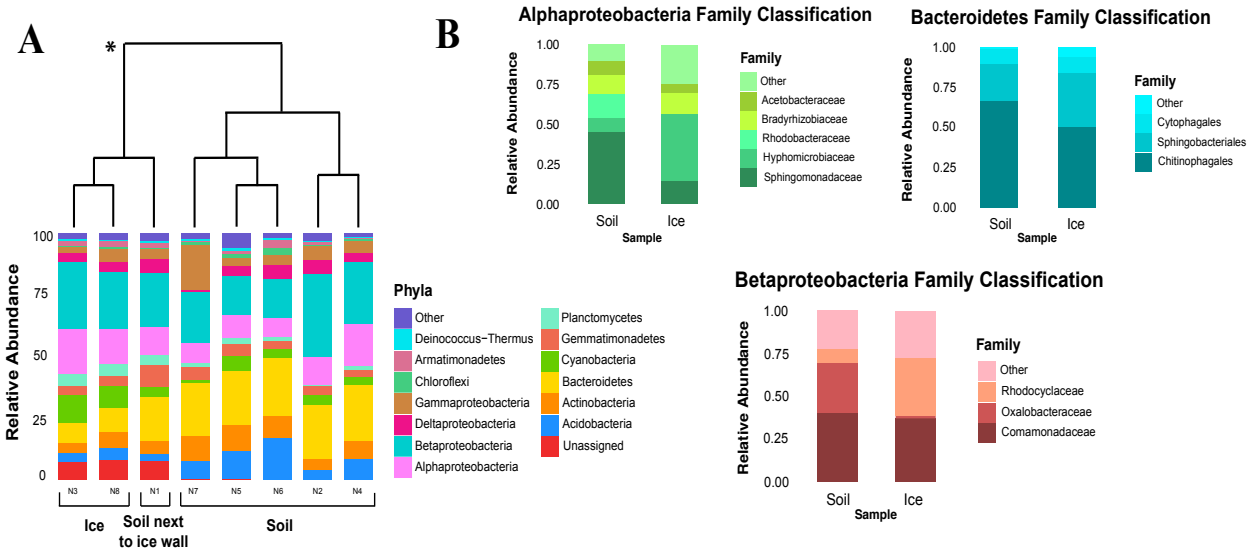

Figure 2.2. Phylogenetic analysis of 16S rRNA gene sequences obtained from ice and soil samples close to the summit of Mt. Kilimanjaro. (A) Cluster analysis of the phylogenetic structure and the relative abundance of bacterial phyla in the ice and soil samples as assigned by Greengenes to Illumina MiSeq sequences. Proteobacteria were split into classes for greater detail. The asterisk indicates statistical significant difference in composition (ANOSIM, P<0.05). (B) Classification at lower taxonomic level is reported for Alphaproteobacteria, Bacteroidetes and Betaproteobacteria.

Illumina sequencing data revealed that both soil and ice 18S communities were limited to 9 phyla. Ice communities were heavily dominated by Cercozoa, representing 72% of total sequences (Figure 2.3), while in the soil they were the most abundant taxonomic group together with green algae (Chlorophyta), at about 34% abundance each. The Chlorophyceae comprised about 9% and 34 % of total sequences in the ice and soil respectively and were most closely related to the genera *Chlamydomonas*, *Chloromonas* and *Stigeoclonium*.

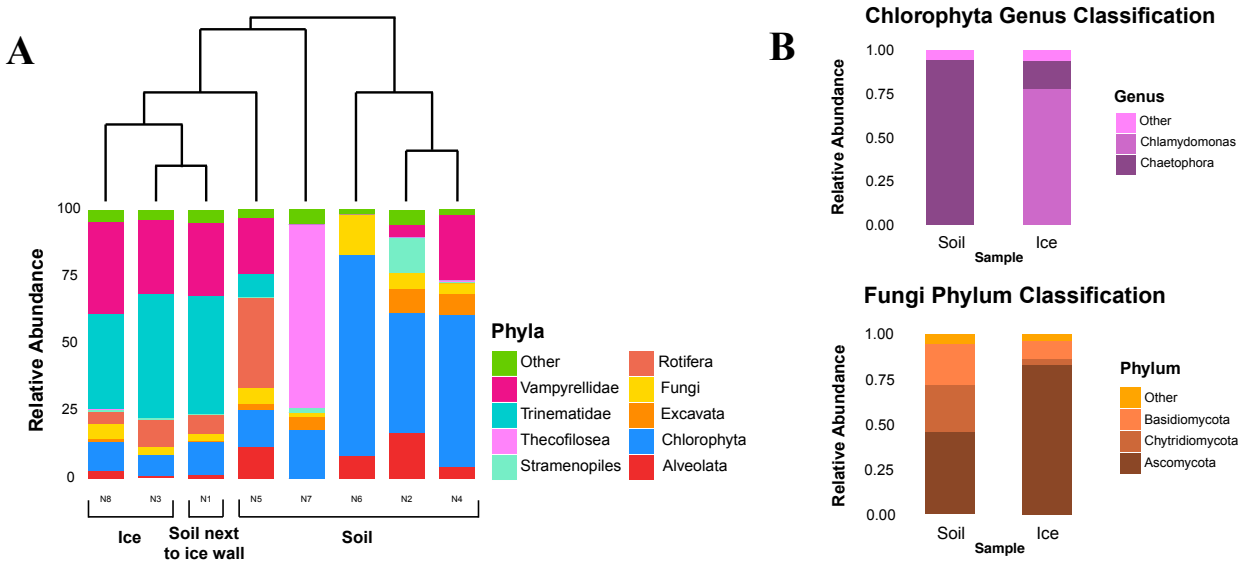

Figure 2.3. Phylogenetic analysis of 18S rRNA gene sequences obtained from ice and soil samples close to the summit of Mt. Kilimanjaro. A) Cluster analysis of the phylogenetic structure and the relative abundance of eukaryotic phyla in the ice and soil samples as assigned by Silva 104 database to Illumina MiSeq sequences. Cercozoa were split into classes (Thecofilosea), orders (Vampyrellida) and families (Trinematidae) for greater detail (B) Classification at lower taxonomic level is reported for Chlorophyta and Fungi.

Within the Cercozoa, most sequences fell within the Trinematidae and the Vampirellida. Longer reads from Sanger sequencing showed that the Trinematidae family clustered in very few OTUs closely related to the known organisms *Trinema lineare* and *Trinema enchelis* (Figure 2.4). In contrast, long-read sequences within the Vampirellida order were very diverse and all fell into a previously undescribed clade (wedge in Figure 2.4).

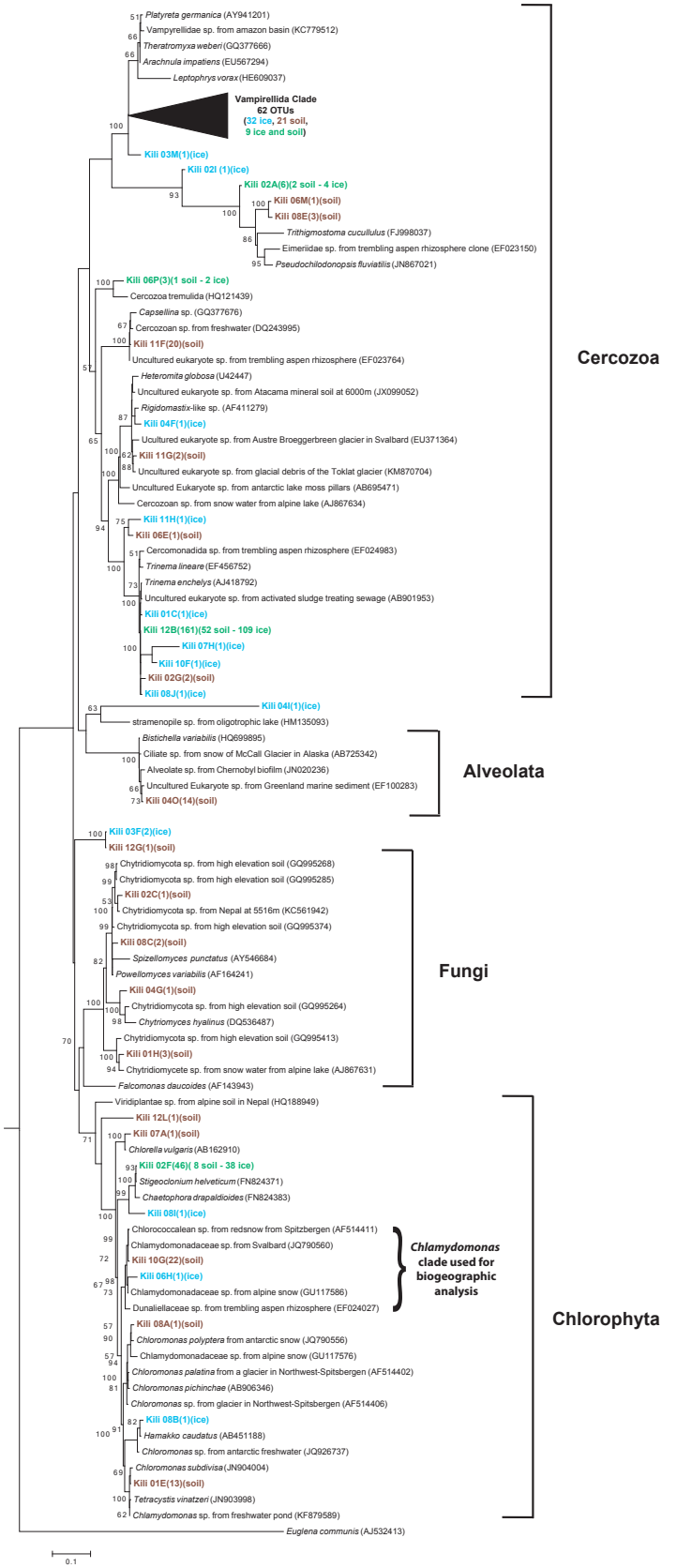

Figure 2.4. Phylogenetic analysis of 18S rRNA gene long-read sequences retrieved in ice and soil samples close to Mt. Kilimanjaro summit. Maximum Likelihood consensus phylogenetic tree includes 18S rRNA gene sequences from ice and soil close to the summit of Mt. Kilimanjaro and their closest GenBank BLAST and ARB matches. The accession numbers of most closely related taxa are listed parenthetically. Tree is rooted with the sequence of Euglena communis *(AJ532413). Kilimanjaro phylotypes are bolded and followed by the number of sequences in each phylotype. Kilimanjaro phylotypes color code is as follows: blue, sequences retrieved in ice: brown, sequences retrieved in soil: green, sequences retrieved in both ice and soil. Node support is given as maximum likelihood values (n. of bootstrap replicates) when equal or greater than 50%. The scale bar corresponds to 0.1 substitutions per site. Major groups are shown to the right. The parenthesis indicates the* Chlamydomonas *clade that was used for the biogeographic analysis.*

A total of 15 archaeal Sanger sequences that clustered into 3 OTUs were also retrieved in one of the soil samples (N7) with closest relatives being uncultured Haloarchaea from alkaline-saline desert and Antarctic soils (Figure A2.6), however we did not use Archaea-specific primers in this study.

Environmental classification of OTUs

We used the approach of Herbold et al. 2014 to classify bacterial phylotypes into "endemic" and "non-endemic" and within the non-endemic whether they are "cryophilic", "non-cryophilic" or "polythermal" (Table A2.1). Of the 60 non-endemic bacterial OTUs found on Mt. Kilimanjaro summit, 46 (77%) were found to be cosmopolitan, meaning that all database entries that matched the representative sequence for that OTU had been previously observed in both perennially cold and temperate environments. Only 6 bacterial OTUs appeared to be endemic, meaning that they were less than 97 % identical to any phylotype in the NCBI database.

Biogeographic analysis

Within the Bacteria and the Eukarya, two genera common to glacial and periglacial environments were selected to test their biogeographical distribution by comparison of genetic distance versus geographic distance. The analysis of the *Polaromonas* clade revealed that there was a slight (rM = 0.08) but significant (Mantel test, P = 0.002) increase in genetic distance with geographic distance, however spatial structuring was not evident for 11 out of 14 distance classes (Figure 2.5). An expanded dataset of Darcy et al. 2011 was used (Table A2.2) including 96 long-read sequences from 25 geographically well separated glacial and periglacial environments (Figure A2.2), which makes it the most inclusive long-read *Polaromonas* biogeographical analysis performed to date. The biogeographic analysis on the *Chlamydomonas* clade showed a slightly stronger (rM = 0.125, P = 0.001) increase in genetic distance with geographic distance, but spatial structuring was not evident for 8 out of 11 distance classes (Figure 2.6).

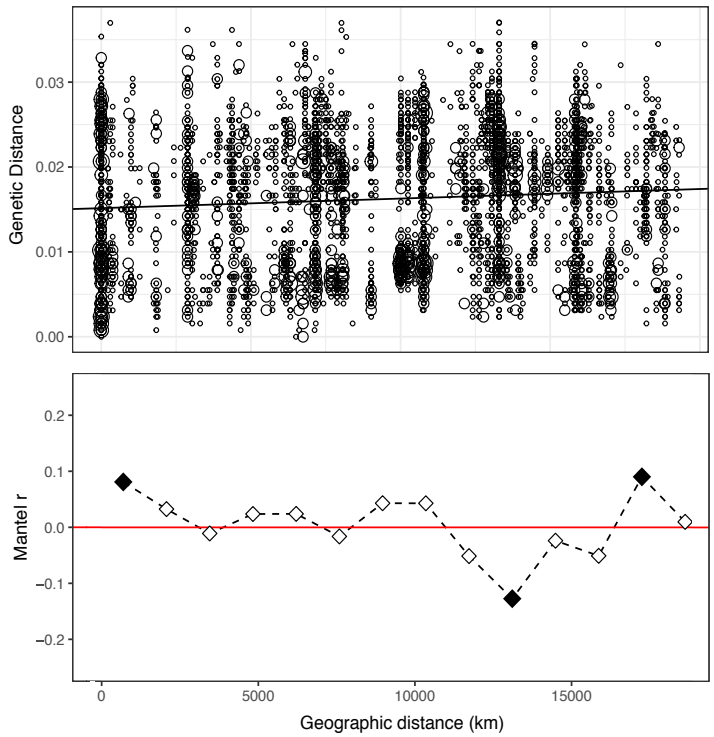

Figure 2.5. Genetic distance by geographical distance relationships for the dominant Polaromonas *clade from Mt. Kilimanjaro summit. Upper panel shows pairwise comparisons of genetic distance among* Polaromonas *sequences (n=4560) and geographic distance among different glacial environments (n=25). The largest geographical distance in this study was between John Evans Glacier, Nunavut and Kamb Ice Stream, Antarctica (19307km). There was a slight (rM = 0.08) but significant (Mantel test, P = 0.002) increase in genetic distance with geographic distance. Circle size is proportional to the number of pairwise comparisons at each point on the plot. Lower panel shows Mantel correlogram of genetic distance among the same sequences separated into 14 distance classes. The midpoint of each distance class is plotted. Shaded squares indicate significant tests in the Mantel correlogram after applying the Bonferroni correction. Spatial structuring was not evident for 11 out of 14 distance classes, supporting the contention that* Polaromonas *phylotypes are globally distributed.*

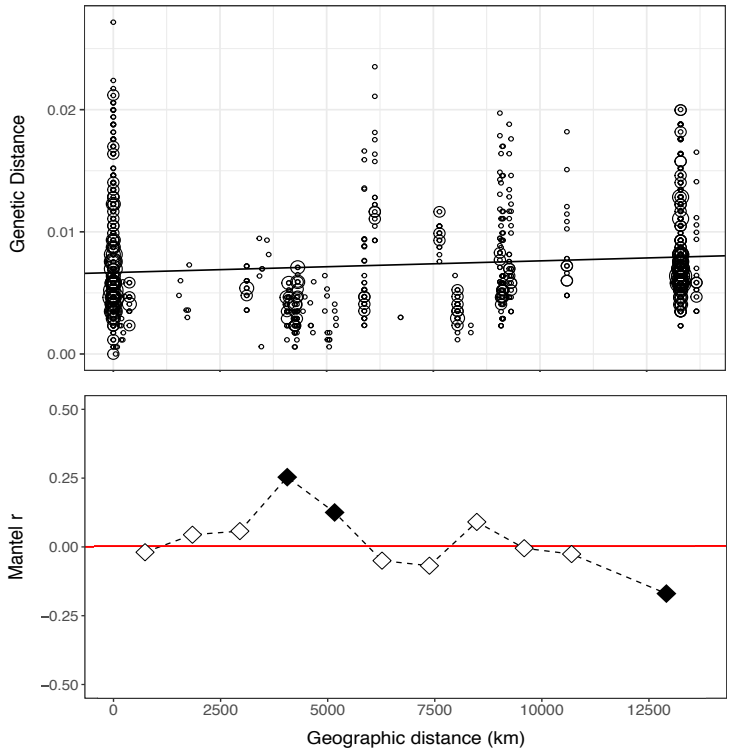

Figure 2.6. Genetic distance by geographical distance relationships for the dominant Chlamydomonas *clade from Mt. Kilimanjaro summit. Upper panel shows pairwise comparisons of genetic distance among* Chlamydomonas *sequences (n=1326) and geographic distance among different glacial environments (n=15). The largest geographical distance in this study was between Kilimanjaro and Harding icefield, Alaska (13667km). There was a slight (rM = 0.125) but significant (Mantel test, P = 0.001) increase in genetic distance with geographic distance. Circle size is proportional to the number of pairwise comparisons at each point on the plot. Lower panel shows Mantel correlogram of genetic distance among the same sequences separated into 11 distance classes. The midpoint of each distance class is plotted. Shaded squares indicate significant tests in the Mantel correlogram after applying the Bonferroni correction. Spatial structuring was not evident for 8 out of 11 distance classes.*

Soil characteristics

Soil moisture levels decreased with distance from the glacier wall (Table 2.1) and are comparable with other high elevation and periglacial sites (Costello et al. 2009, Niederberger et al. 2008). Soil pH did not show a trend and ranged from 7.5 to 7.9 across the transect (Table 2.1). DOC and TDN away from the glacier (> 5m) are similar to levels found for Antarctic bare, fell-field soils (Roberts et al. 2009) and high elevation sites in Peru and Colorado (King et al. 2008). In general, nutrient levels were higher in samples taken close to the glacier, especially TDN (Table 2.1).

Discussion

Despite their ecological importance to downstream ecosystems, the study of microbial diversity at high elevations has lagged behind the study of other ecosystems. In particular, no work, using modern molecular approaches, has been done at high elevations in Africa. The present study was initiated to probe the diversity of soil- and ice-dwelling microbes near the top of Mt. Kilimanjaro. To our knowledge, this is the first investigation of the microbial diversity on Mt. Kilimanjaro or the African continent at elevations above 5000 m. This investigation provides a reference for future studies of microbial community structure and function in this extreme environment and other high-elevation sites in Africa.

Data from Illumina and Sanger libraries demonstrated that communities in both soil and ice are represented by a phylogenetically broad spectrum of microorganisms. Richness was high for both soil and ice communities for Bacteria (Table 2.2) and was close to that previously reported for other cold environments such as Antarctica and the Himalayas (Rhodes et al. 2013, Yergeau et al. 2007). Glaciers can support very active microbial communities that sequester nutrients from the atmosphere (Anesio et al. 2009, Steven et al. 2008, Vincent et al. 2004), which may explain the high diversity encountered in ice samples. The majority of

bacterial OTUs were not closely related to cultured microorganisms and among the closest matching sequences were environmental sequences from cold (high-elevation and high-latitude), arid and heavy metal contaminated environments. These comprised putative psychrophilic, xerophilic, endolithic, radioresistant and halophilic microorganisms (Figure A2.1). Diversity of Eukarya was a third of that for Bacteria according to the Shannon Index and Observed OTUs (Table 2.2), but was comparable to Eukaryotic diversity in polar waters (Luo et al. 2016) and sea ice (Majaneva et al. 2012).

Bacterial communities in ice and soil samples were significantly different (ANOSIM, $P < 0.05$). However, the soil sample closest to the ice wall harbored bacterial and eukaryotic communities closely resembling those of the ice samples. This may suggest that the main microbial input for the soils adjacent to the glaciers is the glacier itself and that these ice microbes are replaced by a soil community as the soils age. Ice microorganisms may not survive as well in soils because of exposure to environmental stressors such as freeze-thaw cycles and desiccation, stressors that are partially alleviated in the more constant ice environment. A likely consequence may therefore be the establishment in the soil of new phylotypes from the atmosphere.

Taxonomic diversity of Bacteria

Overall, both Illumina and Sanger technologies yielded similar patterns of taxonomic diversity (Figure A2.7) as has been noted in other studies (Harris et al. 2013) with the taxonomic resolution achieved dependent on the abundance of reference sequences in the particular region of the rRNA tree of life. Sanger sequences were much longer (1345-1715 vs 150-250 bp) and were therefore used to identify sequences at a finer taxonomic level.

Betaproteobacteria (especially members of the Comamonadaceae) are the dominant members of the community similar to other recently deglaciated soils

(Darcy et al. 2011, Nemergut et al. 2007, Sattin et al. 2009). Comamonadaceae are phylogenetically similar in soil and ice (Figure A2.1), indicating that ice may be seeding the youngest soils as previously seen in the High Andes of Peru (Nemergut et al. 2007). Many Comamonadaceae are heterotrophs that are able to metabolize both recalcitrant (Park and Ka 2003) and more labile carbon sources (Willems 2014) and could therefore use multiple aeolian deposited carbon sources. It is still unclear if many Comamonadaceae (e.g. *Polaromonas*) are indigenous to ice or if they are transients from the upper atmosphere. They are found in almost all glacial habitats studied to date (Darcy et al. 2011), perhaps indicating that they are being constantly deposited in glacial systems, but not necessarily growing there.

The other most abundant bacterial OTUs in soil were in the Chitinophagaceae (Bacteroidetes) but these were in lower relative abundance in the ice. Closely related sequences have been previously retrieved from similar environments such as oligotrophic volcanic cave sediments from Mt. Erebus, Antarctica (Tebo et al. 2015), fumarole soils at high elevation in the Andes (Costello et al. 2009), pyroclastic deposits in Alaska (Zeglin et al. 2016), and debris-covered glaciers in the Alps (Franzetti et al. 2013). Chitinophagaceae have the ability to degrade complex polymers such as chitin, which provides microbes with C and N (Moorhead et al. 2012). Chitin may come from Fungi and Arthropods, which often occur in recently deglaciated soils (Zumsteg et al. 2012). Members of the Chitinophagaceae may be opportunists, relying on Aeolian inputs and some species have a dormant stage (Kämpfer et al. 2006), which may allow them to persist in the atmosphere and survive in periglacial habitats once they are deposited.

All cyanobacteria sequences retrieved in ice and soil are closely related to non-heterocystous nitrogen fixers (*Oscillatoria*, *Microcoleus*, *Leptolyngbya*) commonly found in cold ecosystems (Jungblut et al. 2010). Nitrogen is often a limiting nutrient in poorly developed early successional soils (Knelman et al. 2014).

Other sites at similar elevations in Peru and Nepal have also recently been shown to have similar cyanobacteria (Schmidt et al. 2011, Nemergut et al. 2007, Schmidt et al. 2008a) and cyanobacteria have long been known to colonize barren habitats in the Arctic and Antarctica (Quesada and Vincent 2012). However, very dry, high-elevation sites like the stratovolcanoes in the Atacama Desert completely lack cyanobacteria, except in wet areas near active fumaroles (Costello et al. 2009, Lynch et al. 2012, Solon et al. 2018). Cyanobacteria were more abundant in the ice wall and the soil adjacent to the glacier than soil, possibly indicating that they depend on access to a constant source of water (provided by meltwater of the glacier), which is not available in soils further from the glacier.

Among the Alphaproteobacteria, the most abundant Hyphomicrobiaceae family was only found within ice samples and the soil closest to the ice wall and are known for being oligocarbophilic, thriving only in the presence of low carbon concentrations (Oren and Xu 2014), which can explain their being restricted to the ice and soils near the ice. Sphingomonadaceae were the second most abundant family within the Alphaproteobacteria and were more abundant in soils. Sequences from this family are commonly detected in glacial habitats and have been reported in Arctic cryoconite (Edwards et al. 2011), Antarctic ice sheet (Alekhina et al. 2007) and Arctic glaciers (Zeng et al. 2013). Members of the Deinococcus-Thermus group were present in our site, as well as Antarctica Dry Valleys (De la Torre et al. 2003) and sites over 5400 meters in the Andes (Schmidt et al. 2009).

Taxonomic diversity of Eukarya

Among the Eukarya, Cercozoa dominated the community and the most unexpected diversity found was in the order Vampyrellida (family Leptophryidae). These sequences are distinctly different from any taxa in Genbank (Figure 2.4), suggesting that this unique environment harbors a previously unknown Vampyrellida community. Vampyrellida are only rarely recovered in environmental

DNA surveys (Berney et al. 2004) and have only been previously found at high elevations in soils from fumaroles on Volcán Socompa (Schmidt et al. 2018). Our sequences were however less than 95% identical to those found on Volcán Socompa suggesting high endemicity of this group on Kilimanjaro. Given the presence of fumarolic activity on Mt. Kilimanjaro, it is possible that this group is associated with thermal environments. The Vampyrellida are very diverse and are generally known from aquatic habitats and soil where they are predators of algae, fungi, protozoa and small metazoans (Berney et al. 2013). Vampyrellida have multiple nuclei (Berney et al. 2013), which could lead to a bias and overestimation in their relative abundance. However, our Sanger clone libraries showed many distinct OTUs suggesting that multiple representation of the same organism was minimal. Many Cercozoa (including some Vampyrellida) are very resistant to drought (Harder et al. 2016) and can respond quickly to periods of transient nutrient and water availability (Berney et al. 2013), which may account for their abundance in the soils studied here. Food preferences may also be an important basis for lineage differentiation in Vampyrellida (Hess et al. 2012). Their relative abundance was significantly higher in the ice samples (Figure 2.3 and Figure A2.8), suggesting that those in the soil may have come from the ice.

The most abundant OTU in the Eukarya was very closely affiliated with the known Cercozoans *Trinema lineare* and *Trinema enchelis* (order Euglyphida) (Heger et al. 2010), common testatae amebae in moist environments. The same taxa of testatae amebae have recently been found for the first time in glaciers in the Andes (Santibáñez et al. 2015) and in glacier forefield soils in the Alps (Lazzaro et al. 2015). Heterotrophic Cercozoa seem to be more abundant than their potential food sources in our samples. However, inverted trophic pyramids have been seen before at high elevation where producers are scarce (Costello et al. 2009) or absent (Lynch et al. 2012). The high abundance of these predatory microeukaryotes could be

sustained by feeding on fungi and algae in addition to bactivory (Krashevska et al. 2008).

These soils, like other even higher-elevation volcanoes (Costello et al. 2009, Lynch et al. 2012), harbored some of the simplest fungal communities yet described compared to, for example the 15 lineages recovered in the Dry Valleys of Antarctica (Chan et al. 2013). Environmental extremes characteristic of high elevation, such as freeze-thaw cycles and high UV levels may limit fungal communities on Kilimanjaro compared to polar regions. Chytrids dominated our Sanger fungal libraries similar to previous studies of high-elevation and high-latitude soils (Freeman et al. 2009). Most chytrids were in the Spizellomycetes, which are commonly found in harsh soil environments such as arid grasslands (Lozupone and Klein 2002), and glacial till (Wakefield et al. 2010). They can resist extremes of temperature, pH, and desiccation (Gleason et al. 2010) and can decompose pollen or be parasitic. These features may explain their presence on Mt. Kilimanjaro and their wide geographical distribution.

Green algae sequences found in ice and soils of Kilimanjaro are closely related to those found in similar environments such as alpine snow (Remias et al. 2010), Arctic glaciers (Leya et al. 2003) and Antarctic snow (Remias et al. 2013). Their high relative abundance and the scarcity of cyanobacterial sequences in these soils suggests that they may play a significant role as primary producers in this habitat and in early stages of succession following glacial retreat as has been previously suggested for green algae in high-altitude ecosystems (Schmidt and Darcy 2015).

Taxonomic diversity of Archaea

Archaea sequences distantly related (92%) to the Haloarchaea class were only retrieved with Sanger sequencing in one of the soil samples (N7) (Figure A2.6). Scarcity of liquid water at this elevation can drive the formation of salty pockets

within the soils that can explain the presence of Haloarchaea but more work is needed to understand the true phylogenetic diversity of Archaea on the top of Mt. Kilimanjaro. It is known that Archaea are more difficult to detect in cold (Ayton et al. 2010, Cary et al. 2010) and hot deserts (Chanal et al. 2006), which may explain our findings.

Biogeography and spatial scale

Kilimanjaro is the most isolated high altitude peak on Earth (Duane et al. 2008) and approximately 5700 km from the closest mountain range with similar and higher elevation peaks (the Himalayas) (Google earth data, last accessed on 10 March 2018) making this site ideal for testing ideas related to microbial dispersal and biogeography. Despite being separated by large distances and climatic barriers, it has been proposed that cold high-elevation and high-latitude environments are connected through the upper atmosphere (Darcy et al. 2011) but data is lacking for Africa. Small microbes are easily dispersed over long distances, but their long-term survival requires a number of distinct adaptations, similar to those required to survive in icy environments (Pearce et al. 2009). The top of Mt. Kilimanjaro may be an oasis where microbes in transit in the upper atmosphere persist, but more work is needed to determine if they can grow on Kilimanjaro. The present study is a first step towards gaining an understanding of the global dispersal of microbial phylotypes to high elevation sites in Africa.

It is currently unknown how Aeolian deposition affects microbial composition and if Kilimanjaro's great distance from similar environments is reflected in more speciation. Our survey showed that most bacterial diversity at our site is cosmopolitan, as defined by Herbold et al. 2014, with the vast majority of OTUs having close matches with sequences from very distant both cold and temperate environments showing little endemism in this cold isolated environment. More evidence of minimal endemism of this isolated site comes from the biogeographical

analysis of one of the most abundant clades within the Bacteria, *Polaromonas* (Figure 2.5). Our analysis showed remarkably little increase in genetic diversity with geographic distance, pointing towards a uniform distribution of *Polaromonas* phylotypes across the cryosphere. These results confirm the conclusion of previous work that *Polaromonas* phylotypes are globally distributed in the cryosphere (Darcy et al. 2011). Interaction with local microbiota may drive microevolution and post-selection niche separation within *Polaromonas* on the top of Mt. Kilimanjaro.

The finding in Kilimanjaro samples of both cosmopolitan and endemic microorganisms helps corroborate the hypothesis that the distribution of microbial species in the cryosphere is the outcome of the continuum between two opposing theories in microbial ecology: endemism and cosmopolitanism (Martiny et al. 2006). Moreover, the closest matches of 14% of sequences retrieved from our samples were from heavy metal contaminated sites. Since our site is not known to contain relevant quantities of heavy metal, it may be possible that tolerance and resistance mechanisms to heavy metals overlap with those employed by microorganisms to cope with stresses found in extreme environments such as high radiation, freeze-thaw cycles, low nutrient and water levels (Chanal et al. 2006, Dib et al. 2008).

It is likely that larger eukaryotic microbes might be more dispersal limited than smaller Bacteria. Previous work in the cryosphere has shown that larger microbes like algae and zoosporic fungi show distinct biogeographic patterns at global and regional scales (Schmidt et al. 2011, De Wever et al. 2009, Naff et al. 2013) and this could be the case for the most abundant phylum retrieved from our samples, the Cercozoa, which showed a highly diverse new clade within the Vampyrellida. Successful dispersal of small microbes is due to their high abundance and long residence time in the atmosphere (Schmidt et al. 2014), which may not be the case for eukaryotic microorganisms due to their average larger size. Evidence of a pattern of genetic isolation by genetic distance was revealed by the

phylo-geographic analysis of a highly abundant *Chlamydomonas* clade. However, despite the large distances, the pattern revealed was weak (Mantel rM = 0.125, P = 0.001) and it is in accordance with recent molecular studies that suggest that red snow algae may be readily dispersed across the Earth (Lutz et al. 2016, Brown et al. 2016). Our analyses suggest that *Chlamydomonas* may be successful at global dispersal, however in a more limited fashion compared to most bacteria.

Conclusions and Implications

Microbial diversity in the extreme environments on top of Mt. Kilimanjaro proved to be higher than expected compared to high volcanoes in drier regions (Costello et al. 2009, Lynch et al. 2012, Solon et al. 2018), especially for the Bacteria. In addition, the ice bacterial community was significantly different from the soil community, suggesting that soils are predominately seeded by wind-transported terrestrial sources. The combination of cosmopolitan bacterial diversity and weak biogeographical patterns for the clades analyzed suggest that the effect of distance and local environmental conditions is overwhelmed by continuous dispersal. Taken together, considering the isolation of Mt. Kilimanjaro from other perennially cold environments, our results strongly support the concept of a global distribution of microbial ecotypes throughout the cold terrestrial biosphere as has been suggested by other studies of Bacteria (Schmidt et al. 2011, Darcy et al. 2011, Jungblut et al. 2010). This finding suggests that there may be gene flow between the polar regions or that the rate of evolutionary divergence has been slow relative to the timescales of isolation. In depth molecular comparisons of microbial diversity with other distant high elevation sites could provide further insights into dispersal mechanisms and may help elucidate how microbes adapt and diversify in response to environmental pressures. Unravelling the full extent of diversity in these environments would provide a unique opportunity to investigate microbial

endemism and evolution as well as roles of inoculum from the atmosphere, fumaroles and glaciers for microbial communities in recently deglaciated soils.

As glacier recession continues unabated, understanding the biodiversity and function of organisms in these glaciers and periglacial soils is important, before these ecosystems are changed forever. One of the main concerns is the loss of biodiversity and potentially the loss from the biosphere of a pool of genes adapted to surviving and thriving in the cold (Anesio and Laybourn-Parry 2012). Our work adds to a growing body of knowledge about microbial diversity in high elevation ecosystems that are currently responding to a rapidly changing climate. The presence of high bacterial and eukaryotic diversity indicates that specific life forms have evolved to thrive in this extreme environment and may provide a model for viable habitats for life on early Mars since similar conditions may have existed or still exist in sediments adjacent to and beneath the Martian north polar ice cap (Ponce et al. 2011, Preston and Dartnell 2014).

CHAPTER III

GROWTH OF HIGH-ELEVATION *CRYPTOCOCCUS* SP. DURING EXTREME FREEZE-THAW CYCLES

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Abstract

Soils above 6000 m.a.s.l. are among the most extreme environments on Earth, especially on high, dry volcanoes where soil temperatures cycle between -10 and 30°C on a typical summer day. Previous studies have shown that such sites are dominated by yeast in the cryophilic *Cryptococcus* group, but it is unclear if they can actually grow (or are just surviving) under extreme freeze-thaw conditions. We carried out a series of experiments to determine if *Cryptococcus* could grow during freeze-thaw cycles similar to those measured under field conditions. We found that *Cryptococcus* phylotypes increased in relative abundance in soils subjected to 48 days of freeze-thaw cycles, becoming the dominant organisms in the soil. In addition, pure cultures of *Cryptococcus* isolated from these same soils were able to grow in liquid cultures subjected to daily freeze-thaw cycles, despite the fact that the culture medium froze solid every night. Furthermore, we showed that this organism is metabolically versatile and phylogenetically almost identical to strains from Antarctic Dry Valley soils. Taken together these results indicate that this organism has unique metabolic and temperature adaptations that make it able to thrive in one of the harshest and climatically volatile places on Earth.

Introduction

Recent research indicates that some of the most extreme soil ecosystems on Earth are found at high elevations in dry valleys and slopes of the high Andes (Schmidt et al. 2009, Schmidt et al. 2012). In fact, the driest high mountains on Earth occur just to the east of the Atacama Desert, where numerous, massive strato-volcanoes rise majestically from the Puna de Atacama (Costello et al. 2009). The highest of these volcanoes is the brooding mass of Volcán Llullaillaco (el. 6739 m.), the location of the highest elevation archaeological sites on Earth (Reinhard 1999, Reinhard and Ceruti 2010) and "arguably the best naturally preserved assemblage of mummies found anywhere in the world" (Wilson et al. 2013). From a microbiological perspective these mummies are also of great interest because they showed almost no signs of decay after being buried without preserving chemicals for 500 years. The lack of natural decay of these mummies may indicate an environment that is too cold and dry for the proliferation of decaying microbes and inspired several microbiological expeditions to these mountains over the last ten years (Costello et al. 2009, Lynch et al. 2012).

The present study builds on our initial studies of the biogeochemistry and microbiology of the high slopes of Volcán Llullaillaco and nearby Volcán Socompa (Costello et al. 2009, Lynch et al. 2012). These studies showed that the soils on these volcanoes contained microbial communities of extremely low diversity, except in areas near fumaroles (Costello et al. 2009). Perhaps most surprising was the extremely low diversity of eukaryotic microbes found in non-fumarolic soils on these volcanoes. The dominant eukaryotes in culture-independent studies of both volcanoes are related to endolithic and xerotolerant yeasts in the genus *Cryptococcus* (Basidiomycota) that are closely related to organisms isolated from soils of the Dry Valleys of Antarctica and the Himalayas (Schmidt et al. 2012). These yeasts from the Dry Valleys of Antarctica and the Himalayas have been

known for many years (Goto and Sugiyama 1970, Vishniac and Hempfling 1979a), but their ecological role in extreme soils systems is still largely unknown. The genus *Cryptococcus* is very common in soils of extreme polar regions, dominating Antarctic eukaryotic libraries (Buzzini et al. 2012) and representing one third of species in the Arctic libraries (Buzzini et al. 2012). Recent studies in European and South American glaciers have also found an extremely high abundance of *Cryptococcus,* up to 39% and 33% of total yeast species, respectively (Buzzini et al. 2012). Given their ubiquity in extreme high-elevation and high-latitude soils it could be that they are just dormant cells that are globally distributed from less extreme systems. Conversely, they may be important functional components of extreme soils, but little evidence of their functional role exists (Vishniac and Hempfling 1979b).

Daily temperature cycling across the freezing point is considered to be a key challenge for microbial growth and survival at high-elevations and high-latitudes. Previous studies have shown that freeze-thaw cycles frequency influence microbial communities in the cryosphere (Yergeau and Kowalchuk 2008) and limit net primary productivity in the extreme soils of the Dry Valleys of Antarctica, where daily fluctuations vary more than 20°C per day during the austral summer (Cary et al. 2010). Mineral soils at high elevation on Llullaillaco and other nearby volcanoes can experience even more extreme diurnal temperature fluctuation than soils of the Antarctica Dry Valleys (Lynch et al. 2012, Schmidt 1999, Schubert 2014). The purpose of this study is to investigate: 1) whether *Cryptococcus* can grow during extreme freeze-thaw cycles typical of Volcán Llullaillaco; 2) the metabolic capabilities and growth rate of *Cryptococcus* across a range of temperatures and during repeated freeze-thaw cycles, and 3) the phylogenetic placement of this species in relation to *Cryptococcus* from other extreme environments.

Materials and methods

Sample collection

Soils used in this study were collected at an elevation of 6030 meters above sea level (m.a.s.l.) as described elsewhere (Lynch et al. 2012). At this elevation soils are subject to year-round, freeze-thaw cycles and we endeavored to replicate these conditions in the laboratory. It is especially important to mimic the sub-zero cooling rates experienced in the field. Soil buffers changes in temperature compared to air temperatures and therefore the rate at which field soils cool at night is much slower than that of air. This is an important consideration when designing freeze-thaw experiments because the small volume of soil used in laboratory experiments can cool at a much faster rate than could ever occur in the field, and it has been shown that the sub-zero cooling rate of soils is one of the most important parameters controlling microbial survival during freeze-thaw stress (Henry 2007, Lipson et al. 2000, Schmidt et al. 2009). We therefore used a specially designed chamber (DarwinChamber.com) to mimic the sub-zero freezing rate experienced by field soils at a depth of 4 cm at high elevations. Figure 1 shows the rate of sub-zero soil cooling in the chamber compared to field data (4 cm depth) obtained from an elevation of 5800 m.a.s.l. on Volcán Llullaillaco during February (Lynch et al. 2012).

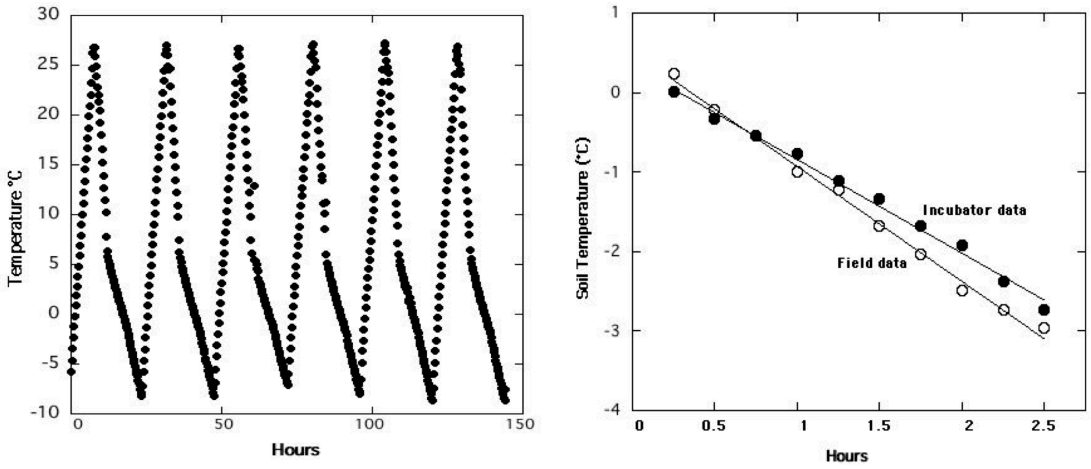

Figure 3.1. Example of freeze-thaw cycles as recorded in the growth chamber used in this study. The chamber was cycling up to a high of 27°C during the day and a low of -9°C at night with an amplitude of 36°C in 24 hrs (left panel). The right panel shows the period of most rapid sub-zero cooling in the chamber ("Incubator data") and in field soils (4 cm depth) on Volcán Llullaillaco ("Field data"). We endeavored to mimic field-cooling rates because this is a critical factor overlooked in most freeze-thaw experiments and is an important factor determining survival of microbes during natural freeze-thaw cycles (Lipson et al. 2000; Schmidt et al. 2009).

Five g of soils were used in each microcosm (4 replicates per treatment). Treatments were: freeze-thaw with added water (70% water holding capacity = freeze-thaw + water) and freeze-thaw at field water content (0.24% water = freeze-thaw dry). An additional 5 replicates of untreated soils were used as controls. The chamber was run for 48 days, cycling up to a high of 27°C during the day and a low of -9°C at night with an amplitude of 36°C in 24 hrs (Figure 3.1).

DNA extraction, amplification and amplicon sequencing

Total DNA was extracted with Power Soil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA). Amplification of the eukaryotic 18S rRNA was performed using the oligonucleotide primers Euk_1391f/EukBr (Earth Microbiome Project, accessible at http://www.earthmicrobiome.org/emp-standard-protocols/18s/). All forward and reverse primers were modified to include a unique 12 nucleotide barcode. PCR reaction mixtures contained 0.5 µL of forward primer (10 µM), 0.5 µL of reverse primer (10 µM), 1µL of template and 12.5 µL of MM Gotaq Hot start Colorless Master Mix (Promega Corporation, Madison, WI, USA). The reaction

volume was adjusted to a total of 25 µL with ultrapure DNase/RNase free water. Thermal cycles consisted of an initial denaturation of 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec; 57°C for 60 sec; and 72°C for 90 sec; with a final elongation step of 72°C for 10 min. To prepare amplicons for sequencing, amplicon purification and normalization was done with Invitrogen SequalPrep Normalization Kit (Invitrogen Inc., CA, USA). Amplicons were combined into a single pool and sequenced using the Illumina MiSeq platform (BioFrontiers Institute, Boulder, Colorado) using pair-end 2x150 bp chemistry.

Sequencing and statistical analyses

Forward-oriented sequences were demultiplexed, quality filtered and processed using the QIIME pipeline (Caporaso et al. 2010b). Paired-end sequences did not work for these reads and only the read corresponding to the 1391F primer was used. This read was selected because it overlaps more with most sequences within the NCBI and SILVA databases (Darcy and Schmidt 2016). Singletons were excluded from further analysis and sequences with >97% SSU rRNA gene sequence similarity were clustered into an OTU via UCLUST. Representative sequences for each OTU were chosen for classification and the Silva 104 reference database was employed to assign taxonomy identification to each single OTU. Sequences were aligned with PyNAST (Caporaso et al. 2010a) and a phylogeny was built with the FastTree algorithm (Price et al.2009). OTU tables were rarified to 1488, the lowest number of sequences in a sample and were used to assess Alpha diversity and relative abundance of all taxa. In order to evaluate the significance of phylogenetic differentiation across different conditions, pairwise distance matrices based on weighted Unifrac (Lozupone and Knight 2005) were generated for the entire communities. A one-way Analysis of Similarity test (ANOSIM) from the R vegan package (Oksanen et al. 2013) was used to test significance of difference in Beta diversity between untreated samples and treatments (freeze-thaw and freeze-thaw

+ water addition). Additionally, Principal Coordinate Analysis (PCoA) ordination was constructed based on both OTU tables and weighted Unifrac distance matrixes in order to visualize differences among community compositions of treatments among samples.

Culture isolation

To obtain isolates approximately 0.05 grams of soil that had been exposed to 48 days of freeze-thaw cycling (as described above) were streaked onto agar media containing 4 g of Llullaillaco soil; 1 g of KH_2PO_4; 1 g of $MgSO_4$ $7H_20$; 0.04 g of NH_4NO_3; 0.1 g of Carboxymethyl-cellulose; 0.05 g of Chitin; 0.1 g of Tryptone; 0.1 g of yeast extract and 15 g of agar per liter of water. The pH of the medium was 5.5 and all cultures were incubated at 6°C. Isolates were subcultured several times to obtain pure cultures.

Growth rates

In order to establish the optimal growth temperature of the *Cryptococcus* isolate, subsamples of the isolated cultures from liquid medium were grown at different temperatures. Three replicates were used at each temperature tested (-6, -2, 4, 10, 16, 22, 27°C). All inoculums (grown at 6°C) were taken from the same tube to ensure that the yeast used for all the temperature treatments were at the same metabolic state at the beginning of the experiment. Growth was measured through absorbance at 630nm to assess cell density changes in a fixed volume. Absorbance was measured in a 96-wells plate using Synergy HT Multi-Detection Microplate reader (Biotek, Winooski, Vermont). Correction was made for negative controls, which consisted of sterile media. Data were fitted using an exponential growth function (Darcy and Schmidt 2016) using Kaleidagraph (Synergy Software, Reading, PA) and the maximum specific growth rate (μ) was determined for each growth temperature. Growth of the *Cryptococcus* isolate in liquid medium was also tested under freeze-thaw cycles following the same cycle program described above.

Growth data at different temperatures were fitted with a simplified form of the integrated Schoolfield equation (Schmidt et al. 2008b, Schoolfield et al. 1981) to test if growth cultures follow Arrhenius temperature responses (growth rate is exponentially proportional to temperature) at low temperature and growth inhibition at high temperatures.

Cryptococcus *sequencing and phylogenetics*

Biomass for sequencing isolates was grown on agar plates in permissive medium (as described above). The small subunit ribosomal DNA (SSU) was amplified directly from individual yeast colonies without prior DNA extraction using 4Fa-Short (5'-ATCCGGTTGATCCTGC-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was performed with the AmpliTaq Gold® 360 kit (Thermo Fisher Scientific, Waltham, USA) following manufacturer's protocols. The PCR profile was: 95°C initial denaturation for 10 min; 35 cycles of 95°C, 1 min; 53°C, 30 sec; 72°C, 2.5 min; with a final elongation step of 72°C for 10 min. PCR products were cleaned using the QIAquick Gel Extraction kit (Qiagen Inc., Valencia, USA) according to manufacturer's protocols and sent to Functional Biosciences (Madison, WI, USA) for subsequent sequencing using the T7 and M13 primers. The sequences were edited into contigs using Geneious 5.4.6 (Drummond et al. 2011). Edited sequence was identified with a National Center for Biotechnology Information (NCBI) BLASTN search and the sequence was a match (100%) to an uncultured eukaryote clone (JX099190) from Lynch et al. (2012). To place the yeast in phylogenetic context, published sequences from Lynch et al. (2012) and sequences from 2 outgroup taxa were downloaded from NCBI and aligned using the MUSCLE algorithm (Edgar 2004) in Geneious 5.4.6 (Drummond et al. 2011). Sequences were clustered with select database guide sequences into 97% identity operational taxonomic units (OTUs) using the average neighbor algorithm implementation in mothur (Schloss et al. 2009). The resulting alignment of 1879 sites and 21

sequences was used for Bayesian inference (BI) and Maximum Likelihood (ML) analyses. The BI analysis was performed in MrBayes version 3.2 (Huelsenbeck and Ronquist 2001) using the GTR+G+I model and default priors. The BI analysis was run to convergence for 5M generations with 25% burn-in and chains sampled every 1000 generations. Changes in temperature did not change tree topology or resulting posterior probabilities so the default of 0.1 was utilized. The ML analysis was conducted using a general time reversible (GTR) model of evolution with a gamma distribution (g) and a proportion of invariable sites (I) with PhyML version 3.0 (Guindon et al. 2010) in SeaView version 4.3.4 (Gouy et al. 2010). Node support was estimated using 500 bootstrap replicates.

Substrate utilization

For characterization of potential substrate utilization by the isolated *Cryptococcus* sp., Biolog YT microplates (BIOLOG®, Hayward CA) were used. *Cryptococcus* grown in permissive medium was pelleted and resuspended in sterile water. Plates were inoculated with 100 µL of *Cryptococcus* cell suspension and were incubated at 10°C for 4 days in the dark. Sterile water was used for control wells. After this period metabolism of each compound was measured by both visual inspection of a colorimetric indicator and through absorbance measured using a 96-wells plate using Synergy HT Multi-Detection Microplate reader (Biotek, Winooski, Vermont). Compounds with absorbance values 1.5 - 2 times greater than that of sterile water were considered as potential carbon sources.

Results

Community shifts during freeze-thaw cycles

An experiment was conducted to determine the effects of repeated freeze-thaw cycles on the structure of the eukaryotic community and the relative abundance of *Cryptococcus* phylotypes in the community. Initially, five replicate

soil samples from Llullaillaco showed high variability in community structure (Figure 3.2). In contrast, after 2 months of freeze-thaw cycling, microcosms held at 70% of water holding capacity (freeze-thaw + water) and at field moisture levels (freeze-thaw dry) showed significantly altered microbial communities that were dominated by a *Cryptococcus* phylotype (Figure 3.2). ANOSIM analysis (Oksanen et al. 2013) showed that *Beta* diversity of the communities was significantly different between untreated microcosms and microcosms subjected to freeze-thaw cycles both for freeze-thaw + water and freeze thaw dry samples (with a Global R of 0.2 ($p < 0.05$) for both comparisons). The observed community shift was obviously driven by the complete dominance of *Cryptococcus* in both treatments. While these data indicate that *Cryptococcus* likely grew during the experiment, it is important to note that these are relative abundance data and therefore are not definitive proof of growth.

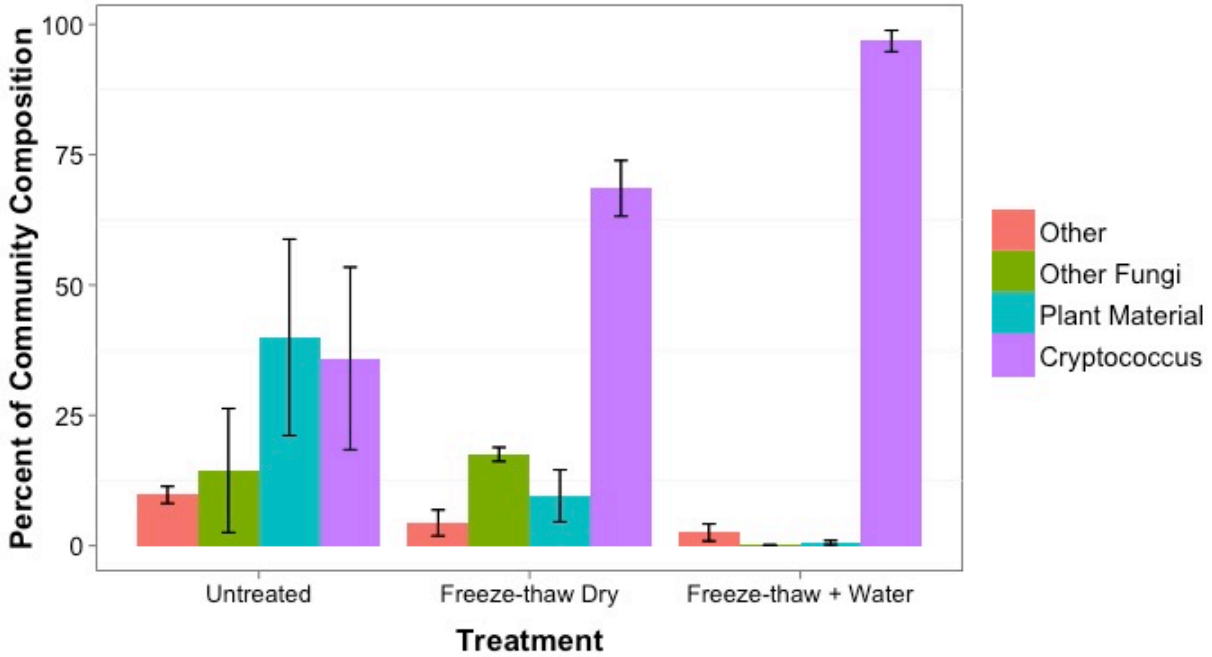

Figure 3.2. Relative abundance of eukaryotic phylotypes after 48 days of freeze-thaw cycles, based on PCR amplifications of the 18S rRNA gene using Euk1391f/EukBr primer sets. The microbial community differed significantly between untreated and freeze-thaw dry samples and between untreated and freeze-thaw + water samples (ANOSIM, Global R of 0.2 ($p < 0.05$) for both comparisons). Plant material represents Aeolian deposited material because no plants grow at elevations above 5000 m.a.sl. on Llullaillaco and these soils were collected at elevation of 6030 m.a.s.l. Each bar is the mean of four replicate microcosms (error bars are standard error of the mean).

Phylogenetics and growth of Cryptococcus *cultures*

The yeast isolated from Llullaillaco after 48 days of freeze-thaw had the identical long-read 18S (1879 sites) as the dominant phylotype from Volcán Llullaillaco (Genbank # JX099190). These sequences indicate that the isolated yeast clusters within a *Cryptococcus* clade and its most closely related organisms are *C. friedmanni*, *C. albidus*, *C. antarcticus*, *C. bhutanensis* and *C. vishniacii*. This clade contains members that have been found only at high altitude or high latitude with the exception of *C. albidus*, that has been found also in more temperate sites (Figure 3.3).

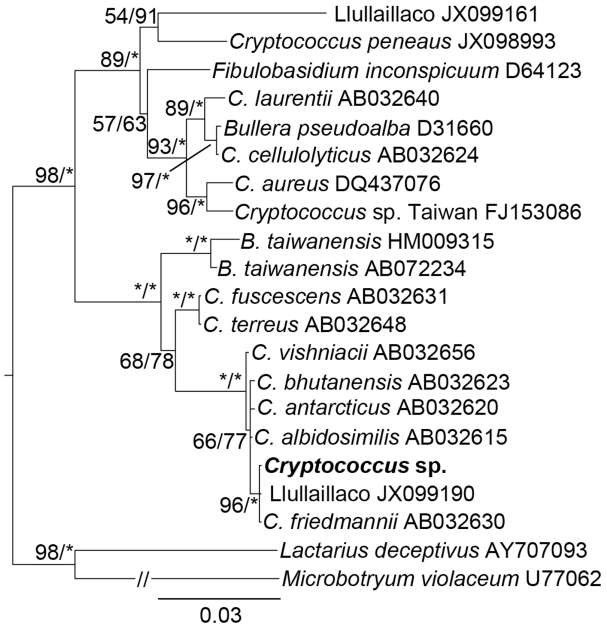

Figure 3.3. Bayesian consensus tree of SSU data from environmental sequences from Llullaillaco volcano (JX099161 and JX099190) and representatives of Cryptococcus *isolates including the* Cryptococcus *used in this study (in bold type). Node support is given as maximum likelihood bootstrap values (500 bootstrap replicates) / Bayesian posterior probability (as a percent). Note that the closest phylotypes to the* Cryptococcus *isolate used in this study are an environmental sequence (JX099190) from Volcán Llullaillaco and* C. friedmannii *(AB032630) from Antarctica. Data are from this study, Schmidt et al. (2012), and Lynch et al. (2012).*

To determine if the *Cryptococcus* sp. isolate could grow during extreme freeze-thaw cycles, replicate cultures were subjected to the same freeze-thaw cycles described above. Under these conditions the culture grew at a rate of 0.013 h^{-1} (Figure 3.4). Experiments were also done to determine the optimal growth temperature of the isolate and to compare its growth rate at various temperatures to the rate under freeze-thaw cycling (Figure 3.5). The isolate was able to grow at the lowest temperatures tested (-2 and -6°C) but not at 27°C and the growth rate at

0°C was approximately the same as the rate under freeze-thaw conditions (Figure 3.5). The Schoolfield equation (Schmidt et al. 2008b, Schoolfield et al. 1981) showed that rates exhibited Arrhenius behavior (growth rate being exponentially proportional to temperature) at low temperatures and enzyme inhibition at temperatures above the optimal temperature of about 17°C (Figure 3.5).

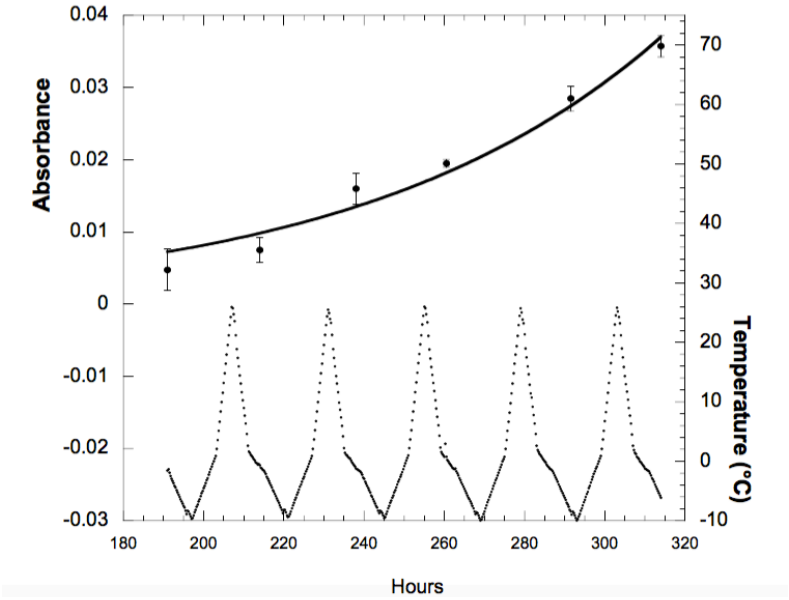

Figure 3.4. Exponential growth of the Cryptococcus *sp. isolate (top panel) during freeze-thaw cycles of 27°C to -10°C as measured with data loggers in the growth chamber (bottom panel). Temperatures in the actual growth medium (measured by thermocouple) were comparable to these in the chamber, but showed a lag of about 1 hour (data not shown). All cultures froze solid every night and completely melted every day. It is not clear from these data if growth was continuous or occurred only during periods of liquid water since all sub-samples were taken when cultures were melted. Each point represents the mean of four replicate cultures (error bars are standard error of the mean).*

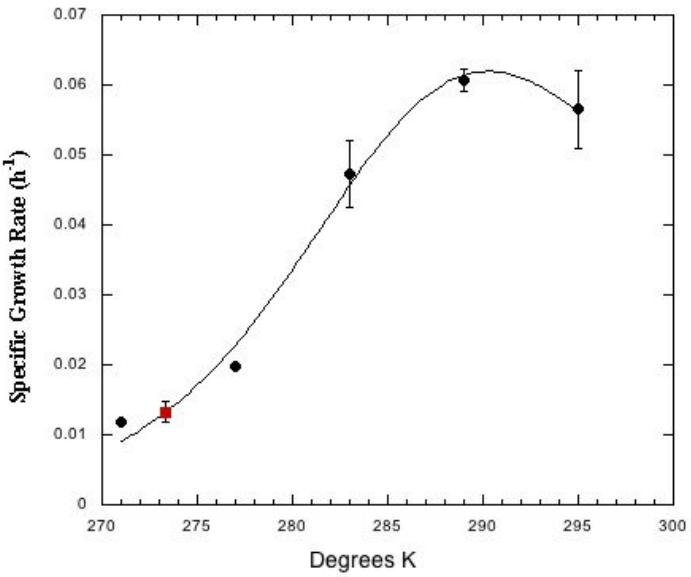

Figure 3.5. Temperature response curve for Cryptococcus *growth rates over a range of temperatures. The rates plotted are the maximum specific growth rate (μ with units of h^{-1}) observed at each temperature. The curve fit is a non-linear regression fit of the Schoolfield equation (Schoolfield et al. 1981) to the data. R^2 values for curve fit is > 0.98. Each point represents the mean of three replicate cultures (error bars are standard error of the mean). The red point represents the growth rate of* Cryptococcus *during freeze-thaw cycles (from the curve in Fig. 4). The x-axis is in degrees Kelvin and can be converted to degrees Centigrade by subtracting 273.15 from each value.*

Substrate Utilization

Table 3.1 shows the results obtained from 3 independent replicate experiments of substrate utilization by the *Cryptococcus* isolate using Biolog YT microplates (BIOLOG®, Hayward CA).

Table 3.1. Potential carbon sources used by Cryptococcus *sp. isolate measured with Biolog YT microplates*

Potential carbon source	Utilization
2-Keto-D-gluconic acid	++
Acetic acid	0
Acetoin plus D-xylose	+
Adonitol	++
Alpha-D-lactose plus D-xylose	++
Alpha-D-glucose	++
Alpha-keto-glutaric acid	+
Alpha-methyl-D-glucoside	++
Amygdalin	0
Arbutin	++
Beta-methyl-D-glucoside	++
Bromo succinic acid	++
D-Arabinol	+
D-Arabinose	++
D-Arabitol	0
D-Cellobiose	++
D-Galactose	+
D-Galactose plus D-xylose	+
D-Gluconic acid	++
D-Glucosamine	0
D-Glucuronic acid plus D-xylose	++
D-Mannitol	++
D-Melezitose	++
D-Melibiose	0
D-Melibiose plus D	+
D-Psicose	+
D-Raffinose	0
D-Ribose	+
D-Sorbitol	++
D-Trehalose	++
D-Xylose	++
Dextrin	++
Dextrin plus D-xylose	++
Formic acid	
Fumaric acid	++
Gamma-amino-butyric acid	+
Gentiobiose	++
Glycerol	++
i-Erythritol	0
Inulin	0
L-Arabinose	++
L-Aspartic acid	0
L-Glutamic acid	++
L-Malic acid	++
L-Proline	0
L-Rhamnose	++
L-Sorbose	0
m-Inositol plus D-xylose	+

Potential carbon source	Utilization
Maltitol	++
Maltose	++
Maltotriose	++
N-Acetyl-D-glucosamine	0
N-Acetyl-L-glutamic acid plus D-xylose	+
Palatinose	++
Propanediol plus D-xylose	+
Propionic acid	0
Quinic acid plus D-xylose	++
Salicin	+
Stachyose	0
Succinic acid	0
Succinic acid mono-methyl ester	+
Succinic acid mono-methyl ester plus D-xylose	+
Sucrose	++
Turanose	++
Tween 80	+

The *Cryptococcus* isolate was able to utilize many sugars and other plant-derived compounds including aromatic compounds (arbutin and salicin), a hydrolysable tannin (quinic acid) and plant-derived polymers (cellulose and dextrin) (Table 3.1).

Discussion

Field data from Volcán Llullaillaco and other nearby volcanoes show extraordinary temperature fluctuation faced by microbial life in these high elevation soils (Lynch et al. 2012, Schmidt 1999, Schubert 2014), but it is currently not known whether organisms found at these sites are able to grow there or if they are just dormant cells deposited by Aeolian processes. Therefore, the first goal of this study was to determine if the dominant eukaryotic organism found in these soils by previous workers (Costello et al. 2009; Lynch et al. 2012) could increase in relative abundance during freeze-thaw cycles similar to those that would occur just under the surface of the soil (4 cm depth). Exposure of soil microcosms to repeated freeze-thaw cycles at field moisture levels (freeze-thaw dry) and in the presence of added water (freeze-thaw + water) resulted in a significantly different community structure (ANOSIM) compared to untreated microcosms. The community shift was driven by *Cryptococcus*, especially in samples subjected to freeze-thaw cycles with added water (freeze-thaw + water). These results provide evidence that *Cryptococcus* has the capacity to grow during freeze-thaw cycles, and that it may be able to mainly do that during periods of higher soil moisture. Such periods of higher soil moisture do occur on Llullaillaco and other nearby volcanoes during snow-melt after infrequent snow storms despite the tendency for snow to sublimate directly back to the atmosphere at extreme elevations (Schubert 2014). At the same time, its prevalence also in freeze-thaw dry samples leads to the hypothesis that *Cryptococcus* may be partially active even at extremely low water content.

To confirm that *Cryptococcus* can grow during freeze-thaw cycles we isolated a strain of *Cryptococcus* from Llullaillaco soils and tested its ability to grow during freeze-thaw cycles in our freeze-thaw incubator. Before doing these experiments we confirmed that the isolate we used was a strong genetic match to the *Cryptococcus* phylotypes in soils from Llullaillaco. Figure 3.3 shows that the isolate used in our experiments is almost genetically identical to environmental sequences obtained directly from Llullaillaco soils by Lynch et al. (2012) and to *Cryptococcus* "species" isolated from Antarctic soils (*C. friedmannii, C. antarcticus, C. vishniacii*) and the Himalayas (*C. bhutanensis*). The close identity of all of these cryospheric strains of *Cryptococcus* is interesting in its own right and indicates that further phylogeographic work with these organisms may yield new insights into the functional significance of *Cryptococcus* in vast regions of the cryosphere. However, for the present study Figure 3.3 simply proves that we are working with a relevant strain of this organism because it is closely affiliated with environmental DNA sequences and isolates from extreme environments (1879 bp, 99% similarity).

The primary purpose of our study was to establish if *Cryptococcus* could actively growth under low temperatures and during extreme freeze-thaw cycles commonly observed at high elevation sites. Experiments with our isolated strain of *Cryptococcus* sp. showed that it can grow at the lowest constant temperature tested (-6°C), and even more surprisingly, that it could grow during freeze-thaw cycles with temperatures ranging from a high of 27°C to a low of -10°C (Figure 3.4). To our knowledge this is the first definitive demonstration of growth of an organism during repeated extreme freeze-thaw cycles and lends even stronger support to our hypothesis that *Cryptococcus* can function in high altitude environments where daily freeze-thaw cycles occur year-round. The fact that our strain can grow at temperatures below zero is also an indication that it may be adapted to life at high elevations, however many other studies have demonstrated the growth of a wide

variety of yeasts at sub-zero temperatures (reviewed in Buzzini et al. 2012), whereas this study is the first to show growth during extreme temperature cycles.

We conducted further experiments with this organism and determined that its temperature optimum for growth is about 17°C (Figure 3.5) and that it could not grow at a constant temperature of 27°C. It can therefore be classified as a psychrotroph rather than a true psychrophile (Morita 1975). Being a psychrotroph makes sense for an organism that experiences extreme temperature fluctuations in the field, while true psychrophiles would be better suited to the more stable temperature regimes such as in permafrost (Rhodes et al. 2013). There is also evidence that adaptation to oligotrophic conditions (e.g. nitrogen and carbon starvation) and other types of stress, including osmotic stress, may pre-adapt yeast to freeze-thaw tolerance (Park et al. 1997). In other words it is very likely that cross-resistance to many types of stress may pre-adapt cells to freeze-thaw stress in both Eukayra and Bacteria (Park et al. 1997, Leenanon and Drake 2001, Bang and Drake 2002, Dubernet et al. 2002). This sort of cross-resistance may be especially relevant to the extreme soils of Llullaillaco where high UV radiation, extreme desiccation, low nutrient availability and freeze-thaw cycles co-occur every day of the year at elevations above 6000 m.a.s.l.

Our experiments with varying temperatures and water levels (Figure 3.2 and 3.4) give a preliminary indication of how *Cryptococcus* may be able to function in extreme high elevation soils, but it is still unclear what substrates they have access to for carbon and energy in these extremely oligotrophic soils. The soils we have studied so far on Llullaillaco contain among the lowest carbon contents of any soils yet studied on Earth even lower than soils from the Dry Valleys of Antarctica (Lynch et al. 2012). However, Aeolian plant and animal matter is routinely transported from lower elevations to high elevations and can support simple ecosystems in plant-free, high-elevation ecosystems (Swan 1992). To survive in an

Aeolian-supported environment one might expect organisms that are generalist in their metabolic requirements so that they can take advantage of whatever nutrients come their way. Our preliminary work (Table 3.1) with *Cryptococcus* hints that it is indeed a generalist, as suggested before for this genus (Benham 1956), at least in terms of the breadth of carbon substrates that it can utilize for growth (Table 3.1). *Cryptococcus* is able to grow oxidizing sugars such as glucose, maltose, maltotriose, gentiobiose, cellobiose and trehalose and may therefore be able to grow in response to sugars released following breakdown of Aeolian deposited plant material. The freezing and thawing of soil may be key in increasing nutrients and carbon availability since it has long been known that freeze-thaw cycles damage and destroy microbial cells (Skogland et al. 1988) providing nutrients to surviving microbes. It is therefore possible that *Cryptococcus* also thrives thanks to nutrients and carbon released following freeze-thaw cycles. Uptake of trehalose may also help *Cryptococcus* adapt to anhydrobiosis. Trehalose accumulation is known to be a strategy employed by both bacteria (Welsh and Herbert 1999) and eukaryotes (Gadd et al. 1987) to cope with desiccation stress. *Cryptococcus* can also use plant aromatic compounds such as arbutin and salicin (Table 3.1) further indicating that it may depend on Aeolian deposited plant material. It is also relevant that our deep sequencing of the 18S gene at this site revealed the presence of exogenous plant material in the soils used in our soil experiment, but that this plant material was not detected after incubation in the presence of water, perhaps indicating that *Cryptococcus* was involved in the degradation of this material (Figure 3.2). In contrast, the inability of *Cryptococcus* to oxidize N-Acetyl-glucosamine (the monomer from chitin) suggests that this organism may not be involved in the breakdown of Aeolian deposited arthropods - another large source of carbon to many Aeolian habitats (Swan 1992). Overall, the assimilation profile of our isolate is very similar to that of the Antarctic yeast *Cryptococcus friedmannii*, whose 18S rRNA is

also the closest match to our isolate (Figure 3.3). For example, *C. friedmannii*, can also utilize cellobiose and Vishniac (1985) claimed that this trait distinguished it from all the other basydiomycetous yeasts. Vishniac (1985) proposed that *C. friedmannii* is a secondary consumer within cryptoendolithic communities, but to our knowledge there are no cryptoendolithic communities (or in fact any known phototrophs) in the volcanic glass soils of Llullaillaco and therefore our working hypothesis is that *Cryptococcus* on Llullaillaco are primarily supported by Aeolian plant material and not autochthonously produced organic carbon.

Conclusion

Taken together our data indicate that *Cryptococcus* species on Volcán Llullaillaco are well adapted to a life in soils that undergo extreme diurnal temperature fluctuations and exogenously supplied carbon compounds. During freeze-thaw cycles *Cryptococcus* was able to grow at a specific growth rate of 0.013 hr^{-1} that corresponds to a doubling time of 50 hours or about 2 days. This means that in the presence of some exogenous plant matter, *Cryptococcus* populations could potentially double in size every two days as long as snow melt water was available. At present we don't know how many such periods of water availability would occur per year at 6000 m.a.s.l., but climbers and scientists have observed such periods on Llullaillaco and other nearby volcanoes (Halloy 1991, Costello 2009, Schmidt 1999, personal observation 2009). Therefore, our working hypothesis is that *Cryptococcus* (and perhaps other soils microbes) are able to persist in the extreme soils above 6000 m.a.s.l. by increasing in numbers during rare times of water and substrate availability and then entering long periods of dormancy in between such events. More work is needed on the survival strategies and *in situ* dynamics of these hardy yeasts to confirm this hypothesis.

CHAPTER IV

NIEVES PENITENTES ARE A NEW HABITAT FOR SNOW ALGAE IN ONE OF THE MOST EXTREME HIGH-ELEVATION ENVIRONMENTS ON EARTH

By Vimercati L, Solon, A. J., Krinsky, A., Arán, P., Porazinska, D. L., Darcy, J. L., Dorador, C. and Schmidt, S. K. 2019.

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Abstract

Nieves penitentes are pinnacle-shaped ice structures found at high elevations in the dry Andes. Here we report, using molecular and microscopic approaches, the first description of snow algae communities inhabiting penitente ice at 5,277 m. a.s.l., demonstrating a new habitat for snow algae in one of the most extreme environments on Earth. Red ice patches on penitentes contained a microbial community dominated by algae in the genera *Chlamydomonas* and *Chloromonas*, both of which were closely related to known snow algae from alpine and polar environments. In contrast, we obtained few snow algae sequences from clear ice, but we did find cyanobacteria sequences and evidence of aeolian-deposited organic matter. Tephra ("soil") within and just downhill from the penitente field hosted more complex bacterial and eukaryotic communities that were significantly different from ice communities in terms of both alpha and beta diversity. In this environment penitentes provide both water and shelter from harsh winds, high UV radiation, and thermal fluctuations, creating an oasis in an otherwise extreme landscape. Intriguingly, recent planetary investigations have suggested the existence of penitente-like structures on other planetary bodies of our solar system. Therefore, penitentes and the harsh environment that surrounds them provide a new terrestrial analogue for astrobiological studies of life beyond Earth.

Introduction

Nieves Penitentes are surface ablation ice forms that are commonly found in the dry Andes at high elevations (above 4000 m.a.s.l.) (Lliboitry 1954, Matthes 1934) and were first mentioned in the scientific literature by Darwin (1839). The name "penitente" is a Spanish word (meaning "penitent one") that comes from the resemblance of a field of penitentes to a procession of monks in white robes doing penance (Betterton 2001). They form when snowfields are subjected to the unique combination of high radiation, low humidity and dry winds causing differential ablation that lead to the formation of spire-like ice pinnacles that can range in size from a few centimeters to over 5 meters in height. The key climatic condition that leads to their formation is that the dew point temperature is always below 0°C (Corripio and Purves 2005). Penitente initiation and morphogenesis requires cold temperature and once the process starts the surface geometry of the evolving penitente produces a positive feedback mechanism and radiation is trapped by multiple reflections among the walls (Corripio and Purves 2005). Thus, once they start to form, further growth in height can occur by melting downward of the wells between the spires (Bergeron et al. 2006).

The high-elevation desert areas where penitentes form are characterized by some of the most life-limiting conditions found on Earth, such as extreme solar radiation and dryness, dramatic temperature fluctuations, and a thin atmosphere (Lynch et al. 2012). The study of this harsh environment and penitentes has relevance to the field of Astrobiology since this environment is perhaps the best analogue for Mars-like soils on Earth (Schmidt et al. 2018) and penitentes have been detected on Pluto and other planetary bodies such as Europa (Hobley et al. 2013, Moores et al. 2017). Given the harshness of the environments where they are found, nieves penitentes may represent "oases" for life, because, along with

fumaroles, they represent intermittent water sources in these very arid environments (Costello et al. 2009, Solon et al. 2018).

There is currently no information on microbial life inhabiting these ice structures, although a preliminary study of volcanic soils (tephra) within two penitente fields indicated that they increased microbial diversity in tephra associated with a penitente field at 5277 m.a.s.l. on Volcán Llullaillaco, but not in a penitente field at 5825 m.a.s.l. on nearby Volcán Socompa (Solon et al. 2018). However, no previous studies have explored the microbial diversity in and on the ice of penitentes. The present study is the first sampling of life in penitente ice and was undertaken because we noticed snow algae like patches of red ice on the sides of some penitente spires and in the "wells" between penitentes on Volcán Llullaillaco in March of 2016. Here we show that these red ice patches were dominated by algae whose 18S rRNA gene sequences most closely match sequences of snow algae from other sites around the world. Additionally, we describe the total eukaryotic and bacterial microbial community found within these unique ice structures and in the tephra soils that receive meltwater from penitentes.

Materials and Methods

Site Location and Sample Collection

The penitentes field described here (Figure 4.1) is situated at 5277 m.a.s.l. on the Chilean side of Volcán Llullaillaco (24°43′40″ S, 68°34′20″W, 6739 m.a.s.l.), the second highest volcano in the world. All penitentes fields on Llullaillaco occur above 5000 m.a.s.l., which is about the elevational limit for vascular plants on this mountain as well (Arroyo et al. 1988). The penitentes were roughly 1-1.5 m in height (Figure 4.1). A total of 7 samples were collected from the ice structures on March 12th, 2016: 2 samples from clear ice and 5 samples from visibly red patches (Figure 4.1). Ice samples were collected by scraping the ice wall with a sterile

stainless-steel spoon and were placed in sterile whirlpool bags. These ice samples were melted and filtered onto 0.22 µm Whatman Nucleopore membranes (GE Healthcare, Pittsburg, PA, USA) the same day that they were collected.

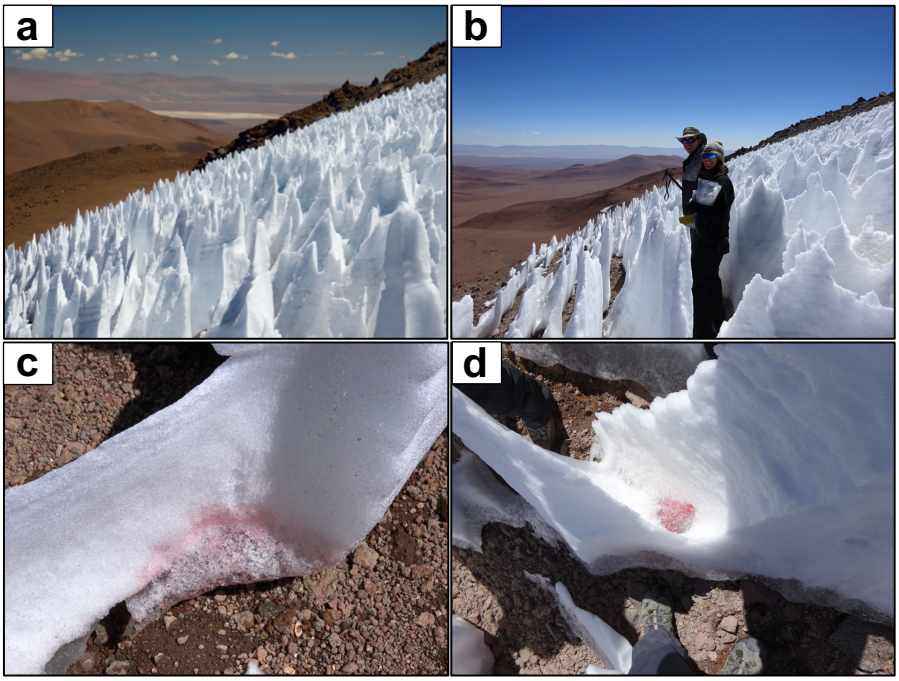

Figure 4.1. ***a*** *and* ***b****, Photographs of Llullaillaco penitente field at 5277 m.a.s.l, Jack Darcy and Lara Vimercati are pictured for scale in* ***b****.* ***c*** *and* ***d****, Close up pictures of red patches in penitente ice close to the tephra ground. Figures* ***1c*** *and* ***d*** *have been modified with Adobe Photoshop CS5 to enhance the color of the red patch. Input levels of image were adjusted using the quick selection tool to select the area of interest (red ice) and selective color percentages were adjusted by increasing magenta and yellow to make red ice stand out. Photos by G. Zimmerman and S. K. Schmidt.*

A total of 9 tephra samples were collected within and downhill from the penitente field; 5 samples from wet soils between the penitentes structures and 4 samples were from a horizontal transect 10 m down slope from the penitente field. All soil samples consisted of the top 4 cm of soil. All samples were collected with a sterile stainless-steel spoon and were placed in sterile whirlpool bags. They were stored on ice until the descent from the mountain and transported to Antofagasta where they were frozen at -20°C until they were shipped to University of Colorado

at Boulder where they were stored at -80°C until being processed as described below.

DNA extraction, PCR, and sequencing

Total environmental genomic DNA from samples collected within the penitentes field was extracted using PowerWater® DNA Isolation Kit for clear ice and filtered red ice samples and PowerSoil® DNA Isolation Kit for soil samples adjacent to penitents (MoBio Inc., Carlsbad, CA USA), following the manufacturer's instructions. Extracted genomic DNA was amplified using the oligonucleotide primer set F515 and R806 for Bacteria (Caporaso et al. 2012), and the 1391F-EukBr primer set for Eukarya. Both primer sets can be accessed from the Earth Microbiome Project website (http://press.igsb.anl.gov/earthmicrobiome/emp-standard-protocols). Amplified DNA was pooled, normalized to equimolar concentrations using SequalPrep Normalization Plate Kits (Invitrogen Corp., Carlsbad, CA, USA), barcoded and then sequenced using the Illumina MiSeq V2 pair-end technology (2x150 bp chemistry) at the BioFrontiers Sequencing Core Facility at the University of Colorado at Boulder.

Bioinformatics and statistical analysis

Raw 16S/18S rRNA gene sequences were demultiplexed, quality filtered and processed using the QIIME v1.9.1 bioinformatics package (Caporaso et al. 2010b). 16S rRNA gene paired-end reads were joined, but this process did not work for 18S rRNA gene reads due to insufficient overlap, so only the read corresponding to the 1391F primer was used (150 bp). This read was selected because it overlaps more with most sequences within the NCBI and SILVA databases (Darcy and Schmidt 2016). Singletons were excluded from further analysis and sequences with > 97% similarity were clustered into an OTU via UCLUST (Edgar 2010). Representative sequences for each OTU were chosen for classification and the GreenGenes 13.5 (DeSantis et al. 2006) and SILVA Ref NR 97 database (Quast et al. 2012) for 16S

and 18S rRNA gene sequences, respectively, were employed to assign taxonomy identification to each single OTU. All mitochondrial and chloroplast OTUs based on this classification were removed from the bacterial data set and all bacterial OTUs were removed from the eukaryotic data set. All sequences belonging to the Enterobacteriales order, a lineage commonly seen in the gut, were also removed from the dataset as likely reflecting lab or sampling contaminants. The taxonomic assignments of the top OTUs from each habitat were verified by using BLAST to search NCBI, and refined as needed. Sequences were aligned with PyNAST (Caporaso et al. 2010a) and a phylogeny was built with the FastTree algorithm (Price et al. 2009). OTU tables were rarefied to the lowest number of sequences in the lowest populated sample to make comparisons more robust and were used to assess alpha diversity and relative abundance of all taxa. Rarefaction curves, which plot the number of OTUs as a function of the number of samples, were also calculated using the QIIME v1.9.1 bioinformatics package. Alpha diversity significance was determined by a one-way analysis of variance (ANOVA) with function AOV. A community-level distance matrix with pairwise weighted Unifrac beta-diversity values (Lozupone and Knight 2005, Lozupone et al. 2007) was generated and analyzed using an ADONIS model (Anderson 2001, Oksanen et al. 2013) to partition the variance in community composition. Test of homogeneity of multivariate dispersion (PERMDISP) was determined with function BetaDisper to evaluate whether the difference supported by ADONIS was due to differences between clusters or differences between the variations within clusters. Principal Coordinate Analysis (PCoA) ordination was constructed based on weighted Unifrac distance matrices in order to visualize differences among community compositions of soil and ice samples. All statistical tests were performed using the Vegan package of R (Oksanen et al. 2013).

Nucleotide sequence accession numbers

16S rRNA gene sequences from Illumina MiSeq libraries determined by this study were deposited in the GenBank database under accession numbers MK242070 to MK243365, while 18S rRNA gene sequences were deposited in the SRA (Short Read Archive) database under Bioproject PRJNA530025.

Results

Alpha and beta diversity patterns

Alpha and beta diversity patterns were significantly different between ice and soil samples for both eukaryotic (18S) and bacterial (16S) communities. Rarefaction curves (Figure 4.2) show that soils within and close (10 m down slope) to the penitente field have significantly higher 18S and 16S OTU richness (alpha diversity) than ice (clear and red) communities (ANOVA $p < 0.001$, $F = 63$; $p < 0.001$, $F = 66.24$; respectively) (Figure 4.2a, 4.2b). Analysis of both 18S and 16S beta diversity showed clustering of communities based on habitat type (ice vs. soil) (ADONIS $p < 0.001$, $R^2 = 0.63$; $p < 0.001$, $R^2 = 0.46$; respectively) (Figure 4.3a, 4.3b). The test for homogeneity of dispersion was not significant for both 18S and 16S communities (PERMDISP $p > 0.3$, $F = 1.28$; $p > 0.2$, $F = 1.54$; respectively), showing that the main factor driving community clustering was the difference between habitat types rather than within habitat types.

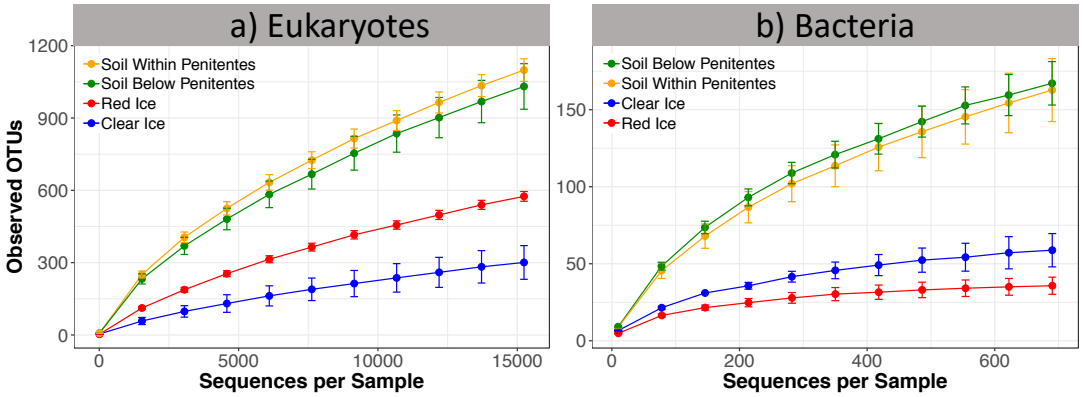

Figure 4.2. Alpha rarefaction curves based on the number of OTUs observed for penitente ice and soils within and below penitentes based on a 97% similarity clustering. ***a****, Eukaryotes and* ***b****, Bacteria. Soil within and below penitentes had 2x the number of eukaryotic OTUs and about 4x the number of bacterial OTUs compared to the penitente ice (18S ANOVA $p < 0.001$, $F = 63$; 16S ANOVA $p < 0.001$, $F = 66.24$). Data shown are means ± s.e. (Soil within Penitentes n=5; Soil below Penitentes n=4; Red Ice n=5; Clear Ice n=2). Note that the scale of the y-axis changes between the panels.*

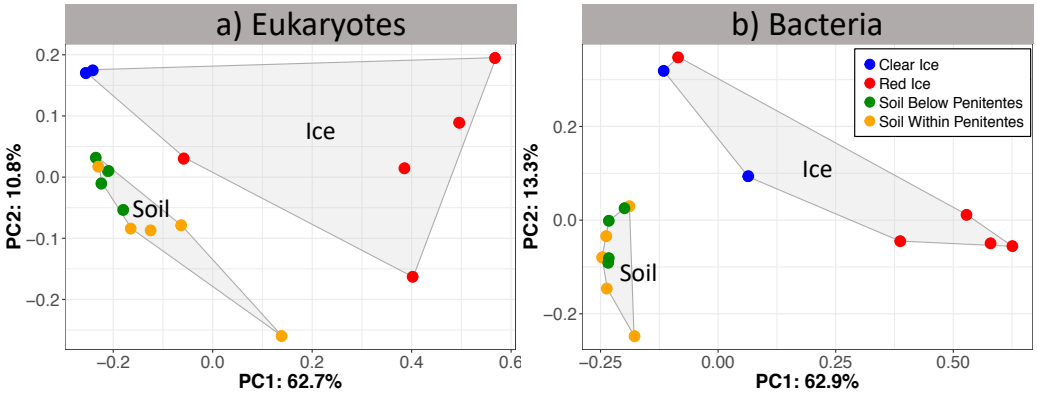

*Figure 4.3. Cluster diagram-based PCoA plot using weighted Unifrac of Eukaryotes (****a****) and Bacteria (****b****). Both eukaryotic and bacterial soil communities and ice communities are significantly different from each other (18S ADONIS $p < 0.001$, $R^2 = 0.63$; 16S ADONIS $p < 0.001$, $R^2 = 0.46$). Ovals surround total soil and total ice communities in both figures.*

Eukaryotic taxonomic composition

Red ice samples were dominated by algal sequences with 3 OTUs accounting for ~85% of total relative abundance (Figure 4.4). The most abundant algal OTU in red ice is a 100% match for the snow algae *Chlamydomonas nivalis* (Table 4.1) and its closest environmental matches were from a variety of globally distributed glacial environments including Mt. Kilimanjaro (KX771811, 100% identity; Vimercati et al.

2019a), snowpack in the Swiss Alps (KT184441, 100% identity), red snow from Svalbard (JQ790560, 100% identity), Antarctica (GU117587, 100% identity; Remias et al. 2010), Alaska (LC371441, 100% identity; Segawa et al. 2018), Greenland (AB902971, 98.6% identity), and the Pamir region of Tajikistan (AB903025, 100% identity). OTUs affiliated with *C. nivalis* are also found in soil samples within and below penitentes, albeit at lower abundance (32% and 5%, respectively). The second most abundant 18S OTU in red ice is distantly related to the genus *Chloromonas* (only 96% identity to *Chloromonas fukushimae* AB906342, Matsuzaki et al. 2014), suggesting it belongs to a previously uncharacterized new species or genus since most *Chloromonas* species share more than 99% identity in the hypervariable V3-V4 region of the 18S rRNA gene (Remias et al. 2018).

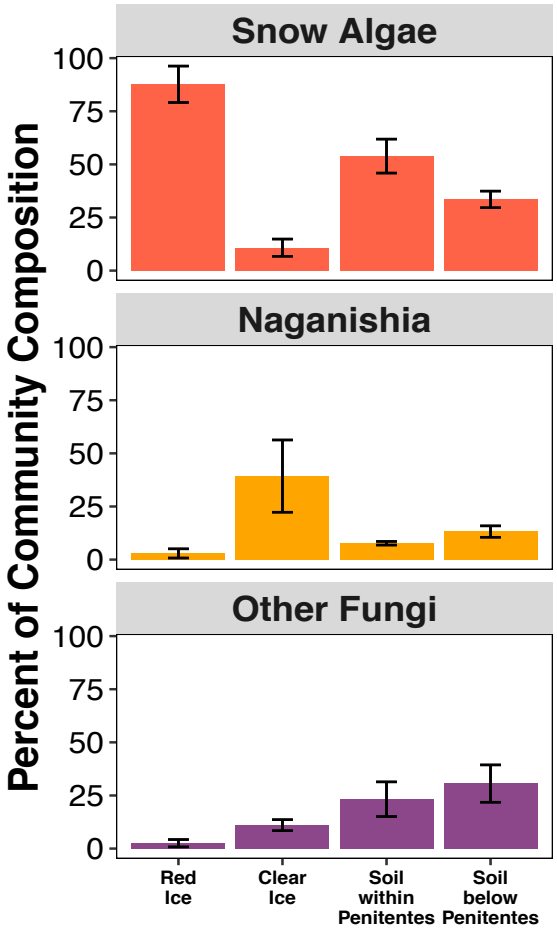

Figure 4.2. Relative abundance of the main eukaryotic phylotypes as a percentage of total sequences retrieved from penitente ice and soil samples based on PCR amplifications of the 18S rRNA gene using Euk1391F/EukBr primer sets. Snow algae make up most of the diversity in red ice samples and they are abundant in soil samples within and just downhill from the penitente field.

Table 4.1. Sequences count, GenBank ID of the closest Blast hit, identity % and tentative taxonomic affiliation of most abundant phylotypes retrieved in red and clear penitentes ice samples. Top: Eukaryotes; Bottom: Bacteria.

Eukaryotic Sequence Count

Red Ice	Clear Ice	GenBank ID	Identity%	Closest Blast Hit
8879.4 ± 1486	363.5 ± 1.5	JQ790560	100	*Chlamydomonas nivalis* (algae)
1355.6 ± 263.3	940 ± 345	AB906342	96	*Chloromonas* sp.*1* (algae)
1499 ± 1430	0	LC012710	95	*Chloromonas* sp. *2* (algae)
785 ± 642	4737 ± 3210	JX844984	99	*Periptyctus* sp. (insect)
415.4 ± 313	5682 ± 2429	AB032630	100	*Naganishia friedmannii* (fungi)
132.8 ± 118	249.5 ± 116.5	MG569511	100	Dothideomycetes (fungi)
0	588 ± 558	KX757779	99	Magnoliophyta (plant)
57.6 ± 40.31	292.5 ± 157.5	MH628292	100	*Deschampsia antarctica* (plant)

Bacterial Sequence Count

Red Ice	Clear Ice	GenBank ID	Identity%	Closest Blast Hit
313 ± 86	0	KR846626	77	Unassigned (uncultured bacterium)
0	73 ± 73	MF527140	100	*Microcoleus* sp. (Cyanobacteria)
1.8 ± 1.8	45 ± 45	JQ776471	100	Nostocales (Cyanobacteria)
14.6 ± 13.4	4.5 ± 1.5	MK041527	100	Pseudomonadales (γ-Proteobacteria)
9 ± 7.3	80 ± 60	MF670897	100	Chitinophagaceae (Bacteroidetes)
11.4 ± 10.4	37 ± 35	KP109895	100	Burkholderiales (β-Proteobacteria)
26.6 ± 14.6	11.5 ± 11.5	MH196469	100	Myxococcales (Δ-Proteobacteria)
2.2 ± 1.9	74 ± 72	MG642784	100	Bacillales (Firmicutes)

The closest un-cultured matches include glacial sequences from Antarctica (AB903009, 94% identity), the Tibetan plateau (DQ104086, 93% identity; Zhang et al. 2009) and Alaska (LC371418, 92% identity; Segawa et al. 2018). This novel algal OTU was also found in soils within and below penitentes, with a relative abundance of 16% and 20%, respectively.

The third most abundant algal OTU in red ice samples is only distantly related to *Chloromonas* sp. Hakkoda-1 with only 95% identity (LC012710, Matsuzaki et al. 2015), also suggesting it belongs to an undescribed new species. The closest environmental matches for this phylotype are from Pyrenean glacial ice (JX456233, 95% identity; García-Descalzo et al. 2013) and red snow in Antarctica (AB903008, 92% identity). This OTU is also found in soils within and below penitentes with a relative abundance of 6% and 8% respectively. Microscopic examination of red ice samples also revealed the presence of cysts resembling those of many snow algae (Figure 4.5).

Figure 4.5. Photomicrograph (1000x magnification) of a spore of a Chlamydomonas *sp. from red penitente ice.*

The 18S community composition of clear ice samples is significantly different from the community of the red ice and is dominated by a basidiomycete yeast related to *Naganishia friedmannii* (AB032630, 100% identity) (Figure 4.4) and an OTU with a 99% match for Coleoptera (JX844984, Robertson et al. 2013). Another relatively abundant fungal OTU is a 100% match to a Dothideomycete isolated from aerosol of a low-elevation vegetated region (MG569511).

Bacterial taxonomic composition

The most abundant 16S OTU (54% relative abundance) within red ice samples is unclassified and its closest match in the NCBI database is only at 77% identity (KR846626). This unclassified OTU was followed in decreasing abundance by OTUs within the Pseudomonadales, Actinomycetales, Burkholderiales and Rhodospirillales orders. In contrast, the most abundant OTUs in clear ice samples are mainly Cyanobacteria (Oscillatoriales and Nostocales orders; Table 4.1), Bacteroidetes (Saprospirales), Proteobacteria (Rhizobiales, Burkholderiales and Xanthomonadales), Firmicutes, Acidobacteria and Actinobacteria (Actinomycetales).

The dominant cyanobacterium was a 100% match for a *Microcoleus* sp. (Oscillatoriales) from a soil biocrust from Spain (MF527140, Muñoz-Martín et al. 2019) and the second most abundant cyanobacterium was in the Nostocales and is a 100% match with sequences from other cold environments, such as the High Andes (GQ306061, Schmidt et al. 2009), Antarctic soil crusts (MF468245), a Chilean glacier foreland (JQ776471, Raggio et al. 2012) and soils from the High Himalayas (HQ189094, Schmidt et al. 2011).

Soils within and below penitentes ice structures have closely overlapping communities dominated by OTUs within the Acidobacteria, Actinobacteria (Actinomycetales), Bacteroidetes (Saprospirales), Verrucomicrobia (Chthoniobacterales) and Chloroflexi (Ktedonobacteria). The most abundant Acidobacteria phylotype is also 100% identical to sequences detected in perennially cold environments such as the top of Mt. Kilimanjaro (KX771519, Vimercati et al. 2019a) and soil biocrusts from Antarctica (MF468273).

Discussion

Our analysis represents the first description of microbial life in ice structures known as nieves penitentes. These structures, commonly found in the driest parts of central and southern Andes, are of particular interest because they can be an intermittent source of melt water to tephra in one of the most extreme dry environments on Earth (Lynch et al. 2012, Solon et al. 2018). Penitentes are formed during the metamorphosis of snow beds (areas where windblown snow accumulates) into rows of evenly-spaced ice spires (Figure 4.1). As such, they represent a unique and previously unstudied environment for microbial life.

Our study revealed for the first time that snow algal communities similar to those in many other snow or ice environments are present in the ice of nieves penitentes at least in the penitente field at 5277 m.a.s.l. on Volcán Llullaillaco. The

most abundant 18S OTUs from red patches and in soils associated with the penitente field are snow algae with closest matches to the genera *Chlamydomonas* and *Chloromonas*. Although there have been no previous reports of snow algae in penitente ice, there have been descriptions of snow algae in snow and on glaciers in the Andes (Nedbalová and Sklenár 2008, Takeuchi and Kohshima 2004). In addition, Solon et al. (2018) previously reported the presence of snow algae in the tephra soils within penitente fields on Volcán Llullaillaco and nearby Volcán Socompa. Conversely, no snow algae (or any other algae) or cyanobacteria have been found in numerous surveys of dry tephra soils that make up most of the barren landscape at elevations above 5100 m.a.s.l. on both Llullaillaco and Socompa (Costello et al. 2009, Lynch et al. 2012, Solon et al. 2018). We therefore assume that the snow algae from the penitentes are the source of algae found in the soils within and near the penitentes. That is, snow algae have complex life cycles that include residence in soil for part of the year (Remias 2012) and their putative life cycle includes the formation of thick-walled cysts making them resistant to high irradiance, dryness, and freezing (Hoham and Duval 2001, Holzinger and Lütz 2006, Komárek and Nedbalová 2007, Remias et al. 2010). These and other attributes discussed below would likely allow them to survive in tephra associated with penitentes and on the ice surface of penitentes themselves. In addition, cysts of snow algae such as *Chloromonas nivalis* can be photosynthetically active (Remias et al. 2005, 2010), but it is not clear if they can photosynthesize outside of the snow environment in dry soils.

It is also of importance that the snow algae found on Volcán Llullaillaco are red in color. The red color is likely due to a suite of pigments including carotenoids (Müller et al. 1998, Remias et al. 2005), which shield the photosynthetic machinery from over illumination (Remias 2012) and act as a pool of antioxidative compounds (Grossman et al. 2004). These pigments dissipate excess energy as heat and red

pigments radiate higher amounts of heat compared to other colors (Dial et al. 2018). Interestingly, increased carotenoid production has also been linked by previous studies to freeze-thaw tolerance (Dieser et al. 2010). This may be especially relevant to the harsh environments of Llullaillaco where extreme thermal fluctuations occur every day of the year at elevations above 5000 m.a.s.l. (Lynch et al. 2012, Vimercati et al. 2016). Red snow algae also tend to inhabit drier, more nutrient-poor environments compared to algae of other colors (Lutz et al. 2014). Red snow is also indicative of a more mature carotenoid-rich resting stage, compared to green motile trophic stages (Lutz et al. 2014). We did not observe any green patches in the penitente field but that could be because we visited the site for only several hours: there may be green motile stages present at this site earlier in the development of penitentes. We also could not find any colored patches in penitentes structures above 5400 m.a.s.l. on either Llullaillaco or Socompa: higher elevations are colder and drier, possibly establishing an upper elevation limit to snow algae distribution (Dial et al. 2016), but more intensive surveys would be needed to verify this hypothesis. Interestingly, sequences of algae such as *Mesotaenium berggrenii* and *Ancylonema* sp., that are known to live on bare glacial surfaces (Remias et al. 2009) including ice fields in South America (Takeuchi and Kohshima 2004), were not found in the present study. This may be because these algae lack a motile stage in their life cycle and therefore are not able to migrate into transitory structures such as penitentes.

Our finding of microbial communities dominated by red-pigmented algae also brings up the intriguing question of whether these communities may be contributing to the formation of penitentes. Models incorporating physical processes have provided insight into penitente formation (Betterton 2001, Bergeron et al. 2006, Claudin et al. 2015), however, organic biomass, particularly snow algae, may play a role in penitente formation as has been suggested for the formation of other

snow/ice structures (Ganey et al. 2017, Lutz et al. 2014, Takeuchi et al. 2001). Snow and glacier algae can influence physical glacial surface processes by changing glacial albedo much like dust deposits (Takeuchi 2009). That is, snow algae can reduce surface albedo with reductions up to 80% and increase melting of surrounding ice (Dial et al. 2018, Lutz et al. 2014, Ryan et al. 2018). But it is unlikely that snow algae can migrate through ice and therefore it is more likely that they move up through the snow pack as the penitentes are morphing from snow to ice. Obviously more work is needed to determine at what point in the formation of penitentes the snow algae are present. It will take detailed field observations to understand the temporal dynamics of algal migration and penitente formation.

Eukaryotic community

The closest environmental matches of the most abundant algal OTU in red ice from penitentes and in soils between penitentes were related to snow algal phylotypes globally distributed in the cryosphere, including arctic, Antarctic, and alpine ecosystems. This finding lends further support to the concept of global distribution of red snow algae ecotypes throughout the cold terrestrial biosphere. A number of studies using next generation sequencing based on both ITS and 18S rRNA genes have elucidated the geographic distribution patterns of red snow algae in polar regions showing that genera such as *Chlamydomonas* are cosmopolitan. For example, algal blooms causing red snow in polar regions comprise mainly cosmopolitan phylotypes (Lutz et al. 2016, Segawa et al. 2018). Propagule distribution can explain their global distribution in the cryosphere; Brown et al. (2016) suggested that red snow algae may be readily dispersed across the large distances as their size (~15 µm in diameter) (Remias et al. 2005) is compatible with long-range aerial transport. Future studies combining both morphological data and sequencing of the ITS2 marker gene will be necessary to fully describe algal phylotypes found in penitentes. It has been shown that 18S rRNA gene amplicons

do not adequately identify taxa on the species level in several taxonomic groups (Xiao et al. 2014).

Red snow algae decrease in relative abundance in soil compared to red ice, especially in soils further from penitentes, giving way to organisms such as the basidiomycete yeast *Naganishia* in the soils below penitentes (13% of sequences). However, *Naganishia* is not as dominant as it is in nearby extremely dry tephra where it can make up 90% of the eukaryotic community (Lynch et al. 2012; Solon et al. 2018). Chytrids were also detected in soils within and below the penitente field, as noted for other moist high-elevation soils (Freeman et al. 2009). Chytrids are also found in association with snow algae (Brown, Olson, and Jumpponen 2015, Naff et al. 2013), but we did not detect any chytrids in our penitente ice samples.

Clear ice samples host a very different community than that seen in red ice and soil samples, with the two most abundant OTUs being matches for *Naganishia* and Coleoptera. *Naganishia* may be active within the penitentes in areas not dominated by algal blooms; alternatively, their high abundance may be a reflection of wind-transported cells from the tephra soils surrounding the penitente field. The second most abundant OTU, matching Coleoptera, likely reflects aeolian deposition of dead organic matter from lower elevation environments as has been shown for other low biomass, high-elevation environments, including tephra from a penitente field on Volcán Socompa (Solon et al. 2018), and clear glacial ice in the Dry Valleys of Antarctica (Sommers et al. 2019).

Bacterial community

Our molecular analysis revealed that cyanobacteria within the Nostocales and Oscillatoriales order comprise 28% of the total bacterial community in clear ice and they are also detected in soils surrounding penitentes, however at extremely low relative abundances (0.6-1.4%). This is the first report of cyanobacterial sequences in any high-elevation habitat investigated on Llullaillaco (cf. Lynch et al.

2012, Solon et al. 2018). On the other hand, cyanobacterial sequences were not retrieved in red ice patches. This may indicate that penitentes provide enough water for cyanobacterial communities to develop, but snow algae may outcompete cyanobacteria as primary producers in these environments because of their ability to colonize snow and ice thanks to their flagellated states, which are absent in cyanobacteria. It is known that cyanobacteria are relatively rare in melting snowfields and other non-stable cryosystems, possibly because they are unable to keep pace with constant losses in meltwater (Vincent 2000, Quesada and Vincent 2012). A broader scale study of penitentes fields will be necessary to further investigate the relative role of competition and priority effects in structuring the community of primary producers in penitente ice. Interestingly, the bacterial OTU with the highest relative abundance (54%) was unclassified according to GreenGenes and its closest match on NCBI was only at 77% identity. This may suggest that penitentes harbor novel bacterial diversity, however a long read sequencing approach will be needed to allow fine-scale phylogeny of these communities.

Astrobiological implications

Recent studies of high-elevation volcanic soils (tephra) of the Atacama region have made a strong case that this environment is perhaps the best earthly analogue for surface and near-surface soils on Mars (Pulschen et al. 2015; Schmidt et al. 2018; Solon et al. 2018). This is because high-elevation (>5000 m.a.s.l.) areas of the Atacama are subjected to the highest levels of UV radiation (Cabrol et al. 2014), the most extreme daily freeze-thaw cycles (Lynch et al. 2012), and one of the driest climates on Earth (Costello et al. 2009). This combination of extreme conditions has selected for some of the simplest microbial communities yet studied in a surface soil ecosystem on Earth (Lynch et al. 2012; Solon et al. 2018). Despite these extreme conditions, the study reported here shows for the first time that penitentes harbor

snow algae communities, at least up to an elevation of 5277 m.a.s.l. on Volcán Llullaillaco. This finding along with the presence of complex microbial communities in soils affected by penitente melt water indicate that penitentes are oases of life in the otherwise barren expanses of high elevation tephra on Volcán Llullaillaco. These findings are of importance to the field of Astrobiology because they add to our understanding of the cold-dry limits to life on Earth and because penitente-like formations have recently been discovered on Pluto (Moores et al. 2017) and perhaps on Europa (Hobley et al. 2013).

Conclusions and future studies

We employed high-throughput sequencing to characterize cryophilic communities dwelling in penitente ice structures and adjacent soils on Volcán Llullaillaco. Penitente ice supports the only known photosynthetic communities (mainly algae) in this harsh high-elevation environment. This work differs from previous work that showed the presence of algae in soils associated with penitentes (Solon et al. 2018), in two distinct ways: 1) by showing that snow algae are present in the penitentes themselves, and 2) that some of these algae are related to known snow algae. This first report of snow algae occurring in penitente ice opens the door to future work that will address the altitudinal limits of these communities. There are also intriguing unanswered questions relating to whether snow algae are contributing to the formation of penitentes or whether they just migrate into them while they are forming? Finally, the microbiomes of penitentes and the surrounding harsh environment deserve further study as extraterrestrial analogue systems. All of these unanswered questions will be a challenge to address given the remoteness and harshness of the stark landscapes in which penitentes occur.

CHAPTER V

RESPONSE OF AN EXTREMOPHILIC MICROBIOME TO INCREASED WATER AND NUTRIENTS IN THE HYPER-ARID SOILS OF A HIGH-ELEVATION ATACAMA VOLCANO: IMPLICATIONS FOR THE COLD-DRY LIMITS OF MICROBIAL LIFE ON EARTH

By Vimercati L., Bueno de Mesquita C. P. and. Schmidt S. K. 2019. *In preparation*.

Abstract

Soils on the world's highest volcanoes (> 5000 m.a.s.l.) represent some of the harshest ecosystems yet discovered on Earth. Microbial life in these environments has to cope with a complex interplay of parameters, such as a high UV flux, extreme diurnal freeze-thaw cycles, low atmospheric pressure and extremely low nutrient and water availability. Only a very limited spectrum of bacterial and fungal lineages seems to have overcome the harshness of this environment and may have evolved the ability to function *in situ*. However, these communities may lay dormant for most of the time and then spring to life only when enough water and nutrients become available during occasional snowfalls and aeolian depositions. We applied experimental water and nutrient amendments to high-elevation soils (> 5000 m.a.s.l.) from Volcán Llullaillaco in both the field and in lab microcosms to investigate how microbial communities respond when resource limitations are alleviated. The dominant taxon in these soils, the extremophilic yeast *Naganishia* sp., increased in relative abundance after water + nutrient additions in microcosms, and marginally in the field after only 6 days. Community structure of both Bacteria and Eukarya changed significantly with water and water + nutrient additions in the microcosms and taxonomic richness declined with amendments of water and nutrients. These results indicate that only a fraction of the detected community is able to become active under realistic favorable conditions, and that water alone can

dramatically change community structure. Given the harshness of this environment, our study sheds light on which extremophilic organisms are most likely to respond when favorable conditions occur and provides new insights into the types of organisms that can respond to nutrient and water pulses in extreme earthly environments and perhaps in extraterrestrial environments as well.

Introduction

Recent research indicates that some of the most extreme soil ecosystems on Earth are found at high elevations in the dry valleys and slopes of the high Andes (Costello et al. 2009, Lynch et al. 2012, Vimercati et al. 2016, Solon et al. 2018). In fact, the driest high mountains on Earth occur just to the east of the Atacama Desert, where numerous, massive stratovolcanoes rise majestically from the Puna de Atacama (Costello et al. 2009). Geographical barriers in this area restrict the flow of atmospheric moisture, which in turn results in some of the most inhospitable proto-mineral soils on the planet that contain nearly undetectable levels of soil water, organic carbon stocks, microbial biomass pools and microbial extracellular enzyme activities (Lynch et al. 2012). These sites are characterized by a thin atmosphere, high UV radiation, extreme aridity, low year-round air temperatures, and extreme diurnal soil temperature fluctuations (Costello et al. 2009; Lynch et al. 2012, Cabrol et al. 2014). The highest of Atacama volcanoes is Volcán Llullaillaco (6739 m.a.s.l.), the location of the highest archaeological sites on Earth (Reinhard 1999, Reinhard and Ceruti 2010) and some of the best naturally preserved mummies anywhere in the world (Wilson et al. 2013). The lack of decay of these mummies suggests that this environment may be too dry and cold for growth and activity of decaying microbes. Initial studies of the biogeochemistry and microbiology of the high slopes of Volcán Llullaillaco and nearby Volcán Socompa (Costello et al. 2009; Lynch et al. 2012, Solon et al. 2018) showed that the soils on

these volcanoes contained microbial communities of extremely low diversity, except in areas near fumaroles and penitentes (Costello et al. 2009, Solon et al. 2018, Vimercati et. al. 2019b) and had exceptionally low organic carbon content (0.005 to 0.017%) (Lynch et al. 2012). The functional nature of these communities remains debatable given the harshness of this environment and the low levels of biomass and diversity (Lynch et al. 2014). However, microbial communities are definitely functioning at sites that receive supplemental water and nutrients (from fumaroles and/or melting ice) at elevations even above 6000 m.a.s.l. (Solon et al. 2018).

High-elevation desert soils are also of great interest to the field of Astrobiology, as the present climate of Mars can be compared with that of a very high mountain on Earth (Graham 2004). Today the Martian surface is very cold, hyper-arid and experiences extreme thermal fluctuations. This was not always the case and Mars orbiters and landers have obtained evidence that liquid water existed on the surface of Mars, possibly as recently as 300,000 years ago (Boynton et al. 2009). Despite the presence of water in the distant past, it is likely that any existing - and past - microbial life on Mars would have to adapt to the conditions currently found on the surface. Extreme high-elevation systems such as the high Andes can therefore be a model system for potential life on Mars and their study can provide insights for the search of life on other habitable planets in our universe (Schmidt et al. 2018).

The true extent of the microbial growth and activity in extreme high-elevation environments is still unclear. Understanding factors that control the proportion of active microbes in the environment is critical to determine which microorganisms make a living in these harsh environments and are truly extremophiles and which are dormant propagules from elsewhere. It was generally assumed that low diversity and biomass of extreme cryophilic environments is due to lack of water (Borin et al. 2010, Cary et al. 2010, Costello et al. 2009, Lynch et al.

2012, 2014, Schmidt et al. 2011). However, recent studies have challenged this assumption by showing that nutrient additions to periglacial soils (> 5000 m.a.s.l.) in the high Andes of Perú dramatically accelerated microbial community succession (Knelman et al. 2014, Darcy et al. 2018). A few similar studies of soils of the Antarctic Dry Valleys also indicate that nutrients may be limiting to microbial activity and diversity in cold-dry ecosystems. For instance, a natural gradient in water availability did not produce predictable responses of bacterial community characteristics across local and regional scales in the Taylor and Wright Valleys (Van Horn et al. 2013), while later mesocosm experiments showed that nutrient additions had a much stronger effect on microbial activity and community structure than water additions (Van Horn et al. 2014). High elevation soils on Llullaillaco have some of the lowest nutrient levels ever measured in a terrestrial environment, which may indicate that these environments are more nutrient than water limited. It is possible that microbial activity can occur in this extreme environment during periods of transient water availability only if nutrients are available to support microbial growth.

A significant, but under-studied, trait of this harsh ecosystem is the daily fluctuation in temperature that drives diurnal freeze-thaw cycles (Lynch et al. 2012, Schmidt et al. 2009). The thin atmosphere combined with intense solar radiation creates freeze-thaw cycles that are among the most extreme yet observed for soils on Earth's surface. Daily temperature cycling across the freezing point is a key challenge for microbial growth and survival at high elevations and high latitudes. Previous studies have shown that freeze–thaw cycle frequency influences microbial communities in the cryosphere (Yergeau and Kowalchuk 2008) and limits net primary productivity in the extreme soils of the Dry Valleys of Antarctica, where daily fluctuations vary more than 20°C per day during the austral summer (Cary et al. 2010). Mineral soils at high elevation on Llullaillaco and other nearby volcanoes

can experience even more extreme diurnal temperature fluctuation than soils of the Antarctica Dry Valleys (Lynch et al. 2012, Schmidt 1999, Schubert 2014). In order to gain insight into how microbial communities shift and grow in response to freeze-thaw stress, recent work exposed soil microcosms to prolonged freeze-thaw cycles using a chamber that replicates field cycles with an amplitude of 36°C every 24 hours. That study revealed that the original soil communities significantly shifted upon repeated freeze-thaw cycling (Vimercati et al. 2016). This study also showed that the basidiomycete yeast *Naganishia freidmannii* (formerly *Cryptococcus* sp.), which is the dominant microeukaryote at high elevations on Volcán Llullaillaco, increased in relative abundance in soils subjected to freeze-thaw cycles when water was available and could grow relatively rapidly in pure culture during freeze-thaw cycles (Vimercati et al. 2016).

In the present study we build on the previous work of Vimercati et al. (2016) to characterize how microbial communities from arguably the harshest ecosystem on Earth respond under freeze-thaw stress when water and nutrient limitations are alleviated to gain insights into the taxonomic and functional diversity of active microbes under realistic favorable conditions. Specifically, we carried out experiments where we amended high-elevation soils (> 5100 m.a.s.l.) from Volcán Llullaillaco both in the field and in controlled microcosms in the lab to determine which community members respond to just water addition (simulating a snow melt event) from those that respond to water and nutrients (simulating snow melt combined with the presence of aeolian deposited nutrients).

We hypothesized that 1) based on our previous work, *Naganishia* would increase in abundance as water and nutrients first become available. 2) The microbial community would undergo significant changes in richness and structure during freeze-thaw cycles when both water and water + nutrients become available. Water + nutrient addition is expected to allow for a more pronounced change and

response of microorganisms than when only water is available. 3) Only a small fraction of the total community be able to be active under realistic favorable environmental conditions.

Materials and Methods

Soil microcosms manipulation

Soils previously collected at 5103 m.a.s.l. (March 8, 2016) from the slopes of Volcán Llullaillaco (24°44.043, 68° 34.573) were homogenized and added (13 g dry weight equivalent in each plate) to sterile microcosm plates (55 mm diameter Petri dishes, Fisher Scientific 8-757-13A). Microcosms were incubated in a temperature-controlled chamber (Model TH024-LT, Darwin Chamber Co.) with a light-dark cycle of 12 h, and freeze-thaw temperature cycling that mimics the thermal fluctuations experienced by field soils at a depth of 4 cm at high elevations on Llullaillaco (Lynch et al. 2012, Vimercati et al. 2016). Light in the chamber was supplied by full-spectrum fluorescent bulbs (Sylvania GRO-LUX® F15T8/GRO/AQ/RP, and Philips F32T8/TL741). Freeze-thaw cycles were programmed so that the rate of soil freezing did not exceed rates of soil freezing recorded near the field site from which the soils were collected (Lynch et al., 2012, Vimercati et al., 2016, Schmidt et al. 2017).

Treatments were randomly assigned to each of the microcosm plates yielding 4 replicates per treatment. Treatments were 1) sterile deionized water addition (+W) to simulate occasional snowfalls (70% water holding capacity, 24-26% H_2O) and 2) sterile deionized water + nutrients addition (+WN) to simulate aeolian deposition when water is available (inorganic nitrogen, phosphorus and organic carbon). Ammonium chloride (NH_4Cl), monopotassium phosphate (KH_2PO_4) and sucrose were used as sources of N, P, and C, at concentrations of 75 µg/g, 75 µg/g, and 100 µg/g, respectively. The concentrations used were based on preliminary laboratory experiments that determined that these amounts ensure an adequate

amount to overcome nutrient limitations without changing the ionic balance of the soil solution (Schmidt et al. 2012). In addition, we tested the cumulative effects of repeated wet-dry cycles (as would occur on the mountain) by simulating 3 discrete snowfall episodes in both water addition (+W) and water + nutrients addition (+WN) microcosms. Controls consisted of microcosm plates (4 replicates) that did not receive any nutrients or water but were incubated in the freeze-thaw chamber and were sampled after the 1st (C1) and 3rd (C3) snowfall events. 4 replicates of microcosm soils that were left completely untreated were representative of the starting community. The arrangement of the plates in the incubator was randomized every 3 to 4 days to account for minor variations in temperature and light within the chamber.

The first subset of soil microcosms (4 replicates per treatment) was destructively sampled at 5 days after the first water and water + nutrients addition (C1, W1, WN1). The remaining soil microcosms were left in the chamber for an additional 6 days before receiving another water addition (2nd snowfall event) to restore the amount lost due to evaporation in the chamber after the first simulated snowfall event (up to 70% water holding capacity). Preliminary experiments have shown that approximately 90% of added water is lost after a total of 11 days in the chamber. Microcosm plates were sealed with parafilm (Bemis Company Inc, Neenah, WI, USA) after the 2nd and the 3rd water additions in order to decrease water evaporation rates. The second subset of soil microcosms (2nd snowfall event) was destructively sampled at 9 days after the second water addition (W2, WN2). The remaining soil microcosms were left in the chamber for an additional 13 days before receiving another water addition (3rd snowfall event) and were destructively sampled 9 days after the third water addition (C3, W3, WN3). To summarize, a total of 36 samples were included in the experiment: 4 Starting soils, 8 Control soils, 4 +W (x3 simulated snowfall events), 4 +WN (x3 simulated snowfall events).

Environmental manipulation

The field experiment was conducted in March 2016 at a site about 100 meters elevation above basecamp on the west-facing slope (24°44.043, 68° 34.573, 5103 m.a.s.l.) of Volcán Llullaillaco. Square plots of about 0.5m x 0.5m were established as shown in Figure 5.1. Control and treatments were paired, with a total of 8 controls and 8 treatments. Controls were plots that received no amendment and treatments were plots that received 1) sterile deionized water addition (+W) and 2) sterile deionized water + nutrients addition (+WN). We used the same type and amount of nutrients as the microcosm manipulations described above. Plots amended with water and water + nutrients were sampled after 6 days after the initial amendments. Conditions were cloudless during the experimental period and soil temperatures at 4cm depth ranged from + 26.79˚C (± 1.77) to - 2.60˚C (± 0.43) within 24 hours (Figure A5.1).

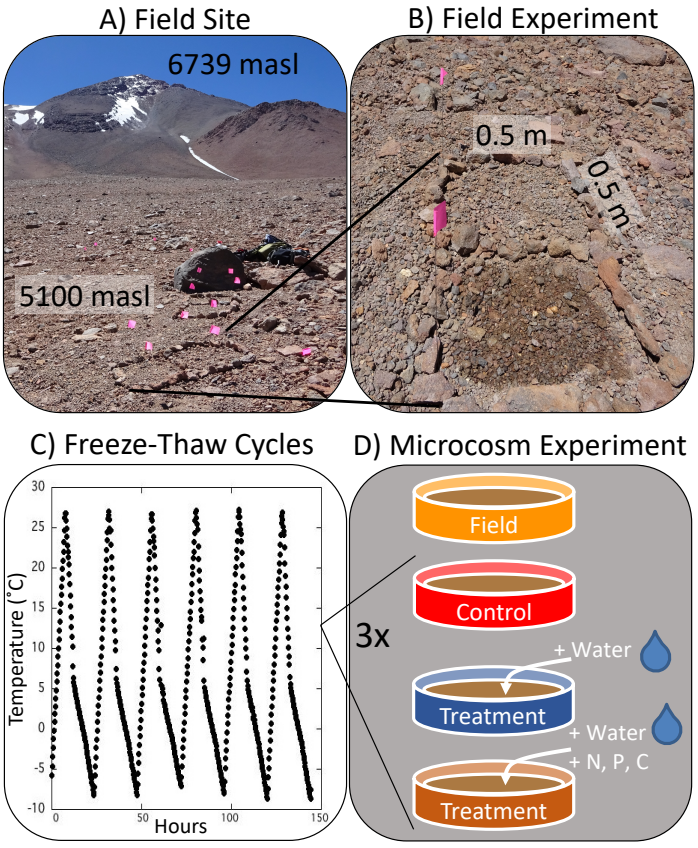

Figure 5.1. Study site and experimental design. a) Location of soil sampling site at 5103 m.a.s.l. on Volcán Llullaillaco (24°44.043, 68° 34.573). One of the false summits (~6400 m.a.s.l.) of Volcán Llullaillaco is visible in the background. b) Close-up view of one of the plots of the environmental manipulation experiment: the top plot is a control, while the bottom plot received water addition. c) Temperature cycling as recorded in the environmental chamber used in this study. The chamber was cycling up to a high of 27 °C during the day and a low of −9 °C at night with an amplitude of 36 °C in 24 h. d) Soil microcosm experimental design.

DNA extraction and sequencing

Total DNA was extracted with PowerSoil® DNA Isolation Kit (MoBio Inc., Carlsbad, CA, USA), following the manufacturer's instructions. For the amplification of the bacterial and archaeal 16S rRNA gene region we used the oligonucleotide primer set F515 and R806 (Caporaso et al. 2011), while for the amplification of the eukaryotic 18S rRNA gene we used the Euk_1391f/EukBr primer set (Earth Microbiome Project, accessible at http://www.earthmicrobiome.org /emp-standard-protocols/16S-18S/). Amplified DNA was pooled, normalized to equimolar concentrations using SequalPrep Normalization Plate Kits (Invitrogen Corp., Carlsbad, CA, USA), barcoded and then sequenced using the Illumina MiSeq V2 pair-end technology (2x150 bp chemistry) at the BioFrontiers Sequencing Core Facility at the University of Colorado at Boulder.

Data processing and statistical analysis

Forward-oriented sequences were demultiplexed, quality filtered and processed using the Quantitative Insights into Microbial Ecology (QIIME) v1.9.1 bioinformatics package (Caporaso et al. 2010b). 16S rRNA gene paired-end reads were joined, but this process did not work for 18S rRNA gene reads due to insufficient overlap, so only the read corresponding to the 1391F primer was used. This read was selected because it overlaps more with most sequences within the NCBI and SILVA databases (Darcy and Schmidt 2016). Singletons were excluded from further analysis and sequences with > 97% identity were clustered into OTUs via UCLUST (Edgar, 2010). Representative sequences for each OTU were chosen for taxonomic classification with the Greengenes 13.5 (DeSantis et al. 2006) and Silva Ref NR 97 databases (Quast et al. 2012) for 16S and 18S rRNA gene sequences, respectively. All mitochondrial and chloroplast OTUs based on this classification were removed from the bacterial data set and all bacterial OTUs were removed from the eukaryotic data set. The taxonomic assignments of the top OTUs from each

treatment were verified by using BLAST to search NCBI, and refined as needed. Sequences were aligned with PyNAST (Caporaso et al. 2010a) and a phylogeny was built with the FastTree algorithm (Price et al. 2009). OTU tables were rarified to the number of sequences in the lowest populated sample to make comparisons more robust and were used to assess alpha diversity and relative abundance of all taxa. Richness of OTUs was estimated for the rarefied 16S and 18S rRNA gene datasets in QIIME. We analyzed differences in OTU richness with a two-way analysis of variance (ANOVA) with the R function 'aov', including an interaction term, to test if water and/or water + nutrient additions significantly affected richness after multiple simulated snowfall events. We used Tukey's Honest Significant Differences (HSD) for *post-hoc* pairwise comparisons. A community-level distance matrix with pairwise weighted Unifrac beta-diversity values was generated and analyzed with permutational multivariate analysis of variance (PERMANOVA) corrected for multiple comparisons using the 'adonis' function (Oksanen et al. 2013) to partition the variance in community composition attributable to water versus nutrients addition. *Post-hoc* pairwise comparisons were generated with 'RVAideMemoire' R package (Hervé 2018) to ask how assemblage structures differed among treatments. We tested homogeneity of multivariate dispersion (PERMDISP) with the 'betadisper' function to evaluate whether the difference supported by PERMANOVA was due to differences between clusters or differences between the variations within clusters. Principal Coordinates Analysis (PCoA) ordination was constructed based on weighted Unifrac distance matrices in order to visualize differences among community compositions of treatments. Similarity percentage analysis (SIMPER) was used to determine the OTUs that contributed most to the observed dissimilarity among treatments. All statistical tests, unless otherwise stated, were performed using the "vegan" R package (Oksanen et al. 2013), in R version 3.6.1. (R Core Team 2013).

Results

Naganishia friedmannii *response*

Because our previous work showed that *N. friedmannii* increased in relative abundance after 2 months of freeze-thaw cycles (Vimercati et al. 2016), we wished to determine if this organism could respond on a shorter (more realistic to field scenarios) time frame (less than a week). *N. friedmannii* (100% match to AB032630) was the most abundant OTU in the starting soils of both the field and microcosm experiments (Figure 5.2) and significantly increased in relative abundance after only 6 days in the +WN microcosm (Tukey's HSD p =0.001), but not in the microcosm receiving only water (Figure 5.2). *N. friedmannii* also increased in the field experiment after only 5 days in the +WN plots compared the starting soil, but this increase was not significant (ANOVA, p = 0.10). In the microcosms, *N. friedmannii* did not continue to increase in relative abundance on subsequent water and nutrient additions and in fact decreased significantly compared to the control (C3 vs W3, Tukey's HSD p = 0.003) in microcosms receiving only water after the third addition (Figure 5.3). Due to logistical constraints, we were unable to resample the field experiment after the initial 6 day results reported above.

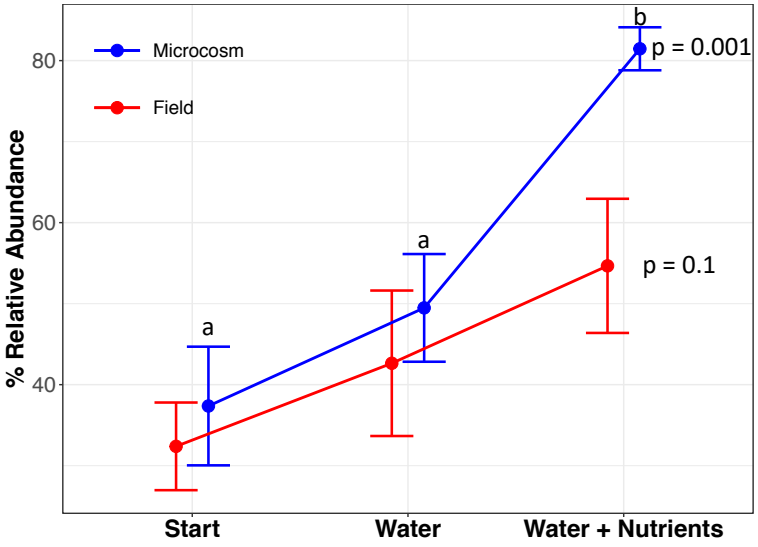

Figure 5.2. Mean (±SE) relative abundance of Naganishia *sp. among the treatments in the microcosms (after first water addition and first water + nutrient addition), and in the field (samples collected 6 days after water and water + nutrient additions). The relative abundance of* Naganishia *sp. increased significantly in the water + nutrient treatment in the microcosms (Tukey HSD, $p < 0.05$), and marginally in the field experiment (Tukey HSD, $p = 0.1$).*

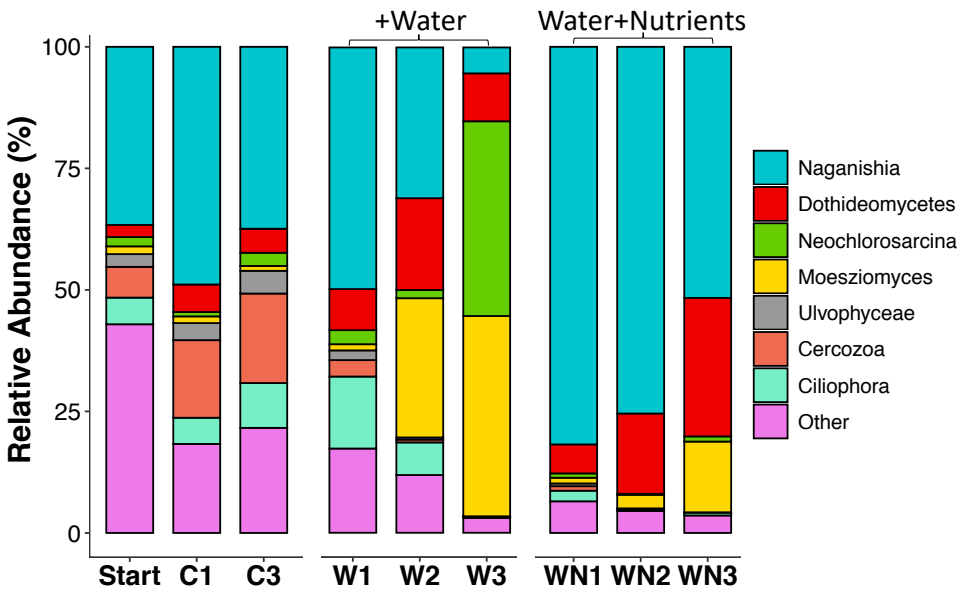

Figure 5.3. Taxonomy of eukaryotic community shift in response to W and +WN amendments. Stacked bar graphs showing relative abundances of dominant eukaryotic taxa (from 18S sequencing data). Taxonomy is from the Greengenes database. Bars represent means (n=4). Most of the "Other" category in the starting soil is plant material (Vimercati et al. 2016). A stacked bar graph of bacterial community shifts can be found in Supplementary Figure 1. Naganishia, Neochlorosarcina, Moesziomyces, *and Ulvophyceae are each represented by one OTU. Dothideomycetes are all in the Pleosporales order.*

Alpha and beta diversity patterns of soil microcosms

Alpha and beta diversity patterns were significantly affected by both water and water + nutrients additions under freeze-thaw cycles for both bacterial (16S) and eukaryotic (18S) communities. 16S OTU richness (alpha diversity) was significantly affected by the different treatments (ANOVA $p < 0.001$, $F = 7.5$) (Figure 5.4a). More specifically, *post-hoc* comparisons showed that both +W3 and +WN3 16S OTU richness was significantly lower than that of starting soil (Tukey's HSD $p < 0.02$; $p < 0.001$, respectively) and control 3 microcosm soils ($p < 0.01$; $p < 0.001$, respectively) (Figure 5.4a). 18S OTU richness was also significantly affected by the different treatments (ANOVA $p < 0.001$, $F = 19.1$) (Figure 5.4b). Specifically, *post-hoc* comparisons showed that OTU richness of all +W and +WN treatments was significantly lower than that of both starting and control 3 microcosm soils (Tukey's HSD $p < 0.01$) (Figure 5.4b).

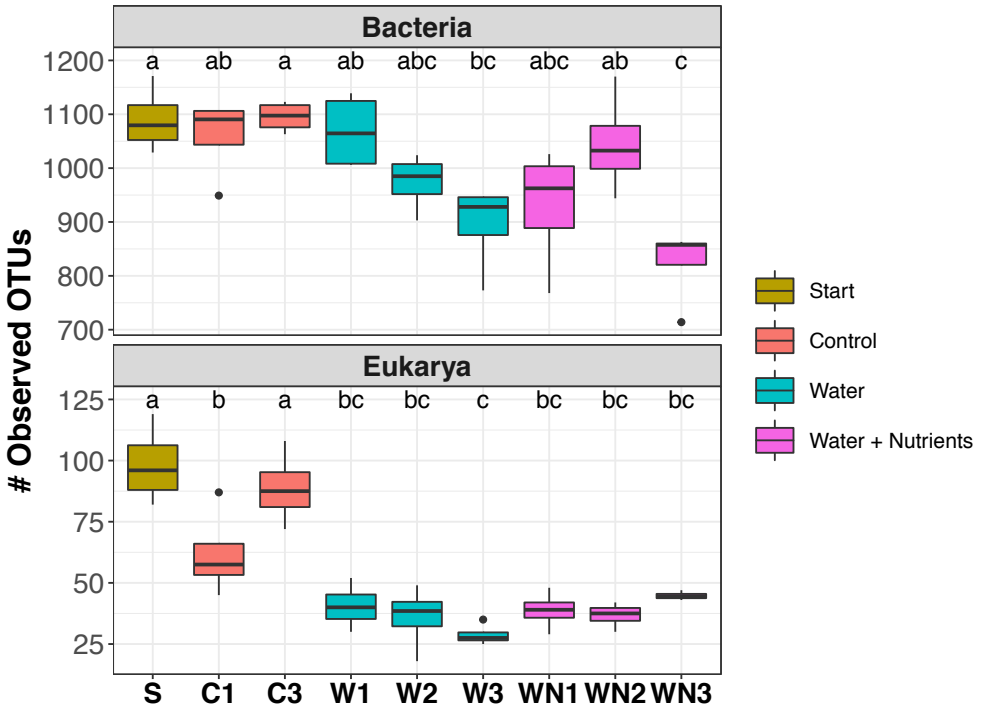

Figure 5.4. Alpha diversity (OTU richness) box plots for eukaryotic and bacterial communities. a) Bacterial community. Significant decrease in alpha diversity after 3rd water addition (Tukey HSD, $p < 0.05$). b) Eukaryotic community. Significant decrease in alpha diversity after 3rd water addition and in all water + nutrients additions (Tukey HSD, $p < 0.05$). The dark horizontal line inside the box represents the median. The long vertical black lines for each box represent the maximum and minimum values of OTU richness and circles represent outliers. Boxes with same letter are not significantly different. Different letters represent significant differences among treatments (Tukey HSD, $p < 0.05$). Note the difference in the y-axis scale between the two panels.

Analysis of both 16S and 18S beta diversity showed clustering of communities based on treatment (PERMANOVA $p < 0.001$, $R^2 = 0.75$; $p < 0.001$, $R^2 = 0.77$; respectively). More specifically, bacterial assemblages of all treatments were significantly different from each other except for starting and control microcosm soils, +W2 and +W3, and +WN1 and +WN2. Eukaryotic microbial assemblages of all treatments were significantly different from each other except for +W2 and +WN3. 16S PCoA ordination revealed that microbial communities are increasingly different from both starting and control soil microcosms with each water addition in both treatments (Figure 5.5). 18S PCoA ordination also showed a similar pattern (Figure 5.6). Microbial communities of starting soil microcosms clustered separately from those of control soil microcosms, showing that thermal fluctuation by itself affects

the community (PERMANOVA $p < 0.05$). The test for homogeneity of dispersion was not significant for 16S communities (PERMDISP $p = 0.078$, $F = 1.92$), showing that the main factor driving community clustering was the difference between treatments rather than within treatment. On the other hand, 18S communities from different treatments displayed significantly different within-treatment variance (PERMDISP $p < 0.05$, $F = 8.26$).

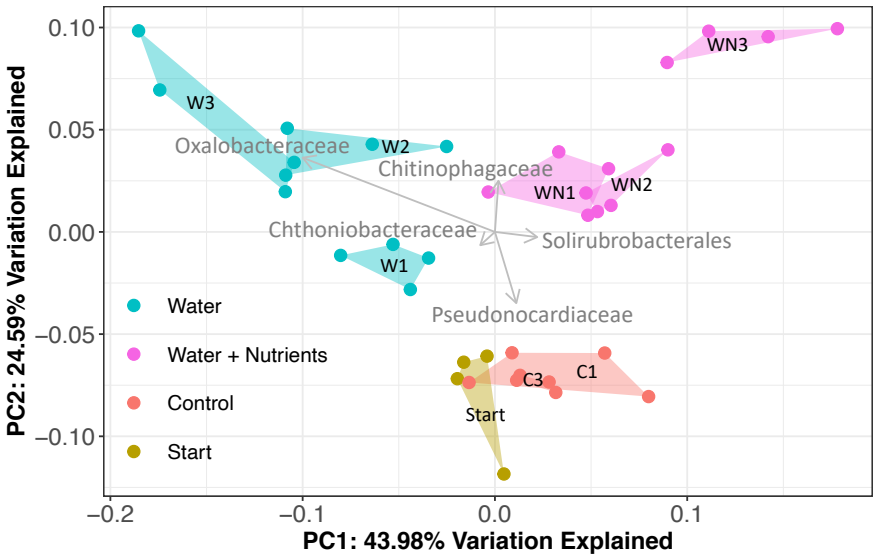

Figure 5.5. Principal Coordinates Analysis (PCoA) ordination plot of bacterial communities in soil microcosms. Repeated water additions on water and nutrients amended microcosms exposed to freeze-thaw cycles significantly changed the bacterial community (PERMANOVA $p < 0.001$, $R^2 = 0.74$). The vector length is proportional to the correlation between the PCoA axes and the OTUs that contributed most to the observed dissimilarity among treatments (SIMPER). Communities became increasingly different from controls following multiple amendments.

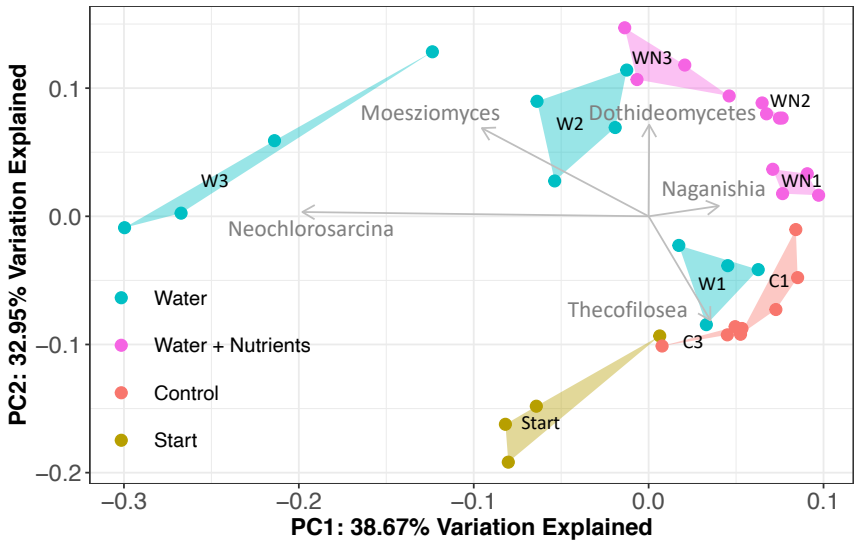

Figure 5.6. Principal Coordinates Analysis (PCoA) ordination plot of eukaryotic communities in soil microcosms. Repeated water additions on water and nutrients amended microcosms exposed to freeze-thaw cycles significantly changed the eukaryotic community (PERMANOVA $p < 0.001$, $R^2 = 0.75$). The vector length is proportional to the correlation between the PCoA axes and the OTUs that contributed most to the observed dissimilarity among treatments (SIMPER). Communities became increasingly different from controls following multiple amendments.

Taxonomic shifts of soil microcosms

Bacterial community response

Bacterial sequences in all treatments were dominated by 6 phyla: Actinobacteria, Proteobacteria, Bacteroidetes, Chloroflexi, Verrucomicrobia and Acidobacteria (Figure A5.2). The mean dissimilarity of the bacterial communities across all treatments was 35% (SIMPER analysis, Table 5.1). Pairwise comparisons between treatments had a mean dissimilarity range of 28-46%. Five OTUs accounted for most of the community compositional differences between treatments, overall contributing to 39% of total relative abundance. The top five OTUs that explained the most variance between each treatment pair were identified as members of the Oxalobacteraceae (Betaproteobacteria), Chitinophagaceae (Bacteroidetes), Solirubrobacterales (Actinobacteria), Pseudonocardiaceae (Actinobacteria) and Chthoniobacteraceae (Verrucomicrobia) (Figure 5.5). The Oxalobacteraceae OTU, whose closest match in the NCBI database was a *Noviherbaspirillum* sp. (KM260006, 100% identity), significantly increased in relative abundance to 9.4% on the third +W treatment compared to both control (0.7%) and starting (1.4%) microcosms (Tukey's HSD $p < 0.05$). The Chitinophagaceae OTU increased in relative abundance in +W (12.1%) and +WN (9.7%) treatments compared to starting soil microcosms (8.4%). The Solirubrobacterales OTU had the greatest increase in abundance in +WN treatments (13.6%) compared to starting soil microcosms (11.1%); however, they decreased in abundance with multiple water additions in +W treatments (9.2 to 5.1%). Pseudonocardiaceae declined in relative abundance with multiple water additions in both +W (4.2 to 0.9%) and +WN (2.9 to 1%) treatments compared to both starting (5.2%) and control (5.2%) soil microcosms. A number of additional OTUs explained variance among multiple water additions in the two treatments of this study: Chloracidobacteria significantly increased after the first water addition

to +W1 treatment (Tukey's HSD $p < 0.05$); Chloroflexi significantly declined with multiple water additions in +W treatments (Tukey's HSD $p < 0.05$); Actinobacteria within the *Phycicoccus* genus significantly increased (Tukey's HSD $p < 0.05$) in +WN3 treatment.

Table 5.1. Similarity percentage analysis (SIMPER) among microcosm treatments for 16S data.

Comparison	OTU ID	Phylum	Family	% Contribution	Cumulative %
Field - Water	denovo6723	Betaproteobacteria	Oxalobacteraceae	7.08	7.08
Mean = 35%	denovo7247	Actinobacteria	Pseudonocardiaceae	3.58	10.66
	denovo3383	Actinobacteria	Solirubrobacterales	2.82	13.48
DFT - Water	denovo6723	Betaproteobacteria	Oxalobacteraceae	6.82	6.82
Mean = 36%	denovo3383	Actinobacteria	Solirubrobacterales	3.87	10.69
	denovo7247	Actinobacteria	Pseudonocardiaceae	3.42	14.11
Field - Nutrients	denovo7247	Actinobacteria	Pseudonocardiaceae	3.57	3.57
Mean = 36%	denovo11840	Verrucomicrobia	Chthoniobacteraceae	3.00	6.56
	denovo8881	Actinobacteria	Phycicoccus	2.65	9.22
DFT - Nutrients	denovo7247	Actinobacteria	Pseudonocardiaceae	3.56	3.56
Mean = 35%	denovo7213	Bacteroidetes	Chitinophagaceae	2.89	6.45
	denovo15971	Bacteroidetes	Chitinophagaceae	2.86	9.31
Water - Nutrients	denovo6723	Betaproteobacteria	Oxalobacteraceae	7.01	7.01
Mean = 37%	denovo3383	Actinobacteria	Solirubrobacterales	4.72	11.73
	denovo11840	Verrucomicrobia	Chthoniobacteraceae	2.55	14.28

Eukaryotic community response

There was a profound shift in the eukaryotic community in both +W and +WN treatments (Figure 5.3). The mean dissimilarity of eukaryotic communities across all treatments was 60% (SIMPER analysis, Table 5.2) and pairwise comparisons between treatments had a mean dissimilarity range of 52-67 %. Six OTUs were the main drivers of the compositional difference among treatments, overall contributing to 74% of total relative abundance.

Naganishia friedmannii response to multiple water and water + nutrients additions was described above. Multiple water additions to +W treatments resulted in a significant increase of the fungal species *Moesziomyces antarcticus* (MH188429,

100%), whose relative abundance significantly increased (Tukey's HSD $p < 0.05$) in +W2 (29%) and +W3 (41%) treatments. By the third water addition, +W3 samples showed a significant increase (Tukey's HSD $p < 0.05$) in relative abundance (%) of the Chlorophyta *Neocholorosarcina negevensis* (MG022670, 100% identity). A fungal OTU within the Dothideomycetes class significantly (Tukey's HSD $p < 0.05$) increased in abundance with multiple water additions in +WN samples (5.3% in +WN1, 15.4% in +WN2, 17.2% in +WN3). This OTU is a 100% match with *Alternaria atra* (MH864090; Vu et al. 2019). Cercozoa within the Thecofilosea class significantly declined (Tukey's HSD $p < 0.05$) in all treatments. Two green algae OTUs within the Chlamydomonadales order were only detected in starting microcosm soils (5.7 and 8.5%) but were absent from all other treatments. The first of these OTUs was only distantly related to the genus *Ploeotila* (96.8% identity, HQ404867), while the second OTU was a 100% match for a *Chlamydomonas* sp. (LC380251, Nakada et al. 2019).

Table 5.2. Similarity percentage analysis (SIMPER) among microcosm treatments for 18S data.

Comparison	OTU ID	Phylum	Closest Taxonomy	% Contribution	Cumulative %
Field - Water	denovo259	Basidiomycota	*Moeszyomyces sp.*	16.76	16.76
Mean = 68%	denovo1264	Basidiomycota	*Cryptococcus sp.*	15.27	32.03
	denovo251	Chlorophyta	*Neochlorosarcina sp.*	10.29	42.32
DFT - Water	denovo259	Basidiomycota	*Moeszyomyces sp.*	18.29	18.29
Mean = 63%	denovo1264	Basidiomycota	*Cryptococcus sp.*	17.16	35.45
	denovo251	Chlorophyta	*Neochlorosarcina sp.*	11.39	46.84
Field - Nutrients	denovo1264	Basidiomycota	*Cryptococcus sp.*	29.33	29.33
Mean = 59%	denovo1929	Dothideomycetes	Pleosporales (order)	9.90	39.23
	denovo264	Chlorophyta	*Chlamydomonas sp.*	6.11	45.34
DFT - Nutrients	denovo1264	Basidiomycota	*Cryptococcus sp.*	28.04	28.04
Mean = 52%	denovo1929	Dothideomycetes	Pleosporales (order)	10.27	38.31
	denovo598	Cercozoa	*Thecophilosea sp.*	9.53	47.84
Water - Nutrients	denovo1264	Basidiomycota	*Cryptococcus sp.*	36.05	36.05
Mean = 59%	denovo259	Basidiomycota	*Moeszyomyces sp.*	17.79	53.84
	denovo251	Chlorophyta	*Neochlorosarcina sp.*	12.24	66.08

Environmental community response to water and nutrient additions

In the field experiment, a single application of water and water + nutrients did not result in a significant shift in the overall community composition for either Bacteria or Eukarya (PERMANOVA, $p > 0.05$). However, as discussed above, there was a marginally significant increase in the relative abundance of *Naganishia friedmannii* in the water + nutrient treatment (Tukey HSD, $p = 0.1$). In the bacterial community, an Oxalobacteraceae OTU showed a marginally significant increase in relative abundance (0.5% to 1.1%) significant (ANOVA $p = 0.09$). This was the same OTU that increased in relative abundance after multiple water additions in the microcosm experiment.

Discussion

In order to gain insight into how microbial communities of extreme soils in the high elevations of the Atacama respond to water and nutrients availability under environmentally relevant thermal fluctuations we performed manipulative experiments using tephra soils collected at 5100 m.a.s.l. on Volcán Llullaillaco. While our previous work showed that these sites are too dry to support microbial activity at ambient water availabilities (0.25% water) (Lynch et al. 2012, Schmidt et al. 2018), our work here addressed the question of whether members of the microbial community could respond during extreme freeze-thaw cycles to increases in water availability that occur after rare snowfall events and with and without nutrient addition.

Our study showed that both bacterial and eukaryotic communities were significantly affected by water and water + nutrient additions under field thermal conditions, which supports our second hypothesis (Figure 5.5 and 5.6). Given the extremely low abundance of nutrients in this soil, we predicted a greater response when both water and nutrients become available, but our experiment also shows

that a significant response is detected when just water first becomes available. Water alone, independent of nutrients availability, is therefore a key abiotic factor driving the response of the microbial community, as previously shown in the same environment but at higher elevation (Vimercati et al. 2016). Microorganisms inhabiting these soils may be able to take advantage of the low nutrients found in this environment as soon as there is water, which could explain why there is an effect on the community just by water addition or simply being placed in the freeze-thaw chamber: more nutrients become available in these hyper oligotrophic soils. We observed that a single application of water or water + nutrients was enough to cause a significant change in both bacterial and eukaryotic community structure. This result suggests that these communities, which have to rely on stochastic inputs of water and nutrients in their environment, have adapted to quickly respond when resources are available.

The community shifts seen in the microcosms were not seen in the field, which may be explained by the communities not having enough time to actively respond to the newly available resources. Cloudless conditions and extreme aridity may have caused the amended water to evaporate too fast for the community to take advantage of the water and nutrients pulse. Snowfalls are known to occur on Llullaillaco but little is known about the persistence of snow cover and it is possible that soil communities have water availability for a longer amount of time than that provided by our environmental manipulation.

Naganishia friedmannii *response in microcosms and in the field*

An OTU closely related to the basidiomycete yeast *Naganishia friedmannii* showed the highest increase in relative abundance after the first amendment with water and especially water + nutrients to the soil microcosms, which supports our first hypothesis. This *Naganishia* sp. is most closely related to the endolithic and xerotolerant *Naganishia friedmannii* isolated from the Dry Valleys of Antarctica

and high elevation sites of the Himalayas (Schmidt et al. 2012, Vimercati et al. 2016). *Naganishia* sp. is abundant in polar and high elevation environments (Buzzini et al. 2012) and it has been reported to exhibit polyextremophilic aptitudes (Sannino et al. 2017) that range from high resistance to dehydration-rehydration (Khroustalyova et al. 2019), extreme temperature fluctuations (Vimercati et al. 2016), exposure to UV, desiccation and stratospheric conditions (Pulschen et al. 2018). Its ubiquity in extreme high-elevation and high-latitude soils and its ability to withstand and grow under multiple stressors suggest that *Naganishia* sp. may be an important functional component of these extreme environments. Our soil microcosm experiment showed that *Naganishia* sp. is the first microeukaryote to take advantage of water + nutrient additions further lending support to the idea that this extremophilic yeast may be intermittently metabolically active in this barren landscape. Our working hypothesis is that *Naganishia* sp. is a versatile opportunist, able to persist in these extreme soils by increasing in numbers during rare times of water and substrate availability and possibly entering long periods of dormancy in between such events (Vimercati et al. 2016, Schmidt et al. 2017). *Naganishia* sp. also tended to increase in relative abundance in our field manipulation experiment when water and especially water + nutrients became available. These results suggest that *Naganishia* sp. may have increased in abundance during those 6 days when water and added nutrients were available and further lends support to the idea that this yeast is able to quickly take advantage of episodic increases in moisture and nutrients content in the environment.

Bacterial community response in soil microcosms

Oxalobacteraceae

Among the taxa that were mostly responsible for the community shifts we saw that Oxalobacteraceae within the *Noviherbaspirillum* genus significantly

increased in soil microcosms with multiple water additions. The Oxalobacteraceae family is very widespread in cryophilic and oligotrophic environments such as glacier fed-streams (Wilhelm et al. 2013), glacier forefields (Bajerski et al. 2013), cryoconites (Zhang et al. 2011) and high elevation periglacial soils (Vimercati et al. 2019a). This family is metabolically diverse and some genera are adapted to oligotrophic conditions (Baldani et al. 2014), which may allow them to use different aeolian deposited carbon sources. Some members of the *Noviherbaspirillum* genus have also been shown to be resistant to gamma radiation (Cheptsov et al. 2017) and close relatives of this OTU carry the *nif* genes (Baldani et al. 2014), which indicates that this OTU may make nitrogen available in this nutrient limited environment. Their lower relative abundance in soil microcosms that received both nutrient and water additions adds evidence for this group being oligotrophic and therefore being outcompeted by different phylotypes that show a faster response to carbon and nutrients alleviation.

Bacterial phototrophs

Cyanobacteria, which are found in very low abundance in these soils, did not respond to water amendments in either the field or microcosm experiments. Cyanobacteria have been found in the hyperarid low elevation soils of the Atacama (Wierzchos et al. 2015) and are associated with water sources such as fumarole and penitentes at high elevations on Atacama volcanoes (Costello et al. 2009, Solon et al. 2018; Vimercati et al. 2019b), but they are undetectable in the most extreme higher-elevation sites (6000 to 6300 m.a.s.l.) on Volcánoes Llulaillaco and Socompa (Lynch et al. 2012, Solon et al. 2018). Therefore, they may not be even transiently active in the soils studied here, but may instead be dormant propagules blown in from other environments.

Eukaryotic community response in soil microcosms

In addition to the response of *Naganishia* discussed previously, several other eukaryotes responded significantly to the resource amendments, especially after multiple treatments. A summary of these responses and information about these organisms is presented in Table 5.3.

Table 5.3. Summary of key fungal taxa, their responses to water and nutrient additions, their rate of response (fast = responded after first addition; slow = responded after multiple additions), their potential general evolutionary strategy, and their mechanisms of stress resistance from the literature.

Taxon	Organism Type	+Water	+Water+Nutrients	Increase Rate	Strategy	Mechanisms of stress resistance
Naganishia	Basidiomycete yeast	Neutral	Increase	Fast	r-selected	Dormancy
Moesziomyces	Basidiomycete fungus	Increase	Increase	Slow	K-selected	Cold-active enzymes, biosurfactants
Dothideomycetes	Ascomycete fungus	Increase	Increase	Slow	K-selected	Extracellular polymeric substances, melanin, compatible solutes
Neochlorosarcina	Alga	Increase	Neutral	Slow	K-selected	Extracellular polymeric substances, carotenoids, dormancy, motility

Dothideomycetes

Fungi within the Dothideomycete class significantly increased with multiple water additions in soil microcosms that were amended with nutrients. Many Dothideomycetes are known to be resistant to a number of extreme environmental conditions by developing avoidance strategies (Selbmann et al. 2015). For example, they can tolerate oligotrophy, repeated freeze-thaw stress (Onofri et al. 2007, 2008), radiation and dehydration (Onofri et al. 2012) by producing extracellular polymeric substances (EPS), melanin and compatible solutes. Many genera can form deeply melanized, meristematic colonies and produce extracellular polysaccharides to ameliorate UV and osmotic stresses (Selbmann et al. 2005). Their compact colony morphology helps minimize exposure to external stressors by decreasing their surface to volume ratio and appear to have optimized a slow but persistent growth strategy for extremely cold and barren habitats (Ruibal et al. 2009). They are characterized by simple life cycles that can be completed during short periods of time when favorable conditions prevail (Selbmann et al. 2015). Oligotrophy of the extreme environments where they are found and the high metabolic costs to

synthetize compounds related to stress resistance account for their slow growth velocity (Selbmann et al. 2014) and help explaining why the relative abundance of this taxon increases significantly only after the second and third water additions to the +WN treatments in this experiment.

Moesziomyces

An OTU closely related to the fungal species *Moesziomyces antarcticus* (formerly *Candida antarctica*) significantly increased in relative abundance with multiple water additions to soil microcosms, with the highest increase after the 3rd water addition (+WN3). This yeast was originally isolated from Lake Vanda in the Wright Valley, Antarctica (Goto et al. 1969) and produces cold-active enzymes and biosurfactants (Perfumo et al. 2018). Biosurfactants have been shown to have different functions such as anti-agglomeration effects on ice particles (Perfumo et al. 2018). Biosurfactants can also be part of cell envelopes and participate, together with other components such as exopolysaccharides, in protection against high salinity, temperature, and osmotic stress (Ewert and Deming 2013). Our study provides new evidence that along with its ability to withstand cold temperatures through cold adapted enzymes and other molecules, this yeast is also able to increase in relative abundance under extreme thermal fluctuations and very low nutrient contents.

Neochlorosarcina sp.

By the third water addition to +W microcosms we observed a significant increase in relative abundance of an OTU that was a 100% match with *Noecholorosarcina negevensis,* a member of the Chlorophyceae. This alga is commonly found in biological soil crusts of drylands (Büdel et al. 2016). Layers of algae within the Chlorophyceae have been identified as being the closest phototrophs to the soil surface within gypsum deposits at low elevations in the Atacama Desert (Wierzchos et al. 2015). Many Chlorophyceae have the capability to

synthesize very high amounts of complex molecules, such as carotenoids and EPSs, that enhance cell resistance under unfavorable environmental conditions including excess light, UV, water and nutrition stress (Hanagata and Dubinsky, 1999; Orosa et al., 2000, 2001). Other strategies for survival in harsh conditions include the development of dormant stages or avoidance through motility (Pushkareva et al. 2016). Previous work has shown that in arid environments, high relative humidity is enough to induce metabolic activity of some algae (Palmer and Friedmann 1990), however it is unclear whether these microalgae may be functioning *in situ* once water becomes available following a snowmelt event. More work will be needed to determine if *Neochlorosarcina* sp. is a functional phototroph in this environment. Of broader significance this is the first report of phototrophs on the high elevation slopes of Volcán Llullaillaco not associated with semi-permanent water structures such as fumaroles or penitentes (Costello et al. 2009, Solon et al. 2018, Vimercati et al. 2019b). This ecosystem, was previously thought to be completely heterotrophic or chemolithotrophic dependent (Lynch et al. 2014). It was believed that in this harsh ecosystem, energy entered through the system through aeolian deposited carbon sources and chemolithotrophic fixation of inorganic compounds; however, these new findings suggest that it may also enter the system via photosynthesis. However, it is important to note that this work was conducted using soils from 5100 m.a.s.l. whereas the studies mentioned above were conducted on soils from between 5300 and 6300 m.a.s.l. Therefore, more work is needed to determine if there is an elevational limit to phototrophic life not associated with penitents or fumaroles on these volcanoes.

Together, the results from the bacterial and eukaryote communities support our third hypothesis that only a small fraction of the community is active under favorable environmental conditions. Interestingly, there were more eukaryotic taxa

than bacterial taxa that responded to the resource amendments, suggesting that this is a eukaryotic dominated system.

Astrobiological implications

This work has implications for the prospect of life on Mars, which transitioned from an earlier wetter environment to today's extreme hyperaridity (Fairén et al. 2010). Recent studies have demonstrated that microbial activity can be sustained even in the driest soils of the lower Atacama Desert for very long periods of time (Schulze-Makuch et al. 2018), following an episodic increase in moisture. Our findings expand the range of hyperarid and cold environments temporary habitable for terrestrial life, which by extension applies to other planetary bodies, such as Mars. This transitory habitat with microorganisms active for short periods of time at the highest elevations of the Atacama can serve as a reasonable working model for Mars.

Conclusions

Life is nearly ubiquitous on Earth and microbial communities have been found in some of the harshest ecosystems. 16S and18S rDNA gene surveys have revealed their presence, however the activation and the actual metabolic status of microbial communities in these harsh environments remains poorly understood. Experimental manipulation is a key tool to reveal the potential of these communities and evaluate their response to less restricting conditions. We studied the response of a highly specialized extremophilic community from the high elevations of an Atacama volcano to episodic availability of water and water coupled with nutrients. Our study suggests that occasional snowfall events coupled with nutrients provided by aeolian deposition can provide a habitable environment for microorganisms that can become metabolically active in this harsh ecosystem. Along with a 16S and 18S rDNA survey our investigation provided new insights

into the question of which microbial extremophiles would potentially be active just in response to increased water and which need the additional input of nutrients. Only a small number of taxonomic groups drove the community shift and they likely reflect the active fraction of the community under favorable conditions. Determining the physiological state of microbial cells found in extremely dry and temperature fluctuating environments, specifically whether microorganisms are dormant or metabolically active, is of fundamental importance for the NASA Astrobiology Roadmap (Des Marais et al., 2008) that aims to characterize the boundaries of life in putative Earth analogues, and in particular the dynamics of organism survival and reproduction in conditions currently present on the surface of Mars.

CHAPTER VI

DRAFT GENOME SEQUENCE OF THE POLYEXTREMOPHILIC YEAST *NAGANISHIA FRIEDMANNI*

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2019. *In preparation*.

Abstract

Here we report the draft genome sequence of *Naganishia friedmannii* (formerly *Cryptococcus friedmannii*), a basidiomycete yeast that is commonly found in some of the most extreme environments of the Earth's cryosphere. We isolated *N. friedmannii* from soils > 6000 m.a.s.l. on Volcán Llullaillaco, Chile. The genome is 18.6 Megabases (Mb) long and contains 5,638 predicted protein-coding genes. The availability of the genome sequence is a valuable resource to gain insight into its polyextremophilic lifestyle.

Introduction

Naganishia friedmannii is an extremely stress tolerant basidiomycete yeast that has been proposed as a model organism for exobiology and studies on stress resistance in eukaryotes (Pulschen et al. 2015, Vimercati et al. 2016). This yeast is very abundant in some of the most extreme environments of the Earth's cryosphere (Buzzini et al. 2012) and its survival limits have been investigated in a number of studies demonstrating its high tolerance of UV-B and UV-C radiation, extreme temperature fluctuations, desiccation and exposure to the stratosphere (Pulschen et al. 2015, Vimercati et al. 2016, Pulschen et al. 2018). The analysis of its genome is important for understanding fungal genome evolution and stress adaptation.

We sequenced, assembled and annotated the genome of *N. friedmannii* and the characteristics of the genome and of the predicted proteome are described and discussed in light of its polyextremophilic nature.

Materials and Methods:

Growth conditions and DNA preparation

N. friedmannii was isolated from inorganic tephra soils collected at 6030 m.a.s.l. on Volcán Llullaillaco as described elsewhere (Vimercati et al. 2016). Genomic DNA was extracted from mid-exponential cells grown in a liquid medium at the optimal temperature of 17°C containing: 1g of KH_2PO_4; 1g of $MgSO_4$ $7H_2O$; 0.04 g of NH_4NO_3; 0.1 g of carboxymethyl-cellulose; 0.05g of chitin; 0.1 g of tryptone; 0.1 g of yeast extract per liter of water (Vimercati et al. 2016). The genomic DNA was extracted using a PowerSoil® DNA isolation kit (MO BIO, Carlsbad, CA USA). We assessed DNA quality and quantity by using the Qubit version 2.0 fluorometer with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific).

Genome sequencing and assembly

The genome was sequenced on an Illumina MiSeq instrument (250-bp paired-end reads) and assembled using SPAdes version 3.9.1 (Bankevich et al. 2012) with default settings. We assessed genome completeness using both fungi (fungi_odb9) and Basidiomycota (basidiomycota_odb9) databases with BUSCO version 3 (Simão et al. 2015, Waterhouse et al. 2017). All sequence reads generated and the genome assembly are available in the NCBI under the BioProject (PRJNA540513). Assembled contigs less than 300 base pairs (bp) were removed from the assembly. To remove bacterial contamination (in particular a *Pseudomonas* contaminate) in the assembly, a k-mer binning approach was taken as described elsewhere (Quandt et al. 2017). Briefly, tetramer frequencies were calculated for all 3,000 bp windows in the assembly. A physical representation of these frequency matrices was

rendered using an Emergent Self Organizing Map (ESOM) program, and best BLASTn (REF) based taxonomic assignment was overlaid on the map. The target region of the map containing contig windows that were mostly annotated as fungi and excluding the region annotated as *Pseudomonas*.

Gene prediction and annotation

Structural and functional annotation of the polished assembly was conducted using the Funannotate pipeline v1.5.2 (Palmer and Stajich 2017), which includes masking (Repeatmasker tool), *ab initio* gene-prediction training using Augustus (with *Cryptococcus neoformans* used as the training species) and Genmark, gene prediction, and the assignment of functional annotation to protein-coding gene models. Closely related Basidiomycota protein models were also provided to Funannotate as evidence for protein prediction (which, along with all commands used, are available here: https://github.com/Quandt-Mycology-Lab/Lab_Codes_and_workflows/tree/master/funannotate). Prior to running Funannotate "annotate" command, predicted sequences were annotated with eggNOG v4.0 (Powell et al., 2014) and Interproscan 5 (Jones et al., 2014). Secondary metabolite clusters were identified using antiSMASH v5.0. Finally, Funannotate "annotate" was run with default settings plus the output from these other annotation software programs, including PFAM (Finn et al. 2014), InterPro (Jones et al. 2014), UniProtKB (Apweiler et al. 2004), Merops (Rawlings et al. 2015) and CAZymes (Lombard et al. 2013) to assign functional annotations (Palmer and Stajich 2017). Genes from predicted proteins were mapped in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

A database of proteins of interest was constructed by collecting the protein sequences of the closest organisms for which the genome has been annotated from UniProtKB (Apweiler et al. 2004). These were used as queries in BLASTp (v2.5.0)

(Altschul et al. 1997) for the investigation of protein homologues in *Naganishia friedmannii*. The results were filtered according to the cut-off value < e^{-40} criteria.

Comparative Analysis

A BLAST all vs all proteome analysis with an e-value cutoff of < 0.0001 was conducted using the 'micropan' R package (Snipen and Liland 2018) to compare the full predicted proteome of *N. friedmannii* with seven proteomes of validly described members of the Tremellomycete class (*Filobasidium wieringae, Cryptococcus neoformans, Cryptococcus gattii, Kwoniella heveanensis, Naematelia encephala, Tremella mesenterica* and *Saitozyma podzolica*). PFAM protein family expansions and contractions of *N. friedmannii* were identified by performing a comparative analysis using the Funannotate pipeline v1.5.2 (Palmer and Stajich 2017) against nine fungal species known for their ability to tolerate similar environmental stresses as *N. friedmannii* or their close phylogenetic relationships (*Filobasidium wierigae, Cryptococcus neoformans, Cryptococcus gattii, Kwoniella bestiolae, Exophiala aquamarina, Hortaea werneckii, Rhodotorula toruloides, Rhodotorula graminis* and *Cryomyces antarcticus*).

Results and Discussion:

The assembled draft genome of *N. friedmannii* consisted of 18.6 Mb over 102 contigs with an average size of 183,229 bp. The size of the assembly is comparable with that of *F. wieringae* (19.8 Mb), the closest relative whose genome has been sequenced and annotated. The longest contig was 935,392 bp long and the N_{50} contig length was 386,378 bp. GC content was 51.08%, with a read depth of 350x. Analysis with BUSCO version 3 showed that genome assembly was 90.3% complete (C:90.3% [S:89.9%, D:0.4%], F:3.4%, M:6.3%, n:1335) compared to the Basidiomycota database and 93.4% complete (C:93.4% [S:93.4%, D:0.0%], F:1.7%, M:4.9%, n:290) compared to the fungi database. We identified 5,638 protein-coding genes using the

Funannotate pipeline v1.5.2 (Palmer 2016) and further evaluation of the predicted proteins revealed 242 putative carbohydrate-activating enzymes (CAZYmes) and 167 MEROPS associated predictions. For all the predicted proteins, 2468 genes were mapped in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Evaluation of the predicted proteome revealed the presence of a number of proteins known to play a central role in coping with environmental stressors (Table 6.1) and homologues of 9 "meiosis-specific" genes (Malik et al. 2008) (Spo11, Hop1, Hop2, Mnd1, Dmc1, Msh4, Msh5, Mer3, Rec8). Interestingly, sexual reproduction has not been observed in *N. friedmannii* or any of its closest relatives from Antarctica or the Himalayas (e.g. *N. antarcticus*, *N. bhutanensis*, *N. vishniacii*, Barnett et al. 2000). Additionally, some of the same genes required for virulence in the related pathogen *C. neoformans* (Brown et al. 2007) are also found in the genome of *N. friedmannii* (e-value < e^{-40}). These include genes involved in trehalose biosynthesis, the glutathione system, capsule biosynthesis, and melanin production, pointing to a possible connection between adaptations needed for virulence and extremophilism. Alternatively, these may be shared ancestral characters in the Tremellomycetes. A number of stresses faced by a pathogenic cell invading a host (Brown et al. 2007) are similar to those *N. friedmannii* encounters in its hostile environments. Stress tolerance and virulence may depend on various overlapping genes and molecular strategies.

Table 6.1. Summary of selected proteins found in Naganishia friedmannii *involved in coping with environmental stressors. Shown are the adaptive strategies the proteins are involved in, and the closest matching organism and E-value.*

Protein name	Adaptive strategy	E-value of Closest Match	Closest Match
Trehalose phosphate synthase	Prevent protein denaturation, heat tolerance	0	*Naematelia encephala*
Trehalose phosphate phosphatase		0	*Naematelia encephala*
Trehalase		0	*Saitozyma podzolica*
Laccase 1	Melanin Synthesis	0	*Naematelia encephala*
Laccase 2		0	*Naematelia encephala*
Phytoene dehydrogenase	Carotenoid Synthesis	0	*Saitozyma podzolica*
Phytoene synthase		1E-146	*Kwoniella shandongensis*
Glutathione synthase	Resistance to oxidative and nitrosative stress	0	*Kwoniella shandongensis*
Glutathione reductase		0	*Kwoniella shandongensis*
Glutathione peroxidase		2E-61	*Cryptococcus neoformans*

BLAST all vs all analysis showed that an average of 64% of the proteins in the proteome of *N. friedmannii* match (e-value < 0.0001) with other closely related members of the class Tremellomycetes (Table 6.2). Among the eight organisms considered here, *N. friedmannii* has the lowest number of proteins (5,638) and this number is only slightly lower than its closest relative, *F. wieringae* (5,732). A total of 1,115 proteins did not match with proteins from any of the organisms used for comparative analysis. Among unique proteins, only about half (623) can be classified into at least one protein family, according to the PFAM database (Finn et al. 2014). The comparative analysis of PFAM domains identified a number of protein families that have a significantly lower number of proteins in *N. friedmannii* than in the fungal species considered, mostly involved in amino acid biosynthesis and cell transport. There were 112 proteins that were present in all of the other 9 organisms but not in *N. friedmannii*. In contrast, there were only 23 proteins that were more abundant in *N. friedmannii* than the other organisms.

Table 6.2. Summary of proteome relatedness among Naganishia friedmannii *and seven other members of the Tremellomycete class. Shown are the total number of proteins, the number of proteins shared (blast e value < 0.0001) between each organism and* N. friedmannii*, and the percent of the* N. friedmannii *proteome that is shared with each organism.*

Fungal species	**# of proteins**	**# shared with *N. friedmannii***	**% of *N. friedmannii***
Naganishia friedmannii	5638	NA	NA
Cryptococcus neoformans	6743	3652	64.77
Filobasidium wieringae	5732	3469	61.53
Cryptococcus gattii	6560	3553	63.02
Kwoniella heveanensis	8508	3811	67.59
Naematelia encephala	7963	3677	65.22
Saitozyma podzolica	10205	3726	66.09
Tremella mesenterica	8061	3546	62.89

Its relatively smaller genome size compared to other close relatives and the smaller number of protein family expansions suggests that *N. friedmannii* presents low functional redundancy and a relatively streamlined genome. A more in-depth analysis of *N. friedmannii* genome will enable us to better understand its survival mechanisms in some of the harshest environments of the cryosphere.

CHAPTER VII

CONCLUSIONS

Conclusions

To conclude, the main findings of this body of work are the following:

1) The top of Mt. Kilimanjaro, an analogue of the middle epoch of Mars, supports diverse and rich microbial assemblages. The bacterial community is mostly cosmopolitan and shows evidence of high rates of dispersal, while the eukaryotic community showed more evidence of dispersal limitations and apparent endemism.

2) Microcosm and pure culture studies showed that *Naganishia friedmannii* (formerly *Cryptococcus friedmannii*) is able to grow during extreme diurnal freeze-thaw cycles and is a metabolically versatile opportunitroph. This organism is also the dominant microeukaryote in soils > 6000 m.a.s.l. on volcanoes (Socompa and Llullaillaco) in the Atacama region.

3) Transient ice pinnacles known as “nieves penitentes” (commonly found at high elevations in the dry Andes) are oases of microbial life in an otherwise hostile environment. The eukaryotic community in red ice is dominated by algae in the genera *Chlamydomonas* and *Chloromonas*, some of which are related (100% identity) to known snow algae from alpine and polar environments, whereas others are more distantly related (95-96% identity) to known algae.

4) Extremophilic soil communities from a high elevation Atacama volcano respond when water and nutrient limitations are alleviated, with a change in richness and structure. Only a small number of taxonomic groups drive the community shift and they likely reflect the active fraction of the community under transiently favorable conditions. *Naganishia friedmannii* shows the highest increase in relative abundance after the first amendment with water and nutrients.

5) The draft genome sequence of the polyextremophilic yeast *Naganishia friedmannii* presents a number of genes that are of relevance in explaining its

ability to survive in extreme environments. Its genome size is reduced compared to most closely related fungi and contains a high number of unique proteins that may help explain its unique phenotype.

Overall, the research reported here expands the current knowledge of the diversity and adaptation of extremophilic communities in cold-dry environments on Earth that are considered analogues of other planetary surfaces. Previously unexplored environments of the cryosphere host diverse and rich communities and a number of microorganisms are especially adapted to survive in these hostile environments.

Alleviation of resource limitation has proved to trigger a response from extremophilic communities, where only a selected number of extremophiles appear to grow under less restrictive conditions. This work has also led to the isolation and characterization of one of the most polyextremophilic yeasts known to date, *N. friedmannii*. Physiological and genomic approaches are beginning to reveal some of the traits that this organism uses to survive in some of the most extreme environments on Earth.

Linking what we know about microbial life in extreme environments to habitability potential on other planets remains challenging. Future work should address more in-depth characterization of these environments through meta-genomics and -transcriptomics studies to identify adaptation mechanisms and reveal which microbes are metabolically active in these extreme environments and are potential model organisms for astrobiology.

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Chapter II Appendix

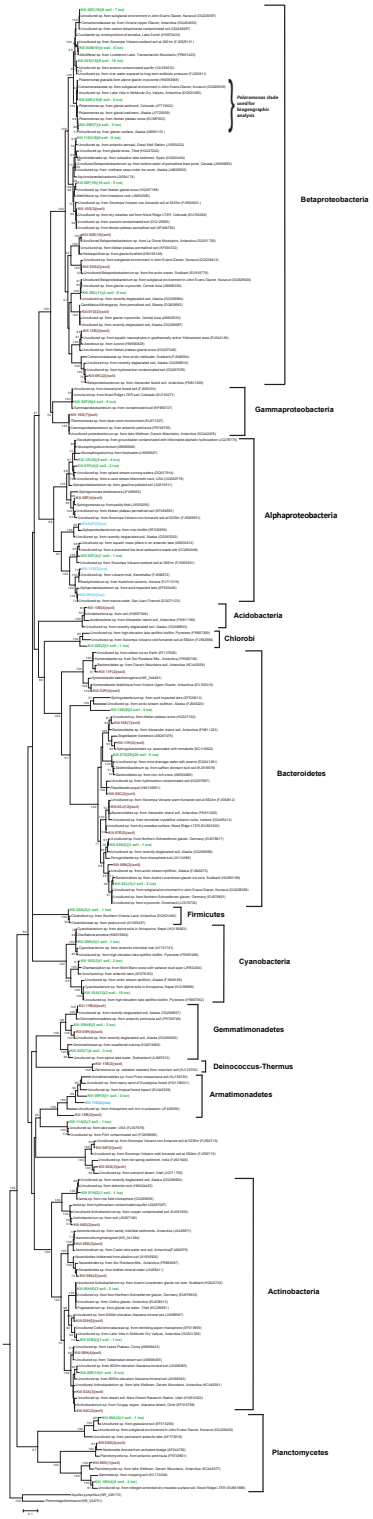


Figure A2.1. Phylogenetic analysis of 16S rRNA long-read gene sequences retrieved in ice and soil samples close to Mt. Kilimanjaro summit. Maximum Likelihood consensus phylogenetic tree includes 16S rRNA gene sequences retrieved from ice and soil samples close to the summit of Mt. Kilimanjaro and their closest GenBank BLAST and ARB matches. Only OTUs containing > 2 sequences are included. The accession numbers of most closely related taxa are listed parenthetically. Tree is rooted with the sequences of Aquifex pyrophilus *(NR_029172) and* Thermatoga thermarum *(NR_024751). Kilimanjaro phylotypes are bolded and followed by the number of sequences in each phylotype. Kilimanjaro phylotypes color code is as follows: blue, sequences retrieved in ice; brown, sequences retrieved in soil; green, sequences retrieved in both ice and soil. Node support is given as maximum likelihood values (n. of bootstrap replicates) when equal or greater than 50%. The scalebar corresponds to 0.1 substitutions per site. Major groups are shown to the right. The parenthesis indicates the* Polaromonas *clade that was used for the biogeographic analysis.*

Figure A2.2. Locations of worldwide sampling sites of Polaromonas *sequences. Alphabetic labels (A – Y) for location markers correspond to those of Table A2.1.*

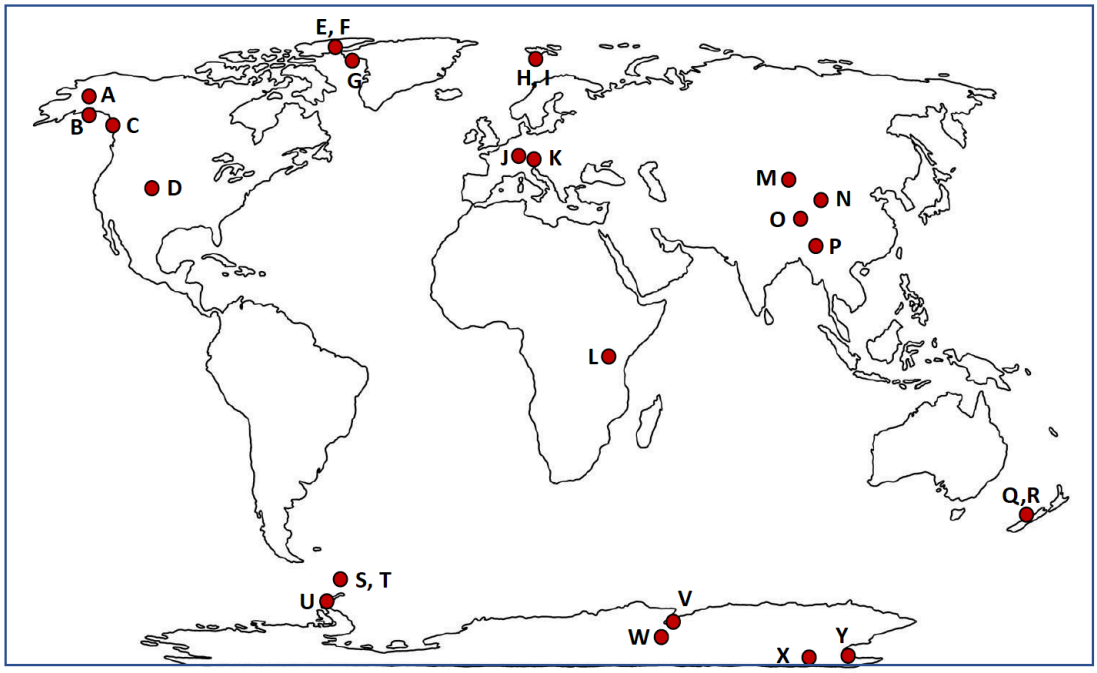
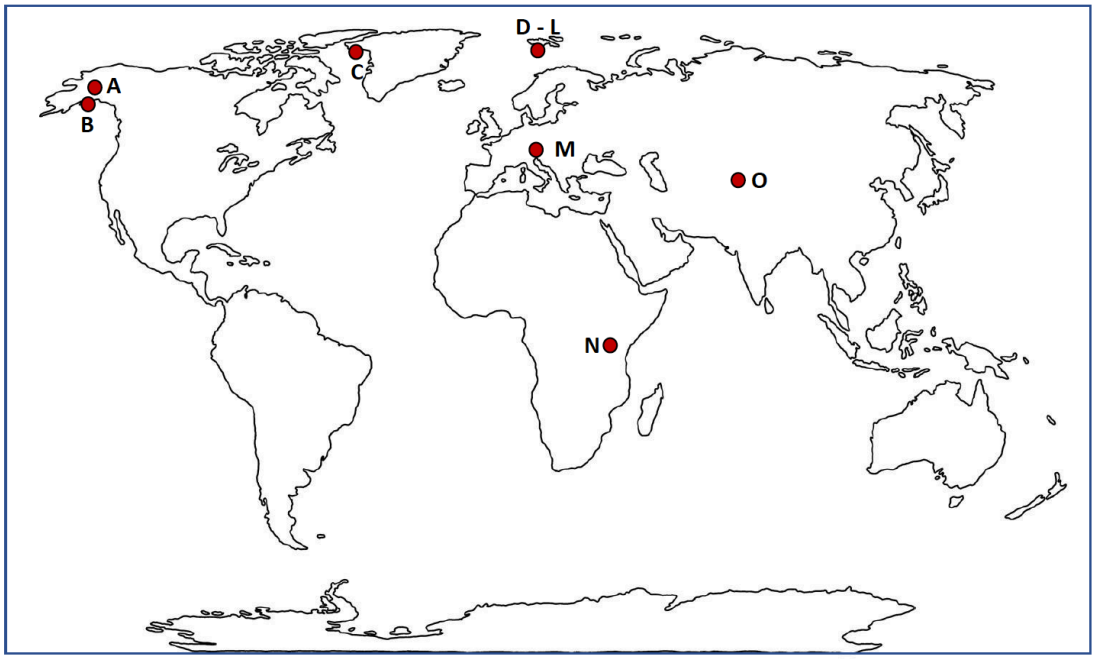


Figure A2.3. Locations of worldwide sampling sites of Chlamydomonas *sequences. Alphabetic labels (A – O) for location markers correspond to those of Table A2.3.*



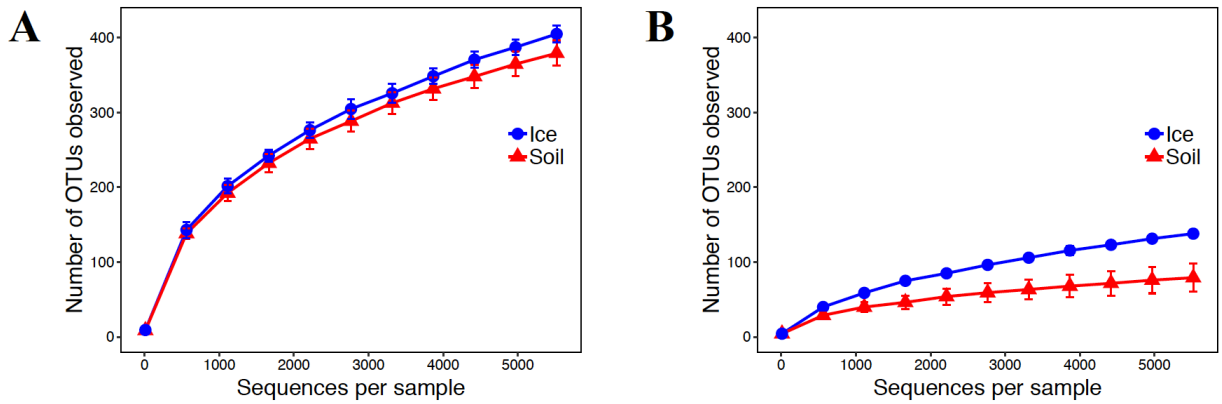


Figure A2.4. Alpha rarefaction curves based on the number of OTUs observed of 16S (A) and 18S (B) rRNA gene sequences obtained from ice and soil samples close to the summit of Mt. Kilimanjaro. Data shown are means ± s.e. The number of bacterial OTUs observed is comparable in ice and soil samples and close to saturation at the sequencing depth used. On the other hand, fewer eukaryotic OTUs are present in soil samples compared to ice. The metric reveals that sampling saturation is reached at a smaller sequencing depth for soil samples compared to ice for eukaryotes.

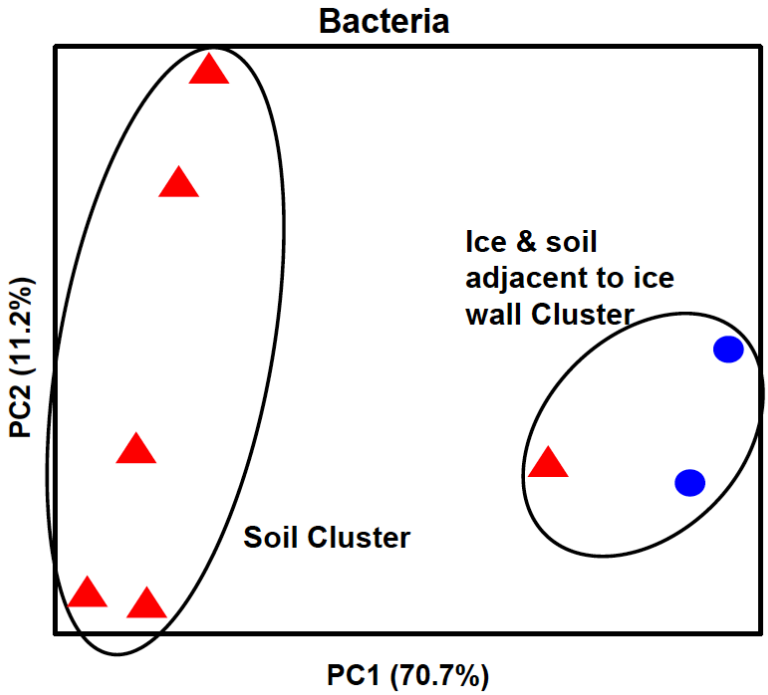


Figure A2.5. Cluster diagram-based PCoA (Principal Coordinate Analysis) using weighted Unifrac of Bacteria of ice and soil samples retrieved close to Mt. Kilimanjaro summit. The variance explained in each axis is given in parenthesis. Colors indicate samples from ice (blue) and soil (red). Bacterial ice communities are significantly different from soil communities (ANOSIM R = 0.8, P = 0.03). Bacterial communities from the same habitat are more similar to each other than they are to the other habitat type with the exception of the soil sample closest to the ice wall that clusters with the ice samples.

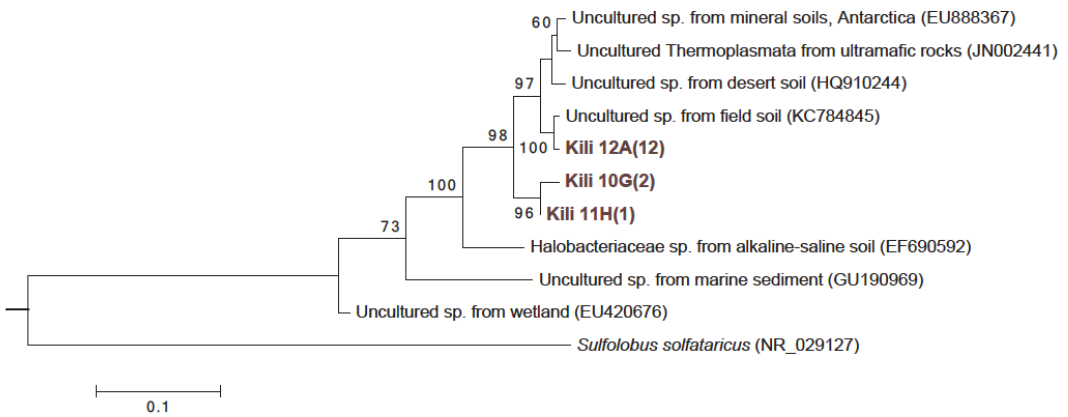


Figure A2.6. Phylogenetic analysis of 16S rRNA gene archaeal sequences retrieved in a soil sample (N7) close to Mt. Kilimanjaro summit. Maximum likelihood consensus phylogenetic tree includes 16S rRNA gene archaeal sequences from soil close to the summit of Mt. Kilimanjaro and their closest GenBank BLAST matches. The accession number of most closely related taxa are listed parenthetically. Tree is rooted with the sequence of Sulfolobus solfataricus *(NR_029127). Kilimanjaro phylotypes are bolded in brown and followed by the number of sequences in each phylotype of the library. Node support is given as maximum likelihood values (n. of bootstrap replicates) when equal or greater that 50%. The scale bar corresponds to 0.1 substitutions per site.*

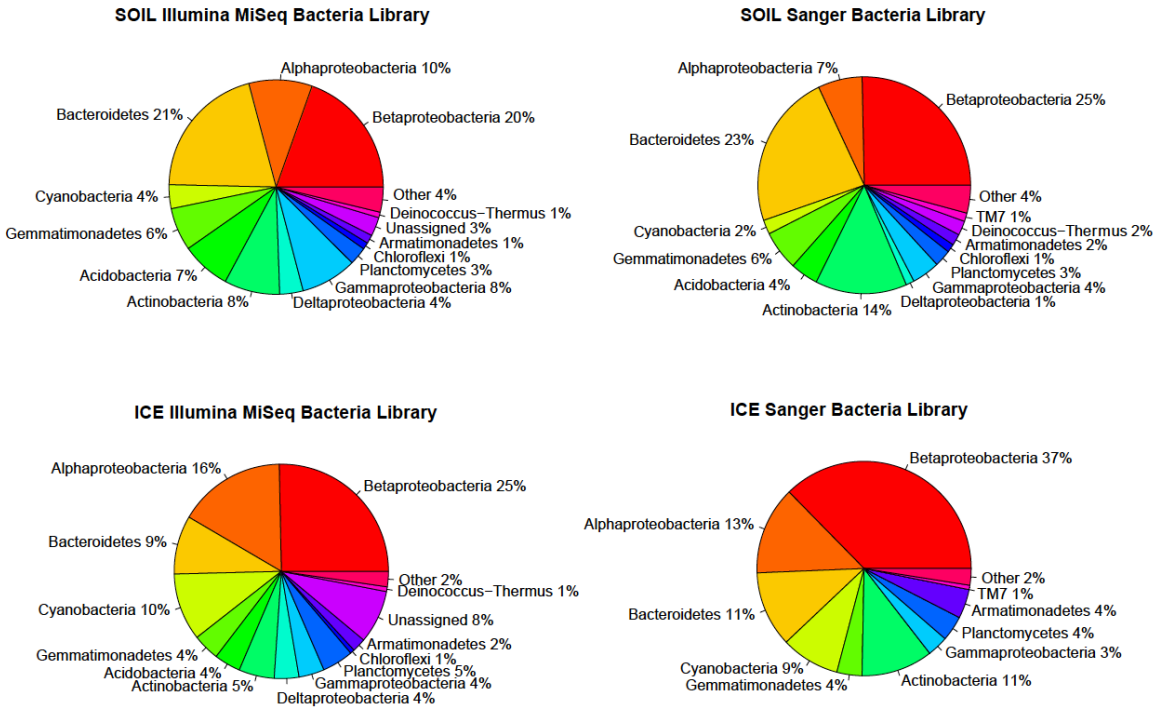


Figure A2. 7. Comparison between broad-level phylogenetic affiliation of Kilimanjaro ice (samples N3 and N8) and soil (samples N1, N5 and N7) bacterial sequences obtained with Illumina MiSeq and Sanger sequencing technologies.

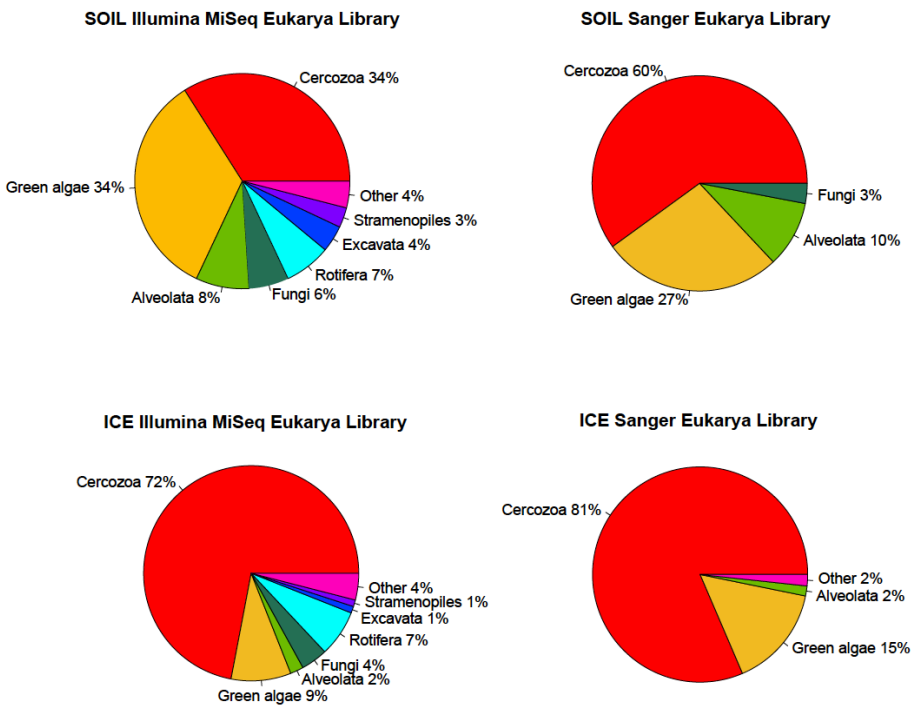


Figure A2. 8. Comparison between broad-level phylogenetic affiliation of Kilimanjaro ice (samples N3 and N8) and soil (samples N1, N5 and N7) Eukaryotic sequences obtained with Illumina MiSeq and Sanger sequencing technologies. Illumina Miseq (pie charts on left) show more phyla than Sanger libraries (pie charts on the right). Eukaryotic libraries are heavily dominated by Cercozoa in both habitats, in particular in the ice.

Table A2. 1. Environmental classification of long-read bacterial OTUs from ice and soil samples close to the summit of Mt. Kilimanjaro.

ENDEMIC						**NON-ENDEMIC**			
Polythermal OTUs	Total n. = 46	**Non-Cryophilic OTUs**	Total n. = 11	**Cryophilic OTUs**	Total n. = 3	**Subnovel OTUs**	Total n. = 3	**Novel OTUs**	Total n. = 3
OTU	Phylum	OTU	Phylum	OTU	Phylum	OTU	Phylum	OTU	Phylum
Kili05C(16)	Betaproteobacteria	Kili12C(6)	Alphaproteobacteria	Kili10X(7)	Bacteroidetes	Kili10D(6)	Bacteroidetes	Kili02Z(2)	Chlorobi
Kili04B(10)	Betaproteobacteria	Kili04F(2)	Alphaproteobacteria	Kili05N(2)	Bacteroidetes	Kili02G(3)	Unclassified	Kili08F(5)	Armatimonadetes
Kili02D(15)	Betaproteobacteria	Kili11E(3)	Alphaproteobacteria	Kili02B(2)	Actinobacteria	Kili02H(2)	Actinobacteria	Kili11G(2)	Armatimonadetes
Kili04E(15)	Betaproteobacteria	Kili08H(4)	Alphaproteobacteria						
Kili08E(7)	Betaproteobacteria	Kili11H(2)	Bacteroidetes						
Kili11D(15)	Betaproteobacteria	Kili11E(2)	Deinococcus						
Kili06F(15)	Betaproteobacteria	Kili11A(2)	Unclassified						
Kili10G(3)	Betaproteobacteria	Kili04D(2)	Actinobacteria						
Kili02E(10)	Betaproteobacteria	Kili06A(2)	Planctomycetes						
Kili03G(2)	Betaproteobacteria	Kili05G(2)	Planctomycetes						
Kili09C(11)	Betaproteobacteria	Kili10H(4)	Planctomycetes						
Kili01G(2)	Betaproteobacteria								
Kili12E(2)	Betaproteobacteria								
Kili09C(2)	Betaproteobacteria								
Kili02F(9)	Gammaproteobacteria								
Kili10G(7)	Gammaproteobacteria								
Kili01F(4)	Alphaproteobacteria								
Kili08F(4)	Alphaproteobacteria								
Kili03F(2)	Alphaproteobacteria								
Kili10E(4)	Acidobacteria								
Kili11F(2)	Bacteroidetes								
Kili02P(4)	Bacteroidetes								
Kili07(29)	Bacteroidetes								
Kili03C(2)	Bacteroidetes								
Kili02J(12)	Bacteroidetes								
Kili07E(2)	Bacteroidetes								
Kili09A(3)	Bacteroidetes								
Kili02L(3)	Bacteroidetes								
Kili02A(2)	Firmicutes								
Kili09H(2)	Cyanobacteria								
Kili10G(3)	Cyanobacteria								
Kili10A(12)	Cyanobacteria								
Kili11B(4)	Gemmatimonadetes								
Kili03H(6)	Gemmatimonadetes								
Kili05H(4)	Gemmatimonadetes								
Kili02G(7)	Gemmatimonadetes								
Kili10B(2)	Armatimonadetes								
Kili04F(2)	Unclassified								
Kili01H(2)	Actinobacteria								
Kili05D(3)	Actinobacteria								
Kili08A(2)	Actinobacteria								
Kili05H(5)	Actinobacteria								
Kili06H(4)	Actinobacteria								
Kili06E(10)	Actinobacteria								
Kili02A(3)	Actinobacteria								
Kili02C(2)	Actinobacteria								

Table A2. 2. Locations, accession numbers, and publication sources for Polaromonas *sequences.*

REGION	SITE	GEOGRAPHIC COORDINATES	ACCESSION N.	PUBLICATION SOURCE	ON SUPP. FIG. 2
Alaska, USA	Toklat Glacier	63.39 N 149.91 W	JF719324-28, 30-38, JF729309	Darcy *et al.*, 2011	A
	Byron Glacier	60.74 N 148.85 W	AB991151	Murakami *et al.*, 2015	B
	Mendenhall Glacier	58.435837 N 134.5546 W	GQ396863, 949, 971	Sattin *et al.*, 2011	C
Colorado, USA	Arikaree Glacier	40.057276 N 105.6432 W	JF719322, 3, 9	Darcy *et al.*, 2011	D
Nunavut, Canada	Johns Evans Glacier	79.66 N 74 W	DQ228403, 9	Skidmore *et al.*, 2007	E
Nunavut, Canada	Johns Evans Glacier	79.63 N 74.38 W	DQ628932-40, DQ530258	Cheng *et al.*, 2007	F
Greenland	Qaanaaq Glacier	77.5033 N 69.1458 W	LC076717	Uetake *et al.*, (unpubl.)	G
Svalbard	Hans Glacier	77.04 N 15.39 E	KU586648, KU586652	Gawor *et al.*, 2016	H
	Werenskiold Glacier	77.075 N 15.34 E	MG098816	Ciok *et al.* (unpubl.)	I
Germany	Schneeferner Glacier	47.42 N 10.98 E	EU978852	Simon *et al.*, 2009	J
Austria	Pitztaler Joechl Glacier	46.55 N 10.53 E	NR_109012-13	Margesin *et al.*, 2012	K
Tanzania	Kilimanjaro, 5772 m.a.s.l.	3.04839 S 37.21628 E	KX771285-86, KX771324-26, KX771367, KX771372, KX771376, KX771395, KX771538, KX771602-13	This study	L
Tianshan, China	Glacier n.1	43.15 N 86.87 E	EF423322, 30, 40	Wang *et al.* (unpubl.)	M
	Glacier n.1	43.15 N 86.87 E	FJ979854, 9	Zhang *et al.* (unpubl.)	M
China	Glacier in Gansu Province	39.7 N 96.62 E	JX950030, 31	Liu *et al.* (unpubl.)	N
Tibet, China	Puruogangri Ice Field	33.89 N 89.15 E	DQ227793	Zhang *et al.*, 2008	O
	Glacier	29.45 N 96.5 E	JX949585	Liu *et al.* (unpubl.)	P
New Zealand	Franz Joseph Glacier	43.48 S 170.21 E	AY315174, 5, 8	Foght *et al.*, 2004	Q
	Fox Glacier	45.51 S 170.14 E	AY315176, 7	Foght *et al.*, 2004	R
Antarctica	Carlini station, King George Island	62.14 S 58.4 W	KY190582, KY190658, KY190735, KY190746, KY190773, KY190785	Vazquez *et al.* (unpubl.)	S
	Baranowski Glacier, King George Island	62.12 S 58.27 W	MG098808	Ciok *et al.* (unpubl.)	T
	Palmer Station, Anvers Island	64.46 S 64.02 W	5 sequences, unpubl.	Vimercati *et al.* (unpubl.)	U
	Davis Station	68.34 S 77.58 E	JX196642	Xiong *et al.* (unpubl.)	V
	Collins Glacier	73.21 S 66.97 E	EU636025-27, EU636029	Garcia-Echauri *et al.*, 2011	W
	Kamb Ice Stream	82.25 S 145 E	FJ477327	Lanoil *et al.*, 2009	X
	Lake Vida, Dry Valleys	77.23 S 161.56 E	DQ521547	Mosier *et al.*, 2007	Y

Table A2.3. Locations, accession numbers, and publication sources for Chlamydomonas *sequences.*

REGION	SITE	GEOGRAPHIC COORDINATES	ACCESSION N.	PUBLICATION SOURCE	ON SUPP. FIG. 3
Alaska	Toklat Glacier	63.39 N 149.91 W	KM870616-7, KM870646, KM870652, KM870656, KM870662, KM870665-6, KM870675, KM870675, KM870679, KM870685, KM870691, KM870694, KM870731, KM870743-4, KM870752, KM870766, KM870769, KM870771	Darcy et al., 2011	A
	Harding Icefield	60.0031 N 150.007 W	AB902998	Ito et al. (unpubl.)	B
Greenland	Qaanaq Glacier	77.8973 N 64.877 W	AB902971	Ito et al. (unpubl.)	C
Svalbard		77.66 N 14.82 E	JQ790560	Remias et al. (unpubl.)	D
		78.16 N 17.89 E	JQ790557	Remias et al. (unpubl.)	E
		78.18 N 15.48 E	JQ790558	Remias et al. (unpubl.)	F
		79.63 N 10.97 E	AF514411	Leya et al. (unpubl.)	G
		79.27 N 11.77 E	AF514412	Leya et al. (unpubl.)	H
		79.81 N 11.83 E	GU117586	Remias et al., 2010	I
		77.57 N 16.9 E	JQ790559	Remias et al. (unpubl.)	J
		78.97 N 11.58 E	AF514407	Leya et al. (unpubl.)	K
		79.74 N 10.83 E	GU117588	Remias et al., 2010	L
Austria	Tyrol	46.92 N 10.93 E	GU117577	Remias et al., 2010	M
Tanzania	Kilimanjaro, 5772 m.a.s.l.	3.04 S 37.21 E	KX771779-800, KX772005	This study	N
Tajikistan	Pamir	38.7851 N, 72.272 E	AB902973	Ito et al. (unpubl.)	O

Chapter V Appendix

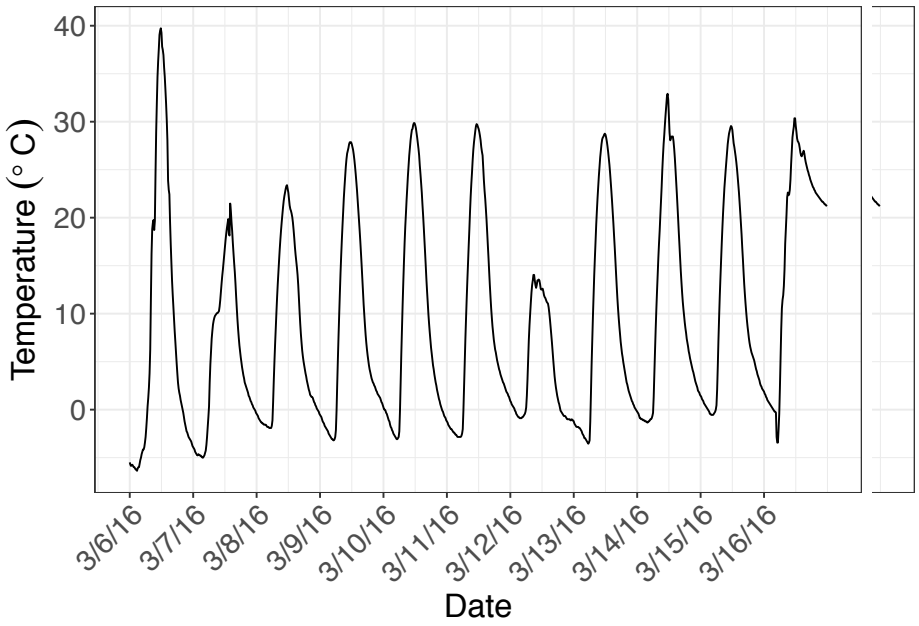


Figure A5.1. Diurnal fluctuations of soil temperature (4 cm depth), during the week of 6 to 16 March 2016 at 100 meters elevation above basecamp on the west-facing slope (24°44.043, 68° 34.573, 5103 m.a.s.l.) of Volcán Llullaillaco.

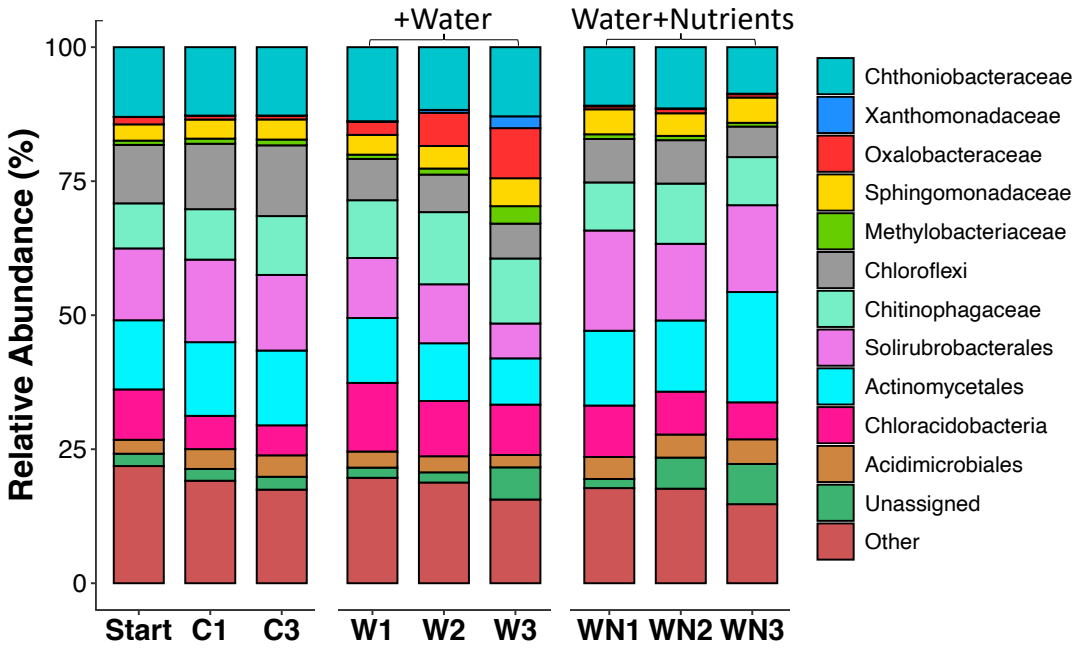


Figure A5.2. Taxonomy of bacterial community shift in response to +W and +WN amendments. Stacked bar graphs showing relative abundances of dominant bacterial families (from 16S sequencing data). Taxonomy is from the Greengenes database; some families could not be specified and thus are presented as orders or phyla. Bars represent means (n=4). Asterisks represent significant differences in relative abundance of taxa among treatments (Tukey HSD, $p < 0.05$).