# MICROBIALLY MEDIATED BIOGEOCHEMICAL CYCLES

# IN POLAR ICE COVERED LAKES

by

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# DEDICATION

Dedicated to my father, Steve, for nature walks and helping me drill my first ice fishing holes through ice covered lakes; my mother, Terri, for her encouragement and sacrifice throughout my many years of school; and the rest of my family, Jordyn, Betty, Jerry, for their generosity, support, and love.

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# GLOSSARY AND NOMENCLATURE

Subglacial Lake Whillans – SLW

West Antarctic Ice Sheet – WAIS

Ross Ice Shelf – RIS

Whillans Ice Stream – WIS: The ice stream of focus in this dissertation.

Ice Stream: An ice stream is a type of fast flowing ice mass that moves at rates of several hundred meters per year promoted by sliding over deformable, saturated, subglacial till.

Grounding Zone – GZ: The point at which an ice sheet transitions from being a grounded ice sheet to a floating ice shelf over a marine water cavity.

Subaerial: Literally meaning under the air, a description of features that are located on the Earth's land surface and exposed to the atmosphere.

McMurdo Station – MCM

McMurdo Dry Valleys – MDV

Lake Fryxell – FRX

Taylor Valley – TV

Non-Purgeable Organic Carbon – NPOC

Excitation Emission Spectroscopy – EEMS

Total Organic Carbon – TOC

Parallel Factor Analysis – PARAFAC

# ABSTRACT

Lakes are important sites for globally-relevant biogeochemical cycles mediated by microorganisms. In the Arctic, seasonally ice covered thermokarst lakes are a large component in Earth's carbon cycle due to their methane emissions from organic carbon degradation. In the Antarctic, over 400 unexplored lakes exist beneath the Antarctic ice sheet with unknown biogeochemical contributions to the Earth system. This dissertation seeks to investigate the biogeochemical role of microorganisms in the lake habitat and how they interact with the seasonal and permanent ice covers of lakes in polar environments. Microbiologically clean hot water drilling was used to access a subglacial lake beneath Antarctica's ice to collect, for the first time, intact sediment and water samples. Laboratory experiments on Arctic and Antarctic, seasonally and perennially, respectively, ice covered lakes were used to investigate the impact of lake ice freezing regimes on microorganisms. My results show that subglacial lake sediments beneath the West Antarctic Ice Sheet contain solute ratios that suggest relict marine sediments were deposited during previous interglacial periods. Microbial activity overprints the marine geochemical signature to produce fluxes of ions into the Subglacial Lake Whillans water column, which ultimately drains to the Southern Ocean. Microbial activity in Subglacial Lake Whillans is partially fueled by biologically-formed methane diffusing from below our deepest collected (~38 cm) subglacial sediment samples. The ice above Subglacial Lake Whillans appears to be an important source of molecular oxygen for microorganisms to drive oxidative physiologies. My experimental evidence shows microorganisms incorporate into lake ice cover to, potentially, avoid increasing stressors from progressive lake ice freezing. Taken together, the results from this dissertation reinforce the hypothesis that subglacial environments beneath the Antarctic ice sheet are habitats for life. Further, the microorganisms in subglacial lakes participate in globally-relevant biogeochemical cycles. Here, I extend the extent of the biosphere and show sediments at the base of ice sheets are an active component of the Earth system.

# CHAPTER ONE

# INTRODUCTION: SEDIMENT MICROBIAL PROCESSES IN ICE-COVERED LAKES

## Lakes and Their Interactions with Ice: Global Importance

Approximately 2% of Earth's land surface is covered by lakes (Downing & Duarte 2006). Many of these lakes (15 – 40%) are located in Arctic and Subarctic regions (Duguay *et al.* 2003). Arctic landscapes, such as those on the North Slope of Alaska, are densely populated by shallow, methane-rich, thermokarst lakes (Matheus Carnevali *et al.* 2015; Hussey & Michelson 1966). These Arctic thermokarst lakes form and expand due to thawing of permafrost (Sepulveda-Jauregui *et al.* 2015). At the opposite pole, over 400 subglacial lakes have been identified beneath the Antarctic ice sheet (Wright & Siegert 2012). Some of these Antarctic subglacial lakes rival the size and depth of the Laurentian Great Lakes (e.g., Subglacial Lake Vostok). The ice-free regions of Antarctica, such as the McMurdo Dry Valleys (MDV), are characterized by a mosaic of habitat types, with perennial ice-covered lakes being a prominent feature (Spigel & Priscu 1998). The Tibetan Plateau, sometimes referred to as Earth's third pole, contains numerous glacially fed and proglacial lakes (Zhang *et al.* 2011). The polar regions contain abundant lakes that interact with ice, forming an important connection between the cryosphere and aquatic ecosystems on Earth (Brown & Duguay 2010).

Lakes are an important component of the landscape and seasonally ice covered lakes will be altered the most by a changing climate (Williamson *et al.* 2009; Saros *et al.* 2012). Climate change and the associated warming due to increases in greenhouse gas release will continue to alter the lake ice phenology. These changes to ice cover phenology will alter ecosystem services (e.g., biodiversity, food, water quality) and human recreation (e.g., ice fishing and ice hockey) provided by lakes with seasonal ice cover (Schallenberg *et al.* 2013; Brammer *et al.* 2014).

Seasonal lake ice phenological changes will alter the chemical and physical nature of the water column (See Figure 1 for the physical differences between summer and winter conditions of a typical seasonally ice covered lake), likely causing impacts on microbially mediated biogeochemical cycles (Bertilsson *et al.* 2013). For example, the ecological association between photosynthetic diatoms and their symbiotic bacteria *Pseudomonas syringae*. The ice nucleation active *P. syringae* catalyzes ice formation, which carries the symbiotic couple up into the photic zone where the diatom can photosynthesize and, presumably, *P. syringae* is provided organic carbon for heterotrophic growth (D'souza *et al.* 2013). This relationship is an example of where the carbon cycle and microbial interactions with ice on a seasonally ice covered lake are linked (D'souza *et al.* 2013). Further studies show that progressive lake ice freezing during the transition to winter decreases the abundance and diversity of planktonic microorganisms in the water column of lakes (Bertilsson *et al.* 2013; Safi *et al.* 2012). Additionally, lake sediment microbial activity is linked to primary productivity in the water column, which is influenced by seasonal stratification and lake ice cover duration

(Figure 1, Dean 1999; Jackson *et al.* 2007). The effects of climate change on lake ice cover will impact elemental cycling carried out by lake microorganisms (Jackson *et al.* 2007; Schroth *et al.* 2015; Post *et al.* 2009; Williamson *et al.* 2009).

Similar to the seasonally ice covered Arctic, high mountain and temperate lakes, perennially ice covered lakes are also impacted by changing climate (Fountain *et al*. *submitted*). The perennially ice covered lakes of the McMurdo Dry Valleys (MDV) region (Figure 1) are shown to be increasing in volume due to increased summer glacial melt water inputs (Fountain *et al. submitted*). Sub-aerial lake ice cover is different than the "ice cover" on a subglacial lake, as subglacial lakes are beneath much thicker ice masses (Figure 1). Ice cover on subglacial lakes is also removed, but on a much longer time scale than sub-aerial lakes. The West Antarctic Ice Sheet (WAIS) has experienced periods of expansive areal extent during glacial periods and significant retreat during interglacial periods (Oppenheimer 1998). The WAIS is a marine-based ice sheet and mostly (>50%) grounded below sea level (Fretwell *et al.* 2013). During interglacial periods, the WAIS showed significant retreat resulting in large regions of open ocean conditions (Scherer *et al.* 1998). The subglacial lakes that are present beneath the ice sheet now will be exposed to ocean sedimentation and inputs from primary productivity in the water column during the next interglacial period. However, the persistence of a subglacial lake is unknown during WAIS retreat due to the physical changes (e.g. sediment movement, depositional environment) associated with deglaciation. This loss of ice cover over a subglacial lake or disappearance of the lake altogether due to deglaciation will drastically change the active microbial populations and biogeochemical

functioning. Climate changes that regulate glacial-interglacial cycles, and thus ice mass extent, impact subglacial lakes and their associated microbially-mediated biogeochemical cycles, but on a much different timescale (thousands of years) compared to sub-aerial lakes (annual).

Glacial runoff during the summer melt season impacts glacially fed lakes and provides another example of how lakes interact with ice masses. Glaciers in Montana's high elevation environments provide lakes with a different nutrient regime compared to lakes fed by snow packs (Slemmons & Saros 2012). As glaciers disappear from the Montana landscape, the inputs to lake water column and lake sediment microbial communities will be different, potentially changing the community function within the lakes (Slemmons & Saros 2012). Glacially fed lakes on the Tibetan Plateau show an influence of glacial runoff stream-sourced microorganisms on the lake water column, but geochemistry and nitrogen availability is the strongest determinant of microbial community composition (Liu *et al.* 2014). As the glacial inputs decrease, the geochemistry of the lake may change and alter bacterial community composition and function. The glacially-derived impacts on the lake are in addition to those imparted by the seasonal ice cover that develops on high elevation lakes during the winter season. The changes or loss of ice masses which interact with lakes may alter the microorganisms inhabiting the lakes and their contribution to global elemental cycles.

Currently, lakes in North America are collectively sinks for carbon because burial processes are greater than $CO_2$ efflux (Butman *et al.* 2016). Ice covered conditions have a primary control on the carbon remineralization pathways due to the physical changes to

the lake (Bertilsson *et al.* 2013). The balance between carbon remineralization to $CO_2$ and burial is determined in the lake sediments by microorganisms. How ice cover changes impact the lake sediment microbial ecosystems and their role as a source or sink of carbon to the atmosphere is unclear. It becomes important to understand how and if water column and sediment microorganisms rely on, or interact with lake ice covers, as microbial communities are the engines that cycle nutrients and elements within lakes (Falkowski *et al.* 2008).

## The Lacustrine Sedimentary Environment

Lake sediments are excellent records of change during the transition from a glacial to an interglacial period (Krause & Whitlock 2013). Not only do lake sediments record change, but they are also biogeochemically active sites, where microbial activity participates in the global carbon, nitrogen and metal cycles (Deutzmann *et al.* 2011; Vitousek *et al.* 1997; Schroth *et al.* 2015) and actively exchange these metabolic products and reactants with the lake water column (Figure 1). Some lakes are growing in size and volume, such as in the McMurdo Dry Valleys, due to increased melt, and in the Arctic, due to permafrost collapse (Sepulveda-Jauregui *et al.* 2015; Fountain *et al.* Submitted). A lake's expansion increases its lake water column interaction with new sediments which provides more energy sources and carbon substrates for microorganisms in the lake water column (Sepulveda-Jauregui *et al.* 2015).

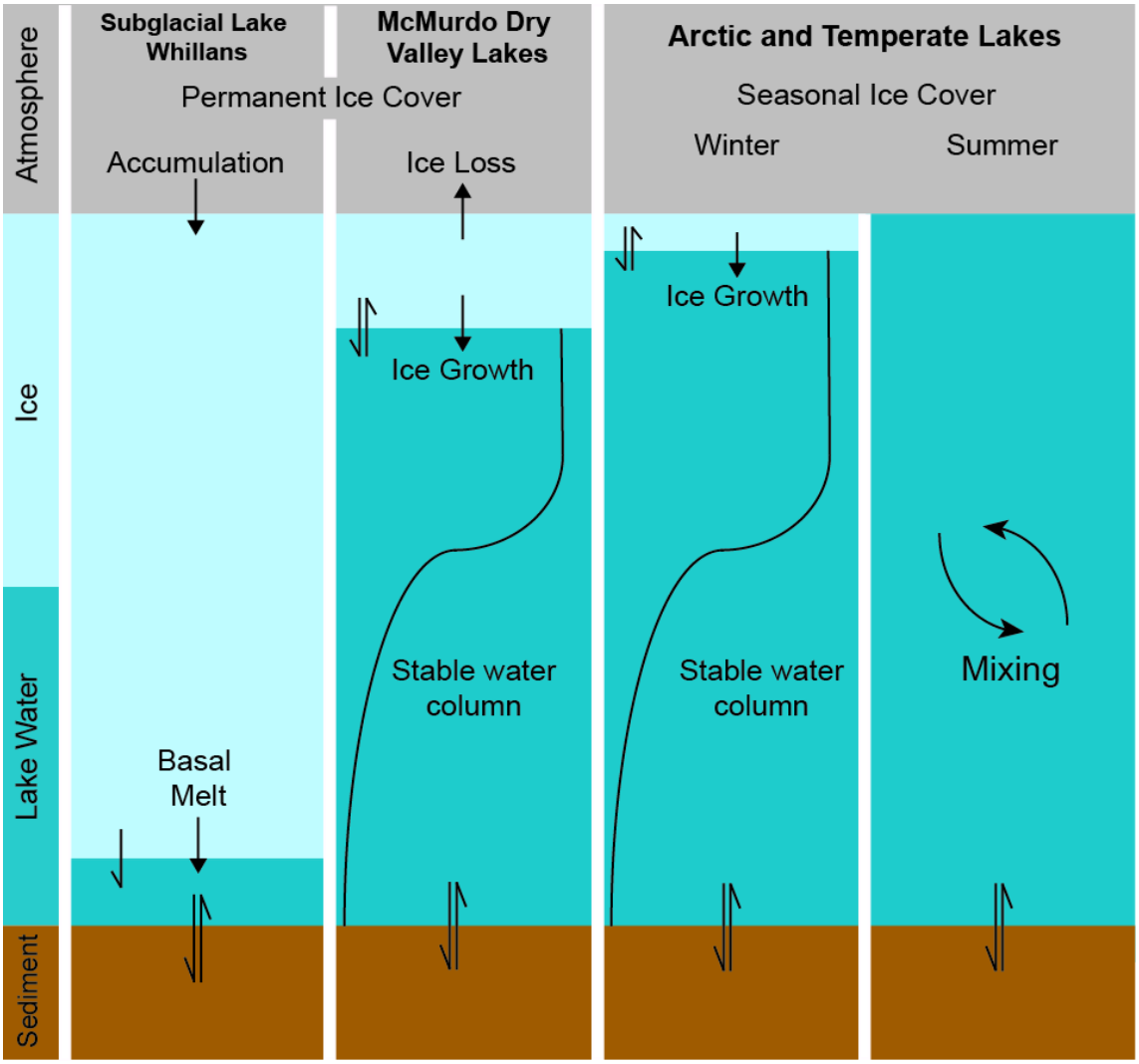



Figure 1. Overview of the three types of lakes studied in this dissertation (not drawn to scale). The three lakes all have different ice cover regimes that exchange gases, ions, nutrients and microorganisms (represented by the double reaction arrows, except in SLW where it is thought that high geothermal heat flux results in net melting at the base of the ice sheet, thus the flux of material is only out of the ice sheet) with the water column. All lakes are shown interacting with the sediments to emphasize the importance of lake sediment fluxes and exchanges of gases, ions, nutrients and microorganisms (double reaction arrow). SLW has 800 m of ice sheet overlying ~2.2 m of water column, which was accessed by clean, hot water drilling (Chapter 2, Appendix 1). SLW lake sediments contain metabolically active microorganisms that influence the flux of ions (Chapter 3) and gases (Chapter 4) to the water column. The MDV lakes have variable ice and water column thicknesses. The MDV lake of interest, Lake Fryxell, has ~4 m of ice cover overlying a ~18 m water column. The Arctic and temperate lake columns represent lakes near Barrow, AK and Montana, highlighting the winter and summer ice cover regimes (Chapter 5).

Table 1. Physical properties and surface area to volume ratio for lakes studied in this dissertation (bold) and other major lakes around the world.

| Lake | Location | Surface area ($km^2$) | Volume ($km^3$) | SA:vol ratio | Citation |
|---|---|---|---|---|---|
| **Subglacial Lake Whillans** | **West Antarctica** | **60** | **0.12** | **500** | **Siegfried et al 2014** |
| **Ikroavik** | **Arctic** | **7.4** | **0.015** | **493** | **Hussey and Michelson 1966** |
| **Emakisoun** | **Arctic** | **2.2** | **0.0048** | **458** | **Hussey and Michelson 1966** |
| Lake N1 | Arctic | 0.05 | 0.00017 | 294 | Toolik Lake LTER |
| **Dailey** | **USA** | **0.88** | **0.0035** | **251** | **fwp.mt.gov** |
| Great Salt Lake | USA | 3750 | 19 | 197 | worldlakes.org |
| Toolik Lake | Arctic | 1.5 | 0.01 | 150 | Toolik Lake LTER |
| Mono | USA | 175 | 2.8 | 63 | worldlakes.org |
| Erie | N. America | 25700 | 484 | 53 | worldlakes.org |
| Subglacial Lake Ellsworth | West Antarctica | 28.9 | 1.37 | 21 | Ross et al., 2011 |
| Huron | N. America | 59600 | 3540 | 17 | worldlakes.org |
| Great Slave Lake | Canada | 27000 | 2088 | 13 | worldlakes.org |
| Michigan | N. America | 57800 | 4920 | 12 | worldlakes.org |
| Titicaca | S. America | 8400 | 932 | 9 | worldlakes.org |
| Superior | N. America | 82100 | 12100 | 7 | worldlakes.org |
| Subglacial Lake Vostok | East Antarctica | 15690 | 5400 | 3 | Rogers et al., 2013 |
| Baikal | Russia | 31500 | 23600 | 1 | worldlakes.org |

Sediment associated processes such as diffusion, resuspension, and bioturbation all have marked influences on lake processes and the strength of these factors on the water column is determined by lake morphology (Figure 1, Carpenter 1983). The ratio of lake surface area to volume approximates the importance or influence sediments will have on the biogeochemistry of the lake (Table 1). A high surface area to volume ratio indicates a strong sedimentary influence on the lake water column. The lakes I have studied (Highlighted in bold in Table 1), have a large surface area to volume ratio compared to many other lakes around the world. The high surface area to volume relationship illustrates the importance of sedimentary microbial processes in the lakes I have studied.

Microbial activity in sediments typically follows classical ecological niche-partitioning where thermodynamically favorable electron donors and acceptors are first

utilized by the organisms with the greatest competitive advantage (Postma & Jakobsen 1996; Froelich *et al.* 1979). This competition between microorganisms creates zones within the sediment profile and these zones are inhabited by distinct physiological guilds of microorganisms. At the top of the sediment, if oxic, near the sediment-water interface, microorganisms that utilize molecular oxygen as a terminal electron acceptor outcompete other physiological guilds (Falkowski *et al*. 2008). As oxygen concentrations decrease with depth, other less energetically-favorable terminal electron acceptors determine the functional group of microorganisms present (Froelich *et al*. 1979). The distribution of microbial populations change porewater concentrations of geochemical species directly related to the physiological process (e.g., sulfate reduction generates sulfide and depletes the sulfate pool, Jørgensen 1982). These physiological processes of microbial metabolism are coupled to water column processes and ice cover (Ellis-Evans 1984).

## Subglacial Sediments

The subglacial environment has only recently been recognized as a habitat for microorganisms (Sharp *et al.* 1999, Skidmore *et al.* 2000) and a diversity of active physiological groups of microorganisms are present in subglacial habitats (i.e., Skidmore *et al.* 2005; Christner *et al.* 2014; Foght *et al.* 2004). These subglacial habitats are characterized by both aerobic and anaerobic niches, filled by functional groups of microorganisms carrying out many different physiological processes. Mountain and outlet glaciers harbor chemolithoautotrophic sulfide and iron oxidizing microorganisms in their subglacial sediment (Lanoil *et al*. 2009; Mitchell *et al*. 2013). Heterotrophic

microorganisms are also known to be present beneath the Antarctic Ice Sheet (Lanoil *et al.* 2009; Christner *et al.* 2014). These particular physiologies require oxygen to be present in the subglacial environment for both inorganic carbon fixation and organic carbon oxidation. In subglacial habitats with oxygen delivery, aerobic microorganisms are significant because their metabolisms have high free energy yields when coupling the oxidation of organic carbon to the reduction of oxygen, allowing ample metabolic energy. Despite being cold, dark and low-energy, deep biosphere environments, subglacial sediments with oxygen delivery show a potential to have high energy oxidative physiologies present (Falkowski *et al*. 2008; Christner *et al* 2014).

Oxic respiration by subglacial microorganisms can drive subglacial sediments to anoxic conditions, but hydrological conditions which isolate parts of the glacial bed from water flow will decrease oxygen supply and create zones of anoxia as well (Tranter *et al.* 2005). Once oxygen is depleted in the subglacial environment, anaerobic microorganisms become prevalent. These anaerobic microorganisms are shown to be endogenous to the subglacial environment (Hamilton *et al.* 2013). Most notably, methanogenic archaea have been found in numerous subglacial environments (Boyd *et al.* 2010; Wadham *et al.* 2012; Stibal *et al.* 2012; Dieser *et al.* 2014) and link the activity of subglacial microorganisms with globally-relevant biogeochemical cycles.

Subglacial water transport through subglacial sediments is highly variable among different glacier and ice sheet types. Although subglacial water transport is not the focus of this introduction or dissertation, it exerts a first order control on the spatial distribution of functional groups of microorganisms which persist depending on the dissolved

constituents of the subglacial water (e.g., $O_2$, $NO_3^-$) (Tranter *et al.* 2005). As subglacial water is routed through distributed drainage channels (e.g., mountain glaciers), flow rate decreases which allows microorganisms to consume the dissolved oxygen (Tranter *et al.* 2005). Water and dissolved oxygen are transported to the base of ice masses through a variety of pathways, such as moulins in the Greenland Ice Sheet (GrIS), englacial conduits in temperate glaciers, or lateral transport from melt-water streams along lateral glacial margins (Benn & Evans 2010; Tranter 2004; Tranter *et al.* 2005). Transport of gases, solutes, microorganisms or particulates to the base of the Antarctic Ice Sheet occurs through the continual downward conveyor belt due to accumulation on top and basal melt at the bottom, releasing what was once deposited at the surface. These hydrologic and glacial dynamics control the distribution and activity of aerobic and anaerobic microorganisms in subglacial sediments (Foght *et al.* 2004; Boyd *et al.* 2011; Montross *et al.* 2013; Tranter *et al.* 2005).

Microorganisms in both oxic and anoxic subglacial environments are also known to increase the rate of mineral weathering (Montross *et al.* 2013; Tranter *et al.* 2005). Biotic weathering is a pervasive force in non-glaciated catchments, where plant roots, fungi, and lichens physically and chemically weather rocks and thereby increase the dissolved solute load of river systems draining to the ocean (Uroz *et al.* 2009). Microorganisms actively weather rocks by removing electrons from the mineral (e.g., sulfide oxidation from pyrite) (Mitchell *et al.* 2013). Also, microorganisms will actively colonize minerals that contain limiting nutrients such as phosphate contained within apatite (Rogers *et al.* 1998; Rogers & Bennett 2004). However, microorganisms do not

need to be in contact with a mineral surface to enhance mineral weathering (Hutchens *et al.* 2006). One pathway of indirect microbial weathering involves byproducts of microbial metabolism (e.g., fermentation produces organic acids). These byproducts interact with the sedimentary matrix resulting in indirect weathering of minerals due to the local decrease in pH caused by fermentative organisms (Uroz *et al.* 2009). Both direct and indirect microbially-mediated weathering processes are present in the subglacial environment and ultimately influence the dissolved load in the subglacial discharge (Montross *et al.* 2013; Mitchell *et al.* 2013). The discovery of active microorganisms in the subglacial environment shows that, despite being a permanently cold environment, these microorganisms can contribute to the global weathering budget and match chemical denundation rates of temperate catchments (Tranter 2004).

## A Hybrid Environment: The Subglacial Lake

Subglacial lakes form beneath ice sheets where water collects in a hydropotential low (e.g., Subglacial Lake Whillans) or geological features retain water (e.g., Subglacial Lake Ellsworth and Vostok) (Fricker *et al.* 2011; Lukin & Bulat 2011; Ross *et al.* 2011). Subglacial lakes also form below ice caps where active volcanism produces meltwater and the caldera bounds the lake (e.g., Subglacial Lake Grímsvötn) (Marteinsson *et al.* 2013; Gaidos *et al.* 2009). Subglacial lakes possess some similar features to subaerial lakes such as the interaction between the water column and sediments. On the other hand, subglacial lakes are exposed to glacial activity which shears and carries new sediment into the lake basin (Powell & Hodson 2014). The glacial activity can also divert water

flow away from the lake or promote new drainage paths into the lake (Carter *et al.* 2013). These physical processes create a dynamic lake state in contrast to the stability of permanently ice-covered lakes of the McMurdo Dry Valleys (Figure 1).

## Significance of My Research

Permanently cold environments constitute 80% of Earth's biosphere (Russell 1990) and microorganisms are the biological machines that drive Earth's biogeochemical cycles (Falkowski *et al.* 2008). Thus, microorganisms adapted for life in the cold are a significant component of the biogeochemical cycles on Earth. My studies of microbial processes in an Antarctic subglacial lake are changing the way the Antarctic continent is viewed. Previous research demonstrated that microorganisms could exist beneath the Antarctic Ice Sheet (e.g. Lanoil *et al*. 2009), but their abundance, activity and function was largely unknown. Data collected for this dissertation contributed to the conclusion that microorganisms are metabolically active and likely to be widespread beneath the Antarctic Ice Sheet. This conclusion vastly expands Earth's active biosphere. Antarctic subglacial lakes, like the ice-covered lakes in Minnesota, Montana, the Arctic and the Tibetan Plateau, are important contributors to global biogeochemical cycles. The extent and significance of microorganisms in polar ice covered lakes and lake sediments drive my research and the chapters of this dissertation.

## Hypotheses and Objectives

The following three hypotheses have driven my research on biogeochemistry of polar ice covered lakes.

*Hypothesis 1:* Access to subglacial aquatic environments can be achieved through microbiologically-clean hot water drilling.

*Hypothesis 1 Objectives:*

1. Develop a method to measure low-level concentrations of bacterial cell biomarkers.
2. Determine the efficacy of the filtration system to remove microbial cells, particulates and organic carbon.
3. Monitor the filtration system during drilling operations into subglacial aquatic environments.
4. Characterize the borehole water prior to subglacial lake breakthrough.

*Hypothesis 2:* Subglacial lakes contain active microbial communities that modify the geochemistry of their environment.

*Hypothesis 2 Objectives:*

1. Characterize the sources of geochemical species in the lake water column and sediment porewater.
2. Analyze geochemical data for evidence of microbial activity.
3. Calculate fluxes of geochemical species to the water column to determine the sediment microbial community impact on SLW water.

*Hypothesis 3:* Ice supports microorganisms living in lakes and subglacial aquatic environments.

*Hypothesis 3 Objectives:*

1. Determine distribution of microbial functional groups in the lake and sediments.
2. Quantify the geochemical segregation between seasonal and perennial lake ice covers using mesocosm experiments.
3. Quantify the bacterial segregation between seasonal and perennial lake ice covers using mesocosm experiments.
4. Quantify the amount of oxygen delivered to and consumed in subglacial lakes.

## Structure of this Dissertation

There are four chapters in this dissertation that are structured for submission or are already submitted to peer-reviewed journals. The first paper (Chapter 2) describes the results of our clean access monitoring during two field seasons and three hot water drill boreholes. This paper represents a follow-up study to the laboratory-based study we published in 2013, which is included in Appendix 1 of this dissertation. As the first paper in the dissertation, it serves the role of reminding readers that first and foremost we considered clean sampling a priority. The next paper (Chapter 3) is a description of the solutes and an analysis of their sources in the Subglacial Lake Whillans water column and sediments. This paper expands the dataset and geochemical discussion presented in our initial report on a microbial ecosystem beneath the WAIS (Christner *et al.* 2014).

Chapter four is the final Subglacial Lake Whillans paper in the dissertation and describes the microbial methane transformations in Subglacial Lake Whillans. It is a geochemical-based approach describing and quantifying the methane production and consumption in SLW. Molecular microbiology data supports this primarily geochemical study showing active methane oxidizers are abundant at the sediment-water interface. The final paper in the dissertation (Chapter 5) shows that bacteria are incorporated into the ice cover of both seasonally and perennially ice-covered lakes in Montana, Antarctica, and the Arctic. It does demonstrate that a biological response to lake freezing is prevalent across lakes from both poles. The final chapter (Chapter 6) is a summary of the conclusions of this dissertation as well as the future directions for work in subglacial aquatic habitats. The summary uses the theme “Biological benefits of ice” as a way to tie these first five chapters together as well as the two appendices and other ice-related papers I have published while a graduate student. Appendix two is a paper detailing the role of biological ice nucleation in hailstone formation. It does not deal with a lake environment, but does have a strong aspect of microbial interactions with ice, thus appropriate for this dissertation. It also occupied a significant portion of my time in graduate school and the dissertation would not seem complete without it. Lastly, I have been a part of several other studies, including one in the Arctic (Matheus Carnevali *et al.* 2015) and several on the Tibetan Plateau (Liu *et al.* 2014, Liu *et al.* 2015). These papers are not included in the dissertation, but have been brought into the discussion of the global importance of lakes and their interactions with ice to highlight the entire breadth and scope of the work I completed while a graduate student.

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# CHAPTER TWO

## FIELD TESTING OF A CLEAN, HOT WATER DRILL USED FOR ACCESS TO SUBGLACIAL AQUATIC ENVIRONMENTS

### Contribution of Authors and Co-Authors

Author: Alexander B. Michaud

Contributions: Collected, processed and analyzed samples. In particular, ran ATP samples and performed leaching experiment. Analyzed data, wrote the manuscript, and prepared figures and tables.

Co-Author: Trista J. Vick-Majors

Contributions: Collected, processed and analyzed samples. In particular, ran DOC and EEMS samples, cell counts, and leaching experiment. Analyzed data and helped to write the manuscript.

Co-Author: Mark L. Skidmore

Contributions: Collected, processed and analyzed samples. Reviewed the manuscript and provided feedback.

Co-Author: John C. Priscu

Contributions: Wrote the proposal to fund the project (NSF-OPP 0838933, 1346250, 1439774). Collected samples. Reviewed the manuscript and provided feedback.

Manuscript Information Page

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## Abstract

Antarctic subglacial aquatic environments are an important component in the Earth system with biological activity contributing to globally important biogeochemical cycles. In order to access these environments cleanly, an internationally accepted drilling strategy was developed. A hot water drill with a filtration system for decontaminating snow melt was built and previously tested. Here we report the microbiological and chemical results from the clean access samples collected during hot water drilling access to three subglacial aquatic ecosystems, as recommended by the committee on Subglacial Antarctic Lake Exploration. We show that Antarctic surface snow is an ideal starting material for clean access to subglacial aquatic ecosystems and that hot water drilling and the filtration system effectively remove microorganisms from the snow melt. Point sources of contamination to the drill water were evaluated and shown to contribute negligible amounts of carbon to the drill water. We provide recommendations for future clean access subglacial lake research based on our data and experiences.


## Introduction

Antarctic subglacial aquatic environments (ASAE) are pristine habitats of great scientific importance (Skidmore 2011). These aquatic habitats beneath the thick ice sheets of Antarctica contain microbial ecosystems, which comprise a globally important reservoir of microbial cells (Christner *et al.* 2014, Priscu *et al.* 2008). These microorganisms contribute to global biogeochemical cycles of carbon, nitrogen, and other elements. Subglacial environments also possess sedimentary packages of paleoenvironmental and paleoclimatic importance (Bentley *et al.* 2013, Scherer *et al.* 1998). Further, the direct measurement of geothermal heat and basal sliding at the bed of ice sheets greatly improves global models of ice sheet mass-balance (Fisher *et al.* 2015, Engelhardt *et al.* 1990, Engelhardt & Kamb 1998). These globally-important datasets are dependent on access to the subglacial environment.

Subglacial aquatic environments can be accessed by mechanical drilling or melting (Rack 2016, Bulat 2016); each approach has advantages and disadvantages. Mechanical drilling is slow and requires drilling fluid to lubricate the drill bit and help carry ice chips up and out of the borehole. Drilling fluids are usually high molecular weight hydrocarbons (i.e., kerosene, foranes) and maintaining sterility is difficult (Bulat 2016). The drilling fluid keeps the borehole from refreezing; temperatures at the mid-depth of the ice sheet can reach ~-30 to -40°C and typically lead to fast borehole refreezing rates (Bulat 2016, Makinson *et al.* 2016), limiting the ability to access aquatic habitats at the bed of the ice sheet. Mechanical drilling has been used to access Subglacial Lake Vostok (SLV) following the collection of ice cores. Mechanical drilling for ice cores, while slow, can yield ice core records representing thousands of years of past climate data (Buizert *et al.* 2015, Petit *et al.* 1999).The SLV lake water microbiology results remain questionable due to contamination issues (Alekhina *et al.* 2007). Hot water drilling has been used to access Subglacial Lake Whillans (SLW; Antarctica) and Subglacial Lake Grímsvötn (Iceland) and was attempted at Subglacial Lake Ellsworth (SLE; Antarctica) (Makinson *et al.* 2016, Rack 2016, Gaidos *et al.* 2009). Regardless of the method used to sample ASAE, environmental stewardship and sample integrity must guide scientific sampling plans.

The protection of and standards for responsible exploration of ASAE were detailed in a report by the National Research Council (NRC, 2007). The NRC 2007 report emphasized that while the further understanding of ASAE should proceed through direct exploration and sampling of subglacial aquatic habitats, sampling and instrumentation of

the subglacial environment must keep Antarctic stewardship at the forefront of planning (NRC 2007). In addition, stewardship, sample and scientific integrity must drive engineering and science plans for access to ASAE. Following the publication of the NRC report, the Scientific Committee on Antarctic Research (SCAR), the international governing body on scientific research in Antarctica, assembled a group of international scientists to develop a Code of Conduct based on the NRC report (Doran & Vincent 2011). Concurrent with the SCAR Code of Conduct document and the NRC report, the Whillans Ice Stream Subglacial Access Research Drilling (WISSARD) project developed and tested a clean access filtration system to generate hot drill water for access to the ASAE (Priscu *et al*. 2013). The WISSARD hot-water drill (Burnett *et al.* 2014, Rack *et al.* 2014, Blythe *et al.* 2014) integrated mechanical filtration, ultraviolet (UV) lights, and pasteurization components to maintain drill water cleanliness. This clean access filtration system was tested in controlled laboratory experiments to better understand the efficacy of each component of the filtration system (Priscu *et al.* 2013), before use on the West Antarctic Ice Sheet. These laboratory tests provided evidence that the filtration system would effectively clean the drill water of microorganisms and organic carbon to internationally acceptable levels and protect forward contamination of the ASAE (Priscu *et al.* 2013). Recommendation 13 of the NRC 2007 report and the SCAR Code of Conduct document emphasize that methods should be developed for drilling and sampling during access to the ASAE. Concurrent with this recommendation, the WISSARD project implemented a clean access, filtration system sampling plan to monitor the cleanliness of the drill water during subglacial access. The WISSARD

project has now completed two field seasons in Antarctica. During these field sampling campaigns, one subglacial lake and two sub-ice shelf, marine cavity habitats were sampled. Here, we adhere to the SCAR Code of Conduct document (Doran & Vincent 2011) and the NRC 2007 report by presenting results from our field-based monitoring of the clean access hot-water drill and potential point sources of contamination, as well as discuss recommendations based on our experiences for future drilling projects to ASAE.

## Methods

### Drill System

The same drill system was used throughout our field campaigns, except that consumables (filters) were replaced between uses. The WISSARD hot water drill uses melted snow as a water source. Snow is melted in an open tank which includes a closed loop of coils that recirculates glycol from the generators to harvest waste heat for snow melting (Blythe *et al.* 2014). The melted snow is pumped into a large holding tank (Fig. 1, “Water Tank”). The water moved from the water tank to the filtration system, within the shipping container (Fig. 1, “Filtration Unit”). A sampling port (port 1) was installed immediately before the water entered the first filter unit (Fig. 1). Once in the filtration unit, the water passed through 3 µm and 0.2 µm filters, and then separate 185 nm and 245 nm UV light banks. After the filtration unit, the water was heated by four Alkota model 12257K pressure washers (water heaters) to ~90°C (Fig. 1, “Water Heaters”; Rack *et al.* 2014). After heating, the water traveled to the reel container unit where it entered the main drill hose and went down borehole to the drill head (Fig. 1). Two sampling ports

were installed after the heating units. The first was immediately after the water left the heaters (port 7) and the other just before the water entered the 1 km drill hose (Fig. 1, “Port 8”). We sampled either port 7 or 8 during the different field seasons. Here, we present and discuss port 7 and 8 as the same sample since the water has passed through both the filtration system and heaters when sampled from these ports. The only difference between port 7 and 8 is ~ 15 m of hose that carries water from port 7 to port 8. We label and present port 7 and 8 only as port 8 throughout. Water flowed up the borehole after being discharged from the drill head to a return water pump located ~100 m beneath the surface. The return water pump brought water to the surface, where a sampling port was installed (Fig. 1, “Port 9”), then the water traveled to the water tank for holding. The drill was operated in a recirculating format when the drill water returned to water tank to be refiltered and reheated. This set up was used at SLW and GZ field sites. At the MIS site, the drill water was not returned to the water tank, and therefore this was considered a non-recirculating system.

McMurdo Ice Shelf

During the 2012-2013 Antarctic field season we tested the drill and filtration system near McMurdo Station. The drill site was on the McMurdo Ice Shelf (MIS) (77.8902°S, 167.0083°E) approximately 8 km from the edge of the MIS, and another 40 km from the sea-ice to open water transition (Vick-Majors *et al.* 2015). Drilling operations took place from 17 Dec 2012 to 18 Dec 2012. The ice was 56 m thick above an 872 m water column. The drill was operated in a non-recirculating format to avoid contamination of hoses and equipment with seawater prior to drilling at Subglacial Lake

Whillans (SLW). We sampled the melted snow, before it entered the filtration system and heaters (port 1) and before the heated drill water went down borehole (port 8; Figure 1) at three time points which represented the beginning of drilling operations (drill at 0 m), half-way through the ice (27 m), and just after breaking through the base of the ice shelf. We collected samples for cellular adenosine-5'-triphosphate (ATP) and cell counts in acid-washed, combusted (4 h, 450°C) glass bottles.

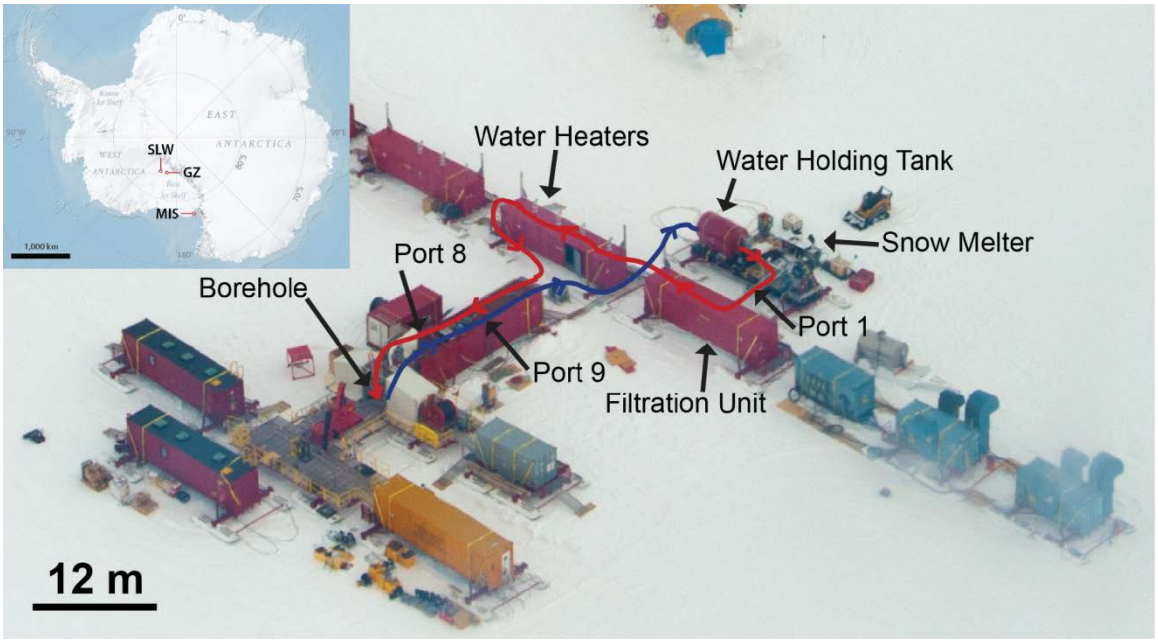



Figure 1. SLW hot water drill set up with sampling ports and drill system components labeled. The red line indicates down borehole water in the direction of the arrowheads on the line. The blue line indicates water coming up the borehole from the return water pump in the direction of the arrowheads on the line. The inset map indicates the three field sites where clean access samples were taken. Inset map by Brad Herried, Polar Geospatial Center. Camp overview photo courtesy of JT Thomas.

Subglacial Lake Whillans

The drill system was traversed to the SLW drill site (84.240°S, 153.694°W), where drilling operations were conducted from 27-30 January 2013 (Tulaczyk *et al.*

2014). The filtration system was monitored using a similar sampling plan to the MIS test, but the drill was run in re-circulating format with a return pump suspended ~100m below the snow surface in the borehole. We sampled ports 1, 8 and cold drill water returning from the borehole (port 9) at the beginning of drilling and prior to breakthrough to SLW. The same set of samples (ATP and cell counts) collected during the MIS test was collected at SLW.

Grounding Zone

The hot-water drill was used to access the sub-Ross Ice Shelf marine water cavity during the 2014-2015 Antarctic field season. The drill site (-84.335° S, -163.613° W) was approximately 13 km downstream from the Whillans Ice Stream grounded margin. The drill and filtration system were set up in the same configuration as the SLW site. We sampled the drill water at the beginning of drilling (0 m), ~50% through the ice shelf (~350 m), and ~75% through the ice shelf (~600 m). Samples were collected at ports 1 and 8 for the same measurements as sampled at the MIS and SLW drill site.

Leaching Experiments

Sample sections of the WISSARD drill hose inner liner (Teflon) were tested for chemical tolerance at simulated hot, drill water temperatures (~90°C). Prior to testing, three 10 cm segments of hose liner were cut with a razor blade, cleaned with Alconox soap and rinsed 6 times with Milli-Q water. Each hose segment was incubated in a separate, pre-combusted (450° C for ~4 h), foil-covered bottle containing 500 mL of Milli-Q water at 90°C (± 5°C) for 106.5 hours, along with triplicate control bottles

containing no hose. Samples (~20 mL) were collected from each incubation bottle at 12.50, 24.25, 36.50, 59.50, 87.50, and 106.50 h by pouring water into pre-combusted 40 ml amber vials. Time zero samples were determined by decanting Milli-Q water directly into sample vials.

A Niskin bottle (5 L) with a Teflon coated spring closing mechanism was rinsed several times with MilliQ water, then filled with MilliQ water and closed. The water in the Niskin bottle was sampled once a day for three days and samples were placed in acid leached and furnaced amber glass vials and stored at 4°C until analysis.

Sample Analysis

Procedures for measuring the concentrations of cells in water from the field tests followed the same protocols outlined by Priscu et al. 2013. Briefly, water collected for direct cell counting was fixed with sodium-borate buffered formalin (5% final concentration), 10 or 100 mL of sample was filtered onto 0.2 μm black polycarbonate filters, stained with SYBR Gold (Molecular Probes, Inc.), and enumerated by counting 30 fields of view or 300 cells using an epifluorescence microscope (Zeiss, Akioskop 50). SYBR Gold stains intracellular DNA and represents cells with intact cell membranes and cellular DNA. The concentration of cellular ATP, a proxy for metabolically viable microbial biomass, was measured using the luciferin/luciferase assay (Lundin 2000). Extracellular, dissolved ATP was removed by filtration through a 0.2 μm syringe filter (Pall Corp.). 100 mL of sample water was filtered, and cells trapped on the filter were lysed by adding 200 μL of lysis reagent (Biothema Corp.). Measurements were made with a 20/20n luminometer (Turner Bio Systems) after the addition of 50 μL of the

luciferase/luciferin reagent. Light emission was integrated over a 10 s period for each measurement.

Non-purgeable organic carbon (NPOC) samples from the hose leaching experiments were analyzed with a Sievers Portable 900 TOC analyzer with an inorganic carbon remover unit run in-line. NPOC samples from the Niskin bottle leaching tests were measured on a Shimadzu TOC-V using the high-sensitivity oxidation catalyst after acidification and sparging (Vick-Majors *et al.* 2015).

## Results

### Geochemistry

The pH of input snow and sampling ports at SLW and GZ field sites was slightly acidic (~5). The conductivity of input snow and sampling ports was low (<6 µS $cm^{-1}$; Table 1).

Table 1. The pH and conductivity for sampled ports at SLW and GZ drill sites.

| | Sample | pH | EC (25°C; µS $cm^{-1}$) |
|---|---|---|---|
| SLW | Input Snow | 5.9 | 3.3 |
| | Port 1 | 5.8 | 4.1 |
| | Port 8 | 5.1 | 5.6 |
| | Port 9 | 5.2 | 4.4 |
| GZ | Input Snow | 5.1 | 3.5 |
| | Port 1 | 5.1 | 4.4 |
| | Port 8 | 5.1 | 4.7 |

ATP

Port 1 ATP levels were the highest at all time points and drill sites (Fig. 2). ATP levels decreased to near background levels at port 8, after water passed through the filtration and pasteurization components before going down the borehole. The blank ATP concentrations are not significantly different from the port 8 ATP concentrations ($p < 0.05$) at the three drill sites. The return water coming up from the borehole was sampled at port 9 only at SLW and the ATP concentration was within the same order of magnitude as before going down borehole (Fig. 2). The $T_1$ and $T_2$ ATP levels at port 8 were less than port 1 at all drill sites. Some sample sites at specific timepoints and ports do not show bars indicating no samples for ATP analysis were collected (Fig. 2).

Cell Counts

Cell counts were highest in port 1 samples from all drill sites (Fig. 3). After passing through the filtration system and pasteurization steps, cells decreased by 25-fold. Cell concentrations decreased 5-fold from port 1 to port 8 at the SLW drill site $T_0$ sampling (Fig. 3). The MIS site had cell count samples taken at all three time points throughout the drilling process and showed that port 8 was always less than port 1. Some sample sites at specific timepoints and ports do not show bars indicating no samples for cell counts were collected (Fig. 2).

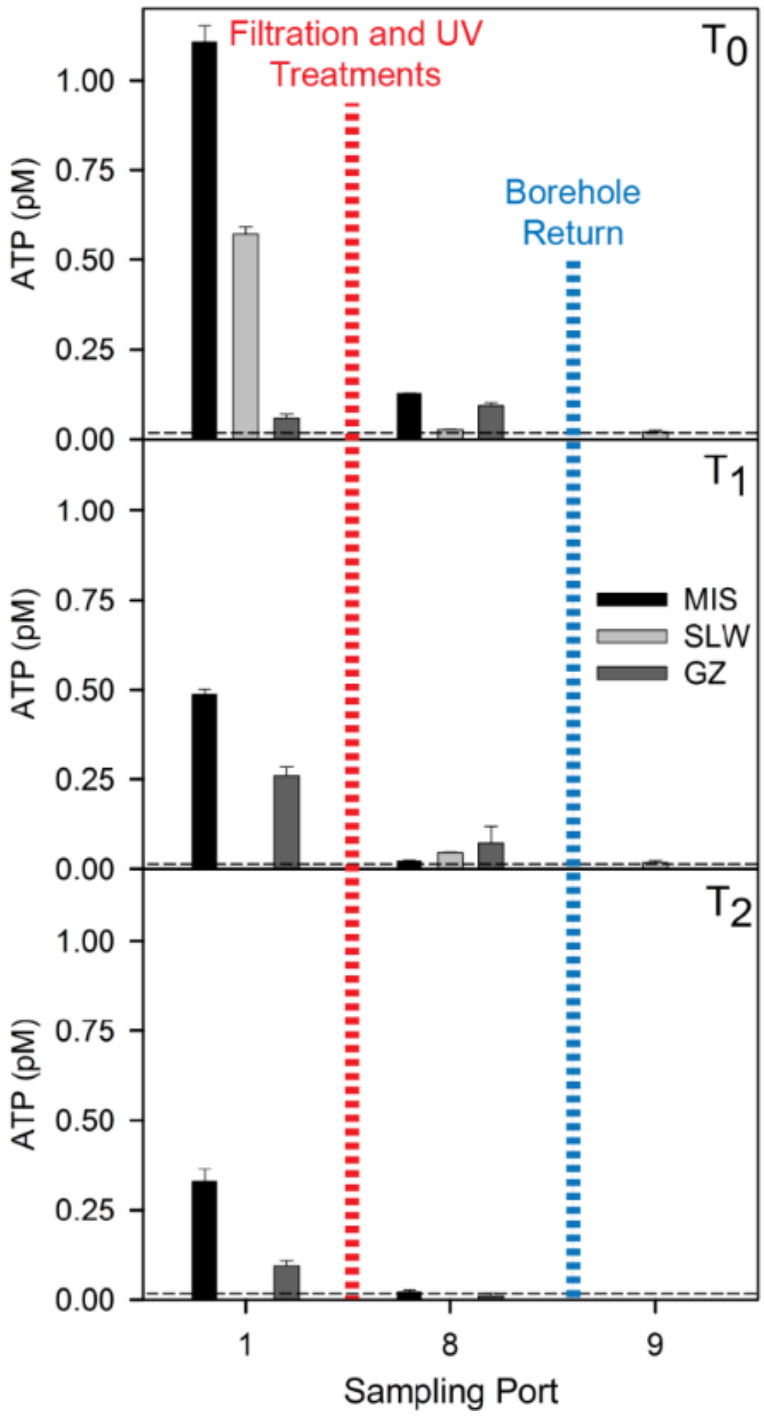



Figure 2. ATP concentration from all three drill sites and time points throughout the drilling process. Horizontal dashed line is average blank value from the three drill sites. The red text indicates hot water, as in Figure 1, going down borehole and the blue represents cold water returning from the borehole and going to the water tank.

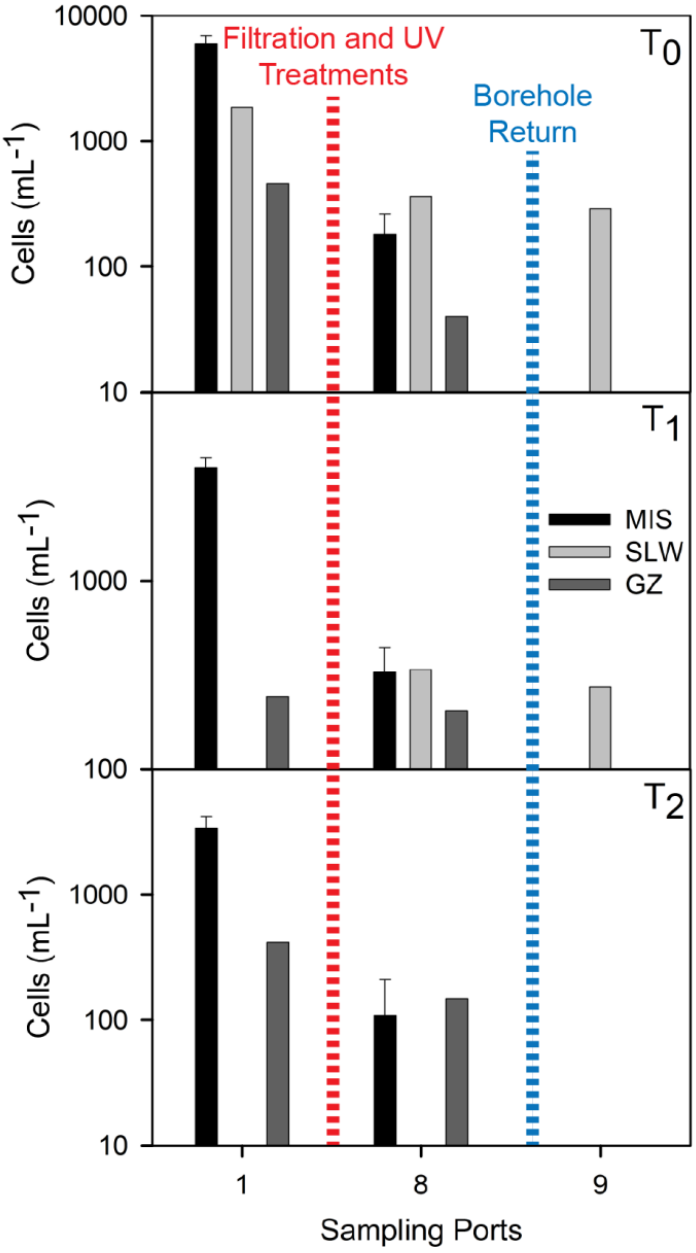



Figure 3. Cell concentrations from all three drill sites and time points throughout the drilling process. Cell concentrations from all three drill sites and time points throughout the drilling process. The red text indicates hot water, as in Figure 1, going down borehole and the blue represents cold water returning from the borehole and going to the water tank.

Leaching Tests

The drill hose liner leached NPOC into the water compared to a control bottle without a hose section. The NPOC leached rapidly from the liner, within the first 12 hours of incubation, but further NPOC accumulation was not observed over the next 100 hours (Fig. 4A). All measurements of NPOC from the Niskin bottle leaching tests were below the methodological limit of detection of 100 ppb.

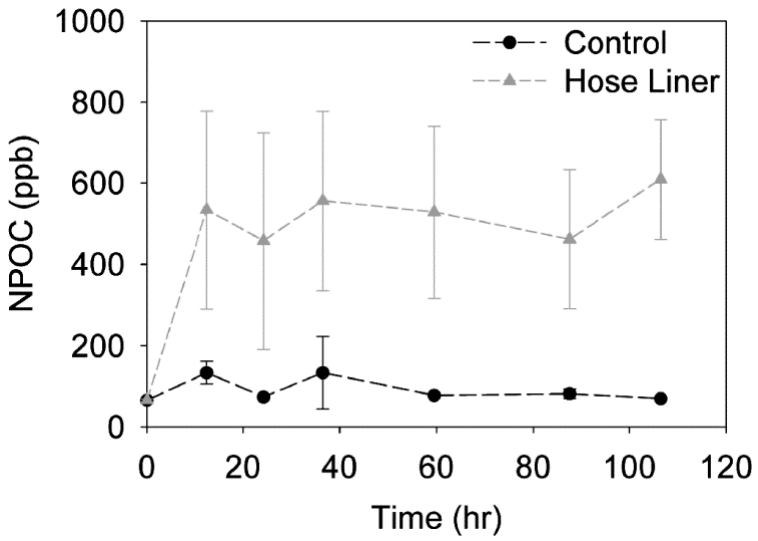



Figure 4. Non-purgeable organic carbon leaching from the drill hose liner compared to the control.

## Discussion

### Clean Access System Performance in Antarctica

The observations and measurements made at our three drill sites show that the clean-access, hot-water drill filtration system reduces cell concentration and viability of microorganisms, effectively preventing contamination of the lake from exogenous

microorganisms and organic carbon. The reduction in cell and ATP concentrations was at least one order of magnitude, up to a 33-fold decrease (Fig. 3). The laboratory tests revealed 4-order of magnitude reductions in ATP and cell concentrations when passed through the filtration system (Priscu *et al.* 2013), but these laboratory experiments were conducted with different source water, with much higher starting cell concentrations. The starting material at all three field sites in Antarctic was surface snow, which contains a low abundance of microbial cells (Pearce *et al.* 2009). South Pole snow is shown to contain between 200 – 5000 cell $mL^{-1}$ (average: 3140 ± 771 cell $mL^{-1}$) of snowmelt (Carpenter *et al.* 2000). The port 1 cell concentrations agree well with the results of Carpenter *et al*., (2000) from South Pole snow and may represent the concentration of cells in source snow. The melted source snow that enters the filtration system has low microbial biomass ($<10^4$ cells $mL^{-1}$) compared to marine and fresh water habitats on Earth (~ $10^5$ cells $mL^{-1}$; Whitman *et al.* 1998). Input snow from the MIS test was 3 and 13 times higher in microbial cells than SLW and GZ input snow, respectively. The MIS site is close (~48 km) to the open ocean (Vick-Majors *et al.* 2015) and airfield activities of McMurdo station. The combined influence of anthropogenic activity and the nearby open ocean may contribute to the higher port 1 cell concentrations. The MIS drill non-recirculating format was similar to the single pass bead experiment conducted in Priscu *et al*., 2013, in which a solution of microorganism-sized fluorescent beads (2 -5 µm diameter) were passed through the filtration system and the outlet went to waste. The MIS results and bead experiment show that the filtration system was effective in removing cells even in a non-recirculating format.

The Antarctic snow is also a good starting material for clean, hot water drilling due to its low concentration of solutes (Table 1). The conductivity measurements show that the starting snow was similar to distilled water in terms of ionic strength and the drill hose and plumbing introduced less than ~2 μS $cm^{-1}$ of ionic impurities. The MIS drill site was run in a non-recirculating method to avoid sea water being taken into the drill system. The conductivity measurements taken from SLW and GZ show that sea water was not introduced into the drill at the MIS site. Our results show that the new drill hose may leach NPOC into the drill water upon initial use, but that the NPOC is leached out quickly and does not increase after the first 12 hours (Fig. 4). Therefore, the NPOC would have leached during the initial drill test at MIS, with minimal potential for NPOC additions at SLW or GZ. Our leaching experiments soaked the entire liner in the heated incubation water, whereas only the smaller inner diameter of the liner would be in contact with hot drill water during drilling, thus our estimates of leachable NPOC may be ~2-fold higher than during initial drill use.

DNA sequence data from borehole water and SLW lake water shows the assemblage of microorganisms present in these samples is significantly different (Christner *et al.* 2014). The sequence results also show that drill water has a lower diversity of species compared to the subglacial water and sediments. Further analysis of DNA sequence data from the MIS and GZ drill site shows that drill waters, sampled at port 8, are also significantly different from the DNA sequences found in the subglacial water and sediment samples (Achberger *et al*. unpublished data). These results imply that

the microorganisms found in the subglacial water and sediments were not from the drill water.

## Recommendations for Future Drilling Projects

Our experience and observations from three drill sites has produced three recommendations to minimize biological and chemical contamination of subglacial aquatic habitats. We recommend that the drill hose should be flushed with hot water before drilling begins toward the subglacial aquatic environment, camp layout should consider prevailing weather conditions, samples should be collected from the drill system and analyzed on site to monitor drill performance and subglacial aquatic environment sampling should be done in an interdisciplinary way to incorporate geophysics datasets on ice thickness and overburden pressures.

As shown by the leaching experiments, a new drill hose will contribute NPOC to the drill water. The drill hose should be flushed with clean, hot water for several hours prior to drilling into ASAE. Our results show the addition of NPOC to the drill water is minimal, but this precaution will help prevent NPOC leaching into the drill water. This water should be discarded and not used for return water pump hole or another hole that may connect to the subglacial access borehole.

Plans for the deep field drill sites should incorporate prevailing weather conditions, particularly prevailing wind direction, in planning the camp layout. Diesel-powered generators are required for electricity to power boiler units. Exhaust from generators, camp equipment, and planes cannot be avoided, but camp layout planning can

reduce exposure of hot-water drilling source water, borehole operations, and scientific laboratories to the contamination potential of exhaust. At our GZ field site the ski-way was set up near the source snow. The ski-way proximity to the source snow did not seem to impact the cell concentrations of input snow at GZ, but tracked vehicle traffic was greater around the GZ field site snow source and small rubber pieces from the tractor tracks were observed on the snow surface. The influence of airfield operations at the MIS site may have caused the increased cell counts. Engine exhaust is not the only contaminating factor for source snow. Human and equipment travel (See darker colored snow near large yellow tent in Fig. 1) in close proximity to source snow is also a source of contamination that can be ameliorated by planning. Also at SLW we limited the area around the yellow tractor (Fig. 1) to drill staff only, so we did not contaminate the source snow area. Antarctic snow is a microbiologically and chemically clean source of water (Table 1, Fig. 3), but can be contaminated by human activities in the field. Field camp layout planning and meteorological data can keep the source snow clean and provide an ideal drill-water source.

A final recommendation is the need to sample and process samples for clean access evaluation in the field. A clean-access, hot-water drill should be engineered and plumbed such that samples can be taken during drilling operations. Samples should be collected, at a minimum, from ports installed before water enters the first stage of cleaning, then after all cleaning steps, but before going down-borehole for drilling. We were able to process our ATP and cell count data (Fig. 1, 2) immediately after collection so we knew, on site, that the filtration system was operating properly. A similar strategy

for other drilling systems, to our sampling port installation at ports 1 and 8 is recommended. This sampling strategy provides data on the efficacy of the drill filtration and cleaning system. This recommendation is a minimum requirement and is suggested because it will not overburden field teams, but also provide a reliable, real-time clean access dataset.

Finally, we recommend that subglacial aquatic access research projects incorporate geophysics data as part of the clean access strategy. Data generated by geophysicists on ice thickness and thus, overburden pressure on subglacial water is integral to planning microbiologically clean access to subglacial habitats. One of the most important clean access considerations is the fate of drill water after breakthrough. Geophysical data can be used to develop a plan to reduce borehole hydrostatic head pressure prior to breakthrough, creating a pressure differential that causes subglacial water to rise into the borehole (Tulaczyk *et al.* 2014). We used this technique at the SLW drill site following recommendation 4.6 of the “Drilling and SAE-entry” section from the SCAR-SALE action group code of conduct report (Doran & Vincent 2011). Shallow lakes or wetlands beneath the ice sheet can also be sampled through this method if sampling tools do not fit into shallow lakes.

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# CHAPTER THREE

# SOLUTE SOURCES AND GEOCHEMICAL PROCESSES IN SUBGLACIAL LAKE WHILLANS, WEST ANTARCTICA

## Contribution of Authors and Co-Authors

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Contributions: Collected, processed, and analyzed samples. In particular, analyzed samples for major ion concentration and performed solid phase minerology. Analyzed the data, wrote the manuscript and prepared figures and tables.

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Contributions: Collected and processed samples. Helped to analyze data and write the manuscript.

Co-Author: Andrew C. Mitchell

Contributions: Assisted in sample collection and processing. Reviewed the manuscript and provided feedback on data analysis.

Co-Author: Trista J. Vick-Majors

Contributions: Collected and processed samples. Reviewed the manuscript and provided feedback.

Co-Author: John C. Priscu

Contributions: Wrote the proposal to fund the project (NSF-OPP 0838933, 1346250, 1439774). Collected samples. Reviewed the manuscript and provided feedback.

Co-Author: Carlo Barbante

Contributions: Assisted in sample collection and processing. Assisted in running the trace element analysis. Reviewed the manuscript and provided feedback.

Contribution of Authors and Co-Authors-Continued

Co-Author: Clara Turetta

Contributions: Ran the trace element analysis. Reviewed the manuscript and provided feedback.

Co-Author: Will vanGelder

Contributions: Ran the ion-exchange experiments. Reviewed the manuscript and provided feedback.

Manuscript Information Page

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## Abstract

Subglacial Lake Whillans (SLW), West Antarctica, is an active component of the subglacial hydrological network located beneath 800 m of ice. The fill and drain behavior of SLW leads to long (years – decades) water residence times relative to mountain glacier systems. Here, we present the aqueous geochemistry of the SLW water column and porewaters from a 36 cm long sediment core. Stable isotopes indicate that the water is primarily sourced from basal-ice melt with a minor contribution from seawater that reaches a maximum of ~6% in porewater at the bottom of the sediment core. Silicate weathering products dominate the crustal (non-seawater) component of lake and porewater solutes, and there is evidence for cation exchange processes within the clay-rich lake sediments. The crustal solute component ranges from 6 meq $L^{-1}$ in lake waters to 17 meq $L^{-1}$ in the deepest porewaters. The porewater profiles of the major dissolved ions indicate a more concentrated solute source at depth (>38 cm). The combination of significant seawater and crustal components to SLW lake and sediment porewaters in concert with ion-exchange processes result in a weathering regime that contrasts with other subglacial systems. The results also indicate cycling of marine water sourced from the sediments back to the ocean during lake drainage events.


## Introduction

Research over the past two decades has demonstrated abundant water beneath the Antarctic ice sheets, with subglacial water estimates of $10^4 – 10^5$ $km^3$ (Priscu et al., 2008). Water-rock interactions in glaciated catchments provide abundant reaction sites for chemical and biological weathering, which generates dissolved aqueous species (Tranter, 2004). Water beneath the West Antarctic Ice Sheet (WAIS) resides and flows in hydrologic networks of streams, lakes, and saturated sediment, leading to prolonged water-rock interactions and solute generation, with the waters ultimately draining to the ocean (Fricker et al., 2007; Skidmore et al., 2010; Carter and Fricker, 2012). These subglacial aqueous environments represent a source of suspended and dissolved biotic and abiotic components and fresh water to the Southern Ocean with potential impacts on

ocean productivity, geochemistry, and circulation (Priscu et al., 2008; Statham et al., 2008).

Active subglacial lakes, such as those along the Siple Coast of West Antarctica, are an important component of the subglacial hydrologic system because they form reservoirs along the hydraulic drainage path to the Ross Sea. Subglacial Lake Whillans (SLW) and other lakes in the Siple Coast region are characterized as "active lakes" (Smith et al., 2009). The filling phase of the lake hydrological cycle occurs over ~2-4 years, slowly increasing the depth and volume of the lake (Siegfried et al., 2014). Drainage events punctuate the filling cycle, moving large (~0.15 $km^3$, in the case of SLW) volumes of water to the grounded margin of the Whillans Ice Stream and the Ross Sea (Carter and Fricker, 2012). Sediment-water interaction times beneath the Whillans Ice Stream are on the order of thousands of years (Christoffersen et al., 2014). This contrasts with the hydrologic conditions beneath valley glaciers and marginal regions of the Greenland Ice Sheet where chemical weathering processes have been more widely studied (Tranter et al., 2002; Tranter, 2004; Wadham et al., 2010). In these (non-Antarctic) subglacial systems, the main water source is surficial meltwater and subglacial water rock-contact times are typically much shorter, hours to months (Skidmore and Sharp, 1999; Tranter et al., 2002; Wadham et al., 2010). Another important distinction between the non-Antarctic subglacial systems and SLW is that during interglacial periods portions of the WAIS basin, including the present-day location of SLW, were periodically inundated by ocean water and marine sediments accumulated (Scherer et al., 1998; Studinger et al., 2001). These deeper, relict marine sediment may have a

biogeochemical legacy that has yet to be explored. Given their solute rich and likely anoxic character, redox gradients may be maintained to perpetuate microbial metabolism in the deep, cold biosphere beneath the WAIS. Here we present the first detailed description of solute sources and geochemical processes for the water column and sediment porewaters from an Antarctic subglacial lake.

## Methods

Samples of water and sediment were collected from SLW, (84.240°S, 153.694°W) 801 m beneath the WAIS, which was accessed using a clean, hot water drill (Priscu et al., 2013). Water samples were collected from the middle of the ~2.2 m water column of SLW using a 10 L Niskin sampling bottle and a 36 cm sediment core was retrieved with a multicorer (60 cm long x 6 cm diameter). Lake water samples for dissolved major ions and trace elements were 0.4 µm polycarbonate filters and 0.2 µm PTFE filters, respectively. Samples of sediment porewater were extracted at 2 cm intervals using Rhizons, equipped with 0.2 µm pore size filters, and distributed into cleaned vials. Samples for major ion and trace metal determinations were stored frozen before analysis via ion chromatography and inductively coupled plasma sector field mass spectrometry (ICP-SFMS), respectively (Turetta et al., 2004; Montross et al., 2013). Stable isotopes (H and O) of the water were measured on unfrozen samples via cavity ring-down spectrometry. Silica was determined colorimetrically (Iler 1979).

## Supplemental Methods

### Field Sample Collection

Subglacial Lake Whillans (SLW) water and sediment were collected through a ~0.6 m diameter borehole which was created with a clean, hot water drilling system (Priscu et al., 2013; Tulaczyk et al., 2014). Lake water samples were collected with a 10 L Niskin bottle and sediments were recovered using a gravity multicorer (Uwitec). Full details of clean access system and the drilling and sample recovery are described in Priscu et al., (2013) and Tulaczyk et al., (2014).

### Sample Processing

SLW sediment porewater samples were collected from multicore 3B (MC-3B) using a Rhizon porewater sampler (Rhizosphere). Rhizon porewater samplers were prepared by soaking in MilliQ (18.2 MΩ) water prior to installation through a pre-drilled hole in the sediment core liner. A 10 mL syringe was attached to the outlet and the plunger locked to maintain a vacuum (Seeberg-Elverfeldt et al., 2005). After 14 h of porewater extraction through the 0.2 µm filter on the end of the Rhizon, the porewater was dispensed into cleaned bottles/vials (Major ions: 10% HCl acid washed and 6 MilliQ rinses; Trace elements: 10% trace metal grade $HNO_3$ acid washed and 6 MilliQ rinses; Water stable isotopes: new, filled with no headspace). SLW water samples for major ion analysis were filtered through a 0.4 µm polycarbonate filter (Whatman) and TE/REE samples were filtered through a 0.2 µm PTFE membrane syringe filter. The filtrate, or raw water in the case of the water stable isotope samples, was dispensed into a bottle

cleaned as described above. Major ion and TE samples were frozen at -20°C and shipped to Montana State University and University of Venice, respectively, for analysis. Stable isotope samples were stored at 4°C and were shipped to University of Washington-Seattle for analysis. Procedural blanks were handled and processed in parallel to SLW samples. Briefly, MilliQ water from McMurdo Station was taken into the field in a 10% HCl acid washed and 6-time MilliQ rinsed HDPE bottle and was run through the same sampling set up for SLW water samples. For the porewater procedural blanks, rhizons were inserted into the MilliQ bottle and ~5 mL of water was pulled through the Rhizon into the syringe and allowed to reside in the syringe for 14 h to replicate syringe residence time. Procedural blank water was then dispensed into the three sample containers for subsequent analysis as described above.

Major Ion Analysis

Major anions and cations were measured using ion chromatography (Metrohm) with an ASupp 5 anion and C4 cation column (Metrohm). Sample concentrations were calculated from a 5-point standard curve for each analyte. Procedural blank samples were analyzed in parallel with samples and were negligible, but subtracted from sample concentrations, such that the values presented are blank-corrected.

Silicon Analysis

Silicon was determined using the Molybdenum-blue colorimetric assay (Iler, 1979). The protocol was modified for a 1mL final reaction volume and the ammonium molybdate amount was doubled. Porewater was extracted from the sediment by

transferring ~5 g of sediment to a 15 mL clean centrifuge tube and spinning at 4500 xg for 30 mins. The porewater was pipetted from the sediment surface and 0.22 µm syringe filtered through Durapore PVDF syringe filters (Millipore). Absorbance was measured at 810 nm. Filter blanks were analyzed in parallel and silicon from filtration was negligible. Concentrations in samples were determined from a 5-point standard curve made from a certified, NIST-traceable Si standard (Sigma-Aldrich). The methodological limit of detection was 0.5 µM following EPA methods (Oblinger-Childress et al., 1999).

Stable Isotope Analysis

Stable isotope measurements ($\delta D$ and $\delta^{18}O$) on water samples were made using a Picarro cavity ring-down laser spectrometer at the Isolab (University of Washington, Seattle). The porewater samples (100 µL) were analyzed in random order (i.e., non-sequential depth and two separate sets of analyses were conducted for each depth on different days). There was good agreement between the values generated in the two separate runs. The results reported are the average of the two runs using standard $\delta$ notation in per mille relative to Vienna Standard Mean Ocean Water (VSMOW).

Trace Element Analysis

Trace element measurements were performed by inductively coupled plasma sector field mass spectrometer (ICP-SFMS; Element2, Finnigan-MAT, Bremen, Germany). The instrument was installed in a dedicated laboratory with the sample introduction area protected by a laminar flow cabinet. The sample introduction system utilized a desolvation unit (Aridus, Cetac Technologies, Omaha, NE, USA) coupled with

a PFA μ-flow nebulizer (100 μL $min^{-1}$); the sample flows in a teflon spray chamber heated up to 95°C to prevent droplet accumulation, then it is swept by an Ar flow into a semi-permeable membrane (heated up to 165°C) to significantly reduce the formation of oxides (Turetta et al., 2004). The introduction system was connected to the ESI SC2-E2 autosampler (Elemental Scientific, Omaha, NE, USA). The sample introduction system and the autosampler were maintained and handled under a laminar flow hood. Intensity optimization was carried out daily, using a tuning solution of ultrapure water containing 1 ng/ml of In. An accurate mass calibration was performed immediately prior to analysis in low, medium and high resolution mode using a solution containing elements with m/z values covering the whole mass range of interest. The accuracy of the measurements was determined using a certified reference material (TM-RAIN95) in which the trace element concentrations were measured.

Ion Exchange Experiments

Solutions were prepared containing single cations $Mg^{2+}$, $Ca^{2+}$, $Na^{+}$ and $K^{+}$ in 20 milli-equivalent concentrations. One-half gram of sediment was suspended in the solutions in triplicate, (with three separate vials per cation solution) and placed on a shaker table (300 RPM) for 24 hours to keep sediment in suspension, in a 4°C room in order to simulate near *in situ* subglacial temperatures. Sediment was also suspended in 18.2 MΩ water to determine the concentration of cations that would go into solution in water from processes other than exchange. After 24 hours of shaking, suspensions were centrifuged at 8000 RPM for 10 mins in a refrigerated centrifuge and the supernatant separated and then filtered through 0.2 μm nylon membrane syringe filters. Cation

concentrations of the supernatant were measured via ion chromatography as described above.

### Scanning Electron Microscopy and Energy Dispersive X-Ray Spectroscopy

Bulk sediment was wet sieved sequentially through 1 mm, 0.589 mm, 250 µm, 125 µm, and 63 µm sieves. The size fractions were dried for 3 h at 90°C. Grains from the 63 – 125 µm fraction were sparsely distributed onto black, sticky carbon tape, mounted on a 50 mm scanning electron microscope (SEM) stub. The grains were coated with iridium using an Emitech K575x sputter coater (Quorum Technologies) operated at 20 mA for 60 s resulting in an Ir coating of approximately 10 nm. Mineral grain elemental composition was determined on a JEOL JSM-6100 SEM equipped with energy dispersive X-ray (EDX) spectroscopy and a NORAN SiLi detector. Elemental mapping was conducted using a RONTEC XFlash 1000 x-ray detector. Rontec electronics and software were used to collect elemental spectra and identify peaks. EDX was performed at 20 kV and spectra were identified using a dichotomous key (Severin, 2004). Two hundred and six grains from the 63 – 125 µm fraction were analyzed for their elemental composition (Data Repository Table 2).

## Results and Discussion

### SLW Seawater Component

The lake water had a $\delta^{18}O\text{-}H_2O$ value of -38.0‰ (Christner et al., 2014) with porewater values increasing down core to -36.5‰ at 37 cm depth, the core bottom (Fig.

1A). δD-$H_2O$ values showed a similar trend towards more positive values at depth (Fig. 1A). Lake water δD-$H_2O$ values were comparable to meteoric ice from other Antarctic outlet glaciers (Souchez et al., 2004). A two component mixing model of δ18O-H2O was used to determine the percentage of glacial melt water and seawater in the SLW lake and porewaters (Phillips and Gregg, 2001), with $\delta^{18}$O-H2O of -39‰, measured from deep glacial ice upstream of SLW (Vogel, 2004), as the glacial ice end member and 0‰ as the seawater end member. This analysis indicated the lakewater was comprised of 97.4% glacial melt and 2.6% seawater, with the proportion of seawater increasing down core to maximum of 6.4% at the core bottom, and the balance, 93.6% from glacial melt. Chloride concentrations also increased down core from 3.5 meq $L^{-1}$ in the lake to 22.5 meq $L^{-1}$ at the core bottom, equivalent to 4.1% of seawater $Cl^-$ concentration (Fig. 1B). The $Cl^-$ concentration profile is consistent with the stable isotope data indicating that the porewaters and solutes are partially derived from seawater. Chloride is a conservative tracer and the linearity of the porewater $Cl^-$ profile suggests that this system is in steady-state, relative to the upward diffusion of $Cl^-$ from a more concentrated source at depth (>38 cm). The seawater influence is unlikely to be contemporary, as tidally-driven pumping of modern, ice-shelf cavity seawater is limited to a distance of ~10 km upstream from the grounding line (Walker et al., 2013), whereas SLW is ~100 km upstream. The seawater signal measured at SLW is likely due to the lasting influence of past seawater inundation and deposition of marine sediments. The other major ions ($Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $SO_4^{2-}$ and $HCO_3^-$) also show trends similar to $Cl^-$ with increased concentrations at

depth and profiles that indicate upward diffusion from a more concentrated source (>36 cm depth) (Fig. 1B, Data Repository Table 1).

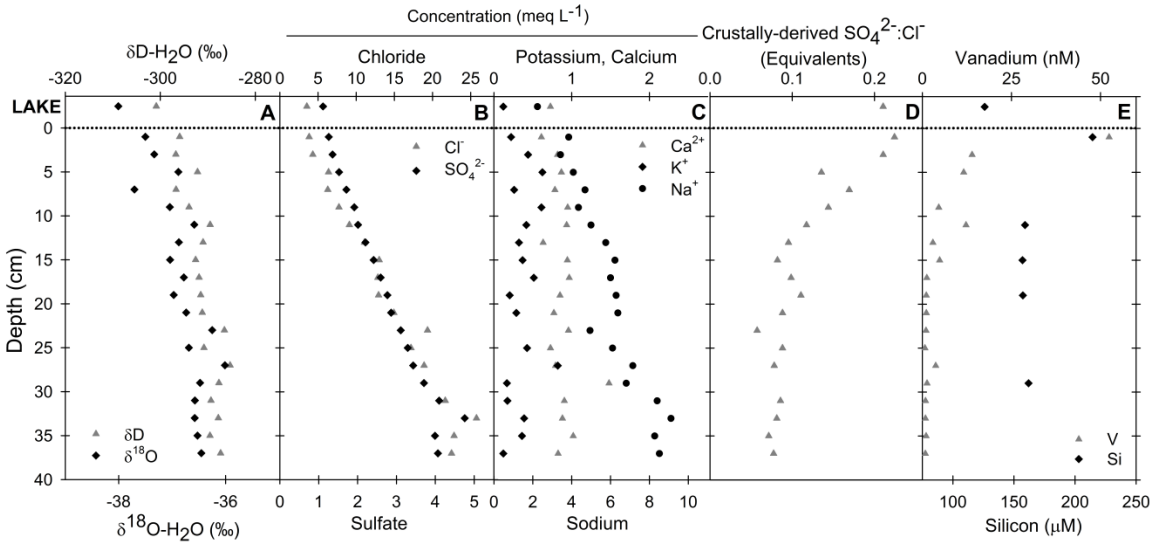



Figure 1. Profiles of Subglacial Lake Whillans water column and porewater water stable isotopes (A); whole water chloride and sulfate (B); crustally-derived, non-seawater sourced calcium, potassium and sodium (C), crustally-derived sulfate to chloride ratio (D), and vanadium and silicon (E). The dotted line indicates the sediment-water interface.

The geochemistry of SLW and its sediment porewaters are atypical for subglacial systems as there is a significant component of seawater-sourced solute (Fig. 2). The seawater component of total solute in SLW sediment porewater increases from 57% in the lake to 77% at 37 cm depth. The seawater component has a notable influence on SLW geochemistry and distinguishes SLW from subglacial waters of other Antarctic, valley and outlet glaciers (Fig. 2A), with the exception being the subglacial outburst waters near Casey Station, which have 34% seawater solute (Goodwin, 1988). The SLW waters exhibit a compositional shift after subtracting the seawater component (Fig. 2). Other subglacial porewater samples from the Siple Coast (i.e., Kamb Ice Stream (KIS) and

Bindschadler Ice Stream (BIS)) are not influenced by the seawater correction as only 3% of the solute is of marine origin (Skidmore et al., 2010; Fig. 2).

Data Repository Table 1. Whole water major cation and anion concentrations from the SLW water column and sediment porewater

| Depth (cm) | Chloride (μeq $L^{-1}$) | Sulfate (μeq $L^{-1}$) | Sodium (μeq $L^{-1}$) | Potassium (μeq $L^{-1}$) | Calcium (μeq $L^{-1}$) | Magnesium (μeq $L^{-1}$) | Bicarbonate† (μeq $L^{-1}$) | Silicon (μM) | Vanadium (nM) |
|---|---|---|---|---|---|---|---|---|---|
| Lake‡ | 3537 | 1111 | 5276 | 186 | 859 | 507 | 2111 | 126 | 19 |
| 1 | 3841 | 1230 | 6977 | 293 | 860 | 494 | 3222 | 214 | 52 |
| 3 | 4316 | 1356 | 7130 | 520 | 980 | 642 | 3553 | ND | 14 |
| 5 | 6377 | 1524 | 9557 | 743 | 1106 | 762 | 4216 | ND | 12 |
| 7 | 6276 | 1713 | 10077 | 377 | 1019 | 692 | 4098 | ND | ND |
| 9 | 7743 | 1916 | 10995 | 756 | 1238 | 1006 | 4279 | ND | 4.6 |
| 11 | 9109 | 2012 | 12815 | 587 | 1276 | 1066 | 4563 | 159 | 12 |
| 13 | 11078 | 2204 | 15264 | 529 | 1046 | 1165 | 4664 | ND | 2.9 |
| 15 | 13012 | 2414 | 17399 | 612 | 1431 | 1293 | 5247 | 157 | 4.8 |
| 17 | 12838 | 2595 | 17023 | 753 | 1449 | 1345 | 5070 | ND | 1.2 |
| 19 | 12931 | 2768 | 17389 | 444 | 1334 | 1401 | 4802 | 157 | 1.1 |
| 21 | 14939 | 2863 | 19203 | 567 | 1329 | 1515 | 4742 | ND | 1.1 |
| 23 | 19346 | 3111 | 21555 | *2911 | 1682 | 1624 | 5236 | ND | 1 |
| 25 | 18205 | 3291 | 21997 | 773 | 1419 | 1698 | 4015 | ND | 0.7 |
| 27 | 18892 | 3431 | 23369 | 1175 | 1499 | 1797 | 5437 | ND | 3.7 |
| 29 | *10015 | 3707 | 15407 | 354 | 1855 | 1788 | 5603 | 162 | 1.3 |
| 31 | 21664 | 4099 | 27003 | 579 | 1716 | 2098 | 5551 | ND | 0.9 |
| 33 | 25757 | 4758 | 31225 | 870 | 1844 | 2404 | 5744 | ND | 0.9 |
| 35 | 22820 | 3992 | 27866 | 788 | 1874 | 2277 | 5908 | ND | 1 |
| 37 | 22493 | 4068 | 27828 | 542 | 1666 | 2274 | 5665 | ND | 0.8 |

## SLW Solute from Crustal Weathering

The seawater component of the lake and porewaters was determined by applying standard seawater ratios of major ions to $Cl^-$ (Holland, 1978). We define whole water as the total solute load measured in the sample. The total solute load with the seawater component subtracted out is defined as the crustally derived, non-seawater component. Following subtraction of the seawater component, significant crustally derived $K^+$ and $Na^+$ were evident in the SLW lake water and throughout the porewater profile, likely from silicate mineral weathering (Fig. 1C) when compared to other subglacial waters (Fig. 2A). This is consistent with the mineralogical data on the sediments, for example,

the 63 - 125µm size fraction was primarily composed of feldspar (35% of grains analyzed; n=206; Data Repository Table 2). Aqueous $Ca^{2+}$ and $Mg^{2+}$ are typically sourced mainly from silicate or carbonate weathering, with a greater proportion of $Ca^{2+}$ from carbonates than for $Mg^{2+}$ (Meybeck, 1987). The concentrations for $Mg^{2+}$ are negative after applying the seawater correction, indicating a $Mg^{2+}$ sink from solution exists in the sediment. Cation-exchange reactions on clay minerals are the most likely sink given the high proportion (50% by dry weight; Data Respository Table 3) of silt and clay-sized particles in SLW sediments, which is similar to the till composition found upstream of SLW (Tulaczyk et al., 1998).

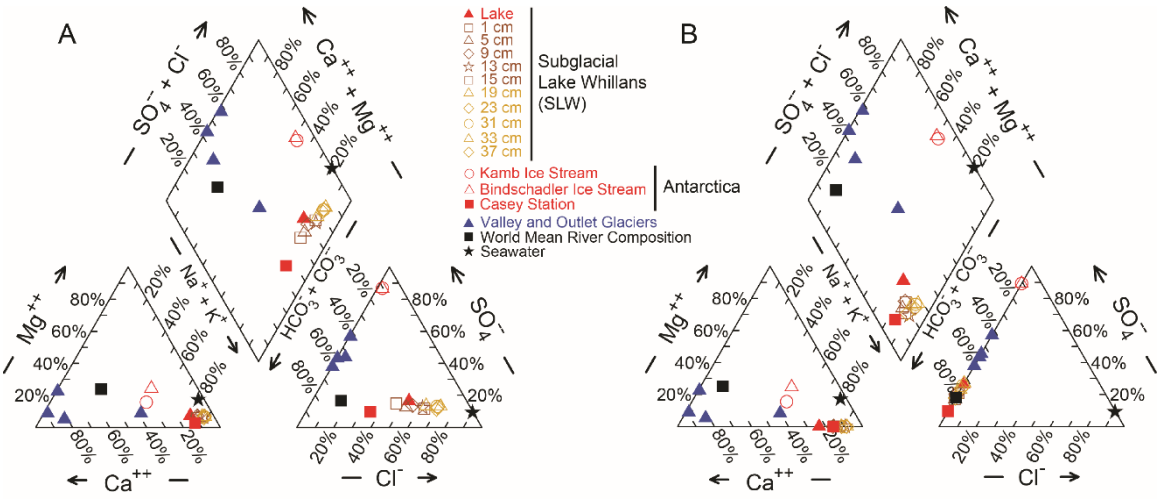



Figure 2. Piper plots of SLW lake water and porewater geochemical composition compared with Antarctic subglacial sediment porewater from KIS and BIS, subglacial water from Antarctica, Greenland, Arctic and Alpine valley glacier systems, world mean river runoff, and seawater. Symbols are colored such that red, brown, and light brown symbols are from Antarctica; blue triangles are non-Antarctic valley and outlet glaciers (Bench Glacier, AK, USA; Robertson Glacier, AB, Canada; Haut Glacier d'Arolla, Switzerland; Bristol Glacier, Greenland) samples. Open symbols are sediment porewater samples. Closed symbols are subglacial water samples, seawater and world mean river runoff. Whole water values (A) and crustally-derived, non-seawater component (B).

Clay minerals are prevalent in SLW sediments and provide cation exchange reaction sites that can modify $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ ion concentrations derived from mineral dissolution and seawater sources. Ion exchange experiments on SLW sediments using a NaCl exchange solution with a similar concentration (20 meq $L^{-1}$) to that at the bottom of the sediment core resulted in 0.9 meq $g^{-1}$ $Ca^{2+}$ and 0.6 meq $g^{-1}$ $Mg^{2+}$ desorbing from clay minerals. The exchange experiments demonstrate that clay minerals are a sink for $Ca^{2+}$ and $Mg^{2+}$ from SLW porewaters.

Data Repository Table 2. SLW sediment grain composition from 63 – 125 µm size fraction (n = 206).

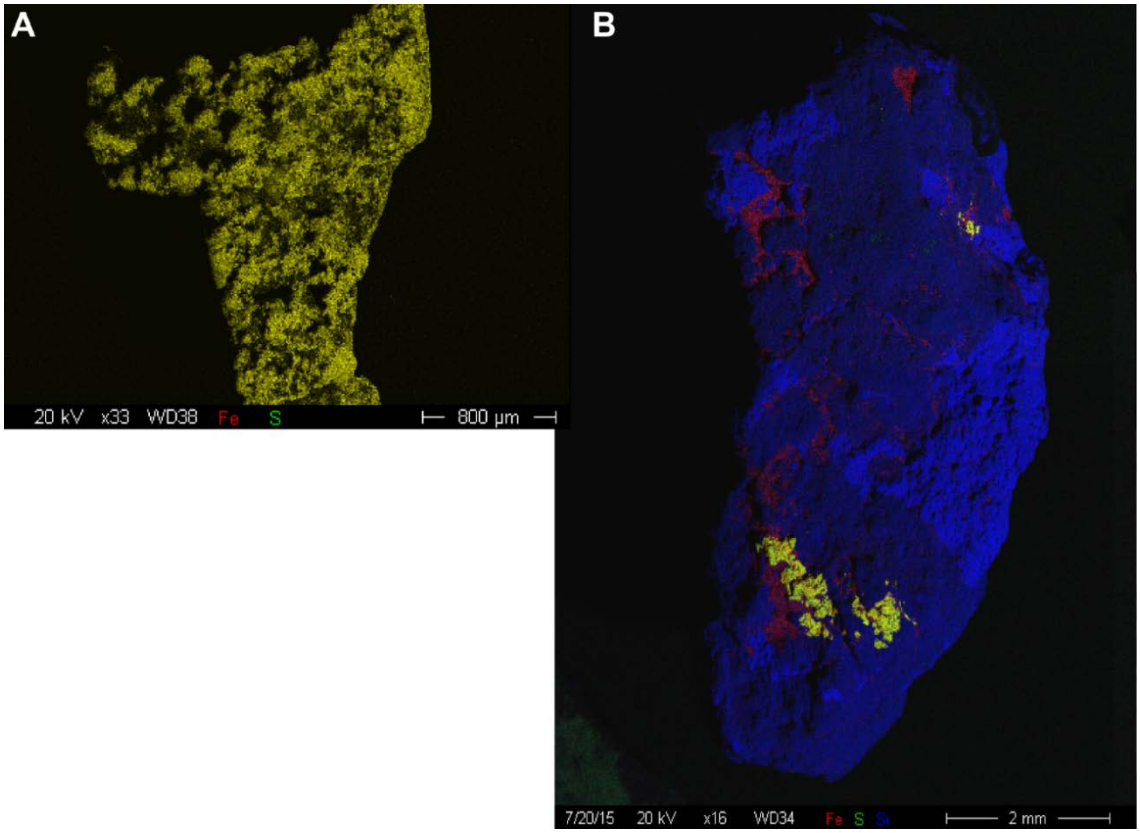
| **Mineral type** | **Percent (%)** |
|---|---|
| Alkali Feldspar | 4 |
| Biotite | 2 |
| Chlorite | 1 |
| Garnet | 2 |
| Hornblende | 0.5 |
| K-Feldspar | 12 |
| Plagioclase | 19 |
| Quartz | 59 |
| Unknown | 0.5 |

Data Repository Table 3. Grain size distribution of SLW sediments.

| **Grain size diameter (µm)** | **Wentworth size class** | **wt %** |
|---|---|---|
| >250 | Medium sand and coarser | 20.9 |
| 125-250 | Fine sand | 12.9 |
| 63-125 | Very fine sand | 16.3 |
| <63 | Silt and clay | 49.9 |

Weathering Regime in
Subglacial Lake Whillans

Calcium is the dominant cation in Alpine subglacial waters irrespective of lithology due to the rapid dissolution kinetics of carbonates (Anderson et al., 1997). By contrast, crustally derived $Ca^{2+}$ is not a dominant ion in SLW, as demonstrated by the low $Ca^{2+}$:Σcation molar ratio (0.04-0.13) compared to the world mean for glacial and non-glacial catchments (~0.45; Anderson et al., 1997). Samples from Antarctic subglacial waters beneath the KIS, BIS and Casey Station also have low crustally derived $Ca^{2+}$:Σcation ratios; 0.22, 0.17, and 0.09, respectively (Skidmore et al., 2010). The low $Ca^{2+}$:Σcation ratios from Antarctic subglacial aqueous samples are generated through a different weathering regime from $Ca^{2+}$-dominated Alpine glacial systems. These lower $Ca^{2+}$:Σcation ratios are most likely due to increased silicate weathering and ion exchange reactions during longer water-rock interaction times in Antarctic subglacial environments. Long water residence time produces proportionately greater amounts of crustally sourced $Na^{+}$ and $K^{+}$ to the subglacial waters (Wadham et al., 2010). Clay minerals in SLW are also a product of long-term silicate weathering and are not observed in the short-residence time valley and outlet glacier systems (Griggs, 2013). The $Mg^{2+}$ and $Ca^{2+}$ concentrations in SLW waters are lower than one would expect from such long residence time waters, and the deficit is assumed to be due to cation exchange on clay minerals, leading to the low $Ca^{2+}$:Σcation ratio.




Data Repository Figure 4. Elemental maps of two pyrite-containing clasts from the SLW sediments. A. Pyrite grain as determined by the predominantly Fe and S composition. Iron is imaged in red and sulfur in green and when these two elements co-occur, the resultant color is yellow. B. Quartz grain with small pyrite inclusions. The blue background is a predominantly silica matrix, with iron in red and sulfur in green. When the iron and sulfur co-occur it results in yellow. Fe-rich veins are visible as red bands in the upper part of B.

Silicate weathering can occur abiotically, but given the presence of an active microbial community in SLW (Christner et al., 2014), and previous research demonstrating the key role of microbes in weathering of glacial sediments (Montross et al., 2013), biotic weathering may also occur. $CO_2$ released from heterotrophic activity in the SLW water column (Christner et al., 2014) will dissolve and produce carbonic acid, which is responsible for indirect weathering of minerals (Montross et al., 2013) and

contributes in part to the $HCO_3^-$ in the lake and sediment porewaters. The balance of $HCO_3^-$ may come from carbonate dissolution and possibly hydrothermal sources given the high geothermal heat flux in the region (Fisher et al., 2015). Direct weathering of sulfur-containing minerals (i.e., pyrite), which are present in SLW sediments (Data Repository Fig. 1), through sulfide-oxidation produces protons and dissolved $SO_4^{2-}$ (Fig. 1B). Microbial sulfide-oxidation is inferred from the excess of crustally-derived $SO_4^{2-}$ with respect to $Cl^-$ in the top 15 cm of the core (Fig. 1D) and has been demonstrated in other glacial sediments (Mitchell et al., 2013; Boyd et al., 2014). Microbial community and functional gene analysis of SLW and KIS sediment support the possibility of microbial pyrite oxidation as microbial phylotypes and sulfide-oxidizing functional genes related to sulfide-oxidizing bacteria are present and abundant in these environments (Lanoil et al., 2009; Christner et al., 2014; Purcell et al., 2014). These microbially-driven sulfide oxidizing processes are likely the source of crustally-derived, non-seawater sulfate in SLW.

Microbial sulfide-oxidation is most energetically favorable in the presence of $O_2$. To estimate the depth below which sulfide oxidation is unlikely to occur, we estimated the reduction/oxidation (redox) state of the sediment using the charge of the element V and thus its aqueous concentration. Redox sensitive elements, such as V, are useful indicators of the redox state of the sediments (Morford and Emerson, 1999). As dissolved $O_2$ concentrations decrease, Vanadate ($H_2VO_4^-$) is reduced to vanadyl ($V(IV)O^{2+}$) and V is removed via precipitation (Morford and Emerson, 1999). Therefore, the decrease in V concentrations to below detection below 15 cm of the SLW sediments implies decreasing

$O_2$ concentrations with depth (Fig. 1E). The $O_2$-consuming processes, are likely microbial, involving either heterotrophic or lithotrophic activity such as sulfide- and ammonia-oxidation, processes which are thought to be important in SLW (Christner et al., 2014). The silicon profile is similar to that of V and shows that silica concentrations are highest in the surficial (0-2 cm) sediments (Fig. 1E). Increased microbial activity in the surficial sediments may be due to the presence of oxidizing conditions, unlike those at depth. Microbial activity in the sediments is therefore likely to be important in liberating solutes via both direct and indirect biotic weathering processes.

## Conclusions

Crustally derived solute in SLW is generated primarily from the weathering of silicate minerals, a process that is likely microbially mediated. Ion exchange reactions with clay minerals also modify the proportions of cations in solution. Seawater is also an important component of SLW solute, in contrast to KIS and BIS porewaters and subglacial waters from other non-Antarctic systems. The major ion profiles all indicate a more concentrated solute source in porewater of sediments below the base of our 36 cm core, which we were unable to sample. These deeper porewaters likely have an increased seawater component, resulting from marine inundation of the SLW region when the WAIS was less extensive. SLW represents a subglacial system where contemporary chemical and biological weathering processes are active, but seawater solute is a component of the water that is ultimately cycled back to the Southern Ocean during lake drainage.

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# CHAPTER FOUR

# MICROBIAL METHANE TRANSFORMATIONS BENEATH THE WEST ANTARCTIC ICE SHEET

## Contribution of Authors and Co-Authors

Author: Alexander B. Michaud

Contributions: Collected, processed, and analyzed the samples. In particular, ran gas quantification and stable isotope analysis. Performed DNA extractions and functional gene analysis. Analyzed data, wrote the manuscript, and prepared figures and tables.

Co-Author: John E. Dore

Contributions: Analyzed the samples and helped to write the manuscript.

Co-Author: Trista J. Vick-Majors

Contributions: Collected and processed the samples. Reviewed the manuscript and provided feedback.

Co-Author: Mark L. Skidmore

Contributions: Collected and processed the samples. Provided instrumentation for stable isotope analysis. Reviewed the manuscript and provided feedback.

Co-Author: John C. Priscu

Contributions: Wrote the proposal to fund the project (NSF-OPP 0838933, 1346250, 1439774). Collected and processed samples. Reviewed the manuscript and provided feedback.

Manuscript Information Page

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## Abstract

Models indicate that subglacial sediments in West Antarctica contain a large $CH_4$ reservoir. The quantity, source and fate of $CH_4$ beneath the ice sheet have yet to be investigated empirically. We show, using $\delta^{13}C$- and $\delta D$-$CH_4$ measurements, that sub-ice sheet $CH_4$ is likely produced via biological reduction of $CO_2$ with $H_2$. $CH_4$ concentration gradients and $\delta^{13}C$-$CH_4$ values indicate that it is consumed in the surficial sediments and may provide an abundant reduced carbon and energy source to the subglacial ecosystem. Aerobic microbial $CH_4$ oxidation may diminish the release of this potent greenhouse gas from West Antarctica to the atmosphere during periods of deglaciation.


## Introduction

Anaerobic habitats exist beneath the Antarctic ice sheet and may be globally important for biological $CH_4$ production *(1, 2)*. A large $CH_4$ reservoir, formed through acetoclastic methanogenesis of relict marine carbon, has been proposed to exist beneath the Antarctic ice sheet that could add significant $CH_4$ to the atmosphere upon deglaciation *(2)*. Despite this claim, no empirical evidence exists for the presence or dynamics of this $CH_4$ pool. We present, based on direct sampling of a lake beneath the West Antarctic Ice Sheet, $CH_4$ concentration, microbial transformations and role of this gas in the subglacial microbial ecosystem.

## Methods

### Methods Summary

We used a microbiologically-clean hot water drill to directly sample the water column and shallow sediments (~40 cm) of Subglacial Lake Whillans (SLW; 84.240° S, 153.694° W), to assess the $CH_4$ dynamics beneath 800 m of the West Antarctic Ice Sheet

(WAIS) *(3, 4)*. Water samples were collected with a 10 L Niskin bottle from the middle of the ~2.2 m deep water column and returned to the surface with minimal exposure to the atmosphere. Sediment cores were retrieved with a multicorer (60 cm x 5.95 cm). Core sub-samples for gas analysis were placed in gas tight glass vials and 6N NaOH was added to prevent biological activity during storage. $CH_4$ concentration was determined via gas chromatography using flame ionization detection. Stable isotopes of $CH_4$ were determined on a Picarro Cavity Ring Down Laser Spectrometer ($\delta^{13}C$) and isotope ratio mass spectrometer at the UC Davis Stable Isotope Facility ($\delta D$). DNA was extracted from the sediments and primers were used to amplify the *pmoA* and *mcrA* gene sequences, which were then cloned and sequenced (See Supplemental Methods for full details).

## Supplemental Methods

### Sample Collection

Subglacial Lake Whillans water column and sediment was sampled through a ~0.6 m diameter and 801 m deep borehole created with a clean, hot water drill on 29 January 2013. The clean access hot water drill system has been shown to reduce cell concentrations to acceptable levels based on the National Research Council 2007 report on subglacial lake access *(3)*. For complete drilling and sampling details see Tulaczyk et al., 2014 *(4)*.

Methane Quantification
and Stable Isotope Analysis

Sediment from a multicore (MC-2A) was sampled every 2 cm by extruding and subsampling the new layer. The sediment was immediately placed in a 20 ml sterile serum vial using a sterile cut off 5 ml syringe, and then the vial was stoppered with a butyl rubber stopper and crimped with an aluminum cap. Three vials were capped in the field to capture atmospheric air as blank vials. Ten milliliters of 2.5% NaOH was added to each sample bottle and the three atmospheric air blanks *(5)*. All vials were stored upside down at 4°C for transport back to Montana State University for gas quantification. Methane was quantified on a gas chromatograph (Hewlett Packard 5890) equipped with a flame ionization detector. Dissolved methane concentrations were calculated based on methane Bunsen coefficient and solubility based on porewater volumes determined from mass loss after drying the sediment at 95°C until the mass stopped decreasing (~24h) and porewater conductivity.

Multicore 3A (MC-3A) was collected from SLW and frozen (-20°C). The frozen core was thawed at 4°C overnight in a class 1000 clean, cold room at the Montana State University Sub-Zero laboratory. The core was extruded and cut every 2 cm and sediment for methane stable isotope analysis was subsampled and fixed in the same method as MC-2A. One milliliter of room temperature headspace gas from the fixed sediment vials was transferred to a gas-tight bag and diluted 1:100 with zero air. The gas-tight sample bag was connected to a Picarro Ring-Down Cavity Spectrometer and sample was introduced to the instrument at a flow-rate of 100 mL per minute and isotopic values were measured every second. $\delta^{13}C\text{-}CH_4$ values were averaged over ≥30 s of

measurements. After each sample, atmospheric air was measured for at least 5 min until atmospheric baseline values had stabilized. The $\delta D\text{-}CH_4$ values were measured at the University of California Davis Stable Isotope Facility (UCD-SIF) using a PreCon concentration system (ThermoScientific) in line with a Delta V plus isotope ratio mass spectrometer (ThermoScientific). Two $\delta^{13}C\text{-}CH_4$ samples (SLW-MC-3A-18-20 cm and 34-36cm) were also run at UCD-SIF to compare to values obtained on the Picarro Ring-Down Cavity Spectrometer. There was a <4% difference in the $\delta^{13}C\text{-}CH_4$ values reported from the two methods. The carbon and hydrogen stable isotope ratios are reported in standard δ-notation ($\delta^{13}C$, $\delta D$) with respect to VPDB and VSMOW standards, respectively. Running average of the methane concentration and isotope profile was calculated in SigmaPlot using a loess smoothing function with a first order polynomial and a sampling frequency of 0.45.

Molecular Analyses

DNA was extracted from SLW sediments *(6)*. *pmoA* clone libraries were constructed by PCR amplification using A189F and m680R. The PCR was set up using 0.1 µL of ExTaq at 5 units $\mu L^{-1}$ (Takara), 2.5 µL of 10x ExTaq buffer (Takara), 2 µL dNTP mixture at 2.5 mM per nucleotide (Takara), 2.5 µL of A189F and Mb661R primers, 2 µL molecular biology-grade bovine serum albumen (BSA; New England BioLabs Inc.), 4 µL of template DNA, and 11.4 µL of PCR-grade water for a final reaction volume of 25 µL. The PCR thermocycling conditions were 1 cycle of 98°C for 2 min; 40 cycles of 98°C for 15 s, 55°C for 1 min, and 72°C for 1 min; followed by a final 72°C for 7 min. PCR products were run on a 1.5% agarose gel and the 491 basepair

*pmoA* fragment was excised from the gel followed by DNA purification using a Wizard SV gel clean-up system (Promega). Cleaned *pmoA* fragments were immediately ligated and cloned with a TA Cloning kit (Invitrogen). Positive clones were transferred to LB+ampicillin broth and grown overnight at 37°C, then sequenced (Functional Bioscience). The *pmoA* sequences were processed by removing the forward and reverse primer sequences, then grouped into operation taxonomic units (OTU) using a 90% similarity cutoff using the program Mothur *(7).* A representative sequence from each OTU was queried against the NCBI database and nearest neighbor sequences were recovered for each OTU. These representative OTU sequences and *pmoA* sequences from representative members of Type 1 and 2 methanotrophs were aligned using ClustalW and a phylogenetic tree was built using the maximum-likelihood method with 1000 bootstrap replications *(8).*

We attempted to amplify *mcrA* gene sequences from SLW sediment DNA extracts. We used two sets of primers to amplify the diversity of *mcrA*-containing methanogens *(9)* and a nested PCR amplification scheme. The two primer pairs used to detect the *mcrA* gene sequence were mcrIRD and ANME-1-mcrI *(9).* These primer pairs are capable of detecting all known and several novel *mcrA* gene clusters *(9).* The first reaction was set up using 0.1 µL of Takara ExTaq, 2.5 µL 10x ExTaq buffer, 2 µL dNTP mixture, 2.5 µL of forward and reverse primer, 2 µL of BSA, 9.4 µL PCR-grade water and 4 µL DNA extract for a final reaction volume of 25 µL. This first reaction was run with an initial denaturation step at 98°C for 2 minutes followed by 40 cycles of 98°C for 15 s, 53°C for 1 min and 72°C for 1 min, and a final elongation at 72°C for 7 min. The

second reaction was set up using 0.25 µL Takara ExTaq, 5 µL 10x ExTaq buffer, 4 µL dNTP mixture, 5 µL of forward and reverse primers, 4 µL of BSA, 21.75 µL of PCR-grade water and 4 µL of product from the first reaction. The second reaction was run with the same thermocycler program as the first reaction. Both reactions were run the same for the two primer sets, except the ANME-1-mcrI primer pair used an annealing temperature of 63°C *(9)*.

## Methane Oxidation Rate

### Modeling and Oxygen Budget

The flux into the 0 – 2 cm surficial sediment layer was calculated using Fick's first law. The diffusion coefficient for methane at 0°C *(10)* was corrected for porosity and tortuousity of SLW sediments *(11)*. See Results and Discussion section for details on modeling the rate of methane oxidation.

Oxygen consumption by methane oxidation follows the stoichiometric relationship:

$$CH_4 + (2 - x)O_2 \leftrightarrow (1 - x)CO_2 + xCH_2O + (2 - x)H_2O \qquad \text{Eq. 1}$$

where *x* is the fraction of carbon from the methane incorporated into biomass. We assumed the fraction of carbon from the methane that was incorporated into biomass was 0.5, which has been shown to be a good approximation for both types of methanotrophs and is a median value across many habitats *(13–15)*. Based on these approximations, we model oxygen consumption as 1.5 mol $O_2$ per 1 mol $CH_4$ consumed.

Two oxygen inputs to SLW were in our model, basal ice melt and water flow into the lake. Basal ice melt releases atmospheric gases into the subglacial aquatic hydrologic

network and can be calculated based on direct measurements of geothermal heat flux at SLW *(16)*. The geothermal heat flux measured at SLW is calculated to melt 1.8 mm $y^{-1}$ *(16)*. This basal ice melt rate delivers 1.0 x $10^6$ mol $O_2$ $y^{-1}$ from the entire 60 $km^2$ SLW area. This basal ice melt oxygen release rate assumes oxygen is 20.95% of atmospheric gas. The density of glacial ice is ~918 kg $m^{-3}$, which leaves ~10% of the ice as atmospheric gas bubble inclusions. The basal ice melt is assumed to only come from the 60 $km^2$ area of SLW. Advection of water into SLW is modeled based on the flow rate derived from the increase in volume for the year before we sampled SLW [0.007 $km^3$ $y^{-1}$; *(17)*]. We assumed that the concentration of oxygen in the inflow water to be the same as that measured in the SLW water column [72 µM; *(18)*]. Advection into the lake supplies 5.0 x $10^5$ mol $O_2$ $y^{-1}$. The standing pool of oxygen in the lake at the time of sampling was 9.5 x $10^6$ mol $O_2$. The total oxygen supply based on basal ice melt and advection into the lake is 1.5 x $10^6$ mol $O_2$ $y^{-1}$ into SLW.

## Results and Discussion

The $CH_4$ concentration in SLW ranged from 24 nM in the lake water to 300 µM in the deepest (37 cm) sediment porewater sample (Figure 1). Fick's first law was used to calculate a $CH_4$ flux using the concentration gradient in the top 15 cm of sediment. The estimated flux to the surficial sediment (0 – 2 cm) of SLW is 6.8 mmol $CH_4$ $m^{-2}$ $y^{-1}$. Modeled ice sheet bed conditions beneath the WAIS indicate that water saturated till, similar to that in SLW, constitutes 50% of the areal extent of WAIS (9.85 x $10^5$ $km^2$; *5, 6*). We estimate a $CH_4$ flux of 0.1 Tg $CH_4$ $y^{-1}$ from water-saturated till beneath the WAIS,

assuming that the $CH_4$ flux we computed for SLW is consistent beneath the WAIS. This flux represents about 0.05% of total natural $CH_4$ emission sources to the atmosphere *(21)*.

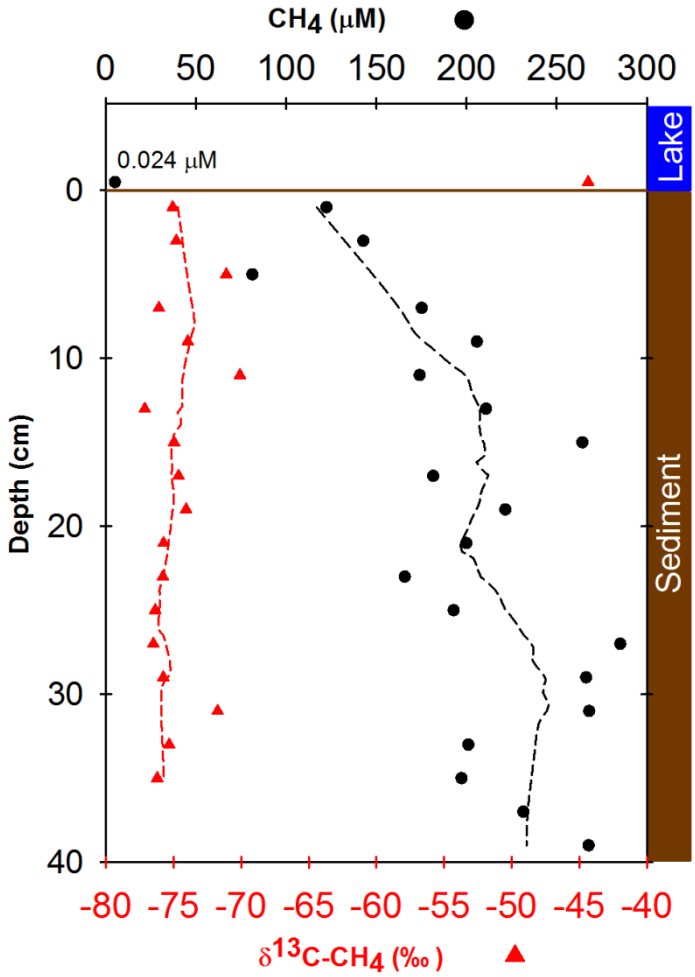



Figure 1. Methane concentration and $\delta^{13}C$-$CH_4$ values from SLW lake water and sediment porewater. Dotted lines indicates the running average using a Loess smoothing function.

$CH_4$ in the SLW sediment has an average $\delta^{13}C$-$CH_4$ value of -74.7‰ with a range from -77.1 to -70.1‰ (Figure 1 and 2). The biplot of $\delta^{13}C$- and $\delta D$-$CH_4$ values places SLW $CH_4$ within the hydrogenotrophic methanogenesis field (Figure 2). These $\delta^{13}C$- and $\delta D$-$CH_4$ values indicate $CH_4$ in SLW sediments is likely produced via biological

reduction of $CO_2$ with $H_2$ *(38)*. These findings are consistent with sediments in maritime Antarctic perennially and seasonally ice-covered lakes on Signy Island and in the McMurdo Dry Valleys, where $CO_2$ reduction also appears to be the dominant methanogenesis pathway *(22, 23)*. The methanogens that utilize $CO_2$ and $H_2$ to make methane are commonly found in deep marine sediments and other low sulfate environments *(24)*. Substrates ($CO_2$ and $H_2$) for $CH_4$ production are likely present beneath the WAIS. Bicarbonate concentrations in SLW are similar to those of sea water (~2 mM) and increase with depth to (~6 mM) at 37 cm depth *(Michaud et al., in press)*. Hydrogen was not directly measured on SLW sediments, but it can be generated abiotically from freshly-crushed and wetted subglacial siliceous bedrock, radiolysis of water, hydrothermal input, and biologically via fermentation *(24-27)*.

The source of $CH_4$ in SLW likely originates from sediment depths below our deepest sampling depth (37 cm below the sediment surface) with $CH_4$ diffusing towards the sediment-water interface. Samples from habitats with active Archaeal methanogenesis typically contain amplifiable and high copy numbers ($>10^4$ copies $g^{-1}$) of the *mcrA* gene *(28, 29)*. We were unable to amplify the *mcrA* gene from the SLW sediment core implying that methanogenic Archaea were low or below detection in the upper 38 cm of the sediment column of SLW. This supports the contention that the source of the $CH_4$ in our shallow core lies below our sampling depth.

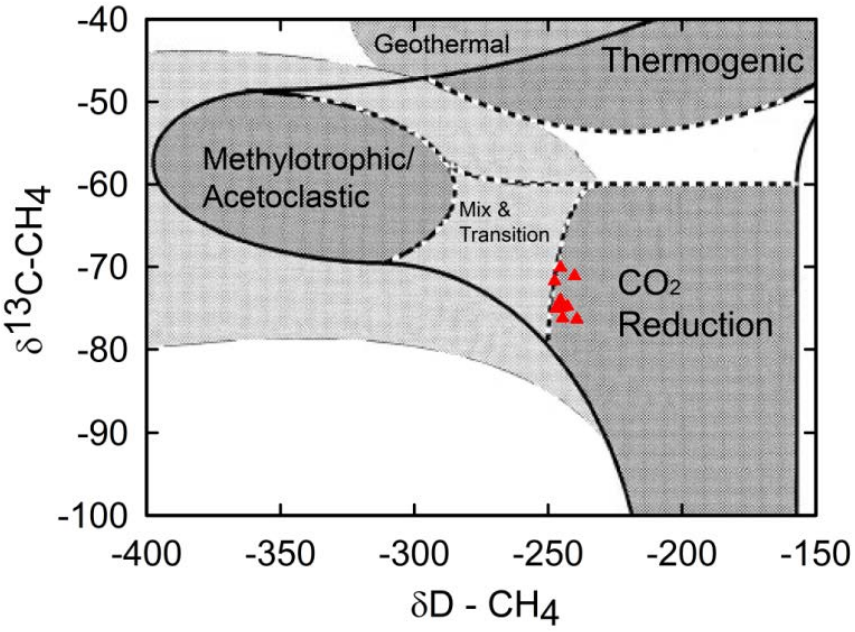



Figure 2. Methane stable isotope biplot for nine depths in the SLW sediment porewater (red triangles). The shaded areas delineate microbial and thermogenic endmembers as well as regions of mixed sources. Modified from Whiticar 1999 *(38)*. $\delta^{13}$C-$CH_4$ values in this plot are the same as Figure 1.

The low water column $CH_4$ levels suggest that aerobic $CH_4$ oxidation in surficial sediment and water column consumes a significant fraction (>99%) of the flux of sedimentary $CH_4$ (Figure 1). Aerobic $CH_4$ oxidation has also been shown to be responsible for consuming >98% of $CH_4$ near the margin of the Greenland Ice Sheet *(30)*. The large decrease in $CH_4$ concentration from the 0 – 2 cm sediments to the water column is commensurate with a large, positive shift (30.7‰) in the $\delta^{13}$C-$CH_4$. We used the Rayleigh distillation model to calculate a kinetic isotope fractionation factor (KIFF) of $\alpha = 1.004$ associated with the $CH_4$ oxidation process. This model assumes a closed system (i.e. no other inputs and measured isotope values are not affected by mixing) and the only sink for sediment $CH_4$ is bacterial oxidation. The KIFF calculated for $CH_4$

oxidation in SLW is at the lower range of laboratory cultures, but similar to field measurements from cold, marine habitats [$\alpha$ = 1.003 – 1.035 *(31, 32)*].

We amplified the alpha subunit of the particulate methane monooxygenase gene (*pmoA*) in sediments from 0 – 16 cm depth, but it was not detected below 16 cm. The *pmoA* genes recovered from SLW showed high (>90%) DNA similarity to the putative aerobic $CH_4$ oxidizing taxa recovered from deep sequencing of the surficial sediment microbial community [Figure 3, *(18)*]. *Methylobacter tundrapaludum* was an abundant member (2.6%) of the 0 – 2 cm sediment microbial community and is characterized by possessing a type-1 methane monooxygenase enzyme *(18, 33)*. Type-1 methanotrophs, *M. tundrapaludum* in particular, are known cold-adapted methanotrophs and dominate high $CH_4$ (µM – mM concentration) freshwater environments *(13)*. These molecular data imply that aerobic $CH_4$ oxidation is occurring in the surficial sediment (0 – 2 cm) and water column near the sediment-water interface *(18)*. Methane-oxidizing activity in SLW manifests in the water column as a 4 order of magnitude decrease in $CH_4$ concentration and a positive 30.7‰ shift in the $\delta^{13}C$-$CH_4$ value (Figure 1). Anaerobic $CH_4$ oxidation is a potential pathway for $CH_4$ oxidation but we found no $SO_4^{2-}$ concentration, $CH_4$ concentration or isotope evidence for this process in the 38 cm sediment column we sampled. The SLW sediments exhibit a linear increase of sulfate down core to a maximum value of ~ 7% of seawater sulfate concentration, indicating no sulfate reduction zone. The low sulfate is unlikely to support sulfate-mediated anaerobic $CH_4$ oxidation *(10, Michaud et al., in press)*. Additionally, the $CH_4$ concentration and isotopic

profiles in the sediment does not exhibit shifts indicative of anaerobic $CH_4$ oxidation *(34)*.

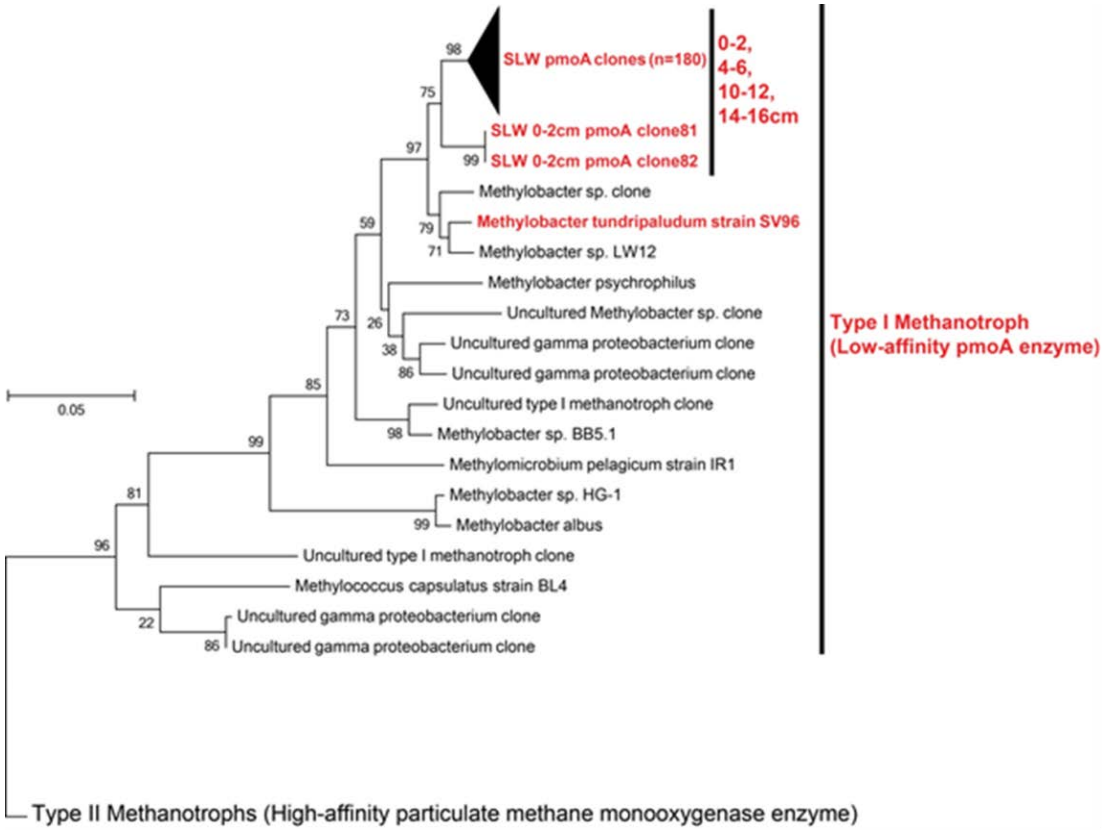



Figure 3. Maximum-likliehood phylogenetic tree of *pmoA* sequences from SLW sediment DNA extracts. Red text indicates *pmoA* clones from SLW and abundant methanotroph, *Methylobacter tundrapaludum* SV96, found in the SLW 16S rDNA community analysis *(16)*.

We modeled the rate of biological $CH_4$ consumption using the equation;

$$\frac{\partial C}{\partial t} = (F_{diff} \times A) - (R \times V) \qquad \text{Eq. 1}$$

where, $\frac{\partial C}{\partial t}$ is the change in methane concentration over time, $F_{diff}$ is the diffusional flux into the 0 – 2 cm surficial sediment, A is the area of SLW, R is the rate of $CH_4$

consumption, and V is volume of SLW lake plus the surficial (0 – 2 cm) sediment. Assuming steady-state conditions ($\frac{\partial C}{\partial t} = 0$) equation 1 simplifies to:

$$R = \frac{F_{diff}}{H_L + (H_{SS} \times \varphi)} \quad \text{Eq. 2}$$

where, $H_L$ and $H_{SS}$ are the height of the lake and surficial sediments, respectively, and φ is the sediment porosity. Based on Eq. 1 and 2, R is equal to 3 mmol $CH_4$ $m^{-3}$ $y^{-1}$. The rate of $CH_4$ removal (R) is the sum of both oxidation of $CH_4$ to $CO_2$ and the incorporation of $CH_4$ as a carbon source for biomass synthesis. An accepted value for the fraction of $CH_4$ going to biomass is 0.5 for both type 1 and 2 methanotrophs *(13, 14)* indicating that methanotrophs could be oxidizing 1.5 mmol $CH_4$ $m^{-3}$ $y^{-1}$ and incorporating 1.5 mmol $CH_4$ $m^{-3}$ $y^{-1}$. This rate of aerobic $CH_4$ oxidation is ten to one hundred fold less than aerobic $CH_4$ oxidation measured in cold (~4°C), surficial marine sediments, Amazonian rivers and deep sea, $CH_4$ cold seep-associated carbonates *(35–37)*. The formation of biomass due to $CH_4$ oxidation in the sediment occurs at a rate (29.7 ng C $L_{porewater}^{-1}$ $d^{-1}$), similar to that measured in the SLW water column for dark carbon fixation (32.9 ng C $L^{-1}$ $d^{-1}$; *25*).

The stoichiometric oxygen demand of the modeled $CH_4$ oxidation rate is 6.2 x $10^5$ mol $O_2$ $yr^{-1}$. The input of oxygen to the lake is basal ice melt, which releases atmospheric gases to the lake, and advection into the lake during the filling phase of the lake cycle *(17)*. Basal melt, which has been estimated at 1.8 mm $y^{-1}$ *(16)*, delivers 1.0 x $10^6$ mol $O_2$ $yr^{-1}$, and advection into the lake provides 5 x $10^5$ mol $O_2$ $yr^{-1}$, assuming the incoming water has the same concentration measured in the SLW water column *(16)*. The oxygen demand from $CH_4$ oxidation is less than the oxygen sources to the lake, with the input of oxygen from basal ice melt alone being sufficient to support the $CH_4$ oxidation rates we

computed. Methanotrophs are oxidizing $CH_4$ and likely producing biomass at a rate comparable to chemoautotrophic activity in the lake water, indicating that $CH_4$ is a carbon and energy source to the SLW sediment-water interface and a biological sink of oxygen in the SLW ecosystem. $CH_4$ supply to subglacial aquatic environments may represent a significant source of reduced carbon and electrons to fuel microorganisms at the bed of the WAIS where basal melting supplies oxygen.

## Conclusions

Our results show that the $CH_4$ pool beneath SLW is formed by hydrogenotrophic metabolism ($CO_2$ reduction with $H_2$; *29*) and used by aerobic methanotrophs as a carbon and energy source in the sediments. Methanogenesis may be widespread beneath the WAIS, but our data indicate that acetoclastic methanogenesis of relict marine carbon is not the main pathway of methane formation. Aerobic methanotrophy converts the sedimentary $CH_4$ in SLW to $CO_2$, keeping the $CH_4$:$CO_2$ ratio low, and similar to that of freshwater Arctic lakes where methanotrophs play an important role in regulating the ratio of greenhouse gases emitted to the atmosphere *(39)*. The geothermal heat flux recorded for SLW, which is higher than the global continental average *(16)*, may play an important role in delivering atmospheric oxygen to the subglacial environment through basal ice melt, which can support methanotrophy over a wide area of the ice sheet bed. Biological $CH_4$ oxidation occurs beneath the WAIS, and this metabolic process may mitigate the atmospheric release of stored $CH_4$ during periods of deglaciation.

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# CHAPTER FIVE

## BACTERIAL RESPONSE TO PROGRESSIVE FREEZING PROCESS IN PERENNIALLY AND SEASONALLY ICE-COVERED LAKES

### Contribution of Authors and Co-Authors

Author: Pamela Santibáñez

Contributions: Supported this research under a Fulbright-CONICYT student scholarship, defined the experimental design, participated in fieldwork, helped to implement and execute the mesocosm experiments and measurements, cut the ice for ions, TOC and bacteria counts, counted bacterial samples for all the mesocosms and ice covers from Barrow lakes, analyzed the data and wrote the manuscript.

Co-Author: Alexander B. Michaud

Contributions: Defined the experimental design, implemented and executed the mesocosm experiments and measurements, cut the ice for ions, TOC and bacteria counts, performed ion analyses and TOC, analyzed the data, discussed the results and implications, and edited the manuscript.

Co-Author: Trista J. Vick-Majors

Contributions: Helped to implement and to execute the mesocosm experiments and measurements, discussed the results and implications, and edited and commented on the manuscript.

Co-Author: Juliana D'Andrilli

Contributions: Helped to execute the mesocosm experiments, performed EEMS analyses and commented on the manuscript.

Co-Author: Amy Chiuchiolo

Contributions: Participated in fieldwork and retrieved water samples from Fryxell, and helped to execute the mesocosm experiments.

Contribution of Authors and Co-Authors - Continued

Co-Author: John C. Priscu

Contributions: Supported this research under the National Science Foundation grant 1115245, the Astrobiology of Icy Worlds program at JPL, and a NASA Astrobiology Science and Technology for Exploring Planets (ASTEP) award (Project Narvak, NNN13D036T). Helped define the experimental design, discussed the results and implications and edited the manuscript.

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## Abstract

Lakes represent important sites of biogeochemical transformations in both polar and temperate environments. Bacteria have direct roles in lake biogeochemical cycles; however, the response of bacteria to seasonal and perennial ice covers on lakes are poorly constrained. The aim of this study was to investigate the distribution of biological and chemical constituents between ice and liquid phases during progressive freezing. We simulated ice formation with controlled freezing mesocosm experiments using water from perennially (Antarctica) and seasonally (Arctic and Montana, USA) ice covered lakes and computed concentration factors and effective segregation coefficients for ions, total organic carbon, and bacteria. Mesocosm experiments revealed that effective segregation coefficients between liquid water and ice were higher for bacteria than for ions and total organic carbon. The organic matter incorporated into the ice was characterized by labile, protein-like material, which differed from the humic-like compounds that remained in the liquid fraction. We observed bacterial aggregates in the natural lake ice cover and in the ice formed during our experiments suggesting a bacterial response to progressive freezing. The wide range of bacterial segregation coefficients (0.8 -4.4) identified in our study illustrates that the incorporation of bacteria into lake ice cover is also dependent on physical (i.e., freezing rate) and chemical processes (i.e., initial solute concentration) which vary between lakes and throughout the freezing process.


## Introduction

Surface lakes contain ~180,000 $km^3$, or ~0.01% of Earth's water, but in spite of their relatively small volume, these inland waters comprise a major component of global biogeochemical cycles [*Shiklomanov*, 1993; *Downing and Duarte*, 2006; *Tranvik et al.*, 2009]. Microbial activity, through the mobilization and regeneration of biologically important elements [*Newton et al.*, 2011] and the emission of greenhouse gases to the atmosphere [*Liu and Xu*, 2015], are key to the role of lakes in biogeochemical processes. Some of the highest densities of fresh surface water on Earth occur at high latitudes, in boreal environments (e.g. Lapierre *et al.* 2013) or at high altitudes, where lakes are either perennially or seasonally (~40% of the year; Walsh *et al.* 1998) ice-covered. Ice cover

alters the physical and chemical structure of a lake compared to ice-free conditions by reducing heat exchange and preventing atmospheric mixing. Little is known about the impacts of ice cover and ice cover formation on microbial community structure [*Safi et al.*, 2012; *Bertilsson et al.*, 2013], although microbial responses to ice cover almost certainly influence lake biogeochemistry.

Seasonal lake ice formation in cold environments is a progressive process governed by intense radiative and turbulent heat losses from the warmer lake surface to the colder atmosphere [*Lock,* 1990; *Petrenko,* 1999]. These physical controls first cause a primary layer of ice to form over the entire lake surface. A secondary layer is composed of ice crystal growth downward from the primary layer and parallel to the temperature gradient. The continuously cold conditions that exist in Antarctica produce perennial ice covers where freezing of lake water onto the bottom is in a dynamic equilibrium with ablation losses at the surface [*Adams et al.*, 1998; *Fritsen et al.*, 1998].

Lake water contains suspended and dissolved impurities, such as gas bubbles, solutes and insoluble particles. During lake ice formation, solute and particles are largely rejected from the solid phase [*Weeks and Lofgren,* 1967; *Clayton et al.*, 1990; *Killawee et al.*, 1998; *Rempel and Worster*, 1999]. As the ice front advances, these suspended and dissolved impurities may subsequently be incorporated into the ice as inclusions of concentrated solution, trapped in grain boundaries, or incorporated in the ice lattice [*Petrenko,* 1999]. Interactions between the ice and impurities depend on the speed of the ice front (freezing rate) and the sizes and charges of the impurities [*Gilpin,* 1980; *Shangguan et al.*, 1992; *Lipp and Körber,* 1993; *Killawee et al.*, 1998; *Park et al.*, 2006].

The concentration factor (CF) and the effective segregation coefficient ($K_{eff}$) are important ice-growth parameters that describe the distribution of solutes and particles (impurities) between liquid and solid phases during progressive-freezing and crystal growth [*Garandet et al.*, 1994; *Killawee et al.*, 1998; *Leppäranta*, 2015]. The concentration factor (CF) describes the progressive evolution of solute and bacteria concentration in the liquid phase [*Garandet et al.*, 1994; *Killawee et al.*, 1998; *Gu et al.*, 2005]:

$$CF = \frac{C_{L\,(T_i)}}{C_{L\,(T_0)}} \qquad \text{[eq. 1]}$$

where, $C_{L\,(T_i)}$ is the solute concentration in the liquid phase at time $i$ and $C_{L\,(T_0)}$ the initial solute concentration in the liquid-phase. The effective segregation coefficient ($K_{eff}$) describes the incorporation of solute and bacterial cells into ice (higher $K_{eff}$ indicates more segregation from the liquid phase to the ice phase), and describes the flux of matter away from ($K_{eff} < 1$, solute rejection) or into ($K_{eff} > 1$, preferential incorporation) the solid phase [*Garandet et al.*, 1994; *Killawee et al.*, 1998; *Liu et al.*, 1999], such that:

$$K_{eff} = \frac{C_{s\,(ice)}}{C_{L(bw)}} \qquad \text{[eq. 2]}$$

where, $C_{s\,(ice)}$ is the solute or bacterial cell concentration in the solid phase and $C_{L\,(bw)}$ is the concentration in the bulk liquid phase. The chemical evolution of the liquid phase during the freezing processes in freshwater lakes is relatively well understood; however, despite the ecological importance of microorganisms, little is known about the relative incorporation of microbial cells into ice cover or their concentration in the water column [*Danz et al.*, 2007; *Schindler*, 2009; *Newton et al.*, 2011. An estimated segregation

coefficient for Antarctic surface lakes [*Priscu et al.,* 1999] was used to estimate bacterial abundances in an Antarctic subglacial lake based on samples of accretion ice (i.e. subglacial lake water frozen onto the overlying glacier ice) [*Christner et al.*, 2006], and a better understanding of bacterial segregation coefficients has important implications for modeling difficult to access icy environments on Earth and in the solar system.

It has been assumed that lake ice does not support living non-particle or aggregate associated microorganisms and that microbes, due to their small size, are excluded from the ice cover as it forms [*Gilpin*, 1980; *Lock*, 1990; *Leppäranta*, 2015]; however, microorganisms are found in association with icy environments. Microbial life has been reported in meteoric and basal ice from supra-glacial, subglacial and sea-ice environments where ice associated microorganisms are important contributors to biological production and biogeochemical cycles [*Helmke and Weyland*, 1995; *Junge et al.*, 2001; *Hodson et al.*, 2008; *Stibal et al.*, 2009; *Bowman et al.*, 2013*Montross et al.*, 2014]. Given the annual occurrence of seasonal lake ice formation and extent of icy environments, we hypothesized that microorganisms inhabiting high latitude or altitude lakes possess adaptations which enable them to incorporate into the ice cover to avoid the chemical and physical stresses associated with freeze concentration in the water column.

In this study, experimental mesocosms were prepared using lake water from seasonal and perennial ice-cover regimes to evaluate the effect of progressive freezing on the distribution of bacterial cells from two different systems. We present the mesocosm experiments with observational studies from seasonally ice-covered lakes to determine bacterial abundance distribution in lake ice cover. Our objectives were: (i) to calculate the

concentration factor and effective segregation coefficients of bacterial cells over the course of freezing; (ii) to compare bacterial abundance in the ice and liquid phase from seasonally and perennially ice-covered lakes; (iii) to compare bacterial concentration factors and segregation coefficients with those of ions ($F^-$, $Cl^-$, $SO_4^{2-}$, $Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$) and total organic carbon (TOC), and with the quality of organic matter (OM); and (iv) to determine whether bacterial distributions during freezing result from biotic or abiotic responses to freezing using a dead bacterial control.

## Methods

### Lake Ice Analysis

Site and Sample Procedure. Ice-cover cores (7.5 cm diameter) were retrieved in April 2010 from Lakes Emaiksoun (ice-cover thickness of 76.9 cm), Ikroavik (ice-cover thickness of 115.9 cm), and Siqlukaq (ice-cover thickness of 85.4 cm) near Barrow, Alaska on the North Slope (71° 04’ N; 156° 49’ W), and transported to Montana State University at near -20°C where they were stored at -30°C until analysis. All of the lakes in this region are thermokarst lakes formed by the degradation of permafrost [*Hopkins,* 1949]. More than 10,000 such lakes occur in this region covering up to 40% of the coastal plain [*Hinkel et al.,* 2005]. Jeffries et al., (1996) estimated that 60% of the lakes have depths ranging from 1.5 to 2.2 m, and many of them freeze to bottom. Depending on lake water depth, the maximum ice thickness occurs between late-April and May [*Jeffries et al.,* 1996; *Jones et al.,* 2009].

Decontamination Protocols. Ice-cover cores were cut into 5 to 10 cm sections, which were then decontaminated in a clean cold room (class 100/1000, ISO 4/5) at -15°C in the Sub-Zero Research Facility at Montana State University. All surfaces in contact with the ice samples were pre-cleaned with ethanol (95% v/v) and materials were sterilized before entering the clean cold room. Ice sections for ions were decontaminated by mechanically removing the outer 0.5 cm of ice followed by melting at 20°C [*Schwikowski et al.*, 1999]. Ice samples for bacterial enumeration were decontaminated following procedures described by *Christner et al.*, [2005]. Between each bacterial ice-sample, sterile gloves were replaced and the bench and band saw surfaces were cleaned with ethanol (95% v/v). Bacterial samples were transferred to sterile glass vials, fixed with formalin (2% final concentration), and stored at 4°C until microscopic analysis.

## Freeze-Down Mesocosm Experiments

Lake Water Collection. Water was collected from 8 m depth in Lake Fryxell, Taylor Valley, Antarctica (77° 37’ S; 163° 07’ E) during November 2010 and 2011, from 0.5 m in Sukok Lake, North Slope of Alaska, USA (71° 04’ N; 156° 49’ W), during October 2011 and from 0.5 m in Dailey Lake, Montana, USA (46° 15’ N; 110° 48’ W) in October 2013. Lake Fryxell has a perennial ice-cover (~4 m thick), a surface area of 7 $km^2$ and a maximum liquid water depth of ~20 m [*Howard-Williams et al.*, 1998]. Sukok and Dailey Lakes have seasonal ice-covers (~December through April) that reach ~1 m and ~0.5 m thick, and have maximum water column depths of ~2 and 7.2 m, respectively.

Water samples from all the sites were stored at Montana State University at 4°C in the dark until experimental set up.

Experimental Protocols. Lake water experiments were conducted in a 65 L flexible rubber tank lined with a polytetrafluoroethylene (PTFE) liner (Welch Fluorocarbon, Inc.) to prevent contamination from the tank. To reproduce ice-cover progressive freezing and prevent lateral and bottom freezing, we added 10 cm of insulation around the tank sides and 20 cm of insulation on the bottom. The mesocosm experiments were conducted in the Cold Sky chamber in the Sub-Zero Science and Engineering Research Facility at Montana State University. This chamber is equipped with a temperature-controlled ceiling (cold sky) to simulate atmospheric winter temperature gradients. The mesocosms were conducted in the dark to exclude photoautotroph activity at an air temperature of -10°C and cold sky temperature of -50°C. A total of five mesocosms experiments were performed. Two experiments were conducted on water from Lake Fryxell (Fryxell I and II; Supplemental Table 2), one each on Sukok and Dailey Lake, and a biological control experiment using killed bacteria from Dailey Lake (See below for further details).

At the beginning of each experiment, 50 L of water from each lake was poured into the tank. This volume produced a water depth of 21 cm. An initial sample (Time 0; $T_0$) was taken 15 min after pouring the water into the tank and before ice formation (0 cm). Liquid-phase samplings (~80 mL) were performed when the ice was 2.5, 5.0, 7.5, 10.0, and 12.5 cm thick (referenced as, $T_1$, $T_2$, $T_3$, $T_4$, and $T_5$, respectively). Ice thickness was measured ~12 times through 0.5 cm diameter holes drilled through the ice near the

center of the tank during each experiment and used to calculate freezing rate. Liquid samples (~80 mL) were collected at mid-depth in the tank through a 2.5 cm hole with a sterile 25 mL pipette and placed in 125 mL acid-leached (10% HCl for 24 h) and combusted (400°C for 4 h) bottles, capped with an acid-leached Teflon lined cap. All ice-thickness measurements and liquid-phase samples were collected from a dedicated half of the experimental mesocosms, thus preserving the remaining half of the lake ice cover for undisturbed progressive ice freezing and sampling (see below).

The liquid water samples were immediately processed and/or stored for pH, ions, total organic carbon (TOC), OM quality, and bacterial enumeration. Approximately 10 mL of sample was used for pH measurement (Fisher Scientific Accumet AB15). Sample water was syringe-filtered through a sterile nylon filter (0.2 μm) into a Milli-Q rinsed HDPE bottle for ion quantification, and stored at 4°C. For bulk TOC and OM measurements, 20 mL of liquid sample was decanted into an acid-washed (10% HCl for 24 h), Milli-Q rinsed, combusted (400°C for 4 h) glass amber vial, and stored at -20°C in the dark until analysis. For bacterial enumeration, an aliquot of 10 mL was fixed with sodium borate buffered formalin (2% final concentration) and kept in a sealed sterile glass vial at 4°C.

After the final liquid-phase sample ($T_5$) was collected, ice-phase samples were collected by cutting large ice blocks (L: 10 cm, W: 10 cm, H: ~14 cm) from the ice cover of each experiment using clean stainless steel saws. Blocks of ice were placed in sterile Whirl-pak® bags and stored at -30°C until decontamination and analysis. Ice blocks were decontaminated in a clean cold room (class 100/1000, ISO 4/5) at -15°C by mechanically

removing 0.5 cm of the outer part of the block with sterile stainless steel razor blade. The block was then cut into the three replicate sections to determine the concentrations of ions, TOC, OM, and bacterial cells. A clean benchtop band saw was used to cut ice sections. The three replicate ice sections were further cut into five ice samples (A – E) corresponding to the intervals between liquid-phase sample collection points (A: 0 - 2.5 cm, B: 2.5 - 5.0 cm, C: 5.0 - 7.5 cm, D: 7.5 - 10.0 cm, and E: 10.0 - 12.5 cm). Ion ice samples were decontaminated by mechanically removing 0.5 cm of the outer part of the ice section. All surfaces and materials in contact with the samples were pre-cleaned with ethanol (95% v/v) and heat sterilized, respectively. The ion ice samples were melted in a laminar flow hood at 20°C and filtered through nylon syringe filters (0.2 μm mesh) before analysis. The ice samples for TOC and OM were decontaminated by removing the outer 1 cm of ice with the bandsaw in the clean cold room without ethanol cleaning steps. Because an ethanol-free environment is required for TOC and OM samples, the blade from the band saw was cleaned between ice samples by making three cuts on Milli-Q ice (diameter 7 cm). The TOC and OM ice samples were melted before analysis in an acid-washed, combusted beaker in a laminar hood at 20°C in the dark [*Christner et al.*, 2005]. Nitrile gloves were used to manipulate TOC and OM ice samples. Ice samples for bacterial enumeration were decontaminated as described by *Christner et al.*, [2005].

Biological Control Experiment. A control was conducted using Dailey Lake water inoculated with dead bacterial cells to determine if the bacterial response to progressive freezing was passive or biologically mediated. The control water was prepared using a continuous circulation system to filter 60 L of Dailey Lake water twice through an 11 μm

membrane filter (Whatman) and then five times through a sterile 0.2 μm membrane filter (PALL Gelman Sciences) until microscopic analysis showed < 2 x $10^2$ cells $mL^{-1}$. Control bacterial cells were collected via centrifugation from fresh non-filtered Dailey Lake water, killed, and then added to the filtered Dailey Lake water to yield a final cell concentration of ~3 x $10^4$ cell $mL^{-1}$. Cells from 14 L of Dailey Lake water were pelleted in sterile falcon tubes at 4,500 x g for 10 min at 10°C to create a preserved bacterial inoculum. The pellets were re-suspended in Milli-Q water. Sodium borate buffered formalin was then added at 2% final concentration and stored overnight, and then pelleted. To avoid chemical changes in the filtered lake water, the fixed pellet was washed 10 times by resuspension in Milli-Q water and centrifuged at 4,500 x g for 10 min. Following the washing steps, the pellet was re-suspended in 50 mL of 0.2 μm filtered Dailey Lake water and sonicated five times (45 sec each) to disrupt bacterial aggregates that formed during centrifugation. Finally, the 50 mL preserved bacterial cell suspension was poured into 50 L of filtered Dailey Lake water and homogenized.

## Sample Analysis

Ions. Ion concentrations ($F^-$, $Cl^-$, $SO_4^{2-}$, $Na^+$, $K^+$, $Mg^{2+}$, and $Ca^{2+}$) were determined using an ion chromatograph (Metrohm) equipped with an ASupp 5 anion and C4 cation column (Metrohm) with 20 μL sample injection loops. Sample concentrations were calculated from a 5 point standard curve made from dilutions of an ISO-certified standard (Metrohm). Procedural blank samples (Milli-Q water syringe filtered through 0.2 μm nylon filters into 15 mL conical tubes) were analyzed in parallel with the lake and melted

ice samples. The methodological detection limits were 0.08 μg $L^{-1}$ for $F^-$ , 5.6 μg $L^{-1}$ for $Cl^-$, 8.1 μg $L^{-1}$ for $SO_4^{2-}$, 12.7 μg $L^{-1}$ for $Na^+$, 47.1 μg $L^{-1}$ for $K^+$, 100 μg $L^{-1}$ for $Ca^{2+}$ and 100 μg $L^{-1}$ for $Mg^{2+}$ and calculated based on *Oblinger-Childress et al.*, [1999].

Total Organic Carbon. TOC was analyzed using the UV/persulfate oxidation and membrane conductometric detection technique on a Sievers 900 portable TOC Analyzer with an inorganic carbon remover module (GE). This instrument performs an acidification step and purges inorganic carbon with the inorganic carbon remover module. Milli-Q water was flushed through the instrument between each sample to eliminate TOC carryover. Milli-Q water had a TOC concentration < 20 μg $L^{-1}$.

Organic Matter (OM) Characterization. The remaining 5 mL of the TOC sample was used for excitation-emission matrix fluorescence spectroscopy (EEMS) to assess the quality of the fluorescent portion of the OM. We first measured absorbance values (A) at 254 nm across a range excitation values from 190 to 1,100 nm using a 1 cm cuvette in a Genesys 10 Series Spectrophotometer (thermos-Scientific) and VISIONlite software. Samples with absorbance values > 0.3 at any wavelengths from 190 to 1,100 nm were diluted before EEMS analysis [*McKnight et al.*, 2001]. Absorbance measurements were also used to remove primary and secondary inner filter effects during EEMS post-processing [*Acree et al.*, 1991; *Tucker et al.*, 1992]. Spectra were blank-corrected against purified water from a Milli-Q system each day of analysis.

EEMS measurements were obtained with a Horiba Jobin Yvon Fluoromax-4 Spectrofluorometer equipped with a Xenon lamp light source and a 1 cm path-length.

Fluoresence data were collected over excitation wavelengths of 240 - 450 nm at10 nm intervals and emission wavelengths of 300 - 560 nm at 2 nm increments, with a data integration period of 0.25 s, and a 5 nm band pass for both excitation and emission monochromators. Data acquisition was carried out in signal/reference mode and the fluorescence emission signal was normalized with the excitation light intensity. Each day, blank samples (Milli-Q water) were scanned to initialize the instrument and prepare data for post-processing, performed in MATLAB to correct for inner filter effects, Raman scattering, and a blank subtraction. Following post-processing, the position and intensity of fluorescence peaks (excitation and emission maximum values) were evaluated to characterize the OM [*Coble*, 1996]. Parallel Factor Analysis (PARAFAC) was used to further decompose the multi-way EEMS data into modeled fluorescent components using the drEEM toolbox [*Murphy et al.*, 2013].

Bacterial Concentration. Epifluorescence microscopy was used to enumerate SYBR-Gold-stained bacterial DNA as described by *Lisle and Priscu,* [2004]. All bacterial counts were performed at 1000x with a Nikon Eclipse 80i epifluorescence microscope equipped with a metal halide lamp (X-Cite® 120 W), a 450-490 excitation filter (B-2A), and a 515 barrier filter. The total bacterial concentration was estimated on each filter by counting at least 400 cells. Controls (Milli-Q water) were counted along with the samples on a daily basis. In order to estimate attached bacteria in aggregates, at least 50 aggregates or up to 100 microscope fields per filter were counted, whichever occurred first. Then bacteria cells were counted at one plane-of-view in at least 10 bacterial-aggregates and a mean of bacterial cells per aggregate was estimated at each

sample. Because we counted aggregated bacteria from only one plane-of-view of bacterial aggregates, the counts of attached bacteria are likely underestimated.

## Results

### Barrow Lake Ice Cores

Mean bacterial concentrations in the ice were 9.4 x $10^4$ cells $mL^{-1}$ for Emaiksoun, 4.7 x $10^5$ cells $mL^{-1}$ for Ikroavik, and 1.0 x $10^5$ cells $mL^{-1}$ for Siqlukaq. The mean bacterial abundances in the ice phase were 22.9-fold, 5.8-fold, and 28.4-fold lower than in the liquid phase in Emaiksoun, Ikroavik, and Siqlukaq lakes, respectively (Supplemental Table 1, Fig. 1), from ice cores collected in April. The ice thicknesses at Emaiksoun, Ikroavik, and Siqlukaq lakes during the April sampling represented 36%, 57%, and 32% of the entire water column (ice plus liquid water), respectively.

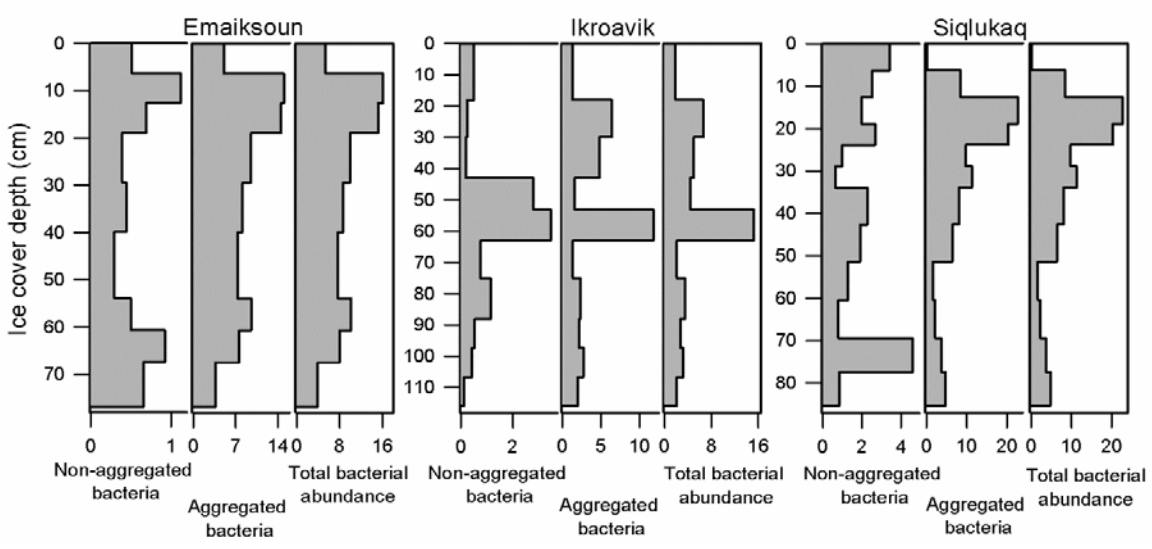



Figure 1. Non-aggregated and aggregated bacterial profiles from the ice cover of Barrow, AK area lakes. Note both horizontal and vertical scales are different for each lake. All concentrations are x $10^4$ cells $mL^{-1}$.

No clear stratigraphy in bacterial abundance (non-aggregated or aggregated cells) was observed in any of the ice cores; however, the abundance of aggregated bacteria was higher than the abundance of non-aggregated bacteria in all three lake ice cores (Fig. 1). The maximum abundance in the Emaiksoun ice cover occurred between 6 and 13 cm depth ($1.6 \times 10^5$ cells $mL^{-1}$) with 95% of the total represented by aggregated bacteria. In Ikroavik, maximum total bacterial abundance occurred in the middle of the lake ice core between 53 and 63 cm ($1.5 \times 10^5$ cells $mL^{-1}$), with aggregated bacteria accounting for 77% of the total, and the lowest abundance in the upper sections of ice between 0 and 18 cm ($1.8 \times 10^4$ cells $mL^{-1}$) with 72% aggregated bacteria. In the Siqlukaq ice cover, the maximum total bacterial abundance occurred between 12 and 24 cm depth ($2.4 \times 10^5$ cells $mL^{-1}$), with 90% of the total represented by aggregated bacteria, and the lowest total bacterial abundance in the upper sections of ice ($3.6 \times 10^4$ cells $mL^{-1}$), with 5% represented by aggregated bacteria. Overall, the upper sections (0-5 cm) of the ice cover contained the lowest bacterial abundances.

Mesocosm Experiments.

Bacterial Abundance in Ice. The ice cover from Sukok and Fryxell I experiments showed greater bacterial concentrations at depths below 10 cm (Fig. 2). In contrast, Dailey and Fryxell II experiments had higher bacterial abundances in the upper section (0 – 6 cm) of the ice, showing a decreasing bacterial abundance in the middle section of ice (~6 – 10 cm). Dailey Lake and the control experiments showed similar patterns of bacterial abundance in the ice cover (Fig. 2).

Supplemental Table 1. Water column composition at sampling locations on April and October 2010.

| | Emaiksoun | | Ikroavik | | Siqlukaq | |
|---|---|---|---|---|---|---|
| | **April - 2010** | **October - 2010** | **April - 2010** | **October - 2010** | **April - 2010** | **October - 2010** |
| Lake depth (cm) | 215 | 208 | 204 | 220 | 265 | 150 |
| Ice cover depth (cm) | 76.9 | 30.1 | 115.9 | 25.0 | 85.4 | 25.0 |
| pH | 6.84 | 6.96 | 6.79 | 7.15 | 7.2 | 6.34 |
| Water temperature ($^{o}$C) | 0.2 | 1.4 | - | 1 | 0.8 | 1.2 |
| Conductivity (mS cm$^{-1}$ at 25$^{o}$ C) | 0.27 | 0.10 | 0.50 | 0.19 | 1.59 | 0.29 |
| DOC (ppm) | 9.22 | 4.76 | 5.29 | 3.89 | 24.34 | 5.22 |
| Individual bacteria (cells mL$^{-1}$) | 2.16 x 10$^{6}$ | 1.56 x 10$^{6}$ | 2.71 x 10$^{5}$ | 2.46 x 10$^{6}$ | 2.92 x 10$^{6}$ | 1.96 x 10$^{6}$ |
| $F^-$ (mg L$^{-1}$) | 0.06 | 0.03 | 0.06 | 0.03 | 0.05 | 0.04 |
| $Cl^-$ (mg L$^{-1}$) | 70.9 | 23.4 | 125.2 | 61.9 | 503.8 | 106.3 |
| $SO_4^{2-}$ (mg L$^{-1}$) | 3.7 | 1.5 | 2.0 | 1.1 | 10.4 | 3.3 |
| $\sum anions$ | 74.6 | 24.9 | 127.2 | 63.1 | 514.2 | 109.6 |
| $Na^+$ (mg L$^{-1}$) | 29.7 | 10.3 | 57.7 | 27.1 | 215.7 | 46.9 |
| $K^+$ (mg L$^{-1}$) | 1.46 | 0.56 | 1.70 | 0.78 | 4.98 | 1.30 |
| $Ca^{2+}$ (mg L$^{-1}$) | 18.7 | 6.0 | 16.6 | 7.1 | 70.6 | 12.4 |
| $Mg^{2+}$ (mg L$^{-1}$) | 9.1 | 3.3 | 11.3 | 4.8 | 42.7 | 8.6 |
| $\sum cations$ | 59.0 | 20.1 | 87.4 | 39.8 | 334.0 | 69.2 |

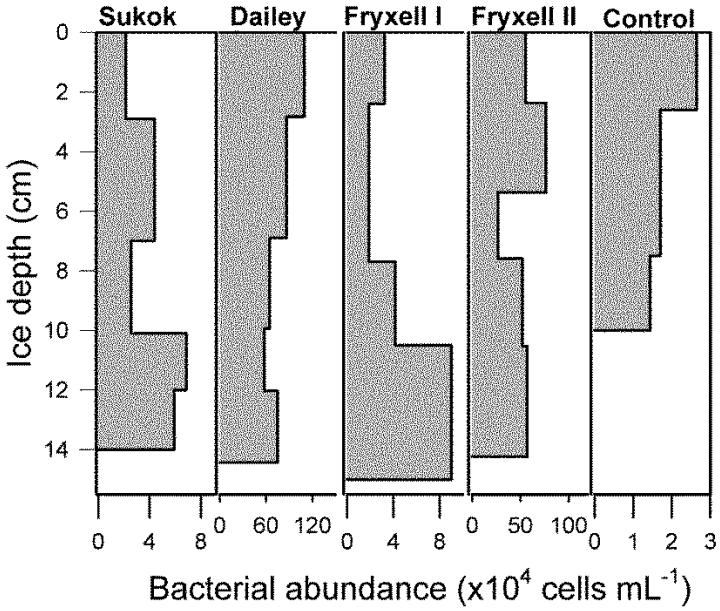
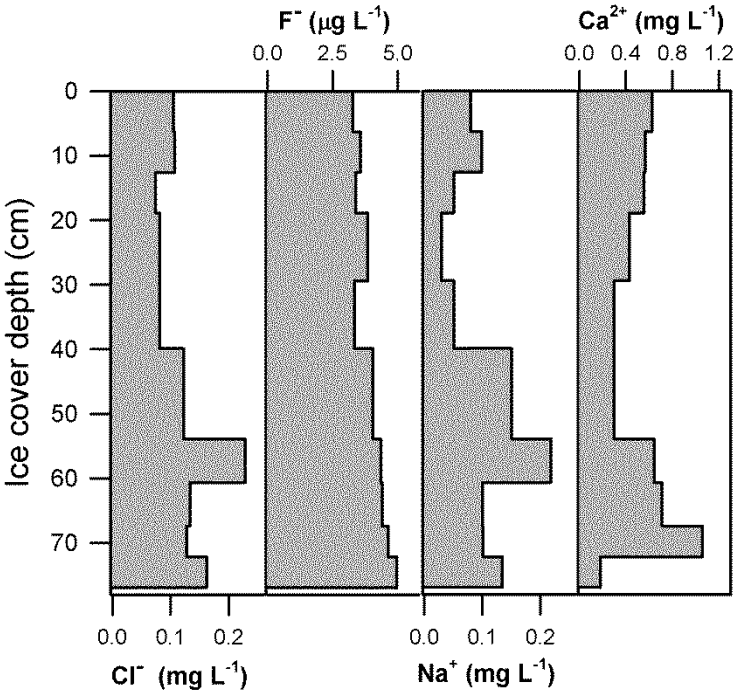



Figure 2. Vertical profiles of bacterial abundance in ice from each mesocosm experiment. Note horizontal scales are different.

Aggregated bacteria were only observed in ice and liquid phases in experiments using waters from seasonally ice-covered lakes (Sukok and Dailey; Fig. 3). Results are only presented from Dailey Lake because the low concentration of bacterial aggregates in the liquid and ice phases and limited liquid-phase sample volume precluded robust quantification of aggregated bacterial abundance in the Sukok mesocosm. The Dailey experiment showed that the contribution of aggregated bacteria to the total bacterial abundance in ice was <10%. However, in the liquid phase the aggregated bacteria constitute 2 – 23% of the total bacterial abundance (Fig. 3).




Supplemental Figure 1. Vertical profiles of ions of Emaiksoun Lake ice cover from April 2010.

Evolution of Liquid Phase: Concentration Factor. An ion is considered conservative when it is concentrated in the liquid-phase and not incorporated into the ice

phase through the progressive freezing process. Ions with higher CF are more conservative (partitioned to the liquid phase) than ions with lower CF (partitioned to the ice phase). No ion was completely conservative in the mesocosm experiments. $Mg^{2+}$ was the most conservative (i.e., highest CF) ions (Fig. 4). Ions were concentrated in the liquid-phase with different efficiencies. CF showed the following trend of ion conservation: $Mg^{2+}>K^{+}>Ca^{2+}>Na^{+}\sim Cl^{-}>SO_4^{2-}>F^{-}$ for Sukok water; $K^{+}> Cl^{-}\sim F^{-}\sim Na^{+}> SO_4^{2-}> Mg^{2+}> Ca^{2+}$ for Dailey water; $Mg^{2+}\sim K^{+}> Cl^{-}\sim Na^{+}> SO_4^{2-}>F^{-}> Ca^{2+}$ for Fryxell I water, $Mg^{2+}> K^{+}\sim Na^{+}\sim Cl^{-}> SO_4^{2-}> F^{-}> Ca^{2+}$ for Fryxell II water; and $Mg^{2+}> Ca^{2+}> Cl^{-}\sim K^{+}\sim Na^{+}> SO_4^{2-}\sim F^{-}$ for the control (Fig. 4).

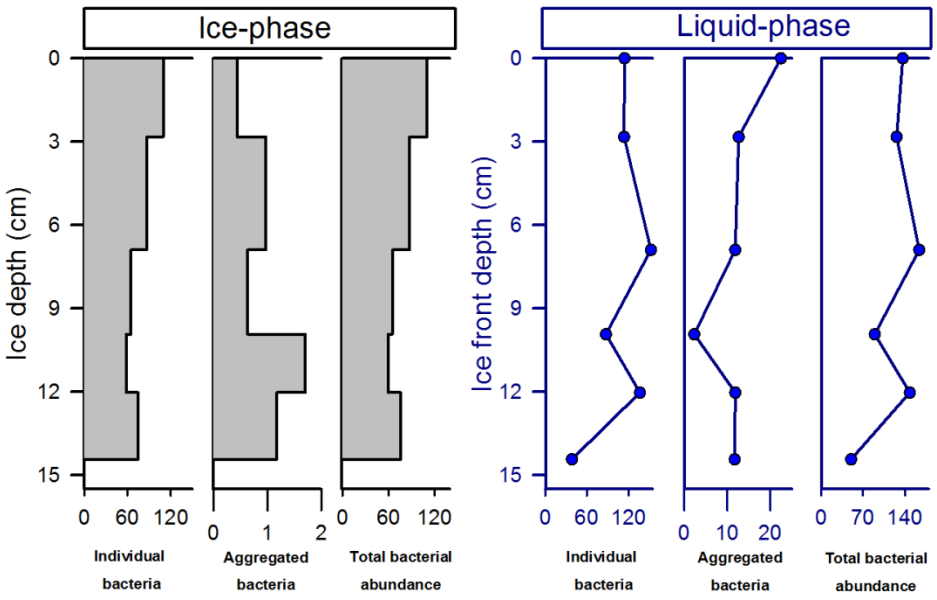



Figure 3. Non-aggregated, aggregated and total bacterial abundance from the ice cover of the Dailey mesocosm in liquid and solid phases. Note horizontal scales are different for attached bacterial abundance. All concentrations are x $10^4$ cells $mL^{-1}$.

In contrast to the ions, non-aggregated bacteria did not concentrate in the liquid phase for the Sukok or Fryxell II experiments at any time during the experiment (CF < 1). For Dailey and Fryxell I, bacteria were concentrated in the water column at the beginning of the freezing process, but did not concentrate in the liquid phase after the ice front proceeded past 8 cm. The control experiment bacteria were concentrated in the liquid phase (CF > 1) throughout the experiment, similar to the ions (Fig. 4e). These data show that living and dead cells respond differently to freezing front progression (Fig. 4a-d). We did not calculate concentration factors for bacterial aggregates due to the lack of aggregated bacteria in the perennially ice covered lake experiments (Fryxell I and II) and low concentrations in Sukok.

Bulk TOC had concentration factors > 1 in all of the mesocosms except for Sukok, indicating that organic carbon was concentrated in the water during the freezing process. In Sukok, bulk TOC did not concentrate (CF < 1); rather, it showed a pattern similar to bacteria (Fig. 4).

Evolution of the Solid Phase: Effective Segregation Coefficient. Most ions had a similar $K_{eff}$ throughout all experiments (Fig. 5, Supplemental Table 3). $Na^+$ and $Cl^-$ had lower $K_{eff}$, indicating weak segregation between the liquid and ice phase, while a higher $K_{eff}$ was observed for $F^-$. All experiments except Sukok had similar patterns of ion $K_{eff}$ throughout the progressive freezing process (Fig. 5).

Supplemental Table 2 Summary of initial conditions of the mesocosm experiments.

| | **Sukok** | **Dailey** | **Fryxell (I)** | **Fryxell (II)** | **Control** | |
|---|---|---|---|---|---|---|
| *Parameters* | | | | | | |
| Date | May 2012 | February 2014 | March 2012 | March 2013 | February 2014 | |
| Freezing rate (mm $hr^{-1}$) | 2.27 | 2.11 | 2.36 | 2.19 | 2.19 | |
| Experiment duration (hr) | 67.41 | 73.5 | 67.34 | 69.92 | 46.9 | |
| Ice-cover regime | Seasonal | Seasonal | Perennnial | Perennial | Seasonal | |
| Started liquid water volume (L) | 50.1 | 50.1 | 50.1 | 50.1 | 48.9 | |
| Ambient temperature ($^oC$) | -10 | -10 | -10 | -10 | -10 | |
| Cold sky temperature ($^oC$) | -50 | -50 | -50 | -50 | -50 | |
| Initial water temperature ($^oC$) | 4.4 | 3.5 | 4.5 | 4.2 | 4.0 | |
| Final water temperature ($^oC$) | -0.24 | -0.21 | -0.29 | -0.36 | -0.23 | |
| | | | | | Before addition of dead bacteria | After addition of dead bacteria |
| Initial conductivity (mS $cm^{-1}$ at 25$^o$ C) | 1.25 | 2.39 | 4.41 | 6.26 | 1.37 | 1.31 |
| Initial pH | 8.0 | 8.9 | 8.25 | 8.01 | 8.97 | 8.95 |
| *Initial concentration* | | | | | | |
| $F^-$ (mg $L^{-1}$) | 0.07 | 0.60 | 1.05 | 1.41 | 0.52 | 0.52 |
| $Cl^-$ (mg $L^{-1}$) | 148.9 | 23.8 | 457.9 | 646.8 | 22.9 | 22.5 |
| $SO_4^{2-}$ (mg $L^{-1}$) | 5.5 | 103.1 | 67.3 | 86.9 | 103.2 | 101.5 |
| $\sum anions$ (mg $L^{-1}$) | 154.4 | 127.5 | 526.2 | 735.1 | 126.6 | 124.5 |
| $Na^+$ (mg $L^{-1}$) | 65.7 | 90.1 | 333.2 | 486.7 | 88.0 | 87.3 |
| $K^+$ (mg $L^{-1}$) | 1.6 | 29.2 | 34.3 | 43.6 | 28.3 | 32.0 |
| $Ca^{2+}$ (mg $L^{-1}$) | 23.0 | 40.9 | 54.2 | 126.8 | 24.7 | 27.4 |
| $Mg^{2+}$ (mg $L^{-1}$) | 12.6 | 273.5 | 29.7 | 73.9 | 204.0 | 224.2 |
| $\sum cations$ (mg $L^{-1}$) | 103.0 | 433.7 | 451.4 | 731.1 | 345.0 | 370.9 |
| Bacteria (cells $mL^{-1}$) | $2.22 \times 10^5$ | $1.14 \times 10^6$ | $4.14 \times 10^4$ | $2.94 \times 10^5$ | $2.23 \times 10^3$ | $1.28 \times 10^4$ |
| TOC (ppm) | 9.23 | 16.2 | 3.06 | 3.54 | - | - |

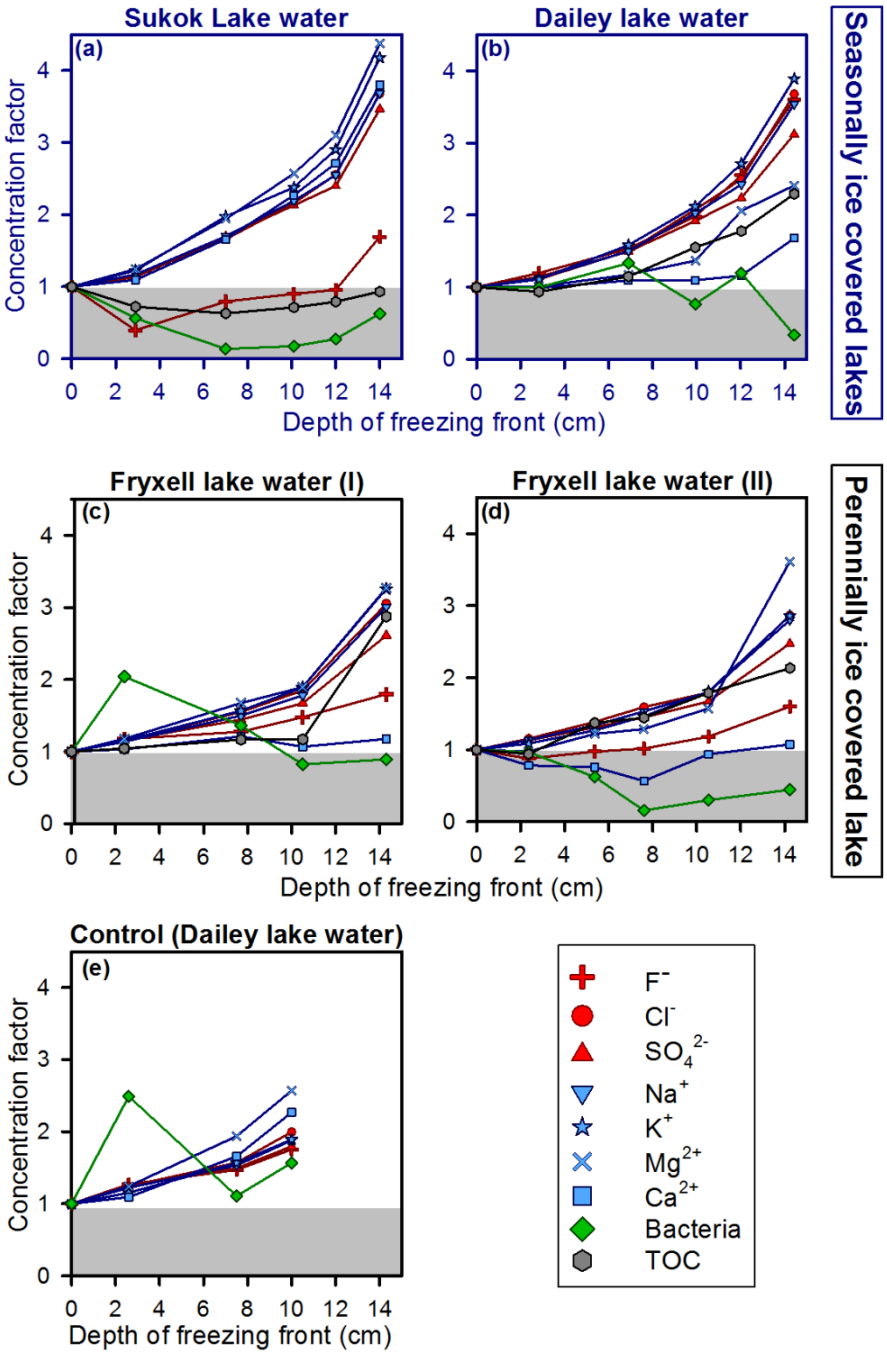
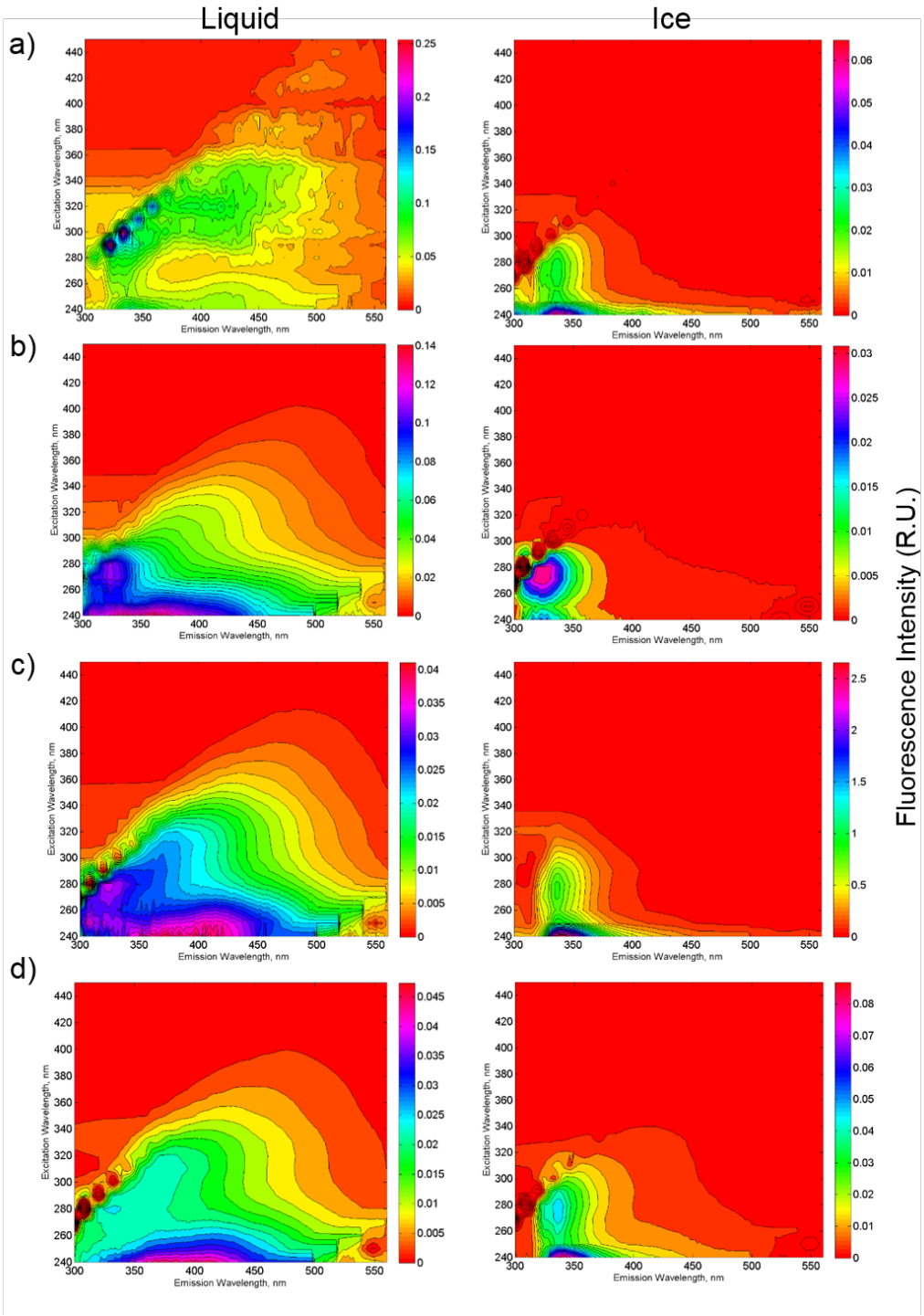



Figure 4. Concentration factor versus depth of freezing front (thickness of the ice; cm) from the five mesocosm experiments. Experiments (a, b) were performed on water from seasonally ice covered lakes: (a) Sukok Lake and (b) Dailey Lake. Experiments (c, d) were performed on water from a perennially ice covered lake (Lake Fryxell) at two different times. (e) The control refers to Dailey Lake sample in which the bacterial cells were killed before the experiment. The horizontal grey bar corresponds to CF < 1 as a consequence of the incorporation into the ice or other losses from precipitation.




Supplemental Figure 2. Excitation emission matrix spectroscopy of the liquid (left column) and thawed solid phase (right column) samples from the (a) Sukok, (b) Dailey, (c) Fryxell I and (d) Fryxell II mesocosm experiments. Fluorescence intensities are in Raman Units on the Z-axis colorscale.

Effective segregation coefficients of non-aggregated bacteria were higher than those calculated for ions in all the experiments, including the control, indicating stronger segregation of bacterial cells than ions to the ice phase during freezing (Supplemental Table 3, Fig 5). We calculated $K_{eff}$ for non-aggregated bacteria only. The Sukok mesocosm had the lowest mean $K_{eff}$ for bacteria (0.77), while Fryxell II had the highest mean (4.43). Segregation coefficients were lower for bacterial cells from seasonally ice-covered lakes than for bacterial cells from perennially ice covered lakes. The Dailey and control mesocosms showed similar $K_{eff}$ (0.93 versus 0.92); however, the bacteria in the Dailey mesocosm formed aggregates (Fig. 3), while the dead bacteria in the control did not (data not shown).

TOC had $K_{eff}$ values similar to bacteria (> 1) in Fryxell II; this was the only experiment where TOC segregated to the solid phase. Sukok, Dailey, and Fryxell I all had TOC $K_{eff}$ values < 1 and typically below the bacterial $K_{eff}$ values. In these experiments, TOC was rejected from the solid phase throughout the progressive freezing process. TOC and bacteria had $K_{eff}$ values greater than ions in all experiments except for the first time point of Sukok. However, subsequent analysis shows there is a component of TOC that is incorporated into the ice in all experiments (See Organic Matter Character and Segregation section).

Supplemental Table 3. Effective segregation coefficient for each experiment. Range 0.0006 – 5.873.

| | Sukok | | Dailey | | Fryxell (I) | | Fryxell (II) | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| $k_{eff}$ | $\bar{x}$ | *Range* | $\bar{x}$ | *Range* | $\bar{x}$ | *Range* | $\bar{x}$ | *Range* | $\bar{x}$ | *Range* |
| $F^-$ | 0.1109 | 0.055 - 0.181 | 0.254 | 0.090 - 0.301 | 0.267 | 0.183 - 0.308 | 0.280 | 0.266 - 0.294 | 0.274 | 0.103 - 0.407 |
| $Cl^-$ | 0.0017 | 0.001 - 0.003 | 0.139 | 0.003 - 0.234 | 0.136 | 0.032 - 0.185 | 0.199 | 0.104 - 0.247 | 0.103 | 0.009 - 0.177 |
| $SO_4^{2-}$ | 0 | 0 | 0.255 | 0.002 - 0.348 | 0.226 | 0.070 - 0.306 | 0.263 | 0.199 - 0.312 | 0.231 | 0.005 - 0.390 |
| $Na^+$ | 0.0011 | 0.0006 - 0.0019 | 0.154 | 0.003 - 0.243 | 0.145 | 0.035 - 0.199 | 0.180 | 0.110 - 0.220 | 0.103 | 0.007 - 0.173 |
| $K^+$ | 0 | 0 | 0.110 | 0.005 - 0.194 | 0.108 | 0.025 - 0.146 | 0.146 | 0.088 - 0.173 | 0.072 | 0.008 - 0.124 |
| $Ca^{2+}$ | 0.004 | 0.002 - 0.008 | 0.107 | 0.011 - 0.167 | 0.185 | 0.073 - 0.252 | 0.240 | 0.196 - 0.311 | 0.048 | 0.006 - 0.116 |
| $Mg^{2+}$ | 0.036 | 0.015 - 0.076 | 0.147 | 0.028 - 0.202 | 0.211 | 0.066 - 0.298 | 0.243 | 0.144 - 0.322 | BDL | BDL |
| Bacteria | 0.767 | 0.172 - 1.446 | 0.928 | 0.423 - 1.946 | 1.145 | 0.334 - 2.643 | 4.429 | 1.944 - 5.873 | 0.920 | 0.72 - 1.21 |
| TOC | 0.199 | 0.115 - 1.401 | 0.573 | 0.477 - 0.706 | 0.667 | 0.387 - 0.984 | 1.479 | 1.219 - 1.765 | - | - |

Organic Matter Character and Segregation. Liquid phase OM fluorescence from the beginning (T0) of each experiment (Sukok, Dailey, Fryxell I, and Fryxell II) was distributed over broad excitation (Ex) and emission (Em) wavelengths (Supplemental Fig. 2 liquid phase). Fluorescence intensities fell within similar ranges for Sukok and Dailey Lake (0-0.25 and 0-0.14 Raman Units; R.U.), while Fryxell I and II were nearly identical (0.005 R.U. difference). The positions and maxima of naturally occurring OM fluorophore peaks varied depending on the lake sampled. All lakes contained OM fluorescence maxima in regions characterized as more-labile (amino acid-like fluorescence) and more-recalcitrant (humic-like fluorescence), depicting OM chemical species with broad ranges of molecular weights, aromatic nature, heterogeneity, and microbial and terrestrial influences. EEMS obtained from the thawed solid phase lake samples contained OM fluorescence at low Ex/Em wavelengths characteristic of a tryptophan-like fluorophore (Supplemental Fig. 2 solid phase).

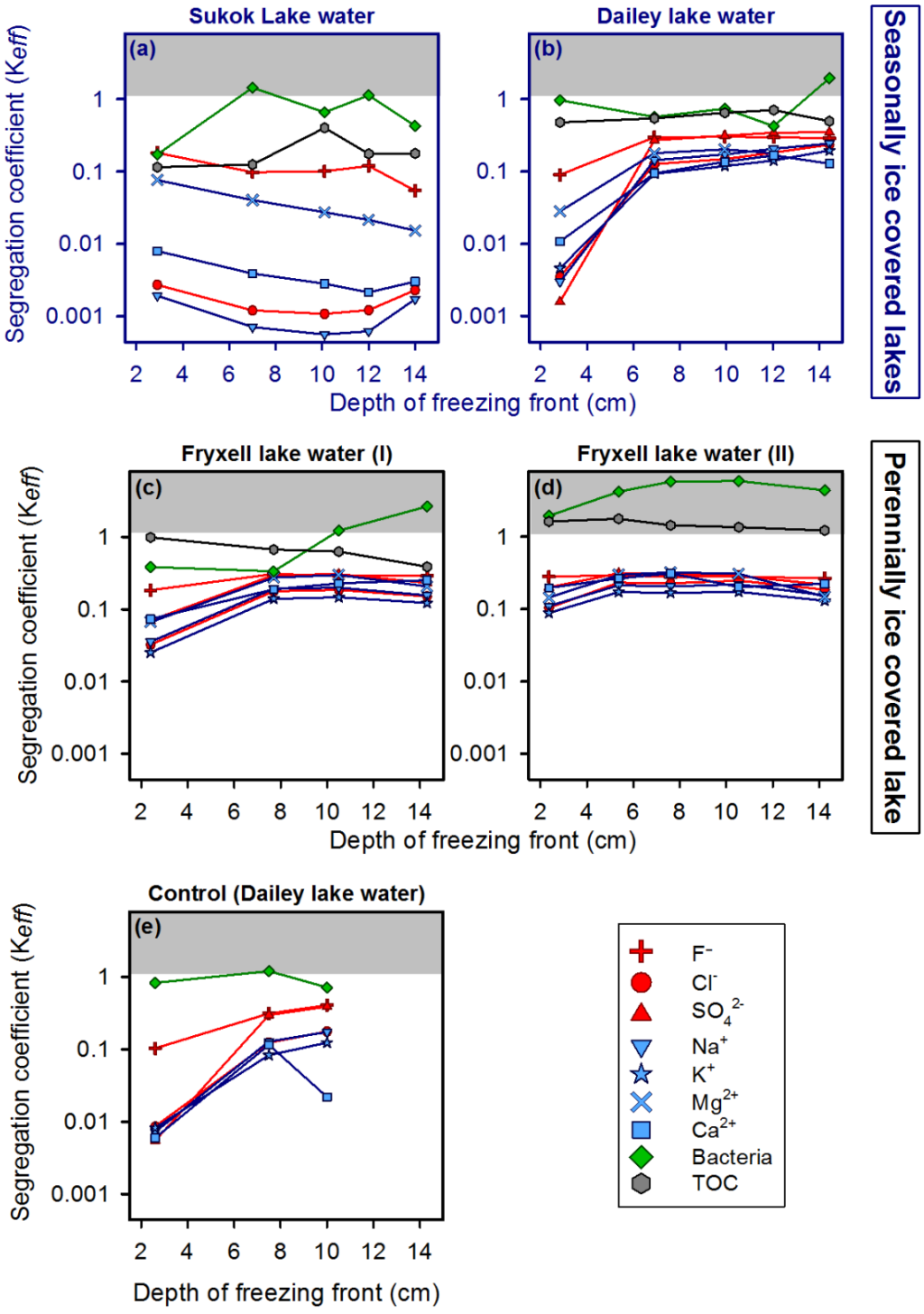



Figure 5. The effective segregation coefficient over the course of each mesocosm experiment. The horizontal grey bar corresponds to $K_{eff} > 1$ as a consequence of higher segregation to the ice. Systems with $K_{eff} < 0.01$ become nearly pure substances upon solidification [*Young and Davis*, 1986].

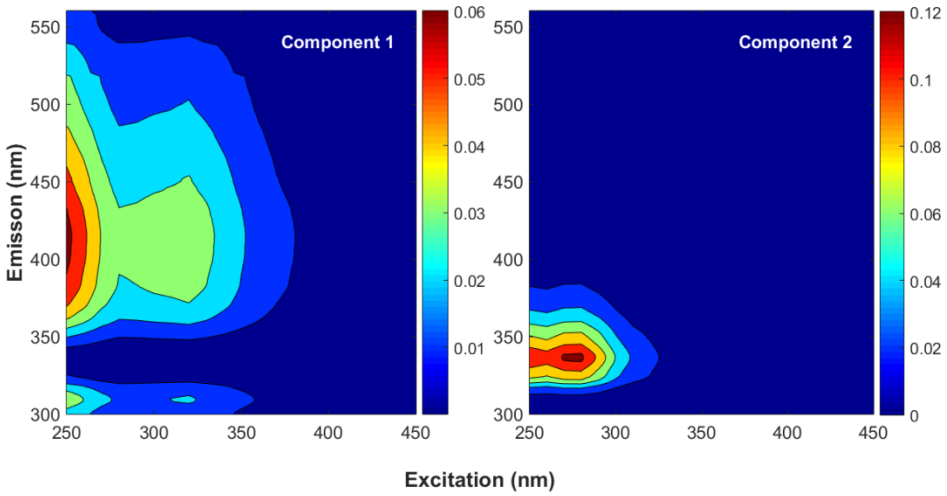



Figure 6. PARAFAC modelled EEMS data showing the predominate organic matter constituent in the lake water (Component 1) and the ice (Component 2).

PARAFAC modeling was applied to all EEMS to identify fluorescent OM components in the lake water and thawed solid phase samples. The PARAFAC model identified two OM components (Fig. 6): Component 1 (C1), found predominantly in the liquid phase samples, and Component (C2), which was dominant in the solid phase. C1 is comprised of a mixture of OM fluorescence mostly situated at higher Ex/Em wavelength ratios (Fig. 6), with the maximum fluorescence intensity within the ranges characteristic of humic-like material [*Fellman et al.*, 2010]. C2 is characteristic of tryptophan-like OM fluorescence, observed both in the lake water and solid phase, but dominant in the solid phase (Fig. 6). Sukok, Fryxell I, and Fryxell II solid phase samples contained no additional regions of maximum OM fluorescence.

## Discussion

Our results revealed that, in contrast to the current understanding of the exclusion of abiotic impurities from the ice front during lake water freezing [*Weeks and Lofgren*, 1967; *Clayton et al.*, 1990; *Killawee et al.*, 1998; *Rempel and Worster*, 1999], bacteria are incorporated into the ice during progressive freezing and aggregated bacteria are abundant in natural lake ice cover. Segregation coefficients for bacterial cells were higher than those calculated for TOC and ions. Living bacterial cells initially concentrated into the liquid fraction, but were incorporated into the ice later in the freezing process. These findings give insights into the influence of ice cover development on bacterial populations in freshwater lakes. Previous studies have shown that lake ice covers can be habitats or refugia for microorganisms; for example liquid-water inclusions associated with sediment particles in perennially ice-covered lakes, are microzones enriched in microbial activity and carbon cycling [*Paerl and Priscu*, 1998; *Priscu et al.*, 1998; *Gordon et al.*, 2000]. Others have documented the response of microbial populations to the freezing process from the liquid phase perspective, showing that stressors imposed during freezing decreased the abundance and diversity of microbial populations within the remaining liquid-phase [*Safi et al.*, 2012].

The mesocosm experiments showed that ions were effectively rejected from the ice in all the experiments, results that corroborate previous studies (e.g., *Killawee et al.*, 1998; *Eichler et al.*, 2001). Solutes are rejected by the advancing ice front and thus become more concentrated in the liquid-phase. Segregation coefficients for solutes are low and depend on solubility (ion charge and size), interaction between solutes,

symmetry and parameters of the crystal lattice [*Lock*, 1990; *Killawee et al.*, 1998; *Eichler et al.*, 2001]. The more conservative ions ($K^+$, $Mg^{2+}$, $Na^+$) in the liquid phase of all the experiments are explained by their low solubility in ice and higher rejection from the ice-phase (Fig. 5) [*Killawee et al.*, 1998; *Eichler et al.*, 2001]. In contrast, the behavior of the less conservative ion ($F^-$) can be explained because it is more readily incorporated into the ice lattice compared to other ions [*Wolfgang-Gross et al.*, 1978; *Wolff*, 1996; *Petrenko*, 1999; *Eichler et al.*, 2001].

TOC segregated in a pattern similar to bacteria and the less conservative ions (i.e., $F^-$), with most of it concentrating in the liquid phase, except in the Fryxell II experiment. These results corroborate observations from Antarctic seasonally ice covered lakes that also showed increasing organic carbon concentrations in the liquid phase during progressive freezing [*Hawes et al.*, 2011]. A fraction of the bulk TOC was selectively incorporated into the solid phase. Only the tryptophan-like fluorescence component was found in the solid phase samples (Fig. 6). This tryptophan-like fluorescence is commonly associated with low molecular weight chemical species, having phenolic and/or quinone-like structures. Such OM species are considered to be more bioavailable to microorganisms in the environment [*Coble et al.*, 1990, 1998]. The more refractory chemical species with high molecular weights remained in the liquid phase (Fig. 6). The trend of labile OM segregating to the solid phase and more refractory OM concentrating in the liquid phase was also reported for several seasonally ice covered Arctic and Antarctic lakes, suggesting the process of lake ice formation has an important role in the carbon cycle of a lake [*Belzile et al.*, 2002; *Hawes et al.*, 2011].

For insoluble particles (including bacteria), the advancing ice front may reject or engulf them as a result of interplay between viscous drag and repulsive forces. Here, we observed higher incorporation (higher $K_{eff}$) of bacteria into the ice relative to ions, indicating that bacteria are incorporated more effectively into the ice than chemical species during the freezing process. The incorporation of bacteria in the ice reveals that critical velocity of engulfment was reached in the experiments and lakes. The critical velocity is reached when viscous drag on the bacterial cell in the liquid phase (attractive force) overcomes the repulsive force (Van der Waal forces) due to ice growth velocity [*Körber and Rau*, 1987; *Körber*, 1988; *Lipp and Körber*, 1993]. The mesocosm experiments had ice growth velocities of ~2 mm $hr^{-1}$ (Supplemental Table 2). At this rate, thermal conductivity or solute concentration in the liquid-phase can explain the engulfment of bacteria and, thus, their high segregation coefficients relative to ions [*Lock*, 1990; *Petrenko*, 1999]. Each experiment was set up under the same conditions, so they should have the same thermal conductivity; thus thermal conductivity cannot explain the engulfment of the bacteria by the freezing front. Indeed, the mean segregation coefficient of bacteria in each experiment is related to the initial total ion concentration (i.e. solute concentration) (Supplemental Table 2 and 3). The sequence of the mean segregation coefficients based on bacteria for each experiment, ranked from highest to lowest, was the same as for ions in each experiment: Fryxell II > Fryxell I > Dailey ≈ control> Sukok, supporting the importance of initial ion concentration in determining bacterial segregation. Solute concentration in the liquid phase is negatively correlated to the critical velocity of engulfment [*Körber*, 1988; *Lipp and Körber*, 1993], where higher

solute concentrations in the liquid phase produce lower critical velocities and higher segregation coefficients, which match our experimental results. We found that higher incorporation ($K_{eff} > 1$; preferential incorporation) of bacterial cells into the ice occurs when the ion concentrations of the liquid phase exceed ~ 27 meq $L^{-1}$ at freezing rates around 2 mm $hr^{-1}$. Our Barrow area lake ice cover data show that bacteria do segregate into the lake ice at low ionic concentrations; however, this is likely due to the slow actual freezing rates for Barrow area lakes (~0.1 – 0.7 mm $h^{-1}$) compared to our experimental freezing rate. While natural lake ice covers have alternative sources of bacteria (e.g., aeolian deposition), some of our observations of aggregated bacteria are from the middle of the ice cover, which indicates they have been incorporated during the freezing process.

We expected segregation coefficients for bacterial populations from seasonally ice covered lakes to be higher than those from perennially ice covered lakes due to selective pressures imposed by annual ice formation. Certain bacteria can produce ice-nucleation, antifreeze and ice-binding proteins to minimize freezing injury [*Kawahara*, 2002; *Raymond et al.*, 2007, 2008; *Achberger et al.*, 2011]. However, mean segregation coefficients of bacteria were lower in seasonally ice covered lakes than in perennially ice covered lakes. Our data indicate that bacterial segregation coefficients were associated with initial ion concentration and we cannot attribute the segregation coefficients to ice-cover regime because seasonally and perennially ice covered lakes had different initial ion concentrations.

The range of calculated segregation coefficients produced by variable initial solute concentration is important to consider when using segregation coefficients to

estimate bacterial cell concentrations in subglacial lakes. The wide range of segregation coefficients (mean bacterial $K_{eff}$ 0.8 – 4.4) we observed would lead to variable estimates in bacteria abundances in the subglacial lakes. Bacterial concentrations in accretion ice from subglacial lakes below the Antarctic ice sheet should reflect those in the actual lake water in a proportion equal to the segregation coefficient that occurs when the water freezes [*Priscu et al.,* 1999; *Priscu and Christner,* 2004; *Christner et al.,* 2006]. Segregation coefficients are affected by different factors such as thermal conductivity, freezing rate and initial solute concentrations [*Lock,* 1990; *Killawee et al.,* 1998]. In our experiments, we controlled freezing rates and only varied the initial solute concentration, which produced a broad range of bacterial segregation coefficients. Thus, estimates of the subglacial lake conductivity and ice freezing rate must be considered when determining an accurate segregation coefficient for estimation of bacterial concentration in the subglacial lake water.

Dailey and the control experiment showed the same pattern of incorporation even though cells were dead in the control experiment. Viability of bacterial cells did not influence bacterial segregation coefficients (0.93 and 0.92, Dailey and Control, respectively; Fig. 5) or distribution of bacteria in the ice cover (Fig. 2). However, experiments with viable bacterial cells had a lower CF than the control (eq.1; Fig. 4). These findings indicate that physical and chemical, rather than biological processes controlled the incorporation of bacteria into the ice. The lower CF in the live versus control mesocosms may be explained by losses of living bacterial cells in the liquid phase due to cell lysis, and/or precipitation of cells to the bottom of the mesocosms. Aggregated

living bacteria were present and quantified in the Dailey experiment, while those in the control did not form aggregates, implying a biological response to freezing. The bacterial losses indicated by the lower CF and unchanged $K_{eff}$ in the Dailey live treatment versus the control may be due to an aggregation response to freezing, although aggregates were similar to the lower end of the range observed in Barrow lake ice cores (5% - 95% of total bacteria). It has been observed that stressors imposed during progressive freezing caused decreases in bacterial abundance and diversity in the liquid phase, in agreement with our abundance observations from all mesocosms with living cells [*Safi et al.*, 2012].

Our study showed that bacterial aggregates are present in natural ice covers of lakes, where the total bacterial abundance was dominated by aggregated bacteria in Emaiksoun, Ikroavick and Siqlukaq. In contrast, mesocosm experiments showed a dominance of non-aggregated bacterial cells. One important difference between the mesocosm experiment and the lake ice cores collected from Barrow, AK lakes was that ice growth rates in the mesocosms were higher than in nature (~2 mm $h^{-1}$ versus ~0.1 - 0.7 mm $h^{-1}$ for Barrow lakes). Bacterial aggregates have been reported and studied within sea-ice, and these aggregates produce extracellular polymeric substances as a cryoprotectant when incorporated into sea-ice [*Krembs et al.*, 2000; *Collins et al.*, 2008; *Krembs and Deming*, 2008]. Bacterial aggregates found in natural ice covers of Barrow lakes revealed that aggregation could be an adaptation for survival in a habitat that freezes solid, similar to the response of sea-ice bacteria. The slower ice growth rates in nature may allow bacteria to utilize this adaptation, whereas our mesocosm experiments

provided physical constraints (rapid ice growth rate) that excluded the possibility of aggregate incorporation into the ice phase.

## Conclusions

The mesocosms were designed to simulate the thermal, chemical, and mechanical stresses experienced by bacterial cells during the freezing of lakes. The incorporation of bacterial cells into lake ice cover was observed in nature. The segregation coefficient for bacterial cells was higher than that of ions; ions were rejected from the ice front more effectively than bacteria and only a small amount of solute was incorporated into the ice matrix. Bacterial cells did not concentrate in the liquid phase, and the resulting loss of bacterial cells can be explained by the incorporation of cells into the ice. Aggregated bacterial cells were more frequently incorporated into the ice phase in natural lake ice freezing than in the mesocosm experiments where the physical control of rapid ice front advance may have excluded much of the aggregate incorporation. Our mesocosm experiments demonstrate that segregation coefficients for bacterial cells vary widely across different lake physicochemical regimes, regardless of cell viability; however, lake ice covers do incorporate bacterial cells and aggregates, overturning the consensus that all lake water constituents are reject during lake ice formation.

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# CHAPTER SIX

# CONCLUSIONS: ICE IS AN IMPORTANT FACTOR IN THE HABITABILITY OF POLAR ICE COVERED LAKES AND BEYOND

When I flew south from New Zealand to Antarctica the first time I was mesmerized by the vast expanse of Antarctica's ice seen from the plane window. The ice looked like a lifeless blanket that had no role in supporting life. Upon landing and further exploring the continent, I was confronted first hand with the adaptability and resilience of organisms, especially microorganisms. Numerous observations of life under and within ice helped me hypothesize that microbial life may be supported by this seemingly lifeless ice blanket. A wide variety of projects in graduate school, from atmospheric microbiology to NASA astrobiology projects in the Arctic to subglacial lakes in Antarctica, helped to me to realize that microorganisms, interact with ice and sometimes use it to their advantage here on Earth and maybe even in other icy bodies in our Solar System and beyond.

## Biological Interactions with Ice

Ice is a pervasive force on Earth. Ice sheets, glaciers, clouds and sea ice are all examples of icy environments that have been a strong selective force on biological organisms. Biological species, in particular microorganisms, have responded to these

selective pressures to produce a wide variety of adaptations that allow organisms to interact with ice.

In chapter 5, we showed that microorganisms incorporate into the ice cover of seasonally and perennially ice covered lakes. The response to the progressive freezing of a lake appears to be the formation of bacterial aggregates that are pervasive in the lake ice covers from Barrow, AK and seen in the laboratory experiments with seasonally ice covered lake water. Bacteria are known to directly interact with ice through the production of an ice binding protein that allows the bacteria to control ice recrystallization and modify the crystal structure to be non-lethal (Achberger *et al.* 2011). A similar type of protein is known to be present on the outer membrane of the well-studied atmospheric and leaf-associated *Pseudomonas syringae* that has the ability to nucleate ice crystals in near zero degree Celsius super cooled water (Morris *et al.* 2008). This microorganism, as well as five other known ice nucleation active bacteria, can contribute to precipitation including snow and rain (Christner *et al.* 2008). In Appendix 2, I show that hail is likely nucleated by biological material, and potentially these ice nucleation active bacteria (Michaud *et al.* 2014). I think the molecular mechanisms of microbial interactions with ice have many open questions that may provide clues to the evolutionary adaptations that underpin the microbe-ice interaction.

## Biological Benefits of Ice

The idea of an extreme environment is subjective to the observer, usually a human scientist. Too often we study ice and conclude how difficult it must be for organisms to

live in icy habitats. Humans may consider an environment, such as ice, to be harsh, but that environment may not be harsh to the microbial inhabitants that are adapted to live there. We should approach the study of ice by also asking: what are the benefits conferred to microorganisms by ice?

Many of the icy systems studied in this dissertation are classified as extreme. Growth optima of microorganisms at low temperatures or high salinities are good evidence that these organisms are adapted to live in these environments, as these microorganisms do not function well outside of the "harsh" environment (Siddiqui *et al.* 2013). Cold temperatures and ice do not preclude microbial activity, but do keep metabolic rates low and slow (Bakermans & Skidmore 2011b, Bakermans & Skidmore 2011a). Sea ice is a particularly challenging environment from the anthropogenic perspective, yet algae and bacteria are found throughout sea ice profiles (Thomas & Dieckmann 2002). Many of the organisms found in sea ice are capable of forming stress-resistant spores or cyst resting forms, but some are adapted for metabolic activity in the environment as seen by the adaptations for efficient photosynthensis in ultra-low light conditions, formation of dense extracellular polysaccharides and membrane modifications that promote fluidity (Thomas & Dieckmann 2002). Ultimately, if an organism is adapted to live in these "extreme" conditions it likely does so to avoid other more stressful or negative conditions. For example, the sea ice algae may incorporate into the ice so that it stays within the photic zone, despite being low light conditions, as opposed to being advected under an ice shelf, which is a permanently dark habitat. As mentioned in the introduction, a symbiotic mechanism of ice incorporation is used by

algae in freshwater lake ice cover to promote positioning in the ice cover for primary production rather than in the low light deep waters (D'souza *et al.* 2013).

Certainly there are cases where ice is harsh, even to a microorganism. Those microorganisms that are present in the liquid vein networks of deep, polar glacial ice encounter high sulfuric acid conditions due to the sulfate exclusion from the ice matrix (c.f., Chapter 5), which generates an acid environment (pH = ~1; Barletta *et al.* 2012). Much of polar ice is also consistently ~ -30°C (Neff 2014). However, the low water activity of liquid vein networks due to cold and high solute concentrations seem to preserve the microbial cells over many thousands of years. We know cells are viable from these deep and old ice cores because they can be revived and cultured (Christner *et al.* 2001; Christner *et al.* 2003). The microorganisms are unlikely to be metabolically active, but rather preserved for when they reach a subglacial lake or other favorable basal habitat upon melting out of the ice sheet base.

Compare this deep, cold, low-energy environment of deep glacial ice to deep marine sediments where the energetic limits of microbial life for long term preservation have been previously explored. In the deep marine subsurface, cell persistence on the thousands to millions of years is precluded by faster biomolecule damage due to high amino acid racemization rates and DNA depurination rates (Lever *et al.* 2015). The rate of these two degradation processes increase with increasing temperature (Lever *et al.* 2015; Price & Sowers 2004). Thus, in ice cores, these two processes are decreased and microbial cell preservation in the deep polar ice may be more efficient than the deep,

warm marine biosphere. Maybe the question needs to be restated to ask what the biological benefits of ice are, rather than how extreme the icy habitat is.

## The Potential Role of Ice in Extraterrestrial Life

Astrobiologists consider the question: What do we look for in the Universe as a signature for life? The answer is not straightforward, as we currently only define life as we know it on Earth. Water is often considered necessary for life, and in fact, NASA has used the mantra of "follow the water" to look for extraterrestrial life. However, findings of this dissertation show ice may be just as important as liquid water. If extraterrestrial life were similar to life on Earth, ice may be a large factor in determining where we find it. In some sense, ice can serve as a membrane, to some extent a pseudo-atmosphere, over a planet or part of a planet. The benefits to the life, most likely, beneath the ice mass may be UV protection and thermal insulation from space, for example. We are beginning to see these types of benefits from thick ice masses overlying subglacial lakes in Antarctica.

Our work in Chapters 2, 3, and 4 is one effort to use terrestrial analogues for the search for life on other planets. In particular, our project in an Antarctic subglacial lake is an analog to the large water ocean found beneath a thick ice cover on Europa, a moon of Jupiter. SLW is not be a perfect analog in terms of the size of the water body or the ice thickness, it is a first attempt at how life might persist on Europa and many aspects of our study are transferable. Our work in Chapter 2 to assess and monitor the cleanliness of sampling a subglacial lake is directly transferrable to a mission to Europa. Forward contamination and potential life detection false-positives are large concerns for mission

planning. In addition to liquid water, the importance of chemical disequilibria has been advanced as a key indicator of habitability outside of Earth (Hand *et al.* 2007), which is a further implication of our work in chapter 4. In chapter 4, we show that life in an Earthly analogue to Europa harnesses chemical disequilibria maintained by the oxidant ($O_2$) release from the overlying ice cover and resupply of reductant ($CH_4$) from microbial processes below. The resulting chemical disequilibrium is harnessed by microorganisms to drive metabolism. While the redox couples may not be methane and oxygen on Europa, the idea is similar such that the ice cover delivers oxidants and some reductant is supplied from below. It is noteworthy to add that geothermal heat in SLW is a strong physical factor in the subglacial environment, not unlike what is thought to occur at the bottom of the Europa water ocean. The ice cover on Europa also blocks a considerable amount of presumably lethal amounts of radiation and insulates the water ocean from cold. The thick (800 m) WAIS overlying SLW plays a similar role, keeping SLW at a "warm" and, presumably, stable -0.5°C. While SLW microbial inhabitants must contend with several stressors, the WAIS does shield subglacial inhabitants from the intense Antarctic UV radiation and provides oxidant delivery due to basal melting from high geothermal heat flux and thermal insulation, similar to the ice cover on Europa. The ice overlying SLW provides many benefits to the microorganisms living in the lake and shows that ice could be important in maintaining life on Europa and beyond.

## Final Remarks

Given the once unknown nature of subglacial lakes in Antarctica, the bulk of this dissertation was discovery-based science with basic hypotheses set forth. At the onset, we were focused on the fundamental question: is life present in an Antarctic subglacial lake? Within 24 hours of breakthrough to the lake, three measurements (intracellular ATP, cell counts, electron transport system activity) showed microbial life was greater than controls and drill water. Our focus shifted to describing the geochemistry of the habitat and the microbial inhabitants. The relatively small amount of sample (30 L of water and 3 ~40 cm sediment cores) we collected and analyzed revealed many insights into how the microbial ecosystem functions beneath the WAIS. The biogeochemical consequence of subglacial lakes is currently understood from a single sampling of one lake. The spatial and temporal variability of microbial biogeochemical functioning and the impact of ice sheet activity on microbial activity are scientific questions that require attention if we are to fully understand the importance of subglacial lakes in the Earth system.

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# APPENDICES

# APPENDIX A

## A MICROBIOLOGICALLY CLEAN STRATEGY FOR ACCESS TO THE WHILLANS ICE STREAM SUBGLACIAL ENVIRONMENT

# APPENDIX A

## A MICROBIOLOGICALLY CLEAN STRATEGY FOR ACCESS TO THE WHILLANS ICE STREAM SUBGLACIAL ENVIRONMENT

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Contributions: Wrote the proposal to fund the project (NSF-OPP 0838933, 1346250, 1439774). Analyzed the data and wrote the paper.

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Contributions: Performed the XTT assays and helped with samples processing. Reviewed the manuscript and provided feedback.

Co-Author: Joel E. Cahoon
Contributions: Helped with fluid flow modeling.

Co-Author: Brent C. Christner
Contributions: Collected and processed samples. Reviewed the manuscript and provided feedback.

Co-Author: Robert L. Edwards
Contributions: Performed the silt test. Reviewed the manuscript and provided feedback.

Co-Author: Warren L. Jones
Contributions: Helped with fluid flow modeling. Reviewed the manuscript and provided feedback.

Co-Author: Alexander B. Michaud
Contributions: Collected and processed samples. Processed and analyzed the ATP data, counted bead-test samples, helped perform the surface cleaning experiments, reviewed the manuscript and provided feedback.

Co-Author: Matthew R. Siegfried
Contributions: Produced figures for the manuscript. Reviewed the manuscript and provided feedback.

Co-Author: Mark L. Skidmore

Contribution of Authors and Co-Authors-Continued

Contributions: Collected and processed samples. Reviewed the manuscript and provided feedback.

Co-Author: Robert H. Spigel
Contributions: Helped with fluid flow modeling.

Co-Author: Gregg W. Switzer
Contributions: Set up the filtration system for experiments.

Co-Author: Slawek Tulaczyk
Contributions: Helped with fluid flow modeling.

Co-Author: Trista J. Vick-Majors
Contributions: Collected and processed samples. Processed and analyzed the bead-test samples, helped perform the surface cleaning experiments, reviewed the manuscript and provided feedback.

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# A microbiologically clean strategy for access to the Whillans Ice Stream subglacial environment

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**Abstract:** The Whillans Ice Stream Subglacial Access Research Drilling (WISSARD) project will test the overarching hypothesis that an active hydrological system exists beneath a West Antarctic ice stream that exerts a major control on ice dynamics, and the metabolic and phylogenetic diversity of the microbial community in subglacial water and sediment. WISSARD will explore Subglacial Lake Whillans (SLW, unofficial name) and its outflow toward the grounding line where it is thought to enter the Ross Ice Shelf seawater cavity. Introducing microbial contamination to the subglacial environment during drilling operations could compromise environmental stewardship and the science objectives of the project, consequently we developed a set of tools and procedures to directly address these issues. WISSARD hot water drilling efforts will include a custom water treatment system designed to remove micron and sub-micron sized particles (biotic and abiotic), irradiate the drilling water with germicidal ultraviolet (UV) radiation, and pasteurize the water to reduce the viability of persisting microbial contamination. Our clean access protocols also include methods to reduce microbial contamination on the surfaces of cables/hoses and down-borehole equipment using germicidal UV exposure and chemical disinfection. This paper presents experimental data showing that our protocols will meet expectations established by international agreement between participating Antarctic nations.

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**Key words:** clean access, environmental stewardship, hot water drilling, subglacial aquatic environments

## Introduction

Recent discoveries of life under the thick ice sheet covering Antarctica have radically changed our view of the interior of the Antarctic continent. Subglacial exploration presents considerable challenges to the way we conduct science in an atmosphere of increasingly stringent environmental concerns (Priscu 2002, Priscu *et al.* 2003). Priscu *et al.* (1999) and Karl *et al.* (1999) were the first to show that there was microbial life in water from Subglacial Lake Vostok that had accreted to the bottom of the ice sheet. Since these seminal reports, others have confirmed the presence of microbial life both within and beneath Antarctica's ice sheet (e.g. Christner *et al.* 2006, Priscu *et al.* 2007, Lanoil *et al.* 2009). As such, the basal zones of ice sheets are now thought to harbour active microbial ecosystems and our view of the extent of Earth's biosphere has expanded substantially (Priscu & Christner 2004, Priscu *et al.* 2008).

The pristine nature of Antarctic subglacial ecosystems has led to international concern about environmental and scientific stewardship during their exploration. Following more than ten years of deliberation by international and national committees (e.g. NRC 2007, Priscu *et al.* 2010), it has become clear that sampling of lakes and sediments beneath Antarctica's ice sheets must be done in such a way that minimizes microbial and chemical contamination to the environment and to the samples being retrieved. All those involved in this research must recognize that environmental stewardship should take precedence over scientific endeavours. We can expect subglacial lakes to be at the forefront of the Antarctic tradition of melding interdisciplinary and international science in exploring one of the last unexplored frontiers on our planet (Priscu 2002).

Largely through the efforts of scientific specialists organized by SCAR, three major subglacial drilling projects are now underway (Priscu *et al.* 2005). These include the



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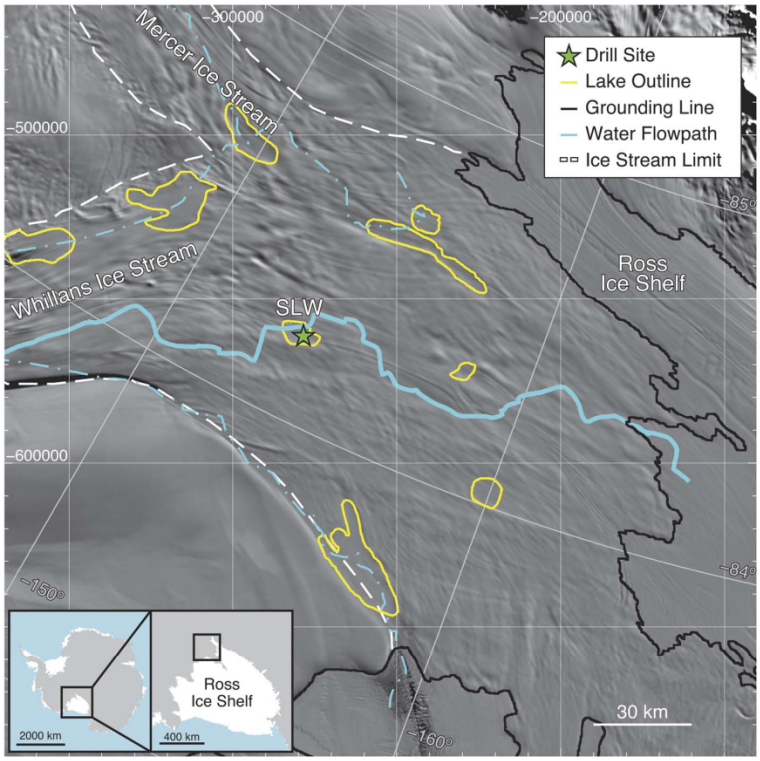



**Fig. 1.** Map showing the location of Subglacial Lake Whillans (green star = SLW, 84.237°S, 153.614°W) and its predicted flow path to the Ross Ice Shelf (heavy blue line). WISSARD sampling sites include the lake itself and near the end of the flow path close to the grounding line (heavy black line). The outlines of other lakes on the Whillans Ice Stream (yellow outlines) and their predicted flow paths (light blue lines) are also presented. Polar stereographic projection with true scale at -71°S; gridlines are spaced at 50 km intervals. Background imagery and grounding line are from the MODIS Mosaic of Antarctica (Scambos *et al.* 2007); lake outlines from MODIS image differencing (Fricker & Scambos 2009); ice stream boundaries were determined from InSAR velocities (Rignot *et al.* 2011); hypothesized water flow paths were determined by tracing continuous local hydropotential minima (Carter *et al.* 2011, Carter & Fricker 2012).

Lake Vostok programme funded by the Russian Antarctic Federation (Lukin & Bulat 2011), the Lake Ellsworth programme funded by the United Kingdom's Natural Environment Research Council (Ross *et al.* 2011), and the Whillans Ice Stream Subglacial Access Research Drilling (WISSARD) project, funded by the United States National Science Foundation (Fricker *et al.* 2011). All of these projects plan to access their subglacial targets within the next few years. Each project has proposed a suite of methods to ensure clean access to their respective systems, the details of which can be found on the Antarctic Treaty System's website as informational papers or comprehensive environmental evaluations (http://www.ats.aq/e/ats_keydocs.htm).

The WISSARD project proposes to sample subglacial lake Whillans (SLW) during the 2012–13 summer field season and the subglacial environment on the lower Whillans Ice Stream in a region near the grounding zone (GZ) during the 2013–14 summer season. The GZ site will be in an area where drainage from SLW is thought to enter the marine environment beneath the Ross Ice Shelf (Fig. 1). Access to the subglacial environment will be accomplished using a hot water drilling system to penetrate the ~800 m of ice overlying the basal water. To ensure that the borehole water

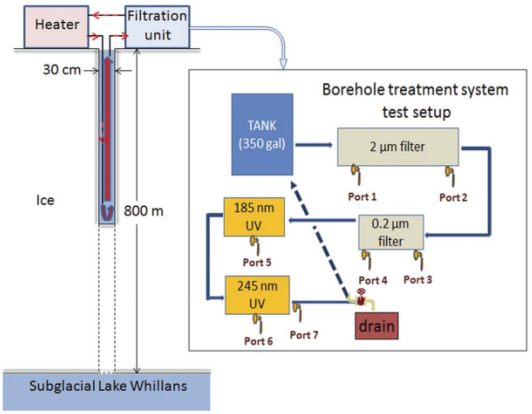



**Fig. 2.** Schematic (left panel, not to scale) showing the flow directions for the field operation of the Whillans Ice Stream Subglacial Access Research Drilling (WISSARD) water treatment system. The filtration and UV-treatment unit will be placed in-line between the borehole water return line and the heater modules. The enlarged inset (right panel) shows the treatment system components as set up for the laboratory tests. See text for details.

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meets the cleanliness requirements promoted by a recent US National Research Council report (NRC 2007) and the scientific integrity of samples (water and sediments) is maintained, the project will include a borehole water treatment system designed to eliminate particles $>0.2\,\mu m$ in diameter, and reduce the concentration of viable microbial cells present in the borehole water (Fig. 2) and attached to deployed equipment and instrumentation.

This document presents: i) results from experiments designed to test the efficacy of the filtration system, and ii) disinfection protocols to be employed at all proposed WISSARD drilling sites where biological clean access is a requirement. These results are discussed within the context of the hydrology of SLW.

## Methods

### *Borehole filtration and germicidal treatment system*

The filtration component of the water treatment system consists of a 2 μm prefilter (pleated polypropylene cartridges, 15 cm diameter x 203 cm long, Champion Process Inc) followed by 0.2 μm filtration through a polyethersulfone membrane cartridge (7 cm diameter x 76 cm long, Champion Process Inc). After filtration, the clarified water was subsequently exposed in sequence to two separate ultraviolet (UV) irradiation modules (Glasco UV) that use 185 nm ozone-producing lamps followed by exposure to 254 nm germicidal lamps (wavelengths denote peak spectral output). Based on data supplied by the manufacturer, the tandem filtration strategy is designed to decrease the number of particles $>0.2\,\mu m$ by 99.98% (i.e. 4-log reduction). The two UV systems provide a 185 nm dosage $>40\,000\,\mu W\text{-sec cm}^{-2}$ and 254 nm germicidal dosage $>175\,000\,\mu W\text{-sec cm}^{-2}$. Doses of $40\,000\,\mu W\text{-sec cm}^{-2}$ are typically used for water disinfection, and depending on the UV transmission properties of the water and the sensitivity of the microorganisms to UV, exposures in this range have been shown to reduce the number of viable cells by 1-log to nearly 6-log (Aquafine Corp). The internal volume of the entire system (filters plus UV units) is 572 l. In a field scenario, the treated water will undergo a final pasteurization step where water temperature will progressively reach 90°C before exiting the 152 m long heating coils of the drilling unit. In summary, the WISSARD borehole water treatment system will use three complementary technologies to reduce microbial contaminants in the borehole water: i) filtration to remove particles $>0.2\,\mu m$, ii) UV irradiation (185 and 254 nm), and iii) pasteurization in the boilers of the hot water drill.

In field operations, hot water is pumped to the bottom of a 30 cm diameter borehole at high pressure (the diameter will be controlled by adjusting the water heating rate), flows upward in the hole and is pumped from the top, as shown schematically in the left-hand panel of Fig. 2. After water is pumped from the top of the borehole, it flows into a 14 000 l holding tank, then through the filtration system followed by the heater modules before again being pumped to the drilling nozzle at the bottom of the borehole. The drilling speed is expected to be $1\,m\,min^{-1}$ through most of the ice and the filtration system will be run continuously during drilling operations. The liquid water in the hole-drill-treatment system thus forms a recirculating system, with a liquid volume that increases as drilling proceeds. A 1900 l insulated melt tank will provide start-up water for the field system by melting snow using heated glycol pumped through a series of heat-radiating immersion plates that form a recirculation system. The components of the treatment system are illustrated in the right-hand panel of Fig. 2 as set up for laboratory testing.

The following laboratory tests were conducted to determine the efficacy of the system:

1) Dye study to determine the system flow characteristics.
2) Fluorescent micro-bead (1–5 μm) and microbial cell removal.
3) Silt (1.2–63 μm particle size) removal.
4) Effect of UV exposure on viability of a model bacterium (*Escherichia coli* strain K-12).
5) Lakewater test to examine the filtration efficiency and disinfecting properties of the UV lamps on populations of natural aquatic microorganisms.
6) Lakewater pasteurization test to assess the influence of the heat generated by the hot water drill on microbial viability.

### *Dye test*

A dye test was performed to examine the hydraulic residence-time characteristics of water in the treatment system. The system was filled with water containing fluorescent Rhodamine WT (water tracing) dye at a concentration of $\sim 9\,\mu g\,l^{-1}$, and then flushed with dye-free tap water at $95\,l\,min^{-1}$ for 25 min. The flow rate of $95\,l\,min^{-1}$ is within the range of flow to be used during actual drilling operations. At this rate, the treatment system has a mean residence time (volume 572 l divided by a flow rate of $95\,l\,min^{-1}$) of 6 min. The test duration of 25 min therefore corresponds to 4.2 residence times. During the course of the test, discrete samples were collected to examine the dilution of the dye at port 7. Data from port 7 represents the integrated flow characteristics of the entire system (filter plus UV lamp canisters). All fluorescence measurements were made on a calibrated Turner Model 112 fluorometer fitted with a C5-60 excitation filter, a C56 emission filter, and F4T-4 lamp.

### *Bead removal experiment*

Fluorescent beads (manufacturer nominal size range: 1–5 μm, mean = 2.2 μm) were added to the feed tank

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containing 13251 of tap water to a final concentration of $\sim 2 \times 10^5$ beads $ml^{-1}$ and mixed thoroughly. The bead mixture in the feed tank was then pumped through the filtration and UV canisters (the UV lamps were off for this test) at $\sim 941 min^{-1}$ until 11131 had passed through the system ($\sim 12$ min). The filtered water was sent to the drain (i.e. it was not recirculated through the system). Samples were then collected in clean quadruplicate vials from the source tank and from ports 1, 2, and 4 along the flow path (Fig. 2). The samples were vortexed vigorously for $\sim 30$ s and prepared for microscopic enumeration by filtering 10 ml of sample onto a black polycarbonate 0.2 μm filter. A Nikon 80i microscope with a Nikon B2-A filter cube was used to enumerate all samples. Percent efficacy of bead removal from each port was calculated as:

((Geometric mean bead count in the tank
- Geometric mean bead count at a selected port)/
Geometric meanbead count in the tank) * 100,

where the geometric mean = the Nth root of the product of N replicates.

*Silt removal experiment*

Local mineral soil was mixed with $\sim 13$ l of tap water and sieved through 63 μm mesh to form a silt/clay slurry. The slurry was added to 4401 of water in the holding tank to a final concentration of $0.395 g l^{-1}$ and pumped through the 2 μm filtration unit at $951 min^{-1}$. The 0.2 μm filter and the UV systems were bypassed in this test. Outflow water from the filtration unit was returned to the holding tank yielding a recirculating system with constant volume. Samples were collected from the tank six times over a 40 min period, with a final sample collected 15 hours after the pump was turned on. Additional samples were collected at ports 1 and 2, four times over the 15 hour experiment. All samples were filtered onto Whatman GF/C filters (effective particle retention 1.2 μm), dried for 24 hours at 100°C and weighed. Standard particle-size classifications (Vanoni 1975) typically specify a range of particle diameters of 4–62 μm for silt, and 0.24–4 μm for clay. The Stokes settling velocity for the largest silt particle (62 μm) is less than $0.2 cm s^{-1}$, and is much smaller than the upward average flow velocity in the 30 cm diameter ice borehole of $2.2 cm s^{-1}$ corresponding to a flow of $951 min^{-1}$. Silt entering the ice hole with meltwater during field operations would therefore be maintained in suspension and carried into the filtration unit.

*Bacterial culture removal and viability test*

Cultures of *Escherichia coli* strain K-12 were grown in 25% nutrient broth and added to the holding tank to a final concentration of $\sim 10^6$ cells $ml^{-1}$. After mixing with 13301 of water in the holding tank for 30 min, triplicate samples were collected from the tank to estimate the bulk bacterial concentration in the test water before it passed through the UV treatment system. The pump was turned on and 2271 was allowed to pass through the UV system in three single pass experiments at flow rates of $191 min^{-1}$, $761 min^{-1}$, and $1521 min^{-1}$, with triplicate samples collected from port 7 (post UV treatment) in autoclaved 20 ml vials. Flushing the system with 2271 before sampling provided adequate time for the UV lamps to reach full output capacity, and allowed water in the UV system to be replaced five times before samples were taken. This ensured that all of the standing water in the UV canisters (total canister volume = 451) was replaced with amended tank water before sampling began. Colony forming units (CFU) from the initial inoculum and each sample were enumerated by standard dilution spread plating on nutrient agar followed by overnight incubation at 37°C, and again after two weeks of incubation. The two week incubation allowed assessment of the potential for the bacteria to recover from UV irradiation. The limit of quantification for this method is 300 CFU $ml^{-1}$ for aqueous samples.

*Lakewater bacterial removal and viability*

Experiments were conducted on lakewater from the Montana State University campus. Lakewater (7601) was sieved through a 125 μm mesh net to remove large debris and brought to 9501 with tap water in the holding tank. The cell count in the original pond water was $1.90 \times 10^6$ cells $ml^{-1}$ and $1.52 \times 10^6$ cells $ml^{-1}$ following dilution in the tank. The UV lamps were switched on for 5 min (allowing them to reach full capacity) before recirculating the water through all filters and both UV lamps at $761 min^{-1}$. Four replicate 10 ml samples were collected from the tank over a period of 90 min at selected times following pump start-up for bacterial counts and determination of intracellular adenosine triphosphate (ATP) concentration. Bacterial samples were collected in 10 ml sterile glass vials, fixed with formalin (5% v/v final concentration), stained using the deoxyribonucleic acid (DNA) dye SYBR Gold™, and enumerated by epifluorescence microscopy (Lisle & Priscu 2004). Cellular ATP concentration, a proxy for microbial biomass and viability (Karl 1980), was determined by concentrating cells in a 10 ml water sample on a 0.2 μm acrodisk syringe filter (Pall Corp). Adenosine triphosphate was extracted with the Microbial ATP Kit HS (BioThema Inc), and luminosity was measured with a Glomax 20/20 luminometer. The limit of detection for this method was $10^{-15}$ mol ATP $ml^{-1}$ based on two standard deviations above the y-intercept in the ATP standard curve.

*Lakewater pasteurization test*

Water collected from a lake on the Louisiana State University campus containing an original cell density of $2.5 \times 10^5$ (estimated by SYBR- Gold™ epifluorescence

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microscopic counts) was serially diluted with filtered lakewater to create two identical sets of cell concentrations ranging from $2.5 \times 10^4$ to 25 total cells $ml^{-1}$. A set of control dilutions was incubated at ~25°C while the other was heated to 85°C for 2 min to mimic the effect of pasteurization in the boilers of the hot water drill. Following exposure to heat, cell viability was assayed by measuring the respiratory reduction of the tetrazolium dye 2, 3-bis-(2-methoxy-4-nitro-5sulphenyl)-(2 H)-tetrazolium-5-carboxanilide (XTT) to formazan (Roslev & King 1993). This tetrazolium salt is reduced by active respiratory electron transport, forming a water soluble formazan dye measureable as absorbance at 490 nm and serves as a proxy for cell viability.

*Surface-based cleaning experiments*

Triplicate flat stainless steel (2.5 cm x 10 cm) and transparent thermoplastic Poly(methyl methacrylate) (PMMA) (5 cm x 10 cm) coupons, representative of materials comprising the down-borehole instrumentation, were contaminated with either *E. coli* K-12 or the endospore-former *Bacillus subtilis*. The coupons were then analysed for cell viability following treatment with 3% hydrogen peroxide. Before inoculation with bacteria, all materials were rinsed with sterile Type 1 (18.2 MΩ $cm^{-1}$) water and autoclaved. The coupons were immersed in cell inocula ($1 \times 10^7$ cell $ml^{-1}$ *E. Coli*, $4 \times 10^6$ cell $ml^{-1}$ *B. subtilis*) for 1 min and allowed to air dry for 5 min before being sprayed five times with 3% $H_2O_2$ (which completely wetted the surfaces) and allowed to react with the bacteria on the coupons for 1 min. Controls consisted of triplicate contaminated coupons not sprayed with $H_2O_2$.

A 10 $cm^2$ area of all control and $H_2O_2$-treated samples were swabbed in triplicate using sterile polyurethane swabs moistened with sterile phosphate-buffered saline (PBS). Swabs were immediately placed into 10 ml of sterile PBS where they were sonicated and vortexed to release cells into the buffer. The 10 ml PBS solution containing the cells was then serially diluted (five dilutions) into sterile PBS. One-hundred μL of each dilution was spread plated in triplicate onto nutrient agar. The cultures were incubated aerobically at 37°C and colonies were enumerated following 18 hours and 72 hours of incubation. The limits of detection for this method, accounting for the area swabbed and the ensuing dilution is 300 CFU $cm^{-2}$.

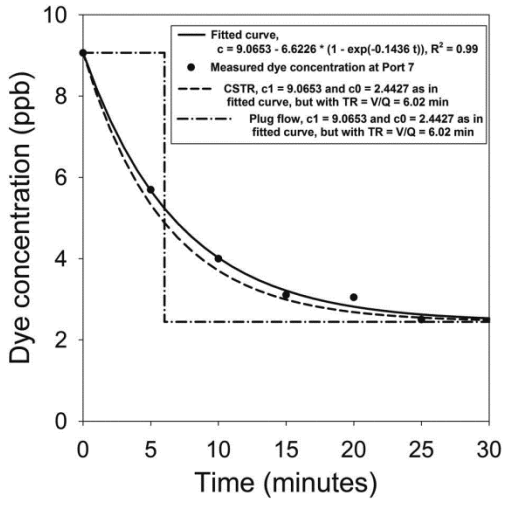



**Fig. 3.** Dye concentrations measured experimentally at Port 7 (filled circles); fitted exponential decay curve (solid line); the curve (dashed line) for a continuously stirred tank reactor (CSTR) with the same initial and final concentrations (C1 and C0) as for the fitted curve, but with the decay constant k computed from the mean residence time (TR = V/Q, = 6.02 min, where V = treatment system volume (572 l) and Q = flow rate (95 l $min^{-1}$), as k = 1/TR); and the curve (dashed-dot line) for a plug-flow reactor with the same initial and final concentrations (C1 and C0) as for the fitted curve, and residence time TR = V/Q = 6.02 min.

## Results

*Dye test results*

A dye test was conducted for 25 min (4.1 times the mean residence time of the system) to examine the rate at which the dye decreased in the system following a clean water flush. This is the same method as that used to experimentally determine the residence time distribution for a reactor (Dankwerts 1953), except that the step-change in dye concentration here is negative. Nevertheless, the results yield the same information.

Changes in dye concentration from test 2 showed an exponential decrease of dye concentration over time (Fig. 3) and were fitted with Eq. (1):

$$C(t) = C1 - (C1 - C0) * (1 - \exp(-kt)), \quad (1)$$

where C(t) = concentration at a given time (t), C1 = initial dye concentration at t = 0, C1 - C0 = the change in concentration between time t = 0 and t = infinity (C0 represents the final, tap-water concentration), and k = exponential decay factor, which denotes 1/residence time (1/TR).

The fit to the experimental points is excellent ($r^2 = 0.99$), and as shown in Fig. 3, is very similar to the theoretical dilution curve predicted for the flushing of a continuously stirred tank reactor (CSTR). This can be expressed in non-dimensional form, in terms of the fraction of fluid in the tank that remains to be replaced or flushed out at time t, by:

$$(C(t) - C0)/(C1 - C0) = 1 - \exp(-t/TR) = 1 - \exp(-kt), \quad (2)$$

with parameters C1 and C0 equal to those of the fitted curve, but decay constant k = 1/TR = 0.166 1 $min^{-1}$. Here TR is the mean residence time (6.0 min), computed from

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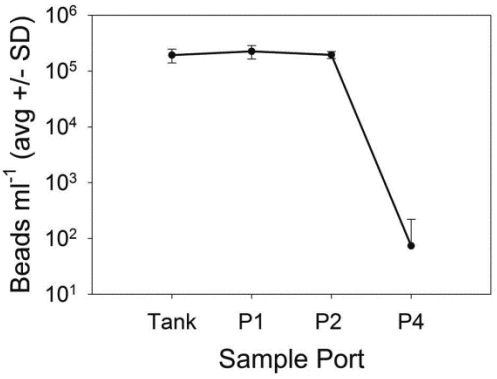



**Fig. 4.** Fluorescent beads counted from the tank and selected sample ports after filtering 11131 through the system. The flow rate was ~941min$^{-1}$. Error bars = standard deviation (SD) ($n = 4$).

the treatment system volume V and the experimental flow rate Q, as TR = V/Q (Dankwerts 1953). Also shown in Fig. 3 is the theoretical flushing curve for plug-flow through the treatment system, which does not provide a good match to the treatment system's observed mixing and dilution behaviour. This is not surprising, given the complexity of internal flow paths through the various components of the treatment system.

The turnover or replacement fractions at various multiples of t/TR can be computed from Eq. (2), showing that one residence time (t/TR = 1) yields a turnover or replacement percent of 63%, and that it requires 3.0 and 4.6 residence times to replace 95% and 99%, respectively, of the fluid in the system. This equates to 95% and 99% replacement times for the fluid in the system of 21 min and 32 min, respectively for the flow of 951min$^{-1}$.

*Bead removal experiment*

Bead concentrations at ports 1 and 2 were not significantly different ($P > 0.05$) from those in the tank after 11131 of water had passed through the 2 μm filter (Figs 2 & 4). However, bead concentration decreased by almost four orders of magnitude at port 4 after the sample water had passed the 0.2 μm filter ($P < 0.05$). The efficacy of bead removal by the 2 μm filter was essentially zero (there was no significant difference in bead concentration between the tank and samples a ports 1 and 2) while the 0.2 μm filter had a removal efficiency of > 99%.

*Silt removal experiment*

The recirculation test using the 2 μm filter only showed that suspended sediment in the holding tank decreased

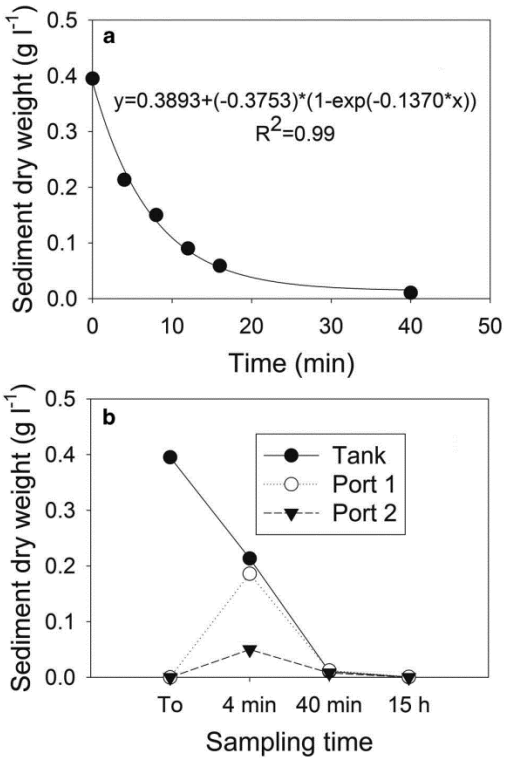



**Fig. 5. a.** Sediment concentration in the tank over time, and **b.** in the tank and ports 1 and 2 at specific times up to 15 hours. Water was recirculated through the tank during this experiment, passing only through the 2 μm filter, at a flow rate of 951min$^{-1}$.

exponentially from 0.4–0.01 g l$^{-1}$ over 40 min at a flow rate of 951min$^{-1}$ (Fig. 5a). The residence time of the system, calculated as 1/k from the fitted equation in Fig. 5a was 7.3 min. Both the shape of the removal curve and the value computed for the e-folding removal time closely match the flushing behaviour of the dye study. By analogy with the flushing described by Eq. (2), 95% and 99% of the sediment in the system would be removed in 22 and 34 min, respectively, under the experimental conditions.

Data from the inlet (port 1) and outlet (port 2) from the 2 μm filter system, showed that the dry weight of the sediment particles (63–1.2 μm in diameter) increased during the first 4 min interval after the pump was turned on, then dropped to background levels in the tank (Fig. 5b). These data indicate rapid mixing before filtration (port 1 data) and that a large number of particles were not retained in the initial pass through the filter. After 15 hours, no silt and clay particles were detectable (detection limit = 0.002 g l$^{-1}$) in samples from the tank,

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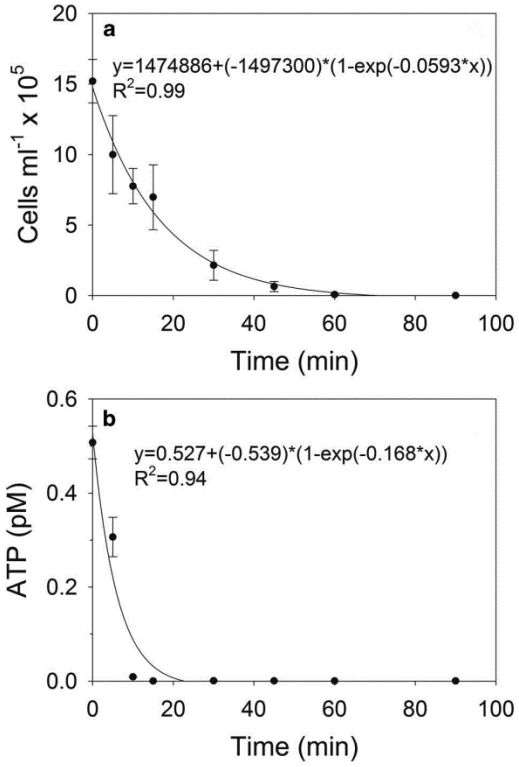



**Fig. 6. a.** Pond water cell, and **b.** adenosine triphosphate (ATP) concentrations in the tank at a flow rate of 76 l min$^{-1}$ with the samples passing through both filter systems and both UV lamps. Each time point represents the mean and standard error (s.e.) ($n = 3$). Cell concentrations and ATP levels were below levels of detection after 60 min and 15 min, respectively.

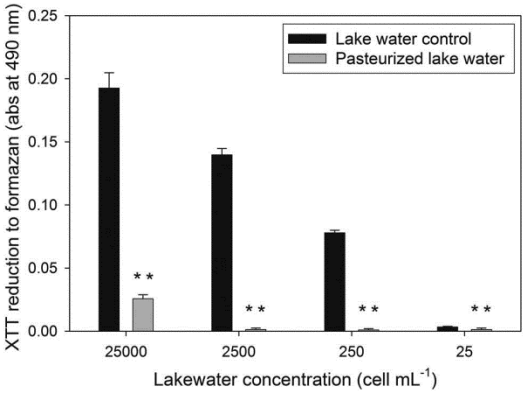



**Fig. 7.** Respiratory potential, detected as tetrazolium dye 2, 3-bis-(2-methoxy-4-nitro-5sulphenyl)-(2 H)-tetrazolium-5-carboxanilide (XTT) reduction to formazan dye (measured as absorption at 490 nm) of pasteurized (85°C for 2 min) lakewater samples relative to unpasteurized lakewater controls (no heat treatment) following 21 hours of incubation with XTT. The data represent a dilution series made from the original lakewater sample. Bars represent mean and 1 SD ($n = 3$). Asterisks denote that pasteurization significantly ($P < 0.001$) reduced cell viability in all dilutions.

port 1, or port 2 (Fig. 5b). The final sampling time at 15 hours is 75 times the theoretical hydraulic retention time of the system.

*UV exposure and cell viability*

Circulation of the *E. coli* suspension through the 185 nm and 254 nm UV irradiation modules reduced the number of viable *E. coli* cells in the tank (initial concentration of 7.2 x 10$^6$ CFU ml$^{-1}$) to below the methodological limit of quantification (< 300 CFU ml$^{-1}$) on a single pass through the UV lamps at flow rates of 19, 76 and 152 l min$^{-1}$, which represents 2.7, 0.68 and 0.34 min of UV exposure as the sample passed through the UV system (based on a UV canister volume of 52 l). After two weeks of incubation at room temperature (~22°C), the CFUs on the plates remained below 300 CFU ml$^{-1}$, providing evidence that the bacteria were killed by the UV treatment as opposed to incurring a level of sub-lethal damage that allowed them to remaining viable and recover.

*Lakewater bacterial removal and viability*

Viable lakewater bacteria within the tank were either physically removed or their DNA was irreversibly damaged by UV radiation during a 90 min experiment, reaching levels similar to that observed in procedural blanks by the end of the experiment (Fig. 6a). Based on the 76 l min$^{-1}$ flow rate used in this experiment and the exponential curve fit shown in Fig. 6a, 99% of all bacteria were removed or killed following 78 min of run time (51 min for 95% removal).

Adenosine triphosphate measurements from the tank over the course of the experiment revealed that cellular ATP decreased by three orders of magnitude (to the limits of detection of the ATP method) following 15 min of circulation (Fig. 6b). Based on the exponential model used to fit the data, 99% of ATP-containing cells were eliminated from the system (through a combination of cell removal by the filters and decomposition by the UV lamps) in 27 min (18 min for 95% removal) at the 76 l min$^{-1}$ flow rate used in the experiment. The almost three times greater reduction in ATP concentration relative to total cell number indicates that intracellular ATP was actually destroyed by the system, presumably by the UV lamps and

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associated ozone. The reduced levels of intracellular ATP could also be the result of the synthesis of ATP-dependent enzymes used to repair UV damaged cells.

*Lakewater pasteurization test*

The pasteurization experiment (85°C for 2 min) conducted on lakewater showed that heating to the temperatures expected in the boiler of the hot water drill (at least 85°C) reduced respiration potential (XTT reduction to formazan dye) of the organisms significantly ($P < 0.001$) at cell densities ranging from 25–25 000 cell $ml^{-1}$ (Fig. 7). These results indicate that the boilers in the hot water drill can produce up to a 2-log reduction in the number of viable cells.

*Surface cleaning experiments*

Following experimental contamination with *E. coli* and *B. subtilis*, the stainless steel coupons averaged (± SD) $8.7 \times 10^4 \pm 8.6 \times 10^4$ CFU $cm^{-2}$ and $7.2 \times 10^4 \pm 4.0 \times 10^4$ CFU $cm^{-2}$, respectively. The averages (± SD) for these same organisms on the plastic coupons were $6.2 \times 10^5 \pm 1.8 \times 10^5$ CFU $cm^{-2}$ and $4.0 \times 10^5 \pm 3.9 \times 10^5$ CFU $cm^{-2}$, respectively. A one-way analysis of variance (ANOVA) followed by a multi-comparison test revealed that a significantly ($P < 0.001$) greater proportion of *E. coli* and *B. subtilis* adhered to the plastic coupons per unit area than the stainless steel coupons. No significant difference ($P > 0.05$) in adherence was evident between the test organisms. Following treatment with $H_2O_2$, plate counts after 18 hours and 72 hours of incubation were below the limits of quantification (300 CFU $cm^{-2}$; i.e. no colonies formed), indicating a 2 to 3-log reduction in the concentration of viable cells.

## Discussion

A flow rate of 95 l $min^{-1}$ and a total volume for the entire treatment system (filtration plus UV components) of 572 l yields a mean retention time of 6 min, which represents the time when 63% of the water in the system is replaced for a CSTR. Timescales for the system for 95% and 99% water replacement are 21 min and 32 min, respectively, at this flow rate. Results from the dye study yielded a dilution curve for the treatment system that was very similar to that for a CSTR. Moreover, the filtration-disinfection-heater-borehole forms a recirculating system, whereby water in the borehole continually recirculates through the treatment system as the borehole volume increases during drilling operations (i.e. the borehole is deepened). The expected drilling rate of $\sim 1$ m $min^{-1}$ (producing an increase in the water volume in a 0.3 m diameter borehole at a rate of 71 l $min^{-1}$) is slower than the upward velocity of water in the 30 cm diameter borehole ($\sim 1.3$ m $min^{-1}$ at a flow rate of 95 l $min^{-1}$). Hence, water in the borehole is being pumped through the filtration system at a faster rate than the liquid volume of the borehole is increasing.

The nature of mixing of water in the borehole itself is uncertain. Assuming an ice borehole depth and diameter of 800 m and 0.3 m, respectively (yielding a borehole volume of 57 $m^3$), and a system pumping rate of 95 l $min^{-1}$, the mean residence time for borehole water would be ten hours. This would be the actual time for complete replacement of the water in the borehole if plug-flow results from water being pumped into the bottom of a relatively smooth borehole and removed from the surface at the same rate where it enters the filtration/UV system and boilers before being returned to the bottom of the borehole. Although flow in the actual borehole will be complicated by buoyancy effects (it is likely to be turbulent; Reynolds number $\sim 4500$), its mixing and replacement-time characteristics will resemble plug-flow much more closely than that of a CSTR (Dankwerts 1953). Hence, it is reasonable to assume that the timescale for complete replacement of borehole water will be of the same order as the mean residence time (ten hours), rather than that given for a CSTR (i.e. 4.6 times the mean residence time for 99% replacement).

As the heated water enters the bottom of the borehole, it will mix with newly melted water. The mixed water will start to flow upward in the borehole to maintain continuity with the rate at which water is being withdrawn at the surface for treatment and reheating. The question of how much, and how often, water in the borehole is treated is difficult to answer, because of this mixing, the complicated flow in the borehole, and the fact that the borehole volume grows with time. If the filtration system is run continuously during borehole drilling, as planned, the water volume in the borehole will be much less than 57 $m^3$ during the early stages of drilling when the borehole depth is considerably shallower than 800 m, and borehole water originally near the surface will be passed through the water treatment system many times before the subglacial environment is entered. However, as the hole becomes very deep, a point will be reached beyond which there is not enough time for water melted at the bottom of the hole to reach the surface before the hole is melted to the base of the ice. Importantly, all of the heated water introduced into the bottom of the borehole will have passed through the treatment system at least once as the borehole reaches its maximum depth of $\sim 800$ m.

Based on our experimental results with sediment removal (Fig. 5b), one pass through the 2 μm filter of the filtration system alone will not eliminate all of the sediment particles in the borehole, particularly if the sinking rate of sediment particles (> 63 μm) exceeds the upward velocity of water through the borehole. However, the UV component of the system, which was shown to reduce *E. coli* by 4-log units following a single pass through the system at 95 l $min^{-1}$ and to reduce cellular ATP levels to below the limits of detection ($10^{-15}$ mol ATP $ml^{-1}$)

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following 15 min at 76 l $min^{-1}$, should significantly reduce the number of viable microbial cells. Hence, both systems working in tandem will effectively eliminate most of the relatively buoyant particles, including cells, in the system and kill a majority of the cells as melting proceeds from the surface to the bottom of the ice stream ($\sim$800 m).

Our pasteurization tests showed that a lake microbial assemblage exposed to 85°C for 2 min significantly ($P < 0.001$) reduced the respiratory potential of the assemblage, resulting in up to a 2-log reduction in the number of viable cells. These conditions are similar to the temperatures and retentions of the boilers on the hot water drill. As such, the heating system alone on the WISSARD drill should kill the vast majority of bacteria that pass through it, particularly if they are not heat tolerant (e.g. not endospore-forming bacteria), which we expect to be the case for most of the bacteria that exist in the ice we will melt to generate the borehole water (e.g. Christner *et al.* 2008, Priscu *et al.* 2008).

In summary, our test results indicate that one passage of a volume of water through the filtration component of the system will reduce the total number of microbes by more than 4-log units. Any cells remaining in the water after filtration will be reduced another 3.5-log units by combined effect of UV irradiance and pasteurization. To place this into context, if we assume an initial borehole cell concentration of $10^6$ cell $ml^{-1}$ (equivalent to coastal ocean waters), the system as tested would be capable of reducing the microbial burden to $< 100$ cells $ml^{-1}$ in the borehole water after one borehole residence time ($\sim$ten hours at a 95 l $min^{-1}$ pumping rate). Because we will treat all down borehole equipment with 3% $H_2O_2$, which produced a 2 to 3-log reduction in our endospore and non-endospore forming test organisms, any cellular contamination introduced into the drilling and water treatment system during our operation is expected to be minimal. In addition to the safeguards discussed above, all hoses and cables on the WISSARD drilling system will pass through a clamp-on high pressure hot water cleaning system followed by a UV collar as they are deployed down the borehole. The UV collar will provide a disinfection dose of 40 000 $\mu$W-sec $cm^{-2}$ at a maximum cable/hose deployment rate of 60 m $min^{-1}$.

In addition to the filtration/UV system and decontamination protocols that will be implemented during borehole drilling, the hydraulic nature of the lake itself provides a safeguard against permanent contamination by drilling procedures. Subglacial Lake Whillans belongs to the category of active subglacial lakes, which are located and defined on the basis of anomalously fast ice surface changes attributed to large water volume fluctuations in subglacial lake basins. Active subglacial lakes were discovered by InSAR (Gray *et al.* 2005) and subsequent studies using satellite altimetry have demonstrated that there are $>120$ such lakes in Antarctica (Wingham *et al.* 2006, Fricker *et al.* 2007, Fricker & Scambos 2009, Smith *et al.* 2009). Active subglacial lakes are different from many previously described Antarctic subglacial lakes (e.g. review in Siegert *et al.* 2005) in a number of important ways. The defining feature of active subglacial lakes is that they actively fill and drain, undergoing volume changes large enough to cause localized, anomalously high (up to several metres) deformation of the ice surface above them that can be detected by a space borne instrument (e.g. Fricker *et al.* 2007). They also tend to occur within fast flowing parts of the Antarctic ice sheet (ice streams and outlet glaciers) as opposed to non-active lakes that are concentrated near ice divides, where ice flow is sluggish (Siegert *et al.* 2005, Smith *et al.* 2009). The active subglacial lakes discovered thus far tend to have a smaller area, and presumably volume, than their non-active counterparts. This may be largely due to observational biases because non-active lakes have been traditionally identified from airborne ice-penetrating radar surveys, which are more likely to encounter large subglacial lakes than small ones (e.g. Siegert *et al.* 2005). At the same time, active subglacial lakes have only been mapped using anomalous ice surface elevation changes occurring within the last decade, when new airborne/satellite altimetry and InSAR data provided sufficiently precise measurements of ice surface topography to reveal their existence. Hence, detection of such lakes is more likely if they have relatively small water residence time (i.e. high water throughput rates combined with low total basin volume).

Analyses of satellite data by Fricker *et al.* (2007) and Fricker & Scambos (2009) indicated that SLW has an area of 59 $km^2 \pm 12$ $km^2$ and it has experienced two drain-fill cycles between 2003 and 2009. These authors estimated that each of the drain-fill cycles resulted in lake volume fluctuations of about 0.1 $km^3$ of water. The WISSARD surface geophysics team completed a high-density survey focused on the SLW basin in the 2010–11 field season. Improved constraints on ice geometry indicate that the subglacial water volume change during fill-drain cycles of SLW is 0.15 $km^3$ (unpublished data), which is 50% greater than the previous estimates of Fricker *et al.* (2007) and Fricker & Scamos (2009). Geophysical estimates of the depth of the lake at the time of the field survey, when the lake was drained, reveals that it is $< 8$ m (Horgan *et al.* 2012, Christianson *et al.* 2012). The relatively shallow depth of the SLW basin is consistent with its location within a region of gently undulating basal topography and low ice surface slope (e.g. Shabtaie *et al.* 1987). Given the area of the lake basin, the lake volume can be estimated to be $< 0.5$ $km^3$. Consequently, it would take fewer than three to four fill-drain cycles to exchange the total lake volume. Given that SLW has undergone two complete fill-drain cycles over a six year period, we estimate a water residence time for the lake of the order of ten years, or less.

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The decadal scale flushing time for SLW is nearly 100 times faster than that predicted for Subglacial Lake Ellsworth (SLE) (750 years; Siegert *et al.* 2012) and about 1000 times faster than the water residence time estimated for Subglacial Lake Vostok (~10 000 years; Bell *et al.* 2002). Clearly, SLW is fundamentally different from SLE and Vostok, which contain large water volumes and do not show evidence of significant water volume changes over the period of instrumental observations. Subglacial Lake Whillans can be considered a small temporary storage basin for water draining beneath the Whillans Ice Stream and any disturbance resulting from drilling and sampling operations should have a minor and transitory impact.

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# APPENDIX B

## BIOLOGICAL ICE NUCLEATION INITIATES HAILSTONE FORMATION

# APPENDIX B

## BIOLOGICAL ICE NUCLEATION INITIATES HAILSTONE FORMATION

### Contribution of Authors and Co-Authors

Author: Alexander B. Michaud
Contributions: Designed research, performed research, analyzed data, and wrote the paper. Partially supported the research through a NSF-IGERT fellowship (DGE 0654336) and a Montana Space Grant Consortium fellowship.

Co-Author: John E. Dore
Contributions: Analyzed data, helped to design research and write the paper.

Co-Author: Deborah Leslie
Contributions: Performed the stable isotope analysis, reviewed the paper and provided edits and comments.

Co-Author: W. Berry Lyons
Contributions: Helped to perform the stable isotope analysis, reviewed the paper and provided edits and comments.

Co-Author: David C. Sands
Contributions: Provided laboratory support, helped to analyze the data, and reviewed the paper and provided edits and comments.

Co-Author: John C. Priscu
Contributions: Supported the work through National Science Foundation grants OPP1027284, OPP0838933 and OPP0839075. Helped to write the paper.

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RESEARCH ARTICLE

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# Biological ice nucleation initiates hailstone formation

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**Key Points:**
- Active biological ice nuclei are present in the embryos of hailstones
- Water stable isotopes reveal warm, subzero freezing of hailstone embryos
- Layered hailstone growth allows direct study of ice nucleation

**Supporting Information:**
- Readme
- Figure S1
- Figure S2
- Figure S3

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**Abstract** Cloud condensation and ice nuclei in the troposphere are required precursors to cloud and precipitation formation, both of which influence the radiative balance of Earth. The initial stage of hailstone formation (i.e., the embryo) and the subsequent layered growth allow hail to be used as a model for the study of nucleation processes in precipitation. By virtue of the preserved particle and isotopic record captured by hailstones, they represent a unique form of precipitation that allows direct characterization of the particles present during atmospheric ice nucleation. Despite the ecological and economic consequences of hail storms, the dynamics of hailstone nucleation, and thus their formation, are not well understood. Our experiments show that hailstone embryos from three Rocky Mountain storms contained biological ice nuclei capable of freezing water at warm, subzero (°C) temperatures, indicating that biological particles can act as nucleation sites for hailstone formation. These results are corroborated by analysis of $\delta D$ and $\delta^{18}O$ from melted hailstone embryos, which show that the hailstones formed at similarly warm temperatures in situ. Low densities of ice nucleation active abiotic particles were also present in hailstone embryos, but their low concentration indicates they were not likely to have catalyzed ice formation at the warm temperatures determined from water stable isotope analysis. Our study provides new data on ice nucleation occurring at the bottom of clouds, an atmospheric region whose processes are critical to global climate models but which has challenged instrument-based measurements.

## 1. Introduction

Atmospheric aerosols play important direct and indirect roles in the radiative balance of Earth. These aerosols directly influence Earth's climate through absorption and reflection of solar radiation in the atmosphere. When these aerosols act as ice nuclei (IN) or cloud condensation nuclei they indirectly affect the climate by forming clouds, which insulate or reflect solar radiation [*Trenberth et al.*, 2007]. Understanding the interactions between aerosol particles and their role in nucleation of tropospheric clouds is critical to developing better global climate models [*Rosenfeld et al.*, 2014].

Convective storms producing large, damaging hail have been a troublesome form of severe weather throughout history [*Changnon and Changnon*, 2000]. Since the early 1800s, many investigators have attempted to explain the causes of hail with the hope of preventing economic losses associated with hailstorms [*Changnon and Ivens*, 1981]. Hailstorms are common in most midlatitude countries, a geographical region hosting large population and agricultural centers [*Leigh and Kuhnel*, 2001; *Changnon et al.*, 2009; *Sioutas et al.*, 2009; *Berthet et al.*, 2011]. In the United States alone, approximately 115,000 hail events occurred between 1996 and 2006 [*National Weather Service*, 2007]. Hail is especially damaging to agriculture because it occurs most frequently during the Northern Hemisphere growing season (May–October) [*Changnon et al.*, 2009]. Thus, hailstone formation has been studied extensively with the goal of prevention. Hail is used in this study to provide data on the processes occurring in the nucleation region of the cloud, a zone which has been challenging to study with instruments [*Rosenfeld et al.*, 2014].

Hailstones are a form of precipitation in which the nucleation event is captured by a distinct unit, the embryo. The embryo is a visually discernable region of a hailstone that typically measures $\approx \leq 5$ mm. Embryos are most commonly (1) frozen-drop embryos, which are produced from the freezing of a raindrop or (2) graupel embryos, which are collections of ice crystals [*Knight and Knight*, 2001]. Newly formed embryos are carried into the main body of a convective storm cloud by warm updrafts [*Knight and Knight*, 2001]. These strong


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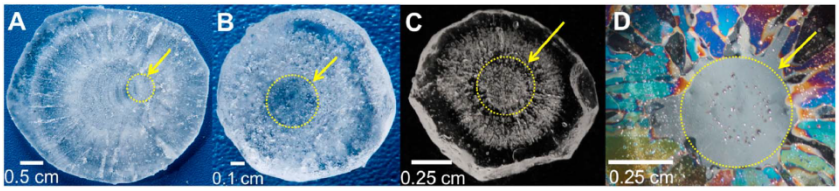



**Figure 1.** Cross-sectional images of representative hailstones from three Rocky Mountain storms. Yellow arrows and dotted circles denote the embryo. (a–c) Photographs taken under transmitted light and (d) photograph taken under cross-polarized light. Figures 1a–1d represent hailstones from the June 2010, July 2010, August 2011, and July 2010 hailstorms, respectively.

updrafts cycle the hailstone within the storm cloud and allow it to accrete supercooled water. This process produces a layered structure to the hailstone (see Figure 1), which eventually falls to the ground once its weight overcomes the force of the updraft [*Knight and Knight*, 2001]. Thus, hailstone layers are a record of the vertical profile of the storm, and the embryo is a frozen sample of what was present during the nucleation event. This chronological formation of the hailstones' embryo and outer layers overcomes the problem of atmospheric scrubbing that occurs during the descent of rain and snow [*Creamean et al.*, 2013; *Cziczo et al.*, 2013].

Hailstones have been used to track the vertical distributions of chemical and particulate concentrations in storm clouds [*Rosinski*, 1966; *MacGregor et al.*, 1990], while the freezing temperature and trajectory of a hailstone embryo and outer growth layers can be recorded through the use of water stable isotopes (δD- and $\delta^{18}O$-$H_2O$) [*Jouzel et al.*, 1975, 1985; *Knight et al.*, 1981; *Federer et al.*, 1982]. The relationship between isotopic composition and temperature is based on the decreasing temperature profile with increasing altitude in the troposphere and the equilibrium isotopic fractionation that takes place during condensation. As an air parcel ascends, cools, and condenses in a convective storm cloud, its vapor phase becomes progressively depleted in the heavy isotopologues (δD- and $\delta^{18}O$-$H_2O$) due to the fact that lower molecular weight isotopologues of water have a higher vapor pressure. Liquid water condensing from this progressively changing air parcel thus also becomes isotopically depleted with increasing altitude and the associated decreasing temperature. During hailstone layer accretion and formation (assuming little or no evaporation takes place), the isotopic signature of the cloud water is preserved and represents an approximate measure of the altitude (and temperature) of layer formation [*Jouzel et al.*, 1985].

An aerosol particle is required for heterogeneous ice nucleation at temperatures associated with lower tropospheric clouds ($> -40$°C) [*Tselioudis et al.*, 1992; *Pruppacher and Klett*, 1998]. Although biological aerosols form a significant fraction of total atmospheric particles, the extent of their role in cloud and ice nucleation remains unclear [*Pöschl et al.*, 2010]. Biological particles (i.e., bacteria, pollen, fungi, and lichens) [see *Hirano and Upper* [1995]; *Després et al.* [2012], and references therein] can be efficient ice nucleators at temperatures $\geq -15$°C. Some abiotic particles are also capable of catalyzing ice nucleation, but their ice nucleation thresholds ($< -15$°C) [*DeMott and Prenni*, 2010] are distinctly colder than those for biological particles; even the most efficient abiotic ice nucleators (e.g., potassium feldspar) require a collectively large surface area or particle number to induce ice nucleation at $> -15$°C [*Pinti et al.*, 2012; *Atkinson et al.*, 2013]. Our study focused on immersion ice nucleation, which occurs when a particle is immersed in a supercooled water droplet, because it is the dominant freezing process in atmospheric clouds [*Murray et al.*, 2012]. Results from hailstone embryo particle composition and water stable isotopes show that biological IN contribute to hailstone formation.

## 2. Methods

### 2.1. Hailstone Collection and Processing

Hailstones were recovered from three northern Rocky Mountain storms near Bozeman, Montana, USA, during the summers of 2010 and 2011. The 30 June 2010 storm produced ~4 cm diameter hailstones, whereas the 21 July 2010 and 29 August 2011 storms produced ~1 cm and ~1.5 cm diameter hailstones,


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respectively (Figure 1). Samples were collected in sterile Whirl-Pac bags (Nasco) immediately after they had landed on the ground. Hailstones were transported frozen to our laboratory and were stored at −30°C until processing and analysis.

All hailstones were processed in the SubZero Research Facility at Montana State University, Bozeman, Montana (http://www.coe.montana.edu/ce/subzero/Index.html), in an International Organization for Standardization Class 6 clean cold chamber, operating at −15°C. June 2010 hailstones processed for biological analysis (ice nucleation assay and culturing) were decontaminated according to *Christner et al.* [2005] except for the final melt step. Briefly, the outer layer was scraped with a sterile razor blade, rinsed with 95% ethanol, and followed by a final rinse with sterile deionized water (MilliQ; 18.2 MΩ·cm resistivity). This process removed 13% ± 2.8% of the total hailstone mass. July 2010 and August 2011 hailstones were decontaminated by scraping the outside with a sterile razor blade because the decontamination protocol of *Christner et al.* [2005] eroded too much of each small hailstone. Hailstone layer separation was achieved by slicing the June hail into cross sections using a hot 28 gauge NiChrome wire, in which a 2.4 A current was passed. The surfaces of cross sections were scraped with a sterile razor blade to eliminate interlayer contamination that may have occurred during hot wire cutting. Cross sectioning exposed the hailstone embryo. Embryos from June 2010 cross sections and July 2010 and August 2011 hailstones were isolated using a sterile razor blade. We visually identified and isolated embryos based on the procedures of *Federer et al.* [1982] and *Knight and Knight* [2001]. This isolation was done by first identifying a region of the hailstone that is surrounded by other concentric layers. Depending on the hailstone's trajectory, the embryo can be in the middle or slightly off center. The embryos were ≤5 mm in diameter. Embryos were stored in sterile 50 mL conical tubes at −30°C under further analysis. Samples were allowed to thaw at 4°C for 18 h before analysis.

### 2.2. Nutrient Analysis

Detection of soluble reactive phosphorus (SRP) in precipitation was used as an indicator of ground-based contamination [*Sheppard*, 2012]. Because phosphorus has no stable gaseous phase, its presence in the atmosphere is almost entirely due to aerosols. SRP concentrations in precipitation are thus low, especially compared to fixed nitrogen species, which have an active atmospheric chemistry. Consequently, a relatively high level of reactive P in precipitation is thus an indicator of ground-based contamination (e.g., from soil organic matter or anthropogenic fertilizer) [see *Sheppard*, 2012]. SRP was determined using the spectrophotometric molybdenum blue assay for SRP [*Murphy and Riley*, 1962] as modified by *Strickland and Parsons* [1968] on melted hailstone embryos. Hailstones from the July storm were cleaned by scraping whole stones as described above. The remaining frozen hailstone material was then melted, and 3 mL of this melt was diluted with MilliQ water up to 10 mL for analysis. Because of the low SRP concentrations expected, a long pathlength (200 cm) liquid waveguide capillary cell (World Precision Instruments) was used in conjunction with a compact spectrometer (Ocean Optics USB4000) for absorbance measurements at 710 nm [*Zhang and Chi*, 2002]. This method as applied had an estimated analytical detection limit of <10 nM SRP.

### 2.3. Bacterial Culturing and Isolate Collection

R2A (Difco), Nutrient Agar (Difco), and King's B Medium [*King et al.*, 1954] were used to culture heterotrophic, aerobic microorganisms from the embryos of the three hailstorms. These types of media were chosen because R2A is low in nutrients and efficient at culturing stressed organisms which have been frozen in ice [*Christner et al.*, 2001]; nutrient agar is an organic carbon and nutrient rich media, and King's B Medium is a selective media for the known ice nucleation active bacterium, *Pseudomonas syringae* [*King et al.*, 1954]. The plates were inoculated by spread plating 100 μL of hailstone embryo meltwater in quintuplicate. Inoculated plates were enumerated after incubation at room temperature (~21°C) for 3 days. Unique colony morphologies from the three types of media were randomly picked and streaked to isolation. Lyse-n-go (Thermo Scientific) was used to extract genomic DNA to determine the 16S rRNA gene sequence of the isolates. Extracted DNA was amplified using polymerase chain reactions (PCR) in 23 μL volumes and included 11 μL GoTaq 2X Green Master Mix (Promega), 1 μL of 20 μM 9bF primer (5′-AGAGTTTGATCCTGGCTCAG-3′), 1 μL of 20 μM 1492R primer (5′-GGTTACCTTGTTACGACTT-3′) [*Lane*, 1991], 2.5 μL of Lyse-n-go lysate, and 7.5 μL nuclease-free water. The reactions tubes were incubated at 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 52°C for 30 s, and 72°C for 1 min, and ended with 72°C for 10 min. PCR amplicons were Sanger sequenced (Functional Biosciences) from the 9bF primer and then processed to 500 base pairs, which resulted in high-quality (> 40 Phred score) sequences. The high-quality, trimmed sequences were then


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queried against the GenBank database to recover the nearest phylogenetic relative of the isolate. Isolates were named based on the nearest cultured neighbor Basic Local Alignment Search Tool hit. A sequence alignment was performed using ClustalW within the program Molecular Evolutionary Genetics Analysis (MEGA4 v.4.0.2) using the default settings [*Tamura et al.*, 2007]. The evolutionary history of the isolates and the GenBank nearest-neighbor relatives was inferred using the Neighbor-Joining method with bootstrap test of phylogeny (1000 replicates) values displayed at the branch nodes. This phylogenetic analysis was also conducted in MEGA4. The 16S rRNA gene sequences for the hailstone embryo isolates have been deposited in GenBank under accession numbers JX237375–JX237452.

### 2.4. Ice Nucleation Assay

The ice nucleation assay was based on *Christner et al.* [2008a], with modifications. An aluminum block was machined to fit a 96-well PCR plate and included channels beneath the plate for recirculating a 25% glycol/water coolant solution. The flow-through channels allowed for a consistent temperature (±0.1°C) across the entire plate. Untreated hailstone embryo meltwater (50 μL) from the July 2010 and August 2011 storms was dispensed into each well of a 96-well PCR plate; the June 2010 sample had 93 wells only due to sample volume limitation. The entire plate was cooled through a gradient of temperatures from −2°C to −12°C over a period of 2 h. The number of drops frozen at each 0.5°C interval (IN spectrum) was recorded. After the first IN spectrum was complete, the plate was allowed to thaw at room temperature and then heat treated at 95°C for 10 min [*Christner et al.*, 2008a]. The IN spectrum was determined again after heat treatment to determine the concentration of biological IN. One cumulative IN spectrum and one biological IN spectrum were conducted for each hailstorm. Calculations for total IN and biological IN were based on equation 15 from *Vali* [1971], and modified for the assay volume (50 μL) and degree interval (0.5°C) recorded. The concentration of biological ice nucleators was estimated, at each temperature interval, by measuring the fraction of IN that was inactivated by the heat treatment step.

A modified ice nucleation assay was also used to determine the ice nucleation activity of isolates recovered from the hailstone embryos [*Lindow et al.*, 1978]. Isolates were repeatedly subcultured to ensure purity of the colony. Once a pure colony was obtained, it was transferred to nutrient broth and grown at room temperature. Upon achieving a turbid suspension of the isolate, 500 μL was transferred into 4.5 mL of sterile Phosphate Buffered Saline (PBS) in a 10 mL screw cap test tube. The diluted cultures were incubated at 4°C for 3 h before the assay to allow for the expression of the ice nucleation protein [*Joly et al.*, 2013]. The ice nucleation assay was performed in triplicate for each isolate. These tubes were bathed in a 25% glycol/water solution and cooled from −2°C to −12°C over a period of 2 h. Tubes were visually inspected for frozen contents every 0.5°C. The positive control was *Pseudomonas syringae* strain CC94 (D.C. Sands, Montana State University) [*Morris et al.*, 2007]. Negative controls included sterile PBS and sterile PBS with 500 μL of R2 broth, Nutrient broth, Tryptic Soy Broth, or Luria Broth. The positive control was active between −2.5°C and −3.5°C, while negative controls did not freeze until ≤ −13°C, a typical limit of detection for this assay [*Christner et al.*, 2008a]. An isolate was scored positive for ice nucleation activity if two of the three culture tubes froze before the negative controls in two separate assays.

### 2.5. Stable Isotope Analysis

Stable isotopes ($\delta^{18}O$ and $\delta D$) from a random selection of hailstone embryos from each storm were analyzed with a Picarro Wavelength Scanned-Cavity Ring Down Spectroscopy Analyzer for Isotopic Water Model L1102-*i* and were only thawed immediately before analysis. The stable isotopic analysis includes a calibration using internal laboratory standards of waters spanning the expected isotopic range of the hail samples. The Picarro internal laboratory standards were deionized water aliquots from Colorado, Florida, Ohio, and Nevada, USA. Internal laboratory standards were calibrated relative to Standard Light Antarctic Precipitation and Vienna Standard Mean Ocean Water, at Ohio State Byrd Polar Ice Core Paleoclimatology Laboratory by a duel inlet mass spectrometer. During a Picarro sample run, each thawed sample was pipetted into a 100 μL polyspring glass insert contained in a 2 mL glass sample vial and sealed with a screw cap to prevent evaporation. To eliminate memory effects between samples, six replicate injections of 2 μL were performed, and the results of the first three injections were discarded. Internal laboratory standards were interspersed throughout the run with samples to correct for memory effects. The isotopic results were corrected based on a rolling calibration such that each sample is calibrated by the standards run closest in time to that of the sample. The accuracy was ≤4% for $\delta D$ and ≤2% for $\delta^{18}O$, calculated by relative standard


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error comparing the Picarro isotopic measurement for an internal laboratory standard and the mass spectrometer isotopic measurement for an internal laboratory standard. Precision was ≤1% for δD and ≤0.5% for $\delta^{18}O$, for 100 measurements using relative standard deviation.

We used the stable isotopic composition ($\delta^{18}O$ and δD) of individual hailstone embryos to estimate in situ temperatures of freezing. Although the variation in equilibrium isotopic fractionation of meteoric waters with temperature is predictable and kinetic fractionation in hailstones is typically small [*Federer et al.*, 1982], fixing the temperature scale is problematic, because direct measurements of the isotopic composition of the vapor feeding the clouds are seldom possible. Consequently, we employed two independent empirical approaches to such calibration for our three storms.

Our first approach was to compare isotopic results with those of the similar and well-studied Alberta, Canada, hailstones of August 1971 (Comparison Method) [*Jouzel et al.*, 1975]. The deuterium-temperature relationship for the Alberta hailstones was previously derived from the Isotope Cloud Model (ICM) and the temperature scale fixed using a crystallographic approach [*Jouzel et al.*, 1975, 1985]. Because the strength and duration of hailstorm updrafts differ, the lowest temperature (i.e., highest altitude) at which hailstone layers form is expected to vary considerably between storms. We assumed that following embryo formation, the warmest temperature at which subsequent layers form should represent the base of the main updraft and should be similar between storms producing similar-sized hailstones. Consequently, we assigned the warmest temperature estimated for nonembryo layers of the Alberta hailstones (−14.7°C) to the warmest (highest δD) nonembryo hailstone layer observed during each of the three Montana storms. After fixing the temperature scales of the storms in this way, we used the ICM-based δD-temperature relationship [*Jouzel et al.*, 1975] to infer the temperatures of formation of all other layers, including the embryos.

Our second approach was to take advantage of the interpolated empirically determined climatology of the isotopic composition of precipitation or water isoscape (Isoscapes Method) [*Bowen and Revenaugh*, 2003]. We used the Online Isotopes in Precipitation Calculator (http://www.waterisotopes.org) [*Bowen and Wilkinson*, 2002; *Bowen and Revenaugh*, 2003; *Bowen et al.*, 2005] to generate monthly climatological isotopic values for our Montana location as a function of elevation. We assumed that ground-based precipitation collections represent a mix of waters from the precipitation-forming layers of the clouds above and assigned a particular isotopic value of precipitation to the average temperature of the 1000 m thick layer extending upward from an altitude equivalent to the isoscapes-predicted elevation plus the above-ground height of the cloud base (lower condensation level). To estimate the lower condensation levels for our storms, we utilized radiosonde-collected temperature and dew point data from the nearest NOAA National Weather Service center in Great Falls, Montana. These sonde casts were conducted at local noon on the day of each storm and exhibited typical prethunderstorm structure.

Despite the simplifying assumptions and recognized limitations of all methods that have been used to fix the isotope-temperature scales for hailstones, our two independent approaches yielded similar overall mean (±SD) temperatures for embryo formation in the Montana storms (−14.3 ± 2.7 and −13.5 ± 4.8°C for the comparison and isoscapes approaches, respectively; $n = 14$). In only one case did the predicted embryo formation temperatures differ by more than 3°C. The range of predicted temperatures was narrower for the comparison method than for the isoscapes method (−18.9 to −9.0°C versus −21.8 to −3.1°C, respectively).

### 2.6. Particle Identification and Analysis

Particles from hailstone embryos were collected via filtration and analyzed with scanning electron microscopy (SEM). Two embryos from the June 2010 storm and five embryos from each of the July 2010 and August 2011 storms were filtered onto a 13 mm, 0.2 μm polytetrafluorethylene membrane filter (Millipore Corp.) after thawing at 4°C for 18 h. All plasticware used for filtration and sample handling was sterilized and then rinsed 20 times with sterile MilliQ water. Once the particles were filter concentrated, we performed a dehydration step using a graded 0.2 μm filtered ethanol series with two, 5 min incubations at each ethanol step (10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100%). The particles were coated before SEM imaging with iridium to a thickness of 5 nm. Images and mineral grain identity were performed on a JEOL JSM-6100 SEM equipped with energy dispersive X-ray spectroscopy (EDX) and a NORAN SiLi detector. Spectrum collection and peak identification were performed using Rontec electronics and software. EDX was performed at 15 kV to obtain spectra of all major elements, and identification of each mineral grain was conducted with a dichotomous key from *Severin* [2004]. The accelerating voltage of 15 kV was sufficient to


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identify mineral grains and not too strong to create a large interaction volume and cause filter background effects. Measurement of mineral grain size was done using the program ImageJ [*Abrámoff et al.*, 2004]. Large biological particles were visually identified (L. Szabo and Y. Jin, personal communication, 2012).

Procedural controls were prepared and analyzed in parallel with the hailstone samples. Small cubes of MilliQ water were frozen and then cleaned with the same technique as the hailstones. These cleaned MilliQ ice cubes were filtered and subjected to the same ethanol dehydration series. Particles on the control filter were imaged and identified with the same SEM-EDX methodology. The total number of particles and composition was insignificant compared to the particles found on the embryo samples. An elemental spectrum was collected from the control filter as a reference for its composition.

Statistical tests comparing freezing temperatures of embryo derived particles, stable isotope derived temperatures, and those data reviewed from other studies were conducted using SigmaPlot v11.0. One-way analysis of variance (ANOVA) was applied between treatment groups, and data passed normality and equal variance tests. Pairwise comparisons were conducted with the Holm-Sidak method. Comparison of the two isotope temperature methods, comparative and isoscapes, was conducted using a two-sided *t* test.

## 3. Results and Discussion

### 3.1. Ground-Based Contamination

We used SRP as an indicator of ground-based contamination in hailstone embryo meltwater. The concentration of SRP in July hailstone embryos was below the limit of detection (<10 nM), demonstrating that our decontamination procedures successfully removed any material that the hailstones may have acquired from soils or vegetation after falling on the ground.

### 3.2. Embryo Freezing Temperatures

Stable isotopes ($\delta$D and $\delta^{18}$O) of hailstone embryo meltwater were measured to determine the in situ temperature of embryo formation. The average in situ embryo formation temperatures from the June 2010, July 2010, and August 2011 storms derived from the stable isotope comparative method were −13.1, −16.5, and −11.4°C, whereas those derived from the isoscapes method were −11.4, −16.9, and −9.1°C (Figure 2). The overall means (±SD) for the two methods were −13.5°C (±4.8) and −14.3°C (±2.7), respectively, which are not statistically different ($t = 0.547$; df = 26; $P > 0.5$). The overall mean embryo freezing temperatures are warmer than typical abiotic ice nucleation temperatures (< −15°C) [*DeMott and Prenni*, 2010] and not significantly different (one-way ANOVA, $P = 0.273$) from the results of other stable isotope studies that investigated the temperatures of embryo freezing and hailstone growth [*Knight et al.*, 1981; *Jouzel et al.*, 1985] (Figure 2). The July 2010 hailstorm embryos formed at a significantly colder temperature than those from the other two storms. Their formation could have been catalyzed by biological ice nuclei or by the most efficient of the abiotic ice nuclei (e.g., K-feldspars). Alternatively, the colder apparent formation temperatures of these relatively small hailstones may result from contamination of the embryos with ice from the innermost growth layers during the difficult embryo isolation process. Most (>91%) of the hailstones examined in earlier studies showed that embryos formed at temperatures > −14°C, but no mechanisms were proposed to explain hailstone embryos freezing at these warm temperatures [*Knight et al.*, 1981]. $\delta$D and $\delta^{18}$O data from hailstone embryos in our study corroborate results from studies of embryo freezing temperatures and correspond to the freezing temperatures of other known biological ice nuclei (Figure 2). The relatively warm freezing temperatures determined from stable isotopes in our study and in previous studies reveal that only the most efficient ice nucleators (e.g., biological ice nuclei) likely initiate hailstone embryo formation.

### 3.3. Biologically Mediated Ice Nucleation

Ice nucleation active biological particles are characteristically active from −2°C to −18°C with the majority of biologically mediated nucleation occurring at temperatures > −15°C (Figure 2) [*Hirano and Upper*, 1995; *Després et al.*, 2012]. Our experimental results show that biological ice nuclei from hailstone embryos began to nucleate ice at temperatures of −5°C, −7.5°C, and −7°C for the June 2010, July 2010, and August 2011 storms, respectively (Figure 2; see "Biological Ice Nuclei"). The estimated concentrations of biological ice nuclei measured at these temperatures were 3, 6, and 8 biological ice nuclei per mL of embryo meltwater, respectively. These concentrations are equivalent to ~1 biological ice nucleus embryo$^{-1}$ and


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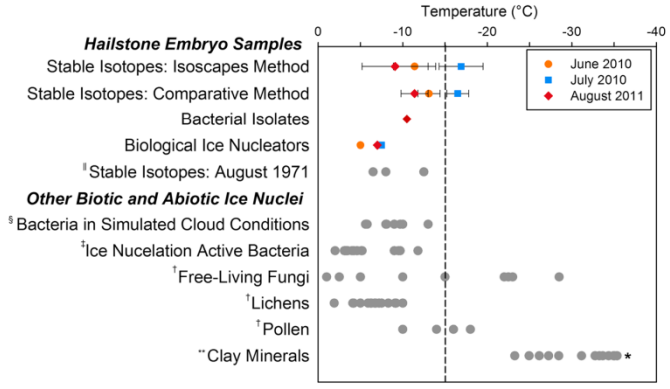



**Figure 2.** Freezing point temperatures computed from four different methods for embryos from hailstorms occurring in the Rocky Mountain region on 30 June 2010 (orange circles), 21 July 2010 (blue squares), and 29 August 2011 (crimson diamonds). The dashed vertical line represents the warmest abiotic nucleation temperature reported by *DeMott and Prenni* [2010]. The single asterisk within the panel indicates that the temperature of nucleation for abiotic particles is significantly different ($P < 0.001$) from biological ice nucleation temperatures and isotopically determined freezing temperatures presented. The right side of the figure is bounded by the warmest temperature at which water will homogenously freeze without a biotic or abiotic ice nucleator at −40°C [*Pruppacher and Klett*, 1998]. Double vertical line denotes data from *Jouzel et al.* [1985]; section sign denotes data from *Möhler et al.* [2007]; double dagger denotes data from *Hirano and Upper* [1995], *Després et al.* [2012], and citations therein; dagger denotes data from *Diehl et al.* [2001], *Diehl et al.* [2002], *Després et al.* [2012] and citation therein; and two asterisks denote data from *Pinti et al.* [2012].

are approximately 10 times higher than reported values for rain at comparable temperatures [*Christner et al.*, 2008a], revealing a relatively high in situ concentration of biological ice nuclei active at warm temperatures in hailstone embryos. Further evidence for abundant biological ice nucleation in hail was found by *Hill et al.* [2014] who used quantitative PCR to determine the number of *ina* gene copies present in hailstones; this gene codes for the proteins responsible for biological ice nucleation activity at relatively warm temperatures (up to −2°C). The comparatively high *ina* gene copy number in hailstones was concluded by *Hill et al.* [2014] to indicate a significant source of warm ice nucleating particles. Particulate matter analysis showed that organic particles constituted between 8 and 34% of the total particles in these samples (Figure 3 and Figures S1 and S2 in the supporting information). Visual identification of particles categorized as organic showed that these particles were composed of fungal spores, pollen grains, and other organic detritus. Fungal urediospores were common biological particles in hailstone embryos (Figure S2 in the supporting information). *Morris et al.* [2013] showed that these spores are ice nucleation active and possess ice nucleating species of bacteria on their surface. These spores may have contributed to nucleation of the hailstones we studied. Rain and snow have also been shown to contain biological particles that are ice nucleation active at warm, subzero temperatures [*Christner et al.*, 2008a, 2008b]. However, rain and snow can scavenge other ice nucleating material during their descent [*Creamean et al.*, 2013; *Cziczo et al.*, 2013], producing a ground-based sample that may not be representative of the particles present in the nucleation region of the cloud. Atmospheric scavenging precludes detailed study of the processes initiating nucleation and leaves the role of biological ice nucleation in rain and snow equivocal. The distinct embryo phase of hailstones preserves the composition of ice nuclei responsible for warm, subzero embryo nucleation and spatially segregates the embryos from outer hailstone growth layers [*Knight and Knight*, 2001].

Bacterial cultures from melted embryos yielded 12–535 colony forming units (CFU) embryo$^{-1}$. The assemblage of bacteria we isolated from hailstone embryos contained similar types of bacteria to those previously recovered from hailstones [*Harrison*, 1898; *Mandrioli et al.*, 1973; *Temkiv et al.*, 2012; *Šantl-Temkiv et al.*, 2013]. A subset (50; 2% of all CFU; $n = 2448$) (Figure S3 in the supporting information) was tested for the ability to nucleate ice. Within the subset of bacteria tested, we isolated one ice nucleation active organism related (>97% rRNA gene sequence similarity) to *Pantoea stewartii* (syn. *Erwinia stewartii*), a


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| Hailstorm | Embryo particle composition (%) | Clay minerals (particles embryo$^{-1}$) | Clay minerals in sampled embryos capable of freezing at ≥-19°C (%) |
|---|---|---|---|
| 30 June 2010 |  | Kaolinite (250) | 0.025 |
| | | Montmorillonite (250) | ND |
| 21 July 2010 |  | Kaolinite (210) | 0.021 |
| 29 August 2011 |  | Kaolinite (212) | 0.021 |
| | | Illite (212) | ND |
| | | Montmorillonite (212) | ND |



**Figure 3.** Particle composition found in hailstone embryos from three Rocky Mountain storms. Diagrams represent percent of particles analyzed by SEM-EDX within each embryo ($n$ = 50). The density of clay minerals per embryo was estimated by multiplying total particles within the embryo by the percent composition shown in the pie diagrams. The percentage of kaolinite particles capable of freezing at warmer than −19°C for each storm was determined by multiplying the density of kaolinite particles within each embryo by the fraction (0.0001%) of particles shown to be active at ≥ −19°C by *Pinti et al.* [2012]. ND = the percentage of ice nucleation active particles was not determined by *Pinti et al.* [2012] for these clay minerals.

known ice nucleation active organism [*Wallin et al.*, 1979; *Mergaert et al.*, 1993]. Our isolate possessed a freezing temperature of −10.5°C in our nucleation experiments (Figure 2), which is colder than the maximum nucleation temperature for *Pseudomonas syringae* (−2°C) [*Morris et al.*, 2014], but is similar to the in situ freezing point that we determined for embryos using stable isotopes of water (Figure 2). Because we only tested 2% of all the CFUs obtained during culturing of hailstone embryo meltwater, it is possible that more ice nucleation active isolates were present in the hailstones samples. The number of colony forming units obtained by our culturing efforts (519 CFU mL$^{-1}$) is similar to the high end of the range reported by *Šantl-Temkiv et al.* [2013] (20–500 CFU mL$^{-1}$) who studied meltwater from whole hailstones (embryos and outer layers) collected from a storm in Europe. Our examination of the small subunit ribosomal RNA gene sequences deposited in GenBank by *Šantl-Temkiv et al.* [2013] showed that the ice nucleation active bacterium (>98.5% 16S rRNA gene sequence similarity) *Pseudomonas fluorescens* represented 1.8% of all sequences they recovered from hailstones. Molecular evidence of ice nucleation active bacteria shows the potential for these bacteria to be active in nucleating hailstones, yet only a fraction of these bacteria may exhibit the ice nucleation phenotype. Our results reveal that ice nucleation active bacteria, albeit in low abundance, are present in hailstone embryos.


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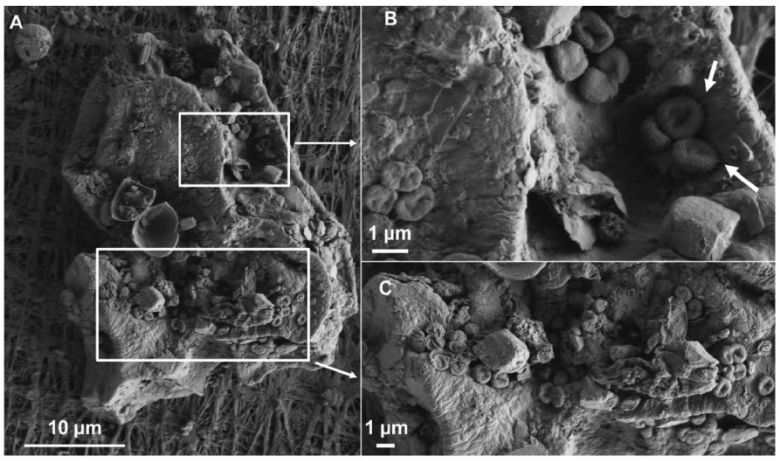



**Figure 4.** (a) A montmorillonite clay mineral particle from a 29 August 2011 embryo showing biological material on the surface. (b) Magnification of white box in Figure 4a. Arrows in Figure 4b reveal the close association between the mineral grain and biological particles, and show filamentous structures which attach the biological particles to the mineral surface. (c) Magnification of white box in Figure 4a showing the diversity of cell morphologies present on the clay grain surface.

### 3.4. Abiotic Ice Nucleation

Abiotic particles have recently been shown to be a component of total ice nuclei in clouds [*Atkinson et al.*, 2013; *Cziczo et al.*, 2013]. To explore the possibility of abiotic ice nucleation in hailstone embryos, we determined whether known abiotic ice nucleation active particles were present. Total (biotic + abiotic) particle concentrations in the hailstones we studied were $1.24 \times 10^4$, $1.04 \times 10^4$, and $1.05 \times 10^4$ particles ($\geq$1.5 µm) embryo$^{-1}$ for hailstones collected from the June 2010, July 2010, and August 2011 storms, respectively. These particle concentrations are similar to insoluble particle concentrations detected in the embryo region of the hailstones studied by *Rosinski and Kerrigan* [1969]. Talc and biotite, which are not known to be significant immersion ice nucleators [*Murray et al.*, 2012], were the most abundant abiotic particles in our study, collectively compromising 16 to 48% of the total particles contained within the embryos of the three storms (Figure 3 and Figure S1 in the supporting information). The sum of the three well-studied ice nucleation active clays illite, kaolinite, and montmorillonite [*Pinti et al.*, 2012] constituted only 2 to 6% of the particles sampled from embryos in our study (Figure 3). Only 0.0001% of kaolinite particles possess an ideal nucleation site and are ice nucleation active at temperatures warmer than −19°C [*Pinti et al.*, 2012]. Therefore, the embryos sampled in our study would require a >4000-fold increase in kaolinite particles to achieve densities likely to produce an ice nucleus at temperatures ≥ −19°C. These three clay types have long been considered the most efficient abiotic ice nuclei in the atmosphere [*Pruppacher and Klett*, 1998], but their ice nucleation activity is likely due to their overwhelming concentration in the atmosphere, especially during dust storms [*Pinti et al.*, 2012], rather than to their high-specific nucleation activity. The relatively low abundance of illite, kaolinite, and montmorillonite in our hailstone embryos indicates that these abiotic particles are unlikely to have initiated nucleation of the embryos in the hailstones we analyzed (Figure 3).

*Atkinson et al.* [2013] contended that potassium feldspar (K-feldspar) minerals are highly ice nucleation active, supporting a report that potassium-rich mica possesses warm ice nucleation activity [*Shen et al.*, 1977]. Atkinson and colleagues further argued that K-feldspar is a more efficient ice nucleator than clay minerals, in contrast to other studies showing that clay minerals are the most efficient abiotic ice nuclei [*Pruppacher and Klett*, 1998; *Pinti et al.*, 2012]. K-feldspar was a minor component of the particle analysis in the embryos we studied, constituting 2 to 10% of the total particles. August embryo samples had the highest concentration of K-feldspar. We used the average size of measured K-feldspar particles to calculate a


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total spherical equivalent surface area and concluded that the August embryos would need a 159-fold increase in surface area to have 1 $cm^2$ and possess 1 active nucleation site at −8°C [*Atkinson et al.*, 2013]. Despite the August storm having the best chance of K-feldspar nucleation, the stable isotope analysis showed these embryos froze within the temperature of freezing range of the biological ice nuclei reported in Figure 2. Although some abiotic particles are potentially ice nucleation active, they require a relatively higher concentration of particles in order to catalyze ice formation when compared to biological particles at the same temperature [*Després et al.*, 2012]. The dependence of high abiotic particle concentration on warm ice nucleation is because of their relatively low ice nucleating fraction [*Després et al.*, 2012]; this dependence on high surface area is applicable to specific clays and K-feldspar particles alike [*Atkinson et al.*, 2013].

Although abiotic particles can contribute to atmospheric ice nucleation, their contribution may be more commonly as a substrate for attachment of ice nucleating bacteria or biological particles rather than as direct nucleation sites [*Conen et al.*, 2011]. Our electron microscopic observations revealed that biological particles were attached to abiotic particles in hailstone embryos (Figure 4 and Figure S2 in the supporting information) and may have been the primary nucleation agents. The results of *Creamean et al.* [2013] support this contention, as they found >50% of precipitation residues contained biological material and dust. Others [*Zimmermann et al.*, 2008; *Ariya et al.*, 2009] have reported anomalously warm (> −19°C) ice nucleation temperatures for mineral particles but did not consider the possibility that attached bacteria or other biological ice nuclei could have been present on their surfaces.

## 4. Conclusions

The chronological structure of ice layers within hailstones provides a model system that allows direct study of the connection between biological ice nucleation, temperatures of nucleation, and the formation of precipitation. The present lack of instrumentation to measure cloud bottom properties and specific aerosols directly has led to a paucity of high-quality data to use in models of cloud dynamics, which are ultimately incorporated into global climate simulations. The major challenge of modeling aerosol effects on cloud properties centers on the nucleation region of the cloud [*Rosenfeld et al.*, 2014]. Specifically, our data reveal that biological particles are abundant within hailstone embryos and are active at warm temperatures; hence, they are critically important ice nuclei within the nucleation region of storm clouds.

## Author Contributions

A.B.M. designed research; A.B.M. performed research; D.L., W.B.L., J.E.D., and A.B.M. performed stable isotope analysis; A.B.M., J.E.D., J.C.P., and D.C.S. analyzed data; A.B.M., J.E.D., and J.C.P. wrote the paper; all authors approved the final draft.

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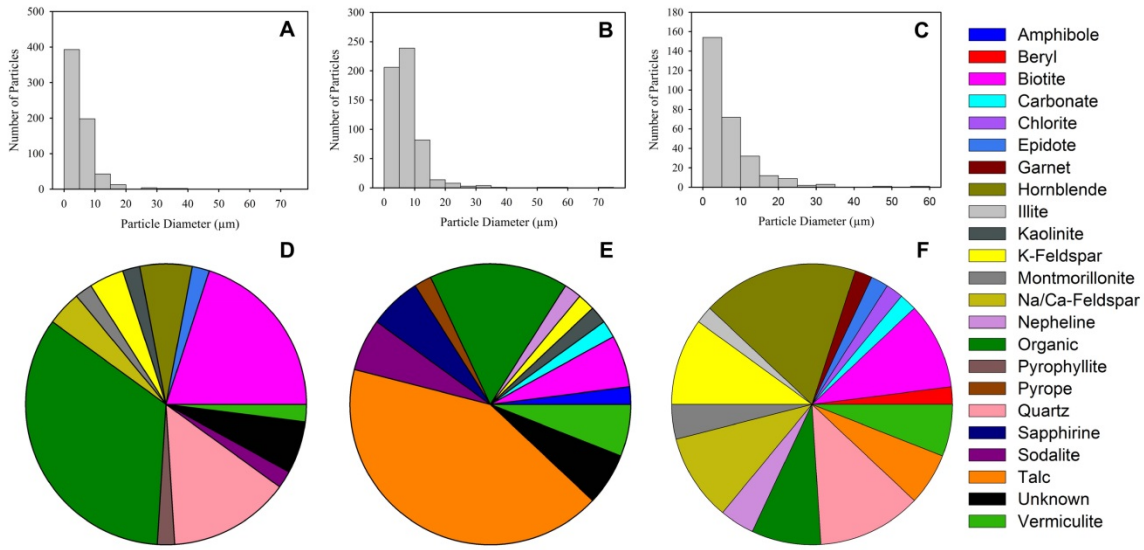

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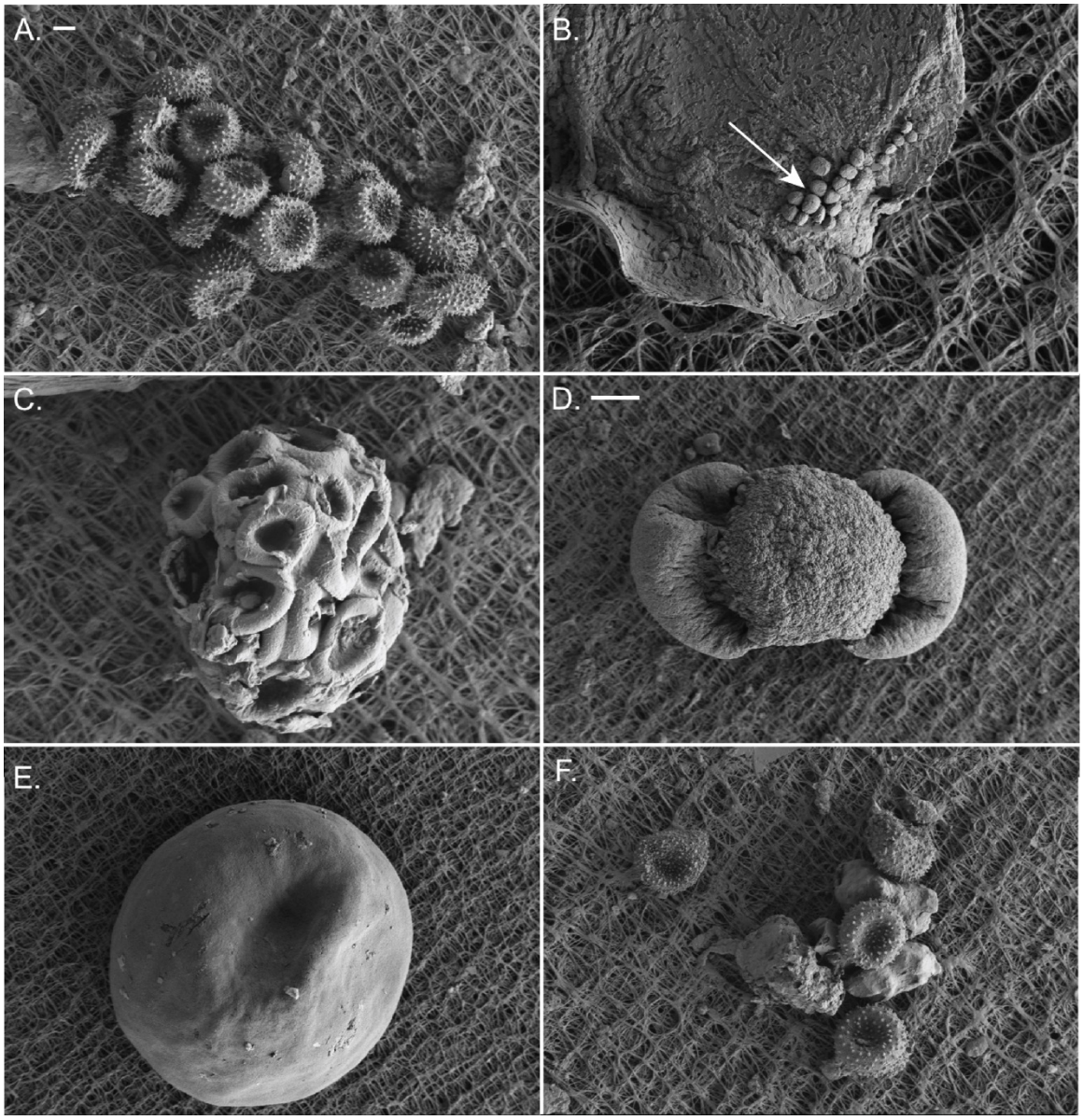
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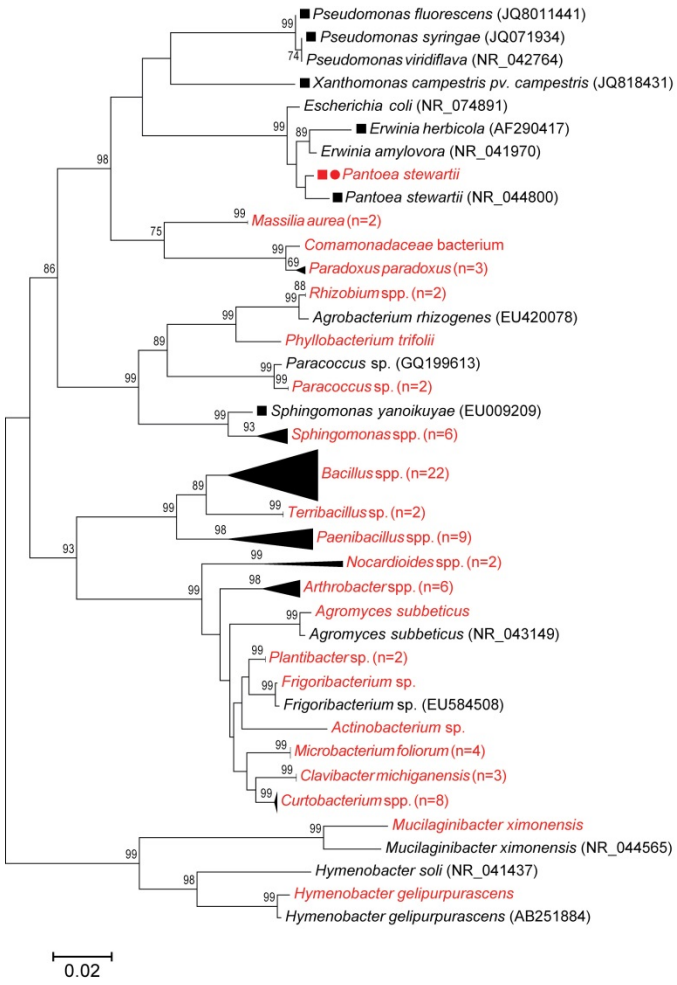
Supplemental Figures




**Supplementary Figure S1.** The particle size and identity from the embryos of the three hailstorms studied. **A**, Distribution of particle diameter for June 2010 hailstone embryos (n=659). **B**, Distribution of particle diameter for July 2010 hailstone embryos (n=560). **C**, Distribution of particle diameter for August 2011 hailstone embryos (n=286). **D**, Relative abundance of particles for June 2010 hailstone embryos (n=50). **E**, Relative abundance of particles for July 2010 hailstone embryos (n=50). **F**, Relative abundance of particles for August 2011 hailstone embryos (n=50).




**Supplementary Figure S2.** SEM images of biological particles found in hailstone embryos. **A**, Cluster of Urediniospores (a cereal and grass rust fungal spore) in June 2010 hailstone embryos (L. Szabo and Y. Jin, personal communication). **B**, Biological particles, indicated by arrow, attached to an inorganic particle, in August 2011 embryos. **C**, Unidentified biological particle in July 2010 embryos. **D**, A pollen grain, likely from the genus *Pinus*, was abundant in July 2010 embryos. **E**, Unidentified biological particle in August 2011 embryos. **F**, Cluster of Urediniospores (a cereal and grass rust fungal spore), similar to panel **A**, but found in July 2010 embryos as well (L. Szabo and Y. Jin, personal communication). Scale bar in panel A is 2μm and applies to panels **A**, **B**, and **C**. Scale bar in panel **D** is 10μm and applies to panels **D**, **E**, and **F**.




**Supplementary Figure S3.** The evolutionary relationships of bacteria isolated from hailstone embryos, known ice nucleation active bacteria, and related organisms. Taxa in red are those from hailstone embryos. Squares preceding taxon names are known INA organisms [*Wallin et al.*, 1979; *Hirano and Upper*, 1995] and circles indicate INA organisms identified in this study. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches only for values greater than 65%. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Scale bar represents 2 substitutions per 100 bases.

# APPENDIX C

## ACKNOWLEDGEMENTS CONTINUED

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## ACKNOWLEDGEMENTS CONTINUED

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