Characterization of active microbial ecosystems in icy astrobiology analog cryo-environments

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Abstract

Ice environments are characterized by permanent subzero temperatures, low nutrient concentrations and low water activity, presenting unique challenges to microbial life. The study of microbes inhabiting ice informs our understanding of the cold limits of life on Earth and extraterrestrial bodies. Lava tube ice caves are analog environments to lava tubes identified on Mars which may contain microbial ecosystems. Glaciers and ice caps are analogs to the icy moons of Jupiter and Saturn which contain thick ice crusts hiding subsurface oceans and may also support a microbial ecosystem. Lava Beds National Monument, located next to the Medicine Lake Volcano in Northern California contains the largest concentration of lava tubes in North America but no study of the microbial ecology of the ice in these caves has been performed. The Devon Island ice cap, and White Glacier in the Canadian high Arctic and Johnsons Glacier in Antarctica are some of the best-studied ice masses in the polar regions, however, little if any information is known of the microbial communities which reside within them. Culture-dependent and independent methods were used to study the microbial ecosystems in all four of these environments. In Lava Beds National Monument, 16S rRNA amplicon sequencing identified ice samples primarily consisting of Actinomycetota, Pseudomonadota, Bacteroidota, Bacillota, and Chloroflexota and culture experiments and metabolic activity assays identified a viable and metabolically active microbial community at subzero temperatures. Furthermore, these communities were found to be more closely related to other cryoenvironments than caves suggesting that ice and cold temperatures exert stronger selection pressures on microbial communities than caves. In the Devon Island ice cap, flow cytometry coupled with live/dead staining identified an ultra-low biomass microbial ecosystem containing 3.62×10^4 cells/mL with a live fraction of 0.8%. Viable cells were also isolated and found to grow at subzero temperatures, high salinity (> 6%), and low pH (< pH 5), including one isolate

capable of growth at -5°C, in 15% NaCl, and pH 3. Metagenomic and metatranscriptomic sequences belonging to diverse metabolic marker genes, including those related to thiosulfate oxidation, aerobic carbon monoxide oxidation, aerobic respiration, fumarate reduction, nitrate and nitrite reduction, nitrogen fixation, oxygenic photosynthesis and carbon fixation, were found indicating a diverse ecosystem composed of lithoautotrophs, photoautotrophs and heterotrophs. Transcripts involved in cold adaptation were also abundant, including cold shock proteins, transcription and translation factors, and membrane and peptidoglycan-altering proteins. The microbial ecosystems of White Glacier and Johnsons Glacier were compared, and their taxonomy was found to be significantly different from each other but shared an overlap in functional potential. Metatranscriptome sequencing of White Glacier revealed an active microbial ecosystem dominated by Cyanobacteria performing oxygenic photosynthesis and carbon fixation. In addition, lithoautotrophic metabolisms including carbon fixation via the 3-hydroxyproprionate cycle, anoxygenic photosynthesis, sulfide oxidation, and nitrate reduction/denitrification support a heterotrophic community performing aerobic respiration and aerobic carbon monoxide oxidation. Metagenome-assembled genomes were also found to be active including Cyanobacteriota, and novel phyla Armatimonadota, Eremiobacterota, and Gemmatimonadota. Cold adaptation genes were found to be abundant in both glaciers and highly expressed in White Glacier. Similarly to the Devon Island ice cap, total biomass within White Glacier was 4.75×10^4 cells/ml with a live fraction of 0.5%. The majority of cultured isolates were capable of growth at subzero temperatures, high salinity (> 6%) and low pH (< pH 5). This study determined that ice environments contain diverse, active microbial ecosystems which are well adapted to life in ice, providing insights into how microbial life is sustained in some of Earth's most extreme environments and how it might survive on Mars or the icy moons.

Résumé

Les environnements glacés sont caractérisés par des températures permanentes inférieures à zéro, de faibles concentrations de nutriments et une faible activité de l'eau, ce qui présente des défis uniques pour la vie microbienne. L'étude des microbes habitant la glace éclaire notre compréhension des limites froides de la vie sur Terre et sur les corps extraterrestres. Les grottes de glace des tubes de lave sont des environnements analogues aux tubes de lave identifiés sur Mars et qui peuvent contenir des écosystèmes microbiens. Les glaciers et les calottes glaciaires sont analogues aux lunes glacées de Jupiter et de Saturne, qui contiennent d'épaisses croûtes de glace cachant les océans souterrains et peuvent également abriter un écosystème microbien. Le monument national des Lava Beds, situé à côté du volcan Medicine Lake en Californie du Nord, contient la plus grande concentration de tubes de lave en Amérique du Nord, mais aucune étude de l'écologie microbienne de la glace de ces grottes n'a été réalisée. La calotte glaciaire de l'île Devon, le glacier White dans l'Extrême-Arctique canadien et le glacier Johnsons en Antarctique comptent parmi les masses de glace les mieux étudiées dans les régions polaires. Cependant, peu ou pas d'informations sont connues sur les communautés microbiennes qui y résident. Des méthodes indépendantes et dépendantes de la culture ont été utilisées pour étudier les écosystèmes microbiens dans ces quatre environnements. Dans le monument national de Lava Beds, le séquençage de l'amplicon d'ARNr 16S a identifié des échantillons de glace constitués principalement d'Actinomycetota, Pseudomonadota, Bacteroidota, Bacillota et Chloroflexota, et des expériences de culture et des tests d'activité métabolique ont identifié une communauté microbienne viable et métaboliquement active à des températures inférieures à zéro. De plus, ces communautés se sont révélées plus étroitement liées à d'autres cryoenvironnements que les grottes, ce qui suggère que la glace et les températures froides exercent des pressions de sélection plus fortes sur les communautés

microbiennes que sur les grottes. Dans la calotte glaciaire de l'île Devon, la cytométrie en flux couplée à la coloration vivante/morte a identifié un écosystème microbien à biomasse ultra faible contenant 3,62 x 104 cellules/mL avec une fraction vivante de 0,8 %. Des cellules viables ont également été isolées et se sont développées à des températures inférieures à zéro, à une salinité élevée (> 6 %) et à un pH faible (< pH 5), y compris un isolat capable de croître à -5 °C, dans 15 % de NaCl et à pH 3. Des séquences métagénomiques et métatranscriptomiques appartenant à divers gènes marqueurs métaboliques, y compris ceux liés à l'oxydation du thiosulfate, à l'oxydation aérobie du monoxyde de carbone, à la respiration aérobie, à la réduction des fumarate, à la réduction des nitrates et des nitrites, à la fixation de l'azote, à la photosynthèse oxygénée et à la fixation du carbone, ont été trouvées, indiquant un écosystème diversifié. composé de lithoautotrophes, photoautotrophes et hétérotrophes. Les transcriptions impliquées dans l'adaptation au froid étaient également abondantes, notamment les protéines de choc froid, les facteurs de transcription et de traduction, ainsi que les protéines altérant la membrane et le peptidoglycane. Les écosystèmes microbiens du glacier White et du glacier Johnsons ont été comparés et leur taxonomie s'est avérée significativement différente l'une de l'autre, mais partageait un chevauchement en termes de potentiel fonctionnel. Le séquençage du métatranscriptome du glacier White a révélé un écosystème microbien actif dominé par des cyanobactéries effectuant la photosynthèse oxygénée et la fixation du carbone. De plus, les métabolismes lithoautotrophes, y compris la fixation du carbone via le cycle du 3-hydroxypropionate, la photosynthèse anoxygénique, l'oxydation des sulfures et la réduction/dénitrification des nitrates, soutiennent une communauté hétérotrophe effectuant une respiration aérobie et une oxydation aérobie du monoxyde de carbone. Les génomes assemblés par métagénome se sont également révélés actifs, notamment Cyanobacteriota et les nouveaux

phyla Armatimonadota, Eremiobacterota et Gemmatimonadota. Les gènes d'adaptation au froid se sont révélés abondants dans les deux glaciers et fortement exprimés dans le glacier White. À l'instar de la calotte glaciaire de l'île Devon, la biomasse totale du glacier White était de 4,75 x 104 cellules/ml avec une fraction vivante de 0,5 %. La majorité des isolats cultivés étaient capables de croître à des températures inférieures à zéro, à une salinité élevée (> 6 %) et à un pH faible (< pH 5). Cette étude a déterminé que les environnements de glace contiennent des écosystèmes microbiens diversifiés et actifs, bien adaptés à la vie dans la glace, fournissant ainsi un aperçu de la façon dont la vie microbienne est maintenue dans certains des environnements les plus extrêmes de la Terre et comment elle pourrait survivre sur Mars ou sur les lunes glacées.

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Contribution to Original Knowledge

This thesis significantly contributes to the current knowledge of microbial life and metabolism in the cryosphere. Specifically:

1. In Chapter 3, I describe the first study of lava tube ice microbial communities. I characterize the taxonomic diversity of these communities, demonstrating they share greater similarities with cryoenvironments than caves. I predict the functional potential of these communities, showing that they may be capable of performing Mars-relevant metabolisms. I isolate and characterize 37 cold-adapted microorganisms, four appear to be novel species and one a novel genus. Finally, I demonstrate that these communities are metabolically active at -5°C.
2. In Chapter 4, I describe the first metagenomic and metatranscriptomic survey of ice anywhere in the world. I characterize an ultralow biomass microbial community's functional gene content and expression from a high Arctic ice cap. I also describe the characterization of 4 isolates, including a *Kocuria sp.* capable of growth at -5°C, 15% NaCl, and pH 3.
3. In Chapter 5, I describe the first metagenomic and metatranscriptomic bipolar comparison of glacial ice microbial communities. I describe the recovery of 30 bacterial genomes, of which 25 appear novel. I characterize the functional gene content of these communities, demonstrating that despite significant taxonomic differences, they share a similar functional potential. I characterize the metatranscriptome from White Glacier in the high Arctic, demonstrating the active microbial community likely relies on primary production from Cyanobacteriota to survive. I characterize 58 cold-adapted isolates, demonstrating that many of them can grow at extremes of salinity and pH.

Contribution of Authors

1. In Chapter 3, I am the first author of the associated published paper. I performed and oversaw all the experiments. I did the sample collection in 2017 and 2018, completed the nucleic acid extractions, prepared samples for 16S rRNA sequencing, performed all culture work, performed direct microscopic counts, performed biolog experiments, performed radiorespiration assays, and completed all the data processing and most of the analysis. I wrote and edited the manuscript. M.Á. Fernández-Martínez provided feedback on the manuscript and performed phylogenetic diversity calculations. L.G. Whyte and R.J. Léveillé conceived and oversaw the study and contributed to the manuscript. All authors provided guidance on the manuscript and data interpretation.
2. In Chapter 4, I am the first author of the associated manuscript. I performed and oversaw all the experiments. I performed the sample collection, ice core processing, nucleic acid extractions, sequencing library preparation and prepared the samples for flow cytometry work. I completed the sequencing data processing and analysis. I performed the initial isolation of cultured strains. I wrote and edited the manuscript and prepared all the figures. Donovan Allen performed characterizations of cultured isolates and taxonomic identification of isolates. Matthew Quinn performed PCR's of the isolates and sent them for 16S sanger sequencing. L.G. Whyte conceived the study, and both L.G. Whyte and R.J. Léveillé provided guidance on the manuscript and data interpretation.
3. In Chapter 5, I am the first author of the associated manuscript. In 2023 I performed sample collection in the Arctic and Antarctica. I conceived of the study and oversaw all the experiments. I processed the ice cores, extracted nucleic acids, and prepared the extractions for sequencing and prepared the samples for flow cytometry work. I initially isolated cultured strains. I performed all the data processing and analysis. I wrote and

edited the manuscript and prepared all the figures. Donovan Allen performed characterizations of cultured isolates and performed biolog measurements and analysis. Matthew Quinn performed PCR's of the isolates and sent them for 16S sanger sequencing. Nastasia Freyria determined if there were statistically significant differences in the taxonomy between the Arctic and Antarctic samples. L.G. Whyte aided with sample collection from White Glacier and both L.G. Whyte and R.J. Léveillé provided guidance on the manuscript and data interpretation.

List of Figures and Tables

List of Abbreviations

AMP: Adenosine Monophosphate

ASV: Amplicon Sequence Variant

ATP: Adenosine Triphosphate

AWCD: Average Well Colour Development

CFU: Colony Forming Units

CPM: Copies per Million reads

NADH: Nicotinamide adenine dinucleotide

DAPI: 4′,6-diamidino-2-phenylindole

DNA: Deoxyribonucleic Acid

DPM: Disintegrations Per Minute

GS-MS: Gas Chromatography-Mass Spectroscopy

GTDB: Genome Taxonomy Database

GTR: General Time Reversible

GYA: Billion Years Ago

JGI IMG/M: Joint Genome Institute Integrated Microbial Genomes & Microbiomes

KEGG: Kyoto Encyclopedia of Genes and Genomes

LABE: Lava Beds National Monument

MA: Million Years

MAG: Metagenome Assembled Genome

MYA: Million Years Ago

mRNA: messenger Ribonucleic Acid

NCBI: National Centre for Biotechnology Information

NJ: Neighbor Joining

NMDS: Non-metric Multidimentional Scaling

OD: Optical Density

OTU: Operational Taxonomic Unit

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PD: Phylogenetic Diversity

RIN: RNA Integrity Number

RNA: Ribonucleic Acid

rRNA: ribosomal Ribonucleic Acid

TDS: Total Dissolved Solids

TOC: Total Organic Carbon

TPM: Transcripts Per Million reads

tRNA: transfer Ribonucleic Acid

UV: Ultra-Violet

Chapter 1. Introduction

1.1 Objectives of this thesis

The overall goals of my research are to characterize the microbial communities present within ice analogous to environments on Mars and the icy moons of the outer solar system and to determine how microbial communities survive and potentially remain metabolically active in such hostile environments. The specific objectives and questions my research seeks to answer are:

1. Characterize the microbial communities within lava tube ice, a subsurface ice habitat hypothesized to exist on Mars and whose microbiology has yet to be studied on Earth. Are the microbial communities within this environment cold-adapted, and does the community contain a metabolically active fraction?
2. Characterize the microbial communities present within an ice cap of the Canadian high Arctic by determining which microorganisms reside there, if the environment supports an active or viable microbial ecosystem and determine the survival strategies required to survive in this environment.
3. Compare the microbial communities inhabiting glacial ice from the High Arctic and Antarctica to gain a bipolar understanding of life in glacial ice. Determine what survival strategies these communities utilize and what metabolisms they employ to survive.

Each group of questions and objectives corresponds to a section of my doctoral thesis described in the following chapters. In Chapter 3, I present the first microbial characterization of lava tube ice, including both culture-dependent and independent characterization. I present results showing that the community is metabolically active at in situ temperatures and conditions

and is cold-adapted, resembling microbial communities in other cryosphere environments more closely than cave environments.

In Chapter 4, I show that DNA and RNA can be extracted and sequenced from extremely low biomass ice obtained from an ice cap in the Canadian High Arctic. I analyze the resulting metagenome and metatranscriptome to determine that this environment supports an active microbial community and employs diverse metabolic lifestyles and many known cold stress response genes to persist in this environment.

In Chapter 5, I determine if microbial communities are metabolically active *in situ* in glacial ice by extracting DNA and RNA from glacial ice cores collected in the Arctic and Antarctica. I reconstruct the genomes of microbial community members by binning metagenomic reads. I map metatranscriptomic sequences to those bins and the wider metagenome to determine which microorganisms remain active within glacial ice and how they survive there. Additionally, I culture isolates to demonstrate further these environments contain a viable microbial community. Finally, I perform a bipolar comparison of the microbial communities in the Arctic and Antarctica.

In addition, I discuss how the results from each chapter impact the search for life on other celestial solar system bodies containing large ice deposits.

Chapter 2. Literature Review

2.1 Microbial ecology of cryoenvironments and ice

Cryoenvironments, typically defined as any environment that regularly or permanently experiences temperatures at or below 0°C (Goordial et al. 2013), comprise the largest fraction of the Earth's biosphere. These environments include the deep sea (90% of the world's oceans' volume is below 5°C), alpine regions, the upper atmosphere, caves, and polar regions. But in addition to cold temperatures, cryoenvironments often experience extremes such as high salinity, high UV, prolonged day/night cycles, and desiccation.

2.1.1 Snow

Snow occurs in many places on the Earth's surface; however, given it only occurs seasonally at mid-latitudes and lower elevations, only Earth's polar and alpine regions are likely to contain microbial communities truly adapted to life in snow. Snow is an extreme environment in that snow, by definition, only exists at subzero temperatures and is very nutrient-poor (Granskog and Kaartokallio 2004; Harding et al. 2011). Furthermore, UV penetration into snow increases photo-oxidative stress and the generation of reactive oxygen species (Grannas et al. 2007; Domine et al. 2008). Perhaps then, it is no surprise that the biomass in snow environments is very low, between 10^3-10^4 total cells/mL (Maccario et al. 2015). These microbial communities are thought to originate by atmospheric deposition. For example, microbial communities of snowpacks on sea ice flows in the high Arctic were similar in composition but different to the sea ice and seawater beneath (Hauptmann et al., 2014), suggesting they originated from the atmosphere. High Arctic snow microbial communities resembled those of

adjacent cyanobacterial mat communities, which could only have been transported there by atmospheric deposition (Harding et al. 2011).

The most well-known microbial community members in snow are Algae, often found in microbial mats (Segawa et al. 2018; Davey et al. 2019; Soto et al. 2023). These microorganisms are often easy to spot because of their impressive green, red, or orange pigmentation. These pigments shield DNA from excess UV radiation present in polar regions. They are thought to stabilize membranes at cold temperatures by creating local differences in membrane polarity or causing areas of flexibility in the membrane by varying the location of the pigments (Britton 1995; Singh et al. 2017; Seel et al. 2020).

The most prominent snow algae are the Chlamydomonadales, members of Chlorophyta. Other algae capable of colouring snow may include Euglenoids, Cryptomonads, Chrysophytes, and Dinoflagellates (Hoham and Remias 2020). If the algae appear red, it is because carotenoid pigments dominate, while green snow indicates the dominance of chlorophyll. Evidence suggests that true snow algae are highly adapted to snow and cannot survive in any other environment (Hoham and Remias 2020); however, the presence of snow algae can significantly alter their environment by increasing the albedo of the snow, encouraging melting and destruction of their habitat (Takeuchi et al. 2006; Ganey et al. 2017). In addition to algae, bacteria can make up a significant fraction of snow microbial communities, especially in the winter when sunlight disappears entirely in the polar regions. Without sunlight, algae cannot photosynthesize, and the snow microbial community becomes dominated by bacteria (Winkel et al. 2022). Of the bacteria found in snow, the vast majority are heterotrophs capable of feeding

on the byproducts of algal primary production (Lutz et al. 2015). The taxa found most often are Actinobacteria, alpha-, beta-, and gamma-proteobacteria, Bacteroidetes, Cyanobacteria, and Firmicutes (Cameron et al. 2015; Maccario et al. 2015; Antony et al. 2016; Winkel et al. 2022).

2.1.2 Ice

Ten percent of the Earth's land area is covered in ice (Benn 2006). This includes glacial ice, ice caps, and the ice sheets of Greenland and Antarctica (Anesio and Laybourn-Parry 2012). Sea ice covers about 9% of Earth's Ocean volume (Hall et al. 2006), equal to about 34 million km^2, roughly three times the area of Canada. Therefore, a significant portion of Earth's total surface area is influenced by ice and deserves considerable attention and it has been estimated that there are between $10^{25} – 10^{29}$ total cells trapped in ice (Irvine-Fynn and Edwards 2014), making it a significant biome on Earth. Microorganisms found in glacial ice must contend with subzero temperatures, nutrient limitation, very low water activity and at greater ice depths, lack of light and oxygen, and extreme pressures (Price 2000, 2007). Ice has only been recognized as a biome relatively recently (Hodson et al. 2008; Anesio and Laybourn-Parry 2012), with most work that laid the groundwork for this determination being performed in the early to mid-2000's. First, in 2000, Buford Price proposed deep Antarctic ice as a habitat for psychrophiles where microorganisms could survive in brine veins and liquid water inclusions around mineral grains (Price 2000; Tung et al. 2006). As water freezes, mineral or ionic impurities are dispelled from the growing ice crystal matrix, eventually concentrating into brine veins along crystal boundaries (Mader 1992; Dani et al. 2012). These brines can concentrate the available nutrients and organic carbon in the bulk water a million-fold (Price 2000).

Research performed in the mid-2000s was able to see the partitioning of microorganisms into brine veins and around mineral inclusions in sea ice and glacial ice (Junge et al. 2004; Mader et al. 2006; Amato and Christner 2009). Junge et al., (2001) developed an approach to directly image cells within sea ice brine inclusions. They were able to directly image cells in brine veins that had been stained with the DAPI DNA stain (Figure 2.1). Tung et al., (2006) imaged microorganisms in liquid inclusions surrounding clay grains in 3 km deep glacial ice from Greenland for the first time (Figure 2.1). Rohde & Price, (2007) then used fluorimetry to count the number of cells within veins and found the cell number to be as high as 66 cells per 100 uM distance of vein.

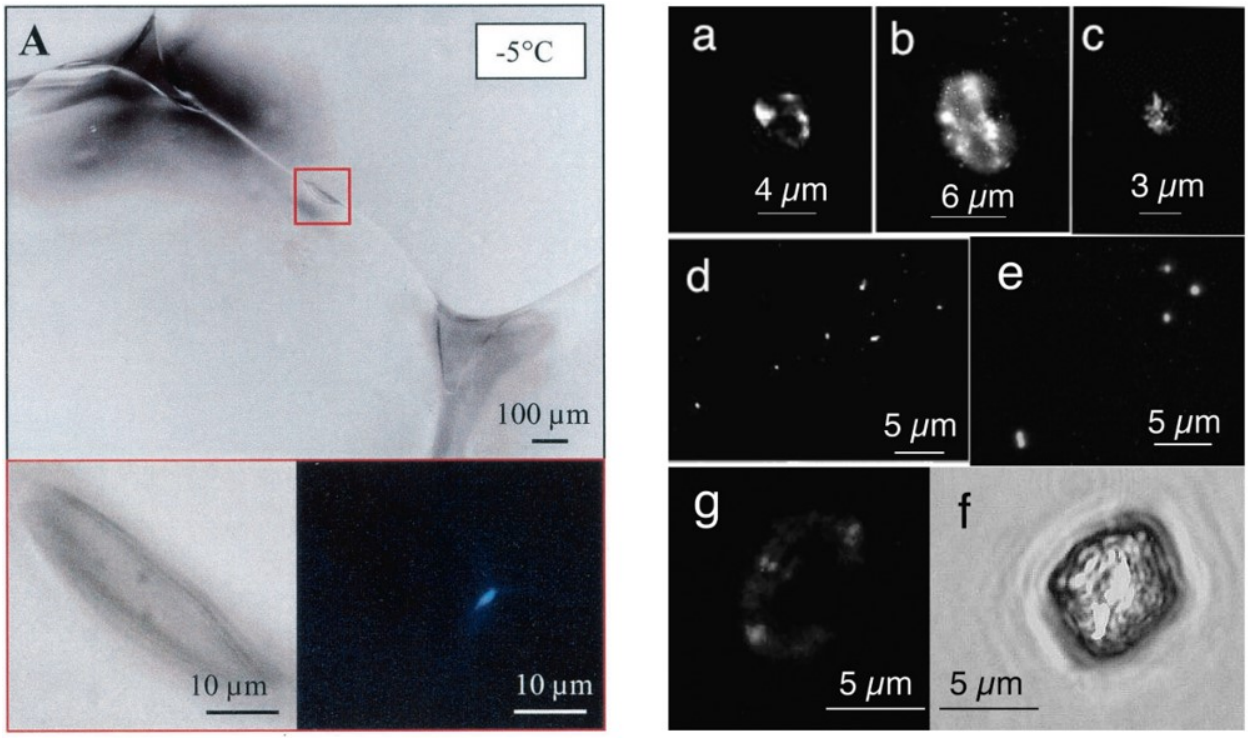

Figure 2.1. Microorganisms imaged in sea ice. **Left Panel:** Microscopic images of wintertime sea ice at -5°C depicting brine veins. The lower left photo is an enlargement of the photo above, and the lower right photo depicts a DAPI-stained bacterium in the brine vein. Adapted from Junge et al., (2004). **Right Panel: a-d.** Microscopic images of DAPI stained, mineral attached (a-c) cells and (d) unattached cells from Greenland englacial ice. **E.** F_{420} autofluorescence image of unattached *M. jannaschii* freshly removed from an anaerobic container. **F-g.** F_{420} image of

methanogens attached to a clay grain in Greenland englacial ice (g) and bright-field image of the same grain (f). Adapted from Tung et al., (2006).

Recognizing that ice, particularly glacial ice, contains microhabitats for microorganisms to inhabit requires that the microorganisms that reside there be taxonomically and functionally characterized. This has been performed extensively on surface and basal (subglacial/bottom) ice samples but much less in englacial ice (middle ice layer). Surface ice is thought of as ice which is directly in contact with the atmosphere or which is subjected to surface processes (i.e. melt) and like snow, surface ice is primarily dominated by algae and, to a lesser extent, cyanobacteria (Figure 2.2). Vincent et al., (2004) found that microbial mats on the surface of the Markham ice shelf on the northern coast of Ellesmere Island were dominated by 88% algae. Cyanobacteria are also widely distributed on ice shelves, including *Nostoc, Phormidium, Oscillatoria, Leptolyngbya*, and *Gloeocapsa* (Mueller et al. 2006). Despite the dominance of algae and Cyanobacteria, other bacterial and archaeal phyla are also present including alpha-, beta-, epsilon- and gamma-proteobacteria, Actinobacteria, and to a small extent, Firmicutes, Verrucomicrobia, Gemmatimonadetes, Crenarcheaota, and Euryarchaeota (Bottos et al. 2008). Bradley et al., (2023) found Actinobacteria, Pseudomonadota, and Planctomycetota to be the dominant and active taxa of glacial surfaces on Greenland and Iceland glaciers. Similar community assemblages are also seen in Antarctic ice shelves (Archer et al. 2015), suggesting these phyla contain unique adaptations to survive on glacial surface habitats.

More recent work characterizing the microbial communities present on the surface of an Icelandic glacier found that of the Eukaryotic community, the fungi phyla Basidiomycota and Chytridiomycota were dominant, comprising between 19% and 50% of all 18S rRNA gene

sequences while the bacterial community was dominated by Bacteroidetes, Actinobacteria, Alphaproteobacteria and Gammaproteobacteria (Winkel et al. 2022).

Subglacial ice is ice found at the bottom of the glacier which picks up debris from the underlying substrate and directly interacts with the bedrock. Subglacial ice is an extreme environment; nevertheless, many microorganisms reside there. This environment is highly oligotrophic and anaerobic, so it has been proposed that most microorganisms that reside there are chemolithoautotrophs (Telling et al. 2015). Indeed, the composition of newly exposed sediments from subglacial environments is dominated by chemolithoautotrophs (Khan et al. 2023; Venkatachalam et al. 2024). Subglacial ice is seeded by microorganisms in the sediments below the ice or from above through cracks and fissures that naturally occur in glaciers and ice sheets (Figure 2.2). Skidmore et al., (2005) determined that the basal ice of John Evans Glacier in the Canadian High Arctic was composed mainly of 50% beta-Proteobacteria and 25% Bacteroidetes which was similar to the composition of basal ice of two New Zealand glaciers (Foght et al. 2004). A further study of basal melt water from the John Evans glacier determined the same phyla to be present but with the addition of Actinobacteria (Cheng & Foght, 2007). A limited analysis of basal ice microorganisms cultured from Taylor Glacier in Antarctica could only recover isolates of the genus *Sporosarcina* of the phylum Firmicutes (Montross et al. 2014).

Englacial ice is defined as the layer devoid of influence by the surface or the substrate beneath the ice (Fountain 2011). The microbiology of the englacial environment may be the least studied of any ice environment. This is problematic because while englacial ice is assumed to be very low biomass per unit volume, it still comprises by far the largest volume of ice within

glaciers and ice sheets (Figure 2.2). Thus, the total biomass within the englacial ice of glaciers and ice sheets is significantly higher than in the supraglacial and subglacial environments. One of the earliest studies to characterize englacial communities was by Tung et al., (2005) who found samples of ice approximately 3 km beneath the surface of the Greenland ice sheet to contain 10^5 cells/ml and also found methanogenic archaea and excess methane at the same depth suggesting that the methanogens produced the methane *in situ*. The most common phyla found in near-surface englacial ice (a few centimetres – 3 meters) are the Proteobacteria (Alpha- and Beta-), Bacteroidetes, Actinobacteria, and Cyanobacteria (Simon et al. 2009; Zeng et al. 2013; Garcia-Lopez et al. 2019). Zhong et al., (2021) determined that the most abundant genera inhabiting two Tibetan englacial ice core samples belonged to the genera *Janthinobacterium, Polaromonas, Herminiimonas, Flavobacterium, Sphingomonas,* and *Methylobacterium* all of which have been found in glacial ice before (An et al. 2010; Chen et al. 2016; Zhong et al. 2018). Furthermore, it was determined that in addition to bacterial taxa, viral taxa were also present in the ice, with many likely capable of infecting the bacterial taxa present. This finding adds a new dynamic to ice microbial ecology, suggesting that viruses may regulate microbial communities in glacial ice and may aid in nutrient cycling by causing cell lysis.

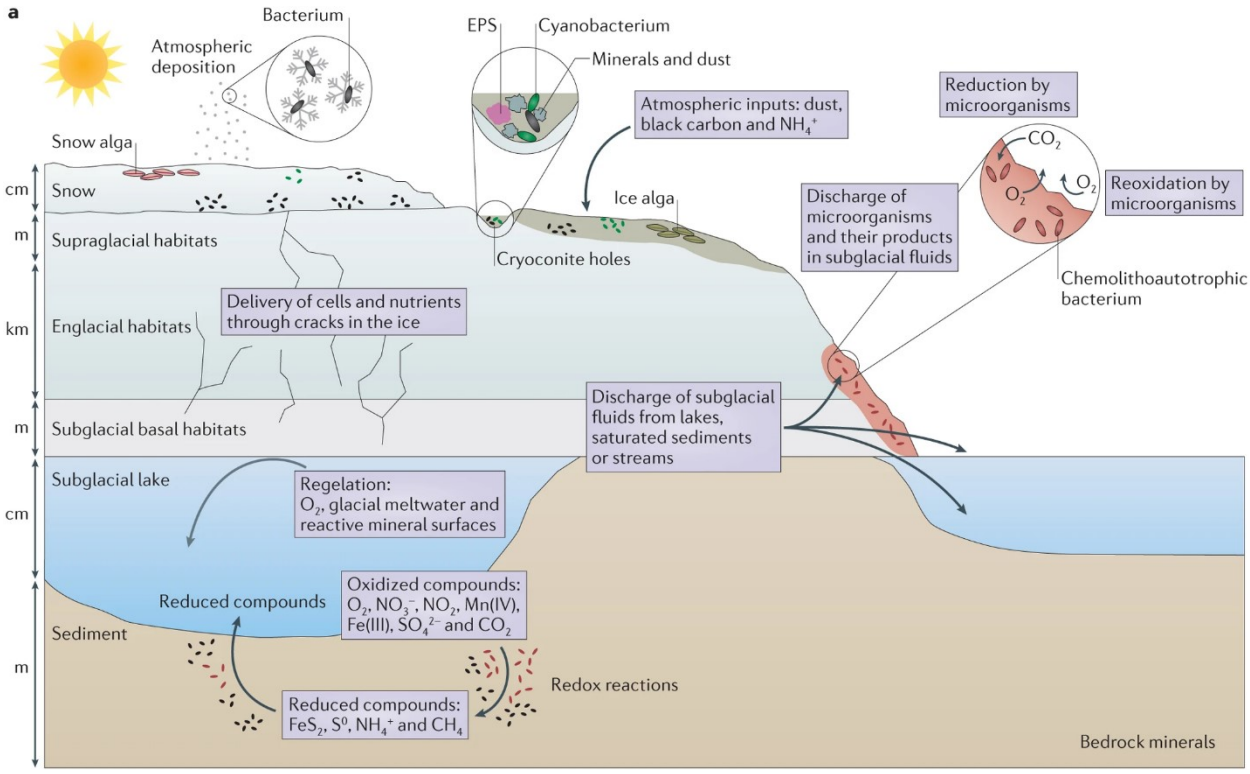

Figure 2.2. The various environments of glacial ecosystems. Adapted from Boetius et al., (2015).

2.2 Introduction to Earth and ice analog environments for astrobiology

Due to the difficulty and extreme cost of visiting other solar system bodies, terrestrial analog environments are necessary to study many processes on other worlds (Martins et al. 2017). Analog environments are terrestrial environments that mimic past or present environments on other solar system bodies in one or more ways, either chemically, geologically, environmentally, or biologically (Léveillé, 2009; Marlow et al., 2008, 2011; Martins et al., 2017). Analog environments can be divided into four main categories. Those that mimic 1) the composition of planetary bodies, either through mineralogy, elemental abundance, organic content, or others, 2) electrochemical analogs that can mimic properties like pH, redox potential, and water activity, 3) environmental analogs that mimic temperature, radiation, wind, salinity,

and aridity and, 4) physical analogs which include material properties like albedo, thermal inertia, particle size distribution, shape, density and porosity (Martins et al. 2017). These environments are used to a) collect samples for astrobiological and geological study and b) test instrumentation, methodologies, and protocols before being tried on a mission.

Analog environments prepare scientists for future planetary science missions by teaching them how life might survive on other planetary bodies, what biosignatures to look for, and how to find them. Nowhere is this truer than in microbiology and the search for life on other planets and moons. While the instruments and methods of analyses of some scientific disciplines lend themselves well to incorporation on robotic spacecraft, it is considerably more challenging to include biological payloads on these missions. This is because the instrumentation used for biology experiments is relatively large and complex and requires a large degree of human input (e.g., nucleic acid sequencing). Indeed, to date, microbiology experiments have only been included on one mission beyond Earth's orbit. This was the biology payload included on the Viking Landers, which touched down on Mars in 1976 (Klein et al. 1976). This payload included a GC-MS gas exchange experiment to detect chemotrophic microorganisms, a pyrolytic release experiment to detect phototrophic microorganisms, and a labelled release experiment to detect heterotrophic microorganisms. Of these, the labelled release experiment was the only one to produce a positive result, but due to disagreements with the results of the other experiments, it was ultimately concluded that microbial life had not been detected.

From an astrobiology standpoint, the main conditions often considered when choosing high-fidelity analog environments are temperature, water activity, and radiation. Many of the

places currently believed most likely to support life in our solar system (Mars, Europa, Enceladus) are extremely cold (avg. -60°C to -200°C) (Read and Lewis 2004; Bland et al. 2012; Ashkenazy 2019), are hyper-saline (Hand and Chyba 2007; Levy 2012; Kang et al. 2022), receive lots of surface radiation, or are hyperarid (Mars) (Goordial and Whyte 2014). As such, many of our best analog environments are located in Earth's polar regions. Specific environments include glaciers and ice sheets (analogs for the ice-covered moons of Jupiter and Saturn) (Garcia-Lopez and Cid 2017), permafrost (analogs for Martian subsurface ice) (Fairén et al. 2010), Arctic cold saline springs (analogs for Mars recurring slope lineae and the subsurface oceans on Europa and Enceladus) (Magnuson et al. 2022, 2023) and the Antarctic McMurdo Dry Valleys (analogs for Martian regolith) (Goordial and Whyte 2014; Goordial et al. 2016, 2017; Chan-Yam et al. 2019; Cassaro et al. 2021). Earth's poles have average yearly temperatures below 0°C, receive higher radiation levels, and in some locations, such as the Antarctic McMurdo Dry Valleys, are extremely dry, receiving less than 10 mm of rain annually (Fountain et al. 2010).

Another environment often used as an astrobiology analog to mimic the extremely low water activity and mineralogy on Mars is the Atacama Desert (Martins et al. 2017). The Atacama Desert is the driest place on Earth (Azua-Bustos et al. 2012). Furthermore, the high elevation of the Atacama Desert means it also receives higher levels of radiation than most other areas of the planet. The mineralogy of the soils in the hyperarid core of the Atacama Desert is also similar to that of the Martian regolith (Martins et al. 2017).

Other environments are considered analogs not because they mimic places in our solar system that may support life but because they mimic environments on early Earth when life began. The early Earth was very different than today; it was hotter, more volcanically active, and contained little oxygen, but still contained vast oceans, although more acidic than today (Frances 2012). On present-day Earth, environments that best mimic the early Earth include hot springs such as those found in Yellowstone, USA (pH 1 to 10, temperature 40 – 92°C) (Fournier 1989) and the Danakil Depression in Ethiopia (pH 0, temperature up to 112°C, 433 g/kg TDS) (Pérez and Chebude 2017; Cavalazzi et al. 2019), and in deep ocean hydrothermal vents such as the Mid Cayman Rise (temperature >398°C) (McDermott et al. 2018) and the Hellenic Volcanic Arc (temperature 315°C) (Valsami-Jones et al. 2005).

Given the abundance of life demonstrated to survive and even metabolize in ice on Earth, it raises the question of whether icy environments elsewhere in the cosmos could also support life. The majority of outer space is too cold to support the existence of water in its liquid form; however, as noted in section 1.3, even within solid ice, liquid water can persist in brine channels, and given the large volume of ice known to exist within our solar system, on places such as Mars or the icy moons of the outer solar system, habitable brine channels or mineral inclusions may be plentiful and widespread.

2.2.1 Mars

Many ice environments have been recognized on Mars (Carrier et al. 2020), including in the polar regions, pore space ice, patchy ice, solid ice, and sediment-rich ground ice (Holt et al. 2008; Byrne et al. 2009; Rummel et al. 2014; Dundas et al. 2018). Furthermore, evidence of

periglacial geomorphology has been seen across the Martian northern mid-latitudes (Byrne et al., 2009), and Mars is hypothesized to have once had extensive glaciers across its surface (Grau Galofre et al. 2020) (Figure 2.3). Furthermore, ice was directly sampled at the site of the NASA Phoenix lander near the Martian north pole (Arvidson et al. 2009). Liquid brine veins could provide a source of concentrated nutrients, salts, and liquid water for putative microorganisms on Mars. Such brine vein habitats could persist to temperatures as low as -56°C, close to Mars's mean annual surface temperature (-60°C) (Read and Lewis 2004) suggesting that liquid veins could persist over much of the year on Mars, even near the surface. Ice also acts as an effective radiation shield. Radiation at the Martian surface (180 – 225 μGy/day, Hassler et al., 2014) is considerable and detrimental to life as we know it (Pavlov et al., 2002), but only 1 meter of ice would be required to provide an effective radiation shield (Córdoba-Jabonero et al. 2005).

On Earth, high-fidelity analog environments for Martian ice can be permafrost (Dickinson et al. 2003; Frolov 2003; Goordial et al. 2016), glacial ecosystems (MacClune et al. 2003; Arfstrom and Hartmann 2005), and lava tube ice (Carrier et al., 2020; Léveillé & Datta, 2010; Popa et al., 2012; Teehera et al., 2017) (Figure 2.3). Martian lava tubes have been modelled to contain stable ice deposits (Williams et al. 2010). Thus, in addition to protecting from extreme temperature fluctuations, erosion, and increased radiation protection, the ice contained within them could provide a water source, making these environments attractive from a habitability standpoint and worthy of further study. Teehera et al. (2017) characterized the microbial communities present in ice melt water from two lava tubes in Hawaii and found Pseudomonadota and Actinomycetota to be dominant, which is consistent with the dominant communities of other oligotrophic cave environments; however, at finer taxonomic resolution, this community is

unique to all other cave environments and suggests that ice imparts unique selective pressures on these communities. Further proving lava tube ice to be a high-fidelity analog environment for Mars, Popa et al., (2012) characterized a novel *Pseudomonas* strain isolated from the basalt-ice interface of a lava tube in Oregon; this strain could use bicarbonate as a source of carbon and ferrous iron (Fe^{2+}) from igneous olivine found in basalt as an electron donor and O_2 as an electron acceptor and capable of growing between 4°C and 31°C. At neutral pH, the microbial oxidation of ferrous iron is favoured in low oxygen environments, and thus, this type of metabolism would likely be favoured in Martian lava tubes where ferrous iron is abundant, carbon could be obtained from atmospheric CO_2 fixation, and water would be present at the ice/rock interface.

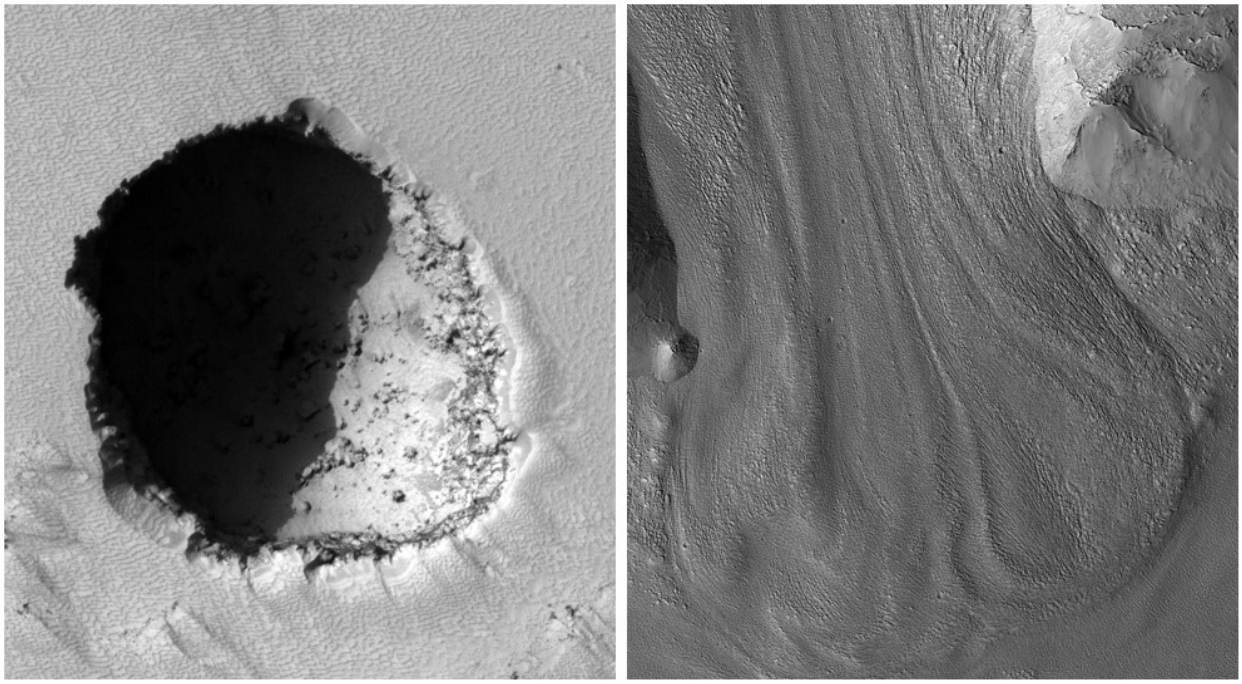

Figure 2.3. Mars possible locations with ice**. Left Image:** Lava tube skylight identified on Mars. **Right Image:** Glacier-like feature identified in the Martian mid-latitudes. Both images were taken using the HiRISE Camera on the Mars Reconnaissance Orbiter. Credit: NASA/JPL/University of Arizona.

2.2.2 Europa, Enceladus and Titan

On Earth, the best analog environments to study the icy surfaces of the moons of the outer solar system are glaciers, ice sheets, ice caps, and sea ice (Hoover and Pikuta 2009; Garcia-Lopez and Cid 2017). The icy moons include Europa, Ganymede, and Callisto, orbiting Jupiter; Titan, Rhea, and Enceladus, orbiting Saturn; and Triton, orbiting Neptune. All these moons have a thick surface crust of water-ice which hides a liquid water ocean beneath (Shematovich 2018). These bodies are expected to sustain liquid oceans because of tidal flexing and heat from their host planet (Tyler 2008). Hydrothermal activity and water-rock interactions at the seabed (Choblet et al. 2022; Bire et al. 2023) may provide the energy needed to support subsurface ocean microbial ecosystems (Hsu et al. 2015). Cryovolcanism, spewing water and ice into space, has been observed on Enceladus (Figure 2.4), meaning water from its subsurface ocean is being ejected into space. Fortuitously, the Cassini spacecraft sent to explore Saturn and its rings contained an ion and neutral mass spectrometer that was used to sample the plume material. The results indicated that the ocean contained mostly salty water, with significant amounts of hydrogen and other molecules such as carbon dioxide, methane, and ammonia (Waite et al. 2009; Kite and Rubin 2016) as well as silicon which supports the idea of hydrothermal activity in the ocean and indicates that many of the ingredients needed for life are present within subsurface ocean. Similar plumes of Europa have not been clearly detected, although phyllosilicates have been detected on the surface and are often associated with organic molecules (Marion et al. 2004). The source for these molecules could be comets and meteorites (Chyba and Phillips 2001) and from the ocean below.

Material from the oceans may be transported into the ice above by various mechanisms, the first being by cryovolcanism whereby cracks in the ice shell create high-pressure volcanoes that spray water from the ocean onto the surrounding surface (Porco et al. 2006) as occurs on Enceladus. While direct sampling of plumes on Europa has yet to occur, evidence does point to their existence (Roth et al. 2014; Sparks et al. 2016; Jia et al. 2018). Another mechanism for material from the oceans to move onto the European surface ice is through upwelling (Sotin et al. 2002). The surfaces of both Europa and Enceladus are thought to be geologically young (<50 MA), which suggests their ice shells are active (Greenberg et al. 1998; Greenberg and Geissler 2002). The activity of the ice shells implies movement, which causes fractures and channels to develop, which could force ocean water into the ice.

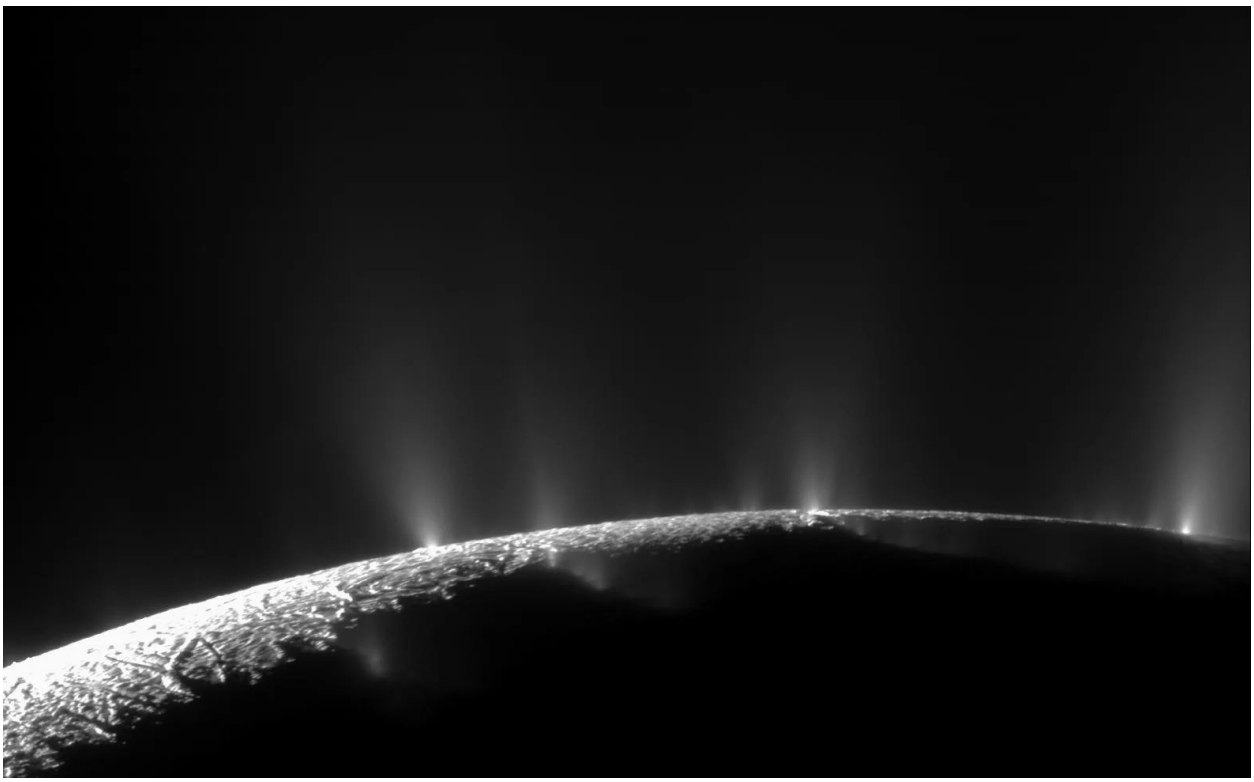

Figure 2.4. Cassini image of the plumes emanating from the south polar region of Enceladus. Credit: NASA/JPL/SSI.

Given that the icy moons contain subsurface oceans and the potential for water to be transported into the ice above, it is highly likely that if life has evolved in the oceans, evidence for it will be present in the ice either as living microorganisms or the biosignatures of dead microorganisms. As discussed in section 1.2.2, ice, particularly glaciers, ice caps, and ice sheets, can support viable microorganisms, and there is evidence that they can also support metabolically active microbial ecosystems. Glaciers, ice sheets, and ice caps are thick, spanning hundreds of meters to several kilometres thick in Greenland and Antarctica. Furthermore, they're relatively stable environments, frozen for thousands of years, unlike sea ice, which is much younger. For these reasons, glaciers, ice caps, and ice sheets are among the best terrestrial analog environments to the icy moons whose ice is anywhere from 5 to 35 km thick (McKinnon 1999; Turtle and Pierazzo 2001; Nimmo et al. 2003, 2007b; Čadek et al. 2016; Hoolst et al. 2016; Beuthe et al. 2016) and millions of years old (Greenberg et al. 1998; Greenberg and Geissler 2002; Han et al. 2012).

2.3 Terrestrial ice analog environments

2.3.1 Polar Glaciers

As stated in section 1.3, glaciers are some of the best analog environments to study if and how life might adapt to ice on other solar system bodies such as Mars or the icy moons. Glaciers tend to form at high elevations, either in polar or alpine regions, and slide to lower elevations due to gravity. Snow accumulates in the winter in the zone of accumulation and generally persists through summer to the following winter, where more snow will accumulate on top of the previous year's, causing the snow to compact over time, first to firn and then to ice. As the glacier slides, the ice in the zone of accumulation will eventually pass an equilibrium line and

enter the zone of ablation, where melting will occur. Because the ice moves quicker at the bottom of the glacier, ice near the glacier's terminus tends to be the oldest ice (Figure 2.5).

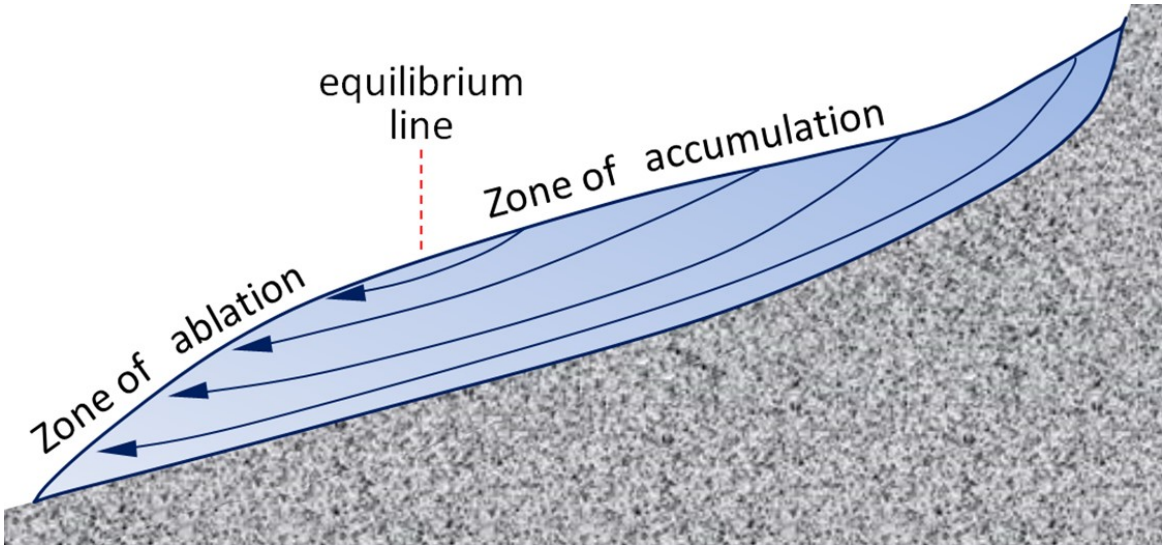

Figure 2.5. Schematic diagram of alpine or polar glacial ice flow. Source: Steven Earle (2015) CC BY 4.0 (https://opentextbc.ca/physicalgeologyearle/wp-content/uploads/sites/145/2016/03/ice-flow-2.png).

2.3.1.1 White Glacier, Axel Heiberg Island, Canada

2.3.1.1.1 Site Description

Many glaciers exist near Expedition Fiord and the McGill Arctic Research Station (MARS) on Axel Heiberg Island in the Canadian High Arctic. This area is classified as a polar desert. The closest location with which average annual meteorological data is available is at Eureka (~100 km east) where mean annual temperatures are -19.7°C and mean annual precipitation ranges from 58 mm a year at sea level to 370 mm a year at 2120 m (Cogley et al. 1996). One of the most well-studied glaciers on the island is White Glacier, situated near MARS (Figure 2.6). White Glacier hosts the longest mass balance record of any alpine glacier in the Canadian Arctic (65 years; 1959-2024). Consequently, it is one of 37 reference glaciers within

the Global Terrestrial Network of Glaciers through the United Nations Framework Convention on Climate Change. The observations are sent to the World Glacial Monitoring Service and, in addition to others, are used to calculate a worldwide glacier mass balance index (Thomson & Copland, 2016). White Glacier is a valley glacier occupying 38.7 km^2 at an elevation that ranges from 1782 m to 100 m above sea level (Thomson & Copland, 2017). The ice thickness is, on average, 200 m but can exceed 400 m in some locations. The summer temperature within the top 2 m of the glacier was previously measured to be between 0°C and -4°C (Blatter 1987).

2.3.1.1.2 Previous Research

Only one microbiological study has been conducted on White glacier ice (Touchette et al. 2023). This study evaluated the community composition and functional diversity of the microbial communities present in the top few centimetres of the ice. This research determined that Pseudomonadota (31%), Actinomycetota (27%), and Cyanobacteria (27%) were the dominant taxa. The Pseudomonadota consisted primarily of Beta (Burkolderiales), Delta (Myxococcales), and Alphaproteobacteria (Hyphomicrobiales). The metagenome revealed a primarily heterotrophic microbial community; however, genes associated with other forms of metabolism, such as photosynthesis, sulfate transport, sulfite reduction, nitrous oxide reduction, and nitrite reduction, were also found. Metabolic activity assays using a prototype metabolic activity assay also found evidence for iron oxidation in the ice. Only five cultured isolates could be recovered from the ice; all 5 were cold-adapted, belonging to the phyla Pseudomonadota or Actinomycetota, with one sharing the closest sequence identity with an isolate previously recovered from a glacial environment (Touchette et al. 2023).

Two studies of White Glacier cryoconite holes have also been undertaken. These studies found cyanobacteria and Chlorophyta to dominate the cryoconite hole ecosystem with heterotrophic bacteria, flagellates, ciliates, rotifers and tardigrades also found (Mueller et al. 2001; Mueller and Pollard 2004), although it is essential to note that both studies only focused on Cyanobacteria and Eukarya and not heterotrophic bacteria. Specifically, they found Oscillatoriales (may include *Oscillatoria* spp., *Lyngbya* spp., *Phormidium* spp., *Leptolyngbya* spp., and *Microcoleus* spp.), *Cylindrocystis brebissonii*, and *Mesotaenium berggrenii* to be the dominant taxa within the cryoconites.

2.3.1.2 Johnsons Glacier, Livingston Island, Antarctica

2.3.1.2.1 Site Description

Livingston Island is the second largest of the South Shetland Islands, which lie just north of the Antarctic Peninsula. Approximately 90% of the island is covered by snow and ice, with ice-free areas near the coast. The mean annual air temperature on the island is -1.5°C (Ramos and Vieira 2009). The Spanish Polar Committee and the Bulgarian Antarctic Institutes have bases on the island on Hurd Peninsula. Hurd Peninsula is dominated by the Hurd and Johnsons Glaciers. Johnsons Glacier (62˚40’ S, 60˚30’ W) is a sea-terminating glacier of approximately 5 km^2 with an altitude between 50 and 330 m above sea level (Navarro et al. 2013) (Figure 2.6). The glacier terminates with a cliff of 50 m above the ocean, with the bottom 5 m below the sea (Sugiyama et al. 2019). The glacier’s mass balance has been continually monitored since the 2001/2002 field season, one of the region’s longest mass/balance records (Navarro et al. 2013). As such, it has been included as a benchmark glacier within the Global Terrestrial Network of Glaciers. The average ice thickness of the glacier is 93 m with a maximum thickness of 160 m

(Benjumea et al. 2003; Navarro et al. 2005, 2009). The glacier also receives sporadic inputs of volcanic ash deposited from the Deception Island Volcano (Furdada et al., 1999), located approximately 35 km away, with the last eruption occurring in 1970, which covered the eastern portion of the island (Baker and Mcreath 1972). The layers of ash can be seen as distinct layers in ice cores taken from the glacier and help validate models of glacial accumulation rate (Furdada et al. 1999) while also being a source of minerals to the glacier.

2.3.1.2.2 Previous Research

Microbiological research of Johnsons Glacier is scarce; a 2020 study assessed the fungal diversity in air and snow samples collected from the glacier (Rosa et al. 2020). In the snow, *Cladosporium*, *Pseudogymnoascus*, *Penicillium*, *Meyerozyma*, *Lecidea*, *Malassezia*, *Hanseniaspora*, *Austroplaca*, *Mortierella*, *Rhodotorula*, *Thelebolus*, *Aspergillus*, *Poaceicola*, *Glarea* and *Lecanora* were the dominant ASVs present. Some overlap was seen in the air and snow assemblages; however, most ASVs were only found in the snow. Furthermore, the snow sample contained higher diversity than the air, suggesting snow presents unique selection pressures on the fungal community.

2.3.1.3 Devon Ice Cap

2.3.1.3.1 Site Description

Devon Island is the largest uninhabited island in the world and lies in Baffin Bay, north of Baffin Island. The Devon Island ice cap (74°30’ to 75°50’ N; 80°00’ to 86°00’ W) occupies the eastern third of the island and is one of the largest ice caps in Canada, covering an area of 14,400 km^2 (Figure 2.6). The summit is 1901 m above sea level and has a maximum ice thickness of

approximately 880 m (Dowdeswell et al. 2004). The ice cap also has a 63-year mass balance record (1961-2024) (Koerner 1970) and is a reference glacier for the Global Terrestrial Network of Glaciers. The mean annual air temperature near the summit is -23°C (Clark et al. 2007). In 2018, the ice cap received considerable attention when radar-sounding data indicated the presence of two highly unique hypersaline, subzero subglacial lakes (Rutishauser et al. 2018); however, further investigations using ground-based techniques did not confirm the existence of the lakes and a reinterpretation of the original radar data suggests that the ice is only underlain by rock (Killingbeck et al. 2024).

2.3.1.3.2 Previous Research

No previous microbiological studies at the Devon Island ice cap have been published.

2.3.2 Lava tubes

Lava tubes may be one of the most easily accessible and habitable near-surface environments on Mars today. Lava tubes occur on Earth all over the world near basaltic terrain. They form after volcanic eruptions when the exterior of lava flows cool quicker than the interior due to contact with the cooler atmosphere and underlying soil, which causes a hollow cavity to form within meters of the surface. Like other caves, lava tubes can provide stable physiochemical conditions conducive to the proliferation of microbial communities. Lava tubes can also accumulate ice deposits through the accumulation of groundwater and surface runoff, which freezes due to the entrapment of colder air within the caves, and like previously stated in section 1.4.1, lava tube ice on Mars may be habitable to microorganisms.

2.3.2.1 Lava Beds National Monument

2.3.2.1.1 Site Description

Lava Beds National Monument (41°42’ N 121°30’ W) is a national park in northern California near the border of Oregon on the northeastern flank of the Medicine Lake Volcano. The park contains the largest concentration of lava tubes in North America, with many caves containing ice deposits (Figure 2.6). Within the park, the annual air temperature fluctuates between just below freezing in the wintertime to 16°C in the summertime, with mean annual rainfall varying between 4 and 13 cm (Adamus et al. 2013).

2.3.2.1.2 Previous Research

There are few studies of lava tube microbiology, and even fewer from Lava Beds National Monument, and none have yet explored the microbiology of the ice within the park caves. Many caves in Lava Beds National Monument contain impressive microbial mats, either yellow, white, or tan, which can cover the entire walls of the caves, a clear demonstration of the habitability of the environment. The only study to examine the microorganisms within these mats compared them to those in overlying soil (Lavoie et al. 2017). They found only an 11% overlap between the OTUs in the microbial mats and overlying soils, suggesting the caves exert a strong selective pressure on the microbial community composition. This study also determined that the dominant phyla within the microbial mats were Actinobacteria, Proteobacteria, and Nitrospirae. Further, it concluded that the cave mats contained many novel taxa, as evidenced by the author’s failure to classify many OTUs beyond the genus level.

Another study examined the geochemical interactions among water, minerals, and microorganisms within the caves of Lava Beds National Monument and found a net accumulation of dissolved organic carbon within the caves which the authors interpreted to mean there was little heterotrophic metabolism occurring within the caves and instead, chemolithoautotrophy may be an important microbial lifestyle in the low light environment of these caves (Kulkarni et al. 2022).

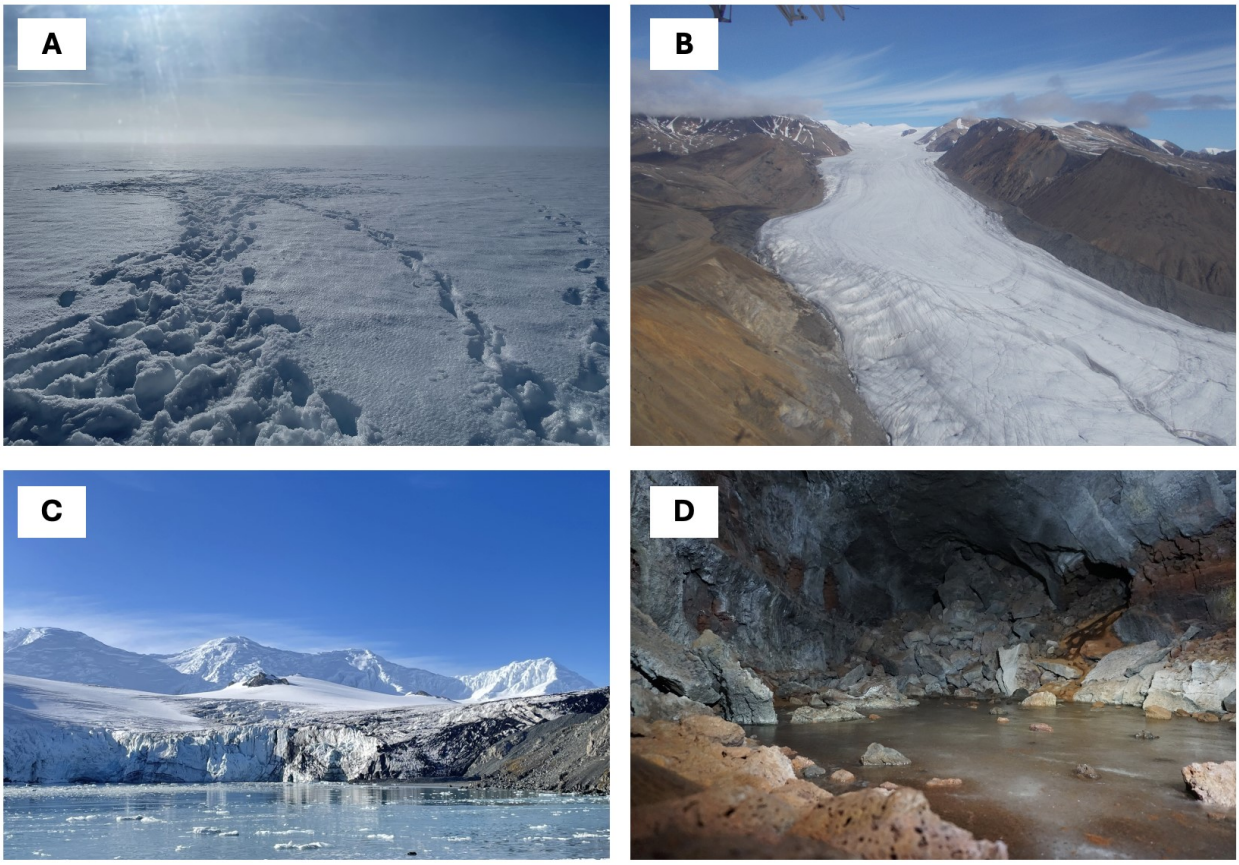

Figure 2.6. Sampling locations of this thesis. **A.** Devon ice cap, Nunavut. Credit: Brady O'Connor. **B.** White Glacier, Axel Heiberg Island, Nunavut. Credit: Scott Sugden. **C.** Johnsons Glacier, Livingston Island, Antarctica. Credit: Brady O'Connor. **D.** Skull Cave, Lava Beds National Monument, California. Credit: Brady O'Connor.

2.4 Microbial adaptations to living in cryoenvironments

The microorganisms found in cryoenvironments, including ice, are frequently classified as polyextremophiles, having to contend with multiple stresses, including subzero temperatures, high salinity, and low water activity, but, despite the harshness, persist and sometimes thrive in these environments. Microorganisms capable of growing in cryoenvironments are typically divided into one of two classes: psychrotrophs or psychrophiles. While both can grow at subzero temperatures, psychrotrophs have an optimum growth temperature above 20°C. Psychrophiles have an optimum growth temperature below 20°C and thus are typically restricted to permanently cold environments (Gounot 1986). As temperatures decrease, so does membrane fluidity, and maintaining a fluid membrane is crucial to cell survival. As a cell's membrane becomes "gel-like" due to a decrease in temperature, physiological processes such as nutrient transport, energy generation, and cell division will cease (Shivaji and Prakash 2010; Doyle et al. 2014). A common strategy to combat a decrease in membrane fluidity is to incorporate unsaturated fatty acids (Margesin et al. 2008; Garba et al. 2016). Cells can also combat low membrane fluidity by incorporating branched side chains, shortening the length of fatty acids, and altering the size and charge of polar head groups (Shivaji and Prakash 2010).

Another issue cold-adapted microorganisms need to deal with is a decrease in protein and RNA flexibility at low temperatures. Without sufficient protein flexibility, the activation energy at catalytic sites increases and reduces reaction rates overall. To combat this, cold-adapted microorganisms employ a variety of modifications to the amino acid structure of their proteins either at the catalytic site or to the whole protein to increase flexibility and access to catalytic reaction sites (Paredes et al. 2011). These include extending highly charged surface loops,

lowering the proportion of proline residues in loop regions, and reducing the number of hydrogen bonds and salt bridges (Doyle et al. 2014). Similarly, RNA can form secondary structures much easier at low temperatures due to the presence of hydrogen bonds, which can interfere with genome replication, transcription, and translation and ultimately lead to cell death (Feller and Gerday 2003). To combat the formation of RNA secondary structures, microorganisms slow translation elongation and transcription initiation is inhibited altogether (Goldstein et al. 1990; Gottesman 2018; Zhang et al. 2018). This is to allow time for the cells to synthesize cold shock proteins which help to stabilize RNA molecules (Phadtare 2004; Jin et al. 2014; Keto-Timonen et al. 2016). Once cold-shock proteins have been created, transcription can start again.

Even at subzero temperatures, microbial life still requires liquid water to survive. Because the only way for water to remain in a liquid state at subzero temperatures is if freezing point depressants such as salts are present, cold-adapted microorganisms must contend with high salt concentrations. Salts in liquid environments decrease water activity and increase osmotic stress, causing water to leave a cell to maintain osmotic equilibrium. There are two ways to counteract osmotic stress. The first strategy is the salt-in strategy, whereby inorganic salts such as K^+ and Cl^- are accumulated in the cell cytoplasm, preventing the accumulation of Na^+ (Gunde-Cimerman et al. 2018). This method has a limitation in that K^+ and Cl^- interfere with cellular function. Therefore, microorganisms using the salt-in strategy must also adapt their cellular machinery to function in high intracellular salt concentrations. As a result, only a select few microorganisms employ this approach. (Weinisch et al. 2018). The other strategy is the "salt-out" strategy using compatible solutes (Shivanand and Mugeraya 2011). Compatible solutes are

organic solutes that a cell either synthesizes or imports to stabilize the movement of solutes across the cell membrane and can be regulated as needed (Kempf and Bremer 1998; Doyle et al. 2014). Unlike K^+ and Cl^-, compatible solutes do not impact cellular machinery, and therefore, no specialized adaptations are needed to utilize this strategy to combat osmotic stress.

The other benefit of accumulating intracellular compatible solutes is to reduce the freezing point of water inside the cell, preventing ice crystal formation (Cowan 2009). One of the major challenges faced by microorganisms living at subzero temperatures is controlling and managing the formation of ice crystals which result in cellular damage (De Maayer et al. 2014). Another method to manage ice crystal formation is through the synthesis of antifreeze or ice-binding proteins. Ice-binding proteins bind to the surface of ice crystals and in doing so, control further ice growth, helping the cell avoid injury (Bar Dolev et al. 2016). The final method used to combat ice crystal formation is to employ ice-nucleating proteins, which anchor to the cell's outer membrane and function to arrange water molecules in ice-like arrangements, which discourages ice crystal formation (Lukas et al. 2022).

2.5 Methods of characterizing microbial communities and their metabolic activity

Estimation of microbial metabolism is an important aspect of microbiology. Environmental microbiology is transitioning from a taxa-centric approach to a function-centric approach (Escalas et al., 2019), whereby the functions a microbial community plays in the environment are more important than the identity of the microbes present. However, to determine if a microbial community can perform specific functions, we must first know if the community is metabolically

active within the environment and not simply dead or dormant. This is why methods to quantify and characterize microbial metabolism were developed.

2.5.1 Metagenome sequencing

Metagenome sequencing is a powerful tool which has transformed environmental microbiology over the past two decades. Metagenome sequencing is defined as a molecular tool used to analyze all the DNA from all the microorganisms within a sample (Ghosh et al. 2019). This allows more than just the taxonomic composition of a community to be determined as amplicon sequencing does but allows the functional potential of the community to be determined as well (Quince et al. 2017) and therein lies the true power of metagenomic sequencing. Furthermore, a decade ago this technique was made even more powerful by the development of algorithms which could reassemble sequences from metagenomes into the original genomes within the sample (termed genome binning) using shared sequence features such as read coverage or nucleotide composition (Sangwan et al. 2016) allowing the study of genomes of unculturable microorganisms (Albertsen et al. 2013). As the cost of next-generation sequencing has steadily decreased over time, the availability and popularity of this technique has become widespread. In a standard metagenomic sequencing workflow, total DNA is extracted from an environmental sample, a sequencing library of this DNA is generated by fragmenting the extracted DNA into smaller, uniform pieces, and sequencing (Quince et al. 2017). While long read metagenomic sequencing is becoming increasingly popular, the majority of metagenomic sequencing is performed by short-read sequencing platforms such as Illumina. The sequencing of short reads usually requires the assembly of these short-reads into longer contiguous sequences (contigs) (Ayling et al. 2020). Once contigs are generated, the taxonomy of these

sequences and thus the taxonmy of the microorganisms within the original sample can be established or, protein coding sequences can be predicted and annotated to determine the identity and each gene present (Quince et al. 2017).

Metagenomics is not without its limitations. First, the sequences of rare taxa and genes are often missed in metagenomic datasets due to loss during library preparation or incomplete sequencing or assembly. Furthermore, the accuracy of taxonomic and functional classification of metagenomic sequences is dependent on incomplete databases (Quince et al. 2017). This problem is likely compounded when working with metagenomic sequences recovered from extreme environments, as sequence databases likely contain even fewer sequences recovered from these environments. Second, metagenomically sequenced DNA can originate from viable, live or dead microorganisms whose DNA remains in the environment after cell death. As such, it is impossible to infer from metagenomic datasets alone which microorganisms within a sample are alive or which genes are metabolically active within an environment, limiting the conclusions that can be drawn from metagenomic datasets alone. To address this shortcoming, metagenomics should be combined with methods to characterize microbial metabolic activity within an environmental sample. In the preceding sections, I will outline some popular methods to characterize microbial metabolic activity within environmental samples.

2.5.2 Redox dye metabolic activity assays

Redox dye assays are a popular way to measure the metabolic activity of individual microorganisms or of an entire community because they're simple to set up and do not require specialized equipment that a typical microbiology lab is unlikely to have. Redox dye assays

work by incubating a microbial isolate or community with a nutrient source and redox dye under the specific conditions being tested. If the microorganisms can metabolize under the tested conditions, they will utilize the nutrients provided. The dye will then be reduced by cytochromes in the electron transport chain or NADH or NADH-dependent oxidoreductases and dehydrogenases of metabolically active cells (Braissant et al. 2020). Reduction of the dye causes its colour to change, which can be quantified by a spectrophotometer or visualized with the 'naked eye'. These assays can be set up manually but are also readily available from commercial companies such as Biolog™. Biolog™ sells the assay in a 96-well plate format where each well tests for the metabolism of a specific nutrient. These nutrients can be carbon, nitrogen, phosphorus, or sulfur molecules. The assays are even capable of determining the chemical sensitivity of an isolate or community (Bochner et al. 2001; Bochner 2003). Assays such as these allow the rapid determination of the ability of a microbial community to metabolize thousands of compounds in a matter of days, allowing for the functional potential of a microbial community to be determined in great detail (Bochner et al. 2001).

The success of redox assays requires that the dye penetrates cell walls and membranes to reach the enzymes or electron transport chain, which reduces it. However, it has been shown that some redox dyes do not penetrate fungi and other eukaryotic cells, limiting their usefulness (Praveen-Kumar and Tarafdar 2003). Some bacteria have even been shown incapable of reducing tetrazolium dyes (the class of dye used in Biolog assays) (Servais et al. 2001) and may even be toxic to others (Servais et al. 2001; Villegas-Mendoza et al. 2015). There has been limited use of redox dyes to evaluate the metabolic activity of archaea; however, some archaeal pure cultures have demonstrated the ability to reduce tetrazolium salts (Oren 1995). Another

disadvantage of redox assays is that they can only be set up *ex-situ.* Therefore, it needs to be clarified how closely the results match the metabolism of a microbial community or isolate in its natural environment. A further limitation is that the assay requires sufficient understanding of the base medium needed to support the metabolism of a microorganism or community; however, this is usually not the case as it has been estimated that on average only 1% of microorganisms can be successfully cultured from environmental samples (Amann et al. 1995; Vartoukian et al. 2010). Finally, redox assays have only been used to assay the metabolism of heterotrophs. No study has developed a redox assay that can measure the metabolism of chemolithoautotrophs or phototrophs.

2.5.3 Adenosine Triphosphate (ATP) Assays

Adenosine triphosphate (ATP) is an essential molecule in all life because of its use as the energy currency of the cell. ATP is generated through catabolic processes and is used in all aspects of a cell's growth and metabolism. Seeing as in environments like soil, ATP has been shown to have a very short half-life (Conklin and Macgregor 1972; Qiu et al. 2016), its presence in an environmental sample must indicate a microbial community is currently or recently metabolically active allowing for its presence as a proxy for metabolic activity. A luciferase ATP assay is the most common way to measure ATP. Luciferase is oxidized to oxyluciferin using ATP and the production of AMP. This reaction releases light and can be measured using a luminometer (Braissant et al. 2020).

While an ATP assay does require some sample processing and some specialized equipment such as a luminometer, one of the advantages of ATP assays over other metabolic activity assays

is that they can be performed directly on environmental samples in the field without the need to isolate or culture microorganisms (Barnett et al. 2016; Mulec and Oarga-Mulec 2016; Christner et al. 2018). Furthermore, there is no need for a sample incubation step to allow the active cell population to transform a molecule. This makes interpretation of the results easier because they more closely reflect the actual level of ATP and, thus, metabolic activity in the environment. Furthermore, because ATP is used by all cells, the assay should be capable of detecting metabolic activity from all forms of life. The assay is also highly sensitive, able to reliably detect as little as 10^{-14} moles (0.01 picomoles) of ATP (Hattori et al. 2003) (emdmillipore.com) making it a great assay to use when trying to detect small changes in activity or activity from very few cells.

There are, however, still disadvantages associated with ATP assays. For one, the presence of ATP in a sample cannot determine which kind of metabolism is being performed. Secondly, ATP extraction from environmental samples is often poor (Karl and Holm-Hansen 1978; Webster et al. 1984). Furthermore, ATP is easily degraded with impurities in the sample matrix degrading it as well as non-neutral pH (Posimo et al. 2014; Šimčíková and Heneberg 2019). It has also been shown that filtration in preparing samples for ATP assays can degrade ATP (Braissant et al. 2020). For these reasons, caution must be taken when concluding the relative metabolic activity of an environmental sample solely from ATP assay results.

2.5.4 Radiorespiration Assays

Radiorespiration assays are a highly sensitive and specific method used to measure metabolic activity in microbial ecosystems, particularly in polar environments (Steven et al.

2007b; Niederberger et al. 2010; Goordial et al. 2016; Chan-Yam et al. 2019; Wood et al. 2024). The assay functions by measuring ^{14}C labelled CO_2 ($^{14}CO_2$) produced during microbial mineralization of a labelled carbon source in a microcosm. $^{14}CO_2$ can be sampled from the microcosm throughout an experiment and measured on a scintillation counter. Typically, labelled carbon sources are glucose or acetic acid because of their ability to be catabolized by most microorganisms; however, other carbon sources can also be used, such as hydrocarbons, including hexadecane and naphthalene (Ellis et al. 2022). While the measurement of $^{14}CO_2$ evolved from catabolism of glucose and acetic acid would only measure heterotrophic microbial activity, it is possible to use the method in reverse to measure autotrophic metabolism through the incorporation of substrates such as ^{14}C labelled bicarbonate into biomass (Llirós et al. 2011). In theory, it is also possible to measure metabolic activity using non-carbon compounds; however, consideration must be given to the half-life of the isotope to be used. Carbon-14 has a half-life of approximately 5000 years and thus is relatively stable in a microcosm experiment; however, sulfur-35, for example, is often used to measure the metabolic activity of sulfate-reducing bacteria (Sand et al. 1975; Colangelo-Lillis et al. 2019) only has a half-life of 87 days and thus is not compatible with long term microcosm experiments.

While radiorespiration is a highly sensitive method of detecting metabolic activity, especially at low temperatures, it also contains some disadvantages. For one, the method requires using a scintillation counter, which can be prohibitively expensive to some laboratories. The method also involves using hazardous radioactive material, which poses a safety hazard and requires specialized training. While the method more closely mimics the *in-situ* environment for measuring metabolic activity than redox dye assays, for example, it still requires the use of

microcosms which cannot perfectly mimic the *in-situ* environment. While sensitive at low temperatures, radiorespiration assays may also require years to detect statistically significant metabolic activity at subzero temperatures (Goordial et al. 2016). Finally, while the rate of metabolic activity can be calculated if the radioactive isotope being detected is part of a gas, it cannot be calculated if the starting radiolabelled isotope is incorporated into biomass because the extraction of the isotope destroys the sample and prevents further measurement.

2.5.5 Metatranscriptomic sequencing

One of the disadvantages of all the methods of measuring metabolic activity that were previously described is that they're only capable of detecting specific functional metabolisms, and they cannot determine which taxa of a community are active and which are not. One way to answer these questions is to use metatranscriptomic sequencing. A metatranscriptome is a transcriptome (RNA) of an entire community of microorganisms rather than just a single isolate. Sequencing of the metatranscriptome from an environmental sample allows for determining which community members are metabolically active and which genes they're expressing *in situ*. To sequence a sample's metatranscriptome, the total RNA of the sample is extracted. Of all the RNA that is extracted, the vast majority (95%-99%) is ribosomal RNA such as 16S or 23S rRNA or tRNAs (Peano et al. 2013) and thus needs to be removed to enrich for the more informative mRNA. Once rRNA and tRNAs have been depleted, the mRNA is reverse transcribed into cDNA, which can be prepped for sequencing like DNA. Depending on the application, after sequencing, the mRNA sequences can either be assembled *de novo* and annotated to determine the function and closest related taxa to the sequences, or they can be aligned to a reference, such as a metagenome if one was sequenced from the same sample. The former approach is typically

used for performing differential abundance analysis, whereas the latter approach is used for surveys of the broad metabolic potential of the entire community. This makes metatranscriptomic sequencing a powerful tool to characterize the metabolic activity of whole microbial communities because it can determine which taxa are active *in situ* and which genes are actively utilized by each taxa.

Despite the power of metatranscriptomic sequencing, there remains many challenges to using the method. Like ATP, mRNA is easily degraded by factors such as high temperature and alkalinity (Jo et al. 2023) and ribonucleases, readily found in the environment and on laboratory surfaces (Green and Sambrook 2019). This necessitates quick sample processing. However, this is often not possible when collecting samples from the field, and thus, methods of preservation in the field have had to be developed (Trivedi et al. 2022). Chemical preservatives such as DNA/RNA Shield from Zymo Research and RNAlater from Qiagen will preserve RNA without cold storage while freezing samples in liquid nitrogen has also been performed (Li et al. 2019). Preservation is critically important, especially in low biomass samples, where minimal degradation can significantly impact the final results. Another challenge is that unlike other methods, such as radiorespiration assays, rates of metabolic activity cannot be determined with metatranscriptome sequencing; only the presence or absence of transcribed genes can be determined. Finally, due to technology limitations, metatranscriptomics can only sometimes capture the entire metatranscriptome. This may be because some transcripts' abundance is too low to capture during sequencing or the sample diversity is too high to capture every unique transcript (Shakya et al. 2019). As such, a metatranscriptome should not be interpreted as

containing all the transcripts of a sample, nor should the relative expression of each gene be considered accurate when library preparation can bias the results (Alberti et al. 2014).

2.6 Conclusion

Ice environments, such as glaciers, ice caps, ice sheets and lava tubes are analogous to putative extraterrestrial environments such as those on Mars, Europa and Enceladus and likely contain active microbial communities despite encountering some of the most extreme conditions on Earth. While previous research has determined the identity of taxa present within these environments and hinted that glaciers can support active microbial communities, to date, no study has definitively determined which microbial taxa are metabolically active within ice, nor have they elucidated the survival strategies employed by ice entrapped microorganisms. Furthermore, while lava tubes containing ice are thought to be a near-surface habitable environment on Mars, research has yet to attempt to characterize the microbial communities of these environments on Earth to determine if they contain active microbial ecosystems and, consequently, if they may be habitable on Mars. Thus, this work aims to characterize the microbial communities of ice cryoenvironments analogous to that found on Mars, Europa, and Enceladus, with particular emphasis placed on describing the metabolically active fraction of these communities using ‘omics techniques and metabolic activity assays to better understand and constrain the limits of life in ice both on Earth and extraterrestrial worlds.

Connecting text

Despite the numerous detections of lava tubes on Mars and modelling evidence that they contain ice which could support a microbial ecosystem, no study has attempted to characterize the microbial communities in this unique environment on Earth. Characterizing these communities is vital to determining if lava tube ice could support a microbial ecosystem on Mars. Lava Beds National Monument, located next to the Medicine Lake Volcano in Northern California contains the largest concentration of lava tubes in North America, many of which contain large ice deposits. Previous studies have focused on the microbial mats which are extensive on the walls of many of the caves however none have explored the communities within the ice. In this chapter, I use a combination of 16S rRNA amplicon sequencing, culturing and metabolic activity assays to determine if these communities are cold-adapted and capable of metabolism at *in situ* subzero temperatures.

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Supplementary Figures S3.1 – S3.7 are available as supplemental material.

Chapter 3. Taxonomic characterization and microbial activity determination of cold adapted microbial communities in lava tube ice caves from Lava Beds National Monument, a high-fidelity Mars analogue environment

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3.1 Abstract

Martian lava tube caves resulting from a time when the planet was still volcanically active are proposed to contain deposits of water-ice, a feature that may increase microbial habitability. In this study, we taxonomically characterized and directly measured metabolic activity of the microbial communities inhabiting lava tube ice from Lava Beds National Monument, an analogue environment to Martian lava tubes. We investigated whether this environment was habitable to microorganisms by determining their taxonomic diversity, metabolic activity, and viability using both culture-dependent and independent techniques. 16S rRNA sequencing recovered 27 distinct phyla from both ice and ice-rock interface samples, primarily consisting of Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes, and Chloroflexi. Radiorespiration and Biolog EcoPlate assays found these microbial communities to be metabolically active at both 5°C and -5°C and able to metabolize diverse sets of heterotrophic carbon substrates at each temperature. Viable cells were predominantly cold adapted, capable of growth at 5°C ($1.3x10^4$ – $2.9x10^7$ cells/ml), and 24 of 38 cultured isolates were capable of growth at -5°C. Furthermore, 14 of these cultured isolated and 16 of the 20 most numerous amplicon sequences we recovered were most closely related to isolates and sequences obtained from other cryophilic environments. Given these results, lava tube ice appears to be a habitable environment, and considering the protections Martian lava tubes offer to microbial communities from harsh surface conditions, similar Martian caves containing ice may be capable of supporting extant, active microbial communities.

3.2 Introduction

Presently, the average temperature on the surface of Mars is -60°C, together with extremely dry conditions, and atmospheric pressures of ~ 6 millibar (1% of that on Earth (Merino et al. 2019)) create an environment inhospitable to life. In addition, surface soils are extremely oxidizing and receive large doses of UV and ionizing radiation due to Mars' thin atmosphere and lack of a magnetic field (Hassler et al. 2014; Merino et al. 2019). Given these harsh surface conditions, Martian caves, specifically lava tubes, may be better environments to search for signs of extinct or extant life (Boston et al. 2001; Léveillé and Datta 2010; Northup et al. 2011; Uckert et al. 2017).

Lava tube caves form in the shallow subsurface of volcanic regions after cooling of a basaltic lava flow's exterior which leaves hollow voids below (Léveillé & Datta, 2010). It is understood that lava tubes can form on any rocky celestial body which is or was volcanically active, with strong evidence to suggest these subsurface features are extensive in the volcanic provinces of Mars (Carr et al. 1977; Hodges and Moore 1994; Wyrick et al. 2004; Cushing et al. 2007; Léveillé and Datta 2010; Farrand et al. 2011; Cushing 2012).

Like terrestrial lava tubes, Martian lava tubes should theoretically provide protection from UV radiation, ionizing radiation and surface dust storms; decrease temperature variation and provide energy sources for microorganisms, such as Fe^{2+}, Mn^{2+}, H_2S, H_2, and CO_2 from alteration of mafic rock (Léveillé & Datta, 2010). Most lava tubes on Mars are geologically old, though there is evidence to suggest volcanism has occurred between 3.7 Gya and 100 to 200 Mya (Werner 2009). Nevertheless, lava tubes are likely much longer lived on Mars than on

Earth due to lower tectonic/seismic activity and rock weathering. In addition, modeling suggests that lava tubes located in the volcanic provinces of Mars may contain stable or accumulating water-ice, even today (Williams et al. 2010), although this has not clearly been observed yet.

On Earth, water can remain liquid within ice by two mechanisms. The first is due to the insolubility of ionic impurities which create micron-sized veins of liquid water to temperatures as low as -90°C (Price and Sowers 2004) while the second forms when mineral grains contact ice forming a liquid film around the mineral surfaces, with both mechanisms accepted to occur on Mars (Rohde and Price 2007). Despite subfreezing temperatures, metabolically active microorganisms have been observed within ice (Carpenter et al. 2000; Rivkina et al. 2000; Jakosky et al. 2003; Junge et al. 2004; Steven et al. 2008; Chan-Yam et al. 2019) and to temperatures as low as -33°C (Bakermans and Skidmore 2011a). Thus, the combination of shelter and bioavailable liquid water within ice could have made Martian lava tubes a potential refuge to microbial life, especially after Mars' climate transitioned from wet and warm to cold and dry approximately 3.7 to 3.0 Gya (Ramirez and Craddock 2018). Therefore, terrestrial lava tubes that present similar characteristics are considered to be important analogues for studying habitability in the Martian subsurface (Boston et al., 2001; Hathaway et al., 2014; Léveillé & Datta, 2010; Northup et al., 2011).

To date, microbial research of lava tubes has largely focused on microbial mats (Hathaway et al. 2014; Riquelme et al. 2015; Lavoie et al. 2017; Gonzalez-Pimentel et al. 2018) and microbe/mineral interactions (Boston et al., 2001 ; Northup et al., 2011 ; Ríos et al., 2011 ; Uckert et al., 2017). However, relatively few studies of lava tubes have focused on

cryoenvironments, i.e. those caves which contain ice or where freezing temperatures exist. Such environments are better analogues for Martian lava tubes given the freezing temperatures likely encountered even in the shallow subsurface. One culture-based study by (Popa et al. 2012) recovered microbes from the basalt/ice interface within a terrestrial lava tube capable of metabolizing iron from the mineral olivine. Teehera et al., (2017) conducted a taxonomic characterization of the microbial communities found associated with secondary minerals and ice from two lava tubes in Hawaii. Despite these limited studies, a knowledge gap remains regarding the identity, viability, and metabolic activity of the microbial communities present within lava tube ice.

The goal of the present study is to determine the structure of the microbial community, together with the cold temperature adaptability and metabolic activity of microorganisms inhabiting lava tube ice from Lava Beds National Monument (LABE). These findings are important to understanding the microbial limits of life in Earth's subsurface, to determine the applicability of using lava tube ice caves as terrestrial analogues for subsurface Martian environments, and to determine if such environments could support life.

3.3 Materials & Methods

3.3.1 Sample site description and sample collection

LABE, whose climate is classified as a semi-arid desert, is located in northern California (41° 42′ 50″ N, 121° 30′ 30″ W) on the north eastern flank of the Medicine Lake Volcano. This area contains the largest concentration of lava tubes in North America, with >800 discovered to date (personal communication with park staff). Many of these caves contain large seasonal or

perennial ice deposits (Figure 3.1). Within LABE the average annual air temperature fluctuates between -0.5°C in the winter and 15.8°C in the summer; mean annual precipitation varies between 12.2 cm in the winter and 4.8 cm in the summer (Adamus et al. 2013). Ice samples were collected during the summer of 2017 and 2018 at sites chosen based on the different type of ice they contained (i.e. perennial vs. seasonal and clear vs. sediment containing). Unfortunately, due to site restrictions not all caves and sites could be sampled both years. A description of each sampling site can be found in Table 3.1. Samples were obtained using flame and ethanol-sterilized chisels and collected in sterile Whirlpak® bags which were kept frozen at -20°C during transport and storage at McGill University to await further analysis.

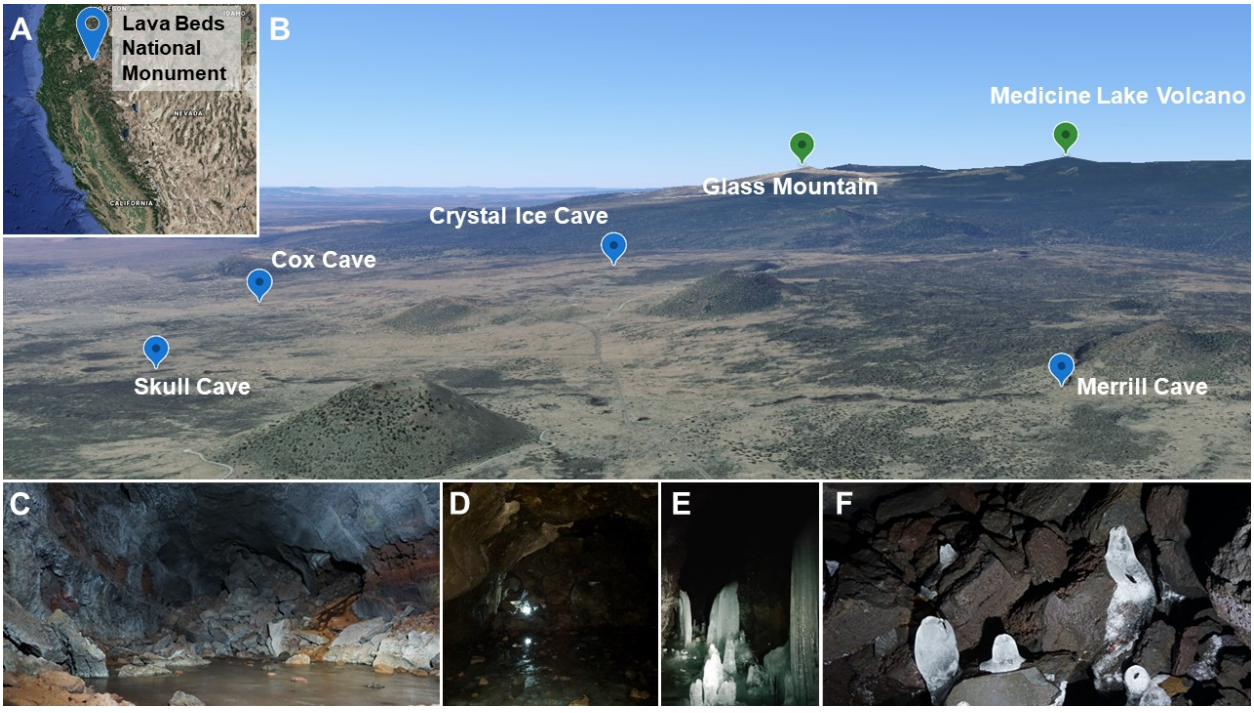

Figure 3.1. Location and pictures of sampling sites. **(A)** Geographic location of Lava Beds National Monument. Credit: Google Earth. **(B)** 3D map with locations of each of the caves sampled in this study. Blue markers denote sampling locations. Green markers denote points of reference. **(C)** Site SC2 2017. An ice floor covered in red oxidized basalt. **(D)** Site CC 2018. An ice floor with some rocks ranging in size from a few centimeters to a few tens of centimeters

large encased in the ice. **(E)** Site CI 2017. Perennial ice stalagmites. **(F)** Site MC 2017. Seasonal ice stalagmites formed on basalt. Maps credit: Google Maps.

Table 3.1. Descriptions of Lava Beds National Monument cave ice samples.

Site	*Cave*	*Type of ice*	*Year sampled*	*Distinguishing feature*
CC 2018	Cox Cave	Perennial ice floor	2018	Ice contains a mix of sediment and small pieces of basalt.
CI 2017	Crystal Ice Cave	Perennial ice stalagmites	2017	Ice is the most prestine we sampled. Very little sediment or debris could be seen within the ice.
MC 2017	Merrill Cave	Seasonal ice stalagmites	2017	Ice is frozen directly to the surface of cm sized basalt.
MC 2018	Merrill Cave	Seasonal ice stalagmites	2018	Ice is frozen directly to the surface of cm sized basalt.
SC1 2017	Skull Cave	Perennial ice floor	2017	A substantial quantity of sediment rests on the surface of the ice floor.
SC2 2017	Skull Cave	Perennial ice floor	2017	A substantial quantity of oxidized basalt powder rests on the surface of the ice floor.
SC2 2018	Skull Cave	Perennial ice floor	2018	A substantial quantity of oxidized basalt powder rests on the surface of the ice floor.

3.3.2 Physiochemical analyses

The pH of the ice samples was measured in the field by submerging pH strips in melted surface ice. The temperature of the ice was determined by either placing a handheld temperature probe directly on the ice surface or placing the probe approximately 4 cm into the ice by chiseling a small divot. Processing of ice samples for further physiochemical analyses was conducted in the laboratory by melting and filtering the ice at room temperature through a 0.45 µm nitrocellulose filter. Total organic carbon (TOC) was then measured by the ultraviolet/persulfate oxidation method on a Sievers Innovox TOC analyzer (General Electric Power and Water, Water and Process Technologies, Boulder, Colorado) following standard

protocols. Analyses of nitrate, ammonia and phosphate concentrations were prepared following the manufacture's instructions and measured colorimetrically on a multi-channel Lachat auto-analyser (Lachat Instruments, Milwaukee, Wisconsin).

3.3.3 Cell enumeration, isolation, cultivation and characterization

To determine the total number of viable microorganisms present within our samples, colony forming units (CFU) were enumerated on R2A agar as follows: melted ice was diluted to 10^{-2},10^{-3}, and 10^{-4} in 0.1% $Na_4P_2O_7$ buffer, pH 7 and plated on agar. To determine the effect of growth temperature on the total CFU, three replicates of each dilution were incubated at three temperatures (5°C for 28 days, 23°C for 14 days, and 37°C for 7 days) before counting the total CFU. To determine if the viable microbial population was cold adapted, we isolated those CFU that presented unique morphologies on plates incubated at 5°C and 23°C from previous analyses. The ability of each isolate to grow at a range of temperatures was further tested on R2A agar at 37°C, 23°C, 5°C, 0°C and at -5°C on R2A+7% sucrose to prevent the agar from freezing. The ability of each isolate to grow in 0%, 3%, 6%, 9%, 12%, 15%, 18%, and 21% NaCl was also tested on R2A agar at 25°C except those isolates which couldn't grow at 25°C which were grown at 5°C. Sanger sequencing was performed to determine the identity of each isolate. First, isolated colonies were lysed by mixing with 250 µl of deionized H_2O and heating in a microwave for 3 minutes. PCR amplification of the 16S rRNA gene was performed using primers 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), which amplify nearly the full length of the gene (~1500 bp). The 16S rRNA PCR cycling conditions were as follows: (1) 95°C for 7 min, (2) 94°C for 45 s, (3) 55°C for 45 s, (4) 72°C for 1 min, (where steps 2 to 4 were repeated 30 times), (5) 72°C for 10 min. Amplicon DNA was sent to

the Plate-forme d'Analyses Génomiques de l'Université Laval (Quebec City, Quebec, Canada) for sequencing. Low-quality sequences were trimmed, and miscalled bases corrected using Sequencher® version 5.4.6 (Ann Arbor, Michigan, USA). Sequences were then compared against the GenBank database using BLASTn to look for the most similar sequence to the query sequence.

3.3.4 Nucleic acid extraction and 16S rRNA sequencing

Five replicate extractions were performed on each ice sample using the Qiagen Dneasy® PowerLyzer® PowerSoil® DNA extraction kit (Qiagen, Venlo, Netherlands), following manufacturer instructions but with slight variations in the final elution step: DNA was eluted in 50 µl (20 µl for samples from Crystal Ice Cave) of molecular grade H_2O rather than elution buffer. Sample replicate extractions were then polled together and DNA concentrations were then checked on a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and concentrated using a ThermoSavant DNA 110 SpeedVac (Thermo Fisher Scientific, Waltham, Massachusetts) if the concentration was found to be too low for sequencing library preparation. Sequencing libraries were prepared following Illumina's 16S metagenomic sequencing library preparation protocol. Primer pair 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT) (Parada et al. 2016), targeting the V4-V5 hypervariable region of the prokaryotic 16S rRNA gene, was used to amplify DNA for sequencing. Sequencing libraries were constructed from the amplified PCR product using the Illumina Nextera XT library preparation kit. Libraries were loaded on an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 (Illumina, California, USA) and sequencing run was carried out for 500 cycles**.**

3.3.5 Sequence analysis

After sequencing, reads were grouped into amplicon sequencing variants (ASVs) using the DADA2 package for R (Callahan et al. 2016). Settings and parameters for sequence analysis were chosen by following the DADA2 Pipeline Tutorial (1.12) (https://benjjneb.github.io/dada2/tutorial.html), with slight variations to better suit our reads. Briefly, to remove low quality base calls, the last 5 and 50 base pairs were trimmed from the forward and reverse reads to a final length of 245 and 200 respectively. Additionally, during the chimera removal step (*isBimeraDenovo*), the argument *minFoldParentOverAbundance* was changed from 1 to 4 to reduce the proportion of reads discarded as chimeras. Alpha and beta diversity indices were calculated in R v 4.0.2 (R Core Team 2020). The 'vegan' package (Oksanen et al. 2019) was employed to calculate the Shannon diversity index (here referred to as H'-tax) and Bray-Curtis distances on ASV distribution tables; 'ape' (Paradis and Schliep 2019) and 'phangorn' (Schliep 2011) were employed to create a Neighbor Joining (NJ) tree (best parsimony results) with 'GTR' nucleotide substitution model on ASV fasta sequences, previously aligned using Clustal Omega software (Sievers et al. 2011); the 'picante' package (Sievers et al. 2011) was employed to calculate Faith's Phylogenetic Diversity (Faith's PD) using the NJ tree and ASV distribution tables, while 'GuniFrac' (Chen 2018) was employed to calculate UniFrac Distances using these same parameters. To determine the functional potential of lava tube ice microbial communities, 16S rRNA ASVs obtained from the lava tube ice were analyzed with the functional prediction pipeline Piphillin (Iwai et al. 2016) employing a 99% sequence identity cutoff against the May 2020 release of the KEGG database (Kanehisa 2000).

3.3.6 Community level physiological profiling

To assess microbial activity and heterotrophic carbon substrate utilization among the microbial communities of LABE ice, samples were incubated in Biolog EcoPlates™ at both 5°C and -5°C. Melted ice samples were diluted 10^{-2} in 0.1% $Na_4P_2O_7$ buffer, pH 7, both to remove sediment particles which could interfere with spectrophotometric readings and to lengthen the time required to detect substrate utilization. 150 µl of diluted sample was then added to the wells and incubated in the dark at 5°C and -5°C. We believe the dye, buffer, and substrates contained within the wells reduced the freezing point of water which prevented freezing of plates incubated at -5°C (see Supplemental Figure 3.1 for the EcoPlate experimental setup). Absorbance changes at 595 nm wavelength were monitored weekly using a SpectraMax® M2e Multi-Mode Microplate Reader (Molecular Devices, San Jose, California). Wells were considered to be positive when their OD_{595} value reached above 0.25 (Garland 1996, 1997). The average well-color development (AWCD) (time to reach plateau phase)(Garland and Mills 1991; Iţcuş et al. 2016), substrate richness I, Shannon-Weaver diversity index (here, referred to as H'-clpp), and Shannon substrate evenness I (Garland 1997; Iţcuş et al. 2016) were calculated in triplicate once the AWCD hit the plateau phase (Pessi et al. 2012), corresponding to 53 days at 5°C and 102 days for -5°C. The AWCD = $\sum$Odi/31, R = number of metabolized substrates, H'-clpp = -$\sum$pi(lnpi), where pi = Odi/$\sum$Odi, and E = (H'-clpp)/lnR. A two-tailed t-test was performed on the data to determine if substrate utilization differed significantly between incubation temperatures. Bray-Curtis distances were calculated on Biolog EcoPlate substrate absorbance data (standardized and log-normalized) found for each sample, together with physicochemical factors measured on these same samples. This distance matrix was calculated and visualized

using non-metric multidimensional scaling (NMDS) with R package 'vegan' (Oksanen et al. 2019).

3.3.7 Measurement of microbial activity using a radiorespiration assay

Radiorespiration assays were configured according to the protocol developed by (Steven et al. 2007b). Microcosms were set up in triplicate with 5 ml of melted ice; the assay was then incubated at both 5°C and -5°C. Sterilized negative controls (autoclaved twice with a 24-hour interval) were also included in triplicate for all samples at both temperatures. Each microcosm was spiked with 20 µl of 0.041 µCi (~90,000 dpm) of 1,2-^{14}C acetic acid and 20 µl of cold acetic acid to a final concentration of 15 mM acetic acid in a total volume of 40 µl. To create the CO_2 trap, 500 µl of 1 M KOH was added to an open top vial within the microcosms. Measurements of CO_2 were taken periodically over a period of 483 days (-5°C incubations) and 447 days (5°C incubations) and checked on a Perkin Elmer Tri-Carb 4810 TR scintillation counter (Waltham, Massachusetts, USA), measuring ^{14}C counts for 1 minute. In the -5°C incubations to determine whether CO_2 was trapped in the ice and avoiding detection, after 483 days, we warmed these incubations to 5°C and mixed them with 37% formaldehyde to prevent detection of new actively respiring microorganisms after thaw. Results were calculated by subtracting values from sterilized controls from the average radioactivity of replicates and expressed as the percentage of starting 1,2-^{14}C acetic acid respired by the microbial community over time.

3.4 Results

3.4.1 Ice characteristics

In situ measurements showed that the temperature of all ice samples was always between 0.5°C and -0.5°C, while pH values ranged between 6 and 7.5 (Table 3.2). Values for total organic carbon (TOC), nitrogen (NO_3^- and NH_3) and phosphorus (PO_4^{3-}) varied greatly among sites and year (Table 3.2). Samples SC1 2017 and SC2 2017 had the highest levels of TOC (176.4 mg/l and 302.7 mg/l respectively) while samples from Crystal Ice Cave (CI 2017) and Merrill Cave collected in (MC 2018) had the lowest levels of TOC (30.6 mg/l and 8.0 mg/l respectively). No related trends were observed between nutrient concentration and biomass.

Table 3.2. Lava tube ice biomass and physiochemical characteristics.

	CC 2018	*CI 2017*	*MC 2017*	*MC 2018*	*SC1 2017*	*SC2 2017*	*SC2 2018*
CFU incubated at 37 °C	$2.40x10^3 \pm 1.80x10^3$	$6.67x10^2 \pm 3.93x10^2$	$8.13x10^3 \pm 7.26x10^2$	$1.33x10^2 \pm 1.09x10^2$	$3.91x10^4 \pm 1.51x10^3$	$2.40x10^3 \pm 6.53x10^2$	$1.33x10^2 \pm 1.09x10^2$
CFU incubated at 23 °C	$3.23x10^6 \pm 1.04x10^5$	$7.60x10^3 \pm 1.89x10^2$	$4.20x10^5 \pm 4.14x10^4$	$2.27x10^4 \pm 2.49x10^3$	$1.05x10^7 \pm 1.65x10^6$	$1.52x10^6 \pm 2.47x10^5$	$4.31x10^6 \pm 3.50x10^5$
CFU incubated at 5 °C	$5.72x10^6 \pm 1.50x10^5$	$2.23x10^4 \pm 5.05x10^3$	$7.69x10^5 \pm 1.70x10^4$	$1.33x10^4 \pm 2.68x10^3$	$2.91x10^7 \pm 3.98x10^6$	$4.83x10^5 \pm 1.78x10^4$	$4.57x10^6 \pm 9.68x10^4$
Temperature (°C) (ice)	-0.4	0	0.2	N.D.	0.9	0.3	-0.3
pH	N.D.	6.5	7	N.D.	6.5	7.5	N.D.
TOC (mg/L)	86.94	30.57	50.49	8.01	176.44	302.72	36.91
NH_4^+ (mg/L)	0.11	U.D.	$4.97x10^{-3}$	$1.75x10^{-3}$	0.02	U.D.	0.02
NO_3^- (mg/L)	0.14	0.03	0.04	0.01	0.27	N.D.	$4.00x10^{-3}$
PO_4^{3-} (mg/L)	0.88	0.02	0.03	0.07	N.D.	N.D.	0.3

CFU, colony forming units. TOC, Total organic carbon. N.D., No data. U.D., Undetectable. For CFU, standard error is included as a (±) value.

3.4.2 Microbial cell enumeration, isolation, and characterization

The majority of colony forming units (CFUs) incubated at 5°C, 23°C and 37°C were observed on plates incubated at 5°C (23% - 74%). The total number of CFU determined at this temperature ranged from 1.3 x 10^4 CFU/ml in sample MC 2018 to 2.9 x 10^7 CFU/ml in sample SC1 2017 (Table 3.2). Based on morphology, 38 unique colonies obtained from the 2017 samples were isolated from plates incubated at 5°C and 23°C and chosen for further identification and characterization. Of the 38 isolates, 37 grew at 5°C and 24 grew at -5°C. 29 isolates grew in concentrations of 3% NaCl while 13 isolates grew in concentrations of 9% NaCl, none grew in concentrations of 12% to 21% (Table 3.3). Sanger sequencing of the 16S rRNA gene of these isolates revealed that all isolates belonged to the phyla Actinobacteria (17), Firmicutes (9), Bacteroidetes (6), and Proteobacteria (6, specifically the Alpha, Beta, and Gamma classes) (Supplemental Figure 3.2). According to the NCBI database, we determined at least 14 of our isolates shared closest sequence similarity with bacteria isolated from various cryophilic environments, including the Arctic, Antarctica, and various glaciers (Table 3.3). Only two isolates showed closest similarity to sequences obtained from a cave (*Micromonospora palomenae* strain BBHARD22 and *Devosia limi* strain SZGX-3). Additionally, another isolate isolated from Merrill Cave in 2017 may be a candidate novel genus, since it showed a 94% sequence similarity to *Paenibacillus frigoriresistens* strain YIM 016 as the highest hit.

Table 3.3. Characterization of lava tube ice cultured isolates.

ID	Closest BLAST hit[a]	Phyla	Isolation environment of closest BLAST hit	% Similarity	Temp. growth range (°C)	Salinity growth range (% NaCl)	Site	GenBank accession number
SC1-G	Williamsia marianensis strain DSM 44944	Actinobacteria	Marine sediment (NR_118613.1)	99%	-5 - 25	0 - 3	SC1 2017	MW296063
SC1-H	Bacillus simplex strain AN6	Actinobacteria	Lodgepole Pine, Canada (MG561818.1)	99%	5 - 37	0 - 6	SC1 2017	MW296064
SC1-I	Cryobacterium psychrotolerans strain 4ABT14[c]	Actinobacteria	Arctic (no other info) (MH482242.1)	98%	-5 - 25	0 - 9	SC1 2017	MW296059
SC1-F	Arthrobacter rhombi strain MLS-8-5 16S	Actinobacteria	Soil, China (KT997469.1)	99%	-5 - 25	0 - 9	SC1 2017	MW296065
SC1-A	Paenibacillus antarcticus strain AS-99[c]	Firmicutes	Colour Lake, Axel Heiberg Island, Canada (KR857410.1)	98%	-5 - 25	0 - 3	SC1 2017	MW296066
SC1-K	Paenibacillus macquariensis strain 18JY54-12	Firmicutes	No info on NCBI (MH497649.1)	99%	-5 - 25	0 - 3	SC1 2017	MW296067
MC1-D	Micromonospora palomenae strain BBHARD22	Actinobacteria	Cave sediment, USA (MH182597.1)	100%	25 - 37	0	MC 2017	MW296068
MC1-A	Pseudomonas mandelii strain SY03134(1)	γ-proteobacteria	Qilian Mountain, China (KT369882.1)	100%	-5 - 25	0 - 3	MC 2017	MW296069
MC1-B	Flavobacterium glaciei strain 0499	Bacteroidetes	No. 1 glacier, China (NR_043891.1)	99%	0 - 25	0 - 3	MC 2017	MW296070
MC1-C	Flavobacterium sinopsychrotolerans strain 20ABA3	Bacteroidetes	Arctic (no other info) (MH482347.1)	99%	-5 - 25	0	MC 2017	MW296071
MC1-S	Arthrobacter kerguelensis strain YF28-1(3)	Actinobacteria	Qilian Mountain, China (KT369841.1)	99%	-5 - 25	0 - 6	MC 2017	MW296072
MC1-E	Flavobacterium xinjiangense strain HME8661	Bacteroidetes	Fresh Water (no other info) (KF911333.1)	100%	0 - 25	0	MC 2017	MW296073
MC1-F	Paenibacillus crassostreae strain LPB0068[c]	Firmicutes	No info on NCBI (CP017770.1)	97%	0 - 25	0 - 3	MC 2017	MW296061
MC1-H	Pedobacter steynii strain SE34(1)	Bacteroidetes	Qilian Mountain, China (KT369893.1)	99%	-5 - 25	0 - 6	MC 2017	MW296074
MC1-J	Pseudomonas avellanae strain KS1-13	γ-proteobacteria	Freshwater sediment (no other info)(KX809749.1)	99%	-5 - 25	0 - 3	MC 2017	MW296075
MC1-M	Sporosarcina globispora strain VAL7	Firmicutes	Soil, Antarctica (KF026352.1)	100%	0 - 25	ND	MC 2017	MW296076
MC1-N	Pseudarthrobacter sulfonivorans strain RR26	Actinobacteria	Rice rhizosphere, South Korea (KU512892.1)	99%	-5 - 25	0 - 9[b]	MC 2017	MW296077
MC1-O	Herbaspirillum autotrophicum strain aqa1[c]	β-proteobacteria	Coal-tar contaminated aquafer, United Kingdom (AM942540.1)	98%	-5 - 25	0 - 9[b]	MC 2017	MW296060
MC1-P	Paenibacillus macquariensis strain 18JY54-12	Firmicutes	No info on NCBI (MH497649.1)	99%	-5 - 25	ND	MC 2017	MW296078
MC1-Q	Paenibacillus frigoriresistens strain YIM 016[c]	Firmicutes	Soil, China (NR_109546.1)	94%	0 - 25	ND	MC 2017	MW296062
SC2-H	Microbacterium phyllosphaerae isolate WP02-1-38	Actinobacteria	3000 m depth, West Pacific (EF143430.1)	99%	-5 - 25	0 - 6[b]	SC2 2017	MW296079
SC2-I	Psychrobacter aff. glacinola A1/C-aer/OII	γ-proteobacteria	Lake Fryxell, Antarctica (AJ297439.1)	99%	-5 - 25	0 - 9	SC2 2017	MW296080
SC2-J	Salinibacterium amurskyense strain NJES-28	Actinobacteria	Krill digestive tract, Antarctica (KR140228.1)	100%	-5 - 25	0 - 6	SC2 2017	MW296081
SC2-K	Flavobacterium degerlachei strain 4BR4R10[c]	Bacteroidetes	Arctic (no other info) (MH482269.1)	98%	-5 - 5	0 - 9[b]	SC2 2017	MW296058
SC2-M	Paenibacillus frigoriresistens strain GNHG-3	Firmicutes	Associated with *Salix gordejevii* (MF101000.1)	99%	0 - 25	0	SC2 2017	MW296082
SC2-N	Arthrobacter alpinus strain 12-2(2)	Actinobacteria	Qilian Mountain, China (KT369947.1)	99%	-5 - 25	0 - 6[b]	SC2 2017	MW296083
CI2-A	Devosia psychrophila strain Cr7-05	α-proteobacteria	Glacial cryoconite, Austria (NR_117516.1)	99%	0 - 5	0	CI 2017	MW296084
CI2-C	Rhodococcus fascians strain HLSBA48	Actinobacteria	South China Sea, China (FJ999590.1)	99%	-5 - 5	0 - 9[b]	CI 2017	MW296085
CI2-G	Arthrobacter subterraneus strain MER_TA_10	Actinobacteria	Mars rover spacecraft, USA (KT719419.1)	99%	-5 - 25	0 - 9[b]	CI 2017	MW296086
CI2-H	Rhodococcus fascians strain JPLtot2-4	Actinobacteria	Clean room facility, USA (DQ870746.1)	99%	-5 - 25	0 - 9[b]	CI 2017	MW296087
CI2-J	Cryobacterium roopkundense strain RuGI7	Actinobacteria	Glacial soil, India (NR_104500.1)	99%	0 - 25	0 - 6	CI 2017	MW296088
SC1-X	Microbacteriaceae bacterium CC14M2	Actinobacteria	Amphibian skin, USA (KM187179.1)	99%	0 - 25	ND	SC1 2017	MW296089
SC1-L	Pedobacter bambusae strain THG-G118	Bacteroidetes	Bamboo soil, South Korea (NR_148294.1)	99%	-5 - 25	0 - 9[b]	SC1 2017	MW296090
SC1-O	Rhodococcus yunnanensis strain IHBB 9867	Actinobacteria	Suraj Tal Lake sediment, India (KR085922.1)	100%	-5 - 25	0 - 9[b]	SC1 2017	MW296091
SC1-P	Marisediminicola antarctica strain 20BR6Z1	Actinobacteria	Arctic (no other info) (MH482332.1)	99%	0 - 25	0 - 3	SC1 2017	MW296092
SC1-R	Devosia limi strain SZGX-3	α-proteobacteria	Stalactite surface, China (MF170834.1)	99%	0 - 25	0 - 3	SC1 2017	MW296093
SC1-S	Arthrobacter flavus strain A-133	Actinobacteria	Mudboil, Antarctica (MF467841.1)	100%	-5 - 25	0 - 9[b]	SC1 2017	MW296094
SC1-U	Sporosarcina globispora strain HEN5	Firmicutes	Soil, Antarctica (KF026330.1)	99%	0 - 25	0 - 9[b]	SC1 2017	MW296095

Abbreviations: ND, not determined; Temp, temperature.
[a] The closest BLAST hit to a cultured isolate was chosen for inclusion in the table.
[b] Indicates poor growth.
[c]Indicates a putatively novel species based on a 16S rRNA sequence similarity cut-off of ≥98%.

3.4.3 Prokaryotic community profiling

Illumina amplicon sequencing of the 16S rRNA V4-V5 gene region of microbial communities from LABE ice samples generated, after processing, 838,591 reads grouped into 11,475 unique ASVs. Generally, the majority of ASVs were distributed in 2 or more sites (Supplemental Figure 3.3), except ASVs from MC 2017 and MC 2018 where most of them (6528 or 85%) were unique to that cave (Supplemental Figures 3.3 & 3.4). The community from all samples was composed almost exclusively of bacteria (>99.97% of total sequences), with very few archaeal sequences (<0.02%) detected. The dominant bacterial phyla included the Actinobacteria (30% of total sequences), Proteobacteria (30%), Bacteroidetes (19%), Firmicutes

(5%), and Chloroflexi (5%) (Figure 3.2). The most abundant genera within LABE ice were *Flavobacterium* (7% of total sequences) (Bacteroidetes), *Arthrobacter* (4%) (Actinobacteria), *Sphingomonas* (4%) (Proteobacteria), *Nocardioides* (3%) (Actinobacteria), and *Pseudomonas* (3%) (Proteobacteria), but with a high abundance of unclassified sequences at this taxonomic rank (25%). Of the few archaeal sequences recovered, most belonged to either the phyla Euryarchaeota, or Thaumarchaeota. Of the 20 most abundant ASVs recovered from our sites, 16 shared closest blast similarity to other cryophilic environment derived sequences and none with cave or subterranean derived sequences (Table 3.4). Alpha diversity measures (Shannon and Faith's PD) revealed sample MC 2017 to be the most diverse while beta diversity measures (Bray-Curtis and UniFrac distances) revealed samples CC 2018 and CI 2017 to be the most dissimilar among all samples. (Supplemental Figure 3.5).

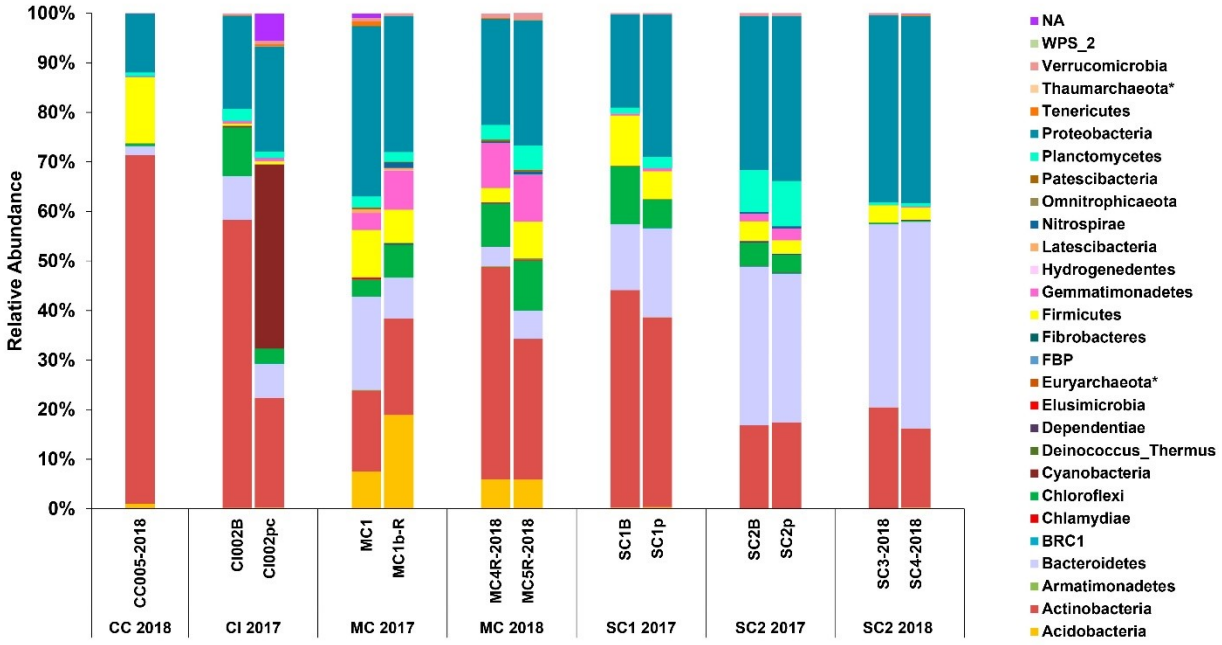

Figure. 3.2. Bacterial and archaeal 16S rRNA community profile of LABE ice. Relative abundance is calculated from sequence read counts. Archaeal phyla are denoted in the legend by an (*).

Piphillin predicted the LABE ice microbial communities to contain complete or almost complete heterotrophic and lithoautotrophic pathways. Lithoautotrophic pathways include C1 compound metabolism (methanotrophy and methanogenesis), sulfur cycling (thiosulfate oxidation and sulfate reduction), nitrogen cycling (nitrogen fixation, nitrification, complete nitrification, denitrification, and dissimilatory nitrate reduction), as well as others related to phototrophy, and manganese oxidation (Supplemental Figure 3.6, Supplemental File 1).

Table 3.4. Most abundant ASVs within lava tube ice.

ID	*Sampling environment of closest BLAST hit*	*Closest BLAST hit*	*Genbank accession number of closest BLAST hit*
ASV_3	Arctic soil	Flavobacterium sp. strain TSA-D2	MK272784.1
ASV_4	Antarctic soil	Arthrobacter sp. strain BIS1075	MN810179.1
ASV_6	Antarctic soil	Pseudarthrobacter sp. YJ56	CP047898.1
ASV_2	Wetland soil	Uncultured bacterium clone B159	KJ817624.1
ASV_9	Antarctic sponge	Algoriphagus sp. strain SER19	MK660307.1
ASV_8	Antarctic soil	Flavobacterium sp. strain Z22-72	MN448526.1
ASV_10	Antarctic rock	Rhodococcus sp. strain Mn531	MN865723.1
ASV_14	Antarctic hydrocarbon polluted soil	Uncultured bacterium clone 7A_10-060	KY190711.1
ASV_16	Antarctic hydrocarbon polluted soil	Uncultured bacterium clone 5A_10-065	KY190543.1
ASV_12	Antarctic sponge	Arthrobacter sp. strain SER44	MK660306.1
ASV_19	Soil	Uncultured bacterium clone 6.12G_103631	KU149322.1
ASV_20	Soil	Uncultured Acidobacteria bacterium clone HEG_08_446	HQ597711.1
ASV_22	McMurdo Dry Valleys, Antarctica algal mat	Uncultured bacterium clone 26539_plate2a10	HM356838.1
ASV_23	Antarctic Marine sediment core	Pseudonocardia sp. strain KRD-170	MH725310.1
ASV_26	Low level radioactive waste site	Uncultured bacterium clone F1_21X	GQ262834.1
ASV_29	Antarctic soil	Knoellia sp. strain R-68061	KY386418.1
ASV_33	Antarctic soil	Antarctic bacterium EM1	FJ517621.1
ASV_31	Arctic and Antarctic petroleum contaminated sea ice	Flavobacterium sp. ice-oil-522	DQ521398.1
ASV_37	Chinese Glacier	Sphingomonas sp. RHLT2-11-1	JX949370.1
ASV_32	Antarctic Soil	Uncultured proteobacterium clone 3B12	KC442648.1

The 20 most abundant ASVs within our dataset were BLAST against the NCBI nucleotide database. ID refers to the identifier of the ASV in our dataset.

3.4.4 Community level physiological profiling of LABE ice microorganisms

A plateau in the AWCD was observed after 53 days and 102 days for 5°C and -5°C incubations, respectively. Biolog EcoPlate data indicated higher overall substrate utilization and

functional diversity in plates incubated at 5°C when compared to plates incubated at -5°C (Supplemental Table 3.2). Microbial communities from site CC 2018 showed the highest substrate utilization, being able to metabolize an average of 26 ± 2.20 and 23.33 ± 2.43 of the 31 substrates at 5°C and -5°C, respectively. This sample also showed the highest functional diversity (*H'-clpp*), with average values of 3.18 ± 0.07 at 5°C and 3.14 ± 0.05 at -5°C (Supplemental Table 3.1). The only sample in which both substrate utilization and functional diversity were higher at -5°C was in CI 2017. This same microbial community utilized tween 80, glycogen, 2-hydroxybenzoic acid, and glycyl-L-glutamic acid only at -5°C and D-mannitol, N-acetyl-D-glucosamine, and L-arginine substrates to a much higher degree at -5°C than 5°C (Figure 3.3). No microbial community showed the capacity to metabolize glucose-1-phosphate, D,L-α-glycerol phosphate, or α-ketobutyric acid neither at -5°C, nor at 5°C. In addition, no microbial community was capable of metabolizing itaconic acid or D-malic acid at -5°C (Figure 3.3).

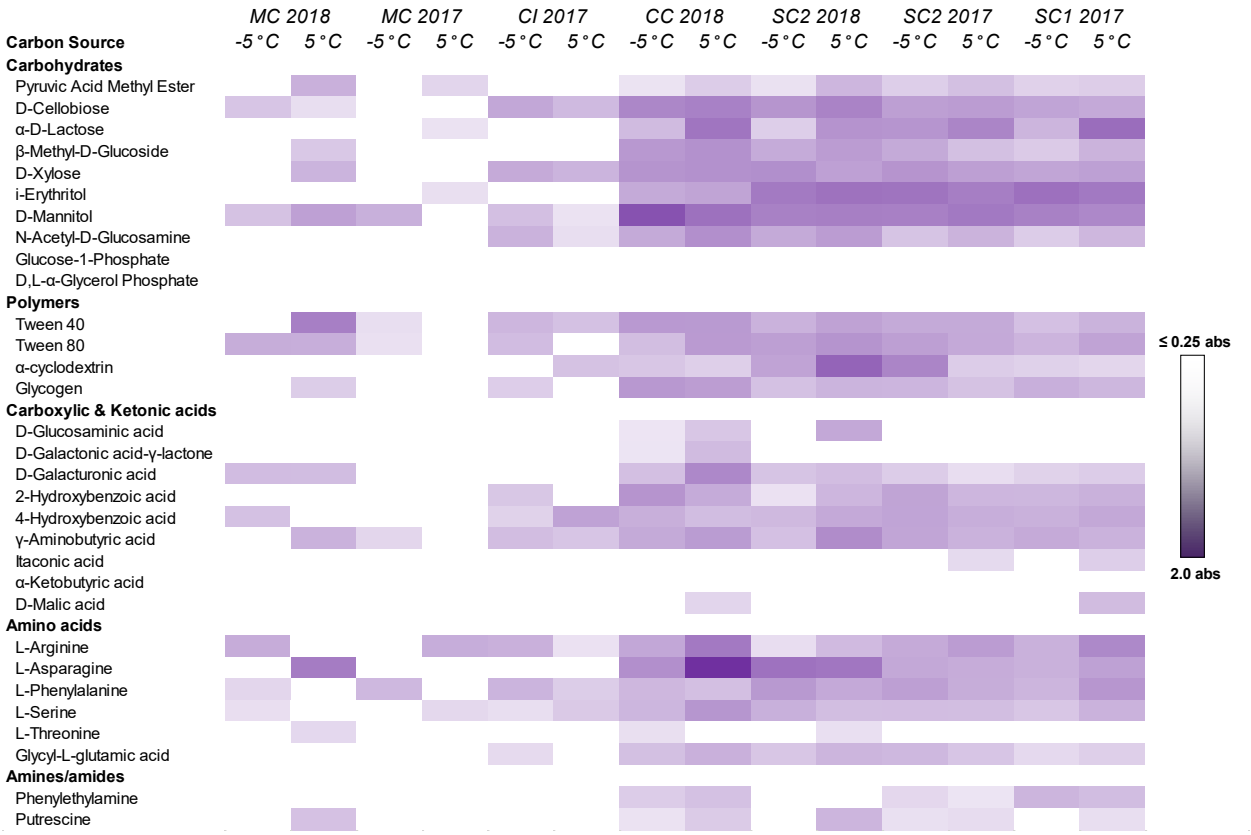

Figure 3.3. Heatmap depicting EcoPlate substrate utilization by LABE ice microbial communities incubated at -5°C and 5°C. The average absorbance of 3 triplicate wells with blanks subtracted was used to calculate the data. Data used to calculate the average absorbance was collected once the AWCD plateaued, corresponding to 53 days for plates incubated at 5°C and 102 days for plates incubated at -5°C.

NMDS ordination of the Biolog EcoPlate results (Supplemental Figure 3.7) showed the location of the samples in the ordination mainly depended on the cave they came from (stress=0.046). Incubations from MC 2017, MC 2018 (except for incubation MC2018 +5°C) and CI 2017 were distantly located from the main group, showing a higher influence of ice temperature on them than on the rest of samples.

3.4.5 Heterotrophic microbial activity measured by a radiorespiration assay

In vitro heterotrophic microbial activity was detected above control levels in all samples by day 77 of the incubation at 5°C (Figure 3.4). By day 447, between 6% (MC 2018) and 12% (SC1 2017) of initial acetate was mineralized. Activity was much lower in samples incubated at -5°C (Figure 3.4). In this case, microbial radiorespiration was only detected above sterile control levels from samples CC 2018 and SC2 2018. By day 483, CC 2018 and SC2 2018 communities had mineralized 1% and 0.5%, respectively, of the initial acetate, compared to control values of 0.6% and 0.3%, respectively. In both samples control and experimental assays were significantly different until day 447 for SC2 2018 when error bars began to overlap.

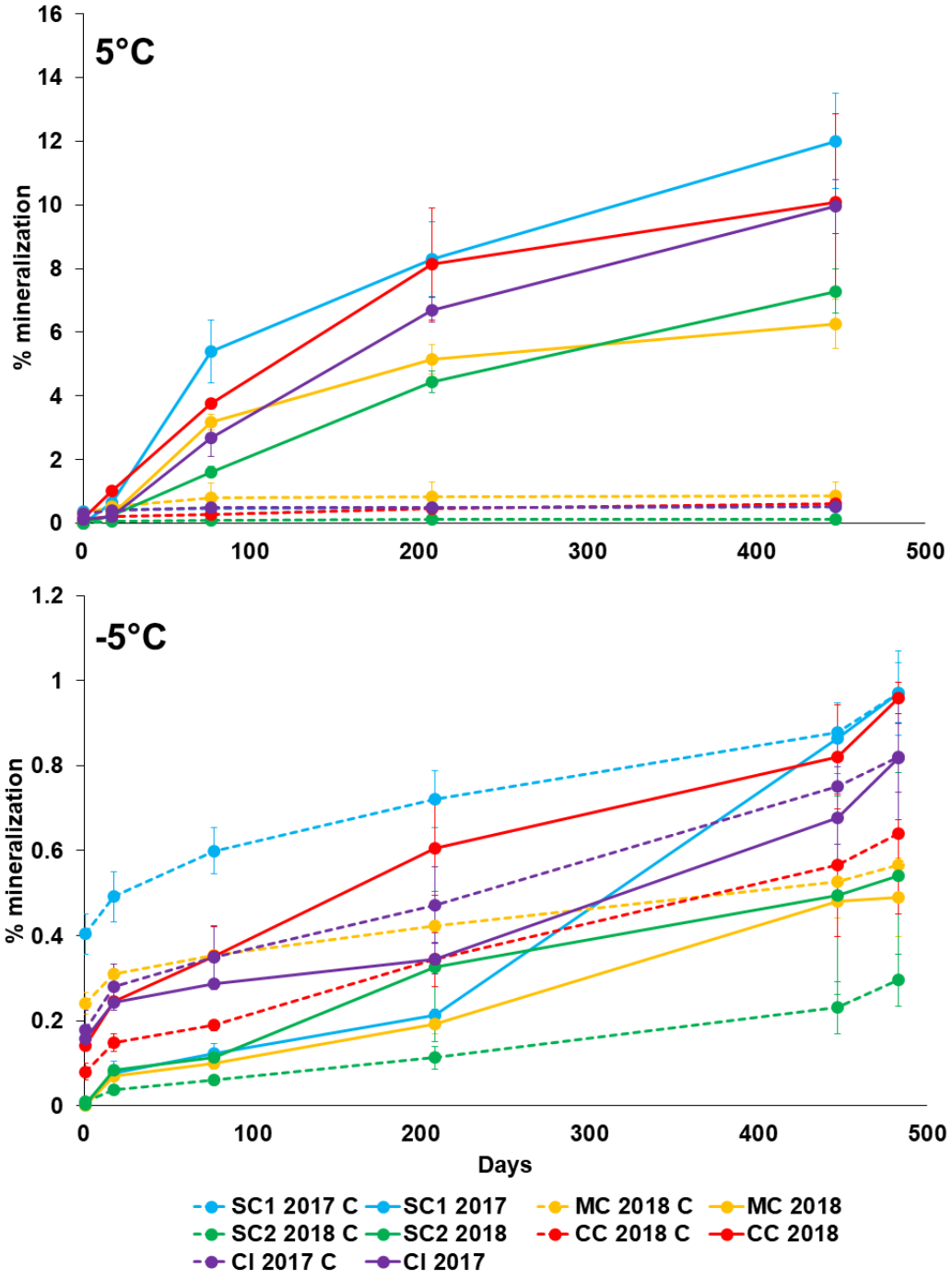

Figure 3.4. Microbial activity measured at -5°C and 5°C by the detection of biomineralized 14C acetate. Data is displayed as the percent of ^{14}C respired as CO_2 of the initially provided ^{14}C. The assay was stopped after 447 days for 5°C incubations and after 483 days for -5°C incubations. Dotted lines refer to control samples and thus abiotic release of CO_2. Error bars are calculated as the standard error based on three replicates. Note the differences in scale of the vertical axis.

3.5 Discussion

3.5.1 LABE ice harbours microorganisms adapted to cold temperatures

Several lines of evidence suggest LABE ice microbial communities are primarily cold adapted. The strongest evidence being that the greatest number of CFUs we recovered were from plates incubated at 5°C as opposed to 23°C or 37°C. Of all the CFU we isolated for further characterization, almost all grew at 0°C or -5°C. Using a single media may only offer a narrow view of the culturable microbial community. However, the R2A media employed in this study has previously resulted in the highest viable plate counts from other cryoenvironments (Christner et al. 2000; Miteva et al. 2004; Steven et al. 2007a; Hansen et al. 2007; Bottos et al. 2008) and therefore is a good indicator of the viable lava tube ice microbial community. Among both cultured isolates and the 20 most abundant ASVs recovered from our samples, most shared closest sequence similarity with sequences recovered from other cryoenvironments such as from the Arctic, Antarctic and mid-latitude glaciers. Extensive study of microbial communities inhabiting Scarisoara Cave ice, a karstic cave, have also suggested a predominantly cold-adapted microbial community (Hillebrand-Voiculescu et al. 2015; Iţcuş et al. 2016; Brad et al. 2018; Itcus et al. 2018; Paun et al. 2019).

Our results add to the evidence that caves ice deposits may constitute reservoirs of novel cold-adapted microorganisms. Based on a full length 16S rRNA sequence similarity cut-off of less than 98.7% as proposed by (Kim et al. 2014) we determined 6 of our cultured isolates to be putatively novel species, all of which grew at -5°C and/or 0°C. At the rank of family and genera 10% and 25% respectively of our ASVs were unassigned. Similarly, study of Scarisoara cave has revealed a large proportion of unassigned and putatively novel OTUs among bacteria,

archaea and fungi (Brad et al. 2018; Itcus et al. 2018). Therefore, future study of these novel microorganisms may help us understand cryophilic biology and evolutionary adaptations which may allow life to persist on other planets.

Members within the dominant Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes, and Chloroflexi phyla revealed by 16S rRNA analyses at these cave sites were also detected in other cave ice environments including Scarisoara Cave (Itcus et al. 2018; Paun et al. 2019) and Hawaiian lava tubes on the side of Mauna Loa Volcano (Teehera et al. 2017). Proteobacteria and Chloroflexi also dominated sediment from three volcanic ice caves from Mount Erebus in Antarctica, with a lesser presence of Actinobacteria and Firmicutes (Tebo et al. 2015). Together, these studies suggest that, at higher taxonomic classifications, microbial communities remain generally consistent among ice containing and subzero cave habitats.

Although phyla recovered from our samples such as Actinobacteria and Proteobacteria are ubiquitous across many environments, the appearance of Firmicutes in LABE ice may be due in part to the ability of members of this phyla to persist in cryophilic environments (Shivaji et al. 2014). The family Planococcaceae, comprising the largest proportion of Firmicutes from our sites, have routinely been isolated from cryophilic environments around the world (Shivaji et al. 2014). In fact, *Planococcus halocryophilus*, isolated from Arctic permafrost (Mykytczuk et al., 2013) can grow at temperatures of -15°C and metabolize to temperatures as low as -25°C (Mykytczuk et al., 2013). Many genera of the Planococcaceae can also form endospores (Shivaji et al. 2014); protective structures which allow the bacterium to enter dormancy, protecting it from nutrient limitation, extreme temperatures, UV radiation, and desiccation. Within our own

dataset we found sequences belonging to numerous Firmicute endospore formers such as *Oxobacter, Anoxybacillus, Syntrophomonas, Anaerobacillus, Coxiella, Bacillus, Paenibacillus, Paenisporosarcina*, and *Sporosarcina*. Of note, we found abundant sequences from our sites showing high sequence similarity to endospore formers, *Paenisporosarcina macmurdoensis* and *Bacillus subtilis*; the first is a psychrophilic bacterium originally isolated from the McMurdo Dry Valleys in Antarctica and the second is a model spore former. *Bacillus subtilis* spores have even survived years in the harsh conditions of space (Horneck et al. 1994) leading to the suggestion that should endospore formers have evolved when Mars' climate was milder then they may still persist on the planet today (Yung et al. 2007).

Given the average surface temperatures of present day Mars are well below 0°C and recent evidence suggests that early climatic conditions on Mars may have been predominantly cold with surficial water stored as snow and ice (Grau Galofre et al. 2020) suggests that extinct or extant Martian life would need to be adapted to cold temperatures. This theory also applies to microorganisms inhabiting Martian lava tubes given modelling of atmospheric temperatures within this environment are also well below 0°C (Williams et al. 2010). Our results depict a community composed of psychrophilic, psychrotrophic and endospore-forming microorganisms more closely related to communities inhabiting other polar cryoenvironments, as opposed to those communities inhabiting temperate cave and subsurface environments. Such an insight points to the strong selective pressure which ice and cold temperatures exerts on microbial communities.

3.5.2 Lava Beds ice supports active microbial communities

We believe this to be the first study to directly measure metabolic activity of lava tube ice associated microorganisms and the first time Biolog EcoPlates have been used to measure subzero microbial metabolic activity. Our results imply the heterotrophic microbial communities of LABE ice are just as capable of metabolic activity at subzero temperatures as at above zero temperatures. In every sample, tested substrates were metabolized at -5°C and, in the majority of samples, there were no significant differences in substrate richness or diversity between the two incubation temperatures (Supplemental Table 3.2). Although microbial communities inhabiting cryophilic environments are assumed to be adapted to life at cold temperatures, the influence of incubation temperature on carbon substrate utilization is still poorly understood. Reports from cryophilic environments such as ice containing caves and Antarctic soil have shown substrate utilization richness and functional diversity increases with decreasing temperature (Pessi et al. 2012; Iţcuş et al. 2016), however, the opposite result was reported from Arctic permafrost (Ernakovich and Wallenstein 2015). Our data do not fully support either result but do extend the range these results can be applied, to including subzero temperatures.

Exploratory ordination analysis (NMDS, Supplemental Figure 3.6) of our sites suggests that higher organic substrate abundance and diversity promotes the development of more diverse microbial communities capable of metabolizing those substrates, no matter the temperature of the site. Therefore, the cave sites which contained higher concentrations of organics also contain more functionally diverse microbial communities, similarly to what has been found for other similar settings as Scarisoara Cave (Iţcuş et al. 2016). Such results suggest that at both above and subzero temperatures, organic carbon content, and presumably substrate richness, may predict heterotrophic functional diversity.

Among sites MC 2017, MC 2018, and CI 2017, many substrates were found to be metabolized preferentially at -5°C but not 5°C (Figure 3.3), thus hinting at potential strategies used by active microbial communities to thrive in lava tube ice. Such substrates which may play a role in cold adaptation include D-mannitol, γ-aminobutyric acid, tween 40 and tween 80. D-mannitol is a well-known compatible solute, capable of providing cryopreservation, and osmoprotection to psychrophiles in addition to carbon, nitrogen, and energy (Weinstein et al. 1997; Tribelli and López 2018). Γ-aminobutyric acid, is a compatible solute produced by plants to withstand various stresses such as cold temperatures (Shelp et al. 1999; Mazzucotelli et al. 2006) and given our results, may do the same in bacteria. Extensive metabolism of tween 40 and tween 80 has been reported by cryophilic microbial communities in environments such as Antarctic and Arctic seawater (Sala et al. 2005, 2008), as well as in Antarctic soils (Pessi et al. 2012). It has been proposed that polyols such as tweens are metabolized by cold adapted microbial communities to grow at cold temperatures and protect themselves from freeze-thaw events (Wynn-Williams 1990; Robinson 2001). Our results support this hypothesis. Tween 80 may specifically play a role in microbial cold tolerance by being incorporated into cell membranes and repressing fatty acid synthesis, thus preserving membrane fluidity (Reitermayer et al. 2018) at low temperatures.

It is surprising that microbial activity was not detected more readily in -5°C radiorespiration assays, given both the sensitivity of the assay in cryophilic environments (Steven et al. 2007b) and that *in situ* microbial activity has been detected from ice and other cryophilic environments all over the world, including Arctic permafrost (Rivkina et al. 2000;

Jakosky et al. 2003; Steven et al. 2008), sea ice (Junge et al. 2004), Antarctic snow (Carpenter et al. 2000) and Antarctic water tracks (Chan-Yam et al. 2019). Although radiolabelled carbon may have been trapped in the ice, analysis after thawing still did not show any significant increase in respired carbon, so perhaps our samples required a longer incubation period or a larger nutrient spike to detect greater activity. Such could explain the observed disparity in metabolic activity between radiorespiration and EcoPlate assays. For example, substrates contained within EcoPlate wells are approximately 100 times more concentrated than within our radiorespiration assays. While EcoPlates can indicate the potential of a microbial community to metabolize substrates at *in situ* temperatures, radiorespiration assays better mimic the actual *in situ* environment (Steven et al. 2007b). However, radiorespiration assays do not perfectly mimic the *in situ* environment, nor do any other laboratory-based metabolic activity assays. Therefore, we suggest further investigation, employing techniques such as in situ stable isotope probing or in situ gas flux analyses should be explored to confirm our laboratory-based assays that demonstrated active microbial metabolic activity.

Despite the uncertainty in the extent that LABE ice microbial communities are metabolically active *in situ*, our results do point to microbial communities from this environment capable of metabolic activity at subzero temperatures. The continued investigation of these communities may increase our understanding of microbial adaptations to living in subzero temperatures. Moreover, such a finding raises the possibility that Martian lava tubes may be a habitable environment for extant microbial life.

3.5.3 Potential metabolisms of Lava Beds ice microbial communities

Our sites are not considered oligotrophic, oligotrophic caves should contain less than 2 mg/L of total organic carbon (Tomczyk-Żak and Zielenkiewicz 2016) and, although many caves meet this definition, lava tubes may be an exception (Northup and Lavoie 2015). Given their proximity to the surface and the propensity of the host rock to fracture, organic carbon can readily enter into lava tubes by transport of meteoric water, plant roots and soil from the surface through cracks and fissures in the walls and ceiling. Deposition of guano and other detritus by seasonal bat and rat populations can also occur (Northup et al. 2011). We suspect most of the organic carbon from LABE caves originated from an allochthonous origin, having observed plant roots, animal deposition and human alterations near our sampling sites. Such sources help explain the abundance of heterotrophic microorganisms found within our sites.

Total organic carbon concentrations measured in the Martian surface are quite low (although likely higher in the subsurface) (Freissinet et al. 2015; Eigenbrode et al. 2018), suggesting any heterotrophic microorganism in existence today within Martian lava tubes would likely need to rely on primary production by chemolithoautotrophs, as has previously been observed in terrestrial cave habitats (Engel 2012). Complete or almost complete pathways for heterotrophic and lithoautotrophic metabolisms were predicted by Piphillin to exist within our samples. Furthermore, various *Pseudomonas* ASVs, all within the 100 most abundant ASVs within our dataset, showed 99% 16S rRNA gene sequence similarity to a facultatively autotrophic iron oxidizing, *Pseudomonas spp.*, recovered from the basalt/ice interface of a lava tube from the nearby Oregon Cascades (Popa et al. 2012). Many *Flavobacterium* and *Arthrobacter* ASVs also contained within the 100 most abundant ASVs showed a 16S rRNA gene sequence similarity of ≥98% to manganese oxidizing bacteria isolated from caves of the

upper Tennessee river basin (Carmichael et al. 2013). We suggest these taxa may derive energy from reduced iron and manganese present within the basaltic host rock of LABE caves. Thus, these taxa may contribute to cave mineral alteration and dissolution (Northup and Lavoie 2001).

Lithoautotrophic microorganisms, such as those recovered from our sites, likely persist in lava tubes owing to a variety of energy sources produced by water-rock interactions and radiolysis (Hays et al. 2017). These interactions may support, among others, iron oxidation, sulfate reduction, hydrogen oxidation, and methanogenesis (Stevens and McKinley 2000; Bach and Edwards 2003; Lin et al. 2006; Lever et al. 2013; Osburn et al. 2014; Simkus et al. 2016). Considering much of the Martian surface is comprised of basalt and ultramafic rock (Boynton et al. 2008; McSween et al. 2009), similar interactions also likely occur there. Molecules such as NO_3^- and SO_4^{2-}, both observed on Mars (Stern et al. 2015; Rapin et al. 2019) may serve as electron acceptors in the absence of oxygen (Hays et al. 2017). Thus, the variety of both inorganic electron donors and acceptors potentially contained within Martian caves suggests primary production by lithoautotrophs, especially chemolithoautotrophs is favourable, should life have ever been present there in the first place.

3.6 Conclusion

Our results demonstrate that microbial communities inhabiting lava tube ice from LABE, analogues to Martian lava tubes, are cold adapted and capable of metabolic activity at subzero temperatures. Furthermore, these communities resemble cryophilic microbial communities more than cave microbial communities. Lithoautotrophic metabolisms predicted to exist within our sites such as methanogenesis, sulfate reduction, nitrate reduction, and iron oxidation are believed

to be plausible metabolisms for survival on Mars. Our results raise the prospect Martian lava tubes may be a habitable environment and a refuge to Martian microorganisms, as the planet's surface transitioned to the inhospitable environment observed today. In this regard, lava tube ice would likely provide relatively stable temperatures, protection from radiation, water from ice, and energy from basaltic minerals of which lava tubes are composed. Our findings serve as a basis for further study of this unique environment.

3.7 Acknowledgements

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Connecting Text

Chapter 3 of this study determined whether there was an active microbial community in lava tube ice, a high-fidelity Mars analog environment. This chapter aimed to determine if another icy analog environment; the Devon Island ice cap in the Canadian high Arctic, an analog for the icy moons Europa and Enceladus contained an active microbial community. I used a combination of culturing, metagenomics and metatranscriptomics to characterize the microorganisms in this environment to determine if a viable and active microbial community was present, and if so, uncover some of the survival strategies they employ to survive in such a harsh environment.

Supplementary Tables S4.1 – S4.3 and Supplementary Figure S4.1 are available as supplemental material.

Chapter 4: Characterization of a potentially active ultra-low biomass microbial community in the near-surface ice of the Devon Island ice cap.

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4.1 Abstract

The icy moons of the outer solar system are considered some of the most habitable places in our solar system due to the presence of liquid water oceans beneath their icy surfaces. If microbial life exists in these oceans, it could be actively transported into the near-surface ice above, which would be easier to access than the oceans on future robotic missions. We, therefore, set out to characterize the microbial communities of the Devon Island ice cap in the Canadian high arctic, an analog environment for the icy moons, and determine if the near-surface ice contains an active microbial ecosystem. Using culture-dependent and independent methods, we demonstrated that the near-surface ice of the Devon Island ice cap contains an active microbial community. Flow cytometry and live/dead staining revealed a total cell concentration of 3.62×10^4 cells/ml and a live fraction of 0.8% (289.6 cells/ml). Isolated cultures demonstrated subzero growth and metabolism in laboratory experiments. The most abundant genus present in a 1.5 meter depth ice core metagenome was *Phyllobacterium,* while the most abundant genus of the metatranscriptome was *Pseudomonas*. Transcripts were found for diverse metabolic marker genes, including those related to thiosulfate oxidation, aerobic carbon monoxide oxidation, aerobic respiration, fumarate reduction, nitrate and nitrite reduction, nitrogen fixation, oxygenic photosynthesis and carbon fixation. Transcripts involved in cold adaptation were also abundant, including cold shock proteins, transcription and translation factors, and membrane and peptidoglycan-altering proteins. These results demonstrate the presence of an active microbial ecosystem within shallow near-surface ice surviving by a combination of heterotrophy and lithoautotrophy. Furthermore, these results suggest that future life detection missions to the icy moons should target the search for life in near-surface ice.

4.2 Introduction

The icy moons of the outer solar system are considered some of the best places to search for life (Taubner et al. 2020). This is because moons such as Europa, Ganymede, and Callisto of the Jupiter system and Enceladus and Titan of the Saturn system are thought to contain large oceans of liquid water hidden below thick outer ice crusts (Lunine 2017). These bodies are hypothesized to maintain liquid oceans due to tidal flexing and heating from their host planet (Nimmo and Pappalardo 2016). Energy to sustain life is hypothesized to come from water-rock interactions at the seafloor (Carrizo et al. 2022) and hydrothermal activity (Hsu et al. 2015). In 2005 water plumes were discovered emanating from Encaledus' south pole (Dougherty et al. 2006; Hansen et al. 2006; Porco et al. 2006; Spahn et al. 2006; Waite et al. 2006). Fortuitously, the Cassini mission, sent to explore Saturn, contained an ion and neutral mass spectrometer and cosmic dust analyzer which was used to sample the aqueous plume material. The results revealed that Enceladus' ocean contains organic carbon and other elements and molecules required for life, including nitrogen, oxygen, carbon dioxide, methane, and molecular hydrogen; the ocean is also salty like on Earth (Waite et al. 2009). The discoveries of crucial building blocks of life in an ocean outside Earth raise the possibility that life may reside within the oceans and even in the ice crusts above.

Given that access to the oceans of icy moons requires drilling or melting through ~5-35 km of ice (Nimmo et al. 2007b; Čadek et al. 2016; Hoolst et al. 2016; Beuthe et al. 2016), if life does exist in the ocean of Enceladus or indeed any other icy moon, it will be very challenging to sample directly. Fortunately, evidence of life may exist in the ice above the oceans, transported from below by two mechanisms. The first is by cracks in the ice shell, creating cryo-volcanoes

that spray ice grains from the ocean below onto the surface (Porco et al. 2006). As mentioned above, these geysers have been confirmed on Enceladus and may also exist on Europa (Roth et al. 2014; Sparks et al. 2016; Jia et al. 2018). The second mechanism is upwelling, pushing water from the ocean through fractures in the ice shell to the surface (Sotin et al. 2002). This is possible because the ice shells of Europa and Enceladus are young (<50 Ma), suggesting geological activity (Schenk and McKinnon 2009; Schmidt et al. 2011; Rhoden et al. 2020; Green et al. 2021). This activity likely creates cracks and conduits for liquid water to move from the oceans into the upper ice layers and onto the surface, where it may become trapped (Nimmo and Gaidos 2002; Nimmo et al. 2007a).

On Earth, microorganisms have been discovered in ice all over the world including sea ice (Junge et al. 2002, 2004), glaciers (Christner et al., 2000; Liu et al., 2022; Touchette et al., 2023; Winkel et al., 2022; Zhong et al., 2021), and ice sheets (Miteva et al. 2004; Miteva and Brenchley 2005). Viable cells have been recovered from all of these environments and even to a depth of several kilometres in Greenland (Miteva et al. 2004; Miteva and Brenchley 2005) and Antarctica (Karl et al. 1999; D'Elia et al. 2008). Furthermore, some microbial isolates have been revived from glacial ice tens of thousands of years old (Zhong et al. 2021) and permafrost 500,000 years old (Johnson et al. 2007). These results suggest that ice may be conducive to preserving viable cells, but they also raise an intriguing question. Do active microbial ecosystems exist in such extreme cold ice environments which are also highly oligotrophic, hypersaline and/or hyperacidic?

There is also some evidence of microbial activity in sea ice and ice sheets. Helmke and Weyland (1995) demonstrated the incorporation of glucose and leucine by sea ice bacteria from the Weddell Sea at temperatures of -2°C, (Bakermans and Skidmore 2011b) demonstrated metabolism in isolates frozen in sea ice at -5°C and Junge, Eicken and Deming (2004) found evidence for microbial activity in hypersaline liquid brines within winter sea ice from the Arctic at -20°C. In ice sheets, Tung, Bramall and Price (2005) found excess methane in layers of ice containing methanogenic Archaea 3 km below the surface of the Greenland ice sheet, implying that the methanogens produced the methane after encapsulation in the ice. Tung et al. (2006) provide further evidence that excesses of CO_2 in the Greenland ice sheet could be accounted for by Fe-reducing bacteria present on microscopic clay grains in the ice.

Microorganisms are thought to be metabolically active in ice due to microscopic brine veins in ice that form as water freezes and solutes are expelled from the crystal matrix, forming brines at the crystal boundaries (Price 2000). While highly salty in sea ice or acidic in ice sheets or glacial ice, these brines provide a source of liquid water and, thus, a potentially habitable environment (Price 2000; Barletta et al. 2012; Sattler et al. 2013; Barletta and Dikes 2015). Indeed, when imaged under a microscope, cells have been found concentrated in sea ice brine veins (Junge et al. 2001, 2004) and modelling of brine pockets in Europa's ice shell supports submillimeter brine veins as a microbial habitat (Wolfenbarger et al. 2022).

The widespread distribution of microorganisms in glacial ice worldwide and evidence that many cells are viable and metabolically active within ice suggests that extant life may be capable of surviving in the ice shells of the icy moons of the outer solar system. Cell abundance on

Enceladus may be as high as $<10^4$ cells/ml, comparable to glaciers and ice sheets on Earth (avg. $10^1 – 10^4$ cells/ml) (Anesio and Laybourn-Parry 2012; Porco et al. 2017; Santibáñez et al. 2018). In ice, these microorganisms would be supplied liquid water in brine veins and shielded from solar and cosmic radiation. They would also be more accessible for future missions than the subsurface oceans. However, before a life detection mission can be sent to explore one of the icy moons, questions related to life on Earth remain to be answered. Firstly, despite the evidence that microorganisms can metabolize within the ice, which taxa are active or what metabolisms and survival methods they use to cope in such extreme conditions are largely unknown. Secondly, can nucleic acids be accurately analyzed and annotated from ultra-low biomass ice on Earth, suggesting the same may be possible on the icy moons?

To answer these questions, we collected surface ice cores from the Devon Island ice cap in the Canadian High Arctic. We chose the Devon Island ice cap for this study after, in 2018 it became a significant analog to the icy moons when research suggested it contained two hypersaline subglacial lakes, analogous to the subsurface oceans on Europa and Enceladus (Rutishauser et al. 2018). Reanalysis has since determined the lakes not to exist (Killingbeck et al. 2024) but due to its geographic isolation (Devon Island is uninhabited), age (>23,000 years) (Boon et al. 2010) and presumed low biomass, the Devon Island ice cap's status as a high-fidelity analog to the icy moons remains. We used a combination of culture-dependent (microbial isolation and physiological characterization) and independent methodologies (metagenomics, metatranscriptomics and flow cytometry) to characterize the ice to determine if it contained a cold-adapted, active, and/or viable microbial ecosystem.

4.3 Methods

4.3.1 Sampling site description and sample collection

The Devon Island ice cap is approximately 14,400 km^2 in area, occupying the eastern third of Devon Island, located in Baffin Bay in Nunavut, Canada and is the largest uninhabited island in the world. Its summit lies at an elevation of 1,920 meters and has a maximum ice thickness of 880 meters (Dowdeswell et al. 2004). The ice cap is considered a polar desert, receiving mean annual precipitation of less than 200 mm (Gardner & Sharp, 2007), with the yearly accumulation rate varying between ~10 cm water equivalents in the Northwest corner and ~50 cm water equivalents in the Southeast margin (Koerner 1970). The mean annual air temperature is -23°C but can reach -40°C in the winter and +10°C in June and July (Clark et al. 2007).

In late June 2022, we travelled to the Northwest corner of the Devon Island Ice Cap (75°25'54" N 84°05'49" W) at an elevation of 1066 meters above sea level (Figure 4.1). The ice thickness at this site is estimated to be between 400 and 600 meters based on previous airborne radar surveys (Dowdeswell et al. 2004). Snow (~50 cm) was removed from the top of the ice, and two 1.5 – 2-meter cores were drilled using a Kovacs 9 cm corer. The temperature of the ice was measured by placing a thermometer against the core as soon as it was removed from the borehole. These cores were immediately cut on the ice cap into 20 cm subsections, stored in sterile whirl-pak bags and transported frozen to our laboratory at McGill University for further processing and analysis.

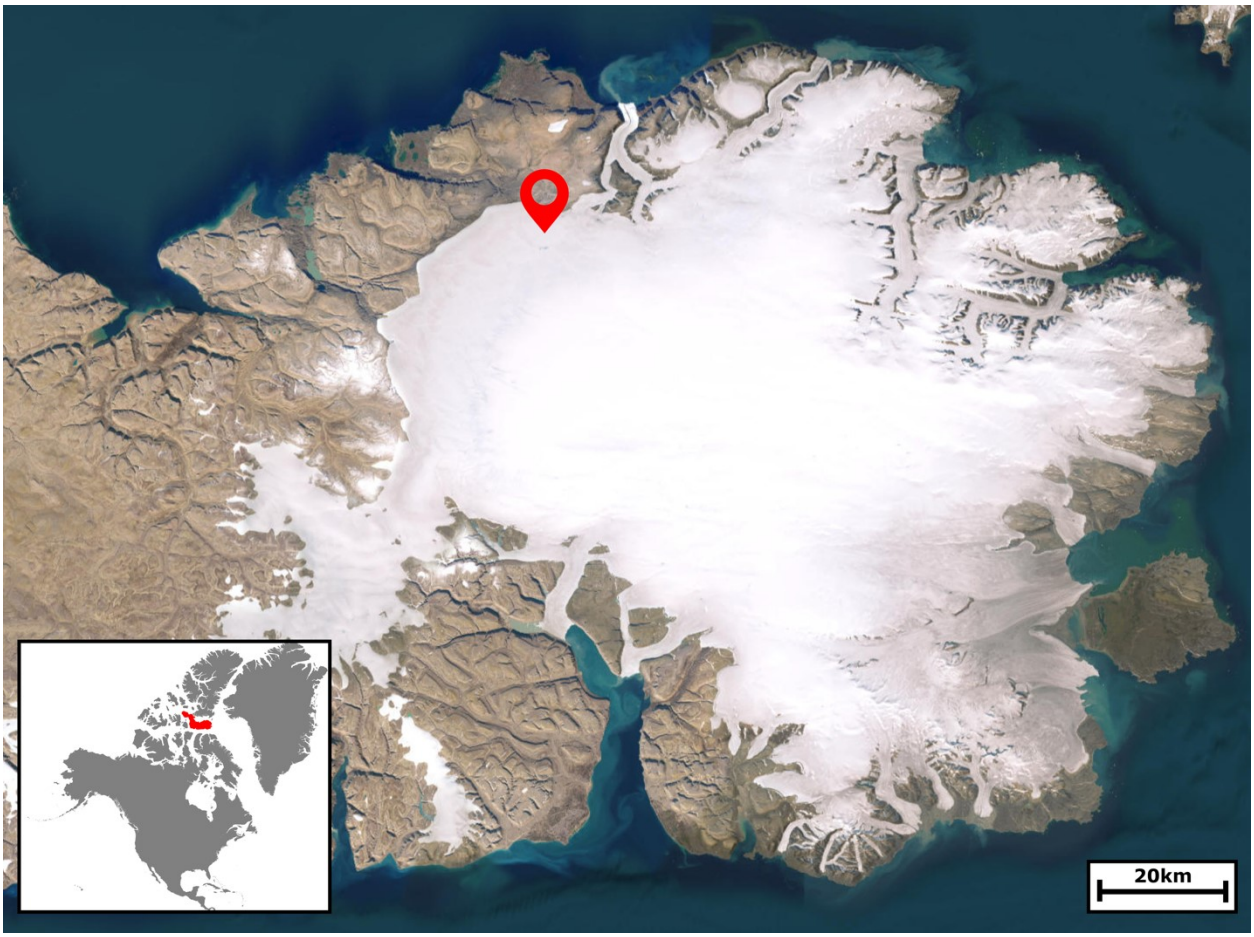

Figure 4.1. Map of the Devon Island ice cap. The ice cores was taken near the northwest margin of the ice cap (red marker).

4.3.2 Core decontamination and processing

A core section at a depth of 1.3-1.5 meters was chosen for this study because it was the deepest section for which we had a replicate section. A negative control artificial core was constructed by freezing double-filtered milli-Q water (18.2 MΩ cm, 25°C) in a whirl-pak into the shape of a cylinder. This artificial core was melted, filtered, and processed the same way as the core from the ice cap. To decontaminate the exterior of the cores, we adapted a method described by (Coelho et al. 2022). All tools and surfaces were cleaned with DNAse, Rnase, 70% ethanol, and UV sterilized for 30 minutes prior to contacting the core. In a biological safety

cabinet, the exterior 5 mm of the core subsections were removed using a saw and then washed with 70% ethanol. The decontaminated core was added to a sterile whirl-pak bag with an equal volume (~1 litre) of DNA/RNA Shield (Zymo Research, Irvine, California) and left to thaw in the dark at 4°C. The core sections were allowed to melt directly into DNA/RNA Shield reagent to prevent microbial metabolism during ice thaw. In this way, as soon as microbial cells melted from the ice, they would be preserved, thus halting all metabolism and preserving the *in situ* metatranscriptome and metagenome. Furthermore, by melting the ice at 4°C instead of room temperature, we hoped to significantly slow any microbial metabolism that might otherwise occur during thaw.

Once the core subsections had thawed, they were filtered onto a 0.22 µm nitrocellulose membrane. The membranes were then submerged in 5 ml of DNA/RNA Shield and vortexed for 2 minutes with 3 mm sterile DNA/RNA free glass beads to dislodge cells from the membranes. The 5 ml of DNA/RNA Shield reagent containing the cells was then passed through a 50 kDa Amicon® Ultra Centrifugal Filter (Millipore, Burlington, Massachusetts) to further concentrate cells into approximately 800 µl.

4.3.3 DNA/RNA extraction and sequencing

400 µL of the final concentrated cell volume after Amicon filtration of both the artificial core and ice cap core were extracted using the ZymoBIOMICS^{TM} DNA/RNA Miniprep Kit (Zymo Research, Irvine, California) using the dual DNA and RNA purification protocol and reducing the final elution volume from 100 µl to 50 µl. The other 400 µL from the Amicon filtration was saved in case a second extraction was required. 400 µl of Ambion^{TM} Nuclease-

Free Water (Invitrogen, Waltham, Massachusetts) was also extracted and used as a negative extraction control. Cleanup of the extractions was performed using the NEB Monarch® DNA and RNA Cleanup Kits (New England Biolabs, Ipswich, Massachusetts), and DNA carryover was removed from the RNA extractions using the Turbo DNA Free Kit (Invitrogen, Waltham, Massachusetts). The DNA and RNA concentration of the sample and negative controls was measured on a Qubit™ 4 Fluorometer using the Qubit™ 1X dsDNA High Sensitivity and RNA High Sensitivity Assay Kits (Invitrogen, Waltham, Massachusetts). The RNA extractions' RNA Integrity Number (RIN) was measured on a Bioanalyzer 2100 using the RNA Pico Kit (Agilent Technologies, Santa Clara, California).

Metagenome library preparation of the sample and negative controls was performed using Illumina's Nextera XT DNA Library Preparation Kit. The metatranscriptome library preparation was performed using the NEBNext rRNA Depletion and Ultra ll Directional RNA Library Prep Kits and protocol for Illumina (New England Biolabs, Ipswich, Massachusetts). The protocol was modified to avoid the RNA fragmentation step because the RIN values indicated that the RNA did not require further fragmentation. The metagenomes and metatranscriptomes were sequenced on a NovaSeq 6000 (Illumina, San Diego, California) SP flowcell (2 x 100 base pairs) at The Centre for Applied Genomics at the SickKids Hospital in Toronto, Ontario, Canada.

4.3.4 Metagenome and metatranscriptome data analyses

Low-quality bases, reads, and adapters were trimmed from both the metagenome and metatranscriptome with Trimmomatic (v.0.33, settings LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15) (Bolger et al. 2014). To remove contaminating sequences, the

metagenome reads from the artificial ice core and the negative extraction control were co-assembled using MegaHit (v.1.2.9, setting meta-sensitive) (Li et al. 2015) and DeconSeq (v.0.4.3) (Schmieder and Edwards 2011) was used to create a database of these contaminant sequences. DeconSeq was then used to remove sequences from the ice cap metagenome and metatranscriptome, which mapped to the negative control co-assembly. The decontaminated ice cap metagenome reads were then assembled using MegaHit. Genome binning was attempted with MetaBat2 (v.2.15) (Kang et al. 2019), MaxBin2 (v.2.2.7) (Wu et al. 2016) and SemiBin2 (v.1.5.1) (Pan et al. 2023) however, no method produced more than a single low-quality metagenome-assembled genome, likely because there were not enough sequence reads. SortMeRNA (v.4.3.6) (Kopylova et al. 2012) was used to remove contaminating rRNA sequences from the ice cap metatranscriptome and the removehuman tool, available with the BBMap package (v.38.92) (Bushnell B. – sourceforge.net/projects/bbmap/) was used to remove contaminating human DNA from the ice cap metatranscriptome.

The protocol of (Bağcı et al. 2021) was followed to functionally annotate the metagenome and metatranscriptome from the ice cap. DIAMOND (v.2.1.8.162, blastx alignment) (Buchfink et al. 2014) was used to align the sequences to the entire Ref-Seq non-redundant protein database from NCBI and MEGAN (v.6.25.6) (Huson et al. 2016, 2018) was used to perform functional binning of the alignments as well as to visualize the data and calculate rarefaction curves. Kaiju (v.1.8.2) (Menzel et al. 2016) was used to determine the taxonomy of sequences within both the metagenome and metatranscriptome datasets.

4.3.5 Ice core cell isolation and characterization

A replicate core from the same depth used for sequencing was decontaminated as stated above, and 500 ml was melted at 4°C in the dark. The melted ice was filtered onto a 0.22 µm nitrocellulose membrane. The microorganisms from the membrane were then resuspended in 10 ml of PBS buffer and plated on various agar media and enrichment broths. Specifically, resuspended cells were plated on R2A, 1/10 R2A, 1/100 R2A, R2B, ¼ R2B, 1/10 R2B, TSA, 1/10 TSA, 1/100 TSA and in broth enrichments of R2A, ¼ R2A, 1/10 R2A, R2B, ¼ R2B, 1/10 R2B, TSB, 1/10 TSB, and 1/100 TSB. Plates were incubated at 5°C for four months, and enrichment cultures were incubated at 5°C for six months with continual shaking before plating on equivalent agar plates. Colonies isolated from plates were then further tested for growth at 37°C, 25°C, 15°C, 10°C, -2°C, and -5°C. Colonies were also tested for growth on plates incubated at 5°C, supplemented with 1%, 3%, 6%, 9%, 12%, 15%, and 18% NaCl, and on plates incubated at 5°C and adjusted to pH 11, pH 10, pH 9, pH 8, pH 7, pH 6, pH 5, pH 4, pH 3. Isolated colonies also had their metabolism characterized using Biolog Gen lll plates (Biolog, California, USA) following the manufacturer's instructions. Cell cultures were diluted to a 95% turbidity in IF-C buffer (Biolog, California, USA), and 100 µl was inoculated into each well of the plates. Absorbance changes were measured weekly at a wavelength of 595 nm with a SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices, San Jose, California, USA). Positive substrate utilization were determined once the Average Well Colour Development (AWCD) of the plate had plateaued (Garland and Mills 1991). Substrate utilization was considered positive if its OD_{595} value reached above 0.25 (Garland 1996, 1997).

Sanger sequencing was attempted to determine the identity of each isolate however was only successful for a small proportion of isolates. First, isolated colonies were lysed by mixing

with 250 mL of deionized H_2O and heating in a microwave for 3 min. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed with primers 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), which amplify nearly the full length of the gene (~1500 bp). The 16S rRNA PCR cycling conditions were as follows: (1) 95°C for 7min, (2) 94°C for 45 s, (3) 55°C for 45 s, (4) 72°C for 1min (where steps 2–4 were repeated 30 times), (5) 72°C for 10 min. Amplicon DNA was sent to the Plateforme d'Analyses Génomiques de l'Université Laval (Quebec City, Quebec, Canada) for sequencing. Low-quality sequences were trimmed, and miscalled bases corrected, with 4Peaks v. 1.8. Sequences were then compared against the GenBank database by using BLASTn to look for the most similar sequences to the query sequences.

4.3.6 Flow cytometry

To quantify the total and live cell concentration in the ice core, an aliquot of resuspended cells in PBS was live/dead stained using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Waltham, Massachusetts) following the manufacturer's instructions. One of two 400 µL aliquots of resuspended cells was left unstained, and 1.5 µL of SYTO® 9 and propidium iodide were added to the other. Flow cytometry measurements were conducted on a Guava easyCyte (Millipore, Burlington, Massachusetts) after samples were incubated for 15 minutes at room temperature in the dark. The adjustments made to the gains in the red and green fluorescent channels, forward and side scatter, and live and unstained (boiled for 10 minutes) culture controls were made to ensure that most of the data points were within the dynamic range. Based on controls, the regions were classified as live and dead. The sample was subjected to 5000 events, with the concentration of viable bacteria being determined by deducting the blank value.

4.3.7 Physicochemical analysis

The temperature of the ice was measured immediately after the cores were removed from the borehole by pressing the probe of a thermometer against the inside surface of the borehole. Analysis of dissolved metals (Al, Ag, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Si, Sr, Ti, Tl, V, Zn), anions (NH_4^+, NO_2^-, NO_3^-, total organic nitrogen, PO_4^{3-}, SO_4^{2-}, Cl^-), total carbon, total organic carbon, pH and electrical conductivity was performed by the Natural Resources Analytical Laboratory at the University of Alberta.

4.4 Results and Discussion

4.4.1 Physicochemical, flow cytometry and culture analysis

To better constrain the physicochemical characteristics of the ice cap, the concentration of nutrients, metals, pH and salinity were measured from the ice (Table 4.1). We could not obtain an accurate measurement for total organic carbon (TOC) because of what we believe to be ethanol contamination of the sample during the core decontamination process. Total inorganic carbon was 0.204 mg/L, ammonium was 98.348 ug/L, nitrate was 768.537 ug/L, and nitrite was 19.059 ug/L. Phosphate and sulfate were measured to be below the limit of quantification but total sulfur was measured to be 1.207 mg/L. While oligotrophic, these values match closely to concentrations in Greenlandic surface ice with the exception of nitrate which is an order of magnitude higher in the Devon ice cap (Stibal, Gozdereliler, et al., 2015). Though the concentration available to microorganisms within liquid veins in ice sheets may be 100x greater than what is reported for bulk water (Barletta and Dikes 2015). The pH was 6.55, and electrical conductivity was measured to be 39.4 µS/cm, well within the lower range for fresh water, suggesting very few salts are present within the ice. The concentration of metals was also

measured but was found to be low or below the limit of detection in most cases (Supplemental Figure 4.1.).

Table 4.1. Cell abundance and physicochemical characteristics of a Devon Island ice cap near-surface ice core.

Parameter	Value
Total cell Abundance (Cells/ml)	3.62×10^4
Live fraction	0.80%
Ice temperature (°C)	-0.1
Total organic carbon (mg/L)	NA
Total inorganic carbon (mg/L)	0.2035
NH_4^+ (ug/L)	98.348
NO_2^- (ug/L)	19.059
NO_3^- (ug/L)	768.5369
PO_4^{3-} (ug/L)	<LOQ
SO_4^{2-} (mg/L)	<LOQ
Total S (mg/L)	1.207
Cl^- (mg/L)	3.788
EC (uS/cm)	39.4
pH	6.55

LOQ = Limit of Quantification

To help determine the habitability of the Devon ice cap, flow cytometry coupled with live/dead staining was performed, and pure cultures attempted to be isolated to provide multiple lines of evidence that the ice contained a viable microbial community. Flow cytometry results (Table 4.1) indicated a total cell concentration of 3.62×10^4 cells/mL, comparable to the average cell concentration in other englacial environments worldwide ($10^1 – 10^4$ cells/ml) (Anesio and Laybourn-Parry 2012; Santibanez-Avila 2016). It is also near the predicted cell concentration for Enceladus' plumes ($10^4 - 10^7$ cells/ml) (Porco et al. 2017) but two orders of magnitude above the concentration hypothesized for Europan surface ice, estimated from cell abundances in Lake Vostoc accretion ice (Christner et al. 2006; Hand 2017). We measured the live cell fraction in the Devon ice cap to be 0.8% (289.6 cells/ml), which is in the range of previous sea ice research

(0.5 – 4%) but is much less than live cells measured from depths 100 – 2,000 meters in the Greenland ice sheet (40 – 85%) (Junge et al. 2004; Miteva et al. 2015). An absence of dust in the Devon ice cap (as observed on 0.2 μm filters) likely explains the difference in live fraction between the two sites as lots of dust was observed in the Greenland core and has been positively correlated to cell abundance and activity (Stibal, Gozdereliler, et al., 2015; Tung et al., 2006).

Microorganisms from the Devon ice cap were cultured to determine if a viable microbial community exists within the ice. Despite considerable efforts using both nutrient-rich and poor media, including media that successfully cultured microorganisms from Greenland ice (Miteva and Brenchley 2005), only four colonies were recovered, all from either TSA or 1/10th TSA agar media (Table 4.2). This finding reinforces the notion that the Devon ice cap is an extreme environment, comparable to some of the most extreme environments on Earth, such as the Antarctic Dry Valleys (6 colonies; Goordial et al. 2016), and even more extreme than 3 km deep Greenland ice (22 – 800 colonies; Miteva, Sheridan and Brenchley 2004; Miteva and Brenchley 2005).

Table 4.2. Characterization of isolates recovered from the near-surface ice of the Devon Island ice cap.

Isolate ID	Isolation media	16S identity	Sequence similarity	Temperature growth range	Salinity growth range	pH growth range
DI3	TSA	NA	NA	-5°C - 37°C	1% - 12%	5 - 9
DI13	TSA	*Kocuria spp.*	100%	-5°C - 37°C	1% - 15%	4 - 9
DI14	TSA	NA	NA	-5°C - 25°C	1% - 15%	3 - 10
DI15	1/10 TSA	*Salinibacterium spp.*	100%	-5°C - 25°C	1% - 12%	4 - 9

Two isolates (DI3 and DI13) exhibited a growth range of 37°C to -5°C, while the other two (DI14 and DI15) had a growth range of 25°C to -5°C. All four isolates were capable of growing

in high salt concentrations (12 – 15% NaCl) and a wide pH range (3 – 10) (Table 4.2). The ability of the four isolates to metabolize at -2°C was tested using Biolog Gen III plates, which assess the ability to metabolize various heterotrophic carbon sources and chemical sensitivity. All four isolates could metabolize substrates at -2°C (Supplemental Figure 4.2), with isolate DI14 demonstrating the greatest capacity by metabolizing 50 of 71 carbon substrates. This isolate also showed the ability to grow across the widest range of salinity (0-15%) and pH (3-10), indicating it is a true polyextremophile, adapted to life in acid veins within ice sheets, which can reach pH 3 (Barletta et al. 2012).

The culture results suggest that the Devon ice cap contains a small but viable microbial community capable of metabolism and growth at subzero temperatures. Among these four taxa, only two were identified successfully: *Kocuria spp.* (DI13) and *Salinibacterium spp.* (DI15). Kocuria (DI13) and Salinibacterium (DI15) have been isolated from cryoenvironments before, including Arctic and Antarctic permafrost, Arctic seawater, Antarctic snow, and Tibetan glacial ice cores (Zhang et al. 2008a; Yan et al. 2012; Gundlapally, Ara and Sisinthy 2015; Raymond-Bouchard et al. 2018). Studies of the genomes of Kocuria have also revealed that it contains many cold adaptation genes (Gundlapally et al. 2015; Raymond-Bouchard et al. 2018). The capability of all four isolates to metabolize and grow at subzero temperatures, high salinity, and low pH suggests they are particularly well-adapted to life in ice.

4.4.2 Metagenomic and metatranscriptomic sequence taxonomy

Metagenomic and metatranscriptomic sequencing resulted in 2.6 and 2.2 million reads, respectively. Due to the small size of both datasets, no metatranscriptome reads aligned to the

metagenome assembly, and as such, both datasets were annotated separately. For the metatranscriptome annotation, only the forward reads were used because the reverse reads had very low-quality scores. Due to the ultra-low biomass nature of the ice, we took a conservative approach to sequence analyses in which, in addition to sequences removed by DeconSeq, we also removed any sequences or contigs that appeared to be contaminants (such as human-associated microorganisms). A complete list of these taxa can be found in the supplemental materials (Supplemental Table 4.3). This likely resulted in removing some sequences belonging to microorganisms that were naturally present in the ice but reduced the possibility of contaminants impacting the results. Rarefaction curves indicated that sequence coverage depth was sufficient for the metatranscriptome but insufficient for the metagenome, indicating there is likely additional taxonomic and functional sequence diversity in our samples not contained within the metagenome dataset (Supplemental Figure. 4.1).

In general, there was little overlap in the genera recovered in the metagenome and metatranscriptome (Figure. 4.2). Given the small number of sequences recovered from both the metagenome and metatranscriptome, this is not surprising, and if we had been able to filter more ice, therefore recovering more sequences, there likely would have been more taxonomic overlap between the datasets. Nevertheless, we were still able to gain significant insights into the Devon Island ice cap microbial ecosystem despite the low sequence output.

An analysis of all genes in the dataset revealed that the metagenome contained a high proportion of sequences belonging to the phylum Pseudomonadota (57%), with the predominant genus being *Phyllobacterium* (36%) (Figure 4.2). It is common to find Pseudomonadota

(proteobacteria) members in glacial snow and surface ice (Liu et al. 2009; Bradley et al. 2023; Touchette et al. 2023). *Phyllobacterium* is a plant-associated bacterium but has been found in glacial environments such as glacial melt streams in Svalbard (Zhang et al. 2022a) and, like other well-known plant-associated bacteria such as *Bradyrhizobium*, *Mesorhizobium*, and *Pannonibacter*, in the snow of glaciers of the Tibetan plateau (Liu et al. 2009). In addition to glacial snow, *Bradyrhizobium*, which we recovered from the Devon ice cap, has also been found in high Arctic glacial cryoconites (Hay et al. 2023). While there are no plants on the ice cap, abundant plant species have been recorded at the True Love Field Camp approximately 26 km away from our sampling site (Barrett and Teeri 1973). It is then possible that *Phyllobacterium*, *Bradyrhizobium* and other taxa detected in the sample originated in nearby Arctic tundra soil and were blown onto the ice cap by wind. Furthermore, the continued detection of these microorganisms on glacial surfaces suggests that they may be adapted to the glacial ice environment. Other genera commonly associated with glaciers and ice sheets were also found in our samples, including sequences belonging to members of the genus *Frigoribacterium* (4.70%), *Glaciihabitans* (4.44%), and *Cryobacterium* (1.57%) (Miteva et al. 2004; Zeng et al. 2013; Li et al. 2014; Anthony Pearce et al. 2020; Liu et al. 2020a) (Figure 4.2). Eukaryotic taxa were also found in the metagenome, including sequences belonging to members of the fungal phyla Ascomycota (5.40%) and Basidiomycota (3.92%), which have also been found in diverse glacial habitats, including soils, ice and meltwaters (Connell et al. 2008; Branda et al. 2010; De García et al. 2010). A single metagenome-assembled genome was recovered which was 28% complete and 0.14% contaminated most closely related to *Comamonas tsuruhatensis* based on classification from GTDB.

The metatranscriptome was also dominated by sequences belonging to the phylum Pseudomonadota (71.37%) and, specifically, the genus *Pseudomonas* (30.83%). *Pseudomonades* are ubiquitous in the environment and have been found in many glacial and cryoenvironments around the world (Reddy et al. 2004; Carrión et al. 2015; Sherpa et al. 2019; Snopková et al. 2020; Chauhan et al. 2023). *Pseudomonas* species can likely survive in cold environments due to their physiological versatility (Chauhan et al. 2023) which may explain why they make up such a large portion of the metatranscriptome of the Devon ice cap. Other phyla present at higher abundances were the gram-positive Actinomycetota (9.55%) and the Bacillota (5.32%), which have commonly been found in cold environments (Boetius et al. 2015). Both Actinomycetota and Bacillota have thick cell walls and are capable of forming spores, which is beneficial in cold, nutrient-poor environments, aiding in long-term survival (Onyenwoke et al. 2004; Schimel et al. 2007; Simon et al. 2009). Cyanobacteriota only comprised 0.81% of the metatranscriptome. Cyanobacteriota are often found on the surface of glaciers in cryoconite holes and snow (Hodson et al. 2008; Stibal, Šabacká and Žárský 2012; Hoham and Remias 2020), but their low abundance in the metatranscriptome we obtained from the Devon ice cap can be attributed to the ~50 cm of snow covering the sampled area, which prevents most sunlight from penetrating the ice, making photosynthesis difficult. Indeed, as part of a different study, light readings taken near the ice cap summit from a depth of 1 – 2 meters beneath the surface and beneath 70 cm of snow were 140 – 10 lux (data unpublished), which confirmed that very little light penetrates deep into the ice. Finally, like the metagenome, the metatranscriptome also contained genera commonly associated with cryoenvironments such as *Cryobacterium*, *Frigoribacterium*, *Glaciihabitans*, *Polaromonas* and *Stenotrophomonas* however, in much lower abundance than the metagenome.

Figure 4.2. Taxonomic classification of all genes of the metagenome and metatranscriptome at the phylum and genus level.

4.4.3 Community functional potential

We know very little about the functional potential of glacially entrapped microbial communities. This fact was noted 15 years ago by Simon et al. (2009) and remains to this day. Thus, we attempted to use metagenomic and metatranscriptomic sequencing to determine the active functional potential of glacially entrapped microbial communities. Despite the small number of sequences we recovered from both datasets, we could still annotate the metagenome and metatranscriptome using the NCBI nr database. This resulted in 2,223 total assignments for the metagenome and 4,049 total assignments for the metatranscriptome. The metagenome contained fewer assignments than the metatranscriptome because we successfully assembled it before annotation, resulting in fewer but longer sequences. Most importantly, we believe this to be the first metatranscriptome ever generated from englacial ice.

The number of unassigned sequences for both datasets was high, with 61.58% for the metagenome and 73.83% for the metatranscriptome. In both datasets, among the assigned sequences, the largest proportion was classified as "metabolism," accounting for 55.06% of the metagenome and 51.47% of the metatranscriptome. Sequences broadly annotated as "information storage and processing" accounted for 23.08% of the metagenome and 22.35% of the metatranscriptome. "Cellular processes and signalling" accounted for 21.86% of the metagenome and 26.18% of the metatranscriptome. These results follow closely to the results generated by Simon et al. (2009), who reported the first metagenomes ever recovered from englacial ice, recovered from the Northern Schneeferner in Germany.

We also mined the metagenome and metatranscriptome for a set of 42 marker genes corresponding to many different forms of metabolism (Figure 4.3). Marker genes in the metagenome and metatranscriptome support the presence of both aerobic and anaerobic forms of metabolism. This is indicated by the presence of the CoxA & CyoA genes, involved in aerobic metabolism in the presence of high concentrations of oxygen, CydA and CcoN genes, involved in aerobic metabolism in the presence of low oxygen concentrations. The marker gene for aerobic oxidation of carbon monoxide (CoxL) was also present in both the metagenome and metatranscriptome, attributed to Beta and Gammaproteobacteria in the metagenome, sharing the closest sequence identity with the genera *Delftia* and *Pseudomonas*. However, in the metatranscriptome, this gene was attributed to Mesorhizobium, an Alphaproteobacterium. Aerobic oxidation of carbon monoxide has been shown to be performed under nutrient starvation conditions in extreme environments (Cordero et al. 2019) and this might explain its activity in the Devon ice cap where nutrient levels are extremely low (Table 4.1). Anaerobic metabolisms present in both the metagenome and metatranscriptome included fumarate reduction, and nitrate/nitrite reduction.

While on the icy moons anaerobic metabolisms are more likely as a microbial lifestyle due to the lack of atmosphere around Europa and Enceladus, aerobic metabolism may still be possible due to radiolysis in the near-surface of the moons (Weber et al. 2023). Radiolysis is caused by the interaction of magnetospheric ions from Jupiter and Saturn with water ice, producing oxygen within the ice, which may be available for aerobic respiration on both Europa and Enceladus. The abundance of oxygen in Europa's ice shell has even been estimated to be $1.2 \times 10^{13} – 1.8 \times 10^{17}$ kg (Hesse et al. 2022). Furthermore, organic compounds were discovered

in Enceladus's plume (Waite et al. 2009), providing a source of carbon to support heterotrophic aerobic respiration in the ice.

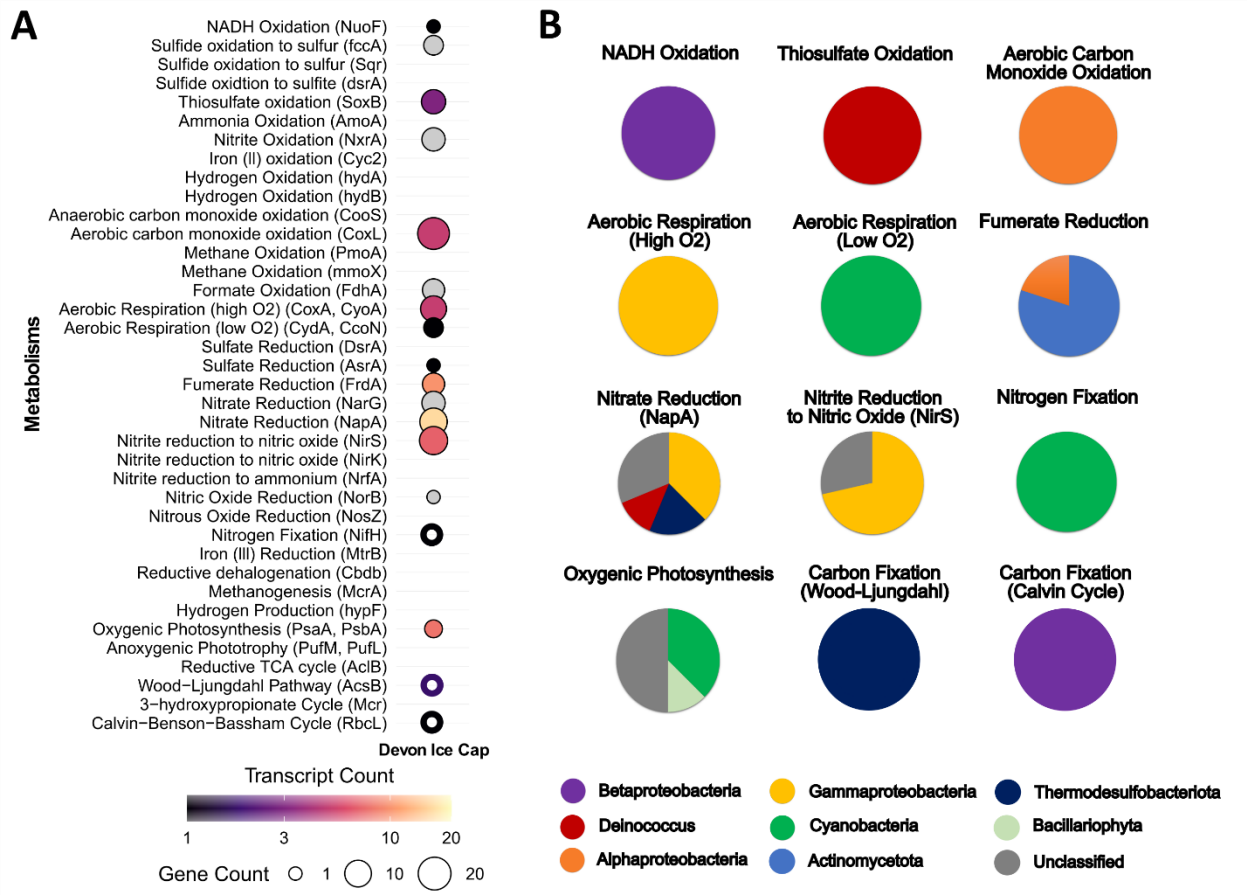

Figure 4.3. Devon Island ice cap detected metabolisms. **A.** Relative abundance of various metabolic marker genes in the metagenome and corresponding mRNAs detected in the metatranscriptome. The size of the circles corresponds to the number of copies of the marker gene in the metagenome, and the colour of the circles corresponds to the number of copies of the marker gene in the metatranscriptome. Gray circles indicate marker genes found in the metagenome but not the metatranscriptome. Circles with holes in the middle indicate marker genes found in the metatranscriptome but not the metagenome. **B.** Phyla and class level taxonomic classification of corresponding metabolic marker genes present in the metatranscriptome.

Notably absent were marker genes associated with methane metabolism, specifically methane oxidation (PmoA, mmoX) and methanogenesis (McrA). This is despite evidence of *in*

situ methanogenesis 90 meters from the bottom of the Greenland ice sheet (Tung et al. 2005) and the popular hypothesis that methane oxidation coupled to sulfate reduction and methanogenesis are the most likely metabolisms to occur on Europa and Enceladus, respectively (Weber et al. 2023).

Nevertheless, marker genes in the metagenome and metatranscriptome do indicate the presence of other autotrophic metabolisms capable of fixing organic carbon and relevant to potential life on Europa and Enceladus. Carbon dioxide has been detected on the surface of Europa and Enceladus, likely produced in the oceans below by serpentinization (Trumbo and Brown 2023; Villanueva et al. 2023; Schwander et al. 2023). Inorganic carbon in the bulk meltwater of the Devon ice cap was measured at 0.2035 mg/L, and carbon dioxide concentrations of 360 ppm have been measured previously in the top of firn cores (Clark et al. 2007). We found marker genes associated with carbon fixation in the metatranscriptome, suggesting carbon fixation is active in the ice. Specifically, transcripts for AcsB, part of the Wood-Ljungdahl pathway, classified as Thermodesulfobacteriota (*Desulfovermiculus*) and RbcL, part of the Calvin-Benson cycle, classified as Gammaproteobacteria (*Delftia*) were found. While the Calvin-Benson cycle is unlikely to have evolved on Europa or Enceladus due to its reliance on photosynthesis and the lack of sunlight in the ice shells (Weber et al. 2023), the Wood-Ljungdahl pathway, possibly the most ancient carbon fixation pathway on Earth (Sousa and Martin 2014) may be a plausible carbon fixation pathway on Europa and Enceladus.

Formate oxidation, detected in the Devon ice cap is another plausible autotrophic metabolism for Enceladus due to the speculation that hydrothermal vents exist on its ocean floor.

Like on Earth, these vents likely supply formate to the ocean, where it is oxidized by microorganisms for energy (Russell et al. 2017). If formate can migrate through the water column to the ice above the ocean (i.e. by cryovolcanism) then it may act as another source of energy in the ice for microbial metabolism.

Nitrogen is one of the essential elements to all life on Earth and has also been detected in the plume of Enceladus providing another source of energy for microbial metabolism. The nitrogen which was detected in the plumes was organic (e.g., HCN, NH_3), making it easily metabolized by microorganisms (McKay et al. 2014). Marker genes involved in various forms of nitrogen metabolism were recovered from the Devon ice cap. In the metagenome, NxrA, involved in nitrite oxidation and NarG, involved in nitrate reduction, were classified as Betaproteobacteria, sharing the closest sequence similarity to *Delftia*. NapA, also involved in nitrate reduction, was classified as Betaproteobacteria (*Delphtia*, *Diaphorobacter*) and Gammaproteobacteria (*Pantoea*) in the metagenome and Deinococcata (*Thermus*), Gammaproteobacteria (*Stutzerimonas*) and Thermodesulfobacteriota (*Desulfonema*) in the metatranscriptome. Denitrification genes were found in both datasets. NirS, encoding part of the reduction of nitrite to nitric oxide, was classified as Betaproteobacteria (*Delftia*) and Gammaproteobacteria (*Pantoea*, *Pseudomonas*) in the metagenome and Gammaproteobacteria (*Stutzerimonas*, *Pseudomonas*) in the metatranscriptome. NorB, encoding part of the nitric oxide reduction pathway, was present only in the metagenome and classified as Gammaproteobacteria (*Pseudomonas*). A lone sequence of the nifH gene encoding part of the nitrogen fixation pathway was classified in the metatranscriptome as Cyanobacteria, most closely related to the genus Leptolyngbya. Nitrogen species were also present in the ice (98.35 µg/L NH_4^+, 19.06

μg/L NO_2^-, and 768.58 μg/L NO_3^-), supporting the metagenomic/metatranscriptomic data suggesting that nitrogen reduction is an active metabolism within the ice. These results also suggest that nitrate and nitrite could act as electron acceptors in Enceladus' ice should the concentration of oxygen produced by radiolysis remain low enough that nitrate/nitrite reduction be energetically favourable.

The presence of marker genes associated with sulfur oxidation pathways, including sulfide oxidation (FecA) in Gammaproteobacteria (Pseudomonas) and thiosulfate oxidation (SoxB) in Actinomycetota (Cutibacterium) and Deinococcata (Thermux), suggests potential sulfur cycling in the Devon Island ice. The detection of assimilatory sulfur reduction (AsrA) in Actinomycetota (Cutibacterium) further supports this notion. With a measured total sulfur concentration of 1.2 mg/L there may be sufficient sulfur species in the ice to sustain sulfur oxidation as an energy source.

The intriguing discovery of both small quantities of sulfur and sulfur oxidation genes (particularly thiosulfate oxidation) in the Devon Island ice metagenome and metatranscriptome holds significant implications for the possibility of life on Europa. Europa receives substantial amounts of sulfur from volcanic eruptions on Io (Anders and Urbassek 2019), and oxidized sulfur species like sulfate have been detected on its surface (Dalton et al. 2005). Given the potential for sulfur oxidation coupled with oxygen reduction to be active in the Devon ice cap, a similar metabolic strategy using sulfate and oxygen produced by radiolysis could be a possible metabolism to sustain microbial life within Europa's icy environment.

Finally, marker genes encoding part of the pathway for oxygenic photosynthesis were present in the metagenome (PsaA) and the metatranscriptome (PsaA, PsaB). In the metagenome it was classified as Chlorophyta (*Koliella*) and in the metatranscriptome, as Cyanobacteria (*Microcoleus*, *Gloeocapsopsis*, *Oscillatoria*), Gyrista (*Pinnularia*), and Heterokonta (*Asterionella*). Photosynthesis is unlikely though to contribute significantly to energy production within the Devon ice cap as light penetration measured near the ice cap summit through the snow at a similar depth to the ice analyzed here was found to be extremely low (10.3 – 142 lux).

The presence of genes and transcripts involved in cold adaptation is widespread in the microorganisms of our Devon Island ice cap core (Figure 4.4). To evaluate whether the Devon Island ice microbial community contained or expressed genes involved in cold adaptation, we searched our datasets for a list of genes known to be involved in cold adaptation as compiled by (Raymond-Bouchard et al. 2018). Cold shock and stress proteins stabilize DNA and RNA and are found widely among bacteria that prefer a wide range of temperatures (Phadtare 2004; Jin et al. 2014; Keto-Timonen et al. 2016). Whereas psychrophiles and thermophiles often contain cold-shock proteins in their genomes, they differ in their thermostability and the functional range in which they provide protection (Keto-Timonen et al. 2016). In the Devon ice cap metagenome, we found 21 copies of genes classified as cold shock or stress-related proteins. Of these, the largest number of copies we identified was the DNA molecular chaperon HSP70 gene. While typically associated with heat shock (Evans et al. 2010), it also has broader housekeeping functions within the cell including cold shock and has been found in the genomes of cold adapted microorganisms (Mayer and Bukau 2005; Pucciarelli et al. 2009; Yusof et al. 2021), which explains its presence in the Devon ice metagenome. Within the metatranscriptome, the most

highly transcribed cold-shock protein was the CspA cold-shock protein. Cold-shock proteins such as CspA function to stabilize RNA, increasing flexibility and prevent unwanted secondary structures (Jiang et al. 1997) and are found widely across Bacteria, Archaea and Eukaria and in the genomes of cryophiles, mesophiles and thermophiles (Phadtare 2004; Jin et al. 2014).

Transcription and translation factors play essential roles in cold adaptation by working with cold shock proteins to acclimatize cells to cold temperatures. Specifically, translation elongation slows after cold shock, and translation initiation is inhibited until cold shock proteins are synthesized to stabilize newly translated proteins (Jones et al. 1987; Goldstein et al. 1990; Zhang et al. 2018). Higher abundances of transcription and translation factors has been observed in a cold adapted permafrost bacteria before compared to their mesophilic counterparts (Raymond-Bouchard et al. 2018) and upregulation of expression of these genes in response to sudden temperature downshifts has been observed in cold-adapted yeasts (Sahara et al. 2002; Homma et al. 2003; Becerra et al. 2003; Strassburg et al. 2010; Touchette et al. 2022). However, we found that transcription initiation and elongation factors were expressed in the ice cores, suggesting that the microbial community within the ice has long since acclimatized to the cold and begun protein synthesis again. The greatest number of copies of any gene within this category was a superfamily ll DNA & RNA helicase and was also the most expressed. These helicases are crucial for stabilizing RNA at low temperatures (Kuhn 2012) but also provide many other functions within the cell, so they are likely always expressed to some degree (Owttrim 2006).

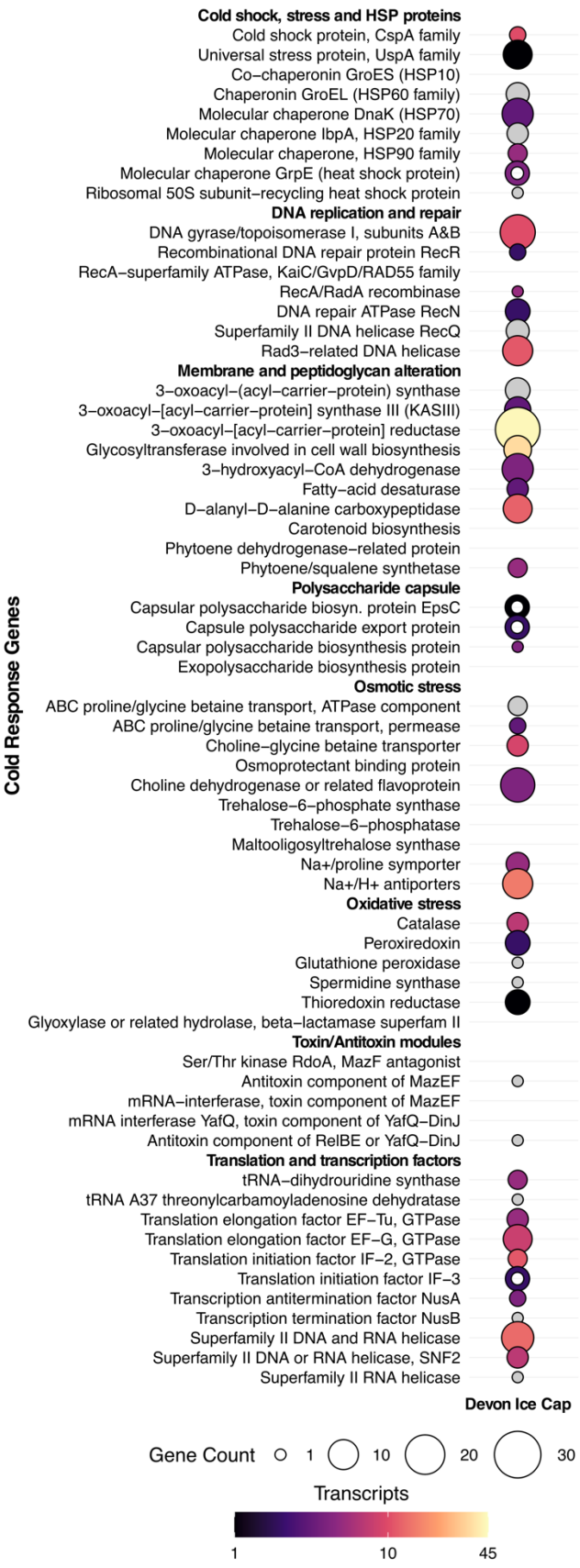
Cold Response Genes
Cold shock, stress and HSP proteins
Cold shock protein, CspA family
Universal stress protein, UspA family
Co−chaperonin GroES (HSP10)
Chaperonin GroEL (HSP60 family)
Molecular chaperone DnaK (HSP70)
Molecular chaperone IbpA, HSP20 family
Molecular chaperone, HSP90 family
Molecular chaperone GrpE (heat shock protein)
Ribosomal 50S subunit−recycling heat shock protein
DNA replication and repair
DNA gyrase/topoisomerase I, subunits A&B
Recombinational DNA repair protein RecR
RecA−superfamily ATPase, KaiC/GvpD/RAD55 family
RecA/RadA recombinase
DNA repair ATPase RecN
Superfamily II DNA helicase RecQ
Rad3−related DNA helicase
Membrane and peptidoglycan alteration
3−oxoacyl−(acyl−carrier−protein) synthase
3−oxoacyl−[acyl−carrier−protein] synthase III (KASIII)
3−oxoacyl−[acyl−carrier−protein] reductase
Glycosyltransferase involved in cell wall biosynthesis
3−hydroxyacyl−CoA dehydrogenase
Fatty−acid desaturase
D−alanyl−D−alanine carboxypeptidase
Carotenoid biosynthesis
Phytoene dehydrogenase−related protein
Phytoene/squalene synthetase
Polysaccharide capsule
Capsular polysaccharide biosyn. protein EpsC
Capsule polysaccharide export protein
Capsular polysaccharide biosynthesis protein
Exopolysaccharide biosynthesis protein
Osmotic stress
ABC proline/glycine betaine transport, ATPase component
ABC proline/glycine betaine transport, permease
Choline−glycine betaine transporter
Osmoprotectant binding protein
Choline dehydrogenase or related flavoprotein
Trehalose−6−phosphate synthase
Trehalose−6−phosphatase
Maltooligosyltrehalose synthase
Na+/proline symporter
Na+/H+ antiporters
Oxidative stress
Catalase
Peroxiredoxin
Glutathione peroxidase
Spermidine synthase
Thioredoxin reductase
Glyoxylase or related hydrolase, beta−lactamase superfam II
Toxin/Antitoxin modules
Ser/Thr kinase RdoA, MazF antagonist
Antitoxin component of MazEF
mRNA−interferase, toxin component of MazEF
mRNA interferase YafQ, toxin component of YafQ−DinJ
Antitoxin component of RelBE or YafQ−DinJ
Translation and transcription factors
tRNA−dihydrouridine synthase
tRNA A37 threonylcarbamoyladenosine dehydratase
Translation elongation factor EF−Tu, GTPase
Translation elongation factor EF−G, GTPase
Translation initiation factor IF−2, GTPase
Translation initiation factor IF−3
Transcription antitermination factor NusA
Transcription termination factor NusB
Superfamily II DNA and RNA helicase
Superfamily II DNA or RNA helicase, SNF2
Superfamily II RNA helicase
Devon Ice Cap
Gene Count
1
10
20
30
Transcripts
1
10
45

Figure 4.4. Devon Island ice cap detected cold adaptation genes. Relative abundance of various genes involved in adaptation to cold temperatures and salinity in the metagenome and metatranscriptome. The size of the circles corresponds to the number of copies of the marker gene in the metagenome, and the colour of the circles corresponds to the number of copies of the marker gene in the metatranscriptome. Gray circles indicate that a gene was found in the metagenome but not the metatranscriptome. Circles with holes in the middle indicate a gene found in the metatranscriptome but not the metagenome.

Maintaining a flexible cell membrane is important when encountering a low-temperature environment. Strategies to accomplish this vary, but one important one is to reduce the number of saturated fatty acids in the cell membrane (Barria et al. 2013). Unsaturated fatty acids add kinks to membranes, making them more flexible and less rigid at low temperatures. Within the Devon ice cap metagenome we found ten copies of the 3-oxoacyl-[acyl-carrier-protein] reductase gene, which is involved in fatty acid biosynthesis but, more specifically, polyunsaturated fatty acid biosynthesis. Furthermore, of all the cold adaptation genes we searched for, the 3-oxoacyl-[acyl-carrier-protein] reductase was the most expressed (Figure 4.4), hinting that it plays a crucial role in maintaining membrane flexibility in ice. Many genes involved in cell wall modifications and biosynthesis were also abundant and expressed in the ice (Figure 4.4). Thirty-four copies of a Glycosyltransferase and twelve copies of the D-alanyl-D-alanine carboxypeptidase gene were found in the metatranscriptome. Upregulation of cell wall modification genes has been reported in cold-adapted microorganisms before such as *Planococcus halocryophilus* and *Rhodococcus* and it is believed that these genes are expressed to thicken the cell wall to better withstand environmental stress (Mykytczuk et al. 2013b; Goordial et al. 2015; Raymond-Bouchard et al. 2018). Given the abundance and expression of genes in this category was the highest in the Devon ice cap, suggests that cell membrane and cell wall modifications may be the most important adaptations to surviving in an ice cap.

Finally, another factor microorganisms found in cold environments must contend with is osmotic stress. For enough water to sustain microbial growth and survival at subzero temperatures, the available water must be highly saline to prevent freezing. In the Devon ice cap, we found typical salt adaptation genes, including Na^+/H^+ antiporters and compatible solute synthesis genes such as choline dehydrogenases to be present and expressed. Cells use antiporters to export Na^+ ions using the proton motive force (Slonczewski et al. 2009) and choline dehydrogenases catalyze the production of the compatible solutes glycine and betaine (Gadda & McAllister-Wilkins, 2003), which were the most abundant cold adaptation genes we analyzed. While salt adaptation is predicted to play a major role in sea ice communities, where cells have been imaged in hypersaline brine veins (Junge et al. 2001). Ice caps and glacial ice, including the ice analyzed in this study contain few salts (Table 4.1, Supplemental Table 4.1) and thus it is unclear what role the expression of salt adaptation genes could be playing in the Devon ice cap. One possibility is that salt adaptation genes are simply co-regulated with other cold adaptation genes as has been demonstrated by the europsychrophile *Planococcus halocryophilus* (Raymond-Bouchard et al. 2017). Given that liquid veins in ice caps are predicted to be acidic (< pH 3) and not salty (Price 2000; Barletta et al. 2012), one possibility is that some mechanisms to combat high salinity also combat acidic conditions. For example, Na^+/H^+ antiporters have been shown to play a role in maintaining pH homeostasis (Pucciarelli et al. 2009).

4.5 Conclusion

The Devon Island ice cap is an analog for the icy moons of the outer solar system and is characterized by ultra-low biomass and nutrients. We have characterized the microbial

community of the near-surface ice using both culture-dependent and independent techniques, including recovering what we believe to be the first metatranscriptome ever generated from glacial ice. These results indicate that glacial ice may be a habitable environment capable of sustaining small populations of active cells. These active cells possess a high degree of functional diversity, potentially capable of thiosulfate oxidation, carbon monoxide oxidation, aerobic respiration, fumerate reduction, nitrate reduction, nitrite reduction, carbon fixation and oxygenic photosynthesis. In addition, the expression of genes involved in cold adaptation was widespread, including cold shock proteins, transcription and translation factors, and membrane and peptidoglycan alteration proteins and enzymes, suggesting the microbial community of the ice cap is well adapted to this environment. We further support the claim that the ice cap contains an active microbial community by the presence of live cells within the ice and by the isolation of viable cells capable of growth and metabolism at subzero temperatures. We've demonstrated using current technology that it is possible to detect microorganisms in samples we believe contain similar concentrations of cells to the near-surface ice of the icy moons should life indeed be present there. As such, our results raise the possibility that extant life could reside in the near-surface ice of the icy moons and future life-detection missions such as the proposed Europa lander mission could detect it.

4.6 Acknowledgements

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Connecting Text

In the previous chapters, I characterized the microbial communities and their activities in unique astrobiology analog ice environments through metabolic activity assays, culturing techniques and meta'omics methods. This chapter shifts focus to a much deeper characterization and comparison of microbial communities in englacial ice from both White Glacier in the Canadian high Arctic and Johnsons Glacier in Antarctica using culture methods, flow cytometry, metagenomics, and metatranscriptomics. I use flow cytometry to estimate the live and total biomass within the glaciers, and culture techniques to assess the viability of microbial communities under extremes of temperature, pH, and salinity. Using metagenomics and metatranscriptomics, I assess the presence and survival strategies, including metabolisms and cold adaptations of active microbial communities and metagenome-assembled genomes within these extreme environments.

Supplemental tables S5.1 – S5.5 and Supplemental figure S5.1 are available as supplemental material.

Chapter 5. Metagenomic and metatranscriptomic characterization of englacial ice from the Arctic and Antarctica finds evidence for metabolically active microbial communities

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5.1 Abstract

Glacial ice, an extreme environment characterized by freezing temperatures, low water activity, and nutrient scarcity, can still support active microbial ecosystems. However, the identity and survival strategies of these microorganisms remain largely unknown. We characterized viable and active microbial communities from Arctic and Antarctic englacial ice using flow cytometry, culturing, metagenomics, and metatranscriptomics. The ice, though low in biomass (10^4 cells/ml), harbours communities capable of growth at subzero temperatures (-5°C), high salinity (>6% NaCl), and low pH (pH 3). The community of both glaciers was significantly different, with MAGs from the Arctic glacier belonging to Cyanobacteriota and novel phyla and MAGs from the Antarctic glacier belonging to Pseudomonadota and Actinomycetota. Despite this, both glacial communities shared key metabolic functions, including aerobic respiration, aerobic carbon monoxide oxidation, sulfide oxidation, and denitrification. However, the Antarctic glacial community lacked genes for carbon fixation, suggesting that without a method of fixing carbon, the scarce nutrients in this environment may make long-term survival challenging. Metatranscriptomics revealed dominant Cyanobacteriota in the Arctic glacier, performing oxygenic photosynthesis and carbon fixation. These, along with active lithoautotrophs performing metabolisms such as carbon fixation via the 3-hydroxyproprionate cycle, anoxygenic photosynthesis, sulfide oxidation, and nitrate reduction/denitrification, support a heterotrophic community performing aerobic respiration and aerobic carbon monoxide oxidation. This study highlights the distinct but functionally similar microbial communities in Arctic and Antarctic glaciers, hinting there may be a core set of metabolisms required for living in ice and suggesting that such ecosystems could persist in glacial ice on Mars or the icy moons.

5.2 Introduction

Climate change disproportionately affects Earth's polar regions. The Arctic is warming at a rate 3 times faster than the global average (0.6 – 0.75°C/decade) (Comiso & Hall, 2014; Post et al., 2019), and, while warming in Antarctica is much slower (0.31°C) (Dalaiden et al. 2022), it is predicted to increase in the coming decades (Siegert et al. 2019). As such, polar glaciers have and will continue to melt quicker than their mid-latitude counterparts. The Northern Canadian Arctic and Greenland are currently losing a combined mass of ~66 gigatons per year, with Antarctica losing ~20.9 gigatons per year (Hugonnet et al. 2021). Among the many issues this creates, is the loss of one of the most poorly understood microbial ecosystems on Earth.

It is estimated that there are between 10^{25} and 10^{29} microorganisms within glacier ice globally, and by some estimates, between 10^{17} to 10^{21} microorganisms are released from glacial ice each year due to glacial melt (Castello & Rogers, 2005; Edwards, 2015), although this estimate is likely to increase as glacial melt accelerates. Therefore, understanding the identity, diversity, and functional potential of englacially entrapped microbial communities is critical to predicting their effect on surrounding ecosystems.

To date, much of our understanding of glacial microbial communities comes from supraglacial or subglacial environments. But very little is known of the englacial environment which is defined as the body of the glacier which is not influenced by surface or subglacial processes (Fountain 2011). This is despite englacial environments containing the largest number of microorganisms globally. One reason for the lack of research is that englacial ice has long been considered too harsh to sustain more than maintenance metabolism (metabolism to repair

DNA damage) (Price and Sowers 2004). However, evidence is growing that active microbial ecosystems do exist in englacial ice, capable of performing more than just maintenance metabolism (Junge et al. 2004; Tung et al. 2005, 2006; Rohde et al. 2008; Bakermans and Skidmore 2011b, 2011a; Martinez-Alonso et al. 2019). Most of these studies only found indirect evidence for englacial microbial metabolism or demonstrated microbial metabolism in ice in laboratory experiments but not *in situ*. As such, a detailed understanding of the survival strategies of active englacial microbial communities remains elusive.

Obtaining a better understanding of how active microbial communities survive in ice is important not only for determining how microbial life survives in such extreme cold environments, how the release of these communities will impact downstream environments as well as inform the search for life elsewhere in our solar system, such as on Mars and the icy moons of the outer solar system where large ice deposits and liquid water exist (Lunine 2017; Dundas et al. 2021). If life ever did evolve on any of these celestial bodies, it may have adapted to life in ice where it could reside today, close to the surface, and relatively accessible for future life detection missions (Hand et al. 2022).

In this work, we combine metagenomics and metatranscriptomics with flow cytometry and culturing to determine if polar glacial ice from both the Arctic and Antarctic contains a viable, potentially active microbial community and what metabolisms and survival strategies these communities employ *in situ*.

5.3 Methods

5.3.1 Sampling site descriptions and sample collection

White Glacier is a 38.7 km^2 valley glacier located ~8 km inland from Expedition Fiord on Axel Heiberg Island in the Canadian High Arctic (79.437380N 90.641717W, Supplemental Figure 5.1). The glacier has an altitude of 100 to 1782 m above sea level (Thomson and Copland 2017). The ice thickness is, on average, 200 m but can exceed 400 m in some locations. The glacier has the longest mass balance record of any alpine glacier in the Canadian Arctic (65 years) and is a world reference glacier within the Global Terrestrial Network of Glaciers. The area around White Glacier is a polar desert, receiving 58 mm of precipitation per year at sea level and 370 mm of precipitation per year at 2120 m (Cogley et al. 1996). The closest location where mean annual temperature data is available is at the Eureka weather station (~100km east), which has a mean annual temperature of -19.7°C.

Johnsons Glacier is located on Livingston Island in the South Shetland Islands of Antarctica near the Juan Carlos l Spanish Antarctic Station (62.67057S 60.36961W, Supplemental Figure 5.1). It is a sea terminating glacier of approximately 5 km^2 with an altitude between 50 and 330 m a.s.l. (Navarro et al. 2013). The glacier's mass balance has been continually monitored since the 2001/2002 field season, one of the region's longest mass/balance records (Navarro et al. 2013). As such, it has been included as a benchmark glacier within the Global Terrestrial Network of Glaciers. The average ice thickness of the glacier is 93 m, with a maximum thickness of 160 m (Benjumea et al. 2003; Navarro et al. 2005, 2009). The glacier also receives sporadic inputs of volcanic ash deposited from the Deception Island Volcano (Furdada et al., 1999), located approximately 35 km away, with the last eruption occurring in

1970, which covered the eastern portion of the island (Baker and Mcreath 1972). Ninety percent of the island is covered in snow and has a mean annual air temperature of -1.5°C.

We travelled to White Glacier in July 2022 and 2023 and Johnsons Glacier in February 2023. Using a Kovacs 9 cm corer, from White Glacier, we collected surface one-meter cores (79.443163N, 90.639496W), and from Johnsons Glacier, we collected surface cores up to a depth of 1.5 meters (62.66925S 60.63998W) (Supplemental Figure 5.1). On the glaciers, the cores were cut into 20 cm subsections, stored in sterile whirl-pak bags, and transported frozen back to McGill University. An IM150 light meter (Illuminati Instruments, Santa Clara, California, USA) was also lowered into the White Glacier core borehole and covered. Light (LUX) and colour temperature measurements were recorded to estimate the percent of light penetration into the ice. The temperature of the ice was measured immediately after the cores were removed from the borehole by pressing the probe of a thermometer against the inside surface of the borehole. Analyses of dissolved metals (Al, Ag, As, B, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Si, Sr, Ti, Tl, V, Zn) (Supplemental Table 5.1), anions (NH_4^+, NO_2^-, NO_3^-, total organic nitrogen, PO_4^{3-}, SO_4^{2-}, Cl^-), total carbon, total organic carbon, pH, and electrical conductivity was performed by the Natural Resources Analytical Laboratory at the University of Alberta (Edmonton, Canada).

5.3.2 Core decontamination and processing

From White Glacier, a core section at a depth of 0.7-0.9 meters was chosen for this study. This depth was deep enough to ensure separation from the surface ice but shallow enough that light could penetrate. From Johnsons Glacier, a core section at a depth of 1.0-1.2 meters was

chosen for this study. A negative control artificial core was constructed by freezing double-filtered milli-Q water (18.2 MΩ cm, 25°C) in a whirl-pak into the shape of a cylinder. This artificial core was melted, filtered, and processed the same way as the White Glacier and Johnsons Glacier cores. To decontaminate the exterior of the cores, we adapted a method described by (Coelho et al. 2022). All tools and surfaces were cleaned with DNAse, RNAse, 70% ethanol, and UV sterilized for 30 minutes prior to contacting the core. In a biological safety cabinet, the exterior 5 mm of the core subsections were removed using a saw and then washed with 70% ethanol. The decontaminated core was added to a sterile whirl-pak bag with an equal volume (~1 litre) of DNA/RNA Shield (Zymo Research, Irvine, California) and left to thaw in the dark at 4°C. The core sections were allowed to melt directly into DNA/RNA Shield reagent to prevent microbial metabolism during ice thaw. In this way, as soon as microbial cells melted from the ice, they would be preserved, thus halting all metabolism and preserving the *in situ* metatranscriptome and metagenome. Furthermore, by melting the ice at 4°C instead of room temperature, we hoped to significantly slow any microbial metabolism that might otherwise occur during thaw. Decontamination and processing of the White Glacier core was performed at McGill University, and in the laboratory facilities at the Juan Carlos l Antarctic base for the Johnsons Glacier core.

Once the core subsections had thawed, they were filtered onto a 0.22 µm nitrocellulose membrane. The membranes were then submerged in 5 ml of DNA/RNA Shield and vortexed for 2 minutes with 3 mm sterile DNA/RNA free glass beads to dislodge cells from the membranes. The 5 ml of DNA/RNA Shield reagent containing the cells was then passed through a 50 kDa

Amicon® Ultra Centrifugal Filter (Millipore, Burlington, Massachusetts) to further concentrate cells into approximately 800 µl.

5.3.3 DNA/RNA extraction and sequencing

400 µL of the final concentrated cell volume after Amicon filtration of both the artificial core and glacial ice cores were extracted using the ZymoBIOMICS™ DNA/RNA Miniprep Kit (Zymo Research, Irvine, California) using the dual DNA and RNA purification protocol and reducing the final elution volume from 100 µl to 50 µl. The other 400 µL from the Amicon filtrations was saved in case a second extraction was required. 400 µl of Ambion™ Nuclease-Free Water (Invitrogen, Waltham, Massachusetts) was also extracted and used as a negative extraction control. Cleanup of the extractions was performed using the NEB Monarch® DNA and RNA Cleanup Kits (New England Biolabs, Ipswich, Massachusetts), and DNA carryover was removed from the RNA extractions using the Turbo DNA Free Kit (Invitrogen, Waltham, Massachusetts). The DNA and RNA concentration of the sample and negative controls was measured on a Qubit™ 4 Fluorometer using the Qubit™ 1X dsDNA High Sensitivity and RNA High Sensitivity Assay Kits (Invitrogen, Waltham, Massachusetts). The RNA extractions' RNA Integrity Number (RIN) was measured on a Bioanalyzer 2100 using the RNA Pico Kit (Agilent Technologies, Santa Clara, California).

Metagenome library preparation of the samples and negative controls was performed using Illumina's Nextera XT DNA Library Preparation Kit. The metatranscriptome library preparation was performed using the NEBNext rRNA Depletion and Ultra ll Directional RNA Library Prep Kits and protocol for Illumina (New England Biolabs, Ipswich, Massachusetts). The protocol

was modified to avoid the RNA fragmentation step because the RIN values indicated that the RNA did not require further fragmentation. The metagenome and metatranscriptome from White Glacier was sequenced on a NovaSeq 6000 (Illumina, San Diego, California) SP flowcell (2 x 100 base pairs) while the metagenome and metatranscriptome from Johnsosn Glacier was sequenced on a NovaSeq X (Illumina, San Diego, California) 10B flowcell (2 x 150 base pairs), both at The Centre for Applied Genomics at the SickKids Hospital in Toronto, Ontario, Canada.

5.3.4 Metagenome and metatranscriptome data analysis

Low-quality bases, reads, and adapters were trimmed from both the metagenome and metatranscriptome with Trimmomatic (v.0.33, settings LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15) (Bolger et al. 2014). To remove contaminating sequences, the metagenome reads from the artificial ice core and the negative extraction control were co-assembled using MegaHit (v.1.2.9, setting meta-sensitive) (Li et al. 2015) and DeconSeq (v.0.4.3) (Schmieder and Edwards 2011) was used to create a database of these contaminant sequences. DeconSeq was then used to remove sequences from both glacier metagenomes and metatranscriptomes, which mapped to the negative control co-assembly. The decontaminated metagenome reads were then assembled using MegaHit. Genome binning was performed with MetaBat2 (v.2.15) (Kang et al. 2019), MaxBin2 (v.2.2.7) (Wu et al. 2016), and SemiBin2 (v.1.5.1) (Pan et al. 2023). dRep (v.3.4.3) (Olm et al. 2017) was used to dereplicate the bins we generated and iRep (v.1.10) (Brown et al. 2016) was used to estimate the *in situ* replication rate of the microbial population each bin represented. SortMeRNA (v.4.3.6) (Kopylova et al. 2012) was used to remove contaminating rRNA sequences from the metatranscriptomes and the removehuman tool, available with the BBMap package (v.38.92) (Bushnell B. –

sourceforge.net/projects/bbmap/) was used to remove contaminating human DNA from the metatranscriptomes. Metatranscriptomes were aligned to the metagenomes using bowtie2 (v.2.5.1) (Langmead and Salzberg 2012) and counted using HTSeq2 (v.2.0.2) (Putri et al. 2022). Functional annotation was performed by uploading the metagenomes to the JGI IMG/M annotation pipeline (Chen et al. 2023). MetaPhlAn 4 (v.4.0.6) (Blanco-Míguez et al. 2023) was used to determine the taxonomy of the assembled metagenomes and processed metatranscriptome reads.

To determine if there was a statistically significant difference in the taxonomy between the communities of White and Johnsons Glaciers the pairwise Multinomial Species Classification Method (CLAM); (Chazdon et al. 2011) was used to classify each metagenome sequence assigned to a taxonomy with MetaPhlAn 4 into the categories: generalist, specialist of White Glacier or specialist of Johnsons Glacier or too rare. We used the clamtest() function in R (v.4.3.3) with a p-value of 0.05 and a coverage limit of 10 sequences as a rarity threshold to separate samples from both poles. The diversity permutation test was used to compare the diversity between both glaciers using random permutations and several alpha diversity indexes (Shannon H, Simpson index, evenness). The diversity t-test was used to compare the diversity between the two samples. This test is an alternative to the diversity permutation test. The Shannon index includes a bias correction in this test.

5.3.5 Ice core cell isolation and characterization

From each glacier, a replicate core from the same depth used for sequencing was decontaminated as stated above, and 500 ml was melted at 4°C in the dark. The melted ice was

filtered onto a 0.22 µm nitrocellulose membrane. The microorganisms from the membrane were then resuspended in 10 ml of PBS buffer and plated on various agar media and enrichment broths. Specifically, resuspended cells were plated on R2A, 1/10 R2A, 1/100 R2A, R2B, ¼ R2B, 1/10 R2B, TSA, 1/10 TSA, 1/100 TSA and in broth enrichments of R2A, ¼ R2A, 1/10 R2A, R2B, ¼ R2B, 1/10 R2B, TSB, 1/10 TSB, and 1/100 TSB. Plates were incubated at 5°C for four months, and enrichment cultures were incubated at 5°C for six months with continual shaking before plating on equivalent agar plates. Colonies isolated from plates were then further tested for growth at 37°C, 25°C, 15°C, 10°C, -2°C, and -5°C. Colonies were also tested for growth on plates incubated at 5°C, supplemented with 1%, 3%, 6%, 9%, 12%, 15%, and 18% NaCl, and on plates incubated at 5°C and adjusted to pH 11, pH 10, pH 9, pH 8, pH 7, pH 6, pH 5, pH 4, pH 3. All colonies isolated from White Glacier and 14 randomly selected colonies isolated from Johnsons Glacier also had their metabolism characterized using Biolog Gen lll plates (Biolog, California, USA). To do this, cell cultures were diluted to a 95% turbidity in IF-C buffer (Biolog, California, USA), and 100 µl was inoculated into each well of the plates. Absorbance changes were measured weekly at a wavelength of 595 nm with a SpectraMax M2e Multi-Mode Microplate Reader (Molecular Devices, San Jose, California, USA). Positive substrate utilization were determined once the Average Well Colour Development (AWCD) of the plate had plateaued (Garland and Mills 1991). Substrate utilization was considered positive if its OD_{595} value reached above 0.25 (Garland 1996, 1997).

Sanger sequencing was attempted to determine the identity of each isolate however was only successful for a small proportion of isolates. First, isolated colonies were lysed by mixing with 250 mL of deionized H_2O and heating in a microwave for 3 min. Polymerase chain reaction

(PCR) amplification of the 16S rRNA gene was performed with primers 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), which amplify nearly the full length of the gene (~1500 bp). The 16S rRNA PCR cycling conditions were as follows: (1) 95°C for 7min, (2) 94°C for 45 s, (3) 55°C for 45 s, (4) 72°C for 1min (where steps 2–4 were repeated 30 times), (5) 72°C for 10 min. Amplicon DNA was sent to the Plate-forme d'Analyses Genomiques de l'Université Laval (Quebec City, Quebec, Canada) for sequencing. Low-quality sequences were trimmed, and miscalled bases corrected, with 4Peaks v. 1.8. Sequences were then compared against the GenBank database by using BLASTn to look for the most similar sequences to the query sequences.

5.3.6 Flow cytometry

To quantify the total and live cell concentration in the ice core, an aliquot of resuspended cells in PBS was live/dead stained using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Waltham, Massachusetts) following the manufacturer's instructions. One of two 400 µL aliquots of resuspended cells was left unstained, and 1.5 µL of SYTO® 9 and propidium iodide were added to the other. Flow cytometry measurements were conducted on a Guava easyCyte (Millipore, Burlington, Massachusetts) after samples were incubated for 15 minutes at room temperature in the dark. The adjustments made to the gains in the red and green fluorescent channels, forward and side scatter, and live and unstained (boiled for 10 minutes) culture controls were made to ensure that most of the data points were within the dynamic range. Based on controls, the regions were classified as live and dead. The sample was subjected to 5000 events, with the concentration of viable bacteria being determined by deducting the blank value.

5.4 Results/Discussion

5.4.1 Polar glacial ice contains a viable and metabolically active microbial community

We used various methods, including both culture-dependent and independent methodologies, to determine if near-surface englacial ice from White Glacier and Johnsons Glacier contains viable and active microbial ecosystems. Flow cytometry coupled with live/dead staining was unsuccessful from Johnsons Glacier, but White Glacier revealed that there were 4.75×10^4 total cells/ml, of which 0.5% (237.5 cells/ml) were alive (Table 5.1). These values are similar but slightly higher than that found for the core from the Devon Island ice cap discussed in Chapter 4 and very similar to glacial surface ice samples from Svalbard (Irvine-Fynn et al. 2012) and an alpine glacier in Tibet (Yao et al. 2008). Generally, englacial ice contains ~10^1 – 10^4 total cells/ml (Anesio and Laybourn-Parry 2012; Santibanez-Avila 2016) where differences in glacial ice cell concentrations reflect differences in atmospheric currents and emission sources (e.g., ocean, dust etc.) (Santibanez-Avila 2016) as well as the ability of microorganisms to withstand freezing into the ice.

Table 5.1. Cell abundance and physicochemical characteristics of White Glacier and Johnsons Glacier ice cores.

Parameter	White Glacier	Johnsons Glacier
Total cell Abundance (Cells/ml)	4.75×10^4	NA
Live fraction	0.50%	NA
Ice temperature (°C)	-0.2	-0.1
Total organic carbon (mg/L)	4.05	2.17
Total inorganic carbon (mg/L)	0.602	0.856
NH_4^+ (ug/L)	36.432	15.498
NO_2^- (ug/L)	1.774	<LOQ
NO_3^- (ug/L)	133.676	<LOQ
PO_4^{3-} (ug/L)	<LOQ	<LOQ
SO_4^{2-} (mg/L)	<LOQ	<LOQ
Total S (mg/L)	0.245	0.059
Cl^- (mg/L)	0.656	0.42
EC (uS/cm)	13.1	3.97
pH	6.96	5.59

LOQ = Limit of Quantification

The proportion of live cells is at the lower end of metabolically active cells determined in Alaskan sea ice (Junge et al. 2004) (0.5 – 4%) but an order of magnitude lower than live cells detected in old ice, 2 km beneath the surface of the Greenland ice cap (39 – 84%) (Miteva et al. 2015). We believe the percentage of viable cells in White Glacier to be at the lower end of sea ice bacteria reported by Junge et al., (2004) due to greater challenges faced by microorganisms in glacial ice compared to sea ice, including colder temperatures, lower water availability, lower nutrient concentrations, and longer entrapment compared to the seasonality of sea ice (Boetius et al. 2015). An absence of dust in White Glacier (as observed on 0.2 μm filters) likely explains the difference in the fraction of live cells between it and the Greenland ice sheet as lots of dust was observed in the Greenland core and has been positively correlated to cell abundance and activity (Tung et al. 2006; Yao et al. 2008; Stibal et al. 2012; Chen et al. 2016).

Despite being unable to determine the total and live cell concentration within the Johnsons Glacier core, we successfully recovered culture isolates from this glacier. Across all culture media used, we recovered 50 isolates. Forty-three of these isolates could grow at -2°C or -5°C, 13 grew above 6% NaCl, 15 grew in pH 4 or lower (Table 5.2) and all 14 isolates tested showed metabolic activity on Biolog plates grown at -2°C (Supplemental Table 5.2). From White Glacier, we only recovered 8 isolates. Three were capable of growth at -2°C or -5°C; one was capable of growth in or above 6%, two grew in pH 4 or lower (Table 5.2), and all 8 showed metabolic activity on Biolog plates at -2°C (Supplemental Table 5.2). From both glaciers, roughly half could not grow above 15°C, suggesting that they're true psychrophiles and many appear acid tolerant which may assist them in living in acid veins within glacial ice which is estimated to be as acidic as pH 3 (Barletta et al. 2012). Overall, Johnsons Glacier contained a larger viable microbial community and a higher proportion of isolates capable of growth at subzero temperatures than did White Glacier. However, these results also demonstrate that englacial ice from both glaciers contains a viable microbial ecosystem, a fact which confirms previous research from Greenland (Christner et al., 2000; Miteva et al., 2004; Miteva & Brenchley, 2005; Sheridan et al., 2003), the Arctic (Touchette et al. 2023), temperate glaciers (Christner et al. 2000, 2003; Ball et al. 2014; Balcazar et al. 2015) and Antarctica (Karl et al. 1999; Christner et al. 2000).

Evidence for a metabolically active microbial community present within the ice of the glaciers comes from two sources. First, using iRep, we estimated the *in situ* replication rate of some of the MAGs recovered from each glacier. This analysis uses the difference in genome

coverage across a MAG and demonstrated that each MAG we analyzed was replicating at the time of sampling (Supplemental Table 5.3). The strongest evidence however comes from the recovery of a metatranscriptome from White Glacier. Transcripts (mRNA sequences) can only be produced by metabolically active microorganisms and so the successful sequencing of a metatranscriptome from White Glacier suggests a metabolically active microbial community exists within the ice there. Unlike White Glacier, metatranscriptome sequencing from Johnsons Glacier was not successful, potentially for several reasons as follows. Firstly, melting and filtering of the ice was performed at the Juan Carlos l research station in Antarctica. While the filters were preserved in DNA/RNA Shield, the cold chain could not be maintained for the trip back to our laboratory, which could have resulted in the degradation of the RNA. Secondly, when preparing RNA libraries from low biomass samples it is very easy to lose the genetic material due to small pipetting errors or because library protocols are not optimized for such low inputs of RNA. Finally, we cannot discount the possibility that there is no metabolically active *in situ* microbial community in Johnsons Glacier, although this would be surprising given that the glacier contains an abundant viable subzero microbial community and iRep analysis suggests that MAGs from Johnsons Glacier were performing cell division when the ice was collected.

Table 5.2. Characterization of isolates recovered from White Glacier and Johnsons Glacier englacial ice.

Sample	Isolate ID	Isolation media	16S identity	Sequence similarity	Isolation environment of closest blast hit	Accession number of closest blast hit	Temperature growth range	Salinity growth range	pH growth range
Johnsons Glacier	JNG 1	R2B	NA	NA	NA	NA	-5°C - 25°C	0% - 1%	5 - 9
Johnsons Glacier	JNG 2	R2B	NA	NA	NA	NA	5°C - 25°C	0% - 3%	4 - 10
Johnsons Glacier	JNG 3	R2B	NA	NA	NA	NA	-5°C - 25°C	0% - 1%	4 - 10
Johnsons Glacier	JNG 4	R2B	NA	NA	NA	NA	-5°C - 25°C	0% - 9%	4 - 10
Johnsons Glacier	JNG 5	R2B	NA	NA	NA	NA	-5°C - 15°C	0% - 1%	5 - 10
Johnsons Glacier	JNG 6	R2B	NA	NA	NA	NA	-5°C - 15°C	0% - 3%	5 - 10
Johnsons Glacier	JNG 12	R2B	NA	NA	NA	NA	5°C - 10°C	0% - 3%	6 - 7
Johnsons Glacier	JNG 13	R2B	NA	NA	NA	NA	-5°C - 5°C	0% - 3%	5 - 10
Johnsons Glacier	JNG 14	1/4 R2B	NA	NA	NA	NA	5°C - 25°C	0% - 9%	3 - 7
Johnsons Glacier	JNG 15	1/4 R2B	NA	NA	NA	NA	-5°C - 15°C	0% - 6%	5 - 7
Johnsons Glacier	JNG 16	1/4 R2B	NA	NA	NA	NA	5 - 10	0% - 3%	6 - 7
Johnsons Glacier	JNG 18	1/4 R2B	Glaciihabitans sp.	99%	Antarctica	MT586022.1	-5°C - 15°C	0% - 1%	6 - 7
Johnsons Glacier	JNG 19	1/4 R2B	NA	NA	NA	NA	-5°C - 25°C	0% - 3%	4 - 9
Johnsons Glacier	JNG 20	1/4 R2B	NA	NA	NA	NA	-5°C - 5°C	0%	6 - 7
Johnsons Glacier	JNG 21	1/4 R2B	NA	NA	NA	NA	-2°C - 15°C	0% - 9%	6 - 9
Johnsons Glacier	JNG 22	1/10 R2B	NA	NA	NA	NA	-5°C - 25°C	0% - 6%	4 - 9
Johnsons Glacier	JNG 24	1/10 R2B	Glaciihabitans sp	99.85%	Antarctica	CP043653.1	-5°C - 25°C	0% - 1%	7
Johnsons Glacier	JNG 25	R2A	NA	NA	NA	NA	-2°C - 25°C	0%	5 - 7
Johnsons Glacier	JNG 26	R2A	NA	NA	NA	NA	-5°C - 25°C	0% - 3%	4 - 9
Johnsons Glacier	JNG 27	R2A	NA	NA	NA	NA	5°C - 25°C	0% - 9%	4 - 9
Johnsons Glacier	JNG 28	R2A	NA	NA	NA	NA	5°C - 37°C	0% - 3%	5 - 8
Johnsons Glacier	JNG 30	R2A	NA	NA	NA	NA	-5°C - 25°C	0% - 9%	4 - 9
Johnsons Glacier	JNG 31	1/10 R2A	NA	NA	NA	NA	-2°C - 15°C	0% - 3%	6 - 8
Johnsons Glacier	JNG 32	1/10 R2A	NA	NA	NA	NA	-2°C - 15°C	0% - 1%	6 - 7
Johnsons Glacier	JNG 33	1/10 R2A	NA	NA	NA	NA	-2°C - 10°C	0% - 3%	6 - 9
Johnsons Glacier	JNG 34	1/10 R2A	NA	NA	NA	NA	-5°C - 15°C	0% - 3%	6 - 9
Johnsons Glacier	JNG 35	1/10 R2A	NA	NA	NA	NA	5°C - 15°C	NA	NA
Johnsons Glacier	JNG 37	1/100 R2A	NA	NA	NA	NA	-5°C - 15°C	NA	NA
Johnsons Glacier	JNG 38	1/100 R2A	NA	NA	NA	NA	-5°C - 25°C	0% - 3%	6 - 7
Johnsons Glacier	JNG 39	1/100 R2A	NA	NA	NA	NA	5°C - 15°C	0% - 3%	6 - 7
Johnsons Glacier	JNG 40	1/100 R2A	NA	NA	NA	NA	5°C	0% - 3%	6 - 7
Johnsons Glacier	JNG 41	1/100 R2A	NA	NA	NA	NA	5°C - 25°C	0% - 3%	6 - 7
Johnsons Glacier	JNG 42	TSA	NA	NA	NA	NA	5°C - 15°C	0%	5 - 10
Johnsons Glacier	JNG 43	TSA	NA	NA	NA	NA	-2°C - 15°C	0%	5 - 10
Johnsons Glacier	JNG 44	TSA	NA	NA	NA	NA	-5°C - 25°C	NA	6 - 10
Johnsons Glacier	JNG 45	TSA	NA	NA	NA	NA	-5°C - 25°C	1% - 6%	5 - 10
Johnsons Glacier	JNG 47	TSA	NA	NA	NA	NA	-5°C - 15°C	0% - 1%	5 - 7
Johnsons Glacier	JNG 48	TSA	NA	NA	NA	NA	-5°C - 37°C	0% - 12%	NA
Johnsons Glacier	JNG 50	1/10 TSA	Pedobacter sp.	99.04%	NA	NA	-5°C - 25°C	0% - 6%	5 - 9
Johnsons Glacier	JNG 51	1/10 TSA	NA	NA	NA	NA	-5°C - 5°C	0%	5 - 9
Johnsons Glacier	JNG 52	1/10 TSA	NA	NA	NA	NA	5°C - 25°C	0% - 1%	5 - 9
Johnsons Glacier	JNG 53	1/10 TSA	NA	NA	NA	NA	-5°C - 25°C	0% - 6%	4 - 9
Johnsons Glacier	JNG 54	1/10 TSA	NA	NA	NA	NA	5°C - 25°C	3% - 6%	4 - 9
Johnsons Glacier	JNG 55	1/100 TSA	NA	NA	NA	NA	-2°C - 10°C	0% - 3%	5 - 9
Johnsons Glacier	JNG 56	1/100 TSA	NA	NA	NA	NA	-2°C - 10°C	0% - 3%	6 - 9
Johnsons Glacier	JNG 57	1/100 TSA	NA	NA	NA	NA	-5°C - 25°C	0% - 6%	4 - 9
Johnsons Glacier	JNG 59	1/100 TSA	NA	NA	NA	NA	5°C	0%	6 - 9
Johnsons Glacier	JNG 60	1/100 TSA	NA	NA	NA	NA	-2°C - 15°C	0% - 1%	5 - 9
Johnsons Glacier	JNG 61	R2A	NA	NA	NA	NA	-5°C - 25°C	0% - 3%	4 - 9
Johnsons Glacier	JNG 62	1/100 TSA	NA	NA	NA	NA	-2°C - 25°C	0% - 6%	4 - 9
White Glacier	WG 19	R2B	Salinibacterium sp.	95.78%	Ice Core (34.48 m)	KF295299.1	-5°C - 5°C	0% - 6%	5 - 10
White Glacier	WG 20	R2B	Nocaridoides sp.	90.65%	Bufo americanus skin	KM187148.1	5°C - 25°C	0%	5 - 10
White Glacier	WG 21	1/10 TSA	Salinibacterium sp.	99.84%	Ice Core (34.48 m)	KF295299.1	5°C - 15°C	0%	5 - 8
White Glacier	WG 22	1/4 R2B	NA	NA	NA	NA	5°C - 15°C	0% - 1%	6 - 9
White Glacier	WG 23	1/10 R2A	Cryobacterium sp.	97.21%	Glacier	ON693774.1	5°C - 15°C	0%	6 - 7
White Glacier	WG 24	R2A	NA	NA	NA	NA	-5°C - 25°C	0% - 12%	4 - 9
White Glacier	WG 25	R2B	NA	NA	NA	NA	-5°C - 25°C	0% - 3%	4 - 10
White Glacier	WG 26	R2B	Cryobacterium sp.	98.25%	Yuzhufeng ice core (66.68 m)	KF295548.1	5°C - 25°C	0% - 1%	6 - 10

5.4.2 The microbial communities of White and Johnsons Glaciers differ greatly in taxonomy

Using MetaPhlAn 4, most of the sequences within the metagenomes were unclassified. Specifically, 79% of the White Glacier metagenome and 53% of the Johnsons Glacier metagenome were unclassified. A high proportion of unclassified sequences have been found before in glacial metagenomes from all over the world (Liu et al. 2022a) and is often the case in extreme environments as sequences from these environments are often underrepresented in sequence databases (Chorlton 2024). Nevertheless, this result suggests that both glaciers contain a novel microbial community. Of the sequences that were able to be classified, White Glacier was dominated by Cyanophyceae (36.9%) and Actinomycetia (26.6%), followed by Betaproteobacteria (6.1%), Coriobacteriia (4.0%), Lentisphaeria (3.3%), and Alphaproteobacteria (2.9%). In contrast, the Johnsons Glacier metagenome was dominated by Betaproteobacteria (73.5%), Alphaproteobacteria (21.0%), and Actinomycetia (3.9%) (Figure 5.1).

Cyanobacteria are well-known constituents of cryoenvironments due to their ability to cope with cold temperatures (Quesada and Vincent 2012; Chrismas et al. 2015, 2016) and often act as the main primary producers because of the limited nutrients in polar environments (Chrismas et al. 2018). In glacial environments, Cyanobacteria are often the dominant taxa on glacial surfaces, on snow and ice and in Cryoconite holes (Hodson et al. 2008; Stibal et al. 2012; Hoham and Remias 2020) and have been recovered previously from the surface of White Glacier (Mueller et al. 2001; Mueller and Pollard 2004; Touchette et al. 2023). They have also been found in deep ice in the Greenland ice sheet (Miteva et al. 2015), so we expected to find them in near-surface englacial ice. Actinomycetia is a phylum of gram-positive bacteria known for the

ability of many members to form spores and degrade complex organic compounds (Miao and Davies 2010). Actinomycetia are well-known constituents of cryoenvironments (Edwards et al., 2013; Stibal et al., 2015; Wu et al., 2012) and, in a spore state, can combat starvation and the high UV radiation encountered in the polar regions. Many members also produce mycelia to search for nutrients and water when they are scarce (Dion and Nautiyal 2008; Zhang et al. 2016).

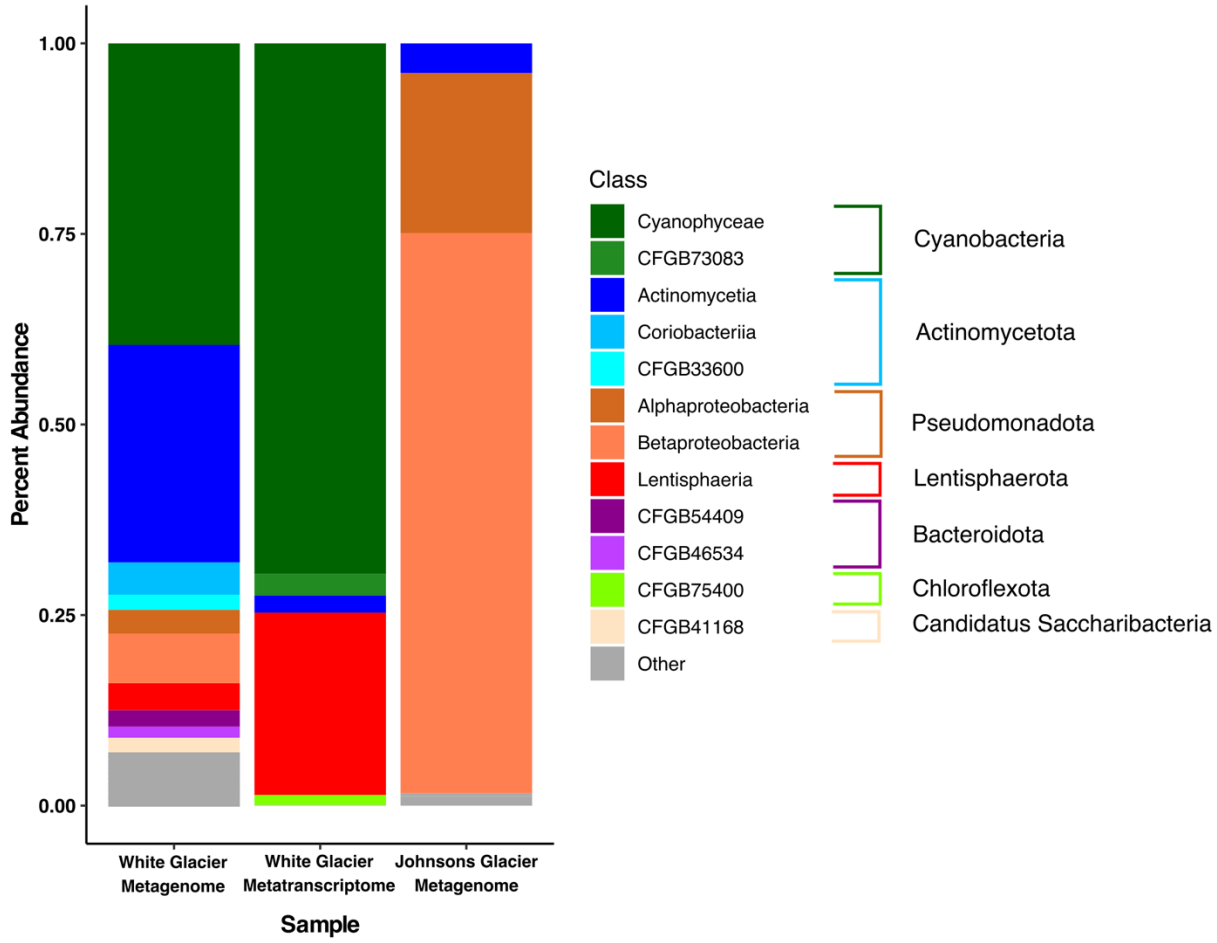

Figure 5.1. Taxonomic composition of the metagenomes and metatranscriptome from White and Johnsons Glacier. Comparison of taxon abundance is based on metabolic marker genes and using MetaPhlAn 4. The estimation was performed on assembled metagenomes and unassembled reads for the metatranscriptome. Phyla with a relative abundance below 1.5% were grouped into "Other".

From the White Glacier metagenome, the predominant taxa at the lowest classifiable rank were *Frigoribacterium* (20.8%), *Phormidesmis* (12.9%), *Microcoleus* (12.1%), and Pseudanabaenaceae (8.0%). From the Johnsons Glacier metagenome, they were Burkholderiaceae (73.0%), *Sphingomonas* (4.9%), and *Frigoribacterium* (3.1%). Sixty-two species were unique to the White Glacier metagenome, 53 species were unique to the Johnsons Glacier metagenome, and 27 taxa were shared between both glaciers (Supplemental Table 5.4). Shared taxa included known cold-adapted microorganisms such as Glaciihabitans_sp_INWT7, Frigoribacterium_sp_CG_9_8, Frigoribacterium_SGB87749, and *Phormidesmis priestleyi*. Despite the species shared between the metagenomes of the two glaciers, statistical analysis demonstrated that the microbial communities remain significantly different. Both randomization tests (diversity permutation test and diversity t-test, see Supplemental Table 5.5) showed significant differences between the diversity of the bipolar samples, with a Shannon index p-value <0.001 in both tests. We further carried out a pairwise CLAM test on both glacier metagenomes to identify polar specialists (Supplemental File). The test indicated that 83.67% of the metagenome sequences assigned to a taxonomy could be considered "Antarctic glacial specialists" and 45.8% as "Arctic glacial specialists". Pseudomonadota, mostly Betaproteobacteria of the genus *Burkholderia* and Alphaproteobacteria of the species *Bradyrhizobium viridifuturi* were considered "Antarctic glacial specialists". In contrast, within Actinomycetota, the genus *Frigoribacterium* was assigned as an "Arctic glacial specialist" along with Cyanobacterota including the genera *Microcoleus* and *Phormidesmis*. Of the White Glacier metagenome sequences assigned to a taxonomy, a total of 54.19% were assigned by the CLAM test as too rare, while for Johnsons Glacier, only 13.01% were assigned as too rare. One explanation for these differences may be the source of microbial biomass which seeds the

glaciers. White Glacier, located in more mountainous terrain than Johnsons Glacier, receives significantly more dust, evidenced by the large number of cryoconite holes on its surface, compared to Johnsons Glacier, which has none. Furthermore, as Johnsons Glacier is an ocean-terminating Glacier, it likely receives inputs of microorganisms from the Antarctic Ocean. The location we sampled was approximately 1.5 km from the ocean, and we found common marine microbial taxa within the Johnsons Glacier metagenome, including *Pseudomonas* sp., *Flavobacterium* sp. And *Salinicoccus*. So, while some common cold-adapted taxa are shared between the two glaciers, they appear largely different, and these differences are likely to be explained by regional geography.

Of the 30 recovered metagenome-assembled genomes, 13 were retrieved from White Glacier, and 17 were retrieved from Johnsons Glacier (Table 5.3). The MAGs from White Glacier were more diverse than those retrieved from Johnsons Glacier (Figure 5.2). Five Cyanobacterial MAGs were retrieved from White Glacier, mirroring their abundance in the metagenome. We also retrieved MAGs belonging to Actinomycetota, Pseudomonadota, Chloroflexota and rare or newly described taxa: Armatimonadota, Eremiobacterota, Patescibacteria, and Gemmatimonadota. No MAGs were shared between White and Johnsons Glacier, with the majority of MAGs recovered from Johnsons Glacier belonging to the Pseudomonadota and Actinomycetota, again mirroring their abundance in the metagenome. Across both glaciers, based on classification using GTDB, the MAGs were novel, with 25 of 30 appearing novel at the species level, 13/30 at the genus level, 4 of 30 at the family level, and one MAG from White Glacier representing a potential novel order (Figure 5.2). Due to the scarcity of genomes from microorganisms recovered from ice, particularly polar ice (Liu et al. 2022a),

these MAGs are a valuable resource for studying survival mechanisms in polar glacial ice. They also offer insights into newly named phyla whose functional potential and environmental niche are not well understood.

Table 5.3. Identities and statistics of medium and high-quality MAGs recovered from White and Johnsons Glaciers.

	Name	Completeness	Contamination	Classification	TPM
White Glacier	MAG 1	92.92	4.11	p__Actinomycetota;c__Actinomycetia;o__Actinomycetales;f__Microbacteriaceae;g__Lacisediminihabitans	7,688
	MAG 2	55.31	1.11	p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Microcoleaceae;g__Microcoleus	142,735
	MAG 3	58.45	6.53	p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Microcoleaceae;g__Microcoleus	63,767
	MAG 4	82.17	8.67	p__Armatimonadota;c__Chthonomonadetes;o__Chthonomonadales;f__Chthonomonadaceae	6,618
	MAG 5	61.6	6.27	p__Eremiobacterota;c__Eremiobacteria;o__Baltobacterales;f__Baltobacteraceae	381
	MAG 6	99.86	0.85	p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Coleofasciculaceae;g__PCC7113	53,488
	MAG 7	93.88	3.41	p__Patescibacteria;c__Microgenomatia;o__Levybacterales;f__UBA12049;g__PPGL01	127
	MAG 8	70	8.33	p__Chloroflexota;c__Anaerolineae;o__SBR1031;f__UBA2796	22,131
	MAG 9	55.82	1.58	p__Gemmatimonadota;c__Gemmatimonadetes;o__Gemmatimonadales;f__Gemmatimonadaceae;g__Gemmatimonas	3,824
	MAG 10	85.51	3.89	p__Chloroflexota;c__Ktedonobacteria;o__Ktedonobacterales;f__Ktedonobacteraceae;g__DTNP01	7,595
	MAG 11	69.52	7.78	p__Pseudomonadota;c__Gammaproteobacteria;o__Steroidobacterales;f__Steroidobacteraceae;g__Bog-1198	2,701
	MAG 12	57.32	9.73	p__Cyanobacteria;c__Cyanobacteriia;o__Cyanobacteriales;f__Nostocaceae	18,681
	MAG 13	75.62	1.01	p__Cyanobacteria;c__Cyanobacteriia;o__Leptolyngbyales;f__Leptolyngbyaceae;g__Phormidesmis_A;s__Phormidesmis_A priestleyi_B	35,304
Johnsons Glacier	MAG 1	98.76	1.2	p__Actinomycetota;c__Actinomycetia;o__Mycobacteriales;f__Mycobacteriaceae;g__X156	N/A
	MAG 2	99.99	1.06	p__Pseudomonadota;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Sphingomonas;s__Sphingomonas aquatilis	N/A
	MAG 3	97.37	1.63	p__Actinomycetota;c__Actinomycetia;o__Mycobacteriales;f__Pseudonocardiaceae;g__Pseudonocardia	N/A
	MAG 4	99.72	5.56	p__Pseudomonadota;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Paraburkholderia;s__Paraburkholderia sp005503145	N/A
	MAG 5	95.09	2.29	p__Pseudomonadota;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas;s__Stenotrophomonas maltophilia_AM	N/A
	MAG 6	77.22	6.22	p__Pseudomonadota;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Rhodoferax	N/A
	MAG 7	78.92	9.39	p__Pseudomonadota;c__Gammaproteobacteria;o__Xanthomonadales;f__Rhodanobacteraceae;g__Rhodanobacter	N/A
	MAG 8	83.89	4.54	p__Pseudomonadota;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas_E	N/A
	MAG 9	89.93	4.47	p__Pseudomonadota;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Polaromonas	N/A
	MAG 10	100	4.81	p__Actinomycetota;c__Actinomycetia;o__Mycobacteriales;f__SCTD01	N/A
	MAG 11	76.91	2.06	p__Actinomycetota;c__Actinomycetia;o__Mycobacteriales;f__SCTD01	N/A
	MAG 12	89.31	4.79	p__Actinomycetota;c__Actinomycetia;o__Actinomycetales;f__Dermatophilaceae;g__UBA4719	N/A
	MAG 13	98.59	0.51	p__Pseudomonadota;c__Alphaproteobacteria;o__Acetobacterales;f__Acetobacteraceae;g__CAITOL01	N/A
	MAG 14	89.71	2.05	p__Bacteroidota;c__Bacteroidia;o__Sphingobacteriales;f__Sphingobacteriaceae;g__Pelobium;s__	N/A
	MAG 15	81.31	1.75	p__Pseudomonadota;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Paraburkholderia;s__Paraburkholderia fungorum	N/A
	MAG 16	100	3.67	p__Pseudomonadota;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae;g__Bradyrhizobium;s__Bradyrhizobium sp003020075	N/A
	MAG 17	90.42	1.84	p__Pseudomonadota;c__Betaproteobacteria;o__Burkholderiales;f__Burkholderiaceae;g__Cupriavidus;s__Cupriavidus pauculus	N/A

Completeness and contamination levels were estimated with CheckM2, and taxonomy was determined using GTDB-tk. Transcripts per million reads (TPM) aligned to each MAG from White Glacier are listed. A heatmap highlights transcript mapping to each MAG. No metatranscriptome was recovered from Johnsons Glacier, and thus, transcripts could not be mapped to the MAGs recovered from this site.

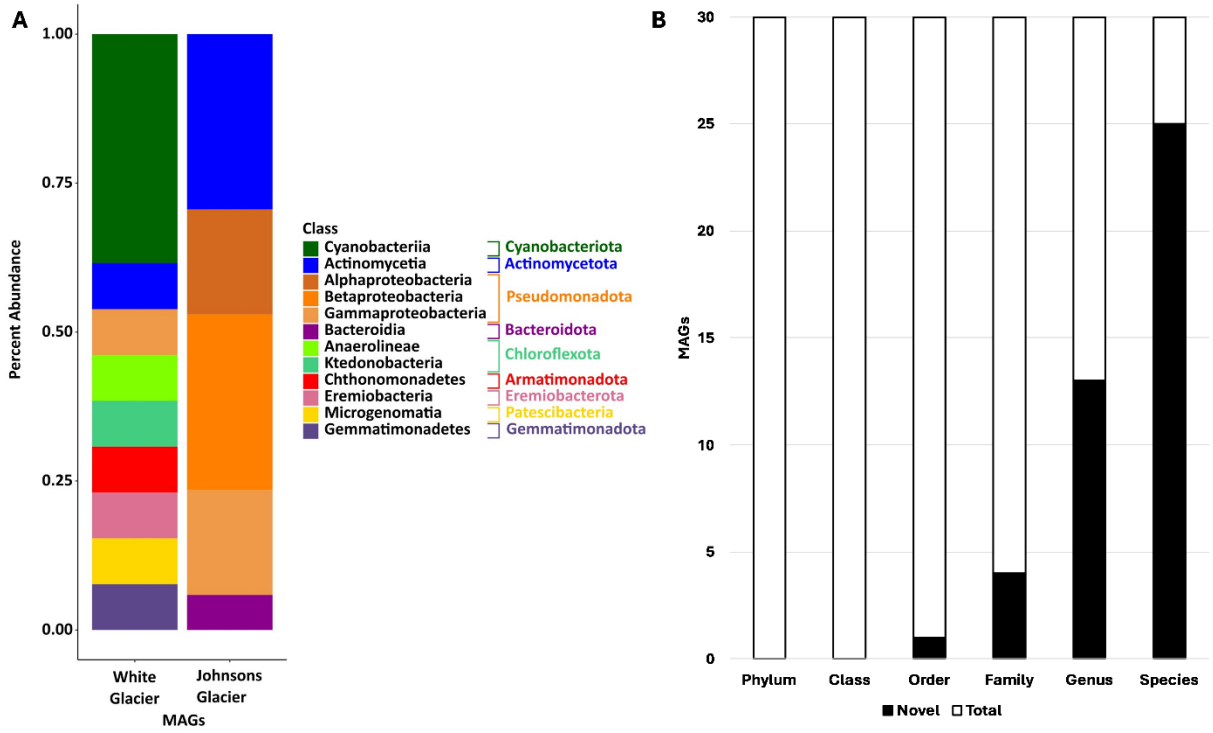

Figure 5.2. Metagenome Assembled Genome Taxonomy and novelty. **A.** Metagenome Assembled Genomes composition from each glacier listed by class and phylum. **B.** Taxonomic novelty of the MAGs recovered in this work. The degree of novelty was determined based on the lowest taxonomic rank with which GTDB-tk could assign a recognized taxon name.

5.4.3 Aerobic and anaerobic metabolisms are present in polar englacial ice

We searched the glacial metagenomes for marker genes representing 38 diverse metabolisms (Figure 5.3). This data revealed the diverse functional potential of these microbial communities. Broadly, metabolic similarities exist between both glaciers despite sharing little taxonomic similarity, suggesting a common survival mode despite their physical differences and geographic isolation.

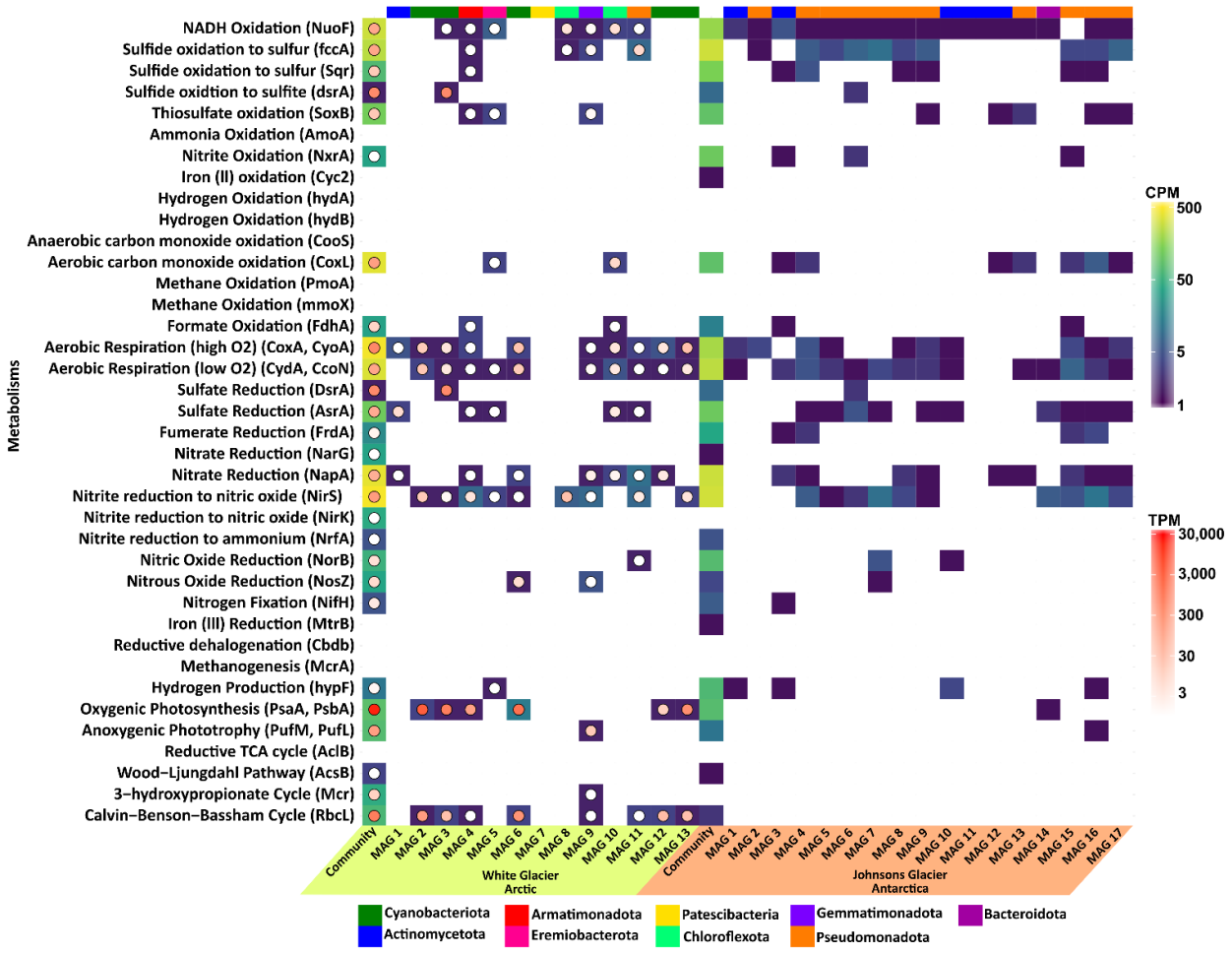

Figure 5.3. Composition of metabolic marker genes from the metagenomes, metatranscriptome and MAGs of White Glacier and Johnsons Glacier**.** Genes in the metagenome and MAGs are represented by the square heatmap and expressed as copies per million genes (CPM). The metatranscriptome aligned to the White Glacier metagenome and MAGs is represented by the circle heatmap overlaying the squares and is expressed as the number of transcripts per million reads (TPM). The colours above each column correspond to the phylum of each MAG.

Aerobic metabolisms were abundant within the metagenomes of both glaciers. The organic carbon concentration measured in the melted ice was 4.05 mg/L and 2.17 mg/L in White Glacier and Johnsons Glacier, respectively (Table 5.1). While these concentrations of organic carbon are considered low, they are similar to other low biomass environments such as open ocean surface waters (0.5 – 3 mg/L) (Mulholland 2003) where microbial respiration is well

known to occur (Robinson and le B. Williams 2007) and thus may still be high enough to support microbial activity. As such, of all metabolic marker genes we searched for, the CoxA/CyoA genes, responsible for aerobic respiration in high oxygen environments, were two of the most abundant (White Glacier: 450.96 cpm; Johnsons Glacier: 210.83 cpm), followed by the CydA/CcoN genes (White Glacier: 346.01 cpm; Johnsons Glacier: 257.41 cpm), responsible for aerobic respiration in low oxygen environments (Figure 5.3). CoxA/CyoA and CydA/CcoN were present in most MAGs recovered from both glaciers. From White Glacier, only MAG 7 (Patescibacteria) and MAG 8 (Anaerolineae) and from Johnsons Glacier only MAG 11 (Mycobacteriales) and MAG 12 (Dermatophilaceae) did not contain any copy of these genes. Such is the case that the near-surface ice of both glaciers are dominated by aerobic metabolisms (Figure 5.3).

CoxL, responsible for the aerobic oxidation of carbon monoxide, provides further evidence that aerobic metabolism dominates in both glaciers. CoxL was the fourth most abundant marker gene we studied from White Glacier (377.17 cpm) and was relatively abundant in Johnsons Glacier (85.80 cpm) (Figure 5.3). In White Glacier, this gene was found in MAG 10 (Ktedonobacteraceae) and MAG 5 (Baltobacteraceae), a member of the under-represented and newly recognized Ermiobacterota phylum, making this the first evidence for aerobic carbon monoxide oxidation in this phylum. From Johnsons Glacier, CoxL was present in seven MAGs (MAG 3, *Pseudonocardia*; MAG 4, *Paraburkolderia*; MAG 12, Dermatophilaceae; MAG 13, Acetobacteraceae; MAG 15, *Paraburkolderia*; MAG 16, *Bradyrhizobium*; and MAG 17, *Cupriavidis*) (Figure 5.3). Autotrophic aerobic carbon monoxide oxidation has been previously reported in members of the *Pseudonocardia* genus (Grostern and Alvarez-Cohen 2013), and

therefore, MAG 3 may be capable of this, making carbon monoxide a source of both energy and carbon in Johnsons Glacier.

Despite the abundance of aerobic metabolisms within both glacier metagenomes, fermentative metabolisms were also present. Fumerate reduction is an important form of anaerobic fermentative metabolism. The FrdA marker gene was present in both glaciers (White Glacier: 19.68 cpm; Johnsons Glacier: 40.45 cpm) but was absent from any MAG from White Glacier and present in four MAGs from Johnsons Glacier (MAG 3, *Pseudonocardia*; MAG 4, *Paraburkolderia*; MAG 15, *Paraburkolderia*; MAG 16, *Bradyrhizobium*) (Figure 5.3). Formate oxidation is another form of anaerobic metabolism which was present in both glaciers (White Glacier: 34.44 cpm; Johnsons Glacier: 14.71 cpm), including MAG 4 (Armatimonadota) and MAG 10 (Ktedonobacteraceae) from White Glacier and MAG 3 (Pseudonocardia) and MAG 15 (*Paraburkolderia fungorum*) from Johnsons Glacier (Figure 5.3).

Overall, the abundance of aerobic metabolisms suggests that the ice of both glaciers is a predominantly aerobic environment and may contain microaerophilic or anoxic micro-environments where anaerobic metabolism can occur.

5.4.4 Photoautotrophic and lithoautotrophic metabolisms are present in polar englacial ice

Both oxygenic photosynthesis and anoxygenic phototrophy marker genes were present in each glacier. The PsaA and PsbA genes, encoding the primary electron-donating protein in photosystem l and the D1 protein in photosystem ll, were moderately abundant in both glaciers (White Glacier: 82 cpm; Johnsons Glacier: 74.77 cpm) and in 6 of 13 MAGs from White Glacier

but only 1 of 17 MAGs in Johnsons Glacier (Figure 5.3). Using the KEGG Mapper Reconstruct tool, the completeness of the oxygenic photosynthesis pathway in both the metagenome and MAGs was determined. The metagenome of White Glacier contained 56 of 66 proteins in the KEGG database associated with oxygenic photosynthesis likely indicating a complete pathway was present while White Glacier MAGs contained 9 to 49 of these proteins. MAG 6 (Coleofasciculaceae) and MAG 13 (Phormidesmis) contained the most proteins of the pathway with 49 and 45, respectively, likely indicating they each contained complete pathways as well. PufM and PufL, which encode anoxygenic photosensitive reaction centres, were almost equally abundant as PsaA/PsbA in White Glacier (72.16 cpm) but less abundant than their oxygenic counterparts in Johnsons Glacier (11.03 cpm). Within White Glacier, only MAG 9 (Gemmatimonadota) contained the PufM/PufL genes while MAG 16 (*Bradyrhizobium*) was the only MAG to contain them from Johnsons Glacier (Figure 5.3). Overall, these results indicate that White Glacier has a larger presence of oxygenic and anoxygenic photosynthesis genes than Johnsons Glacier, with some MAGs in White Glacier potentially capable of complete oxygenic photosynthesis. This suggests a greater potential for photosynthetic activity and diversity in White Glacier's microbial community and the potential for oxygenic photosynthesis to be the major primary production pathway within White Glacier's englacial near-surface ice.

Carbon fixation pathways were mainly found in White Glacier. RbcL encoding the Rubisco enzyme, part of the Calvin-Benson cycle, was present in both glaciers (White Glacier: 62.32 cpm; Johnsons Glacier: 2.45 cpm). RbcL was present in eight MAGs from White Glacier (5 Cyanobacterial MAGs, one Armatimonadota MAG, one Gemmatimonadota MAG, and one Gammaproteobacteria MAG) but was not present in any of the MAGs recovered from Johnsons

Glacier (Figure 5.3). The Wood-Ljungdahl pathway (AdB), performed by diverse bacteria and archaea was also present in both glaciers but in lower abundance than the Calvin-Benson cycle (3.28 cpm for White Glacier & 1.23 cpm for Johnsons Glacier). The 3-hydroxypropionate cycle (Mcr), used by some photosynthetic Chloroflexota and some Gammaproteobacteria (Garritano et al. 2022), was only found in the White Glacier metagenome (45.92 cpm) and MAG 9 (Gemmatimonadota) from White Glacier (Figure 5.3). Overall, the findings highlight a greater diversity and abundance of carbon fixation pathways in White Glacier compared to Johnsons Glacier. This suggests that the microbial communities in White Glacier are better equipped to perform carbon fixation and enhance their ability to gain carbon and energy in such a nutrient-poor environment.

Sulfate was below the limit of quantification, but total sulfur was 0.245 mg/L in White Glacier and 0.059 mg/L in Johnsons Glacier (Table 5.1). The oxidation of diverse sulfur species was found in both glacier metagenomes, including the oxidation of sulfide to sulfur, fccA (White Glacier: 249.26 cpm; Johnsons Glacier: 306.43 cpm), Sqr (White Glacier: 68.87 cpm; Johnsons Glacier 139.73 cpm) and thiosulfate oxidation, SoxB (White Glacier: 122.99 cpm; Johnsons Glacier: 89.48 cpm). The dsrA gene, which oxidizes sulfide to sulfite, was also present in both glaciers but in lower abundance than other sulfur oxidation marker genes (White Glacier: 1.64 cpm; Johnsons Glacier: 8.58 cpm) (Figure 5.3). Sulfur oxidation genes were also present in the MAGs we recovered from both glaciers. Marker genes for the oxidation of sulfur compounds were found in five MAGs recovered from White Glacier and thirteen MAGs from Johnsons Glacier. MAG 4 (Armatimonadota) from White Glacier contained three sulfur oxidation marker genes (fccA, Sqr, and SoxB) as did two Johnsons Glacier MAGs (MAG 9,

Gammaproteobacteria; MAG 16, Alphaproteobacteria) (Figure 5.3) suggesting they possess the ability to utilize diverse sulfur species for energy.

MAG 4 (Steroidobacteraceae), MAG 9 (Gemmatimonas), and MAG 11 (Steroidobacteraceae) from White Glacier not only have the ability to oxidize sulfide to sulfur, but they also contain carbon fixation genes and genes for aerobic respiration. This suggests that all three are facultative lithoautotrophs, contributing to a functionally diverse ecosystem of heterotrophic and lithoautotrophic microbes within White Glacier's englacial ice. This finding marks the second report of sulfur oxidation pathways in glacial ice, with the first observed at Borup Fiord Pass, a unique sulfur spring-dominated glacier in the Canadian High Arctic, where sulfur-oxidizing MAGs and taxa are abundant in surface ice (Trivedi et al. 2020). These results suggest that diverse sulfur species could be a crucial energy source in glacial ice ecosystems, particularly where other energy sources are scarce.

The concentration of nitrogen species (NH_4^+, NO_3^-, NO_2^-) in both glaciers was extremely low, measured in ug/L or below detection limits (Table 5.1). However, marker genes for nitrate/nitrite reduction were abundant. The most prevalent were NirS (White Glacier: 464.08 cpm; Johnsons Glacier: 316.24 cpm), part of the denitrification pathway reducing nitrite to nitric oxide, and NapA (White Glacier: 393.57 cpm; Johnsons Glacier: 297.89 cpm), reducing nitrate to nitrite. NirS was found in 19 of 30 MAGs, and NapA in 16. NarG, another nitrate reducer, was mostly present in White Glacier (36.08 cpm) and barely in Johnsons Glacier (1.22 cpm). NirK was another nitrite reduction gene only in White Glacier (44.28 cpm). Both glaciers had NorB (White Glacier: 52.48 cpm; Johnsons Glacier: 73.54 cpm) and NosZ (White Glacier: 37.72

cpm; Johnsons Glacier: 3.68 cpm), responsible for reducing nitric oxide and nitrous oxide to nitrogen gas. NorB was found in MAG 11 (White Glacier: Steroidobacteraceae) and MAGs 7 and 10 (Johnsons Glacier: Rhodanobacter, Mycobacteriales). NosZ was in MAGs 6 and 9 (White Glacier: Coleofasciculaceae, Gemmatimonas) and MAG 7 (Johnsons Glacier: Rhodanobacter). NifH was barely detected (White Glacier: 4.92 cpm; Johnsons Glacier: 6.13 cpm) and only in MAG 3 (Johnsons Glacier: Pseudonocardia) (Figure 5.3). Many of the MAGs containing nitrogen reduction genes, such as MAG 4 (Chthonomonadaceae) from White Glacier, also contain sulfide oxidation genes, implying these MAGs may be capable of a fully chemotrophic lifestyle and may function under aerobic or anaerobic conditions. Overall, the results suggest the potential for the englacial ice microbial communities to perform complete denitrification, an alternative to oxygen as an electron acceptor in anoxic ice microzones. The low nitrate and nitrite levels in meltwater may indicate nutrient depletion by anaerobic nitrogen reduction.

Many forms of metabolism were absent from the glacial ice environment. While methanogens have been discovered in Greenland glacial ice (Tung et al. 2005), genes encoding methanogenesis were absent from either glacier studied here. Similarly, iron-reducing microorganisms have also been observed in deep Greenland glacial ice (Tung et al., 2006); however, the presence of the MtrB gene encoding iron reduction was absent from White Glacier and only present in Johnsons Glacier in very low abundance (1.23 cpm) (Figure 5.3). The general absence of carbon fixation metabolisms from Johnsons Glacier, including a complete absence from any MAGs we recovered, suggests that without a method of fixing carbon, the

scarce nutrients in Johnsons Glacier may make long-term survival challenging, especially in comparison to White Glacier, where carbon fixation metabolisms are abundant.

While we did not measure the concentration of gases trapped within the ice from either glacier, we hypothesize that sufficient atmospheric gases are present to sustain the observed aerobic respiration, aerobic carbon monoxide oxidation, and carbon fixation pathways. Near-surface glacial ice is known to contain high concentrations of gas bubbles, with porosity typically ranging from 62% to 67% (Bender et al. 1997). As snow compacts into ice within the glacier's accumulation zone, atmospheric gases become trapped in air pockets, which were visibly present in the ice cores we collected. Furthermore, glacial ice retains a degree of porosity to depths of up to 40 meters (Bender et al. 1997), suggesting that atmospheric exchange is likely occurring between the glacial surface and the 0.7 to 1.2-meter depths sampled in this study. This atmospheric exchange, coupled with the presence of air bubbles, could provide a continuous supply of oxygen, carbon monoxide, and carbon dioxide, thereby supporting the aerobic and autotrophic metabolic pathways observed in these otherwise nutrient-limited environments.

5.4.5 White Glacier contains an active microbial community dominated by Cyanobacteria performing carbon fixation and photosynthesis

Metatranscriptome sequencing of near-surface englacial ice from White Glacier indicated an active microbial community transcribing most marker genes in the metagenome and MAGs. The metatranscriptome from White Glacier produced 52 million reads, and after quality filtering, decontamination, and post-processing, it was left with 14,558,236 reads. Of these reads, 7,674,390 aligned to the metagenome. To our knowledge, this is the first metatranscriptome

analysis ever performed from near-surface or englacial ice and some of the most direct evidence that glacial ice can support an active microbial community. *In situ* microbial metabolism within ice has been hypothesized and observed before (Bakermans & Skidmore, 2011a, 2011b; Martin et al., 2008; Rohde et al., 2008; Rohde & Price, 2007; Tung et al., 2005, 2006) but only for sea ice and the Greenland and Antarctic ice sheets. Given the very low nutrient availability in ice, the taxa driving the activity in the above studies are chemolithoautotrophs, namely, methanogens, nitrifying and denitrifying bacteria, and Fe-reducing bacteria. Nevertheless, work has experimentally demonstrated that heterotrophs and phototrophs can also be metabolically active in sea ice (Bakermans and Skidmore 2011b, 2011a; Underwood et al. 2013).

Within the White Glacier metatranscriptome 82.8% of the reads were unclassified but Cyanobacteria was by far the most abundant among the classified taxa. Cyanobacteria compose 70.9% of the classified metatranscriptome, indicating that this taxon is the dominant phyla within the ice core. The second and third most abundant phyla were Lentisphaerae (23.4%) and Actinomycetota (2.2%) (Figure 5.1). At the lowest classifiable rank, the dominant taxa were *Microcoleus* (29.9%), Victivallaceae (23.4%), *Phormidesmis* (21.9%), and Pseudanabaenaceae (15.7%). The dominant Cyanobacterial taxa in the metatranscriptome were *Microcoleus* (12.1%), *Phormidesmis* (12.9%), and Pseudanabaenaceae (8.0%). Cyanobacteria are common in glacial environments owing to their ability to produce exopolysaccharides which prevent water loss and ice crystal formation (Vincent 2000, 2007) and their ability to produce antifreeze compounds, thought to act as cryoprotectants (Raymond and Fritsen 2000). Lentisphaerota contains only six cultured representatives. As such, very little is known about the phylum except that members are ubiquitous in the environment (Zhang et al. 2022b). However, other than a

mesophile recovered from a cold seep (Zhang et al., 2022b), no member of this phyla has been recovered from cold environments or identified as a cryophile. The discovery of the Lentisphaerota in White Glacier ice, especially dominant in the metatranscriptome, may be the first evidence that members of this phyla are cold-adapted.

The activity of Cyanobacteria in White Glacier was also seen in the MAGs we recovered. Of the 13 medium and high-quality MAGs we recovered, 5 were classified as Cyanobacteria. These included two MAG 2 and MAG 3 (*Microcoleus*, 55-58% completeness), MAG 6 (Coleofasiculaceae, 99.86% completeness), MAG 12 (Nostocaceae, 57% completeness), and MAG 13 (*Phormidesmis*,75% completeness). These Cyanobacterial MAGs contained the largest number of aligned transcripts (Table 5.3), further suggesting Cyanobacteria are the predominant active taxa within the ice. These taxa are common in cold environments including Arctic sea ice, Arctic glacial cryoconites, Arctic soils, Antarctic soils and Himalayan alpine soils (Bowman et al. 2011; Strunecky et al. 2012; Strunecký et al. 2013; Čapková et al. 2016; Velichko et al. 2023; Gokul et al. 2023; Hay et al. 2023) but to our knowledge, have not been recovered from within glacial ice.

Genes associated with photosynthesis were highly transcribed within the metatranscriptome. The photosynthetic PsaA and PsbA marker genes were the most transcribed genes in the entire White Glacier metatranscriptome (20,789.46109 tpm) (Figure 5.3), accounting for 3.53% of all aligned transcripts and attributed almost exclusively to Cyanobacteria (Figure 5.4). Of the five Cyanobacterial MAGs we recovered, MAG 2 (*Microcoleus* sp.) showed the highest expression of the PsaA and PsbA genes (3,242.606 tpm),

and MAG 6 (Coleofasciculaceae) showed the second highest expression (1,449.439 tpm). To verify sufficient light penetration in the ice to support photosynthesis in 2023, upon returning to White Glacier on a clear day, we measured light penetration at the depths we sampled in 2022 and measured it to be 3.24% of that at the surface. This suggests a significant fraction of light can penetrate our sampling depth of 0.7 – 0.9 meters to support photosynthesis.

Rubisco encodes an ancient carboxylase enzyme and is used by all Cyanobacteria to fix carbon dioxide into organic carbon (Fischer et al. 2016). It is a component of the Calvin Benson carbon fixation pathway and was highly expressed within White Glacier (824.192 tpm) (Figure 5.3), with almost 70% of these sequences sharing the closest sequence similarity to Cyanobacteria (Figure 5.4). Both the metagenome and metatranscriptome contained almost complete Calvin Benson pathways, and rubisco was expressed in all Cyanobacterial MAGs. The highest expression was found in MAG 6 (Coleofasciculaceae, 275.69 tpm), suggesting that carbon fixation via the Calvin Benson cycle may occur *in situ*.

Given light penetration at the sampled depths appears to be sufficient to support photosynthesis and there is likely continuous replenishment of CO_2 through porous surface ice, primary production by Cyanobacteria is occurring *in situ* and is supporting the wider active microbial community within the ice as has been previously demonstrated in sea ice, where phototrophic microorganisms feed the rest of the community in the ice by releasing dissolved and colloidal organic matter (Underwood et al. 2013).

5.4.6 Metatranscriptomics reveals englacial ice contains other active metabolisms related to carbon, sulfur, and nitrogen cycling

There is the possibility that the metatranscriptome analyses presented here are not representative of *in situ* metabolism but rather *ex-situ* metabolism, either after the ice was collected or during ice melt. The ice cores were kept in the dark after collection, during transport, and while being thawed into DNA/RNA Shield solution, supporting the notion *in situ* metabolism was present prior to sampling; for example, the relatively large number of transcripts related to photosynthesis rules out the possibility that the transcripts within our dataset resulted from ex-situ metabolism. Furthermore, melting of the ice directly into DNA/RNA Shield, the active microbial community would cease all metabolism upon release from the ice and contact with DNA/RNA Shield and, if not, then the low temperature the ice was melted at (4°C) would keep levels of ex-situ metabolism negligible.

In addition to active photosynthesis and rubisco-driven carbon fixation performed by Cyanobacteria, transcripts related to other metabolisms were also abundant in White Glacier. Aerobic respiration under high (CoxA/CyoA) and low oxygen (CydA/CcoN) concentrations were highly expressed (526.59 tpm and 113.89 tpm, respectively) (Figure 5.3). This expression was attributed mainly to Pseudomonadota, Cyanobacteria, and Actinomycetota but also nine other phyla (Planctomycetota, Bacteroidota, Gemmatomonadota, Bacillota, Armatimonadota, Verrucomicrobia, Chloroflexota, Ascomycota, and Nitrospirota) indicating a diverse microbial community is metabolically active within the 1 meter englacial ice environment (Figure 5.4).

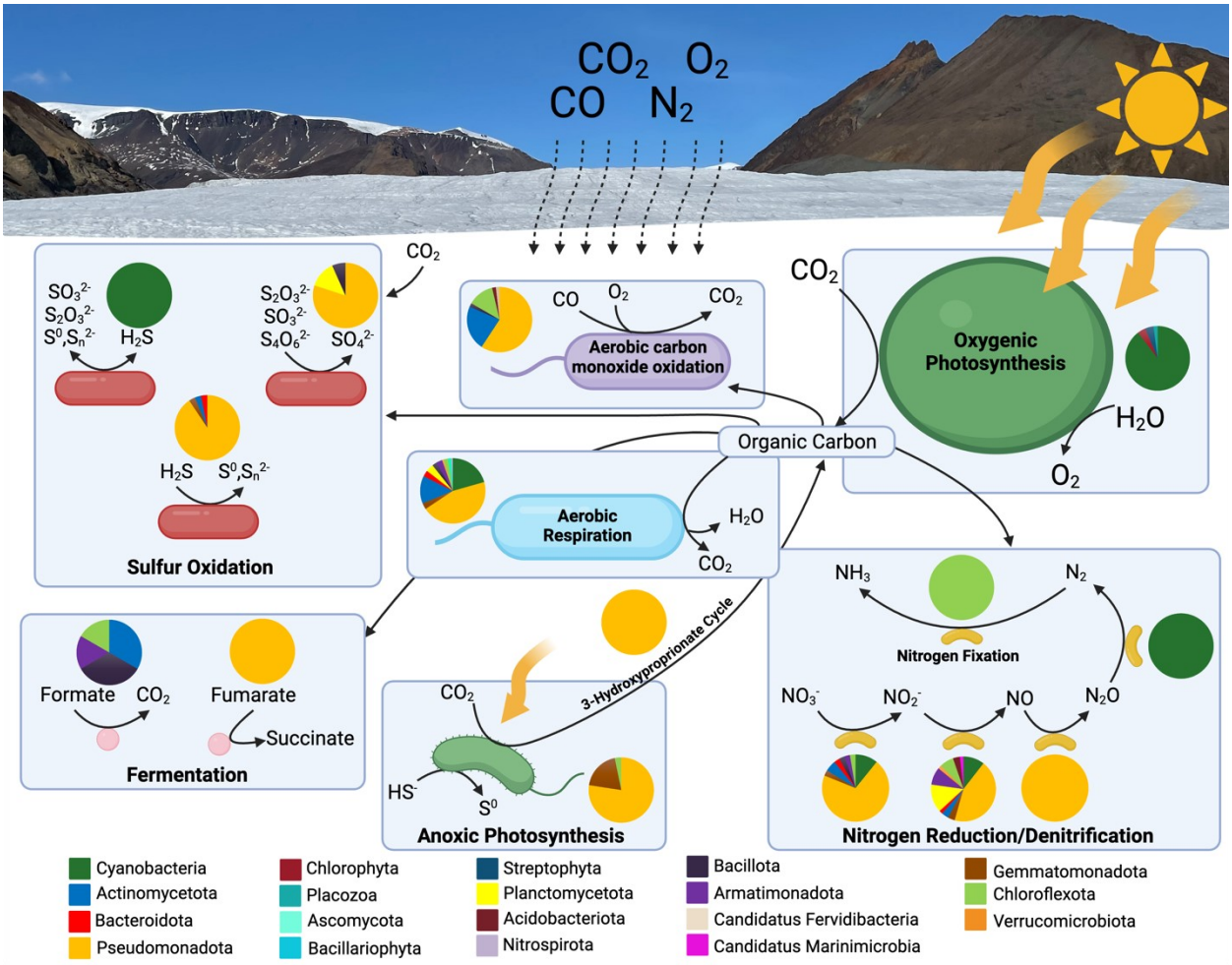

Figure 5.4. Summary of active metabolisms based on metabolic marker gene transcripts found in White Glacier. Phyla performing each metabolism are marked by the pie charts. The size of each of the microorganisms roughly matches the relative level of transcription of the marker gene representing each metabolism. The figure was created using Biorender.com.

Aerobic carbon monoxide oxidation is recognized as a survival mechanism in nutrient-limited environments and is observed in heterotrophic microorganisms during periods of starvation, particularly when organic carbon is scarce (Berney and Cook 2010; Christie-Oleza et al. 2012; Patrauchan et al. 2012; Muthusamy et al. 2017; Islam et al. 2019; Cordero et al. 2019). Consistent with this, the CoxL gene, which encodes the enzyme for aerobic carbon monoxide oxidation, was expressed in White Glacier (160.19 tpm) (Figure 5.3), predominantly by

Pseudomonadota, followed by Actinomycetota and Chloroflexota (Figure 5.4). Carbon monoxide can be oxidized lithoautotrophically, serving as both a carbon and energy source, or heterotrophically as an energy source. In White Glacier, it appears that carbon monoxide is being oxidized heterotrophically.

Chemolithoautotrophic carbon monoxide oxidizers generally require elevated carbon monoxide levels and channel electrons from its oxidation into the Calvin-Benson cycle for carbon dioxide fixation (Cordero et al. 2019). However, the only two MAGs recovered from White Glacier that contained the CoxL gene, MAG 5 (Baltobacteraceae, phyl. Eremiobacterota) and MAG 10 (Ktedonobacteraceae), lacked the Calvin-Benson cycle or any other carbon fixation pathway, indicating they are not lithoautotrophs (Figure 5.3). Additionally, none of the CoxL genes and transcripts from White Glacier matched known cultured chemolithoautotrophic carbon monoxide oxidizers. These findings suggest that heterotrophic carbon monoxide oxidation plays a critical role in microbial survival in glacial ice. Notably, this is the first report indicating that members of the phylum Eremiobacterota may perform aerobic carbon monoxide oxidation. Previously, the only cultured representative of this phylum was known for aerobic anoxygenic photosynthesis and carbon fixation, but not aerobic carbon monoxide fixation, expanding the metabolic versatility of this recently recognized phylum.

In addition to the Calvin-Benson Cycle, transcripts mapped to the Mcr gene for the 3-hydroxypropionate cycle (10.68 tpm; Figure 5.3) were mainly linked to Pseudomonadota (Figure 5.4). MAG 9 from White Glacier, a Gemmatimonas within the Gemmatimonadota phylum, was the only MAG containing and transcribing the Mcr gene (0.33 tpm; Figure 5.3). Although the

MAG was only 55% complete, it contained most of the necessary genes for carbon fixation via the 3-hydroxypropionate cycle and Calvin-Benson Cycle.

Gemmatimonadota is a recently identified phylum with six cultured representatives (Mujakić, Piwosz and Koblížek 2022). All are heterotrophs or photoheterootrophs (Zeng et al. 2015, 2021) and none are known to fix inorganic carbon, except for one MAG from a Siberian soda lake containing the Rubisco enzyme (Vavourakis et al. 2019). This is the first report of the 3-hydroxypropionate cycle in this phylum and due to the low affinity of the Rubisco enzyme for carbon dioxide, the presence of genes for the 3-hydroxyproprionate cycle may indicate the ability of this microorganism to fix carbon dioxide in both low and high- carbon dioxide environments. The presence of genes and transcripts for anoxygenic phototrophy and multiple carbon fixation pathways may indicate a novel survival strategy for this phylum in nutrient-poor environments like White Glacier.

Like the metagenome, sulfur oxidation transcripts were abundant in the metatranscriptome of White Glacier. The most transcribed of these genes was the dsrA gene, responsible for the oxidation of sulfide to sulfite (262.29 tpm; Figure 5.3). Interestingly, this expression was attributed to a single Cyanobacterial MAG (MAG 3, *Microcoleus*) but did not contain marker genes to oxidize any other sulfur compounds. In *Microcoleus*, oxidation of sulfide to sulfite has been observed in hypersaline mats by *Microcoleus chthonoplastes* (Jorgensen et al. 1979; Jorgensen 1994), but this genus is not known to perform oxidation of sulfur species in cold environments. Other forms of sulfur oxidation were also expressed and almost completely attributed to Pseudomonadota (Figure 5.4). These included the fccA gene, responsible for the

oxidation of sulfide to sulfur (117.06 tpm), the soxB gene, responsible for the oxidation of thiosulfate (18.79 tpm), and the sqr gene, also responsible for the oxidation of sulfide to sulfur (16.52 tpm; Figure 5.3). Together, it appears that there is utilization of diverse sulfur species as electron donors to support metabolism within White Glacier.

Similar to the White Glacier metagenome, transcripts mapped to various forms of nitrogen reduction in the metatranscriptome. napA (71.19 tpm), encoding the reduction of nitrate to nitrite, was the relatively most expressed of all the nitrogen reduction marker genes detected (71.19 tpm; Figure 5.3) and attributed primarily to Pseudomonadota (Figure 5.4). napA expression was also observed in six diverse MAGs: MAG 1, *Lacisediminihabitans* (Actinobacteriota); MAG 6, Coleofasciculaceae (Cyanobacteria); MAG 9, *Gemmatimonas* (Gemmatimonadota); MAG 10, Ktedonobacteraceae (Chloroflexota); MAG 11, Steroidobacteraceae (Gammaproteobacteria); and MAG 12, Nostocaceae (Cyanobacteria). The next most expressed nitrogen reduction marker gene was NirS, encoding the reduction of nitrite to nitric oxide (191.61 tpm) attributed to a large number of diverse phyla, including Pseudomonadota, Planctomycetota, Cyanobacteria, Armatimonadota and seven others (Figure 5.4). Six MAGs expressed this gene as well, including MAG 2 and MAG 3, Microcoleus (Cyanobacteria); MAG 5, Chthonomonadaceae (Armatimonadota); MAG 8, Anaerolineae (Chloroflexota); MAG 11, Steroidobacteraceae (Gammaproteobacteria); and MAG 13, *Phormidesmis priestley* (Figure 5.3). In the metatranscriptome steps to complete the rest of the denitrification pathway were also expressed (NorB, NosZ) (11.48 tpm; Figure 5.3)) and attributed to Cyanobacteria and Pseudomonadota (Figure 5.4). The Coleofasciculaceae MAG (MAG 6) was the sole MAG recovered to express NosZ, while none expressed NorB. Globally,

these results indicate that complete denitrification of nitrogen species occurs in White Glacier englacial ice, potentially in anaerobic pockets within the ice, and may be coupled to the oxidation of organic molecules or possibly reduced sulfur species as has been previously reported from sea ice and glacial surface ice (Rysgaard et al. 2008; Wright et al. 2013).

5.4.7 Genes involved in cold adaptation are abundant and expressed in near-surface englacial ice

We also screened the englacial ice metagenomes and metatranscriptomes for a set of 61 genes previously determined to have a higher abundance in the genomes of cold-adapted microorganisms compared to their mesophilic counterparts (Raymond-Bouchard et al. 2018) and known to be involved in adaptation to life at cold temperatures (Figure 5.5). These genes fall into the categories of cold shock, DNA replication and repair, membrane and peptidoglycan alteration, carotenoid biosynthesis, polysaccharide capsule biosynthesis, osmotic stress, oxidative stress, toxin/antitoxin modules, and transcription and translation factors. Genes from each category were present and expressed, suggesting adaptation to cold temperatures.

Cold adaptation genes were abundant in both White Glacier (19,571.90 cpm) and Johnsons Glacier (16,841.60 cpm; Figure 5.5). Like the metabolic marker genes discussed earlier, there was significant overlap in the presence or absence of specific groups of cold adaptation genes (Figure 5.5) suggesting again that despite large taxonomic differences between the two englacial ice microbial communities, they share a similar capacity to adapt to life in the subzero englacial ice environment. Among both glaciers, genes within the categories “membrane and peptidoglycan alteration” (White Glacier: avg. 850.16 cpm; Johnsons Glacier: avg. 678.88 cpm)

and “oxidative stress” (White Glacier: avg. 414.62 cpm; Johnsons Glacier: avg. 377.12 cpm) were the most abundant, while categories “polysaccharide capsule biosynthesis” (White Glacier: avg. 77.90 cpm; Johnsons Glacier: avg. 84.58 cpm) and toxin/antitoxin modules” (White Glacier: avg. 141.36 cpm; Johnsons Glacier: avg. 45.84 cpm; Figure 5.5) were the least abundant. However, when looking solely at transcribed genes from White Glacier, the most active form of cold adaptation was “cold shock and stress-related proteins” (avg. 1,135.23 tpm), followed by membrane and peptidoglycan alteration (avg. 488.97 tpm) which was mostly due to the expression of glycosyltransferases involved in cell wall biosynthesis (Figure 5.5). Differences observed between the two glaciers was in the presence of cold adaptation genes within the MAGs. Johnsons Glacier MAGs contained ~1.5X more genes than White Glacier MAGs related to osmotic and oxidative stress response, which we hypothesize may be due to the proximity of Johnsons Glacier to the Southern Ocean, and potential transport of microorganisms from the adjacent ocean creating a community more adapted to osmotic stress.

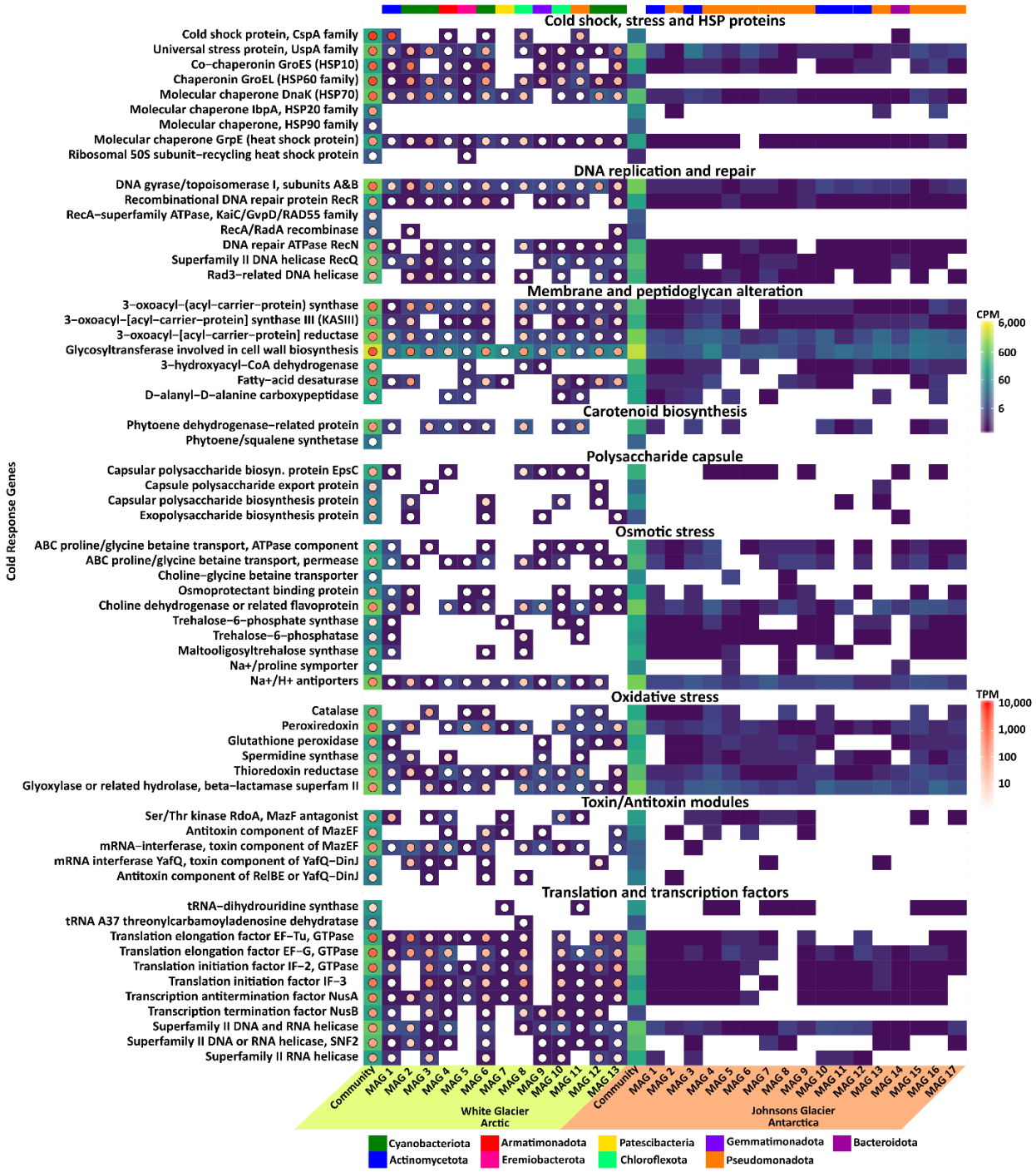

Figure 5.5. Comparison of genes implicated in adaptation to cold temperatures from the metagenomes, metatranscriptome and MAGs of White Glacier and Johnsons Glacier. Genes in the metagenome and MAGs are represented by the square heatmap and expressed as copies per million genes (CPM). The metatranscriptome aligned to the White Glacier metagenome and MAGs is represented by the circle heatmap overlaying the squares and is expressed as the number of transcripts per million reads (TPM). The colours above each column correspond to the phylum of each MAG.

The lack of genes related to carotenoid biosynthesis in either englacial metagenome or derived MAGs was unexpected. Carotenoids are a diverse group of lipid-soluble pigments ranging from yellow to red and distributed widely among bacteria, fungi and plants and commonly found in polar microorganisms (Vincent et al. 2004; Dieser et al. 2010; Kirti et al. 2014; Singh et al. 2017; Vila et al. 2019). While their ability to shield microbial cells from UV damage (Dieser et al. 2010; Guo et al. 2022) may not be required a meter beneath the ice, carotenoids also protect against reactive oxygen species (Guo et al. 2022), and enhance membrane flexibility (Seel et al. 2020), all of which are crucial for survival in cold polar environments. Despite carotenoid synthesis genes not being found in high abundance in either glacier, many of the strains we isolated were brightly pigmented, suggesting that pigments other than carotenoids may be more abundant and potentially beneficial to viable members of the glacial ice community.

Toxin/Antitoxin modules are commonly observed in cold-adapted bacteria (Romaniuk et al. 2017; Jeon et al. 2021; Choi et al. 2022) yet were not found to be abundant or expressed in either englacial ice sample metagenomes/derived MAGs or their parallel metatranscriptomes. This is difficult to explain, given the extreme stresses of subzero temperatures, low water activity and starvation encountered in englacial ice cryoenvironments. Toxin/antitoxin modules are linked proteins/regulatory RNA modules containing a toxin and an antitoxin (Jeon et al. 2021). When combined, the antitoxin protects the cell, but under specific physiological states, the antitoxin can be degraded, leaving the toxin to damage the cell (Brantl and Jahn 2015; Müller et al. 2016; Jeon et al. 2021). One reason for the absence of TA modules from White and Johnsons

Glacier may be that glacial ice provides an extreme but very stable environment which reduces the need for the adaptive and regulatory roles of TA modules. Another possibility is that the energetic cost of maintaining TA modules is too high in a system as energy limited as englacial ice.

5.5 Conclusion

Polar englacial ice supports low-biomass yet functionally diverse and active microbial ecosystems. Despite being taxonomically distinct, these results show that Arctic and Antarctic glaciers exhibit considerable overlap in functional potential. Both environments harbour abundant aerobic and anaerobic metabolisms, including aerobic respiration, aerobic carbon monoxide oxidation, sulfide and thiosulfate oxidation, and nitrate and nitrite reduction. In White Glacier, an active community of heterotrophic microbes engage in aerobic respiration, aerobic carbon monoxide oxidation, and fermentation, supported by active Cyanobacteria performing oxygenic photosynthesis and carbon fixation via the Calvin-Benson cycle. Active lithoautotrophic metabolisms, such as 3-hydroxypropionate carbon fixation, anoxygenic photosynthesis, sulfide and thiosulfate oxidation, and nitrogen reduction/denitrification, contribute additional energy and carbon to this ecosystem. In both glaciers, the abundance of cold adaptation genes and microbial isolates capable of growth in subzero temperatures, high salinity, and low pH further supports the idea that englacial ice can sustain active microbial ecosystems. This study provides critical functional insights into one of Earth's least-studied microbial habitats, offering a baseline characterization of these communities as global warming accelerates glacial melt. The findings also suggest that similar microbial ecosystems could persist or have existed in glacial environments on Mars or the icy moons of the outer solar system.

5.6 Acknowledgements

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Chapter 6. Discussion

The previous chapters have outlined how terrestrial ice, such as lava tube and glacial ice, serve as an analogue for understanding the potential for life in similar environments on Mars, Europa, and Enceladus. Overall, this thesis presents the first in-depth characterization of the unique microbial communities living within lava tube ice in Lava Beds National Monument and glacial ice from the Devon Island ice cap, White Glacier, and Johnsons Glacier. Specifically, my research focused on (i) the taxonomic composition of these communities, (ii) whether they are metabolically active *in situ*, and (iii) the metabolisms that support life in these otherwise extreme cryoenvironments.

6.1 Identifying the microbial community of ice environments analogous to Mars and the icy moons

As the microbial communities of lava tube ice, the Devon Island ice cap, White Glacier and Johnsons Glacier are poorly characterized, or not at all, I sought to elucidate their taxonomic composition using ‘omic sequencing. Despite vast geographic distances between samples and differences in climate and ice type (cave ice vs glacial ice) the taxonomic composition of the microbial communities was similar at higher taxonomic classifications. For example, 16S rRNA sequencing of lava tube ice revealed the microbial communities to be dominated by Actinomycetota (Actinobacteria), Bacteroidota (Bacteroidetes), and Pseudomonadota (proteobacteria) (Figure 3.2). Similarly, the englacial ice environments were dominated by Pseudomonadota, Actinomycetota, and Cyanobacteroidota (Figures 4.2 and 5.1).

Because this is the first characterization of microbial communities from lava tube ice, direct comparisons to other lava tubes are not possible; however, extensive research of the ice in Scarisoara Glacier Cave in Romania and cold-temperature lava tubes in Hawaii have also found Actinomycetota, Bacteroidota, and Pseudomonadota to dominate samples there (Teehera et al. 2017; Itcus et al. 2018; Paun et al. 2019; Mondini et al. 2022). Furthermore, a study of perennial ice from the Pyrenean ice cave in Spain and Obstans ice cave in Austria also found these same three phyla to dominate the ice (Ruiz-Blas et al. 2023; Lange-Enyedi et al. 2024). The results suggest that Pseudomonadota, Bacteroidota, and Actinomycetota are dominant and potentially well-adapted phyla in cold, ice-rich environments, such as lava tubes and glacial caves. Their consistent presence across geographically isolated caves, including Hawaiian and Californian lava tubes and glacier caves in Romania, Spain and Austria, indicates that these microbial groups may play a significant role in colonizing and thriving in these unique and extreme habitats. At the genus level, each of the studies above recovered common cold-adapted genera such as *Polaromonas*, *Cryobacterium*, and *Flavobacterium* and in this study the sequences recovered appear to be more closely related to those from the Arctic and Antarctica (Table 3.4) than from other caves, suggesting that cold temperatures and ice exert stronger selection pressure than caves.

Unlike the 16S rRNA amplicon sequencing strategy used to characterize the microbial communities of lava tube ice, metagenomic sequencing recovered few sequences from ice collected from the Devon Island ice cap. Rarefaction analysis (Supplemental Figure 4.1) of the metagenome revealed insufficient sequencing depth to capture the total microbial diversity within the ice cap. Due to the reliance on PCR amplification, 16S rRNA amplicon sequencing

may have achieved greater depth had it also been used to survey the community. Nevertheless, among the recovered sequences, we found the most abundant phylum to be Pseudomonadota (57%), composed primarily of the plant-associated genus *Phyllobacterium* (36%). *Phyllobacterium* can degrade complex organic compounds and has previously been recovered from glacial ice and snow, mainly in the Himalayas but also in Antarctica (Yao et al. 2008; Liu et al. 2009, 2022b; Lopatina et al. 2013; Zhang et al. 2022a). Although the ice cap is devoid of plants and contains little organic carbon, abundant plant species have been recorded at the True Love Camp approximately 26 km away from our sample site (Barrett and Teeri 1973). It is possible that *Phyllobacterium*, as well as other plant associated taxa recovered in the metagenome, originated in Arctic tundra soil and were blown onto the ice cap by wind. Wind and snow deposition are known to be primary mechanisms by which microorganisms enter glaciers (Miteva 2008; Zhang et al. 2024), suggesting that these processes may have significantly contributed to the microbial diversity observed in this study. Other abundant taxa within the ice cap included the well-known, cold-adapted bacterial genera *Cryobacterium*, *Frigoribacterium*, and *Glaciihabitans*, (Zeng et al. 2013; Li et al. 2014; Cappa et al. 2014; Liu et al. 2019, 2020a, 2020b; Thomas et al. 2020).

The microbial communities of White Glacier in the Canadian high Arctic and Johnsons Glacier in Livingston Island, Antarctica, differed markedly in their composition (Figure 5.1). Cyanobacteria and Actinomycetota largely dominated the White Glacier metagenome, while Johnsons Glacier was dominated almost exclusively by Pseudomonadota, specifically Alpha and Betaproteobacteria. These differences are likely explained by regional geography. White Glacier is located inland among mountainous terrain and receives significant dust inputs to its

surface, whereas Johnsons Glacier is sea-terminating and located in less mountainous terrain; the dust that falls on its surface is volcanic in origin from the Deception Island Volcano. The Antarctic Ocean may also influence the microbial composition of Johnsons Glacier through water vapour deposition of microorganisms. The taxonomic composition of the ice in White Glacier agrees with earlier research, which found abundant Cyanobacteria in cryoconite holes on the glacial surface (Mueller et al. 2001; Mueller and Pollard 2004) and Cyanobacteria and Actinomycetota in surface ice (Touchette et al. 2023). The best comparison to the ice-entrapped microbial communities from Johnsons Glacier is with basal ice communities recovered from nearby Deception Island (Martinez-Alonso et al. 2019). The microbial community there also contained Alpha and Betaproteobacteria with similar abundances as found in Johnsons Glacier. The genus *Burkholderia* (Betaproteobacteria), which was the most abundant classified genera in Johnsons Glacier, has previously been recovered from glacial surfaces (snow and ice) (Ren et al. 2022; Keuschnig et al. 2023). The second and third most abundant classifiable genera in the glacier were *Glaciihabitans* and *Frigoribacterium*, which are well-known constituents of glacial ice and snow (Zeng et al. 2013; Li et al. 2014; Cappa et al. 2014; Thomas et al. 2020) and were also recovered from the Devon Island ice cap and White Glacier as part of this thesis.

Despite the large differences in taxonomic composition among individual ice microbial communities, the geographic breadth covered by this thesis allows for some generalizations about the taxonomic composition of ice microbial communities. Firstly, these communities appear to be most often dominated by the phyla Pseudomonadota and Actinomycetota, which are highly abundant in almost all the ice samples characterized in this thesis. Secondly, the genera *Cryobacterium*, *Glaciihabitans*, and *Frigoribacterium* appear to be common ice constituents,

having been recovered from glacial snow and ice before (Zeng et al. 2013; Cappa et al. 2014; Thomas et al. 2020) and all glaciers and ice caps characterized in this thesis including isolates recovered from lava tube ice in Chapter 3, and the MAGs and isolates recovered from White and Johnsons Glacier in Chapter 5. Many of the taxa recovered here are related to other cold-adapted microorganisms, suggesting that glaciers and ice caps contain microbial communities highly adapted to the unique ice environment and are not simply a record of microbial deposition from adjacent environments (soil, mountains, etc.).

6.2 Determining that ice analog environments contain metabolically active microbial communities

Given the taxonomic evidence for cold-adapted microbial communities across all the ice samples we tested, I then used multiple lines of evidence to show these communities are almost certainly metabolically active *in situ*. In Chapter 3, I used radiorespiration assays to show that the microbial communities in lava tube ice were capable of metabolic activity at -5°C. While radiorespiration assays are very sensitive and have been used to measure metabolism in permafrost (Steven et al., 2007a, 2008), this is the first time this method was used on pure ice and the first time any metabolism has been measured in lava tube ice. This result corroborates the only data available on microbial metabolism in cave ice, which again comes from the Scarisoara Glacier Cave in Romania. In work performed by Paun et al., (2019) and Mondini et al., (2022), 16S rRNA was extracted and converted to cDNA before sequencing, which revealed a potentially active community belonging to the phyla Pseudomonadota, Bacillota, Actinomycetota, and Bacteroidota. Paun et al. estimated metabolic rates which suggested the

active community participated in maintenance metabolism, but growth metabolism was also possible in some ice where organic carbon concentrations were highest.

Metatranscriptome sequencing was employed to determine if an active microbial community exists in the Devon Island ice cap, White Glacier, and Johnsons Glacier ice. Despite the extremely low biomass (~102 live cells/ml) in these environments, I succeeded in recovering metatranscriptomes from the Devon Island ice cap and White Glacier; the metatranscriptome recovered from Devon Island contained fewer than 20,000 sequences after quality filtering and decontamination. I assume that the lack of recovery of metatranscriptome sequences from Johnsons Glacier is similarly due to low biomass within the sample, as iRep, an alternative means of estimating microbial activity (Brown et al. 2016) by estimating bacterial replication rates of MAGs and whose accuracy has been experimentally validated suggested that each MAG tested from Johnsons Glacier was replicating at the time of sample collection.

Metabolically active microbial communities in glaciers and sea ice have been inferred before using laboratory experiments or secondary evidence (Junge et al. 2004; Tung et al. 2005, 2006; Rohde et al. 2008; Bakermans and Skidmore 2011b, 2011a); however, direct *in situ* evidence has not yet been found. Thus, the metatranscriptomes produced in this thesis may be the strongest, most detailed evidence of metabolically active *in situ* microbial communities within englacial ice. In addition, metatranscriptomic data allows us to identify the metabolically active microbial community and their survival mechanisms for the first time.

6.3 Determination of the metabolisms and survival mechanisms utilized by active microbial communities in glacial ice

I used the metagenomic and metatranscriptomic data generated from the Devon Island ice cap, White Glacier, and Johnsons Glacier, including 30 MAGs from the White and Johnsons Glaciers, to characterize gene expression and functional potential for the active englacial microbial communities in these environments. In all three glacial sites, the most abundant marker genes encoded within the metagenomes were associated with metabolisms such as aerobic respiration, aerobic carbon monoxide oxidation, and anaerobic nitrate/nitrite reduction. Aerobic carbon monoxide oxidation can be performed lithoautotrophically to obtain carbon and energy or heterotrophically for energy only. All the known aerobic carbon monoxide taxa in these sites are known only to oxidize carbon monoxide for energy. Transcripts for this metabolism were moderately abundant in White Glacier and the Devon Island ice cap. Aerobic carbon monoxide oxidation has recently been shown to aid the survival of microorganisms under carbon-limiting conditions (Muthusamy et al. 2017; Ji et al. 2017; Islam et al. 2019). Given that polar ice contains few nutrients and atmospheric gases can become trapped in ice or diffuse into near-surface ice, aerobic carbon monoxide oxidation may be an important survival metabolism for microorganisms within ice.

Both White Glacier and the Devon Island ice cap expressed transcripts for nitrate and nitrite reduction. While nitrate reduction was the most transcribed marker gene in the Devon Island ice cap (Figure 4.3), it was only moderately expressed in White Glacier. NapA, responsible for nitrate reduction, was present in seven MAGs from White Glacier but was barely transcribed in any of them. Similarly, NirS, responsible for reducing nitrite to nitric oxide, was

present in nine MAGs from White Glacier, but only transcribed in two: MAG 2, a Microcoleus (Cyanobacteriota) MAG, and MAG 8, an Anaerolineae (Chloroflexota) (Figure 5.3). Notably, the Anaerolineae MAG lacked genes for aerobic respiration (CoxA, CyoA, CydA, CcoN), indicating it is likely a strict anaerobe, as has been previously reported for this class (Yamada et al. 2006). Due to the uneven distribution of gases in ice (Giannelli et al. 2001; Fourteau et al. 2017), dissimilatory nitrate/nitrite reduction in the metagenome and by these MAGs may be favoured in anoxic microzones within the englacial ice. However, transcripts associated with other steps of denitrification (nitric and nitrous oxide reduction) were barely expressed. Furthermore, although oxygen does not inhibit nitrate reduction at concentrations of up to 25 μmol O^2 L^{-1} or sulfate reduction in microoxic conditions (Kalvelage et al. 2011; Schoeffler et al. 2019), the presence of oxygen and the potential for aerobic respiration will promote more energetically favourable aerobic respiration whenever possible, even at nanomolar concentrations of O_2, unless a strict anaerobe such as MAG 8 (Anaerolineae) is present (Berg et al. 2022). As such, aerobic metabolisms dominate over anaerobic metabolisms at the depths of ice that I sampled, which supports better energy yields for microorganisms in an otherwise nutrient-deficient system.

Oxygenic photosynthesis was another important metabolism detected in the metagenomes of all three glacial sites. While, marker genes for oxygenic photosynthesis (PsaA, PsbA) were not the most abundant in the metagenome from any of the three glaciers, they were by far the most transcribed genes in the White Glacier metatranscriptome. Cyanobacteriota were almost entirely responsible for this expression and coupled it to carbon fixation via the Calvin-Benson Cycle. This was also observed in the Cyanobacteriota MAGs I recovered from White Glacier.

Of the 13 total MAGs I recovered, the five Cyanobacteriota MAGs contained the greatest number of aligned transcripts, with the most expression being attributed to MAG 2, a medium-quality *Microcoleus* genome which had more than double the number of transcripts aligned to it as the next most active MAG (Table 5.3). Furthermore, the vast majority of transcripts aligned to these MAGs aligned to PsaA, PsaB and Ribisco, suggesting that oxygenic photosynthesis and carbon fixation via the Calvin-Benson cycle are dominant metabolisms within White Glacier. Cyanobacteria within White Glacier likely act as key primary producers supporting the rest of the active microbial community by supplying organic carbon and energy to be used by heterotrophs, as has been observed in sea ice (Underwood et al. 2013). Whether or to what extent the role of Cyanobacteria as key primary producers persists in ice layers below the photic zone or during the polar night remains an open question.

Some additional primary productivity can be attributed to lithoautotrophic metabolisms driven by sulfur oxidation. Genes encoding the oxidation of diverse sulfur species were found in the metagenomes and MAGs from White Glacier and Johnsons Glacier (Figure 5.3), but only genes encoding the oxidation of thiosulfate were found in the Devon Island ice cap (Figure 4.3). Most transcripts matching the oxidation of reduced sulfur species in White Glacier were related to Pseudomonadota and Plantomycetota (Figure 5.4). In the Devon Island ice cap, these transcripts were most closely related to Deinococcus (Figure 4.3). Within all three glaciers, sulfur oxidation is likely to be performed aerobically due to the likely presence of oxygen in the ice. A minor microbial fraction in White Glacier may also be coupling sulfur oxidation with anoxygenic photosynthesis and fixing carbon dioxide via the 3-hydroxyproprionate cycle. One such microorganism was MAG 9, recovered from White Glacier, belonging to the newly

described phyla Gemmatimonadota, and the genus Gemmatimonas (Figure 5.3). Previously, cultured representatives of this phyla were only known to be heterotrophs or photoheterotrophs (Zeng et al., 2015, 2021), yet the potential for this MAG to couple the oxidation of sulfide with carbon fixation via the 3-hydroxyproprionate or Calvin-Benson cycles and anoxygenic photosynthesis implies a novel lithoautotrophic lifestyle for members of the phylum Gemmatimonadota. Combined with the high abundance of Cyanobacteria and oxygenic photosynthesis transcripts, these results suggest an active litho- and photo-autotrophic microbial community within White Glacier englacial ice that can support the heterotrophic fraction. In contrast, the microbial community in Johnsons Glacier is characterized by a limited potential for lithoautotrophic metabolism, as evidenced by the absence of carbon fixation genes in MAGs and the low presence of such genes in the metagenome. This suggests a predominantly heterotrophic lifestyle, differing significantly from White Glacier, where lithoautotrophic metabolisms are more abundant.

We additionally identified abundant and widespread cold adaptation genes across each glacial sample, which provided evidence for the mechanisms these communities use to survive in extreme englacial environments. Genes involved in membrane and peptidoglycan alteration were the most abundant and expressed, followed by transcription and translation factors and cold shock proteins. Microorganisms experiencing cold temperatures must alter their cell membranes because membranes otherwise lose flexibility at cold temperatures, which decreases their ability to transport nutrients and other molecules in and out of the cell. For this reason, cold-adapted microorganisms often increase membrane fluidity by converting saturated fatty acids to unsaturated and polyunsaturated fatty acids (De Maayer et al. 2014; Ramasamy et al. 2023).

Cold shock proteins and transcription and translation factors complement each other, so, unsurprisingly, both types of proteins are often present and expressed together. At cold temperatures, cold-adapted microorganisms will use transcription and translation factors to slow or halt protein synthesis until cold-shock proteins can be synthesized (Jones et al. 1987; Goldstein et al. 1990; Zhang et al. 2018). These cold-shock proteins prevent mRNA misfolding at temperatures well below their optimum (Keto-Timonen et al. 2016).

Another abundant cold adaptation I detected in both the -omic data and cultured isolates was the osmotic stress response. To survive at subzero temperatures, microorganisms inhabit microenvironments where salts increase the local water activity to minimum levels for survival, which also causes osmotic stress. Accordingly, genes related to osmotic stress response were abundant in the metagenomes of all three glacial sites studied in this thesis. In addition, many cultured isolates recovered from the glaciers could grow at elevated salt concentrations (>6% NaCl) despite conductivity values in the three glaciers being between 3.97 and 39.4 µS/cm (Table 4.1 & 5.1) suggesting they are composed of freshwater containing few salts. It is important to note, however, that while sea ice brines are hypersaline, glaciers contain few salts, and thus, glacial brines are predicted to be hyper-acidic (< pH 3) instead (Price 2000; Dani et al. 2012). The pH in each glacier was slightly to moderately acidic between 6.96 and 5.59 (Table 4.1 & 5.1), and I correspondingly found that many cultured isolates recovered from the three glacial sites could withstand low pH and even grow down to pH 3. Unfortunately, genes involved in acid stress were not analyzed in this study. Still, they should be included in future studies characterizing glacially entrapped microbial communities to fully determine their tolerance to and survival mechanisms in hyper-acidic environments.

6.4 Limitations and future directions

This work comprehensively characterized viable and active microbial communities from ice environments analogous to the ice found on Mars and the icy moons of the outer solar system. While providing one of the most detailed characterizations of these communities to date, the work has raised additional questions. Limitations of the current work and future research directions have already been discussed but will be summarized here.

Generally, this work suffered from a lack of sample replication. While biological and technical replicates from lava tube ice were analyzed in Chapter 3, all the sequencing results presented in Chapters 4 and 5 were based on single replicates. The reason for this was two-fold. Firstly, the logistical and time constraints associated with polar research meant that, at most, only three core replicates could be successfully transported from each field site. From these replicates, ice needed to be divided for physicochemical analysis, culturing, flow cytometry, and sequencing, limiting the amount of material available for each analysis. Secondly, because each ice core required approximately $1,200 CAD worth of DNA/RNA Shield, preserving multiple replicates for sequencing was prohibitively expensive. Future research characterizing ice-entrapped microbial communities should prioritize sequencing an appropriate number of replicates, potentially by only collecting samples at the specific depths of interest and leaving more space and weight for replicate core sections. Home-made preservation solutions such as those developed by Menke et al., (2017) could be tested as a cheaper alternative to expensive commercial nucleic acid preservatives. Using cheaper nucleic acid preservatives would have the added benefit of allowing more ice per replicate to be melted, preserved, concentrated, and sequenced potentially resulting in higher sequencing yields. For example, Simon et al., (2009)

concentrated five litres of glacial ice. Given the low read abundance obtained from the Devon Island ice cap and Johnsons Glacier, sequencing larger ice volumes may result in better sequencing yields.

From both Chapters 4 and 5, I encountered a large proportion of taxonomically unclassified sequences in the datasets, as high as 80% in the White Glacier metatranscriptome. To try to reduce this number I used multiple classifiers including Kaiju (Menzel et al. 2016) and MetaPhlAn 4 (Blanco-Míguez et al. 2023). Ultimately, MetaPhlAn 4's results were the best and presented in this thesis. The high proportion of unclassified reads is likely due to the use of incomplete databases which these classifiers rely on. Furthermore, taxonomic databases are biased towards sequences of model organisms, pathogens or cultured organisms (Quince et al. 2017) which are unlikely to compose a large proportion of the community in glaciers and ice caps studied here. As previously stated in this thesis and by others, the microbial communities of glacial environments are understudied and thus their genomes are under-represented in sequence databases (Liu et al. 2022a). To reduce the number of unclassified sequences from glacier and ice sheets in the future, one could decrease the stringency needed to assign a sequence a match in a taxonomic database. However, this may result in less accurate taxonomic profiles. Ultimately, to solve this issue, more sequencing of microbial communities in glacial ice environments and their inclusion in taxonomic databases will be required.

Another limitation of this work was the inability to account for habitat heterogeneity, such as differences between brines and solid ice and oxic and anoxic environments. This prevented the complete interpretation of the metagenome and metatranscriptome datasets. For example,

nutrient concentrations could only be measured for the bulk meltwater; however, these concentrations are unlikely to be biologically relevant given the majority of microorganisms within ice reside in liquid brine/acid veins or around mineral grains where nutrient concentrations will be much higher than what I measured in the bulk meltwater (Price 2000; Junge et al. 2001; Dani et al. 2012; Barletta et al. 2012). Knowing the nutrient concentrations within these veins is necessary to determine if they're sufficient to support the above-described metabolisms. In future work, attempts should be made to quantify the nutrient concentration within the liquid brine veins and around mineral surfaces to better constrain the availability of nutrients for microbial metabolism. Concentrations of biologically important gases such as carbon dioxide, oxygen, carbon monoxide and methane should also be quantified.

This thesis was also not able to conclusively determine what portion of the microbial community present within the samples was present as a result of transient colonization or are true residents of the ice. In the case of samples collected from the Devon Island ice cap, White Glacier, and Johnsons Glacier, these samples are likely decades old, potentially a few hundred years old in the case of White Glacier (personal communication), and therefore, if they're able to survive that long, the viable and active portion of these communities are at a minimum moderately adapted to this environment, capable of withstanding subzero temperatures, nutrient limitation and low water activity. The samples I collected from ice from Lava Beds National Monument are younger than those collected from the glaciers and, therefore, may contain a higher proportion of transient microbes or even contaminants than the glacial samples. However, previous work from the site has found that the microbial communities within the caves

only share an 11% overlap with OTUs in overlying soils which suggests the cave environment is selecting for microorganisms adapted to the unique cave environment (Lavoie et al. 2017).

Finally, many steps were taken to ensure the metatranscriptomes recovered for this thesis were representative of *in situ* activity, there is still some possibility that the sequenced metatranscriptomes were the result of *ex-situ* metabolism. Combining metatranscriptomics with other experimental methods is desirable to bolster the claims that glacial ice contains an active *in situ* microbial community. One such experiment could be to image metabolizing cells in glacial ice directly. Junge et al., (2001) developed a method to image cells stained with DAPI in sea ice without melting it. This method could be modified to use a metabolic activity stain such as Redox Sensor Green™ (Kalyuzhnaya et al. 2008), thus providing direct evidence of microbial metabolic activity under *in situ* conditions. Another option could be to perform radiorespiration assays on ice samples as I performed in Chapter 3 (Figure 3.4) to measure the rate of activity of heterotrophs in each glacier.

Despite the value in incorporating more replicates, accounting for habitat heterogeneity, and visualizing metabolisms *in situ*, one of the best approaches to understanding the physiological requirements for life in ice is to isolate viable, cold-adapted microorganisms for phenotypic and genomic characterization in pure culture. From lava tube ice, we isolated 37 cold-adapted microorganisms, of which five appear to be novel species and one a novel genus. From the Devon Island ice cap, White Glacier and Johnsons Glacier, 60 isolates were recovered. Many of these isolates can grow in polyextreme conditions of subzero temperatures, high salinity and low pH. Given that cultured isolates from lava tube ice and polar glacial ice are scarce,

studying the isolates recovered as part of this thesis, especially those capable of growth in multiple extremes of temperature, salinity and pH, will help to determine how microorganisms persist in one of Earth's most extreme environments and possibly in ice elsewhere in our solar system.

Chapter 7. Final Conclusion and Summary

The study of microbial life in ice not only expands our knowledge of the limits of life on Earth but also informs the search for life on other worlds. Mars, Jupiter's moon Europa, and Saturn's moon Enceladus are all cold, ice-covered planetary bodies and are some of the best targets to search for life in our solar system. As such, ice in lava tubes, glaciers, and ice caps are some of the best analog environments to understand the potential for microbial life on other worlds. This work utilized culture-dependent and independent methods to characterize microbial communities within these environments to determine (i) if they harbor metabolically active microbial communities and (ii) how these communities survive under such harsh conditions.

In Chapter 3, I characterized the microbial diversity and activity in a unique, never-before-studied lava tube ice environment at Lava Beds National Monument. Using 16S rRNA sequencing, I determined that the resident microbial communities of this environment are Actinomycetota, Pseudomonadota, and Bacteroidota. I determined that viable microorganisms recovered from these sites are cold-adapted and capable of growth at subzero temperatures. Using radiorespiration assays, I also determined that the microbial communities of this environment were metabolically active under *in situ* conditions.

In Chapter 4, I characterized the microbial communities inhabiting the near-surface ice of the Devon Island ice cap in the Canadian High Arctic. Using metagenomics and metatranscriptomics for the first time on englacial ice, I determined there to be a sparse but active microbial community potentially performing metabolisms such as thiosulfate oxidation, aerobic carbon monoxide oxidation, aerobic respiration, fumarate reduction, nitrate and nitrite reduction, nitrogen fixation, oxygenic photosynthesis and carbon fixation. I also isolated and

characterized a *Kocuria spp.* From the Devon Island ice cap capable of growth at -5°C, 15% NaCl, and pH 4, demonstrating the viability of ice-entrapped microorganisms and their ability to withstand some of the most extreme environments on Earth.

In Chapter 5, I characterized and compared the microbial communities inhabiting the near-surface ice of two glaciers: White Glacier in the high Arctic and Johnsons Glacier in Antarctica. These results demonstrated that while the taxonomic makeup of diverse, geographically isolated glaciers are significantly different, their metabolic functional potential contained many overlaps, suggesting a universal survival strategy may exist within englacial ice. Furthermore, using metatranscriptomics, I determined that White Glacier contains an active microbial community supported by Cyanobacterial-driven photoautotrophic primary production and, to a lesser extent, lithotrophic sulfur metabolizing microorganisms. Other active metabolisms within White Glacier included sulfide and thiosulfate oxidation, aerobic carbon monoxide oxidation, aerobic respiration, and nitrate and nitrite reduction.

This thesis demonstrates that various ice environments worldwide contain active microbial communities. It further uncovered the metabolisms and survival strategies employed by active microbial communities to survive in these environments. Finally, it demonstrates the potential for life to survive in analogous ice-rich environments on other worlds, such as Mars or the icy moons Europa and Enceladus.

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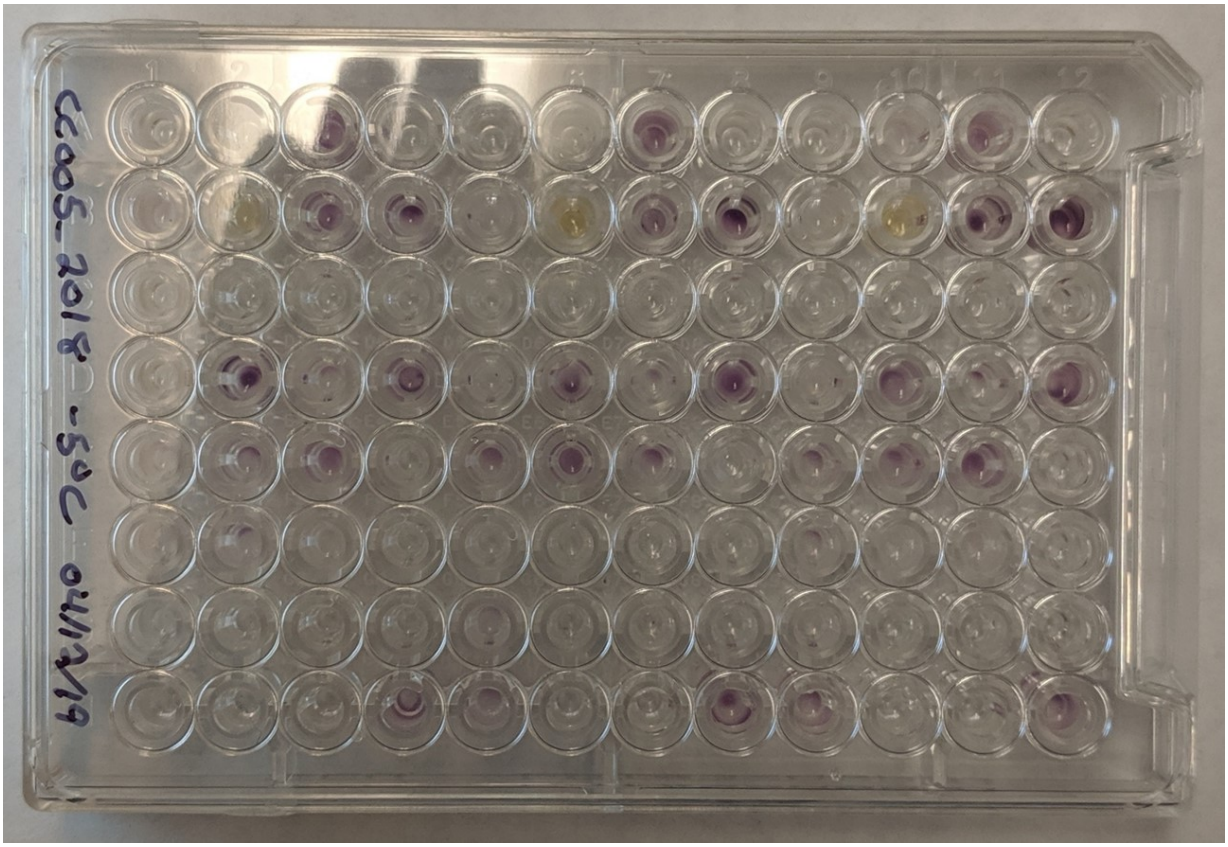
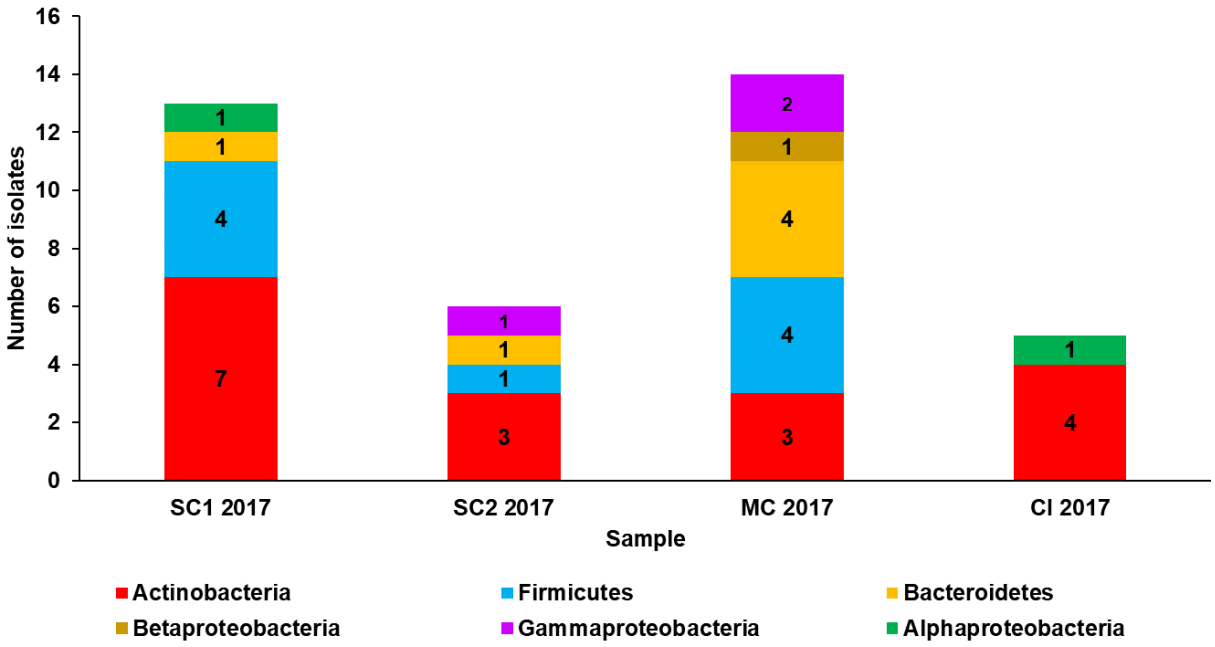
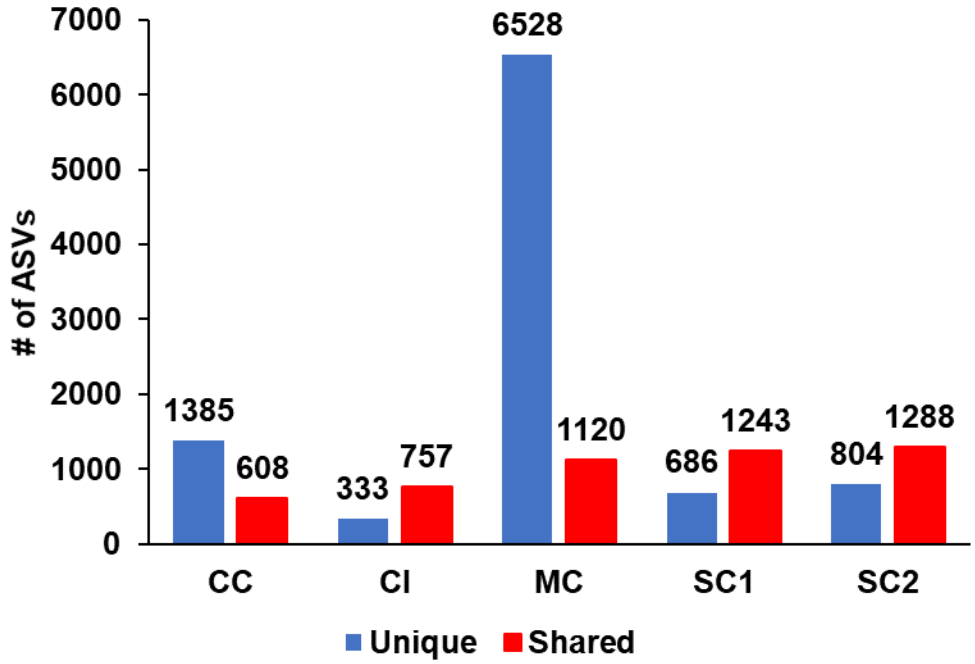
Appendix 1. Supplementary Material for Chapter 3



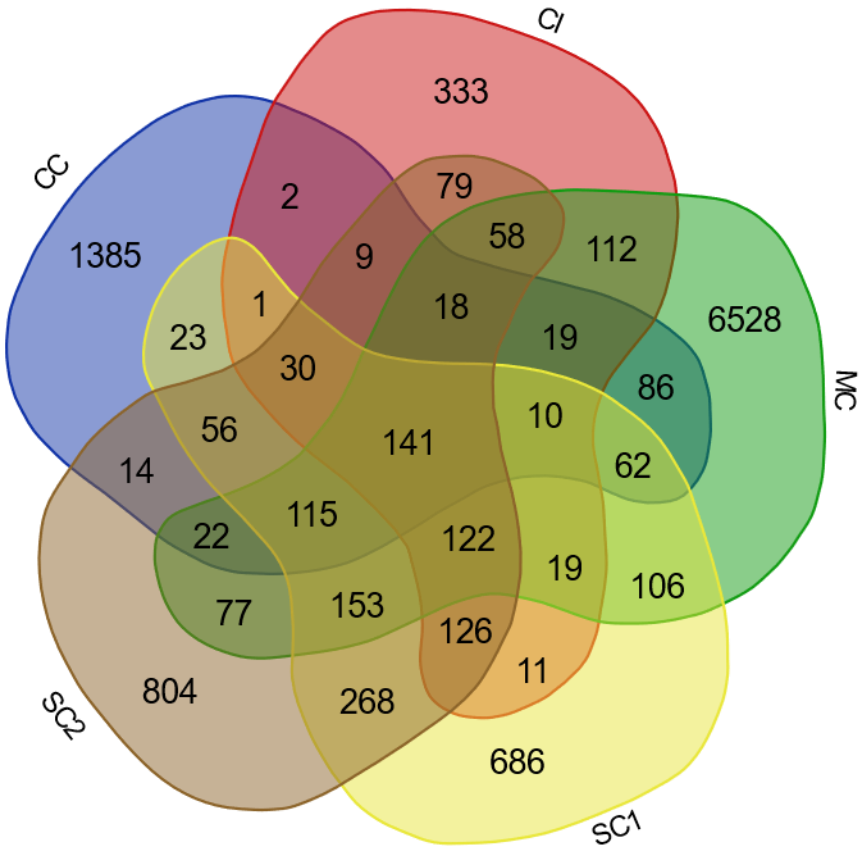
Supplemental Figure 3.1. Representative Biolog Ecoplate used in this study, depicting various positive wells for substrate utilization (purple) at -5°C. 31 heterotrophic carbon sources and a negative control well are replicated 3 times within the plate.



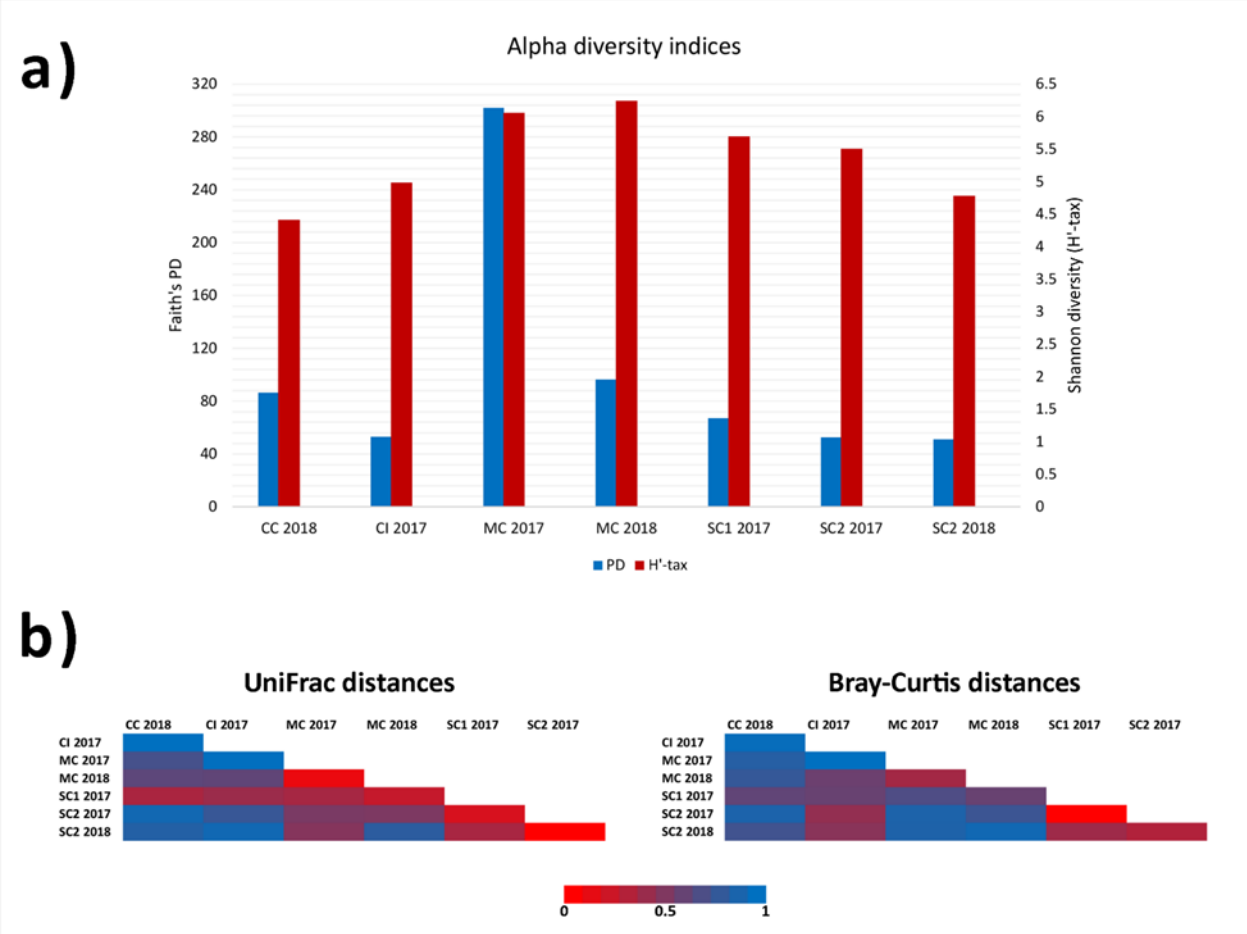
Supplemental Figure 3.2. Taxonomic distribution of cultured phyla from LABE ice samples. Culturing of isolates was only conducted on samples collected in 2017.



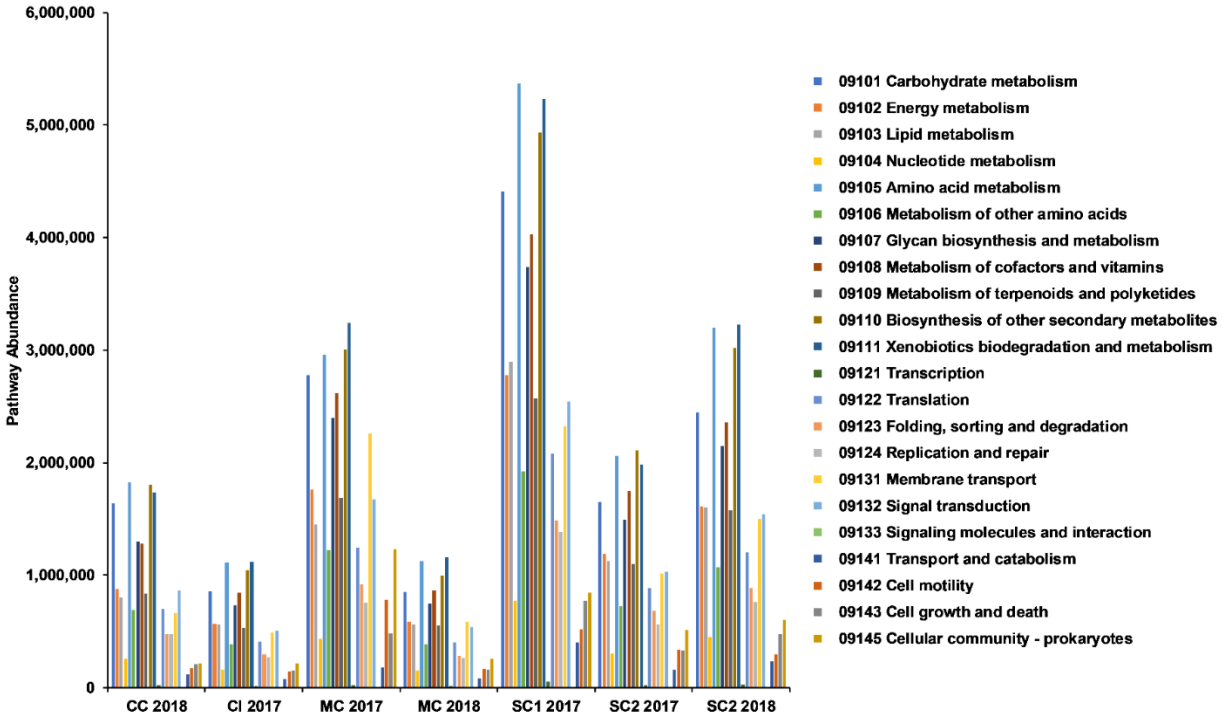
Supplemental Figure 3.3. Distribution of ASVs among cave sites. ASVs unique to each site (blue column) and shared between at least one cave site (red column). Sample results from 2017 and 2018 have been merged.



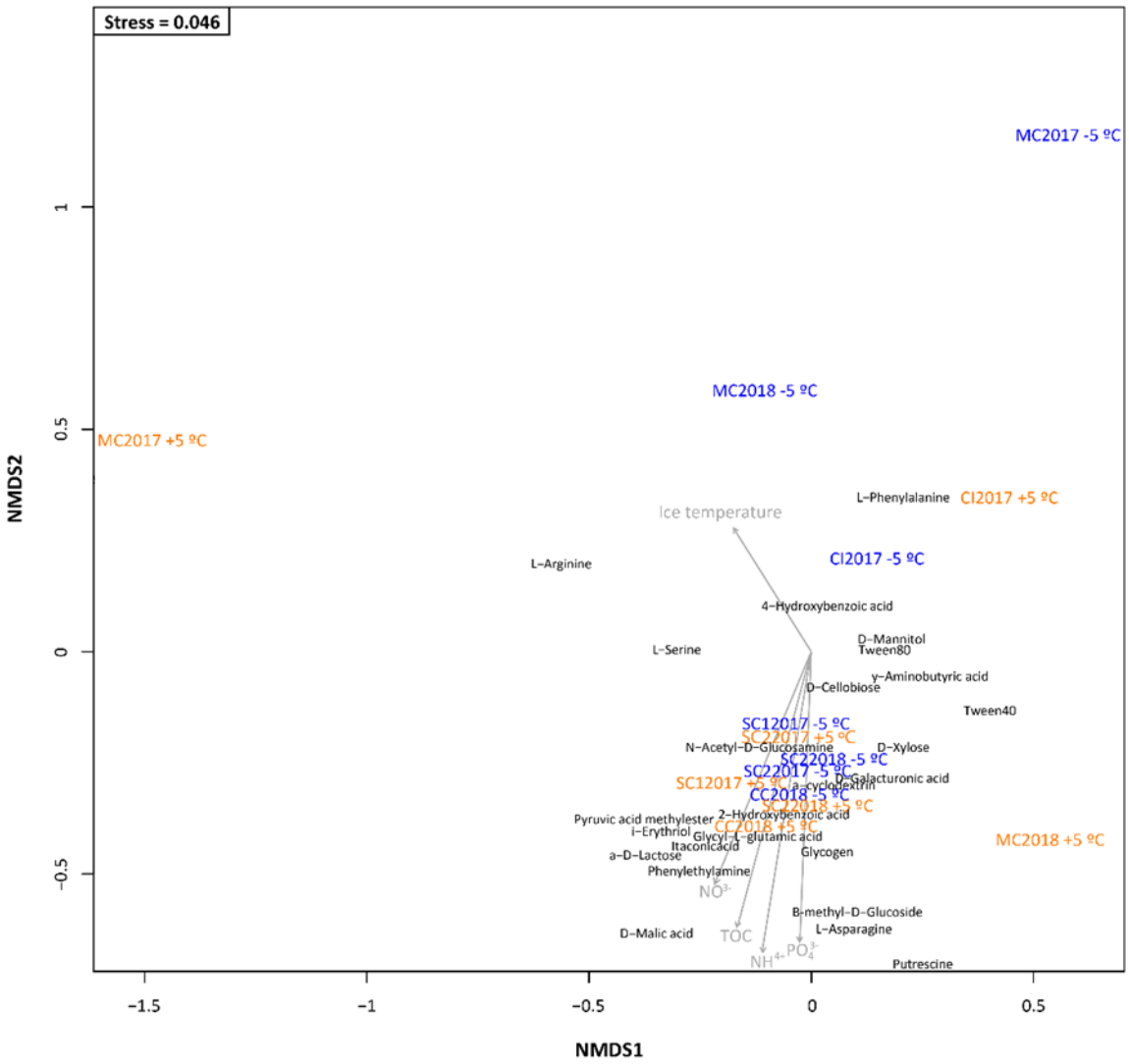
Supplemental Figure 3.4. Venn diagram of ASVs unique to and shared between cave sites. Sites sampled in multiple years were combined.



Supplemental Figure 3.5. Diversity indexes calculated using 16S rRNA ASVs. (a) depicts alpha diversity as measured by Faith's phylogenetic diversity and Shannon diversity. (b) depicts beta diversity heat maps measured by UniFrac and Bray-Curtis distances. A distance of 1 indicates two samples are very dissimilar while a distance of 0 indicates two samples are very similar.



Supplemental Figure 3.6. Prediction of KEGG functional pathways contained within our sites using Piphillin and 16S rRNA ASVs.



Supplemental Figure 3.7. NMDS ordination of Biolog EcoPlates. The location of the samples in the ordination mainly depended on the cave they came from (stress=0.046). Therefore, incubations from CC 2018, SC1 2017, SC2 2017 and SC2 2018, both at 5°C and -5°C temperatures, clustered together and close to the center of the ordination, as most of the substrates did. According to the length and the direction of the arrows, TOC, N-NH4+, N-NO3-, and PO43- were the most influencing factors on the ordination of these samples. On the other hand, incubations from MC 2017, MC 2018 and CI 2017 were located distant to the main group (except for incubation MC2018 +5°C), specially along NMDS2 axis. Samples MC 2017 and MC 2018 were situated further from the rest on the ordination than CI 2017 incubations, with these later incubations specifically related to L-Phenylalanine and 4-Hydroxybenzoic acid. All samples which grouped away from the main cluster seemed to be influenced more by ice temperature than the rest.

Appendix 2. Supplementary Material for Chapter 4

Supplemental Table 4.1. Devon Island ice cap near-surface ice metal concentration.

Metal	Concentration (mg/L)
Ag	<LOQ
Al	0.114
As	0.021
B	<LOQ
Ba	0.006
Be	<LOQ
Ca	0.795
Cd	0.041
Co	<LOQ
Cr	<LOQ
Cu	0.034
Fe	0.099
K	0.567
Li	<LOQ
Mg	0.095
Mn	<LOQ
Mo	<LOQ
Na	3.114
Ni	0.133
P	0.049
Pb	<LOQ
S	1.207
Sb	<LOQ
Se	<LOQ
Si	0.063
Sr	<LOQ
Ti	0.011
Tl	<LOQ
V	<LOQ
Zn	0.117

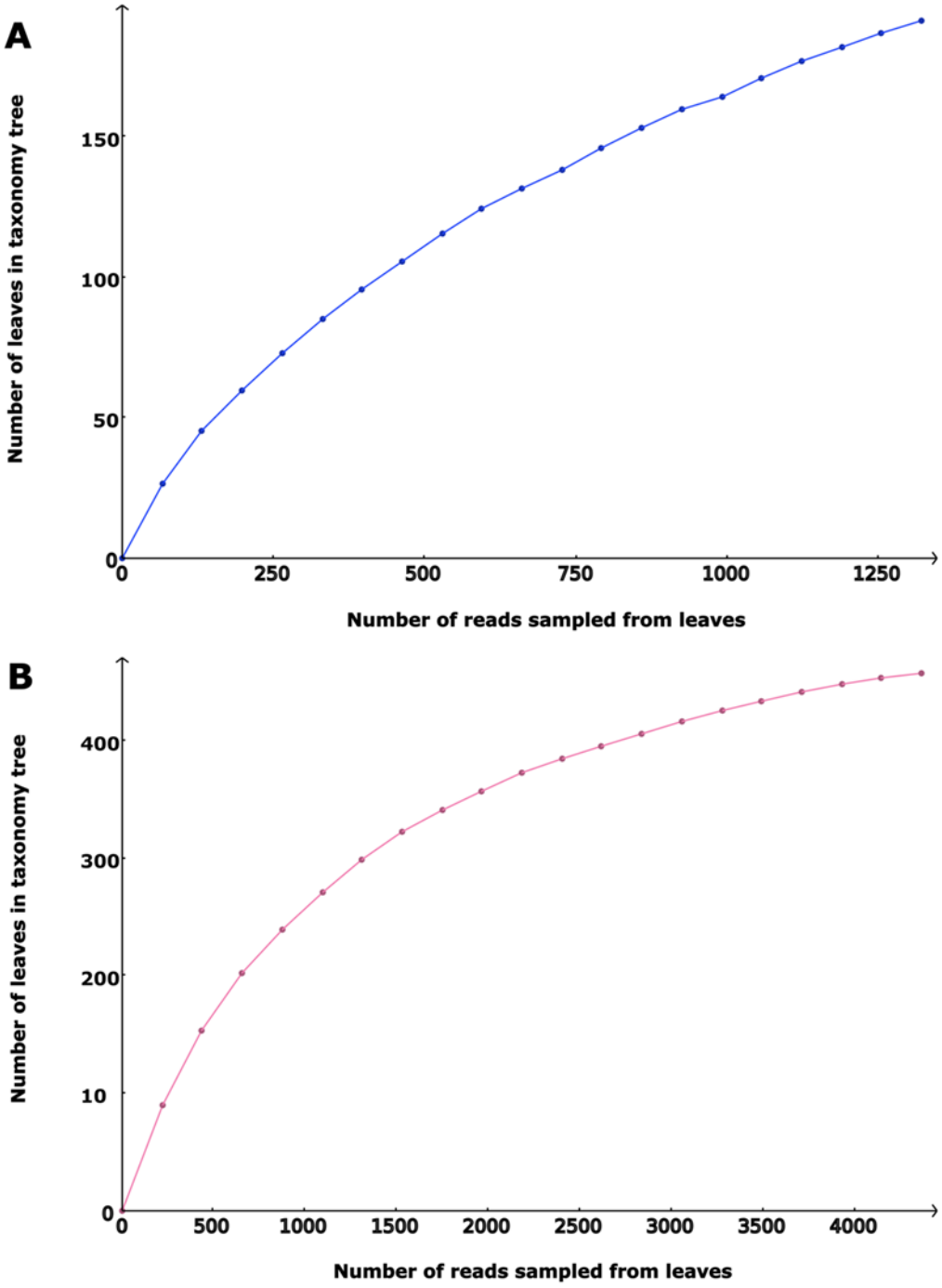
LOQ = Limit of Quantification

Supplemental Table 4.2. Biolog metabolic activity characterization of isolates recovered from the near-surface of the Devon Island ice cap.

	DI3	DI13	DI14	DI15
Dextrin				
D-Maltose			+	
D-Trehalose			+	
D-Cellobiose			+	
Gentobiose				
Sucrose			+	
D-Turanose			+	
Stachyose				
pH 6			+	+
pH 5			+	
D-Raffinose			+	
α-D-Lactose		+		
D-Melibiose		+	+	
β-Methyl Glucoside		+	+	+
D-Salicin			+	+
N-Acetyl-D-Glucosamine			+	
N-Acetyl-β-D-Mannosamine				
N-Acetyl-D-Galactosamine			+	
N-Acetyl Neuraminic Acid		+	+	
1% NaCl		+	+	+
4% NaCl				+
8% NaCl				+
α-D-Glucose		+	+	
D-Mannose	+	+	+	
D-Fructose			+	
D-Galactose		+	+	+
3-Methyl Glucose				
D-Fucose		+		
L-Fucose		+	+	
L-Rhamnose			+	
Inosine		+	+	
1% Sodium Lactate			+	+
Fusidic Acid			+	
D-Serine			+	
D-Sorbitol			+	
D-Mannitol		+	+	+
D-Arabitol			+	
myo-Inositol	+	+	+	
Glycerol			+	+
D-Glucose-6-PO4	+	+	+	
D-Fructose-6-PO4		+	+	
D-Aspartic Acid		+	+	
D-Serine		+	+	
Macrolide			+	
Rifamycin SV			+	
Minocycline			+	
Gelatin				
Glycyl-L-Proline		+	+	
L-Alanine		+	+	
L-Arginine	+	+	+	
L-Aspartic Acid	+	+	+	
L-Glutamic Acid	+	+	+	
L-Histidine	+	+	+	
L-Pyroglutamic Acid		+	+	
L-Serine		+	+	
Lincomycin			+	
Guanidine HCl				
Niaproof 4				
Pectin		+	+	
D-Galacturonic Acid		+	+	
L-Galatonic Acid Lactone		+	+	
D-Gluconic Acid		+	+	
D-Glucuronic acid		+	+	
Glucuronamide	+	+		
Mucic Acid	+	+	+	
Quinic Acid			+	
D-Saccharic acid			+	
Vanomycin			+	
Tetrazolium Violet	+			+
Tetrazolium Blue			+	
p-Hydroxy-Phenylacetic Acid				
Methyl Pyruvate		+	+	
D-Lactic Acid Methyl Ester		+	+	
L-Lactic Acid	+		+	
Citric acid	+	+		
α-Keto-Glutaric Acid	+	+	+	
D-Malic Acid			+	
L-Malic Acid			+	
Bromosuccinic Acid				
Nalidixic Acid			+	+
Lithium Chloride		+		+
Potassium Tellurite				
Tween 40		+		
γ- Aminobutyric Acid				
α-Hydroxybutyric Acid				
β-Hydroxy-D,L-Butyric Acid				
α-Keto-Butyric Acid				
Acetoacetic Acid		+		
Propionic Acid				
Acetic Acid				
Formic Acid				
Aztreonam			+	+
Sodium Butyrate				
Sodium Bromate				

Supplemental Table 4.3. Potential contaminating taxa removed from metagenome and metatranscriptome datasets.

Bilateria	*Staphylococcus*	*Comamonas testosteroni*
Viridiplantae	*Streptococcaceae*	*Corynebacterium suicordis*
Mesangiospermae	*Corynebacterium*	*Cutibacterium acnes*
Neisseriales	*Neisseriales*	*Sphingomonas paucimobilis*
Enterobacteriaceae	*Chlamydia*	*Clostridioides difficile*
Enterococcaceae	*Pseudomonas aeruginosa*	*Dermacoccus nishinomiyaensis*
Acinetobacter	*Pseudomonas stutzeri*	*Vagococcus fluvialis*
Microbacterium	*Pseudomonas syringae*	*Bordetella bronchiseptica*
Burkolderia contaminans	Oethopoxivirue	Human papillomavirus
Genomovirdae		



Supplemental Figure 4.1. A. Rarefaction curve of metagenome. **B.** Rarefaction curve of metatranscriptome. Both curves were generated using MEGAN.

Appendix 3. Supplementary Material for Chapter 5

Supplemental Table 5.1. White and Johnsons Glaciers englacial ice metal concentration.

	Concentration (mg/L)	
Metal	**White Glacier**	**Johnsons Glacier**
Al	0.144	<LOQ
As	<LOQ	<LOQ
B	<LOQ	<LOQ
Ba	<LOQ	<LOQ
Be	<LOQ	<LOQ
Ca	0.355	0.085
Cd	0.004	<LOQ
Co	<LOQ	<LOQ
Cr	<LOQ	<LOQ
Cu	<LOQ	<LOQ
Fe	0.048	<LOQ
K	0.277	<LOQ
Li	<LOQ	0.003
Mg	0.049	0.014
Mn	<LOQ	<LOQ
Mo	<LOQ	<LOQ
Na	0.448	0.322
Ni	0.033	<LOQ
P	<LOQ	0.005
Pb	<LOQ	<LOQ
S	0.245	0.059
Sb	<LOQ	0.003
Se	<LOQ	<LOQ
Si	0.212	0.006
Sr	<LOQ	<LOQ
Ti	<LOQ	<LOQ
Tl	<LOQ	<LOQ
V	<LOQ	<LOQ
Zn	0.036	<LOQ

LOQ = Limit of Quantification

Supplemental Table 5.2. Biolog metabolic activity characterization of isolates recovered from the englacial ice of White and Johnsons Glaciers.

Substrate	WG 19	WG 20	WG 21	WG 22	WG 23	WG 24	WG 25	WG 26	JNG 3	JNG 5	JNG 12	JNG 13	JNG 14	JNG 26	JNG 28	JNG 30	JNG 41	JNG 45	JNG 48	JNG 50	JNG 53	JNG 55
Dextrin																		+				
D-Maltose																		+	+	+		
D-Trehalose				+		+	+						+			+	+	+	+	+		
D-Cellobiose				+														+	+	+		
Gentobiose				+													+	+	+	+		
Sucrose				+		+	+						+			+	+	+	+	+		
D-Turanose				+														+	+	+		
Stachyose				+		+	+											+	+			
pH 6						+	+					+	+			+	+	+		+	+	
pH 5						+	+						+				+				+	
D-Raffinose						+	+											+	+	+		
α-D-Lactose				+													+	+	+	+		
D-Melibiose				+														+	+	+		
β-Methyl Glucoside				+									+				+	+	+	+		
D-Salicin																	+			+		
N-Acetyl-D-Glucosamine				+														+	+	+	+	
N-Acetyl-β-D-Mannosamine				+														+	+	+		
N-Acetyl-D-Galactosamine				+					+								+		+	+		
N-Acetyl Neuraminic Acid									+								+				+	
1% NaCl						+			+			+	+			+	+				+	
4% NaCl						+						+	+			+					+	
8% NaCl																						
α-D-Glucose						+							+			+	+		+			
D-Mannose						+							+			+	+		+	+		
D-Fructose						+							+			+	+		+	+	+	+
D-Galactose						+							+			+	+		+	+		
3-Methyl Glucose																			+	+		
D-Fucose				+						+					+		+			+		
L-Fucose				+															+	+		
L-Rhamnose				+						+						+			+	+		
Inosine				+						+					+		+			+		
1% Sodium Lactate						+	+						+			+	+				+	
Fusidic Acid						+	+					+	+				+					
D-Serine						+						+	+								+	
D-Sorbitol				+		+							+			+	+	+	+		+	
D-Mannitol				+		+							+		+	+	+	+	+	+	+	
D-Arabitol						+	+						+			+	+				+	
myo-Inositol				+						+					+	+	+		+	+	+	+
Glycerol				+		+	+			+			+		+		+		+	+	+	
D-Glucose-6-PO4										+					+		+					
D-Fructose-6-PO4							+			+							+					
D-Aspartic Acid										+												
D-Serine										+					+						+	
Macrolide						+	+				+		+		+		+					
Rifamycin SV						+	+						+			+	+					
Minocycline						+	+					+	+				+					
Gelatin																						
Glycyl-L-Proline																	+					
L-Alanine						+								+			+				+	
L-Arginine													+			+	+				+	
L-Aspartic Acid						+	+				+					+	+					
L-Glutamic Acid						+	+			+	+		+	+		+	+				+	
L-Histidine		+	+		+					+		+		+				+	+	+		
L-Pyroglutamic Acid						+							+			+	+					
L-Serine										+	+					+						
Lincomycin						+	+			+	+		+				+					
Guanidine HCl	+											+										
Niaproof 4																						
Pectin						+	+									+	+					
D-Galacturonic Acid																+	+					
L-Galatonic Acid Lactone											+					+	+					
D-Gluconic Acid						+	+				+					+	+				+	+
D-Glucuronic acid																+	+					
Glucuronamide			+								+											
Mucic Acid			+	+												+	+					
Quinic Acid			+			+	+									+	+				+	+
D-Saccharic acid	+						+									+	+		+			
Vanomycin						+							+		+		+					
Tetrazolium Violet		+	+								+	+			+							
Tetrazolium Blue												+									+	
p-Hydroxy-Phenylacetic Acid																						
Methyl Pyruvate																+	+					
D-Lactic Acid Methyl Ester																	+					
L-Lactic Acid						+											+				+	
Citric acid																+	+				+	
α-Keto-Glutaric Acid															+	+	+					
D-Malic Acid	+		+													+						
L-Malic Acid			+			+	+									+	+				+	+
Bromosuccinic Acid						+	+									+	+				+	+
Nalidixic Acid						+	+						+			+	+				+	
Lithium Chloride																+						
Potassium Tellurite						+	+									+	+				+	
Tween 40		+		+	+	+						+				+	+	+	+	+	+	
γ- Aminobutyric Acid						+															+	
α-Hydroxybutyric Acid																					+	
β-Hydroxy-D,L-Butyric Acid						+							+			+					+	+
α-Keto-Butyric Acid																+						
Acetoacetic Acid		+		+	+							+				+		+	+	+	+	
Propionic Acid																+					+	
Acetic Acid						+	+						+			+					+	
Formic Acid																						
Aztreonam							+						+			+	+				+	+
Sodium Butyrate						+										+						
Sodium Bromate													+			+					+	

Supplemental Table 5.3. *In situ* replication of MAGs from White and Johnsons Glacier using iRep.

Glacier	MAG	iRep Value
White Glacier	MAG 6	1.28
Johnsons Glacier	MAG 1	1.55
Johnsons Glacier	MAG 2	1.58
Johnsons Glacier	MAG 3	1.77
Johnsons Glacier	MAG 5	1.63
Johnsons Glacier	MAG 11	2.43
Johnsons Glacier	MAG 13	2.15
Johnsons Glacier	MAG 14	1.85
Johnsons Glacier	MAG17	1.97

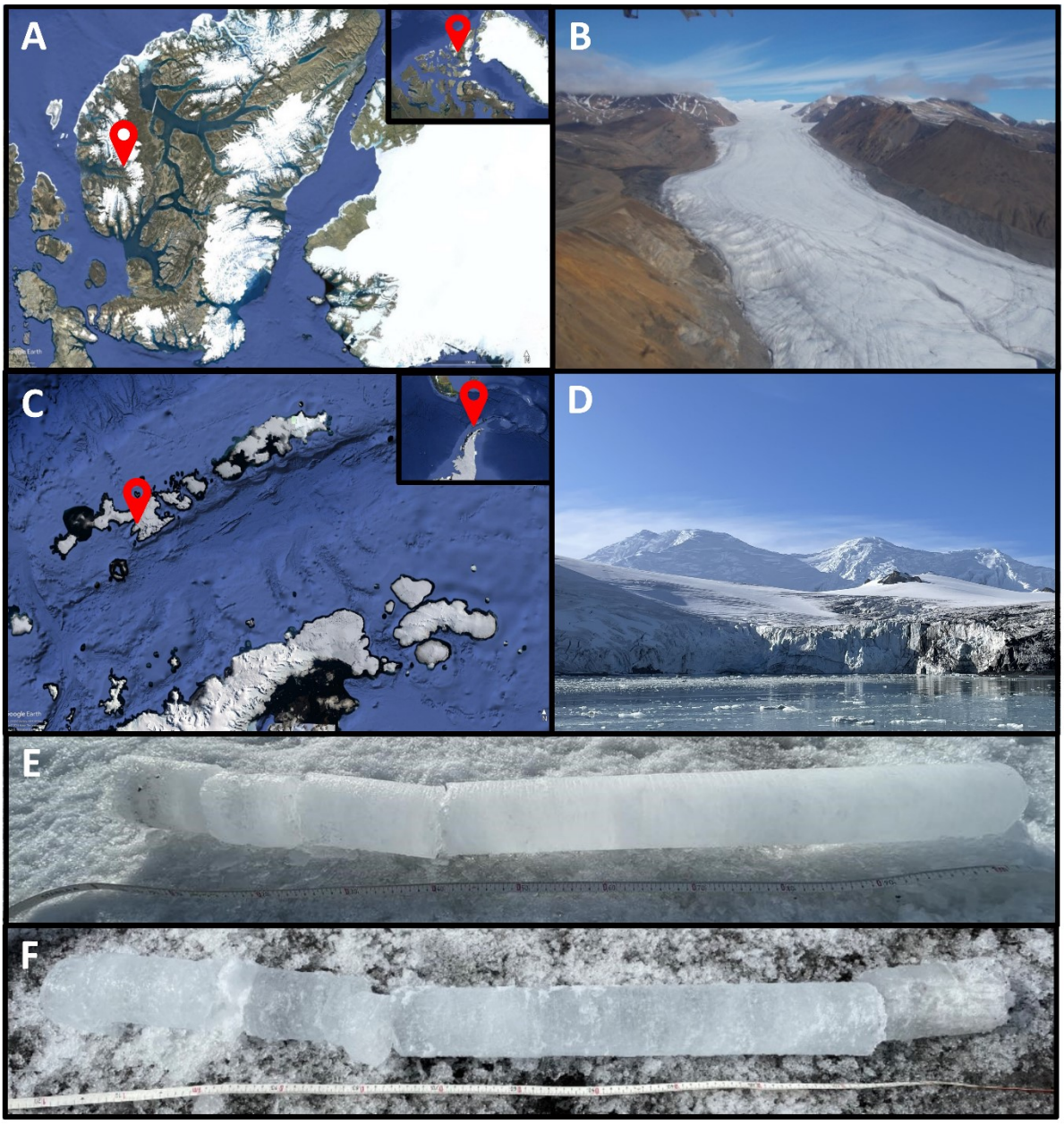
Supplemental Table 5.4. List of unique and shared taxa between White and Johnsons Glaciers.

White Glacier Unique Taxa	Johnsons Glacier Unique Taxa	Shared Taxa
s__TM7_phylum_sp_oral_taxon_350	s__Streptococcus_suis	s__Frigoribacterium_SGB87749
s__Acidovorax_temperans	s__GGB34227_SGB108026	s__Pyrinomonas_methylaliphatogenes
s__Dialister_invisus	s__Pseudomonas_fildesensis	s__Pseudomonas_helleri
s__Betaproteobacteria_bacterium	s__Rhodoferax_ferrireducens	s__GGB64185_SGB86598
s__GGB78370_SGB107507	s__Flavobacterium_sp_CSZ	s__Afipia_broomeae
s__Rhodospirillales_bacterium	s__Psychrobacter_immobilis	s__GGB59240_SGB81015
s__Acinetobacter_SGB86455	s__Microbacterium_aurum	s__Kocuria_indica
s__GGB60172_SGB82097	s__Cupriavidus_basilensis	s__Staphylococcus_epidermidis
s__Pseudolysobacter_antarcticus	s__GGB44028_SGB61402	s__Cutibacterium_acnes
s__GGB63824_SGB86185	s__Flavobacterium_circumlabens	s__Sphingomonas_paucimobilis
s__Variovorax_sp_PAMC28562	s__Pseudomonas_poae	s__Phormidesmis_priestleyi
s__GGB63920_SGB107338	s__Bradyrhizobium_viridifuturi	s__Moraxella_osloensis
s__GGB64512_SGB87014	s__Ralstonia_pickettii	s__Kocuria_tytonis
s__Candidatus_Nanosynsacchari_sp_TM7_ANC_38_39_G1_1	s__Caulobacter_vibrioides	s__GGB79622_SGB56399
s__GGB77798_SGB107851	s__Corynebacterium_singulare	s__Delftia_acidovorans
s__GGB56994_SGB78457	s__Sphingomonas_melonis	s__Kocuria_palustris
s__Methylobacterium_radiotolerans	s__Caulobacter_sp_602_1	s__GGB63823_SGB106877
s__GGB65401_SGB88423	s__Corynebacterium_sp_Marseille_P3884	s__Malassezia_restricta
s__filamentous_cyanobacterium_Phorm_46	s__Acinetobacter_baumannii	s__Glaciihabitans_sp_INWT7
s__Veillonella_parvula	s__Corynebacterium_accolens	s__Kytococcus_sedentarius
s__GGB68979_SGB93007	s__Corynebacterium_aurimucosum	s__GGB78165_SGB107680
s__Solobacterium_moorei	s__Chryseobacterium_gambrini	s__Staphylococcus_hominis
s__GGB77784_SGB107958	s__Paraburkholderia_fungorum	s__GGB58714_SGB80388
s__Pedobacter_psychrophilus	s__Burkholderia_sp_9120	s__Meiothermus_silvanus
s__Pseudomonas_aeruginosa	s__Sphingomonas_adhaesiva	s__Micrococcus_luteus
s__Brachybacterium_paraconglomeratum	s__Pseudomonas_sp_IB20	s__Frigoribacterium_sp_CG_9_8
s__Lancefieldella_rimae	s__Salinicoccus_roseus	s__Dermacoccus_nishinomiyaensis
s__GGB77632_SGB107138	s__Pseudomonas_sp_TNT3	
s__GGB58873_SGB104250	s__Sphingomonas_SGB56342	
s__GGB58876_SGB80565	s__Arthrobacter_psychrochitiniphilus	
s__GGB61111_SGB83162	s__Cupriavidus_pauculus	
s__Deinococcus_radiodurans	s__Pseudomonas_putida	
s__Glacieibacterium_frigidum	s__Corynebacterium_hadale	
s__GGB77574_SGB107205	s__Stenotrophomonas_maltophilia	
s__Kocuria_rhizophila	s__Alpinimonas_psychrophila	
s__Colibacter_massiliensis	s__Afipia_sp_OHSU_II_C1	
s__GGB77435_SGB104288	s__Pseudomonas_oryzihabitans	
s__Streptococcus_mitis	s__Kocuria_rosea	
s__Corynebacterium_suicordis	s__Pseudomonas_sp_N40_2020	
s__GGB65415_SGB108191	s__Herbaspirillum_huttiense	
s__Candidatus_Saccharibacteria_unclassified_SGB19847	s__Cloacibacterium_caeni	
s__GGB58853_SGB80540	s__Burkholderia_sp_4M9327F10	
s__GGB63822_SGB86182	s__Corynebacterium_afermentans	
s__GGB57857_SGB79473	s__Janthinobacterium_lividum	
s__GGB60315_SGB82269	s__Sphingomonas_psychrolutea	
s__Actinomyces_dentalis	s__Pseudomonas_oleovorans	
s__GGB77156_SGB104539	s__Corynebacterium_SGB17105	
s__GGB78578_SGB108364	s__Polaromonas_vacuolata	
s__Olsenella_sp_oral_taxon_807	s__Hymenobacter_nivis	
s__GGB58712_SGB106918	s__Sphingobium_yanoikuyae	
s__Chamaesiphon_minutus	s__Hydrotalea_flava	
s__GGB22433_SGB88768	s__Sphingomonas_zeae	
s__GGB77281_SGB106921	s__Acinetobacter_harbinensis	
s__Microcoleus_SGB104235		
s__GGB77784_SGB107959		
s__GGB65152_SGB88114		
s__GGB77431_SGB104291		
s__Actinomyces_sp_HMT897		
s__Tychonema_bourrellyi		
s__GGB77971_SGB108116		
s__GGB2722_SGB3663		
s__GGB26910_SGB39105		

Supplemental Table 5.5. Diversity permutations for White and Johnsons Glaciers.

	White Glacier	Johnsons Glacier	Perm p(eq)
Taxa S	89	82	**0.0203***
Individuals	80	91	0
Dominance	0.1431	0.5939	**0.0001*****
Shannon H	3.384	1.293	**0.0001*****
Evenness e^H/S	0.3312	0.04445	**0.0001*****
Simpson indx	0.8569	0.4061	**0.0001*****
Menhinick	9.951	8.596	**0.0001*****
Margalef	20.08	17.96	**0.0009****
Equitability J	0.7538	0.2935	**0.0001*****
Fisher alpha	0	400.4	**0.0001*****
Berger-Parker	0.25	0.7473	**0.0001*****

*denotes statistical significance ($p<0.05$)



Supplemental Figure 5.1. A. Location of White Glacier on Axel Heiberg Island in Nunavut, Canada. Photo credit: Google Earth. **B.** Arial view of White Glacier. Photo credit: Scott Sugden. **C.** Location of Johnsons Glacier on Livingston Island, Antarctica. Photo credit: Google Earth. **D.** Photo of Johnsons Glacier. **E.** One of the White Glacier cores used in this study. **F.** One of the Johnsons Glacier cores used in this study.