Microbial community dynamics from permafrost across the Pleistocene-Holocene boundary and response to abrupt climate change

by

Ann Osman Hammad

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ABSTRACT

Permafrost houses active microbial communities adapted to constant sub-zero temperatures and anaerobic conditions. These extreme conditions make permafrost an excellent archive for long-term DNA preservation. Recent studies in the laboratory have shown a shift in permafrost bacterial communities upon thaw while experimental warming of Arctic soils in field experiments, some exceeding a decade, have failed to show significant changes. This discrepancy may reflect that previous studies may have been biased because DNA from non-viable cells is well preserved in permafrost and may be PCR amplified. Here we distinguish between DNA originating from viable cells and total bacterial DNA extracted from permafrost. We examine the response of the active bacterial community composition to the rapid warming that accompanied the end of the Pleistocene, 11,700 years ago. This warming resulted in changes in soil edaphic properties, including pH, TOC and TN as a largely grassland ecosystem was replaced by early boreal forest. Our results show that the viable permafrost bacterial community is significantly different from total DNA and these two assemblages are structured by different environmental parameters. The corollary to these findings is that future climate change is unlikely to shift bacterial communities unless the warming is sufficient to change soil edaphic properties.

PREFACE

This thesis is an original work by Ann Hammad. No part of this thesis has been previously published.

The permafrost cores used in this research were previously collected by Matt Mahony, a former graduate student in Dr. Duane Froese's lab.

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

16S rRNA	16S ribosomal RNA
ACE	Abundance-based coverage estimators
AL	Active layer
^{13}C	Carbon-13
^{14}C	Carbon-14
cal BP	Calendar years before 1950
CCLM4	Consortium for Small-scale modelling climate Lokalmodell
CFU	Colony forming unit
CH_4	Methane
CiPEHR	Carbon in Permafrost Experimental Heating Research
CO_2	Carbon dioxide
CTC	tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride
D	Simpson diversity index
DEDP	Dead *E. coli*, Dead *P. aeruginosa*
DELP	Dead *E. coli*, Live *P. aeruginosa*
DMSO	Dimethyl sulfoxide
DON	Dissolved organic nitrogen
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
eGFP	Enhanced green fluorescent protein
EMA	Ethidium monoazide
gfpF	Green fluorescent protein forward primer
gfpR	Green fluorescent protein reverse primer

GISS	Goddard Institute for Space Studies
Gt	Gigatonne
GtC	Gigatonnes of carbon
H	Holocene
H'	Shannon diversity index
H1	Late Holocene permafrost sample, 8100 Cal yrs BP
H2	Mid Holocene permafrost sample, 9550 Cal yrs BP
H3	Early Holocene permafrost sample, 12150 Cal yrs BP
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
LB	Luria-Bertani
LEDP	Live *E. coli*, Dead *P. aeruginosa*
LELP	Live *E. coli*, Live *P. aeruginosa*
LLII	Lucky Lady II mine
LOI	Loss on ignition
LTER	Long-term Ecological Research
M	Molar
Ma	Megaannum
MAT	Moist acidic tussock
mM	Millimolar
MRPP	Multi-Response Permutation Procedure
MRR	Mark-release-recapture
N	Nitrogen

NaCl	Sodium chloride
Na_2HPO_4	Disodium phosphate
NH_4	Ammonium
nmol	nanomole
N_2O	Nitrous oxide
NO_3	Nitrate
NOAA	National Oceanic and Atmospheric Administration
OC	Organic carbon
OTU	Operational taxonomic unit
P	Pleistocene
P1	Late Pleistocene permafrost sample, 15100 Cal yrs BP
P2	Mid Pleistocene permafrost sample, 16000 Cal yrs BP
P3	Erly Pleistocene permafrost sample, 16200 Cal yrs BP
PBS	Phosphate buffered saline
PCF	Permaforst carbon feedback
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PMA	Propidium monoazide
pmoA	particulate methane monooxygenase enzyme
PO_4	Phosphate
ppb	Parts per billion
ppm	Parts per million
qPCR	Quantitative real-time polymerase chain reaction

RDP	Ribosomal Database Project
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
SOC	Soil organic carbon
SOM	Soil organic matter
TC	Total carbon
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
wt/vol	Weight per volume
ya	Years ago

CHAPTER 1: LITERATURE REVIEW

1.1: Introduction

Climate warming has a more heightened and accelerated effect on the Arctic compared to the rest of the globe due to rising concentrations of greenhouse gases that result in the increase in surface air temperatures lowering sea ice and snow cover (1). Cold environments such as glaciers, sea ice, and permafrost are particularly sensitive to this rapid climate warming since they are poised at the water/ice phase transition. Permafrost thaw can be particularly problematic. Although permafrost covers only about 20% of the global land surface, it has a carbon store of about 1700 Gt (gigatonne) (2). Nearly 50% of Canada's land area is underlain by permafrost, with a carbon store of nearly 200 Gt (3). While permafrost covers only 21% of North America, it accounts for ~ 60% of organic soil carbon in North American soils and nearly 25% of the world's total soil organic carbon (3, 4).

Though this permafrost-affected environment has not been exposed to extensive human influences, it is being affected by global warming due to human activities. The accumulation of greenhouse gases in the atmosphere is resulting in a much more prominent greenhouse effect, where more infrared radiation is absorbed and re-emitted by the Earth's atmosphere inducing a warming climate that causes permafrost to potentially thaw (5). Plant and animal material and microbial biomass that accumulated over the years are preserved in permafrost during cooling events. While plants and animals cannot survive being trapped in permafrost, recent studies reveal viable microorganisms capable of surviving for many hundreds of thousands of years within permafrost (6, 7). Recent studies show that increased temperatures due to global warming will result in the

warming and thaw of permafrost and the release of the stored organic carbon; therefore turning permafrost into a carbon source (3). The newly available organic carbon is utilized by permafrost microorganisms and is then released into the atmosphere as carbon dioxide (CO_2) and methane (CH_4) leading to positive feedbacks into the climate system that may accelerate future warming (8). The resulting changes in the polar environments will not only affect the landscape, but will also affect all macro- and microorganisms existing in these habitats.

Several studies have investigated the response of permafrost microbial communities to thaw but contradictory results have been observed. In some cases, shifts in the microbial communities were observed in laboratory simulated permafrost thaw (9-12) while *in situ* warming experiments showed slight or no shifts in the communities (13-16). The reason for this discrepancy is yet unclear, but one possible explanation is that previous studies were biased due to the "noise" of the preserved DNA in permafrost. The viable communities are changing, but this change might not be detected since they are a small component of the overall DNA being extracted and studied. To address this issue, we developed a protocol that allows the amplification of DNA from viable cells only. This protocol was used to study the composition of viable microbial communities in permafrost and how these microorganisms responded to soil chemical and physical (edaphic) variability resulting from the abrupt climate change that occurred across the Pleistocene/Holocene boundary.

At the end of the Pleistocene, about 10,000 years ago, the Earth's climate system abruptly warmed, leading to a cascade of changes that drove large-scale extinctions and reorganization of ecosystems. We know a considerable amount about the adaptations,

extirpations and extinctions of large mammals and plant communities at the Pleistocene/Holocene boundary, but relatively little about the changes that took place at the microbial level (8).

In spite of the development of several predictive global climate-change models, these models fail to take microbial potential and metabolism in cold environments into account leading to restricted predictive models. In order to develop more reliable models, permafrost microbial ecology must be better studied and understood. Several techniques and approaches have been used to identify permafrost microbial community members, microbial functions and their response to climate change. It is important to note that several variables such as permafrost age, depth and geochemistry must be taken into account when studying permafrost soils due to their ecosystem complexity (17).

The purpose of this research is to study how soil edaphic parameters affect extant microbial communities in permafrost and to use the changing community composition to determine how these communities adapted to abrupt climate change at the end of the Pleistocene. These changes could have important implications for understanding climate feedbacks and providing a compelling analogue for future changes in microbial communities with ongoing and future warming.

In the following sections of this literature review, I will describe global and arctic climate change, arctic permafrost and it's response to climate change, and permafrost microbiology and their response to climate change. I will review the known impacts of a past rapid climate change event, the Pleistocene/Holocene transition. I will also review methods for distinguishing between viable and non-viable microorganisms, a critical component of my M.Sc. thesis. Finally, I will provide an overview of my thesis.

1.2: Global and Arctic Climate Change

The frozen part of the Earth's surface is known as the cryosphere. These surfaces include snow, permafrost, ice on rivers and lakes, ice caps, glaciers, sea ice and ice sheets. Global climate change is likely contributing to changes observed in the Arctic cryosphere. As the climate warms due to atmospheric greenhouse gas accumulation, all of these parts of the cryosphere change by either melting or completely disappearing. Since 1980, the Arctic warmed by twice as much as the rest of the planet and it is expected to undergo the largest increase in temperature in the future (18).

Changes in the Arctic cryosphere will not only affect the Arctic region but will also affect the global climate. Arctic changes can affect the world's climate in two major ways: 1) overall, the Earth will have lower albedo (i.e. it will reflect less energy into space) due to snow and ice melt, thereby causing the planet's surface to warm; 2) methane and carbon dioxide emissions will increase due to increased organic matter decomposition in retreating permafrost and warmer soils and water bodies. These processes act as positive feedbacks by amplifying global climate change (18).

It is important to note that a significant amount of uncertainty remains regarding how fast the Arctic cryosphere will change and how these changes will not only impact the Arctic but Earth as a whole.

1.2.1: Surface Temperatures

According to NASA's Goddard Institute for Space Studies (GISS), the mean global surface temperature has risen by about 0.8°C since 1880 making 2012 the ninth warmest year since 1880. The average increase in global temperature by 2100 is predicted to be at least twice as much as it has been for the past 100 years with average

temperatures increasing by 1.1 to 6.4°C (5). This increase in temperature will be the greatest over land areas and high latitudes, with average Arctic temperatures increasing by twice the global average rate (5).

1.2.2: Carbon Dioxide and Methane Concentrations

Carbon dioxide is the dominant anthropogenic greenhouse gas contributing to global warming. Warming surface temperatures might cause a reduction in the uptake of atmospheric carbon dioxide by land and ocean resulting in a positive feedback for atmospheric carbon dioxide (5). Since high latitudes experience a higher increase in average temperatures, this positive feedback will be much more pronounced in the Arctic with carbon dioxide concentrations higher than average. According to the National Oceanic and Atmospheric Administration (NOAA), the average carbon dioxide level for February 2013 was ~395 ppm. By 2100, atmospheric carbon dioxide concentrations are expected to fall in the range from 700 to 1000 ppm, depending on which model is being used (5).

Methane is also a very important greenhouse gas and since it is 25 times more potent as a greenhouse gas than carbon dioxide, increases in methane emissions is cause for concern. The natural range of atmospheric methane concentrations over the past 650,000 years is 320-790 ppb (5). In the early 1990s, the global atmospheric concentration of methane increased from ~715 ppb to 1732 ppb, increasing to ~1774 ppb in 2005 and ~1803 ppb in 2011 (5, 19). According to the NOAA, global average methane concentrations have started to increase in the Arctic since 2007 due to warm temperatures. The increase in global methane was significantly faster during 2014 than during 2007-2013.

1.3: Arctic Permafrost

The Arctic region covers more than 7 million km^2 and includes parts of Canada, Russia, Alaska, Greenland, Iceland and Scandinavia (20). The Arctic Circle is known for its long, dark, tremendously cold winters and short, cold summers that sometimes only last for 6 weeks per year. The coldest month in the Arctic is February, with surface air temperatures ranging from -20 to -40°C. The warmest month, July, has surface air temperatures ranging from 3-10°C. The Arctic has low total annual precipitation between 60-160 mm, mostly in the form of snow (21).

Since 1970, the upper few meters of permafrost have completely disappeared on peatlands in Scandinavia, Russia and Canada. Other areas with shallow layers of permafrost have completely thawed. The southern limit of permafrost has moved northward by 130 km in Quebec over the last 50 years, and by 30-80 km in western Russia between 1970 and 2005 (18). By the mid and late 21st century, models predict that permafrost near the surface in the Arctic will shrink by 15-30% and 37-81% respectively resulting in the thawing of the upper few meters of the ground (19, 22). Over the 21st century it is predicted that thawing permafrost will release 50-250 gigatonnes of carbon (GtC) due to the release of CO_2 and CH_4 (19).

Permafrost covers about 24% of the Arctic (20); it is ground (soil, rock or sediment) that has been frozen for at least 2 years (23); however, most permafrost is far older, with the oldest permafrost, found in Antarctica, dated to ~20Ma (24) and the oldest in North America dated to ~740,000 ya (25). Within the permafrost, frozen water holds the soil, rock or sediment together forming an impermeable ice layer that shapes the landscape (18). Permafrost regions usually occur at high latitudes and high elevations

with some areas more than 1000 m deep; the thickness ranges from 100-500 m in North America, and greater than 500 m in Siberia (21, 26).

Permafrost is prominently affected by seasonal temperature variations. When the air temperature rises above 0°C in the summer, a thin layer on the ground surface, known as the active layer (about 30-60 cm thick), seasonally thaws and the temperature of the top 10 m of ground (composed of the active layer and upper permafrost) increases by more than 1°C (21, 23, 27). The boundary between the active layer and permafrost is known as the permafrost table and it acts as a physical and biogeochemical barrier that significantly reduces the exposure of permafrost to surface water (27).

Arctic permafrost has a mean annual temperature of -10°C with a temperature range from 0°C to -17°C (27). Arctic permafrost has low water, nitrogen, potassium and phosphorus contents and variable pH due to the different origins of the soils (parent material) (21, 28-30). In addition, Arctic permafrost has carbon stores that range between 4 - 110kg C m^{-2} (31, 32). Permafrost oxygen and nitrogen concentrations are similar to atmospheric concentrations, while methane and carbon dioxide are present at concentrations much higher than in the atmosphere (27, 33). The high concentrations of methane and carbon dioxide are mainly due to the microbial degradation of soil organic carbon in permafrost, which will be further discussed in the upcoming sections.

1.3.1: North American Permafrost

Based on the percentage of underlying permafrost, the North American permafrost region is divided into four zones: the continuous permafrost zone (90-100%), the discontinuous permafrost zone (50-90%), the sporadic permafrost zone (10-50%) and the isolated patches permafrost zone (0-10%) (34). These permafrost zones cover three

main ecological regions: the Arctic (north of the Arctic tree line), the Subarctic (composed of coniferous, open canopy forests) and the Boreal (composed of coniferous or coniferous and deciduous, closed canopy forests) (3).

The soils of the permafrost region of North America are a net sink of 11 metric tonnes of carbon per year and contain 213 Gt of organic carbon, which accounts for 61% of the carbon in all North American soil (4). Approximately 58% of the soil in the North American permafrost region is composed of permafrost-affected soil (soil composed of organic or mineral material with permafrost within 1 or more meters below the surface (23)), of which 83% is permafrost-affected mineral soils and 17% is permafrost-affected organic soils (peatlands) (3). Although organic soils cover a smaller area than mineral soils, they contain about 54% of the region's soil organic carbon (3).

1.3.2: Permafrost and Global Warming

Permafrost contains more than twice the amount of carbon currently in the atmosphere (2). It is predicted that global warming could thaw 25% of the Arctic permafrost by 2100 (10). When the surface temperature increases due to global warming, permafrost-affected mineral and organic soils will thaw resulting in the release of the sequestered organic matter. This will make it more accessible for aerobic and anaerobic microbial decomposition. Furthermore, this will increase the positive feedback of global warming by the release of greenhouse gasses such as carbon dioxide and methane (3).

Several studies have been carried out to observe how the permafrost microbial community will respond to thawing. Metagenomic analysis of permafrost that has been incubated at 5°C for 1 week was used to generate a conceptual model of carbon and nitrogen cycling when permafrost thaws. In this model, methanogenesis occurs in

permafrost and active layer soils at slow rates by cold-adapted methanogens. The methane accumulates in permafrost; when the permafrost thaws, some of that methane is released into the environment and some is consumed by methanotrophs. In addition, as the permafrost thaws, the trapped organic carbon becomes available for microbial degradation and methanogenesis, resulting in the production and release of carbon dioxide and methane into the environment (9, 35, 36). A twelve year incubation study of permafrost soil from Greenland showed a loss of carbon due to microbial decomposition from 9-75% of the initial content, depending on drainage following thawing (37). A three-pool carbon model generated based on this study showed that over the next 50 years of incubation, permafrost carbon pools may lose up to 92% of the initial carbon content under aerobic conditions and 6% under anaerobic conditions (37). Permafrost-affected soils from across the Arctic also showed a similarly high potential for organic carbon microbial degradation upon permafrost thaw, with permafrost carbon pools losing up to 76% of the initial carbon content over the 40 years of incubation (38). Model forecasting by CCLM4 showed that over the next century 24-44% of nitrogen stores in permafrost will be degraded upon thaw depending on the warming scenario followed (39).

1.3.3: Permafrost Carbon Feedback

Terrestrial systems act as carbon sinks and sources, with terrestrial permafrost being one of the largest carbon reservoirs on Earth that is highly susceptible to global warming (40). Permafrost in northern latitudes contain about one third of the global soil carbon (41). The top 3 meters of permafrost contain half of the frozen organic matter and the remaining is in extremely localized deposits that can spread down to 30 meters deep (2). Organic carbon stores in permafrost originate from the ecosystem that occupied the

territory at the time of freezing. Flood plain sedimentation, dust deposition and peat development caused the burying and freezing of plant and animal remains along with other organic material into permafrost during and since the last ice age (40, 42, 43). This burial process is further accelerated by cryoturbation, the vertical mixing of soil during freeze/thaw cycles (42). Since permafrost temperatures are very low, plant and animal remains within these sediments are not decomposed and so they slowly build up to contribute to permafrost's carbon content (40).

The amplification of anthropogenic warming due to carbon emissions from permafrost thaw is known as the permafrost carbon feedback (PCF). When permafrost thaws due to increasing temperatures, the trapped organic matter will be released and consumed by permafrost microorganisms, releasing CO_2 and CH_4 into the atmosphere, in turn amplifying global warming (40, 44). An analysis of estimates indicates that PCF will produce 120 ± 85 Gt of carbon emissions by 2100 increasing global temperatures by 0.29 ± 0.21 °C (i.e. 7.8 ± 5.7%) (45).

Methane. Precipitation and thawed snow cause flooding of permafrost soils and the accumulation of water just above the permafrost table. This water table (where the water accumulates) results in anaerobic conditions where the degradation of soil organic matter (SOM) can only be carried out by anaerobic microorganisms. The presence of carbon dioxide, hydrogen and/or acetate, products that are derived from SOM degradation, are essential for methane production (methanogenesis) by methanogenic Archaea (46). Since microbial fermentation of carbon is much slower than oxidative respiration, organic matter accumulates due to persistent anaerobic conditions and low temperatures in permafrost soils (46).

While some of the methane produced by the methanogenic Archaea in the water table escapes into the atmosphere, the majority diffuses to the upper oxic soil horizons of permafrost soils where it is oxidized by aerobic methane-oxidizing bacteria (46). Between 76 and 90% of the produced methane is oxidized by these methanotrophic bacteria, making biological oxidation a major methane sink in permafrost-affected soils (47).

Methanogenesis also occurs at very slow rates in permafrost by cold-adapted methanogens. Over time, this methane accumulates in permafrost. When permafrost thaws some of this trapped methane is released into the atmosphere and some is consumed by bacterial methanotrophs (9).

Carbon dioxide. While photosynthesis plays an important role in turning permafrost into a carbon dioxide sink, microbial activity in the aerobic active layer, just above the water table, turns permafrost into a source of carbon dioxide. Under aerobic conditions, SOM is respired into carbon dioxide by aerobic soil microorganisms. In addition, methane oxidation by methanotrophic bacteria also results in the release of carbon dioxide from permafrost (46).

Long-term effects of permafrost thaw are not yet understood and we are unable to predict how microbes will use the newly available soil organic matter released by permafrost thawing (17, 48). If most of the organic matter stored in permafrost is challenging to degrade, then this recalcitrant pool of carbon will slowly encourage microbial growth and greenhouse gas production. On the other hand, if the carbon is more labile (e.g. frozen plant litter), the carbon will be readily available for rapid

microbial degradation (48). A recent study showed an increase in CO_2 release from deeper permafrost thawing (44). Permafrost warming will not only affect soil temperature, but will also affect soil hydrology. Soil moisture will increase due to thawing ice in permafrost, resulting in anaerobic conditions favoring the production of CH_4 over CH_4 oxidation (49). Permafrost with low ice content will show the opposite results, with possible higher methanotrophic activity and lower methanogenic activity.

1.3.4: Permafrost Microorganisms

Microorganisms in permafrost are capable of surviving over extended time periods in an extreme environment with continuous subzero temperature, low water activity, low nutrient availability and constant exposure to ground radiation (33, 50). Despite these conditions, a significant abundance and diversity of microorganisms are present in permafrost samples from across the Arctic (50). Viable microorganisms have even been purported to survive for hundreds of thousands of years within permafrost (6, 7). The total number of microorganisms found in permafrost can be as high as 10^9 cells per gram, and the number of viable microorganisms ranges between $10^2 – 10^8$ cells per gram (27, 51-53).

Several studies of the microbial composition of Arctic permafrost have shown the presence of a vast bacterial diversity, much higher than the observed Archaea or fungal diversity. 16S rRNA gene high-throughput sequencing has shown that permafrost contains a wide variety of microbial metabolic groups, including methanogens (acetoclastic and hydrogenotrophic), methanotrophs, endospore formers, cellulose degraders, aerobic and anaerobic heterotrophs, sulfate and iron reducers, and nitrifying and nitrogen fixing bacteria (17, 20, 27, 54).

Highly variable microbial diversity and composition have been found in different permafrost sites, mostly due to different soil and ice conditions that affect where microorganisms can survive in the permafrost. Microorganisms can survive and actively grow in particular areas within permafrost (17). Cryopegs are ancient, closed, aquatic ecosystems found in permafrost. These brine lenses have high salt concentrations that allow them to remain in a liquid state at below freezing temperatures enabling them to act as a habitat for permafrost microorganisms (55). Microbial metabolism was detected in cryopegs at temperatures as low as -15°C; in addition, most probable number analysis detected sulfate reducers, methanogens, acetogens, aerobic and anaerobic heterotrophs (55). Microbial life is also supported in liquid brine veins that form in permafrost ice wedges. Ice wedges are ancient ice that forms due to repeated frost cracking cycles that result in the infiltration of meltwater, snow, and soil into the frost cracks. Brine veins are lines of liquid water with high salt concentrations that form within ice wedges allowing microorganisms to concentrate from the surrounding freezing water (56). Aerobic and facultatively anaerobic bacteria were isolated from a 25,000-year-old permafrost ice wedge; isolates belonged to *Actinobacteria*, *Bacilli* and *Gammaproteobacteria* classes (56). Another habitat within permafrost that can support microbial life is frost boils. Frost boils are small patches of barren or lightly vegetated soil that form due to frost heave, the upward protrusion of soil by water freezing in the soil (57). Depending on the carbon and nitrogen concentrations and the amount of drainage, a heterogeneous microbial composition can be found in frost boils (57).

The use of culture-independent over dependent techniques to study permafrost microbiology has been an ongoing debate. Both methods have indicated that permafrost

accommodates diverse and novel microbial communities (50). Although a wide range of microorganisms have been discovered in permafrost using cultivation-dependent techniques, cultured isolates represent a small proportion of the microbial community. Approximately 0.1-10% and 0.001-0.01% of direct cell counts can be recovered during cultivation of Arctic and Antarctic permafrost, respectively (50). Cultivation-dependent techniques are insufficient to characterize the viable microbial community since simply finding that an organism is viable when cultured does not necessarily imply that they are active, abundant or significant in situ (58). In several cases, cultivation-dependent techniques have shown different microbial communities with differing dominance from cultivation-independent ones (59-61). These differences could be due to biases introduced by DNA extraction and amplification, difficulties in cultivating permafrost bacteria, or the presence of non-viable cells. To address these shortcomings, cultivation-independent techniques must also be performed but that still doesn't mean that cultivation-independent techniques don't have their own limitations. A major challenge of using cultivation-independent techniques to study microorganisms in permafrost is the low biomass. Different types of permafrost seem to have a very wide range of bacterial abundance with counts ranging from 10^5-10^8 cells per gram of dry mass (17, 62). Another drawback of studying microorganisms in permafrost is the fact that permafrost's freezing temperature is ideal for preserving DNA for long periods of time; therefore, not all extracted DNA is coming from active or viable cells.

1.3.4.1: Microbial activity in permafrost

Some microorganisms have been shown to actively metabolize in permafrost, albeit at very low rates (17). Exceptionally low microbial activity in permafrost could still

enormously influence the geochemistry and geophysics of perennially frozen ground on a geological time scale of 1,000 to 10,000 years (63). In active layer soils, microbial activity could be essential for biochemical transformations that result in the reestablishment of soil fertility (63).

Several environmental factors constrain microbial activity at sub-zero temperatures, including low water availability, low temperature (i.e. low kinetic energy of reactants), gamma radiation causing long-term damage, and low soil porosity (affecting diffusion rates within frozen soils) (63). Despite these obstacles, some microorganisms survive in permafrost; two ecological strategies are believed to explain their ability to survive these harsh conditions. One strategy is entry into a dormant resting state, e.g. a spore, which makes the cells resistant to the multiple insults of a cold environment. A second strategy is to metabolize at a minimal rate (64). It has been argued that having a slow metabolism is a much better strategy to survive over long time periods (decades to centuries) in frozen environments such as permafrost, since dormant cells have no active DNA repair, resulting in the degradation of their genomes with time (6).

Recent studies have shown that most permafrost microorganisms are capable of survival, maintenance and even growth at sub-zero temperatures (17). Under carefully controlled laboratory conditions, Panikov *et al.* measured CO_2 and $^{14}CO_2$ release from ^{14}C-glucose amended and intact permafrost at temperatures ranging from 0 to -33°C for a year (65). CO_2 production rates were found to exponentially decline with temperature but remained positive at -39°C. Microbial activity in Alaskan permafrost was also detected using stable isotope probing to detect the incorporation of ^{13}C-acetate into bacterial DNA (66). About 80% of the detected operational taxonomic units (OTUs) synthesized ^{13}C-

labeled DNA at temperatures ranging from 0 to -20°C. Interestingly, phylogenetic analysis of the ^{13}C-labeled 16S rRNA gene revealed that some bacterial community members were active (able to grow/synthesize DNA) across the full range of temperature tested, while others were only active at sub-zero temperatures. These 'sub-zero active bacteria' were members of the *Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes* and *Proteobacteria* phyla (66). Mineralization of [1-^{14}C] acetic acid and [2-^{14}C] glucose was measured in permafrost from the Canadian Arctic from Eureka, Ellesmere Island, Nunavut (67). Mineralization was detected at temperatures as low as -15°C (near ambient permafrost temperature) (67).

Metabolic activity of Siberian permafrost collected from the Kolyma-Indigirka Lowland was determined by measuring the incorporation of ^{14}C-labelled acetate into lipids. Acetate incorporation curves were observed to be similar to typical growth curves, indicating that acetate incorporation could be considered as an indirect measure of growth. Metabolic activity of Siberian permafrost bacteria was observed at temperatures as low as -20°C (68). In addition, metabolic activity of hydrogenotrophic and acetoclastic methanogens was measured by monitoring methanogenesis. Methane production was observed to occur in Kolyma lowland permafrost over a wide range of temperatures (5°C to -16.5°C) (33). The highest rate of methane production was detected at 5°C; the rate of methane formation was reduced by 2-fold as the temperature was lowered to -2°C; a 100-fold reduction was noted as the temperature fell from -2°C to -16.5°C (33). It is important to note that although methanogenesis was occurring at a very low rate at temperatures as low as -16.5°C, methane was being produced nonetheless.

Methanotrophic activity was also observed in another study where activity was detected in Siberian permafrost by measuring the oxidation of $^{14}CH_4$ to $^{14}CO_2$ at -5°C. The highest rate of methane oxidation was observed in the upper permafrost boundary that occasionally thaws in warm years. On the other hand, deep permafrost layers (10,000 to 200,000 years old) showed methane oxidation rates that were one to two orders of magnitude lower than the upper permafrost boundary, but the rate of oxidation was still detectable (69). It is important to note that although several permafrost isolates have been shown to synthesize DNA and proteins at temperatures as low as -15°C, microbial growth thermodynamic models predict that metabolism can occur at temperatures as low as -40°C (70). Based on previously discussed studies, various microbial activities ranging from acetotrophy to methanogensis have been observed in permafrost ranging in temperature from 0°C to -15°C, with some indications that there may be microbial activities at even lower temperatures. It also appears that microbial activity may drop exponentially with permafrost age and temperature; thus, newer, warmer permafrost will have much higher activity than older, colder permafrost.

1.3.4.2: Microbial abundance

Culturable CFU counts for aerobic heterotrophic bacterial cells in permafrost from Eureka, Nunavut, Canada were 3.6 x 10^8 CFU per gram (67). Siberian permafrost also has very similar numbers for aerobic heterotrophic bacteria, with 10^7 – 10^8 cells per gram of dry mass; however, the viable counts only accounted for 0.1 to 10% of that value (71). An aerobic viable bacterial cell count of 6.6 x 10^2 CFU/g was obtained from acidic wetland permafrost in Nunavut using culturing techniques (72). Based on these studies, it seems like the bacterial abundance in permafrost clearly differs from one to another.

These differences could be mainly due to soil physiochemical characteristics along with several geographical biases.

Quantitative PCR of 16S rRNA gene copies of Canadian Arctic permafrost showed archaeal counts of 1.5 x 10^2 16S gene copy numbers per gram of soil (62). In permafrost from an acidic wetland, 2.8 x 10^4 16S archaeal gene copy numbers per gram of soil were detected (72). In both cases, the abundance of Archaea is orders of magnitude less than that of Bacteria but that could be due to lower 16S rRNA gene copy numbers in archaeal genomes than in bacterial genomes (73). This contrasts with more temperate soils, where Archaea are generally approximately 10% of the prokaryotic cells (50).

1.3.4.3: Microbial diversity

Bacterial diversity

Bacterial diversity is higher in permafrost than archaeal diversity. The culturable bacteria isolated from Arctic permafrost belonged to phyla *Firmicutes, Actinobacteria, Proteobacteria, Acidobacteria* and *Gemmatimonadetes*. In Canadian Arctic permafrost, the culturable bacteria isolated from permafrost were dominated by the spore-forming *Firmicutes*; the majority of the isolates were found to be psychrotolerant (about 83%) with some halo- and acidotolerant isolates as well (60). Genera cultured from Canadian Arctic permafrost include *Arthrobacter, Brachybacterium, Kocuria, Micrococcus, Rhodococcus, Flavobacterium, Staphylococcus, Clostridium, Virgibacillus, Pedobacter, Bacillus, Paenibacillus, Planococcus, Sporosarcina,* and *Pseudomonas* (60, 67, 72, 74, 75). The 16S rRNA gene clone libraries and quantitative PCR (qPCR), on the other

hand, displayed the presence of a much more diverse bacterial community with clones belonging to numerous phyla; however, the phylotypes were dominated by sequences related to *Actinobacteria* and *Proteobacteria* and *Bacteroidetes* with the majority of the *Proteobacteria* observed belonging to the *Betaproteobacteria* class(60, 62, 67). Metagenomic analysis and qPCR showed that Gammaproteobacterial methanotrophs were more dominant than Alphaproteobacterial methanotrophs; and detected Gammaproteobacterial methanotrophs related to *Methylococcaceae* (62). Interestingly, the majority of permafrost isolates are psychrotolerant rather than psychrophilic, which could imply their adaptation to the slightly warmer temperatures occurring in the Arctic (60).

In Siberian permafrost sediments (5,000 to 2-3 million years old), cultivation-dependent and -independent techniques revealed the presence of high and low G+C Gram-positive bacteria. The major phyla observed were *Firmicutes, Actinobacteria* and *Proteobacteria*; similar to those observed in the Canadian Arctic. According to several studies, bacterial genera cultured from Siberian permafrost include: *Arthrobacter, Cellulomonas, Microbacterium, Micrococcus, Promicromonospora, Rhodococcus, Streptomyces, Flavobacterium, Bacillus, Exiguobacterium, Paenibacillus, Planococcus, Planomicrobium, Sporosarcina, Aeromonas, Myxococcus, Psychrobacter,* and *Pseudomonas* (61, 76).

Based on these studies, cultivation-dependent techniques showed a much lower bacterial diversity and composition compared to cultivation-independent techniques. Permafrost from different regions shows highly similarly diverse

bacterial communities. It is important to note that similar bacterial compositions are observed in the majority of the permafrost samples, with some exceptions displaying the presence of bacteria unique to permafrost of a particular region. Based on previous studies, the commonly occurring bacterial phyla in Arctic permafrost include *Firmicutes, Actinobacteria, Proteobacteria, Chloroflexi, Acidobacteria, Bacteroidetes* and methanotrophic bacteria such as *Methylobacter* spp., *Methylocella* spp., *Methylomonas* spp., *Methylosphaera* spp. and *Methylocapsa* spp. (17, 51, 60, 62, 77-79). The dominance of these phyla suggests that they are capable of survival in cold conditions and are able to revive and grow at warmer temperatures. For instance, several Firmicutes members such as the *Bacillaceae* and *Clostridiaceae* families form spores aiding their survival in dire conditions (67). In addition, *Actinobacteria* members have a high GC content (65-69%) that has been shown to reduce DNA damage due to freezing temperature and high salinity and to maintain DNA repair (6). Uncharacterized or canadidate phyla have also been found to be abundant in permafrost. Uncharacterized Chloroflexi representatives were found to be ubiquitous in permafrost (9, 72, 78) as well as the candidate phylum AD3 that was observed in an Alaskan boreal forest in high abundance (78).

Archaeal Diversity

Not a lot of studies have been done on permafrost archaeal communities using cultivation-independent techniques. The majority of cultivation-independent characterizations of archaeal diversity are from Canadian high Arctic permafrost. The archaeal phyla *Euryarchaeota*, *Crenarchaeota* and *Thaumarchaeota* were

identified in Arctic permafrost (9, 35, 60, 67, 78). The recovery of viable methanogens from permafrost suggests that permafrost is an adequate environment for methanogenesis (50).

Permafrost from the Canadian Arctic was dominated by the *Euryarchaeota* and/or *Crenarchaeota* phyla with the majority of the phylotypes related to halophilic Archaea (60, 62, 67, 72). Metagenomic analysis revealed sequences that were related to *Methanomicrobia, Methanobacteria, Methanococci* and *Methanopyri* (62). Siberian permafrost was found to be mostly composed of methanogenic species with a wide diversity that comprised *Methanomicrobiaceae, Methanosarcinaceae,* and *Methanosaetaceae* families (80, 81).

1.3.5: Permafrost microorganisms and climate change

Recently, permafrost microbial ecology has been intensively studied in order to address concerns about the impact of permafrost thaw on microbial decomposition of trapped organic matter, that will possibly lead to the release of greenhouse gases (CO_2, CH_4, and N_2O) (17).

Not a lot of work has been done to study permafrost microbial communities and very little is known about these microbial communities' response to permafrost thaw. Microbial communities have been predicted to rapidly respond to permafrost thaw due to the availability of trapped organic matter and vast carbon stores for microbial degradation (9). Mackelprang *et al.* used a deep metagenomic sequencing approach along with methane gas release/oxidation. After only 2 days of permafrost thaw at 5°C, a burst of methane was observed that originated from methane trapped in frozen permafrost. This burst was followed by methane oxidation by methanotrophic bacteria indicating dynamic

methane levels in thawing permafrost. Metagenomic sequencing suggested that the transition from a frozen to a thawed state resulted in rapid shifts in microbial community composition and functional gene abundances. Noteworthy changes that were observed during permafrost thaw were the significant increase in Actinobacteria, bacterial type II methanotrophs, the particulate methane monooxygenase enzyme (*pmoA)* and nitrogen cycling genes.

While the active layer metagenomes were similar to each other before and after thaw, the frozen permafrost samples were dramatically different before thaw but they then rapidly converged to look more like the active layers. The active layers and permafrost samples had significantly different functional gene composition with discrepancies in some major metabolic pathways such as amino-acid transport, anaerobic respiration, energy metabolism, nitrogen fixation and oxidative phosphorylation (9).

Previous studies also undergoing laboratory simulated permafrost thaw have shown a shift in the microbial community, phylogenetic and functional gene abundances, exoenzyme activities, transcriptional response and metabolic diversity (9-12). This rapid shift observed in the microbial community composition of laboratory simulated permafrost thaw could have been due to either an actual shift in the microbial community structure, or due to the co-extraction of preserved DNA from dead cells. This non-viable DNA could be rapidly degraded by low abundance, viable microorganisms after thaw.

On the other hand, *in situ* warming experiments have shown only minor changes in microbial community composition (13-16). In previous field warming experiments, either a strong lag in the response of soil microbial communities was observed (16) or changes in microbial communities were accompanied by parallel changes in soil edaphic

properties (primarily pH; (82, 83)) (Table 4). To resolve this discrepancy, one can study how microbial communities responded to rapid climate change in the past, which will in turn provide insight into the changes that the soil community will experience in this modern era of climate change. The most recent event of rapid climate change occurred ca. 12,600-10,200 cal BP at the Pleistocene-Holocene boundary.

1.3.5.1: Beringia in the Quaternary

Quaternary history has been determined by events that occurred a very long time ago. Eight million years ago, the Indian plate traveling north impacted the southern edge of Asia driving up the Himalayan-Tibetan plateau that affected the atmospheric circulation in the Northern Hemisphere (84, 85). Atmospheric and oceanic circulation changed even more due to the uplift of mountains and the surfacing of the Isthmus of Panama (84, 86). In addition, abnormalities in the earth's orbit and the way it vibrates as it rotates on its axis resulted in periods of more or less solar radiation than typical (84, 87). These changes along with the solar radiation cycles caused the onset of glaciations in the Northern Hemisphere (84). The Quaternary is a geological time period that is subdivided into the Pleistocene (1.7 million years ago) and postglacial Holocene (11,700 years ago) epochs (84, 88). At the end of the Pliocene, the Earth's climate became more sensitive to CO_2-based radiative forcing, ending the warm Pliocene epoch and initiating the cold Pleistocene epoch (89).

The earth's climate during the Pleistocene cycled from glacial conditions, with ice caps forming over North America and northern Europe, to interglacial conditions with limited ice cover and a warm climate (84). The glacial and interglacial stages are further subdivided into stadials (cold periods shorter than glacials) and interstadials (mild periods

shorter than interglacials) (90). It has been estimated that there were between 30 – 50 glacial/interglacial cycles throughout the Quaternary period (90). In Beringia, the region from eastern Siberia to central Yukon, summer temperature for the last glacial period was about 6.4°C colder and interglacial period was about 3.5°C warmer than present temperatures (91). During glacial periods, Beringia, particularly the Yukon, was arid with very low winter snow depth (84); soil was beneficial for grazing animals with high mineral content, high pH, low organic matter, surface salts, well drained, rapid nutrient turnover and deeper permafrost (92, 93). On the other hand, interglacial soil had lower pH, was poorly drained, had low nutrient turnover, and had high insulating organic matter resulting in colder soil and shallower permafrost tables (93). A megafauna mostly composed of grazers and predators such as the short-faced bear and lion existed in the Yukon portion of Beringia during the last glaciation in the Pleistocene (84). Several studies have shown that the Yukon was treeless and vegetated by Arctic-steppe, steppe-tundra, or herbaceous tundra during glacials and by mosses, sedges and shrubs during the interglacials (84, 93).

At the end of the Pleistocene, about 13,000 years ago, the Arctic suddenly plunged back into glacial conditions known as the Younger Dryas stadial (90). The Pleistocene-Holocene transition began from the Younger Dryas. Previous records showed that this return to cold conditions took place in less than 100 years with some areas being characterized by cool-moist conditions while other by cool-dry (94-98). The warming phase that occurred at the end of the Younger Dryas, about 11,000 years ago, lasted only 1,300 years, causing central Greenland temperatures to increase by 7°C or more in only a few decades (90). Concurrently with the end of the Younger Dryas, numerous large

animals went extinct in the Beringia region, including giant ground sloths, camelids, horses, mammoths, ancient bison and mastodons (99).

Following the abrupt end of the Younger Dryas, the Arctic entered the Holocene epoch. During the Holocene, Beringia experienced thousands of years of conditions that were drier, with 40% less moisture, and warmer than today (90, 100). Between 11 – 10 thousand years ago, at the end of the Pleistocene and the beginning of the Holocene, precipitation increased along with the temperature (93). During the early and mid Holocene, summers in most of the Arctic were 1-2°C warmer than present (90). These changes resulted in the recession of glaciers, lowering of river levels, shrinking of vast lakes, desiccation of land, spread of birch shrub tundra along with the disappearance of the mammoth steppe and further the extinction of the Pleistocene megafauna (101). Holocene climate was much more stable and warmer than the late Pleistocene although there were numerous cold intervals that lasted for centuries in the mid and late Holocene (102).

Palaeoclimatic studies based on globally distributed proxy records have shown several global abrupt climate changes throughout the Late Quaternary. Such a period may have transpired in 50 years or less during the late Pleistocene to early Holocene transition, leading to highly dynamic and unstable landscapes (103, 104).

Understanding the sinks and sources of greenhouse gases, such as methane, in thawing permafrost is very important since balancing between methane oxidation and methanogenesis will help provide a better picture of permafrost's role on climate feedback (17). The inability to distinguish between DNA from viable and dead microorganisms (9, 72) could potentially lead to misunderstanding biogeochemical

processes in permafrost; thus, it is critical to distinguish between viable and non-viable sources of DNA in order to understand the organisms underlying carbon and nutrient cycling in permafrost.

1.4: Live/Dead Microbial Cell Distinction

When an organisms dies, the DNA is unstable. The DNA is rapidly degraded into short DNA fragments and is extensively modified by temperature-dependent hydrolytic, oxidative and alkaline processes (105). These spontaneous processes result in the accumulation of strand breaks, base modifications and crosslinks (106). Such processes are highly dependent on temperature, pH, oxygen-free radicals and free water (107, 108). Since reaction rates decrease by approximately an order of magnitude for every 10°C drop in temperature, and because free water concentrations are low, cold environments such as permafrost are excellent archives storing DNA for an extended period of time (109, 110). Ancient environmental DNA of plants, mammals, insects, fungi and bacteria have been discovered in permafrost (6, 106, 110-113).

Studying the microbial ecology of permafrost is especially problematic since some microorganisms remain viable and active within the permafrost (6, 7, 66). Extinct microbial communities can provide insights into the evolutionary and ecological characteristics of microorganism that lived in previous geological epochs (7), while extant microbial communities can provide insight into adaptation to soil conditions resulting from climate changes in past epochs and to the extreme conditions found within the permafrost. Therefore, it is critical to distinguish between DNA originating from extant and extinct microorganisms in permafrost (114).

A major criterion that allows the distinction between live and dead cells is cell membrane integrity. Viable and active microorganisms have intact cell membranes while dead/damaged cells have compromised membranes. Unlike dead cells, live cells can exclude DNA-binding dyes (115). Microscopy and flow cytometry were the most commonly used methods for the discrimination of live-dead microbial cells using propidium iodide as the membrane-impermeable dye (116). Other methods include CTC or tetrazolium reduction to detect cell respiration; use of hydrophilic DNA-binding dyes to detect cells with compromised membrane integrity; amino acid or nucleic acid incorporation to measure bulk activity; stable isotope probing, cellular RNA concentrations or RNA to DNA ratios to detect active nucleic acid synthesis (6, 117-120). Although these approaches allow the quantitation and identification of living and dead cells; they do not enable the extraction of DNA for subsequent genetic or metagenomic characterization.

A newer method combining membrane-impermeable dyes with PCR allows faster and more sensitive live-dead cell discrimination. Propidium monoazide (PMA) is used as the DNA-intercalating agent. When incubated with microbial cells/environmental sample, PMA bound to DNA in dead cells with compromised cell membranes and free DNA. Upon exposure to light at 460 nm, PMA becomes covalently bound to DNA. This light-induced cross-linking to DNA inhibits PCR amplification (115). PMA-PCR has been tested with artificial bacterial cultures, aquatic systems and fecal samples for medical and environmental research (115, 121-123). However, this approach has not been used extensively in soils (or soil-like environments) and the few papers did not fully optimize the method (62).

Some complexities that arise when treating soil with PMA include blocking of light penetration by soil particles and adsorbance of PMA by soil particles. To apply PMA treatments to my permafrost samples, I attempt to address these issues by optimizing a method for using PMA in soils to eliminate ancient/environmental DNA to be able to better assess the observed microbial diversity.

1.5: Diversity Indicators

Diversity measurements play a crucial role in understanding microbial community structure and dynamics, with species as the fundamental unit of analysis (124). Diversity within a community (α diversity) is identified using the species richness (number of species) and species evenness (relative abundance of species) (124). Measuring α diversity allows the comparison of different communities based on total diversity (124). Species-based measures could be quantitative, such as Chao 1 (125), abundance-based coverage estimators (ACE) (126), Shannon index (127) or Simpson index (128).

Non-parametric estimators, like Chao 1 and ACE, are the most reliable for measuring microbial community richness. These estimators are based on mark-release-recapture (MRR) statistics where the proportion of species recaptured (i.e. observed before) to those captured only once is determined(129). In a highly diverse community, there is a low probability that a species will be observed more than once with most species being represented by only one individual(129). In a community with low diversity, there is a high probability that a species will be observed more than once with most species observed multiple times in a sample (129). Chao 1 estimates species richness as

$$S_{Chaol} = S_{obs} + \frac{n_1^2}{2n_2} \qquad \textit{(Eqn 1)}$$

where S_{obs} is the number of observed species, n_1 is the number of singletons (species captured once) and n_2 is the number of doubletons (species captured twice) (125).

While Chao1 focuses on species with one or 2 individual, ACE incorporates data from all species with less than 10 individuals (126). ACE estimates species richness as

$$S_{ACE} = S_{abund} + \frac{S_{rare}}{C_{ACE}} + \frac{F_1}{C_{ACE}} \gamma^2_{ACE} \qquad \textit{(Eqn 2)}$$

where S_{abund} is the number of abundant species (abundance >10), S_{rare} is the number of rare species (abundance <10). The total number of species observed is the sum of S_{abund} and S_{rare}. C_{ACE} is the sample coverage where

$$C_{ACE} = 1 - \frac{F_1}{N_{rare}} \qquad \textit{(Eqn 3)}$$

F_1 is the number of species with i individuals and

$$N_{rare} = \sum_{i=1}^{10} iF_i \qquad \textit{(Eqn 4)}$$

Shannon (H') and Simpson (D) indices are commonly used to measure species diversity in a community. These diversity indices take both, species richness and evenness into account. The Shannon index assumes that all species are represented within a sample and that they are all randomly sampled; it measures entropy, so higher entropy means a higher diversity (127). It is calculated as

$$-\sum_{i=1}^{s} p_i \ln p_i \qquad \textit{(Eqn 5)}$$

where *s* is the number of species observed and *p* is the proportion of individuals belonging to the *i*th species. *p* = n/N where n is the number of individuals of one species and N is the total number of individuals.

The Simpson index, on the other hand, is a dominance index since it gives dominant species more weight; therefore, rare species with a few individuals will not affect the diversity (128). It measures the probability that two randomly selected individuals belong to the same species by

$$\frac{1}{\sum_{i=1}^{s} p_i^2} \qquad \textit{(Eqn 6)}$$

where *s* is the number of species observed and *p* is the proportion of individuals belonging to the *i*th species. *p* = n/N where n is the number of individuals of one species and N is the total number of individuals (128). Simpsons's index is usually reported as it's compliment (1–D) or it's inverse (1/D).

1.6: Research Project

Although there have been several cultivation-independent studies of permafrost microbial communities, only a few take into account the presence of environmental DNA and dead cells in permafrost along with viable and active cells. This makes it difficult to distinguish DNA from active microorganisms that play a significant role in shaping the permafrost environment from DNA released into the environment from dead microorganisms that are no longer part of the permafrost microbial community.

In addition, previous studies look at different depths of permafrost, but none have recognized that permafrost depth is a proxy for time. Studying microbial communities of

permafrost from different ages can shed some light as to how microbial communities changed over time, especially after abrupt climate changes that occurred at the end of the Pleistocene era.

1.6.1: Hypothesis and Predictions

I hypothesize that the abrupt change in climate and the resulting change in edaphic soil parameters at the Pleistocene/Holocene boundary resulted in a dramatic shift in the composition of viable microbial communities in permafrost. Based on this hypothesis, I predict that a) the permafrost viable microbial communities in the same era (Pleistocene or Holocene) are significantly more similar in composition than between eras, regardless of the ages of the soils, b) the shift in the viable microbial communities from the Pleistocene to the Holocene era will lead to a shift in the flux of the greenhouse gases CO_2 and CH_4 when permafrost thaws.

1.6.2: Research objectives

This project aims to answer three main questions: 1) how does the microbial community adapt in permafrost over long periods of time, 2) how does the active permafrost microbial community respond to different edaphic soil parameters caused by climate change, and finally 3) how does the active permafrost microbial community affect greenhouse gas flux upon permafrost thaw?

I compared the viable bacterial community composition in permafrost of different ages from the Pleistocene and Holocene epochs to determine how the permafrost microbial community adapts over long periods of time. Permafrost samples were treated with PMA before DNA extraction to inhibit the PCR amplification of DNA from non-viable cells. Sequence analysis of DNA from viable cells will allow the comparison of the

extant communities from different time points within a single era. By comparing the bacterial composition of different time points, the changes that occurred in the bacterial communities over time can be determined in addition to how rapidly/slowly the bacterial community adapted.

CHAPTER 2: DISTINGUISHING BETWEEN VIABLE AND DEAD MICROBES IN MODERN AND ANCIENT PERMAFROST SAMPLES

2.1: Introduction

Several methods have been developed to allow the distinction between viable and dead bacterial cells. These methods include nucleic acid or amino acid incorporation or frequency of dividing cells to measure bulk activity; use of hydrophilic DNA-binding dyes to detect cells with compromised membrane integrity; tetrazolium or CTC reduction to detect cell respiration; stable isotope probing or cellular RNA concentrations or RNA to DNA ratios to detect active nucleic acid synthesis; and amplification of long DNA fragments that are absent in dead cells (6, 117, 118, 120, 130). However, these methods either do not isolate DNA from viable cells (e.g. bulk activity, DNA-binding dyes, or cell respiration) or are most useful in environments with high microbial activity (e.g. active nucleic acid synthesis) (118) and thus cannot be used in permafrost.

Membrane-impermeant intercalating agents coupled to a monoazide residue, i.e. ethidium monoazide (EMA) (131) or propidium monoazide (PMA) (121) have been used to eliminate DNA from dead cells. When incubated with environmental samples, these agents bind to free DNA and DNA in cells with compromised membranes. Upon light exposure, the azide group becomes covalently cross-linked to the DNA, inhibiting PCR amplification and leading to insolubility and removal during extraction (132). The intercalating agent is excluded from viable cells by the intact membrane; thus, only DNA from living cells is extracted and successfully PCR amplified (133). Although EMA was used initially, PMA is preferred due to its higher charge that allows it to be more readily excluded from cells with intact membranes (115, 132).

While PMA treatment works well in bacterial cultures and aquatic systems (115, 121-123, 132) solid substrates such as soils, sediments, and foods are complex environments that pose difficulties for PMA treatment. For example, particles may prevent exposure to light, thereby preventing dye cross-linking to DNA (123). Most other studies that have successfully optimized PMA treatments in solid media either focused on the discrimination of viable and dead cells from a few taxa rather than the whole microbial community or worked with samples with high microbial activity and abundance (121, 123, 134).

Here, we optimize PMA treatment in pure cultures, inoculated sterile sand, and inoculated sterile soil. This improved protocol is useful for characterization of viable microbial communities in low cell abundance and low biological activity solid substrates such as permafrost and other extreme contaminant-impacted soils and sediments as well as treated foods.

2.2: Materials and Methods

2.2.1: Bacterial strains

PMA treatments were initially optimized with mixtures of live and/or dead pure bacterial strains under various treatment conditions. Two bacterial strains, *Escherichia coli* (ATCC 12014) and *Pseudomonas aeruginosa* (ATCC 10145), were grown in Luria-Bertani (LB) broth (Difco Laboratories, USA) overnight at 37°C with shaking at 175 rpm. To kill cells, cultures in 1X PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4) were incubated at 85°C for 20 min. Cell death was confirmed by re-incubating in LB broth and by streaking 5µl of the cell suspension on LB agar plates and

incubating at 37°C overnight; no growth was observed with heat-inactivated cells. Equal volumes (500µl) of stocks (ca. 10^6 cells ml^{-1}) were mixed and centrifuged at 16,000*xg* for 2 min to pellet the cells. The supernatant was discarded and the cells were re-suspended in 500 µl of 1X PBS.

2.2.2: PMA treatment and lab optimization tests

The following cells were mixed for liquid suspension or added to 1 g of sterile sand or sterile soil (collected from the University of Alberta grounds): live *E. coli*, live *P. aeruginosa* (LELP)*;* live *E. coli*, dead *P. aeruginosa* (LEDP)*;* dead *E. coli*, live *P. aeruginosa* (DELP)*;* or dead *E. coli*, dead *P. aeruginosa* (DEDP). Genomic DNA from each strain (isolated via the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA USA)) was also tested under the same treatment conditions. Prior to inoculation, sand and soil were sterilized with 10% bleach, reduced with 1M sodium thiosulfate, washed with distilled water, and autoclaved (135). Inoculated soil or sand samples were diluted 10^{-1}, 10^{-2}, 10^{-3}, or 10^{-4} (wt/vol) in sterile PBS. Following inoculation with the cells and dilution, either 0, 0.01, 0.02, 0.03 or 0.04 mM propidium monoazide (PMA; Biotium Inc., Hayward, CA USA) in dimethyl sulfoxide (DMSO; Sigma-Aldrich Co., St. Louis, MO USA) was added. Samples were incubated in the dark for 10 min with vigorous shaking every 2 min. PMA was cross-linked to DNA with a 500W halogen lamp (Globe Electric, Quebec, Canada) by placing the samples 20 cm from the light source for 10 min on ice. Dark controls were included for all treatments. All subsequent samples were diluted 1:1000 in sterile PBS and treated with 0.04 mM PMA.

2.2.3: DNA Extraction

Four replicates of each sample were diluted and combined during filtration on to a 47mm diameter, 0.2µm pore size polysulfone (Supor) filter (Pall Corporation, USA) before DNA extraction. Genomic DNA was extracted from 500 µl of overnight culture of strains, liquid suspensions of mixed test cultures, or soil or sand filters using the FastDNA Spin Kit for Soil according to manufacturer's instructions (MP Biomedicals, Santa Ana, CA), with adjustments. DNA extractions had the following adjustments: the samples were homogenized for 45 s at 4,500 rpm twice; after adding the Binding Matrix suspension, the samples were placed in a rotor to allow DNA to bind for 15 min; the tubes were centrifuged for 1 min at 14,000*xg* to collect beads; after spinning, the supernatant was completely removed prior to re-suspension; and ethanol in the spin filter was evaporated by incubating the tubes in a heat block at 70°C for 10-15 min.

2.2.4: Restriction fragment length polymorphism (RFLP) analysis

To determine if DNA from dead cells was present following optimization of PMA protocols, we amplified the V3 variable region of the 16S rRNA gene from the PMA-treated and untreated genomic DNA, PBS only, sand, and soil samples using primers 341F and 518R as previously described (136). The 16S rRNA gene PCR products were digested as instructed by the manufacturer (New England Biolabs, Whitby, Ontario, Canada) using the restriction enzyme *MspA1I,* chosen using the Webcutter program (137) to discriminate between *E. coli* and *P. aeruginosa*. RFLP products were detected by agarose gel electrophoresis as previously reported (138).

2.3: Results

For pure genomic DNA, no PCR amplification was detected following treatment at any PMA concentration (Table 1), while untreated genomic DNA was successfully PCR amplified (data not shown).

16S rRNA genes were PCR amplified for all mixtures of cell culture liquid suspensions (LELP, LEDP, DELP, and DEDP) at 0.01 mM PMA. However, at higher PMA concentrations, PCR products were only generated for samples with live cells (LELP, LEDP, and DELP); no PCR product was obtained for DEDP (Table 1). Mixtures of cell culture liquid suspensions not treated with PMA showed successful PCR amplification (Table 1).

To test the effect of solid substrate presence on PMA effectiveness, mixed cultures were inoculated into sterile sand. 16S rRNA genes were PCR amplified for the two lowest PMA concentrations for all combinations of cells at all dilutions (Table 1). For the two higher PMA concentrations, PCR products were generated for all dilutions of LELP, LEDP, DELP, and for the 10^{-1} and 10^{-2} dilutions of DEDP; but no amplification was observed for the 10^{-3} and 10^{-4} dilutions of DEDP (Table 1). Inoculated sand that was not PMA-treated showed successful PCR amplification for all four combinations of cells at all dilutions (Table 1). We interpret these results to indicate that solid substrates can interfere with crosslinking of PMA to DNA from dead cells at high particle concentrations, but that dilution of particles can alleviate this interference.

To further test the combined impact of shading and adsorbance to mixed size particles and organics, we used sterilized field soils inoculated with the cell culture mixes described above. Amplification was observed for all cell mixes at all soil dilutions for

0.01, 0.02, and 0.03 mM PMA (Table 1). When 0.04 mM PMA was added, PCR products were generated for all mixtures containing live cells and for 10^{-1} and 10^{-2} dilutions for DEDP; but no amplification was observed for 10^{-3} and 10^{-4} dilutions of DEDP (Table 1). We interpret these results to indicate that complex soils can inhibit PMA effectiveness even more than sand, but that higher concentrations of PMA and dilution of soil alleviates this interference.

Table 1. PCR amplification results for lab optimization tests with different PMA concentrations. + indicates that PCR products were generated upon amplification, - indicates that no PCR products were generated upon amplification, na: not applicable, genomic DNA was not tested with sterilized sand or soil.

Treatment	Genomic DNA					LELP					LEDP					DELP					DEDP				
PMA conc. (mM)	0	0.01	0.02	0.03	0.04	0	0.01	0.02	0.03	0.04	0	0.01	0.02	0.03	0.04	0	0.01	0.02	0.03	0.04	0	0.01	0.02	0.03	0.04
LC	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Sand																									
10^{-1}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
10^{-4}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Soil																									
10^{-1}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
10^{-4}	na	na	na	na	na	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

[a] Abbreviations: LC, liquid culture; LELP, live *E. coli*, live *P. aeruginosa*; LEDP, live *E. coli*, dead *P. aeruginosa*; DELP, dead *E. coli*, live *P. aeruginosa*; DEDP, dead *E. coli*, dead *P. aeruginosa*

RFLP digests of the PCR products from untreated samples showed PCR amplification of 16S rRNA genes from both dead and live cells, while samples treated with 0.04 mM PMA showed PCR amplification of 16S rRNA genes only from live cells (Figure 1). The PMA treatment was only able to eliminate DNA from dead cells at the 10^{-3} and 10^{-4} dilutions (Figure 1, Lanes 9-16); lower dilutions showed PCR amplification of DNA from dead cells (Figure 1, Lanes 5-8). Since the lower dilution of 10^{-3} was successful, the subsequent treatments on the environmental samples were carried out with 0.04 mM PMA and 10^{-3} dilution of sediments.

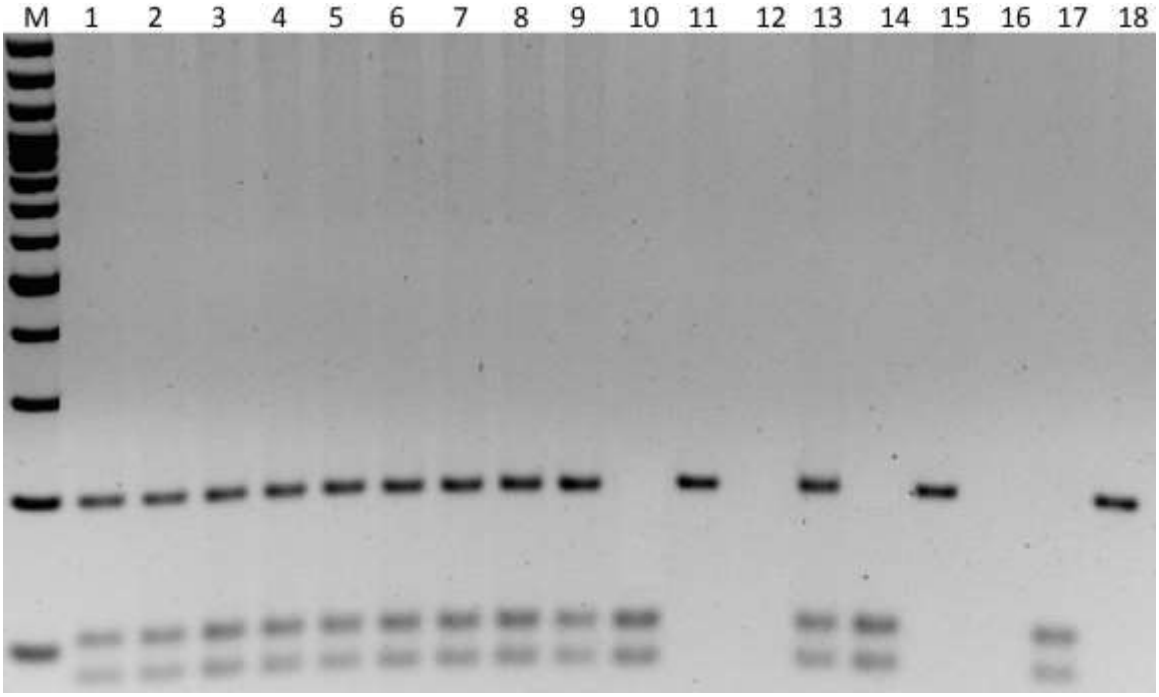

Figure 1. RFLP of PMA-treated and untreated inoculated soil. PCR products of the V3 region of the 16S rRNA genes were digested with *MspA1I.* Treated samples were incubated with 0.04 mM PMA per gram of soil. M: 100bp marker; Lanes 1-4: no PMA treatment; Lanes 5-16: PMA treatment; Lane 17: *E. coli* 16S rRNA gene product, Lane 18: *P. aeruginosa* 16S rRNA gene product. Lanes 1, 5, 9, 13: LELP; Lanes 2, 6, 10, 14:

LEDP; Lanes 3, 7, 11, 15: DELP; and Lanes 4, 8, 12, 16: DEDP. Lanes 5-8, 10^{-1} soil dilution; Lanes 9-12, 10^{-3} soil dilution; Lanes 13-16, 10^{-4} soil dilution

2.4: Discussion

Solid substrates (i.e. sand and soil) were found to inhibit PMA treatment efficacy relative to pure genomic DNA and liquid culture suspensions. A higher concentration of PMA and higher dilution of the samples were necessary to ensure the success of the PMA treatments in solid substrates. This interference is likely due to two major factors, particle shading (as evidenced by the strong effects of both sand and soil) and adsorbance of PMA by sediment (as evidenced by the increased effect of soils over sand). High solid concentrations have been previously shown to significantly impede PMA's efficacy; therefore, solids in soil samples must be diluted to enable successful cross-linking (123). Based on our results, a minimum concentration of 0.04 mM PMA added to samples diluted at least 1:1000 compensates for these effects.

Our optimized protocol is similar to that described by Pisz et al. and applied to permafrost by Yergeau et al. In those papers, they found little difference in treated and untreated samples; however, these papers used qPCR to quantify specific taxa rather than characterizing the entire bacterial community. If the targeted taxa are viable or the taxonomic resolution too low, no change would be observed with qPCR, providing an important caveat for the use of PMA; namely, that the methods used for subsequent characterization may significantly influence the outcome.

CHAPTER 3: ACTIVE BACTERIAL COMMUNITIES FROM PLEISTOCENE AND HOLOCENE AGED PERMAFROST

3.1: Abstract

Experimental warming of Arctic soils in field experiments, some exceeding a decade, have failed to show significant changes in permafrost bacterial communities, while laboratory studies have shown shifts almost immediately upon thaw. One possible explanation for this discrepancy is bias due to preservation of DNA from dead cells in permafrost. We optimized a protocol for distinguishing between DNA originating from viable cells and total bacterial DNA extracted from permafrost. We examine the response of the active bacterial community composition to the rapid warming that accompanied the end of the Pleistocene, 11,700 years ago. This warming resulted in changes in soil edaphic properties, including pH, TOC and TN as a largely grassland ecosystem was replaced by early boreal forest. Our results show that the viable permafrost bacterial community is significantly different from total DNA and these two assemblages are structured by different environmental parameters. The corollary to these findings is that future climate change is unlikely to shift bacterial communities unless the warming is sufficient to change soil edaphic properties.

3.2: Introduction

Permafrost is an exceptional archive of past ecosystems, both because it is a storehouse of well-preserved fossils (139) and because it is an ideal environment for preservation of DNA from dead organisms (106, 110). DNA degradation and modification occurs due to temperature-dependent hydrolytic, oxidative and alkaline processes (106, 107); therefore, the constant sub-zero temperatures, neutral pH and

anaerobic conditions in permafrost make it an excellent archive for storing DNA for extended periods of time. The species compositions of past ecosystems have been inferred from ancient, preserved DNA of extinct plants, mammals, insects, fungi and bacteria from permafrost (6, 106, 111, 113, 140).

At the end of the Pleistocene, about 11,700 years ago (84), the Earth's climate system abruptly warmed, leading to a cascade of changes that drove large-scale extinctions and reorganization of ecosystems. This period is considered an analog for modern, ongoing climate change (141). Due to studies of fossils from permafrost and lacustrine sediments, we know a considerable amount about the adaptations, extirpations and extinctions of large mammals and plant communities at the Pleistocene/Holocene boundary (8). However, relatively little is known about the changes that took place at the microbial level.

Permafrost contains large reserves of frozen labile carbon that is unavailable for respiration (36). Some microorganisms remain viable and active within permafrost (6, 66, 68, 142) and as the Arctic undergoes anthropogenically-driven warming, this labile carbon is vulnerable to degradation by these viable microorganisms (9). Most previous DNA-based studies of permafrost have not distinguished between DNA from viable and dead microorganisms (9, 72) potentially leading to misunderstanding of biogeochemical processes in permafrost. For example, thaw of permafrost has been reported to lead to rapid shifts in microbial community composition (119). This rapid shift could have been due to an actual shift in the microbial community structure or it could be the result of co-extraction of preserved DNA from dead cells. This non-viable DNA could be rapidly degraded by low abundance, viable microorganisms after thaw. These interpretations

have different implications for carbon remineralisation; thus, it is critical to distinguish between viable and non-viable sources of DNA in order to understand the organisms underlying carbon and nutrient cycling in permafrost. Studying the composition of viable microbial communities in permafrost will provide a better understanding of how these microorganisms respond to soil chemical and physical (edaphic) variability resulting from climate change across the Pleistocene/Holocene boundary. This will provide insight into the changes that the soil community is currently undergoing in this modern era of rapid climate change.

In this study, we distinguish between DNA from viable and non-viable permafrost bacteria and show that the extant community is distinct from the total community; thus, only a small percentage of the total community is likely to play a role in carbon mineralization in melting permafrost. We further demonstrate that response of the viable permafrost bacterial community to climate change at the Pleistocene-Holocene boundary was modulated through soil edaphic parameters, possibly providing a mechanism to explain the different results of laboratory based and in situ warming experiments.

3.3: Materials and Methods

3.3.1: Site description and sampling procedure

Active layers (i.e. seasonally frozen sediments overlying permafrost) and perennially frozen sediments were collected in June 2013 and June 2012, respectively, from a placer gold mining site known as the Lucky Lady (LLII) mine (63.74˚ N, 138.86˚ W). LLII is about 50km southeast of Dawson City, Yukon Territory, Canada. Ongoing mining at this southern Klondike goldfield exposed the permafrost below making it easier

to directly access Holocene and Pleistocene permafrost. Vegetation at the sample site is that of a typical boreal forest, composed of *Sphagnum* spp. mosses, labrador tea (*Ledum groenlandicum*), willow (*Salix* sp.), and black spruce (*Picea mariana*).

For the active layers, overlying organic horizons (live and dead plant material) were removed and the samples were taken from a hill inclination of ~15°. Two active layer (seasonally frozen at the time of sampling) cores of 8 cm diameter and 20 cm long were collected 0.8 m apart. For the perennially frozen sediments, three Holocene (H1, H2, and H3, collected at 92, 315, and 667 cm below the surface, respectively) and three Pleistocene (P1, P2, and P3, collected at 997, 1100, and 1125 cm below the surface, respectively) cores of 8 cm diameter and 20 cm long were collected by horizontal drilling with an EDR-260 (ECHO Inc., Lake Zurich, IL, USA) gas powered drill with a diamond bit. Any surface organic matter contaminants that stuck to the cores during drilling were scraped off. Frozen cores were placed in clear bags, transferred to coolers with ice-packs in the field, and stored at -20°C until further analysis.

3.3.2: Core subsampling

Prior to core subsampling, potential contamination was tracked in a similar fashion as described previously (143), except the exterior of each core was sprayed with *E. coli* containing an ampicillin resistant pBAD vector with an enhanced green fluorescent protein (eGFP) 24 hrs before subsampling instead of a pET23a vector. The presence of PCR-amplifiable eGFP DNA was interpreted as potential contamination, and those samples were discarded. When sub-sampling, two sub-samples from each core were

extracted close to the cores' outer surface as positive controls for contamination.

The cores were subsampled as previously described (136) in a class 1000 clean lab at the University of Alberta that had never been used for DNA extraction or amplification. To subsample, the end of the core was laterally excised and a puck was broken off from the core by a hammer and chisel. The samples for DNA extraction were obtained from the center of the puck by hammering sterilized 1/2" hose splicers (metal tubes) into the frozen puck. The subsamples were immediately stored at -20°C until DNA extraction. For DNA extraction, subsamples from each core were combined and homogenized by sieving through a 2mm mesh. Soil samples were either left untreated or treated with PMA as described in the supplementary materials.

3.3.3: Soil Chemistry

Soil chemistry measurements including moisture content, pH, total nitrogen (TN), total phosphorus (TP), nitrate, ammonium, total organic carbon (TOC) and total carbon (TC) were performed on the 2 active layer and 6 permafrost samples using standard methods (144). Soil moisture content was obtained by oven drying the samples at 105°C for 24 hours. Soil pH was determined using a soil to water ratio of 1:2 using an AB15 pH meter (Fisher Scientific, USA). TN and TP were extracted using Kjeldahl digestion as previously described (145, 146) and measured using a SmartChem Discrete Wet Chemistry Analyzer, Model 200 (Westco Scientific, USA) (147, 148). Nitrate and ammonium were extracted using potassium chloride as the extract solution and colorimetrically measured using a SmartChem Discrete Wet Chemistry Analyzer, Model

200 (Westco Scientific, USA) (149, 150). Ammonium was measured using the Berthelot method (151, 152) and nitrate was measured using the Diazo Coupling method (149) TOC was measured using the Loss on Ignition (LOI) procedure (153) using a Lindberg SB Muffle Furnace (Thermo Fisher Scientific Inc., USA). Finally, TC was measured using the dry combustion method (AOACInternational 2000) using a Costech 4010 Elemental Analyzer System (Costech International Strumatzione, Italy). All soil chemistry measurements were carried out in triplicates.

3.3.4: PMA treatment

Soil samples were treated with propidium monoazide (PMA), a DNA intercalating dye that irreversibly cross-links to free DNA upon exposure to light, but cannot penetrate intact membranes. Thus, only DNA from live cells is extractable and amplifiable. For treatment, soils were diluted 1:1000 and incubated with PMA (0.04 mM) in the dark for 10 min with vigorous shaking every 2 min. PMA was cross-linked to DNA with a 500W halogen lamp (Globe Electric, Quebec, Canada) by placing the samples 20 cm from the light source for 10 min on ice. Details of protocol optimization are provided in the supplementary materials.

3.3.5: DNA Extraction

Four replicates of each sample were diluted and combined during filtration on to a 47mm diameter, 0.2µm pore size polysulfone (Supor) filter (Pall Corporation, USA) before DNA extraction. Genomic DNA was extracted using the FastDNA Spin Kit for Soil according to manufacturer's instructions (MP Biomedicals, Santa Ana, CA), with

minor adjustments.

3.3.6: Contamination prevention and detection

To detect the presence of contamination (see above), we used PCR amplification with primers gfpF (5'-ATG GTG AGC AAG GGC GAG-3') and gfpR (5'-TTA CTT GTA CAG CTC GTC CAT GCC-3') (43). The PCR mixture consisted of 0.5 units of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, USA); 0.1 μM of each primer; 2.5μl of 10X HiFi PCR Buffer (Invitrogen, USA); 0.2mM dNTPs (Invitrogen, USA); 3μl of DNA template; and nuclease-free H_2O (Ambion, USA) to a total volume of 25μl. Mixtures were subjected to the following thermal cycling protocol in a S1000 Thermal Cycler (Bio-Rad Laboratories, Inc., California, USA): a denaturing step at 95°C for 1 min; 30 amplification cycles of 30 sec at 95°C, 45 sec at the annealing temperature of 55°C, and 45 sec at 72°C; and a final extension for 10 minutes at 72°C. PCR product was detected in positive control samples, but not in central core samples, indicating a lack of contamination from the outer core (data not shown).

3.3.7: DNA sequencing and analysis of environmental samples

Samples were sequenced commercially by MR DNA (http://www.mrdnalab.com), where the V1-V3 regions of the 16S rRNA gene were amplified with the 27F (5′-AGA GTT TGA TCC TGG CTC AG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') primers and sequenced using the Roche GS-FLX platform (Roche Life Science, USA) (72).

The 16S rRNA gene sequences of the environmental samples were analyzed using

version 1.33.3 of Mothur (154) by following the standard operating procedure (http://www.mothur.org/wiki/454_SOP) for flowgram filtering, noise and chimera removal, identification of operational taxonomic units (OTU), assignment of taxonomy, community comparison, and statistical analysis. Sequences were classified to the class level using the Ribosomal Database Project (RDP) reference taxonomy. Using the average neighbour-clustering algorithm, sequences were assigned to OTUs at 97% similarity. Singleton and doubleton OTUs were removed and the sequences were classified at a RDP cutoff of 60. Following subsampling to 1000 sequences per sample (based on the sample with the fewest number of sequences), the taxonomic data was acquired from Mothur's phylotype classification using the RDP reference taxonomy and the relative abundance of the sequences at the class level was determined. Phylotypes that made up less than 1% of the total number of sequences at the class level were combined and labeled as "other". The α-diversity of bacterial assemblages in PMA-treated and untreated samples was compared using the Mothur implementations of observed OTU richness (SOBS), Chao1 estimated richness (chao command), and Shannon (shannon command) and inverse Simpson's (invsimpson command) diversity indices. The significance of the changes in α-diversity was determined using a 2-sample t-test. Multivariate analysis of the OTU composition was carried out by principal coordinates analysis (PCoA) using PC-ORD v. 6.08 (155). Multi-Response Permutation Procedure (MRPP) tested for significant differences between PMA-treated and untreated samples (156) and was performed in PC-ORD v. 6.08.

3.3.8: ^{14}C Dating

Organic matter was sampled from all 6 permafrost cores for radiocarbon dating. Samples were pre-treated using standard acid-base-acid (ABA) procedures (157). ^{14}C measurements were carried out at the Keck-Carbon Cycle Accelerator Mass Spectrometer (KCCAMS) Facility at the University of California-Irvine using a 500kV compact AMS (National Electrostatics Corporation, WI, USA). Resulting ^{14}C ages were calibrated to calendar years before present (Cal BP) with the IntCal13 calibration curve (158) using the OxCal online program (159).

3.4: Results

3.4.1: Sediment edaphic characteristics

The active layers (AL1 and AL2) showed a lot of variability with very different chemical properties (Table 2). H had significantly higher total nitrogen (TN), total organic carbon (TOC) and water content than P; while P had significantly higher pH than H (Table 2). In general, pH was observed to increase with depth; TN, TOC and water content decreased with depth. Sediment edaphic properties for each individual sample are displayed in Table 2.

Table 2. Soil chemical and physical properties for active layer (AL) and perennially frozen Holocene (H) and Pleistocene (P) layer samples.

Sample	Depth (cm)	Age (Cal yrs BP)	pH[a]	TP (%)	TN (%)[a]	NO_3 (%)[ac]	NH_4 (%)[abc]	TOC (%)[a]	TC (%)	Water (%)[a]
AL1	15	0	7.39 ± 0.01	0.052 ± $1x10^{-3}$	0.142 ± $4x10^{-4}$	0.0005 ± $4x10^{-5}$	0.002 ± $2x10^{-5}$	0.65 ± 0.04	1.05 ± 0.02	26.70 ± 1.21
AL2	19	0	4.61 ± $3x10^{-3}$	0.087 ± $1x10^{-3}$	0.860 ± $2x10^{-3}$	0.0005 ± $5x10^{-5}$	0.001 ± $1x10^{-5}$	7.55 ± 0.43	14.58 ± 2.83	46.84 ± 1.94
H1	92	8100	4.28 ± 0.01	0.068 ± $1x10^{-3}$	0.464 ± $1x10^{-3}$	0.0005 ± $5x10^{-5}$	0.006 ± $6x10^{-5}$	2.17 ± 0.12	5.48 ± 1.06	30.55 ± 1.40
H2	315	9550	6.05 ± 0.01	0.038 ± $1x10^{-3}$	0.470 ± $1x10^{-3}$	0.0003 ± $3x10^{-5}$	0.008 ± $9x10^{-5}$	1.76 ± 0.10	3.65 ± 0.71	40.17 ± 2.74
H3	667	12150	7.66 ± 0.01	0.052 ± $1x10^{-3}$	0.146 ± $4x10^{-4}$	0.0007 ± $6x10^{-5}$	0.004 ± $4x10^{-5}$	0.64 ± 0.04	1.31 ± 0.25	56.01 ± 3.45
P1	997	15100	8.27 ± 0.02	0.049 ± $1x10^{-3}$	0.073 ± $2x10^{-4}$	0.0004 ± $4x10^{-5}$	0.003 ± $3x10^{-5}$	0.34 ± 0.02	1.17 ± 0.23	24.21 ± 0.84
P2	1100	16000	8.01 ± 0.01	0.068 ± $1x10^{-3}$	0.075 ± $2x10^{-4}$	0.0005 ± $4x10^{-5}$	0.003 ± $3x10^{-5}$	0.39 ± 0.02	0.90 ± 0.17	28.72 ± 1.31
P3	1125	16200	8.11 ± $3x10^{-3}$	0.064 ± $1x10^{-3}$	0.068 ± $2x10^{-4}$	0.0004 ± $4x10^{-5}$	0.003 ± $3x10^{-5}$	0.42 ± 0.02	1.00 ± 0.19	25.46 ± 1.43

[1] % dry weight
[2] ± standard error
[3] Abbreviations: TP, total phosphorus; TN, total nitrogen; NO_3, nitrate; NH_4, ammonium; TOC, total organic carbon; TC, total carbon
[a] t-test showed significant difference between Holocene and Pleistocene samples ($p<0.05$)
[b] t-test showed significant difference between Active and Holocene samples ($p<0.05$)
[c] t-test showed significant difference between Active and Pleistocene samples ($p<0.05$)

3.4.2: PMA untreated samples

Untreated samples showed high taxon richness and overall diversity (Table 3). Diversity of AL, H and P samples were not significantly different; AL observed richness was significantly higher than H; and AL estimated richness was significantly higher than both H and P (Table 3).

Samples clustered into three significantly different groups (Figure 2). AL2 clustered with H2 and H3 (group1), H1 clustered with P2 and P3 (group2), and AL1 clustered with P1 (group3). Ice content was the only edaphic property significantly correlated with the samples (Figure 2). Although the untreated samples showed high levels of diversity, only a few bacterial orders showed a significant correlation with the clustering. Only Bacillales was correlated with group 1 and contributed to its separation from groups 2 and 3 along Axis 1. Rhizobiales, Solirubrobacterales, Clostridiales and Actinomycetales was correlated with group 2: Rhizobiales and Solirubrobacterales separated group 2 from group 1 along Axis 1, while Clostridiales and Actinomycetales separated group 2 from group 3 along Axis 2. Finally, Nitrospirales was correlated with group 3 and contributed to the separation of AL1 from P1 along Axis 2 and groups 3 from groups 1 and 2 along Axis 2.

Table 3. α-diversity measures of the 16S rRNA gene libraries for PMA-treated (+PMA) and untreated (-PMA) active layer (AL) and perennially frozen Holocene (H) and Pleistocene (P) layer samples.

Sample	Observed OTUs	Chao1	invSimpson	Shannon	Simpson evenness	Shannon evenness
AL1-PMA	269	533 (441;672)	124 (102;156)	5.15 (5.06;5.25)	0.46	0.92
AL2-PMA	213	457 (364;608)	51 (43;63)	4.62 (4.51;4.73)	0.24	0.86
H1-PMA	211	395 (323;512)	81 (69;98)	4.80 (4.70;4.89)	0.38	0.90
H2-PMA	193	363 (295;476)	55 (47;66)	4.56 (4.45;4.66)	0.29	0.87
H -PMA	187	334 (273;436)	47 (40;58)	4.51 (4.40;4.62)	0.25	0.86
P1-PMA	259	503 (417;636)	98 (80;127)	5.07 (4.97;5.16)	0.38	0.91
P2-PMA	225	397 (332;502)	77 (64;95)	4.84 (4.74;4.94)	0.34	0.89
P3 -PMA	195	324 (271;414)	76 (65;92)	4.73 (4.64;4.82)	0.39	0.90
AL1+PMA	235	453 (372;581)	100 (85;122)	4.96 (4.87;5.05)	0.43	0.91
AL2+PMA	214	335 (288;412)	55 (43;75)	4.77 (4.67;4.88)	0.26	0.89
H1+PMA	126	206 (167;282)	36 (32;42)	4.06 (3.96;4.16)	0.29	0.84
H2+PMA	158	272 (222;360)	34 (29;41)	4.21 (4.10;4.33)	0.21	0.83
H3+PMA	166	275 (228;357)	33 (28;41)	4.26 (4.14;4.37)	0.20	0.83
P1+PMA	82	92 (85;116)	30 (26;34)	3.79 (3.70;3.88)	0.36	0.86
P2+PMA	143	171 (156;204)	69 (59;82)	4.52 (4.44;4.60)	0.48	0.91
P3+PMA	151	193 (172;235)	64 (55;76)	4.52 (4.43;4.60)	0.42	0.90

[a]values in parentheses are 95% confidence intervals

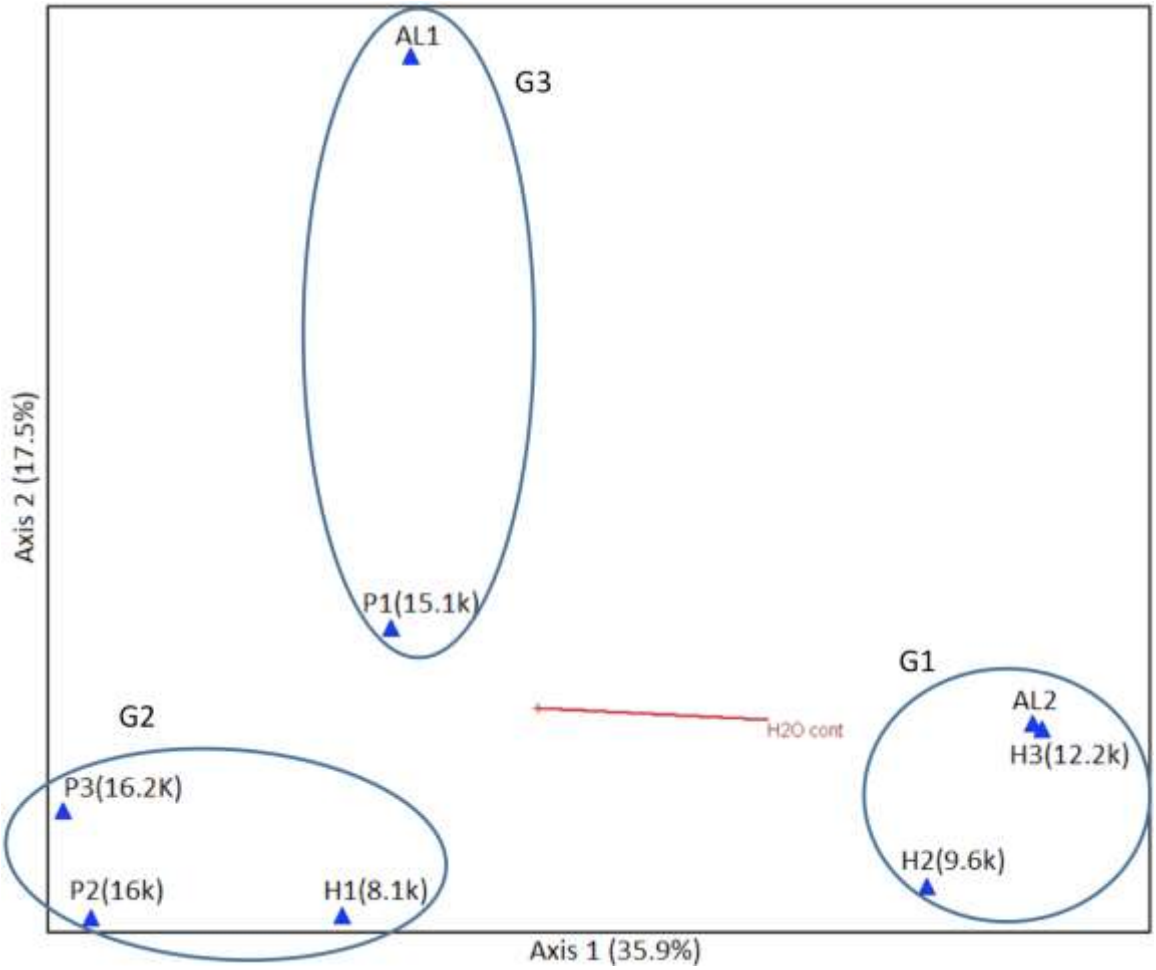

Figure 2. Bacterial communities of untreated active layer (AL), Holocene (H) and Pleistocene (P) clustered using PCoA of the Bray-Curtis distance matrix. Numbers in parentheses indicate sample age. The percentage of variation explained by each axis is indicated on the figure. Clusters displayed are based on MRPP analysis where groups 1, 2 and 3 (G1, G2 and G3) were significantly different from each other (G1-G2 $p<0.05$; G2-G3 $p<0.00001$; G1-G3 $p<0.05$). Soil edaphic parameters that significantly correlate with the axes ($p<0.05$) are displayed as vectors.

3.4.3: PMA treated samples

Unlike in the untreated samples, following PMA treatment, a decreasing trend in observed and estimated richness was observed with increasing age and depth from AL to H to P. AL's estimated and observed richness were significantly higher than that of H and P; AL showed a significantly higher diversity than H but not P (Table 3). Following PMA treatment, there is a significant difference in richness and diversity between the active and perennially frozen layers, but not within the perennially frozen Holocene or Pleistocene sediments. PMA treated H and P samples had a significantly lower richness and diversity than the untreated (Table 3). More hydrated samples were found to have the lowest average percentage of dead OTUs (i.e. OTUs present in the untreated sample but absent from the treated).

Like the untreated samples, the PMA treated samples clustered into three groups. However, in this case, the groups were primarily determined by the geological epoch from which they originated: AL samples clustered together along with H1 (group1), H2 and H3 clustered with each other (group2), and all Pleistocene samples clustered together (group3) (Figure 3). pH, TN and TOC all showed significant correlation with the viable bacterial communities (Figure 3). Permafrost samples did not cluster by age: H1 and H2 were only 1,450 years apart and H3 and P1 were 2,950 years apart and yet very distinct from each other. There was a shift in the bacterial community composition and relative abundance from AL to H and across the P/H boundary, with H1 showing a very similar composition to AL1 and AL2 (Figure 4). In the active layers and H1, Actinomycetales, Rhizobiales and Solirubrobacterales had high relative abundance along with a high diversity and abundance of Acidobacteria orders. These orders were outcompeted by

Bacillales in H2 and H3. In Pleistocene sediments, Actinomycetales, Burkholderiales, Clostridiales and Rhizobiales increased in abundance in P1 while Bacillales dropped. In P2 and P3 Burkholderiales abundance decreased whereas Actinomycetales, Clostridiales and Solirubrobacterales increased P3. Only four orders showed significant correlation with the PMA treated samples (data not shown): Acidobacteria groups 1 and 4 contributed to the separation of the active layer and H1 from the perennially frozen samples; Bacillales equally contributed to the separation of H2 and H3 from the active layers and Pleistocene samples; and Clostridiales separated the Pleistocene samples from H2 and H3 (data not shown).

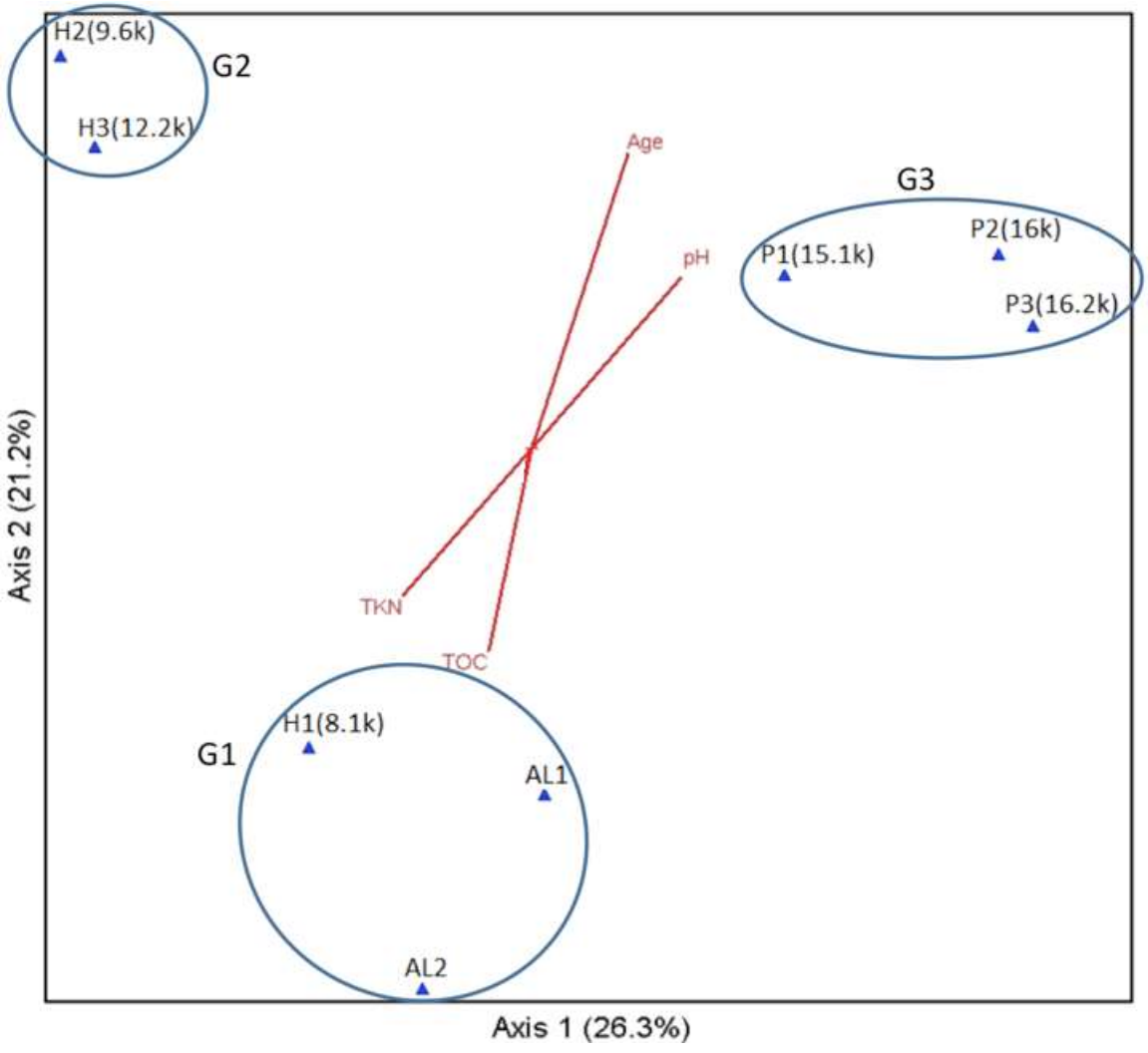

Figure 3. Bacterial communities of PMA-treated active layer (AL), Holocene (H) and Pleistocene (P) bacterial communities clustered using PCoA of the Bray-Curtis distance matrix. Numbers in parentheses indicate sample age. The percentage of variation explained by each axes is indicated on the figure. Clusters displayed are based on MRPP analysis where groups 1, 2 and 3 (G1, G2 and G3) were significantly different from each other (G1-G2 $p<0.00001$; G2-G3 $p<0.00001$; G1-G3 $p<0.05$). Soil edaphic parameters that significantly correlate with the axes ($p<0.05$) are displayed as vectors.

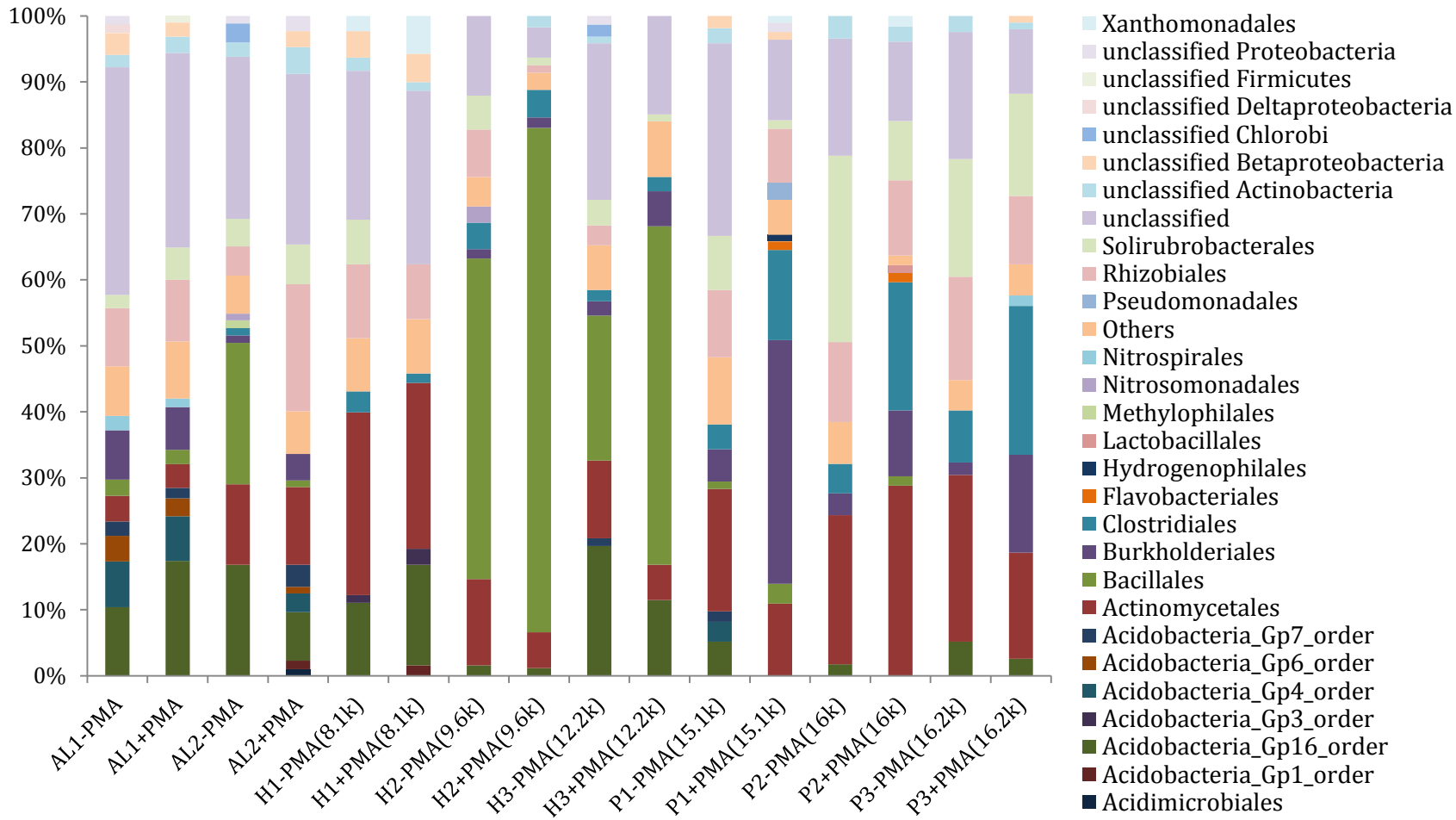

Figure 4. Relative abundance of bacterial 16S rRNA genes taxonomically classified to the order level in PMA-treated (+PMA) and untreated (-PMA) active layer (AL), Holocene (H) and Pleistocene (P). Numbers in parentheses indicate sample age.

3.5: Discussion

The transition from the Pleistocene epoch to the Holocene epoch was marked by a rapid climate change, going from a dry and cold environment to a warm and wet one (102). This warming led to shifts in plant communities, from steppe to tundra, in turn influencing the surrounding soil's chemical and physical properties such as soil water holding capacity, pH, organic matter content, total nitrogen and soluble phosphorus (160). We observed this shift in our samples where distinctive difference between H and P perennially frozen sediment properties were observed (Table 2). However, samples from within an epoch showed no significant variance in the measured environmental variables.

When focusing on DNA from viable cells only, samples clustered according to era/epoch edaphic parameters. Holocene permafrost was clearly distinct from Pleistocene, indicating that the active component of the community is selected for by both permafrost conditions and sediment edaphic parameters at the time the permafrost was formed. In addition, taxonomic analysis showed that bacterial communities in the Pleistocene and Holocene samples were composed of different bacterial orders, but similar orders were observed within an epoch despite the large age differences. As previously reported, the active layer viable bacterial communities were clearly distinct from the permafrost communities (9, 78), except for the H1 sample that clustered with the active layer.

Soil microbial communities play imperative roles in regulating soil biogeochemistry and ecosystem functioning, but their response to climate warming is yet unclear (161). This understanding is particularly lacking in environments such as permafrost where ice-rich ground has high levels of inertia (162). Microbial communities

have been predicted to rapidly respond to permafrost thaw due to the availability of trapped organic matter and vast carbon stores for microbial degradation (9).

Since our data indicate that permafrost bacterial communities shifted across the Pleistocene/Holocene boundary and age alone did not appear to be the cause of this change, it appears that changing temperatures alone do not lead to shifts in the soil bacterial communities. This explains why some experiments show microbial community shifts with warming or thaw while others don't. Previous studies undergoing laboratory simulated permafrost thaw have shown a shift in the microbial community, phylogenetic and functional gene abundances, exoenzyme activities, transcriptional response and metabolic diversity (9-12). On the other hand, *in situ* warming experiments have shown only minor changes in microbial community composition (13-16). In previous field warming experiments, either a strong lag in the response of soil microbial communities was observed (e.g. Lamb et al. 2011 (16)) or changes in microbial communities were accompanied by parallel changes in soil edaphic properties (primarily pH; e.g. Hayden et al, 2012 and Rousk et al, 2012 (82, 83)) (Table 4). In those experiments where warming was insufficient to result in a significant change in the soil edaphic parameters, there was, in turn, no corresponding change in the microbial community (Table 4). A change in pH alone was found to be sufficient to determine microbial community composition both at continental scales and across the Arctic (83, 163-165). One possible explanation for this discrepancy between lab-based and field-based results is that laboratory thaw experiments might lead to a shift in the permafrost microbial communities due to increased effects on edaphic parameters resulting from container effects; in the field-based experiments on the other hand, if a shift is not observed for soil edaphic

parameters, then the microbial community will not shift. Another possible explanation for this discrepancy is that field-based studies have examined total preserved DNA, while lab experiments primarily obtained DNA from viable cells.

Table 4. The effect of warming on microbial community structure and composition in different soil environments

Paper	Environment	Duration of warming	Increase in soil temperature	Soil chemical properties pre-warming		Soil chemical properties post-warming		Conclusions
Rousk *et al.*, 2012(83)	Temperate forest at Harvard Forest LTER-Prospect Hill	3 years	+5°C	Organic soil	pH=3.4 OC=270mg/g TN=11mg/g	Organic soil	pH=3.3 OC=310mg/g TN=13mg/g	Temperature adaptation of bacterial communities suggest community shift but didn't confirm shift
				Mineral soil	pH=4.1 OC=80mg/g TN=4mg/g	Mineral soil	pH=3.9 OC=73mg/g TN=4mg/g	
Kuffner *et al.,* 2012 (166)	Temperate mountain forest soil in North Tyrolean Limestone Alps near Achenkirch, Austria	4 years	+4°C	pH slighty >6		NA		No significant difference in bacterial community structure or composition
DeAngelis *et al.*, 2015 (167)	Temperate forest at Harvard Forest Long-term Ecological Research (LTER) site SWaN	5 years	+5°C	Organic soil	pH=3.72 TC=3314g/m^2	NA		No significant difference in bacterial community structure or composition
				Mineral soil	pH=4.38 TC=3478g/m^2	NA		

		Barre Woods	8 years		Organic soil	pH=4.29 TC=1772g/m^2	NA	No significant difference in bacterial community structure or composition
					Mineral soil	pH=4.42 TC=1810g/m^2	NA	
		Prospect Hill	20 years		Organic soil	pH=3.82 TC=2565g/m^2	NA	Significant difference in bacterial community structure and composition
					Mineral soil	pH=4.41 TC=2859g/m^2	NA	No significant difference in bacterial community structure or composition
Sistla *et al.*, 2013 (168)	Arctic tundra, moist acidic tussock (MAT) near Toolik Lake		20 years	+1-2°C	Surface organic soil	TC=1027g/m^2 TN=27g/m^2 C:N=42*	Surface organic soil	TC=929g/m^2 TN=28g/m^2 C:N=36*	Shift in plant community structure; increased plant biomass and woody dominance
					Deep organic	TC=4547g/m^2 TN=164g/m^2	Deep organic	TC=3907g/m^2 TN=146g/m^2	Shift in plant community structure;

				soil	C:N=32	soil	C:N=28	increased plant biomass and woody dominance; change in fungal:bacterial biomass
				Mineral soil	TC=6382g/m^2* TN=318g/m^2 C:N=20*	Mineral soil	TC=8342g/m^2* TN=377g/m^2 C:N=22*	Shift in plant community structure; increased plant biomass and woody dominance
Rinnan *et al,* 2007 (14)	Subarctic tundra heath near Abisko Scientific Research Station in Swedish Lapland	15 years	+1.2-2°C	pH=7.1		SOM= 70-89% H2O content significantly lower in warmed top 5cm soil than control but not in 5-10cm depth		Significant alterations in microbial biomass accompanied by strong modification in microbial community composition. Warming significantly decreased the relative abundance of fungi compared to bacteria
Biasi *et al,*	Tundra in N-Siberia near Yamburg on	2 years	+0.9°C	Organic	Moisture=58.2%	Organic	Moisture=54.8%	No significant

2008 (13)	Tazovskiy Peninsula				soil	SOM=84.4% C=40.4% N=1.1%	soil	SOM=79.7% C=38.5%* N=1.3%*	differences in soil bacterial composition
					Mineral soil	Moisture=47.6% SOM=30.7% C=14.7% N=0.7%	Mineral soil	Moisture=50.1% SOM=36.1% C=16.9% N=0.9%	
Lamb *et al,* 2011 (16)	High Arctic tundra soils on Alexandra Fiord, Ellesmere Island, Nunavut			16 years	NA	NH_4= 63.2mg/kg NO_3= 26.7mg/kg PO_4= 0.53mg/kg SOC= 268.9mg/kg DON= 32.7mg/kg		NH_4= 65.9mg/kg NO_3= 23.7mg/kg PO_4= 1.09mg/kg* SOC= 303.9mg/kg DON= 37.7mg/kg	No change in soil microbial community or diversity of microbial functional groups
Clemmensen *et al.*, 2012 (169)	Arctic tundra ecosystems	Heath tundra near Abisko		14 years	+0.4-0.6°C	pH=7.1		NA	No change in fungal biomass with warming
		Tussock tundra (MAT) near Toolik Lake			+2.2°C	pH=5.0		NA	Increased fungal biomass
Walker *et al.*, 2008 (170)	High Arctic tundra soils on the East coast of Ellesmere	Lowland sites	Sedge Meadow	13 years	+0.7°C	pH range=surface soils-deeper layers		NA	Significantly different community structure and genotype

	Island, Nunavut					pH=6.6-5.9		richness for *nosZ* and *NifH*
			Cassiope Heath			pH=4.9-5.4	NA	No significant differences in *nosZ* or *NifH* communities
			Riverside Willow			Driest lowland pH=5.2-4.6	NA	Significantly different community structure and genotype richness of *NifH*
		Upland sites	Granitic		NA	pH=4.9-5.5	NA	Significantly different community structure; less significance for genotype richness (may be biologically significant) of *NifH*
			Dolomitic			pH=7.9	NA	Significantly different *nosZ* communities
Zhang *et al,* 2005	Tallgrass prairie at Great Plain Apiaries, near		Clipped	3 years	+2.7°C	NA	No effect on inorganic N	No significant changes

(171)	Norman OK			Unclipped		+1.8°C	NA	inorganic N decreased but not significant	Shift in microbial community structure with fungal dominance. Significant reduction in microbial metaboli potential
Penton *et al,* 2013 (172)	Oklahoma tallgrass prairie soil, Great Plain Apiaries site				>10 years	+1.8-2.7°C	NA	NA	No significant effect on the fungal community structure except for a few groups
	Alaskan permafrost, at Carbon in Permafrost Experimental Heating Research project (CiPEHR) in Eight Mile Lake, Alaska				1 year	+1.5°C	NA	NA	
Hayden *et al.*, 2012 (82)	Australian grassland soil, Pontville in south-eastern Tasmania	0-5cm soil depth	Ambient CO_2	C_4 grass	5 years	+2°C	NH_4^+=0.39 µg/g * NO_3^-= 2.57µg/g Mineral N=2.96 µg/g SOC=3.26% TN=0.24% C:N=13.84	NH_4^+=3.98 µg/g* NO_3^-=6.21 µg/g Mineral N=10.18 µg/g SOC=3.80% TN=0.27% C:N=14.31	Archaeal abundance was significantly affected by warming in C_4 grass and C_3 plants; total archaea increased in warmed C_4 grass and decreased in warmed C_3 plants. Fungal abundance increased with
				C_3 plants			NH_4^+=0.84 µg/g NO_3^-=1.22 µg/g Mineral N=2.06 µg/g*	NH_4^+=3.06 µg/g NO_3^-=2.19 µg/g Mineral N=5.24 µg/g*	

							SOC=2.51% TN=0.18% C:N=13.72	SOC=2.63% TN=0.19% C:N=13.82	warming and warming with elevated CO2. Bacterial abundance slightly decreased in response to warming and warming with elevated CO2. In general, bacterial diversity did not change under warming or warming with elevated CO2.
			Elevated CO_2	C_4 grass			NH_4^+=3.28 μg/g* NO_3^-=2.16 μg/g Mineral N=5.44 μg/g SOC=3.16% TN=0.22% C:N=14.28	NH_4^+=0.69 μg/g* NO_3^-=2.83 μg/g Mineral N=3.52 μg/g SOC=2.93% TN=0.21% C:N=13.95	
				C_3 plants			NH_4^+=0.66 μg/g NO_3^-=1.53 μg/g Mineral N=2.18 μg/g SOC=3.23% TN=0.22% C:N=14.62	NH_4^+=3.89 μg/g NO_3^-=4.74 μg/g Mineral N=8.62 μg/g SOC=3.16% TN=0.22% C:N=13.96	
		5-10cm soil depth	Ambient CO_2	C_4 grass			NH_4^+=2.85 μg/g NO_3^-=2.16 μg/g Mineral N=5.00 μg/g SOC=2.73%	NH_4^+=4.71 μg/g NO_3^-=3.70 μg/g Mineral N=8.41 μg/g SOC=2.98%	

						TN=0.20% C:N=13.71	TN=0.21% C:N=14.19	
				C_3 plants		NH_4^+=3.21 µg/g NO_3^-=2.32 µg/g Mineral N=5.54 µg/g SOC=1.88% TN=0.14% C:N=13.48	NH_4^+=4.75 µg/g NO_3^-=2.59 µg/g Mineral N=7.33 µg/g SOC=1.86% TN=0.13% C:N=14.00	
			Elevated CO_2	C_4 grass		NH_4^+=2.86 µg/g NO_3^-=0.36 µg/g Mineral N=3.22 µg/g SOC=2.46% TN=0.17% C:N=14.46	NH_4^+=2.86 µg/g NO_3^-=2.06 µg/g Mineral N=4.93 µg/g SOC=2.47% TN=0.18% C:N=13.71	
				C_3 plants		NH_4^+=4.03 µg/g NO_3^-=2.11 µg/g Mineral N=6.15 µg/g SOC=2.37% TN=0.16%	NH_4^+=5.33 µg/g NO_3^-=1.83 µg/g Mineral N=7.17 µg/g SOC=2.28% TN=0.17%	

						C:N=14.58	C:N=13.70	
Yergeau *et al.*, 2012 (173)	Sub-Antarctic	Falkland Islands	Vegetated	3 years	+0.7°C	SM=0.27% NH_4^+=24.5mg/kg NO_3^-=0.8mg/kg N=0.9%	SM=0.2% NH_4^+=21.6mg/kg NO_3^-=1.0mg/kg N=0.96%	Shift in microbial community composition and abundance; significant changes in functional genes
			Fell-field			SM=NA NH_4^+=15.7mg/kg NO_3^-=23.5mg/kg N=0.92%	SM=NA NH_4^+=19.3mg/kg NO_3^-=48.4mg/kg N=0.93%	
	Antarctic	Signy Island	Vegetated		+0.5-0.7°C	SM=0.17% NH_4^+=47.6mg/kg NO_3^-=4.2mg/kg N=1.94%	SM=0.17% NH_4^+=44.8mg/kg NO_3^-=3.0mg/kg N=1.96%	
			Fell-field			SM=0.07% NH_4^+=NA NO_3^-=NA N=NA	SM=0.06% NH_4^+=NA NO_3^-=NA N=NA	
		Anchorage Island	Vegetated		+0.6-1.5°C	SM=0.25% NH_4^+=146.9mg/kg	SM=0.26% NH_4^+=89.2mg/kg	

						NO_3^-=7.9mg/kg N=3.3%	NO_3^-=35.6mg/kg N=3.25%	
			Fell-field			NA	NA	
Weedon *et al.*, 2014 (174)	Sub-Arctic peat bog from the Abisko Scientific Research Station in Abisko, Sweden	Spring/summer		9 years	+0.6-1.0°C	Organic matter content>95% C=45%	NA	No significant effect of warming on potential activity or temperature sensitivity of hydrolytic soil enzymes
		Winter			+0.5-2.2°C		NA	

Based on our results and previous studies, significant warming can occur without a shift in the microbial community structure and function; however, once that warming exceeds the threshold needed to achieve significant shifts in soil edaphic parameters, either directly or through shifts in plant community composition, there will be an abrupt and dramatic shift in microbial community composition. Therefore, we should expect that there will be a lack of change in Arctic soil microbial communities until a "tipping point" is reached, at which point we should expect to see a dramatic change.

In contrast to previous studies (67, 71), our study found significantly higher taxon richness and overall diversity in the active layers than in the perennially frozen layers. In those studies, they did not differentiate between DNA from viable and non-viable cells. Thus, it is likely that the lack of difference in diversity measures between seasonally and perennially frozen soils was due to the inclusion of DNA from non-viable cells. When compared to untreated samples, the observed OTUs, estimated richness and diversity are lower after PMA treatment, indicating that previous studies overestimated the richness and diversity of microbial communities.

In untreated samples, the microbial communities clustered based on ice content, rather than age or geological epoch. One possible explanation for this correlation is that hydrolysis is the primary DNA degradation pathway in permafrost (175); thus, soils with higher water content prior to freezing would have higher DNA degradation. Untreated samples appear to cluster based on the level of degradation; thus, it appears that there is taxon-specific death and degradation. For example, the high ice content samples appear to have more sequences related to spore-forming strains than the lower ice content samples. Bacillales in particular were preserved better in high ice content environments

(176); other taxa including Rhizobiales, Solirubrobacterales, Clostridiales, Actinomycetales and Nitrospirales were preferentially lost in these environments. However, Clostridiales is primarily found in drier samples despite also being Gram-positive spore formers. The change in composition is most likely due to DNA degradation and not climate change since only water content was correlated with the untreated (with total DNA) samples, no soil edaphic parameters. On the other hand, treated (DNA from viable cells) samples showed different bacterial composition and diversity based on soil edaphic parameters that are altered by climate change.

A possible explanation for the clustering of H1 with the active layer samples is that it takes time for the viable bacterial community to change from an active one to a permafrost one; in this case, it took more than 8,100 years. At some point between 8,100 ya and 9,950 ya, the soil chemistry (TOC, TN, and pH) and microbial community shifted significantly. The mechanism of this shift is unclear. Another possible explanation could be that cryoturbation (the churning of soils by freezing and thawing) could have mixed the AL community down to the permafrost table where it froze along with the H1 sediments resulting in similar bacterial communities. Further investigation is required to test this theory.

In summary, the differentiation between DNA from viable and non-viable cells is important when looking at cold environments such as permafrost where DNA is preserved (110). Our optimized PMA protocol is not only useful for permafrost-affected environments but is also a very valuable asset for characterization of viable microbial communities in low cell abundance and low biological activity solid substrates such as other extreme systems (e.g. thermal or highly alkaline soil), contaminant-impacted soils,

and deep subsurface sediments. PMA treatments can also be used to detect survival following disinfection treatments in solid substrates such as food, wastewater, and manure.

In this study, the extant bacterial community was quite different from the bacterial community inferred from total DNA; thus, only a small percentage of taxa observed in total DNA will be important for carbon mineralization in melting permafrost. Contemporary warming of permafrost soils has failed to show significant changes in microbial community composition since climatic modulation of soil edaphic parameters drives microbial community composition. In a changing modern climate we are unlikely to see shifts in the microbial community structure unless there is a climate driven shift in the soil edaphic parameters; however, once that "tipping point" is reached, we predict a rapid and dramatic shift in Arctic soil bacterial community composition.

3.6: Acknowledgements

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CHAPTER 4: CONCLUSION

The Earth is currently undergoing a rapid shift in climate with the largest impact in polar regions. At the end of the Pleistocene, about 10,000 years ago, the Earth's climate system abruptly warmed, leading to a cascade of changes that drove large-scale extinctions and reorganization of ecosystems. We know a considerable amount about the adaptations, extirpations and extinctions of large mammals and plant communities at this boundary, but relatively little about the changes that took place at the microbial level; however, such impacts have important implications for feedbacks to the climate system, productivity in Arctic environments, and nutrient and carbon flux and dynamics (8). What little we know about permafrost microbial community responses to climate change is contradictory. Rapid shifts in the microbial community composition have been noted in some cases with laboratory simulated permafrost warming, but other findings have reported a lag or even a lack in response to climate warming in field based experiments. These contradictory observations could be due to the inability to differentiate between DNA from viable cells and preserved DNA from dead cells in field based experiments; whereas preserved DNA was metabolized by viable cells leading to the extraction of DNA from viable cells only in laboratory thaw experiments. Another possibility is the increased effects in soil edaphic parameters resulting from container effects in laboratory thaw experiments leading to a shift in the microbial community; conversely, the lack of a shift in soil edaphic parameters in field based experiments.

The purpose of this research is to use permafrost of Eastern Beringia as an archive of past northern environments and microbial diversity and use the changing community composition to determine how these communities adapted to abrupt climate change at the

end of the Pleistocene. These changes provide a compelling analogue for future changes in microbial communities with ongoing and future warming.

This research is the first to study and compare viable bacterial communities in permafrost based on permafrost age. We hypothesized that viable microbial communities from permafrost of a geological epoch would be more similar than between epochs due to climate's significant cumulative effect on soil edaphic parameters. To test this hypothesis, we examined the composition of extant bacterial communities in modern soils from the active layer, Holocene and Pleistocene-aged permafrost from the Klondike in central Yukon.

First, a method for distinguishing between DNA from viable and non-viable cells was optimized for permafrost-affected sediments using PMA treatments. No significant differences were observed for taxon richness and diversity between treated and untreated active layer samples. On the other hand, perennially frozen layers had significantly fewer taxon richness and diversity following treatment. Bacterial community composition was found to be distinct for the treated and untreated active and perennially frozen layers, with very few shared OTUs.

Second, viable bacterial communities of active, Holocene and Pleistocene perennially frozen sediments were compared to examine their response to the rapid warming that occurred at the end of the Pleistocene. This warming resulted in changes in soil edaphic parameters where soil pH became significantly more acidic in the Holocene with significantly higher TN, NO_3, NH_4, TOC and water content than in the Pleistocene. Gas flux measurements showed no correlations with untreated samples (i.e. total DNA) while CO_2 production significantly correlated with treated samples (i.e. DNA from viable

cells) with the modern samples showing the highest levels of CO_2 production upon permafrost thaw. As hypothesized, viable bacterial communities were observed to be more similar within an epoch than between epochs with active layer, Holocene and Pleistocene samples clustering separately from each other. Taking soil edaphic properties into account, it appears that this distinctive clustering is due to significant changes in soil properties due to abrupt climate change.

APPENDIX A

Although gas flux incubations and measurements were carried out, they did not clearly relate to the sequencing data. The methodology, results and discussion of the gas flux incubations are described below.

Gas flux measurements were carried out to determine rates of CO_2 production, aerobic CH_4 consumption, anaerobic CH_4 consumption and anaerobic CH_4 production in the active layers and Holocene and Pleistocene permafrost samples. Samples were obtained using a 10-inch drill press-DP102L drill (RYOBI, Anderson, SC, USA) by drilling through the center of the puck using a 1.7inch drill bit. Approximately 10g of intact frozen soil were added to autoclaved 160ml media bottles sealed with butyl septa caps. For the anaerobic incubations of CH_4 consumption and CH_4 production, the bottles were sparged with N_2 for 10 mins. For the aerobic and anaerobic CH_4 consumption bottles, 2ml (approximately 8.9×10^{-5} mol) CH_4 gas was added. The bottles were stored at -20°C until the time zero measurements were taken. The bottles were then incubated at 4°C in the dark for 6 months. CH_4 and CO_2 gases in the headspace were measured at time zero, 24hrs after incubation at 4°C and every 2 weeks by gas chromatography- flame ionization detector (Hewlett Packard 5890 Series II Gas Chromatograph, USA). If the

amount of CH_4 in the CH_4 consumption bottles dropped below 10 nmol, 100 nmol of CH_4 were added.

H3 showed anaerobic CH4 production (at a rate of 0.09 ± 0.02 nmol g sediment^{-1} day^{-1}). No other sample produced methane during the course of the experiment. No samples showed any anaerobic methane consumption.

Both active layer as well as H3, P1, and P3 all showed aerobic methane consumption. Surprisingly, H3 and P1 showed CH_4 consumption 25 and 40 days before AL1 and AL2, respectively. P1 showed the highest rate of CH_4 consumption (0.18 ±0.01 nmol/g/day) followed by H3 (0.15 ±0.01 nmol/g/day) then P3 (0.11 ±0.01 nmol/g/day) then AL1 and AL2 (0.08 ±0.003 nmol/g/day and 0.09 ±0.01 nmol/g/day respectively) (Table A1).

Table A1. Rates of aerobic CH_4 consumption and first day of activity for active layer (AL) and perennially frozen Holocene (H) and Pleistocene (P) layer samples.

Sample	First Day of Activity[a]	Rate (nmol/g/day)[b]
AL1	70	0.08 ± 0.003
AL2	70	0.09 ± 0.01
	120[c]	0.33 ± 0.01
H3	45	0.15 ± 0.01
	85[c]	0.17 ± 0.002
	150[d]	0.74 ± 0.02
P1	30	0.18 ± 0.01
	85[c]	0.26 ± 0.02
	120[d]	0.80 ± 0.03
	150[e]	1.20 ± 0.04
P3	70	0.11 ± 0.01
	120[c]	0.63 ± 0.02
	150[d]	0.88 ± 0.03

[a] the 1st day that CH_4 consumption was significantly higher than negative controls
[b] nmol of CH_4 produced per gram of sediment per day ± standard error
[c] 1st acclimation; CH_4 concentration was too low so 100 nmol CH_4 was added
[d] 2nd acclimation
[e] 3rd acclimation

CO_2 production rates were measured in samples incubated for anaerobic CH_4 consumption, anaerobic CH_4 production, aerobic CH_4 consumption and an experimental set with no modifications (Table A2). The aerobic CH_4 consumption and non-modified samples showed a similar trend of CO_2 production with a large drop in metabolic activity from active to perennially frozen layer, then a slight increase during the Holocene (H2), followed by continuous decrease in CO_2 production rates for older/deeper perennially frozen sediments. Anaerobic CH_4 consumption incubations also showed a big drop in CO_2 production rates from active to perennially frozen layer but then remained steady with deeper perennially frozen sediments. In the anaerobic CH_4 production incubations,

CO_2 production rates increased from active to H1 and then gradually decreased with deeper perennially frozen sediments.

Table A2. Rates of CO_2 production and first day of activity for all gas flux experiments for active layer (AL) and perennially frozen Holocene (H) and Pleistocene (P) layer samples.

Sample	CH4 Consumption				CH4 Production		Not modified	
	Aerobic		Anaerobic					
	First Day of Activity[a]	Rate (nmol/g/day)[b]	First Day of Activity[a]	Rate (nmol/g/day)[b]	First Day of Activity[a]	Rate (nmol/g/day)[b]	First Day of Activity[a]	Rate (nmol/g/day)[b]
AL1	30	0.36 ± 0.04	30	0.12 ± 0.03	7	0.47 ± 0.04	30	0.14 ± 0.01
AL2	7	1.74 ± 0.07	7	0.53 ± 0.02	7	0.47 ± 0.01	7	1.38 ± 0.31
H1	30	0.15 ± 0.01	7	0.07 ± 0.004	120	1.06 ± 0.06	0	0
H2	1	0.56 ± 0.02	1	0.17 ± 0.01	7	0.88 ± 0.05	7	0.49 ± 0.10
H3	30	0.54 ± 0.02	7	0.10 ± 0.04	0	0	30	0.28 ± 0.07
P1	30	0.69 ± 0.06	30	0.11 ± 0.003	7	0.25 ± 0.01	7	0.16 ± 0.01
P2	30	0.17 ± 0.02	30	0.06 ± 0.004	7	0.35 ± 0.01	7	0.12 ± 0.04
P3	30	0.65 ± 0.08	30	0.15 ± 0.002	7	0.21 ± 0.0002	7	0.24 ± 0.02

[a] the 1st day that CH_4 consumption was significantly higher than negative controls
[b] nmol of CH_4 produced per gram of sediment per day ± standard error

No correlations between gas flux measurements and untreated samples were observed. For PMA treated samples, only CO_2 production significantly correlated with AL1, AL2 and H1 samples (data not shown).

The sudden burst of CH_4 observed in H3 on day 1 could have been due to the release of trapped CH_4 in permafrost as previously observed (9). The continuous increase in the rate of CH_4 production could be due to the sample's very high ice content that

could have resulted in better anaerobic conditions despite the fact that the bottles were sparged with N_2 to simulate an anaerobic environment or due to a high and continuously increasing abundance of methanogens.

Our samples did not show any anaerobic CH_4 oxidation indicating the absence of anaerobic methanotrophic archaea and bacteria. This could be due to low concentrations of electron acceptors such as sulphate, nitrate, nitrite and ferric iron whose reduction is coupled with anaerobic CH_4 oxidation (177). Similar to previous studies, aerobic CH_4 oxidation was detected in a few samples with the perennially frozen sediments showing higher rates of aerobic CH_4 oxidation than the active layer (9). This observation could be due to higher anaerobic methanotroph abundance in the perennially frozen sediments despite the fact that previous studies have shown higher abundances in the active layer (62). In permafrost, methanotrophic bacteria are mostly responsible for methane oxidation (17).

The big drop in the rate of CO_2 production from active sediments to perennially frozen sediments for the aerobic and anaerobic CH_4 consumption and non-modified test samples has been previously observed (9) since perennially frozen layers have much lower microbial abundance due to long-term exposure to low temperatures, low nutrient availability and low water content (48, 62) and available TOC for microbial respiration. The decrease in CO_2 production rates with deeper/older perennially frozen sediments could also be attributed to the continuous decrease in TOC in deeper sediments. Perennially frozen sediments could have shown higher or equivalent rates of CO_2 production than active layer sediments in anaerobic conditions in the absence of added CH_4 (anaerobic CH_4 production test samples) due to a higher abundance/diversity of

microbes adapted to anaerobic conditions such as permafrost unlike the active layer aerated sediments (17, 178). Acetoclastic (ferment acetate to CH_4 and CO_2) and hydrogenotrophic (use hydrogen to reduce CO_2 to CH_4) methanogens have been previously identified at high abundances in Arctic permafrost (17, 78, 179, 180). The anaerobic CH_4 production H3 sample was the only one to show CH_4 production but no CO_2 production probably due to the absence of acetoclastic methanogens or due to a higher abundance of hydrogenotrophic over acetoclastic methanogens. Further research is required to confirm these predications and to distinguish between the different methanogenic and methanotrophic pathways to better predict CH_4 and CO_2 proportions and fluxes (17, 181).

CO_2 production could have been highest in group1 samples due to high levels of TOC, microbial diversity and activity in the active layers. Although only CO_2 production showed any correlation with the samples, it is too early to deduce anything without further studying the bacterial community composition following permafrost thaw.

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