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Microbial responses and contributions to climate change in Greenland

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Microbial Responses and Contributions to Climate Change in Greenland

By

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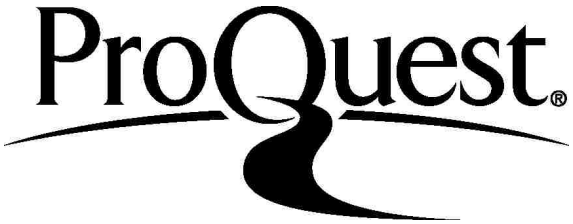

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Microbial Responses and Contributions to Climate Change in Greenland

Chairperson: James E. Gannon, Ph.D.

Despite the fact that microorganisms are the major drivers of global biogeochemical cycles, the relationship of microbial community activity and greenhouse gas production is still largely unexplored. The body of work presented here identifies previously unknown microbial community structure in bare ice, tracks shifts in permafrost active layer microbial communities along a moisture gradient, examines microbial trends throughout the Arctic growth season under climate change scenarios, and goes beyond identifying organisms working to link the structure and function of microbial communities to process level measurements. With deep sequencing of 16S rRNA, this study determined that bare ice collected from the Greenland Ice Sheet contains similar phyla to what has been detected on snow and cryoconite holes. Surprising results from this data set reveled extreme heterogeneity in ice samples even on a relatively small scale of 40 meters. In Zackenberg, GL permafrost active layer samples were collected from a soil moisture gradient. High throughput 16S rRNA sequencing revealed that moist active layer communities are more similar to dry active layer communities than those detected in fen samples. The fen samples were the only samples exhibiting net methane emissions. Given the microbial data, the permafrost in this area would have to collapse and form wetlands in order to become a likely methane source. To better understand microbial responses to climate change scenarios, communities were studied throughout the Arctic growth season on Disko Island, GL under increased snow accumulation and soil warming manipulations *in situ*. Phylogenetic and linear discriminant analyses of 16S rRNA genes and transcripts revealed microbial community succession with seasonal trends and the susceptibility of microbial community structure to increased soil warming and snow accumulation. Additionally, quantitative PCR of key functional genes illustrated that the activity of methane and nitrogen cycling organisms varied seasonally. The activity of methane cyclers corresponded to the peak in methane oxidation observed during the Arctic summer. The activity of nitrogen cyclers correlated to measured N pools. This work represents initial steps in developing a framework that links microbial community structure and activity *in situ* to biogeochemical cycles in the Arctic.

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CHAPTER ONE

The Uncertainty of Carbon Sources and Sinks: Environmental Disruptions in Soil Moisture Pulsing in Permafrost May Impact Microbial Communities and Methane Emissions

ABSTRACT

It remains a difficult challenge predicting carbon sources and carbon sinks. Permafrost has historically been considered a carbon sink, however, with recent and increased permafrost thaw due to climate change, the fate of carbon storage in this ecosystem remains unclear. Evidence gleaned from similar pulsing ecosystems may shed light on possible outcomes of permafrost thaw. It seems probable that pulses of soil moisture largely contribute to microbial activity in permafrost. Understanding how these soil moisture pulses impact methanogen, methanotroph, and methylotroph activity will allow for better predictions relative to net methane flux emissions from permafrost. This review discusses a possible "pulsing ecosystem" model that assists in designing experiments to better understand the relationship between seasonal soil moisture pulses, microbial activity, and net green house gas emissions from thawing permafrost.

INTRODUCTION: THE STATE OF CURRENT CLIMATE CHANGE MODELS

In order to predict net greenhouse gas emissions, sources and sinks of relevant gases need to be determined. Unfortunately, this remains a difficult challenge as climate change continues. For instance, increased air temperatures in the arctic may induce a shift from arctic tundra as a carbon sink to a carbon source (1). This unpredictability is in part due to the high complexity of environmental variables impacting the nature of these

carbon sources and sinks and their role in the global carbon cycle. Due to their complexity, the same variables are often left out of current climate change models.

For example, the global nutrient cycles including the carbon, nitrogen, and phosphorus cycles are all tightly intertwined, each impacting the other. However, the nitrogen and phosphorus cycles are not always considered in climate change models (2). Further, a large driver in the progression of all biogeochemical cycles is microbial activity. No climate change models to date attempt to integrate microbial activity to make more accurate predictions.

The relationship of microbial community activity and greenhouse gas production is still largely unknown (3). In order for this information to be appropriately incorporated into models, more work needs to be done to determine which microbes impact certain cycles, how the shifts in abundance and activity of these microbes impact the biogeochemical cycle directly, and show that the impact leads to a negative or positive net flux of greenhouse gases. This review addresses one possible conceptual model that assists in designing experiments to improve predictability of green house gas emission models in thawing permafrost.

PERMAFROST AND METHANE EMISSIONS

Methane is extremely efficient at absorbing radiation, making it a potent greenhouse gas. It is nearly 25 times more potent than carbon dioxide (4). This has made methane a growing public concern and the focus of much current climate change research (5, 6). This greenhouse gas is emitted from both anthropogenic sources and natural

sources, including rice paddies, wetlands, cow flatulent, and waste-water treatment plants.

Permafrost is frozen artic and alpine soil that naturally emits methane. Large amounts of carbon are stored in the permafrost layer which remains frozen year long; approximately 50% of the global below ground carbon pool may be contained in permafrost (7). This attribute makes permafrost largely a carbon sink, however it remains uncertain if this will be sustained as climate change progresses. Arctic environments are much more sensitive to changes in climate and are predicted to warm much more rapidly than any other environment (8).

The surface and near-surface soil in permafrost thaws seasonally; this portion of the permafrost is termed the active layer. The seasonal thaw of the active layer is normal, however, due to increases in air temperature from climate change the active layer is growing deeper and deeper every year (9, 10). In a twelve-year study in north-east Greenland, the active layer was found to be increasing at a rate greater than one centimeter per year (11). This means that the carbon once stored in this frozen soil, is being made available for decomposition by the microbial community.

A positive feedback loop between thawing permafrost and climate change is quickly developing (Fig 1.). As the active layer becomes increasingly deeper, the microbial community becomes more active and produces more carbon dioxide, methane, and other greenhouse gases. These gases will contribute to climate change, further increasing the air temperature in the Arctic, thus perpetuating the spiraling loop. Additionally, current studies suggest that the internal heat produced by the dormant

microbes would further the thaw of the active layer, again contributing to the spiraling nature of the positive feedback loop (12-14).

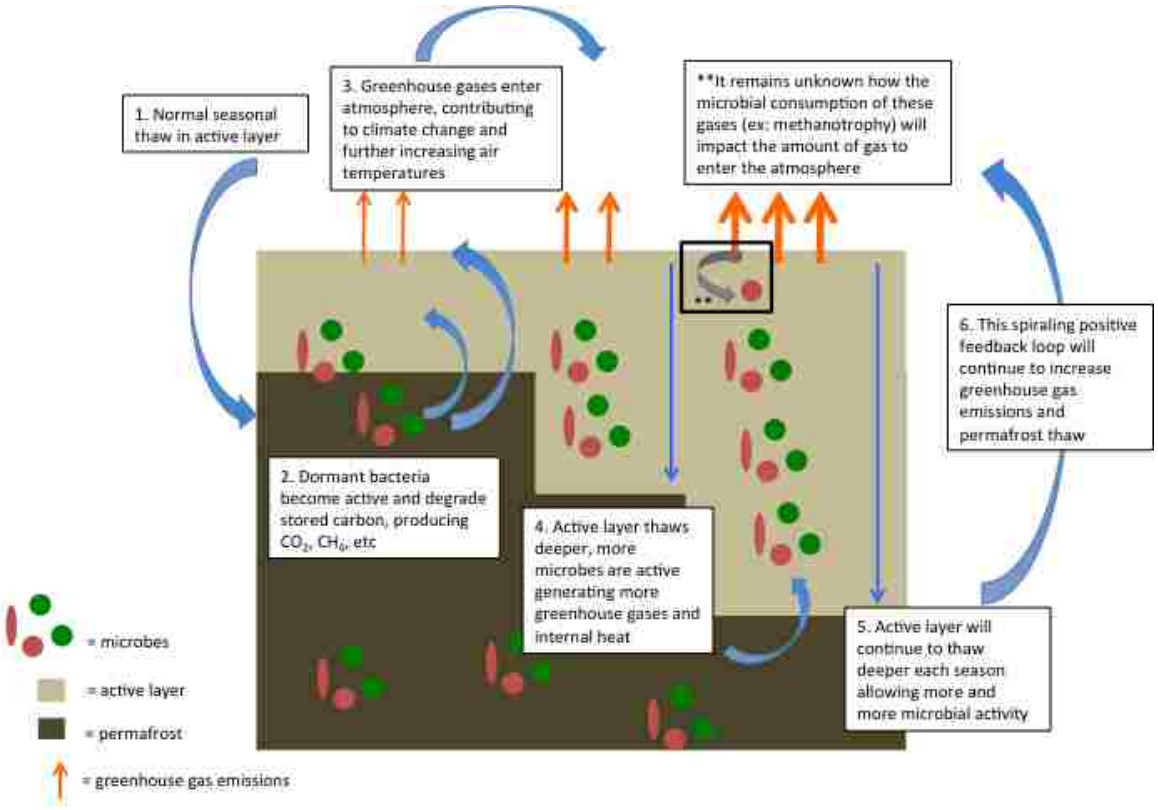

Figure 1. A positive, spiraling feedback loop between permafrost thaw and climate change. As Arctic air temperatures rise, the active layer in permafrost will become increasingly deeper allowing the microbial community to consume previously stored carbon and create greenhouse gases as a by-product.

MICROBIAL ACTIVITY AND NET METHANE FLUX

As stated previously, microbial activity is still largely ignored in climate change predictions. Many studies are underway that examine the microbial communities in the arctic, however much data is needed to inform predictive climate models.

In order to understand the contributions microbial activity has on the net flux of greenhouse gases entering the atmosphere from thawing permafrost, a substantial amount of research is required in order to understand the balance between microbes producing

these gases and microbes that are capable of metabolizing these gases (Fig 1). This relationship between microbial production and consumption of greenhouse gases and the impact this relationship has on net greenhouse gas flux is largely understudied (3). For example, the net flux of methane depends on the balance of activity between methanogens and methanotrophs (15). This balance is impacted by many environmental variables and studies reveal that it is not a linear relationship (16, 17). In order to accurately predict methane fluxes from thawing permafrost, methylotrophs and methanogens, need to be studied extensively *in situ.*

Studies investigating the methane cycle in permafrost tend to focus on methanogens. Despite the current work, the activity of methanotrophs and methylotrophs *in situ* is vaguely understood (18). Studies that attempt to examine both methanogens and methanotrophs have utilized quantitative PCR, which may not be capturing the whole community of methanotrophs. Some organisms, such as *Methylocella,* lack the commonly targeted particulate methane oxidase (*pmoA*) gene (15). Additionally, a studying employing metagenomic sequencing found that *pmoA* made up 80% of the methane oxidation genes but the other 20% were variations of soluble methane oxidase gene (*mmoX*), illustrating that relying solely on *pmoA* does not capture the complete methane oxidase potential (19). Further, these studies tend to rely on a relatively low number of samples collected from one time point and extracted from the field to be examined in the lab. These are extremely valuable studies, however, removing the samples from the environment to do experimental incubations prevents exposure to natural variables such as seasonal soil moisture fluctuations.

Soil moisture is likely to be a key variable impacting methane cycling organisms and should not be ignored. Unfortunately, because of the multitude of environmental variables it can be difficult to define the most informative field studies for microbial focused research. This review attempts to present a unique "pulsing" view of permafrost such that data may be informative for climate change models. A pulsing ecosystem seems a more useful model for predicting changes in permafrost microbial communities, as the normal variations in permafrost soil moisture are likely to change with the progression of climate change. The patterns seen in methane cycling organisms in other pulsing ecosystems may shed light on or allow for predictions to be made as the permafrost ecosystem responds to climate change.

IMPORTANCE OF PULSING ECOSYSTEMS

Pulsing ecosystems, like wetlands, are ecosystems that have fluctuating hydrology. These fluctuations may either be daily or seasonal. Additionally, pulses may reflect the true steady state of an ecosystem. It might be that ecosystem performance is best or enhanced when this natural pulsing is in place, as pulses lead to fluctuations in production and consumption in the community (20, 21).

This idea of a productive, pulsing ecosystem has gained attention in the ecological engineering community. In general, the pulsing water flow in wetlands lead to a fringe zone that has higher oxygen content, promoting methane oxidation. A static wetland lacks this fringe zone and may lead to a decreased occurrence of methane oxidation (22). When designing wetlands as wastewater treatment facilities or restoring natural wetlands,

it may be ideal to restore them in such a way to promote pulsing. The natural pulsing may decrease the amount of methane emissions by promoting methane oxidation.

It is known that fluctuations in water level impact the availability of oxygen in soils, and thus has an effect on the activity of microbes that are linked to nutrient cycling (23-25). Generally, it has been noted that static wetlands emit more methane than pulsing wetlands (22). These key concepts that have attracted environmental engineers may also be useful to scientists studying thawing permafrost. Understanding this pulsing nature of ecosystems as well as the microbial communities within will be helpful in predicting greenhouse gas emissions from permafrost.

PERMAFROST AS A PULSING ECOSYSTEM

The seasonal pulsing soil moisture in permafrost can be considered a pulsing ecosystem. Throughout the winter in the arctic, all soil moisture in permafrost is frozen. However, once the spring season starts, snow begins to melt and the active layer of permafrost begins to thaw. This leads to a rapid increase in soil moisture in the active layer. As spring progresses to summer, snow completely melts, the active layer may reach its final depth, and glacial run-off is at its maximum. This is when soil moisture is the highest. However, the warm summer temperatures around 17°C are likely to start drying the active layer once snow and glacial melt ceases. Once the daylight starts decreasing and the Arctic enters the fall season, the soil moisture once again freezes. Permafrost is an example of an ecosystem with both a very condensed growth season and condensed moisture pulses (Fig 2, Box A).

During the pulse of soil moisture, microbial activity is likely to increase dramatically. It has been shown that active microbes persist in frozen permafrost, however activity is the greatest in the active layer (26). If microbial activity is linked to the pulse of soil moisture and if the pulse changes with climate change then microbial activity will change with it. Figure 2 illustrates some potential shifts in soil moisture pulsing that could occur as climate change progresses. For instance, as air temperature continues to rise in the Arctic, spring may arrive earlier along with earlier snow and glacial melt. The same amount of snow and glacial run-off may persist but may enter the ecosystem at a slower rate (Fig 2, Box B). Under these conditions microbial activity in the active layer of permafrost may last longer than under the normal conditions displayed in Box A. This pattern (Box B) may continue until permafrost does not thaw completely each winter, and either some soil moisture will persist in the environment all year resulting in a year long active microbial community or the permafrost may completely dry out (Fig 2, Box C).

Understanding the normal pattern of soil moisture pulsing in permafrost and how microbial communities and activities are tied to this pulse will allow for better predictions for how microbial communities will respond to climate change. Additionally, integrating what is observed in other pulsing ecosystems will help to substantiate these predictions.

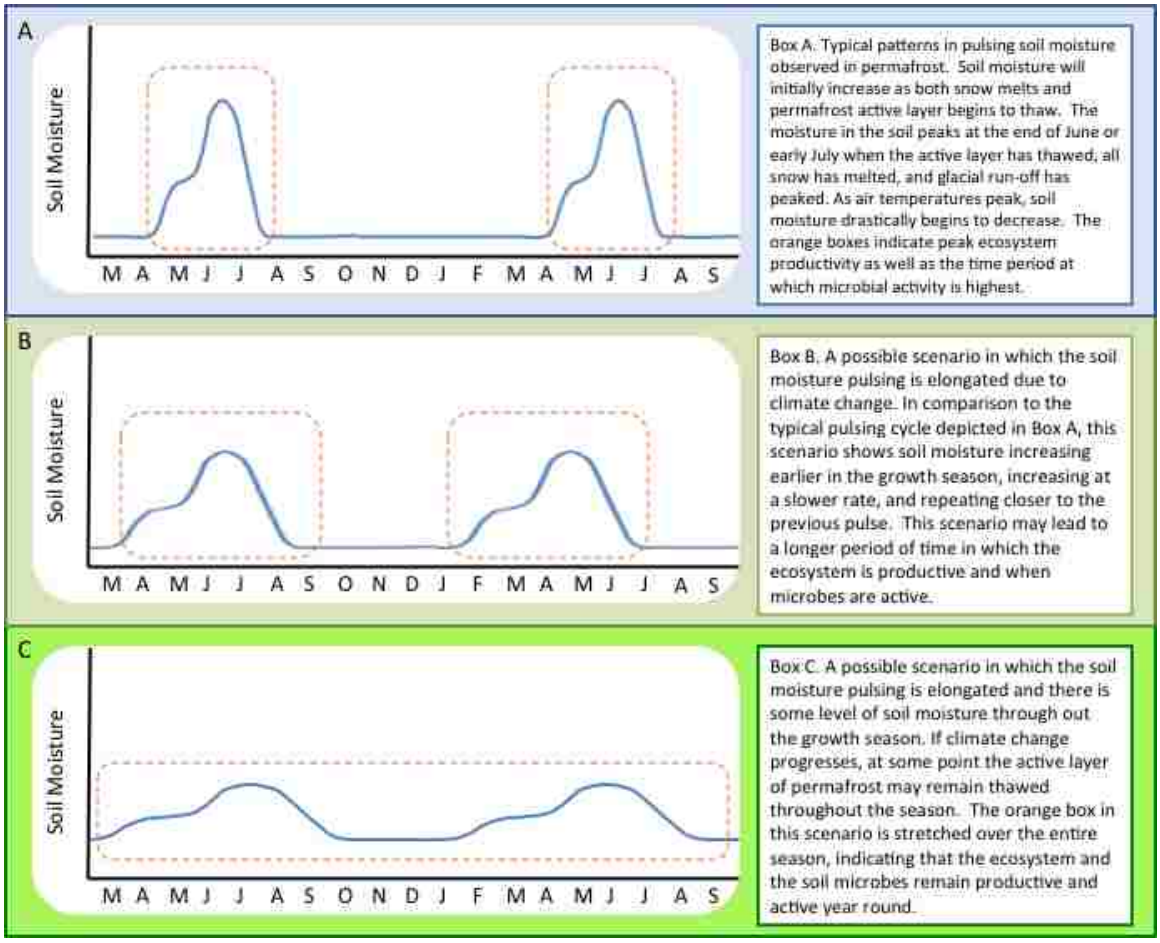

Figure 2. Conceptual projections of soil moisture trends as climate change progresses in the Arctic. Box A illustrates the normal pulse of soil moisture observed in permafrost ecosystems. Box B illustrates one scenario where the pulsing becomes closer together as the growing season elongates. Box C illustrates a long-term scenario where permafrost no longer freezes and some moisture persists in the system permanently.

METHANOGENS AND METHANOTROPHS

Before previous results from similar pulsing ecosystems are integrated into these scenarios, it is important to have some understanding of the target organisms. This review focuses on methane emissions and thus methanogens and methanotrophs. Methanogens are archaea that produce methane and are grouped by terminal electron donors. Possible electron donors include hydrogen, acetate, formate, methanol, methylamines, dimethylsulfur, and alcohols (27). Hydrogenotrophy and acetotrophy are

the most common methanogenic pathways (22). Hydrogenotrophic methanogens use hydrogen as a reducing agent and carbon dioxide as both a carbon source and an electron acceptor. Where as, acetoclastic methanogens convert acetate to methane. Both hydrogenotrophic and acetoclastic methanogens have been detected in active layer permafrost (15).

Methane oxidizing bacteria are both ubiquitous in soil and may be capable of oxidizing up to 90% of the methane emitted from methanogens, making methanotrophs a key regulator of methane emissions (28-32). Methanotrophs are specialized methylotrophs and are differentiated based how they assimilate carbon (33). Type I methanotrophs include organisms from the Gamma-proteobacteria class and type II methanotrophs include organisms from the Alphaproteobacteria class (34). Type I methanotrophs utilize the ribulose monophosphate (RuMP) pathway and are generally more efficient at assimilating carbon than the type II methanotrophs which use the serine pathway (27). Type I methanotrophs are most typically found in the Arctic (16, 35).

Furthermore, there are some methanotrophs that exhibit high affinity for methane and those that have low affinity for methane. Low affinity methane oxidation will occur in areas with an excess of available methane. Type II methanotrophs have lower affinity for methane and require high concentrations of methane (36). Some type I methanotrophs are also low affinity methane oxidizers and in general the organisms responsible for low affinity oxidation are not as well understood (22). Peaks in soil moisture within permafrost may promote methanogenesis and thus a high availability of methane, possibly increasing the of abundance type II methanotrophs.

The first step of methanotrophy involves the enzymes particulate monooxygenase (*pMMO*) or soluble cytoplasmic monooxygenase (*sMMO/mmoX*), which catalyze the reaction from methane to methanol. The particulate monooxygenase is much more common in methanotrophs, and so is the target gene of many studies, but not all methanotrophs have pMMO, such as *Methylocella* (37). Additionally, some methanotrophs are facultative and do not solely utilize methane as a carbon source. *Methylocella* is a facultative methanotroph and can use acetate, ethanol, and methane as a carbon source (37). This raises the importance of studying the methylotroph group as a whole when investigating the methane cycle in permafrost.

A linear relationship between methanotroph and methanogen activity may not exist in part due to the complexities in the methane cycle, or in any nutrient cycle. The formation or consumption of methane is not completed in one simple step. Strict methanotrophs can utilize methane as a carbon source and reduce methane to methanol. But facultative methanotrophs and general methylotrophs may carry out the bulk of the methane cycle because they can utilize any one-carbon molecule as an energy source, methane included, but are not restricted to methane. This means methylotrophs are also capable of converting methanol to formaldehyde, formaldehyde to formate, formate to carbon dioxide, carbon dioxide to formyl, formyl to methylene, and methylene to general methyl groups. Methanogens are then able to utilize methylene and methyl groups to create methane. These factors can complicate studying methanotrophs. It may be that a deep 16S high throughput sequencing effort or metagenomic sequencing is the best way to quantify all methanotrophs in a given sample. Many current studies exclude general

methylotrophs and focus on strict methanotrophs. In doing so, results in an incomplete view of the methane budget.

Beyond these two main groups of methanotrophs, it is believed that more unknown methanotrophs exist. More recently identified methanotrophs that do not fit into these two groups are sometimes called type X methanotrophs and are typically *Verrucomicrobia*. New denitrifying ammonia oxidizers that have been discovered may be capable of methanotrophy (38). Methane oxidation is primarily aerobic, however, anaerobic methane oxidation does occur but little is known about this pathway and it is this pathway that the ammonia oxidizers likely employ (22, 38).

Experiments have revealed that methane oxidation is controlled by temperature, pH, oxygen concentrations, ammonium, ammonia, and the variable that impacts methanotrophs most is likely methane concentration (39, 40). Because methanogenesis is strictly anaerobic, oxygen and water levels may be the variables that impact methanogens the most (41). It is clear that in order to understand net methane emissions from permafrost methanogens and methanotrophs need to be identified and their activity in response to changing methane concentrations, oxygen concentrations, and soil moisture needs to be quantified.

WHAT WE KNOW FROM OTHER ENVIRONMENTS

Applying patterns in methanotroph and methanogen activity discovered in other pulsing environments will help to make predictions as to what may happen in permafrost under the climate change scenarios depicted in Figure 2. In general, fluctuating soil moisture may increase the concentration of oxygen in the soil and help to decrease

methane emissions. Examining other pulsing ecosystems in detail provides evidence for this assumption.

Rice fields were once irrigated by means of year long flooding, but a transition was made by farmers to use pulsing water flow. Methane emissions from rice fields decreased from 1960 to 1970, likely due to this shift in continuous flooding to mid season drainage (42). Decreased methane emissions have been measured in many pulsing rice fields, confirming that this pulsing soil moisture may help to decrease methane emissions (43). These drastic shifts observed in rice field methane emissions sets the stage for what may be observed in other pulsing ecosystems.

Natural wetlands and constructed wetlands also contribute a significant amount of methane to the atmosphere (44). Temperature and seasonal variations impact methane emissions from wetlands. Higher temperatures may enhance both methane oxidation and methanogenesis (45). Higher temperatures may be reducing methane solubility. However, temperature and seasonal variations may be confounded with soil moisture. For example, increased methane oxidation rates in summer months may be due to drier soil and not actually higher temperatures (46). It is hard to tease apart these variables, and this is why more field research is required to fully understand these ecosystems.

Pulsing soil moisture may be a key factor to consider when studying methane cycling organisms *in situ.* It has been found that methanotroph activity and methane oxidation rates are highest at the interface of oxic and anoxic layers in wetlands (35, 47). Small decreases in water level decrease dissolved oxygen and decrease methanotroph activity (48). Water table level is a large factor impacting methane emissions from wetlands (49, 50). Because wetlands have high amounts of methane available, methane

oxidation is carried out by organisms with low-affinity towards methane (22). Methane concentrations controlled methanotrophy more than any other environmental characteristic in one wetland ecosystem (18).

The pulsing ecosystems may not only promote methanotroph activity, but it might also increase methanotroph biomass. In a study comparing permanently flooded wetlands to intermittently flooded wetlands it was found that methanotroph abundance was highest in the surface soil of the pulsing wetland (18). This study concluded that seasonal pulsing enhanced diffusion of oxygen and lowered methanogenesis (18). This trend was observed in flooded forest soils in Norway; it was noted that an increase in water content decreased the abundance of methanotrophs (51).

Research in wetland ecosystems may also allow us to make predictions as to what kind of methanotrophs may be found. Type I methanotrophs are more common in the surface layers where oxygen concentrations are higher and type II methanotrophs are found deeper in sediment where methane concentrations are higher (52).

METHANE CYCLING LINKED TO OTHER NUTRIENT CYCLES

The trends observed in pulsing ecosystems have illustrated the impact pulsing soil moisture will have on methane cycling organisms. Work in these areas also illustrate how tightly linked the methane cycle is with other nutrient cycles. It will be important to understand these trends in the context of other nutrient cycles in the permafrost ecosystem.

Many studies have found that ammonia and ammonium can inhibit methanotrophs from oxidizing methane (51, 53-56). This is likely due to the similar size of methane and

ammonia (55). Similarities in physiology promote competition between methane and ammonia oxidizing bacteria (57). This could be an important pattern to take into account for predictive purposes and possibly even management strategies.

Rice fields have allowed for widespread studies exposing how an increase in organic input may impact methane emissions. As this is an agricultural wetland, fertilizer is frequently used to increase rice yields. It has been shown that high organic input increases methane output from rice soils (58, 59).

Nitrite-dependent anaerobic methane oxidation may be a possible methane sink in wetlands, and has only been recently studied in this ecosystem (38). This may be an important link between the methane and nitrogen cycle. It has not yet been studied in permafrost. Additionally, it was found that the addition of nitrate may initiate this metabolic process and increase carbon dioxide emissions while decreasing methane emissions (38). Other studies have found evidence for anaerobic methane oxidation (18). Furthermore, any addition of nitrogen may increase methane emissions (60-62). It is clear that nitrogen cycling is interrelated with methane production in wetlands (63). In order to get a complete understanding of methane emissions in permafrost it is evident that further disentangling of these nutrient cycles will be required in order to understand and predict the activity of methanogens, methanotrophs, and methylotrophs in permafrost.

CURRENT KNOWLEDGE OF THE METHANE CYCLE IN PERMAFROST

Most studies investigating the microbial community responsible for methane cycling in permafrost have relied on culture based methods, qPCR of *pmoA* and methyl

coenzyme M reductase (*mcrA*) genes, small sample size or samples from one time point, and 16S rRNA studies with low amounts of reads. Current sequencing technology needs to be combined with dynamic and thorough sampling schemes in order to better observe microbial responses to changes and disturbances in permafrost.

However, early permafrost community studies do help to elucidate basic patterns observed in permafrost. For example, one study examining one 2 meter permafrost core split into an active layer sample and permafrost sample, found that both permafrost and the active layer were relatively similar based on metagenomic 16S rRNA assignments and qPCR targeting *mcrA, pmoA,* ammonia monooxygenase (*amoA*)*,* and nitrogenase (*nifH*)(64). Another study examining the same functional genes found that they were all present and abundant in all permafrost samples collected (65). The same study collected samples at varying depths in the permafrost, but the only gene that illustrated a significant decrease with depth was *nifH (65)*.

Additionally, many studies find that the active layer is slightly more diverse than the permafrost (66, 67). But, after two days of thaw permafrost microbial communities start to resemble the active layer communities (26). These early studies have also revealed major phyla present in this soil type. Acidobacteria, Actinobacteria, Bacteroidetes, and Proteobacteria tend to be the major phyla detected in both active layer and permafrost samples (19, 66-68). Yet, the taxonomic portion of studies did not go much further than quantifying who is there at an extremely coarse level. Making observations about who is there, at a much more specific identification level, and how these communities change under certain variables would reveal more information relative to the cycling process.

More recently, research groups have started to focus more on key organisms related to the methane cycle. For example, methanogens have been found to increase in diversity after permafrost thaw in lab incubations (19, 69). However, one of these studies using qPCR found that the *mcrA* gene did not increase in abundance after permafrost thaw (19). Increasing the sample size may help to elucidate these patterns in the future. Another study found that thawing permafrost lead to a shift from hydrogenotrophic methanogenic pathway to acetoclastic methanogenesis (70). As previously stated, type I methanotrophs are the most commonly detected methane oxidizing bacteria in the Arctic (16, 35). Type II methanotrophs are also detected in permafrost, and these organisms as well as the *pmoA* gene were found to increase in abundance after permafrost thaw under lab conditions (19).

Currently, as techniques are advancing, more evidence is building to support the importance of soil moisture and local hydrology as key factors in determining the microbial community structure. It has been found that the water tracks surrounding tundra permafrost polygons have a higher abundance of cyanobacteria than the drier internal permafrost (71). Additionally, fires in northern Alaska have decreased the permafrost soil moisture leading to a decrease in microbial diversity (72).

Even at the process-level, permafrost studies have had a limited scope and tend to focus solely on saturated permafrost as this is where high methane flux occurs (73). A study investigating fens in Alaska found that fens with a higher water table had higher methane flux than a fen with a lower water table (74). This study also tested the impact of soil warming on methane flux, but the water table level impacted methane flux more intensely (74). These results are similar to what has been observed in other pulsing

ecosystems and confirm the possibility of extrapolating observations from pulsing ecosystems in more temperate regions to those in Arctic regions.

It is clear that many gaps need to be filled. These process level observations need to be better linked to microbial community dynamics. A better comparison between well drained and saturated permafrost microbial communities needs to be established. In addition to this, microbial community shifts due to short-term variations in soil moisture and small pockets or microenvironments consisting of high soil moisture need to be examined. Essentially, the effects of soil moisture need to be studied at a much finer scale in order to make predictions on a broad scale across permafrost types.

THE FUTURE OF PERMAFROST

The patterns observed in other pulsing ecosystems allows for predictions to be made about trends in methanogens and methanotrophs under normal soil moisture pulsing and altered soil moisture pulsing in permafrost (Fig 2). Having a more complete understanding of the microbial community in steady state soil moisture pulsing of permafrost will allow for a stronger basis to make predictions (Fig 2, Box A). It is unknown how microbial communities will shift if the growth season elongates in the arctic and soil moisture pulsing becomes dampened and the pulses occur closer together (Fig 2, Box B). If climate change progresses at the rate predicted, permafrost could be completely lost. This could lead to a scenario where there is some soil moisture present year long, with little to no pulsing (Fig 2, Box C). Once permafrost is lost and there is little moisture pulsing, the ecosystem could completely dry out. In order to predict how methane flux will change as a result of these climate change scenarios, it is pertinent to

understand how the microbial communities will respond to these changes, and applying knowledge from other pulsing ecosystems will provide guidance.

It is clear that there are seasonal dynamics observed in wetlands and rice fields (75). Methanogens and methanotrophs are likely to shift seasonally in permafrost, as well. Seasonal dynamics are a conglomerate of different variables ranging from temperature variations, shifts in organic input, variations in CO_2, and shifts in soil moisture. The fact that additions of nitrogen in rice paddies and wetlands increase methane flux support the idea that some variations in permafrost may be a reflection of organic input into the permafrost ecosystem. However, the extensive amount of support showing the impact water fluxes have on rice fields and wetlands may suggest that studying soil moisture shifts in permafrost will reveal more about the microbial community.

Generally, in soil environments, methane migrates upwards whereas oxygen migrates downwards (76). And so methanotrophs are likely to be located in the oxic surface layer (35). Methanogens will reside lower in anoxic sediment layers. Because of this, methylotrophs are likely to be detected closer to the surface of the active layer, where as methanogens will be found deeper where oxygen is absent. It may be possible to use methanotrophs as indicators of likely areas of net positive methane emissions in permafrost. Type I methanotrophs are more common in the surface layers where oxygen concentrations are higher and type II methanotrophs are found deeper in sediment where methane concentrations are higher (52). It is also known from other studies that more type II methanotrophs have a low affinity of methane and only persist in areas of high

methane concentration. Perhaps type II methanotrophs could be used to indicate areas of potential net positive methane emissions from permafrost.

If soil moisture pulsing begins to decrease in amplitude and if the pulses occur closer together, this is likely to prolong microbial activity. However, its difficult to ascertain if this will promote methanogen activity and decrease methanotroph activity. Its been shown that the draw down in water table aerates wetland areas, allowing for methanotrophic activity. If the soil moisture pulses decrease in amplitude, it may decrease the amount of oxygen in the active layer and methanotrophic activity could decrease. The prolonged presence of moisture may create more anaerobic microsites in the soil, promoting methanogenic activity.

If the dampening of moisture pulsing continues to such an extent that the area becomes permanently flooded, resembling a fen, this will increase methane emissions. With more methane available in the soil, type II methanotrophs may increase in abundance. However, if the dampening of moisture pulsing leads to an eventual drying out of the permafrost system, respiration may increase and carbon dioxide emissions will increase and methane emissions will decrease (77, 78). Carbon dioxide is a less potent greenhouse gas, and so Arctic warming may not accelerate as quickly as when there is net methane production. Drying of soil will lead to soil compaction, which may decrease oxygen in the soil and in turn decrease methanotrophic activity.

In wetlands closer to the Arctic, methanotroph diversity was found to be much lower than the diversity observed in rice paddies (35). The microbial communities in the active layer of permafrost are exposed to more changing conditions than communities located deeper in permafrost. This may make the active layer community more variable

and less stable (15). The low diversity of methanotrophs discovered in wetlands near the Arctic may suggest that these communities are less resilient and resistant to changing environmental conditions (35). This means that there is a large need for more research investigating permafrost microbial communities. These communities may be more sensitive to change than initially thought. O'Connor et al, 2010 does propose that water table depth may be a good predictor of methane emissions from permafrost and suggests two models for going about this, neither of which takes into account microbial activity (79). Understanding the balance between methanotroph and methanogen activity is key in understanding and predicting net methane flux.

An extensive review outlines variations seen in community structure across different permafrost types (26). This may mean that different permafrost types may have different reactions to changing variables, further solidifying the need for expansive field research. More studies investigating methanogens and methanotrophs by means of deep 16S rRNA sequencing or metagenomics will improve clarity on the organisms actually detected in this ecosystem. Additionally, these studies need to collect a larger number of samples. Sampling at multiple times throughout the growth season will provide strong evidence of seasonal shifts. Designing studies that manipulate variables *in situ* will help to examine how a variable directly impacts the methane cycling community. After examining studies done in other pulsing ecosystems, its clear that soil moisture is clearly a variable that should be focused on. If microbial activity is linked to pulses in soil moisture in permafrost, it will be much easier to predict possible changes in methane flux due to climate change impacting this ecosystem.

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CHAPTER TWO

The Thawing Frontier: 16S rRNA Analysis of Supraglacial Bare Ice from the Greenland Ice Sheet Reveals Patchy Communities, Potential Organisms of Downstream Inoculation, and Abundant Cyanobacteria

ABSTRACT

The main form of ice loss from Southwest Greenland is melt water runoff. Runoff from this area may increase as ice loss accelerates across the Greenland Ice Sheet. Allochthonous microorganisms will be inoculated into downstream ecosystems as melt water discharges from the ablation zone of the ice sheet. The results of this "wash away effect" are not well understood, especially the effects of foreign microbes entering new ecosystems. The ice samples examined in this study represent the most in-depth 16S rRNA dataset for supraglacial bare ice to date, with at least 22,362 16S rRNA reads from each of the 21 samples collected near Kangerlussuaq, Greenland. The average number of unique OTUs from the sample sites ranged from 439 to 1457. This study found that bare ice is similar to snow and cryoconite hole microbial communities. The major phyla detected across all samples included Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria. Cyanobacteria made up more than 10 percent of the core community in 5 of the 8 sample sites and were the dominant phyla of the core in 3 of the 8 sample sites. Although no significant difference in α-diversity of the sample sites was observed, variation in the proportions of phyla, core community members, and beta diversity within replicates from the same sample site indicate heterogeneous communities even across a small scale, 223.34 m^2. This study pinpoints organisms of interest that will inoculate downstream ecosystems, confirms heterogeneous microbial communities in ice ecosystems, and reveals a higher abundance of

Cyanobacteria than previously thought. Both the patchy communities and the abundance of Cyanobacteria may impact generalizations made towards carbon storage on the Greenland Ice Sheet.

INTRODUCTION

As climate change progresses, increasing ice melt from sea ice and glaciers from around the globe is a gaining attention due to the issues faced with sea level rise and ocean freshening. The Arctic is not only of high concern because of decreasing annual sea ice (1), but also because of the increasing ice melt and fresh water runoff from varying land masses (2-4). The Greenland Ice Sheet (GrIS) poses to be a large source of freshwater runoff.

The amount of total ice loss from the GrIS has been increasing since 1979 (5) and the acceleration of this ice loss over the last 18 years has been around 21.9 ± 1 Gt/yr^2 (6). In addition to the acceleration of ice loss, there have been many extreme and atypical melt events recorded recently. For instance, an extreme melt event was recorded across the entire GrIS during July of 2012 (7). These factors make the GrIS a large potential contributor to sea level rise. If the entire Greenland Ice Sheet were to melt, the sea level could rise between 7 (8) and 22 cm (9).

The increasing freshwater run-off from the GrIS will not only impact ocean circulations (10, 11), but may also directly and indirectly impact ocean food webs. Many aspects of climate change including increasing ocean temperatures, ocean acidification, and ocean freshening will all likely impact marine microbial food webs (12). A seven year study in the Canadian Arctic confirmed the sensitivity of microbial communities to

changing salinity and nitrate levels in the Arctic ocean (13). As this melt water drains from the GrIS, it will enter surrounding rivers, streams, lakes, and coastal waters of Greenland and introduce not only fresh water and nutrients, but also new microbial life. This concept has been referred to as the "wash away effect," (14, 15) and the impacts of these materials being washed into downstream ecosystems is still largely unknown. Further, the microbial life found on the Greenland Ice sheet is not typically autochthonous, but rather is allochthonous and is likely deposited on the ice sheet by Aeolian processes (16).

Dust particles in air have been shown to contain viable bacterial cells (17) which could then be dispersed across the globe. The Greenland Ice Sheet may act as a repository for foreign microbes that have been blown over from other continents, and these organisms could then be introduced into the ocean or surrounding freshwater environments. Identifying organisms present on the surface of the ice sheet will allow for a better understanding of how the wash away effect will impact these downstream ecosystems. This is especially important to understand in southwest Greenland because the primary form of ice loss from this area is in the form of melt water and runoff rather than ice discharge (14).

Despite this impending need to examine the microbial communities that will be inoculating downstream ecosystems, very little research has been directed towards revealing the microbial communities of supraglacial bare ice on the GrIS. Bare ice is ice where cryoconite holes and melt water streams exist. When debris accumulates on the surface of the ice cryoconite holes may develop (18). Bare ice is different from the marginal zone of the ice sheet, which does not contain cryoconite holes or melt streams.

Like wise, bare ice is different from the slush zone of the ice sheet, which is covered in melting snow. Additionally, only the bare ice zone and slush zone receive Aeolian dust deposits (19). In some areas of the GrIS the bare ice can start around 0.4 km from the ice free zone of Greenland (16) and so is relatively close to the marginal zone. Because of the proximity of bare ice to the ice free zone, the abundance of Aeolian dust, and the abundance of melt water streams, bare ice will be a large source of inoculum to downstream ecosystems. Bare ice will inoculate this melt water runoff that is discharging into freshwater lakes, streams, and coastal waters with additional microbial life.

Many current studies focus on the microbial communities within sea ice (20-22) as these communities become a quick source of inoculum for the surrounding waters as sea ice melts (23). Active microbes persist in glaciers (24) and in cryoconite holes (25) on the GrIS. Previous investigations have focused on the communities within cryoconite holes (19, 25, 26). Cryoconite holes attract a significant amount of research because the bacteria found in these holes may directly impact the albedo of the ice leading to increased ice melt. Microbial communities in Arctic snow pack have also been investigated (27, 28). However, there is a current gap in knowledge surrounding the supraglacial bare ice environment.

Supraglacial communities in bare ice on the Greenland Ice Sheet will be some of the first organisms to be transported to downstream environments and so these communities deserve more attention. A handful of current studies have begun to examine microbial communities on the surface of the GrIS, however, no taxa assignments exist. One study determined that dust content explains variation in abundance of microbes, with an

increase in dust particles correlating to an increase in cell counts (29). A similar pattern was observed in snow microbial communities (28).

Spatial variation in microbial communities found on the GrIS has been confirmed on a relatively large scale (several km) comparing the margin and the interior of the ice sheet (16). Specifically, both microbial abundance and activity increase with increasing distance from ice-free land (16, 30). This is due to Aeolian deposition of nutrients and microbes (16) and is supported by the data showing that nutrient levels (14) and organic matter (15) also increase with distance from the edge of the ice sheet. However, it is completely unknown how these communities vary at a smaller scale.

Much of the current research investigating microbial life on the GrIS is working to understand the activity of autotrophs and heterotrophs in order to determine if the ice sheet is a sink or source of carbon dioxide. Some studies point towards the GrIS maintaining a carbon dioxide sink, supported by net autotrophy measurements (16). However, other studies suggest net heterotrophy on the ice sheet (19). Understanding spatial variation at a smaller scale may help to solidify whether certain areas of the GrIS are a net negative or positive carbon sink.

One goal of this study is to identify the members of the microbial community in supraglacial bare ice on the Greenland Ice Sheet near Kangerlussuaq on the southwest side of Greenland. Identifying which autotrophs and heterotrophs are present in this ice and the relative abundance of each will help to identify potential areas of sources or sinks of carbon dioxide. Identifying specific taxa may also reveal potentially harmful organisms that may be of concern if they were to become introduced and active in a new ecosystem. Additionally, this study seeks to establish general spatial patterns of the

communities on a small scale. If the communities are relatively homogenous on a small scale, it may be easy to make broad claims as to whether certain areas of the ice sheet are sources or sinks of carbon dioxide. However, if the communities are heterogeneous or patchy, then making such broad claims may be less reliable.

METHODS

Sample Site and Sample Collection

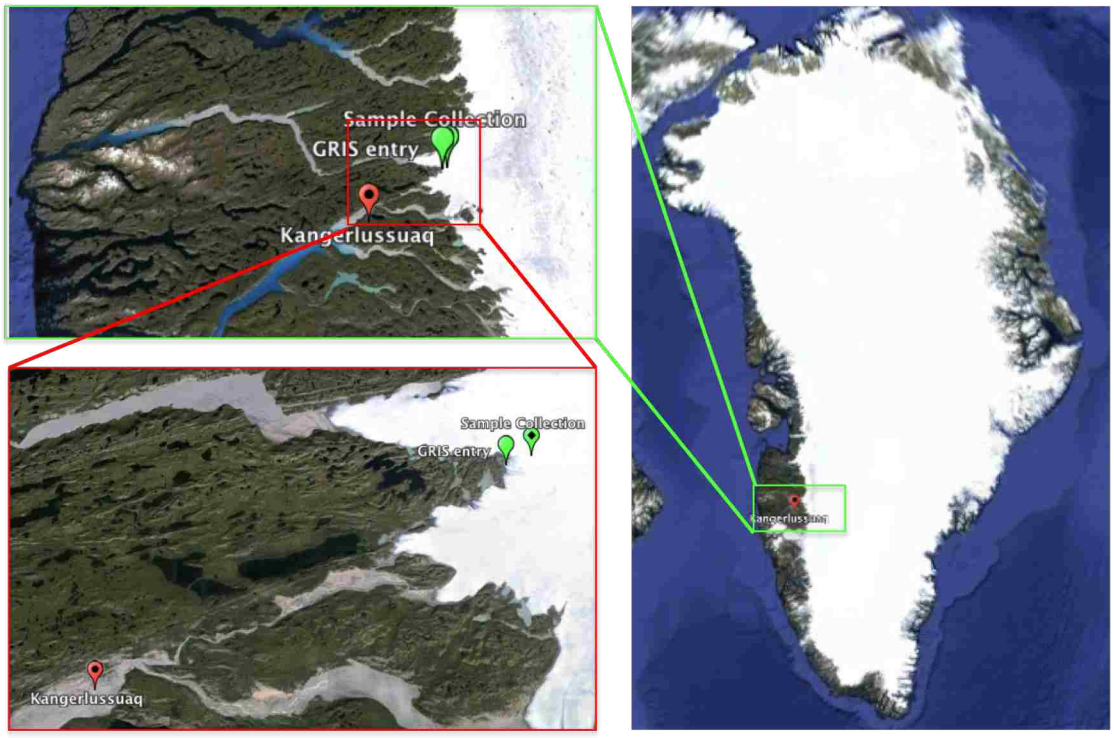

Figure 1. The approximate location of the field site near Kangerlussuaq, Greenland. The research team entered the GrIS just north east of Kangerlussuaq (depicted by green balloon) and the sample site was established one mile from the margin of the ice sheet (depicted by green balloon with black diamond). These images were taken from Google Earth Pro.

Supraglacial ice cores were collected from the Greenland Ice Sheet in July of 2014 near Kangerlussuaq, Greenland. A site approximately one mile from the margin of the ice sheet was selected (Fig. 1). As the topography of the ice sheet in this area is quite variable, a flat and continuous patch of ice that sloped towards the east was used as a

sampling site. The triangular sampling schematic is depicted in Figure 2. The sides of the triangle were 40 m and the height was 39.6 m, and so the overall area of the sample site was approximately 223.34 m^2 (Fig. 2). There were 8 sampling points within the sample site, and at each sampling point three replicate cores were taken no more than 10cm apart. This area consisted of bare ice. There were no melt water streams flowing through the sample area, nor were there any cryoconite holes present. However, there were both melt water streams and cryoconite holes nearby the sample site.

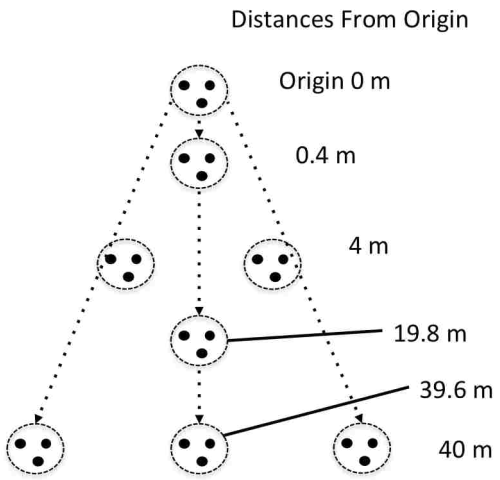

Figure 2. A diagram of the sampling schematic (not to scale). Three replicate cores were collected (dark, filled dots) within in 10 cm of each other at each sampling point (circles). The distance of each sampling point from the origin is noted on the diagram.

Three replicate 6 inch by 2 inch cores were collected at each sampling site, within 10cm of each other. A total of 24 cores were collected. Surface ice cores were drilled using a handheld drill with a custom-built stainless steel corer. Prior to sample collection, the top two cm of the surface ice were aseptically scraped away. No snow or slush was present at this site. The corer was sterilized in a 10% bleach solution between all sample collections. The cores were emptied into in individual Whirl-paks (Nasco, Fort Atkinson, WI) by lightly tapping the outside of the corer with a rubber mallet. The ice cores were never touched by any of the researchers. Samples were transported back the same day to Kangerlussuaq International Science Support (KISS)

station in Kangerlussuaq, Greenland. The samples were then allowed to melt at room temperature for approximately 24 hours.

Nucleic Acid Extraction from Ice

Once samples were melted, the DNA was extracted with the Mo Bio PowerWater Sterivex DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's directions. The entire volume of melt water from the core was used as input into the DNA extraction. These volumes varied from 139 ml – 265 ml.

The DNA was transported on ice back to Copenhagen, Denmark where the DNA concentration was quantified with a Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA). DNA was freeze dried with a ScanVac Cool Safe Freeze Dryer (LaboGene, Lynge, DK) and then transported back to the University of Montana for further analyses. The concentration and purity (260/280 ratio) of all DNA extractions were checked with an Implen NanoPhotometer (Implen, Inc., Los Angeles County, CA). The quality of the extractions were further examined by amplifying a 1X, 1/10, and 1/100 dilution of all samples with universal 16S rRNA gene primers (31).

16S Amplicon Preparation and Sequencing

The V4—V5 region of the 16S rRNA gene was amplified using the generally conserved 536f and 907rN primers developed in this lab (31), which were modified to include barcoded linkers to facilitate data sorting after high-throughput Illumina Mi-Seq sequencing. The PCR reactions included 20µl reactions containing 1 µl of 1X DNA, 0.2µl FastStart Taq (Roche Diagnostics, Indianapolis, IN), 0.4µl 10µg/µl BSA (Roche), 0.04µl 25mM $MgCl_2$, 0.8µl 5mM dNTPs (Invitrogen Life Sciences, Grand Island, NY),

0.4µl 10pmol of each primer. PCR amplification conditions included an initial 3 min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 55°C for 45 sec, elongation at 72°C for 60 sec, and a final elongation at 72°C for 7 minutes. Each sample was amplified in triplicate as per general practice, then the amplicons (PCR products) were pooled and gel purified using the QIAquick Gel Extraction kit (Qiagen, Germantown, MD). The triplicate sets of amplicons from each sample were pooled and 15µl of the mixture from each sample was sequenced at the University of California— Davis Laboratory of Genetic Diversity using the MiSeq platform.

Sequence Analysis

Fastq-join was used to join the paired reads and an 8% maximum difference in the joined regions and a minimum overlap of 6 base pairs was allowed, however, the average overlap was 80 base pairs. Reads that were not paired were not used in further analyses. The Qiime pipeline version 1.8 was used for the following general sequence quality control and downstream analyses (32, 33)Operational taxonomic units (OTUs) were clustered at the 97% similarity level using the subsampled open-reference technique with UClust employing the Green Genes database version 13.8 (34) as a reference. The identified OTUs were classified to the genus level with the RDP classifier which classifies organisms based on the Bergey's *Taxonomic Outline of the Prokaryotes* (35). A phylogenetic tree was generated using the Pynast aligner within the Qiime pipeline.

Paired-end sequence reads from all samples were normalized to 22,362 sequences per sample (the lowest number for any sample) for a more robust bioinformatic analysis.

The Chao1 diversity index, phylogenetic distance (PD), and OTU counts were determined within the Qiime pipeline and used to generate rarefaction curves to assess if the depth of sequencing is adequate for α-diversity (taxon richness) and β-diversity (microbial community similarity between samples) measurements. The Chao1 diversity index, PD, OTU counts, and the Shannon diversity index were used to quantify α-diversity of the samples. Differences between these metrics were determined by ANOVA tests done and verified with a Tukey's honest significance test in R (36).

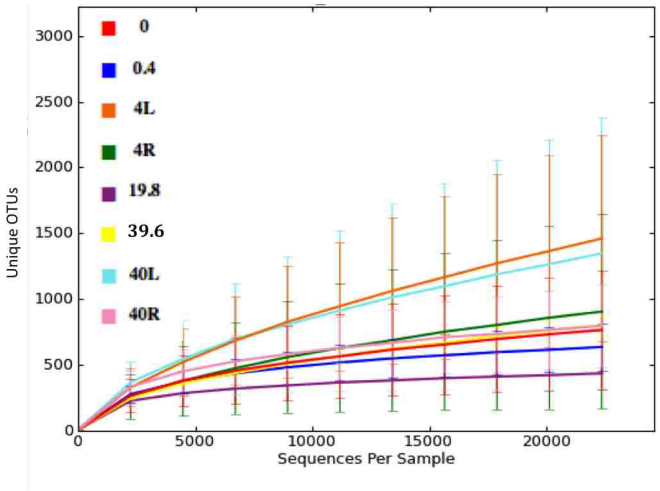

Figure 3. A rarefaction curve depicting the number of observed, unique OTUs at varying sequence depths. The colors represent the different sampling points and the standard deviation between the replicates taken at each sampling point is shown with error bars (origin 0 m (red), 0.4 m from the origin (blue), 4 m to the left of the origin (orange), 4 m to the right of the origin (green), middle of the triangle and 19.8 m from the origin (purple), bottom of the triangle and 39.6 m from the origin (yellow), 40 m to the left of the origin (aqua), and 40 m to the right of the origin (pink).

To visualize β-diversity, weighted UniFrac scores were determined and used for a principal coordinate analysis (PCoA). The Qiime pipeline was also utilized to identify the core community members. To be a member of the core community, a taxon was required to be present in at least half of the samples.

RESULTS

16S rRNA Amplicon Sequencing

As all samples were below detection level of both the Qubit and the Implen NanoPhotometer, the quality of the extractions were further examined by amplifying a 1X, 1/10, and 1/100 dilution of all samples with universal 16S rRNA gene primers. The samples produced PCR product with one micro liter of 1X DNA and so all samples were amplified with barcoded 16S primers and sent out for sequencing.

Of the 24 samples sent out for sequencing, 21 were returned with high quality reads. Two of the three replicates collected at the middle sampling point at 19.8 meters from the origin, did not have any reads. The third sample that did not have any sequencing reads was a replicate from the bottom row of triangle. The lowest number of reads per sample was 22,362 sequences per sample and so the samples were normalized to this sequencing depth.

α-Diversity

A rarefaction curve was used to ensure that the samples were sequenced to an adequate depth for further analyses. The rarefaction metrics tested were the Chao1 α-diversity index (data not shown), phylogenetic distance (PD) (data not shown), and the number of unique operational taxonomic units (OTUs, Fig. 3). All curves began to level off at a sequencing depth of 22,362 indicating that this depth of sequencing was adequate to assess α- and β-diversity of these samples.

Alpha diversity (taxon richness) was measured using multiple diversity indices including the Chao1 diversity index, PD, the number of unique OTUs, and the Shannon diversity index. The average number of unique OTUs from the sample sites ranged from 439 to 1457. Additionally, the Chao1 and Shannon indices ranged from 722.9 to 3779.7

and 4.71 to 6.34 respectively. Phylogenetic distance ranged from 38.44 to 91.13. The middle 19.8 m sample was always at the low range of all diversity indices and the left 4 m sample was at the high range for the Chao1, number of OTUs, and PD. The left 40 m sample was the sample point with the highest Shannon index. Despite these ranges, no significant differences in the α-diversity indices were detected between the sample points (Fig. 4, ANOVA, all p values > 0.1). The α-diversity was variable among the replicates of most sample points, as indicated by the large standard deviation error bars in Figures 3 and 4. The 4 meters right, 4 meters left, and 40 meters left had the largest standard deviations. The middle 19.8 sample did not have any replicates and so was left out of the ANOVA, however the values for that sample are depicted in the graphs in Figure 4.

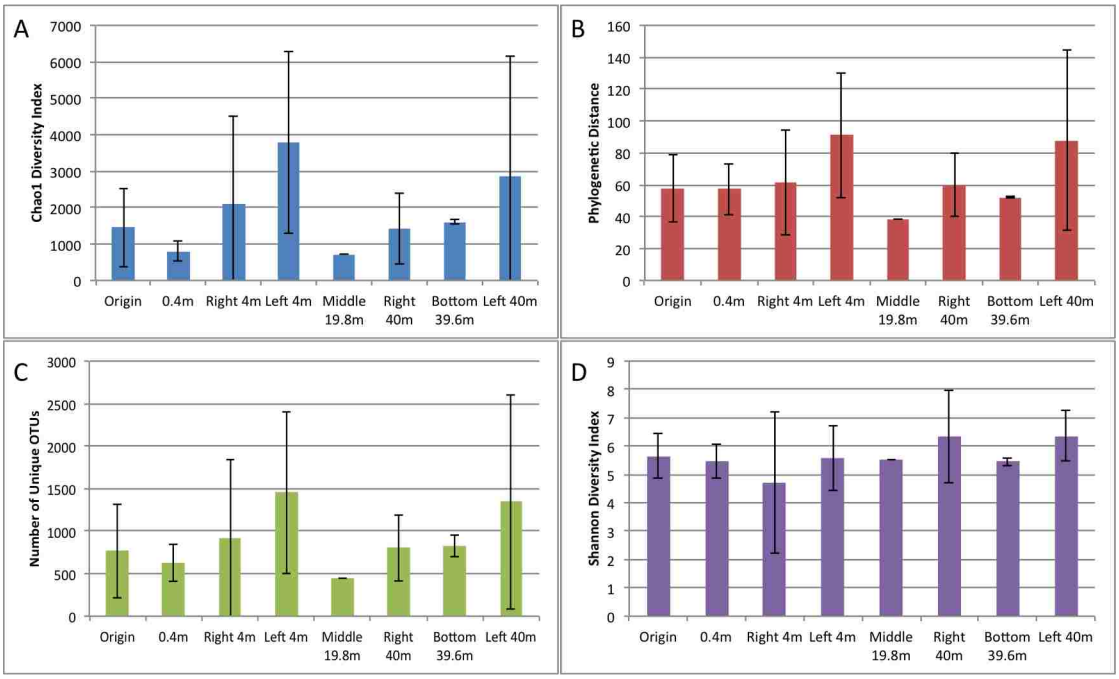

Figure 4. Chao 1 (A), phylogenetic distance (B), number of unique OTUs (C), and Shannon diversity indices (D) of each sample site. Error bars represent the standard deviation between the replicate cores taken at each sample site. There were no significant differences between the sample sites for any of these alpha diversity metrics (ANOVA, all P values > 0.1). The middle sample at 19.8 meters did not have any replicates and so was not included in determining any significant relationship between the samples.

β-Diversity

To assess β-diversity (microbial community similarity between samples) among replicates and samples, weighted UniFrac scores were used in a principal coordinate analysis (PCoA). This analysis revealed variation in both replicates and sample sites. The 4 meters right replicates (green) are the most dispersed, indicating differing β-diversity among these replicates (Fig. 5). Two of the three replicates from the origin (red), 0.4 m (blue), 4 m left (orange), and 40 m left (aqua) cluster near each other with the remaining replicate being more of an outlier. This indicated relatively similar β-diversity among these sample points, however it should be noted that there is some variation among the replicates from each of these sample points. Other than the 4 m right sample point, most samples exhibited replicate similarity.

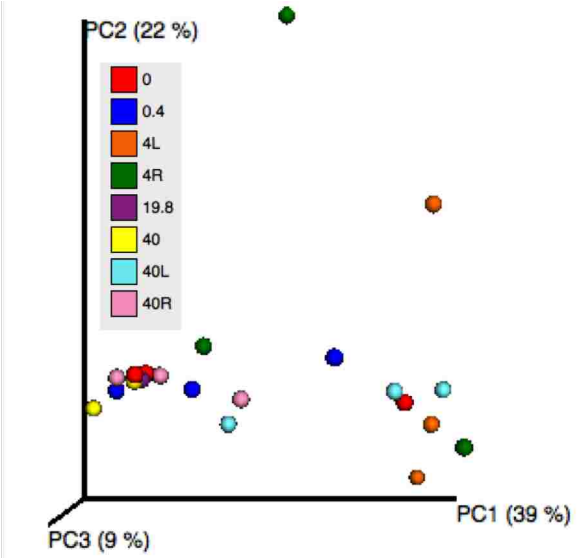

Figure 5. PCoA of weighted UniFrac scores from each sample. The similarity between the samples taken from the sample sites (the origin (red), 0.4 meters from the origin (blue), four meters on the left side of the origin (orange), four meters on the right side of the origin (green), the middle of the triangle at 19.8 meters (purple), the middle of the bottom row of samples (yellow), 40 meters on the left from the origin (aqua), and 40 meters on the right from the origin (pink)) can be assessed based on the distance between the sample points.

The 40 m right sample point, 40 m middle, and the single replicate from 19.8 meters cluster together indicating similar β-diversity among these samples. These three sample sites are fairly close together on the sample area. However, in general the samples sites do not

cluster by proximity, meaning the sites closer to each other in the field site do not cluster near each other in the PCoA.

Community Structure

The major phyla detected across all samples included Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria (Figure 6). Of these major phyla, the most abundant included Cyanobacteria, Firmicutes, and Proteobacteria. The amount of unidentifiable bacteria, labeled as "other," ranged from 0.3 to 18 percent. Nine of the 21 total replicates had communities containing at least 20% Cyanobacteria. Additionally, all samples contained some Cyanobacteria with a range of 0.7 up to 80.5%. Firmicutes ranged from 0.2 to 57.2%. Proteobacteria ranged from 7.2 to 59.7% (Fig. 6).

The replicate samples collected from a 10cm radius have relatively similar phyla proportions, however some replicates appear to have vastly different proportions (Fig. 6). Origin replicate B and C were similar with 41.9% Firmicutes and 19.8% Firmicutes, respectively; and B and C contained 42.0 and 63.9% Proteobacteria. Each of these replicates had very low relative abundances of Cyanobacteria. However, replicate A had a relative abundance of 33.4% Cyanobacteria, 2.3% Firmicutes, but a similar abundance of Proteobacteria at 33.2%. A similar pattern is observed in the 0.4 m with replicate C having a much larger relative abundance of Cyanobacteria. Replicate C had a relative abundance of 33.9% Cyanobacteria, compared to 0.8 and 7.0% Cyanobacteria. The left 4 m replicates have a similar pattern. These replicates however, do not exhibit a large presence of Firmicutes, but have a higher relative abundance of Bacteroidetes compared

to other sample sites. Replicate A was found to contain 2.0% Bacteroidetes, 74.0% Cyanobacteria, and 12.4% Proteobacteria. Replicate B contained 32.8% Bacteroidetes, 20.0% Cyanobacteria, and 18.5% Proteobacteria. Replicate C was made up of 11.1% Bacteroidetes, 30.3% Cyanobacteria, and 39.6% Proteobacteria. Replicate A and C of the 40 m right sample were similar, however replicate B contained more Acidobacteria (24.0% compared to 0.06 and 0.5%). The left 40 m replicates were all relatively similar.

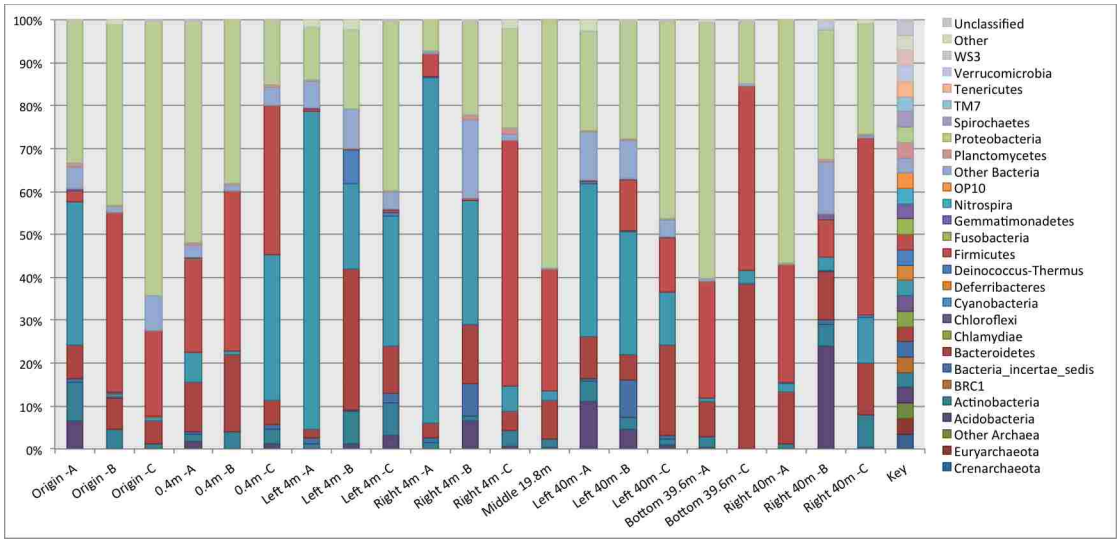

Figure 6. The relative abundance (percent) of phyla detected in all replicates. Major phyla include Acidobacteria (purple), Actinobacteria (dark teal), Cyanobacteria (teal), Bacteroidetes (dark red), Firmicutes (red), and Proteobacteria (light green).

The right 4 m replicates had fairly different proportions of the major phyla. Replicate A contained 80.5% Cyanobacteria. Replicate B had 13.9% Bacteroidetes, 28.9% Cyanobacteria, and 22.1% Proteobacteria, where as replicate C contained 5.7% Cyanobacteria, 57.2% Firmicutes, and 23.1% Proteobacteria. The two bottom replicates were similar to each other in that they did not contain many Cyanobacteria, but they also exhibited differences. Replicate A was dominated by Firmicutes (27.3 %) and Proteobacteria (59.7 %), where as Replicate C was dominated by Bacteroidetes (38.3 %) and Firmicutes (42.9 %) (Fig. 6).

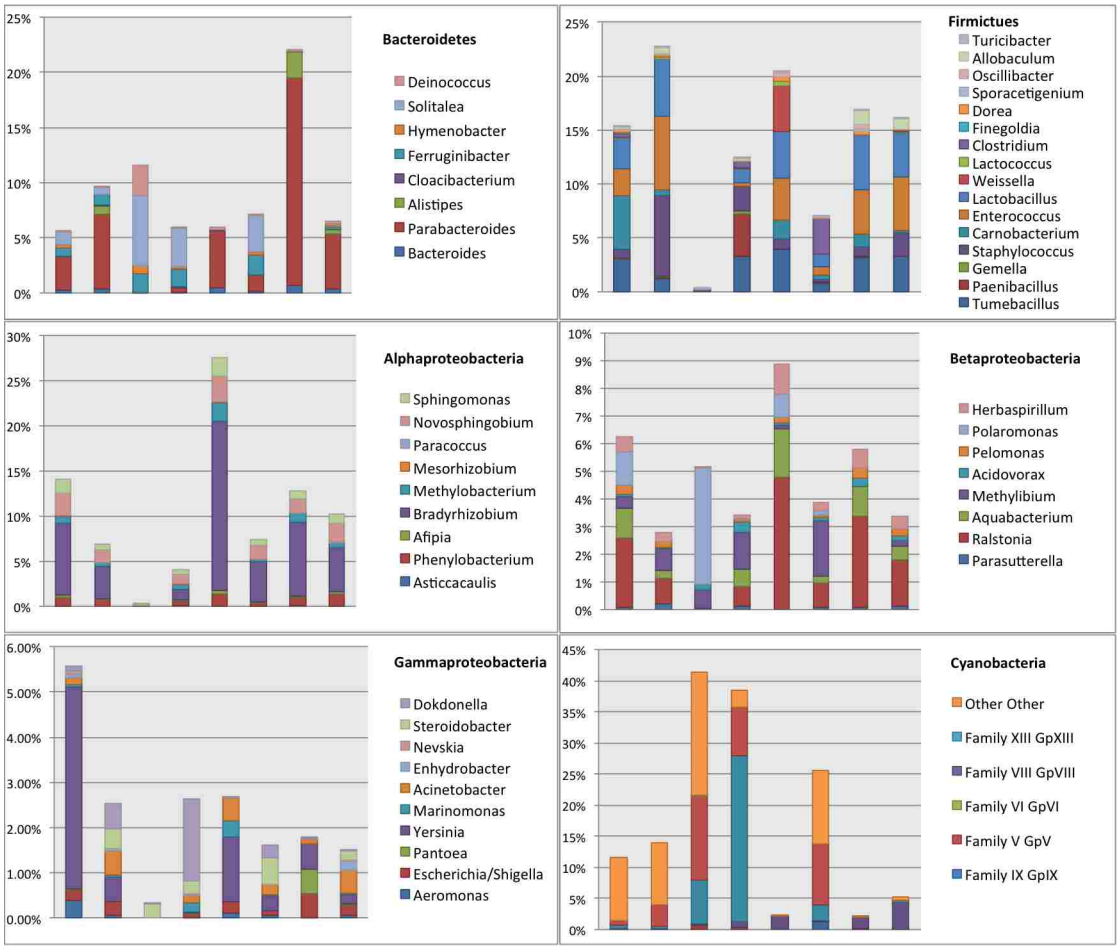

Figure 7. The core community genera within Bacteroidetes, Firmicutes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Cyanobacteria found in sample sites. The percent relative abundance is shown on the y-axis. The x-axis from left to right includes samples from the origin, 0.4 m, left 4 m, right 4 m, 19.8 m, left 40 m, bottom, and right 40 m. The core genera are indicated in the key.

Core Community

To easily examine the most dominant genera found in supraglacial bare ice, a core community was determined by averaging the replicates from each sample and identifying genera that were present in at least half of all samples. The core community genera from some of the major phyla and classes, including Bacteroidetes, Firmicutes,

Alphaproteobacteria, Beta-proteobacteria, Gamma-proteobacteria, and Cyanobacteria are shown in Figure 7.

The relative abundances of core genera varied between sample sites. *Parabacteroidetes* and *Solitalea* are the most common Bacteroidetes core members. The core community of the bottom, middle sample primarily consist of Bacteroidetes, 18.9% of which was *Parabacteroidetes*. Firmicutes made up a large portion of the core community of many samples. The core communities of the origin, 0.4m from the origin, 19.8 m, and right 40 m sample were predominantly Firmicutes. The core genera found within the Firmicutes phylum were primarily *Staphylococcus, Enterococcus, Lactobacillus, Carnobacterium, Tumebacillus, Paenibacillus,* and *Weissella*. Of the Proteobacteria phyla, Alphaproteobacteria were to most dominant class. The core community of the middle 19.8 m sample site was dominated by Alphaproteobacteria, of which 18.7% was *Bradyrhizobium.* Common Betaproteobacteria included *Ralstonia, Polaromonas, Methylibium,* and *Aquabacterium*. Gammaproteobacteria were the least abundant Proteobacteria, but of this class the main genera included *Yersinia, Dokodonella, Acintobacteria,* and *Escherichia/Shigella.*

Cyanobacteria made up more than 10% of the core community in 5 of the 8 sample sites including the origin, 0.4 m from the origin, the left 4 meters, the right 4 meters, and the left 40 meter sample site. This autotrophic phylum was the dominant phyla of 3 of the 8 sample sites. The left 4 m site had a core community that consisted of more than 40% Cyanobacteria. The core community of the right 4 m and left 40 meter were also dominated by Cyanobacteria, with about 37 and 25% respectively.

Additionally, samples that were closer to each other do not necessarily have similar abundances of genera found in the core community.

DISCUSSION

Microbial communities on the Greenland Ice Sheet have raised concern because they may impact albedo, such as in cryoconite holes. However, an overlooked importance of understanding microbial communities of the GrIS is the fact that they will be easily transported as ice melts and will enter downstream ecosystems. In order to predict the future productivity and health of these downstream ecosystems, it is necessary to know what organisms will be introduced as ice continues to melt. An additional value in identifying these organisms is that they will dictate whether the Greenland Ice Sheet is a net sink or source of carbon dioxide. The balance between autotrophs and heterotrophs will determine the net flux of carbon dioxide from this environment. Until now, no previous work has identified bare ice microbial communities at a high resolution. The goal of this study was to determine the make-up and structure of microbial communities in the supraglacial bare ice environment of the GrIS on a small scale.

Various forms of melt water running from the ablation zone eventually running into the ocean may transport dust and nutrients (14) however, this melt water will also pick up microorganisms as it flows. The transport of dust, nutrients, and microbes through melt water has been referred to as the "wash away effect," (14, 15). Previous work has shown that downslope dispersal of microorganisms from arctic soils leads to inoculation of surface waters of downstream lakes (37). In the case of the GrIS, downstream ecosystems may include other areas of the ice sheet, freshwater lakes,

streams, rivers, and ultimately coastal waters. The results of the wash away effect are not well understood, especially the effects of foreign microbes entering new ecosystems. This may be of large concern on the GrIS, because the microorganisms inhabiting the ice sheet are largely allochthonous.

There are several studies that have investigated microbial communities in the Arctic environment. The number of unique OTUs found in sea ice was around 1552 and the Chao1 and Shannon diversity indices were 614 and 3.38, respectively (20). Cryoconite holes in Greenland are being extensively studied due to their contributions to the ice albedo leading to increased ice melt. The Shannon diversity of the microbial communities detected in the holes has been found to be 3.61 (38). This study employed 454 pyrosequencing and obtained 64,597 total reads from all 16 samples and 616 unique OTUs were detected (38). Arctic melt water had a Chao1 diversity index for the microbial community between 103.5-152.2 and a Shannon index between 3.38-3.59 (39). Arctic snow had a Chao1 index between 14.8-59.7 and a Shannon index between 1.93-3.01 (39). Some of the richest snow samples to date were found to have about 1037 unique OTUs (27).

The ice samples examined in this study represent the most in-depth 16S rRNA dataset to date for supraglacial ice, with 22,362 16S rRNA reads from each of the 21 samples. The inability to read the concentration of extracted DNA from these samples and the fact that three samples were returned with no sequences does solidify the fact that the ice environment may harbor relatively low biomass. However, it is clear from the samples that did return high quality sequences, that there is a relatively high diversity of bacteria in this supraglacial bare ice environment. The average number of unique OTUs

from the sample sites ranged from 439 to 1457. Additionally, the Chao1 and Shannon indices ranged from 722.9 to 3779.7 and 4.71 to 6.34 respectively. Despite the range observed in these different α-diversity indices, there was no significant difference in the α-diversity of the different sample sites. The results illustrate a much higher diversity than what has been observed in similar ecosystems described earlier. This may be a reflection of deeper sequencing creating an inability to accurately compare to older studies, or the data reaffirms that most microbial communities, including ice communities, are more diverse than previously thought.

The previous studies that have outlined the α-diversity of a handful of Arctic environments have also provided a baseline of what major phyla to expect in similar communities. In sea ice Proteobacteria, largely Gammaproteobacteria and Flavobacteria, was found to be the dominant microbial phylum, and Cyanobacteria made up nearly 7% of the reads analyzed (20). Proteobacteria were also found to dominate cryoconite holes (40) and Arctic snow (41), however in contrast to sea ice these samples mostly contained Alpha and Betaproteobacteria. More recent work in these environments have found that both Cyanobacteria and Alpha and Betaproteobacteria dominate cryoconite holes in Greenland (42). Additionally, it was found that Alpha, Beta, and Gammaproteobacteria dominated snow on GRIS (27). This body of work shows that bare ice microbial communities are fairly similar to snow and cryoconite microbial communities found on the GrIS. The major phyla detected across all samples included Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria.

Further, this study identifies potential genera of interest or concern when considering what kind of bacteria will be entering new ecosystems. No study of

supraglacial bare ice has identified taxa down to the genus level and so the core community data from this study provides the first glimpse of the major genera inhabiting this environment. One specific genus of concern includes the *Carnobacterium,* which made up about 5% of the origin community. This genus contains 9 different species, one of which, *C. maltaromaticum* is a known fish pathogen (43). *Carnobacteria* are also known to be freeze thaw tolerant (43) and so is able to persist in bare ice. Alphaproteobacteria were a large member of the core communities, specifically, the genus *Bradyrhizobium.* This genus contains many known nitrogen fixers, and the inoculation of this organism into new ecosystems could alter the nitrogen cycle in the downstream environments.

Cyanobacteria were both an overall major phylum in many replicates, and a major member of the core community in many sample sites. Pigmented autotrophs, like Cyanobacteria, may decrease albedo and contribute to increases in ice melt (44) and so tracking the abundance of these organisms across the ice sheet is of high importance. Further, because these organisms are autotrophs, high abundance may indicate an area that is likely to be a carbon dioxide sink. Generally, its been found that the interior of the ice sheet is a carbon dioxide sink and the ice sheet edges are a carbon dioxide source (45). This may be due to the fact that microbial abundance and activity are higher in the interior of the ice sheet than the margin of the ice sheet (16, 30). The concentration of organic matter is also higher in the interior of the ice sheet than the margin (15). These patterns are all likely due to melt-water flow washing microbes off the margin of the ice sheet. Because of these previous findings, and the location of our sample site, it was expected that the core communities of all sample sites would consist primarily of

heterotrophs. However, Cyanobacteria made up more than 10 percent of the core community in 5 of the 8 sample sites and Cyanobacteria were the dominant phylum of 3 of the 8 sample sites. The fact that Cyanobacteria were not found to be the most abundant phyla in all sample sites may help confirm previous indications of areas closer to the edge of the ice sheet being a source of carbon dioxide. However, there may be a larger autotroph presence than previously thought. It might be that due to the wash away effect, and perhaps an acceleration of the wash away effect, more Cyanobacteria are being introduced into the marginal areas of the GrIS. If this is the case, as melt continues to increase the wash away effect, the total surface area contributing as a carbon sink may increase.

Even on a relatively small scale, 40 meters as opposed to 70 km (16), the structure of the communities found in the replicates and sample sites indicate heterogeneity in microbial communities of supraglacial bare ice. At most sample sites, two of the three replicates were similar enough to justify averaging the replicates to establish a core community of the sample site. However, it is still surprising to see differences in both community structure and β-diversity among replicates. Further, it was expected that sample sites closer to each other would be more similar, but this was not the case. The patchiness of these microbial communities emphasizes the need for careful sample collection. Multiple replicates for one sample area should always be collected. This heterogeneity is likely due to the scattered deposition of Aeolian dust or aerosolized soils.

Continuing to measure microbial communities in supraglacial bare ice, melt water, runoff, along with other environmental parameters such as dust content, is important. This work provides an initial, yet quite in-depth, look at the potential of

supraglacial bare ice communities. There are a handful of organisms that may be of concern as they inoculate downstream ecosystems. Additionally, the abundance of Cyanobacteria detected in this study may indicate that the areas of ice close to ice-free land may be less of a carbon source than previously thought. Lastly, the heterogeneity of these samples reinforces the importance of thorough sampling to make claims about the microbial community structure and activity in ice.

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CHAPTER THREE

The Unknown Future of Permafrost: Mounting Evidence for an Unlikely Shift from Methane Sink to Source in Zackenberg, Greenland

ABSTRACT

Current climate change models do not include microbial community data, despite the fact that microbes drive the biogeochemical cycles that produce greenhouse gases. As the net emissions of these gases, such as methane, are dependent on the microorganisms synthesizing and metabolizing these gases, incorporating such data will help to better identify environmental sources and sinks of greenhouse gases. This study examined permafrost active layer samples from a soil moisture gradient established in Zackenberg, Greenland allowing for the collection of samples with varying soil moisture. With deep sequencing of 16S rRNA, this study determines that dry active layer (soil moisture < 25%) microbial communities and moist active layer (soil moisture 25-50%) communities are more similar to each other than to the fen (soil moisture > 50%) microbial communities. Both type I and II methanotrophs were detected in these samples, however, type I methanotrophs were exclusively found in the fen samples. The areas of high soil moisture were also the only areas with net positive methane flux. *Crenothrix*, a type I methanotroph genus, was the most abundant methanotroph genus in the fen samples and could be a candidate to help easily detect likely areas to become sources of methane production, as they are used for similar predictive measures in well water. Additionally, the data presented here help to support previous work that predicts that the Zackenberg area will remain a methane sink even in the face of increasing temperatures and thawing of permafrost.

INTRODUCTION

It has been predicted that in a mere 75 years, nearly 90% of the top three meters of arctic permafrost will completely thaw (1). Significantly, 50% of the global belowground carbon pool may be contained in current permafrost zones (2). This means that as permafrost thaws, the seasonal active layer will increase in depth and labile carbon will become more readily available for microbial degradation. Seasonal thawing of the thin overlaying active layer is normal, however, due to increases in air temperature from climate change, the active layer is growing deeper and deeper every year as permafrost thaws (3, 4). In a twelve-year study, the active layer was found to be increasing at a rate greater than one centimeter per year in Eastern Greenland, the same area where this current work took place (5). These trends, as well as increased air temperatures in the arctic, may induce a shift from arctic tundra as a carbon sink to a carbon source by making a large amount of carbon available to microbial degradation (6).

To make matters worse, a positive feedback loop may be quickly developing between thawing permafrost and climate change. As the active layer becomes increasingly deeper, the microbial community becomes more active and diverse (7, 8) and will likely produce more carbon dioxide, methane, and other greenhouse gases. These gases will contribute to climate change, likely further increasing the air temperature in the Arctic. In addition, current studies suggest that the internal heat produced by the increasing activity of the microbial community may contribute to thaw of the active layer, perpetuating this positive feedback loop (9-11). Of particular concern is methane, which has had increasing atmospheric concentrations since 1750 (12). Methane is extremely efficient at absorbing radiation, making it a powerful greenhouse

gas and nearly 25 times more potent than carbon dioxide (13). Many sources of methane are anthropogenic, however there is growing concern that the permafrost in the arctic will become a significant natural source of great magnitude.

Despite these concerns and the clear link between microbes and greenhouse gases, no climate change models attempt to incorporate microbial activity. As microbes are responsible for both the production and consumption of these gases in permafrost, understanding microbial activity will be key to predicting sources and sinks of greenhouse gases. Unfortunately, the relationship of microbial community activity and greenhouse gas production is still largely unknown (14). In order to understand the contributions microbial activity has on the net flux of greenhouse gases entering the atmosphere, a substantial amount of research is required in understanding the balance between microbes producing these gases and microbes that are capable of metabolizing these gases. For example, the net flux of methane depends on the balance of activity between methanogens and methanotrophs (15). The balance of these organisms is impacted by many environmental variables. Further, most research projects that the relationship between methanogens and methanotrophs is not linear (16, 17) making predictions more difficult. In order to accurately predict methane fluxes from thawing permafrost, the roles and responses of these organisms need to be studied extensively *in situ.*

Working to understand which microbes are present in permafrost and what their roles are in the ecosystem is a growing field. Previous work has established snapshots of what the microbial community looks like in the frozen portion of permafrost and the active layer in a handful of locations in the Arctic. The active layer community primarily

consists of Actinobacteria, Acidobacteria, and Proteobacteria (7, 18-20). It has been found that extracting permafrost cores and allowing them to thaw under lab conditions induces an increase in Actinobacteria and an increase in methanogens (7). However, this particular study was based on a sample size of three and the experiment was done under lab conditions, which may inadvertently exclude important environmental variables. Initiating experiments that mimic the multitude of variables expected in the field or are done *in situ*, may prove to be an extremely important progression for future work.

Additionally, many permafrost studies focus only on fens, a common type of wetland in the arctic, as these are the sources of methane production (21-25). Due to this, studies investigating the methane cycle in permafrost tend to focus on methanogens and the identities and activities of methanotrophs remain vaguely understood (26). Methane oxidizing bacteria are both ubiquitous in soil and may be capable of oxidizing up to 90% of the methane produced by methanogens, making methanotrophs a key player in the regulation of methane emissions (27-31).

Experiments have revealed that methane oxidation is controlled by temperature, pH, and oxygen, ammonium, ammonia concentrations, but the variable that most greatly impacts methanotrophs is likely methane concentration itself (32, 33). Methanogenesis is strictly anaerobic, and so oxygen and water levels may be the variables that regulate methanogens most (34). It is clear that in order to understand net methane emissions from permafrost, methanogens and methanotrophs need to be identified and their activity in response to changing methane concentrations, oxygen concentrations, and soil moisture needs to be quantified and understood in the context of climate change.

Some researchers have recognized that the focus on saturated permafrost may limit complete understanding of these processes and have begun to compare dry and saturated permafrost environments as well. One study in western Greenland compared microbial communities in dry upland permafrost to saturated permafrost and found differing methanotrophic communities in these two environments (35). Additionally, a recent study performed at the same field site in Zackenberg, Greenland as this body of work, found that this area is generally a methane sink (36). The study compared dry and moist tundra permafrost and fen areas and found that the fen areas are the only sources of methane. Further, most of this area of Greenland is made up of dry tundra permafrost. This data, combined with the findings of methane oxidation increasing with an increase in temperature, lead the authors to conclude that this area will continue to be a methane sink well into the future (36). However, it is not clear how permafrost environments and processes will shift as climate change progresses. It is likely that soil moisture distributions within the permafrost will change due to increased precipitation, regression of glaciers, and further permafrost thaw. This means that dry tundra permafrost may not remain dry in the future. Incorporating microbial community data will help to solidify previous findings and offer new predictive measures for what may be in store if dry tundra permafrost becomes moist permafrost due to climate change.

This work goes beyond all prior studies and examines microbial communities and methane fluxes in dry, moist, and saturated active layer ecosystems overlaying permafrost in Zackenberg, Greenland *in situ*. This study addresses the following questions related to methane flux in permafrost: 1) How does the variation in methane flux between dry, moist, and saturated regions of permafrost affect microbial community

composition? 2) How do these variations specifically affect the composition of microbial community members involved in the methane cycle? And 3) How do ratios of methane producers and consumers vary across a moisture gradient in the active layer of permafrost?

METHODS

Sample Site and sample collection

The sample site was established in Zackenberg, Greenland in the spring of 2012. The site was comprised of 32 sampling points over a transect from an elevated grassland with low-moisture down to a wetland area. Eleven plots were set up along this transect, and each with three sampling points as replicates. The first plot (plot 0) was situated at the highest point in the grassland area and only had two sampling points in place for control readings for the gas flux measurements. At each sample point, a metal frame was inserted for the gas flux measurements. Soil sampling was done in July. Samples were collected just outside each of the 32 metal frames using a 5 cm x 10 cm soil core. The top 2-4 cm and at the bottom 8-10 cm of the core were used as separate samples and placed in sterile 2 ml tubes. A total of 64 samples were collected and 250 mg fresh weight soil from each depth was preserved using 750 µl LifeGuard solution (MO BIO Laboratories, Carlsbad, CA) frozen at -18^{o}C, and transported back to Denmark for DNA and RNA extraction.

Gas Flux Measurements and Soil Moisture Calculation

The process level measurements were taken and shared by CENPERM (Center

for Permafrost, University of Copenhagen) collaborators. The metal frames at each sample point had a squared base (20 cm x 20 cm) and were constructed to be airtight when the chamber is placed in the rim for gas measurements. A mobile LGR-DLT 100 (Los Gatos Research, Mountain View, CA) greenhouse gas analyzer was used to measure CH_4 flux. The gas concentrations are measured over 16 minutes (in ppm); the first minute allow the conditions to stabilize, and 15 minutes of recordings, enables flux calculations and correlation analyses. The methane flux measurement process and data conversion is further described in (37). Soil moisture content was measured four times around the base using a hand held moisture sensor (ML2x ThetaProbe, Delta-T Devices Ltd., Cambridge, United Kingdom) (37). Methane flux was measured in June, July, and August in 2012.

Soil Nucleic Acid Extraction

DNA and RNA extraction was performed with the MOBIO PowerMicrobiome RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). This kit has the option of co-isolated DNA removal with reagents provided in the same kit. RNA was converted to cDNA using the RTS DNase kit (MO BIO Laboratories, Carlsbad, CA). A portion of the DNase treated RNA that was not subjected to RT-PCR was kept as a DNase control to ensure that the samples were not contaminated with DNA. These controls were subjected to 16S rRNA PCR to check for possible DNA contamination. The concentration and purity (260/280 ratio) of all DNA extractions were checked with an Implen NanoPhotometer (Implen, Inc., Los Angeles County, CA). The quality of the extractions were further examined by amplifying a 1X, 1/10, and 1/100 dilution of all samples with universal 16S rRNA gene primers (38) as many of the nucleic acid yields were low in concentration. Only cDNA was used for 16S rRNA sequencing.

16S Amplicon Preparation, Sequencing, and Analysis

The V4—V5 region of the 16S rRNA gene was amplified using the generally conserved 536f and 907rN primers developed in this lab (38), which were modified to include barcoded linkers to facilitate data sorting after high-throughput 454 pyrosequencing. The PCR reactions included 20µl reactions containing 1 µl of cDNA, 0.2µl FastStart Taq (Roche Diagnostics, Indianapolis, IN), 0.4µl 10µg/µl BSA (Roche), 0.04µl 25mM $MgCl_2$, 0.8µl 5mM dNTPs (Invitrogen Life Sciences, Grand Island, NY), 0.4µl 10pmol of each primer. PCR amplification conditions included an initial 3 min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 45 sec, primer annealing at 55°C for 45 sec, elongation at 72°C for 60 sec, and a final elongation at 72°C for 7 minutes. Each sample was amplified in triplicate as per general practice, then the amplicons (PCR products) were pooled and gel purified using the QIAquick Gel Extraction kit (Qiagen, Germantown, MD). 49 of the 64 samples contained enough cDNA for sufficient barcoded PCR and gel purification. The gel-purified amplicons (50 µl) were mixed with 90 µl of the Ampure bead solution to further purify the samples. The samples were quantified via 16S and PicoGreen (Thermo Fisher Scientific, Grand Island, NY) qPCR as well as with an Implen Nanophotometer. Samples were pooled based on the average of these quantifications and sent out for 454 paired-end sequencing at CIB Genomics, Utah State.

The Qiime pipeline version 1.8 (39) was for used general sequence quality control and downstream analyses as follows: The sequences were demultiplexed and operational taxonomic units (OTUs) were clustered at the 97% similarity level using denovo OTU

picking. The identified OTUs were classified to the genus level with the RDP classifier (40). A phylogenetic tree was generated using the Pynast aligner within the Qiime pipeline. Paired-end sequence reads from all samples were normalized to 750 sequences per sample (the lowest number for any sample) for a more robust bioinformatic analysis. OTU abundance, the Chao-1 diversity index, and phylogenetic distance were determined within the Qiime pipeline and used to generate rarefaction curves for α-diversity analyses (taxon richness). To visualize β-diversity (microbial community similarity between samples), weighted UniFrac scores were determined and used for a principal coordinate analysis (PCoA). Significance in the clustering was determined with an Adonis test done in Qiime through the Vegan package in R (41). For many analyses, samples were binned based on soil moisture values as previously described (36).

RESULTS

DNA and RNA Extraction and Sequencing

The co-isolated metagenomic DNA (mDNA) recovered using the MOBIO PowerMicrobiome RNA isolation kit proved to be of insufficient quality for sequencing as revealed by poor amplification during the PCR process. For the most part, the cDNA samples from the co-extraction amplified well, only 9 of the 64 samples were not able to amplify. Some of the barcoded PCR products were not reliably quantifiable with the spectrophotometer. Samples were also quantified with PicoGreen and qPCR to evenly multiplex before sequencing. However, the samples that were very low in cDNA concentration, indicated by low spectrophotometer readings, low qPCR copy number, or a large disagreement between these two values, did not return any sequences. Of the 55

samples sent out for 454 paired-end sequencing, 45 samples were returned with at least 750 16S rRNA amplicons.

Methane Fluxes and Other Environmental Data

For the most part, a net positive methane flux was observed in the low fen regions and a net negative methane flux was measured in all other dry and moist areas of this sample site throughout the growth season (Table 1). Plot three had nearly neutral flux rates, and both gradient six and eight varied around neutral flux rates. Although, for most of the growth season plot six exhibited a negative flux and plot eight exhibited a positive methane flux.

Table 1. The averaged net methane flux (μmol CH_4 m^2 hr^{-1} ± SD) measured through out the growth season.

Plot	July	August	September
0	-8.3 ± 4.3	-7.6 ± 4.7	-6.2 ± 2.3
1	-8.6 ± 6.1	-9.3 ± 6.6	-8.6 ± 4.9
2	-6.2 ± 1.5	-6.9 ± 1.8	-7.5 ± 0.9
3	-0.1 ± 0.2	-0.3 ± 0.4	-1.5 ± 0.3
4	-3.9 ± 2.4	-10.1 ± 3.1	-8.9 ± 0.2
5	-1.0 ± 0.8	-3.0 ± 0.5	-3.2 ± 1.2
6	-2.6 ± 2.3	0.2 ± 1.2	-2.5 ± 1.1
7	-3.1 ± 1.0	-2.6 ± 1.2	-3.7 ± 1.1
8	-0.1 ± 0.4	0.1 ± 0.1	-0.3 ± 0.2
9	19.7 ± 14.5	23.8 ± 22.9	12.9 ± 10.4
10	226.7 ± 158.5	281.6 ± 187.2	107.7 ± 70.0

Figure one depicts the soil moisture measurements along the sampling transect (Fig. 1). Plots eight, nine, and ten, had the highest soil moisture and have been binned as fen sights due to the soil moisture being above 55%. Gradients two through seven have been binned as moist plots because the soil moisture in these areas ranges from 25-55%. And gradients zero and one have been binned as dry plots because the soil moisture lies below 25%. Environmental data from this field site are further analyzed in a recent publication (36).

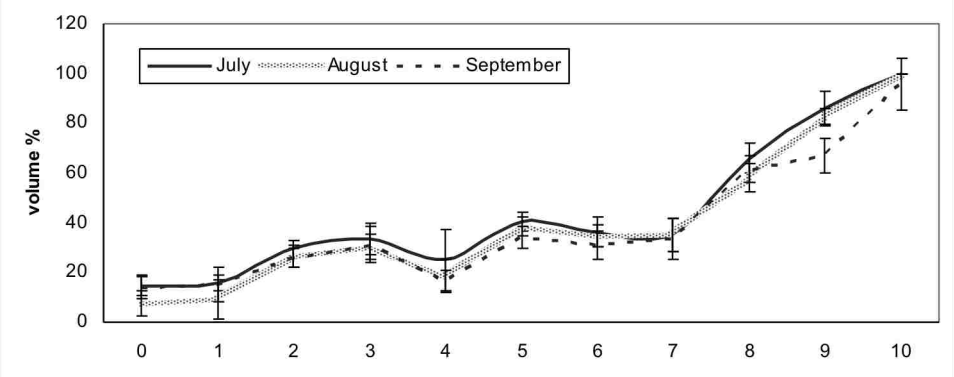

Figure 1. Soil moisture values (% volume, error bars represent SD) measured along the gradient throughout the growth season.

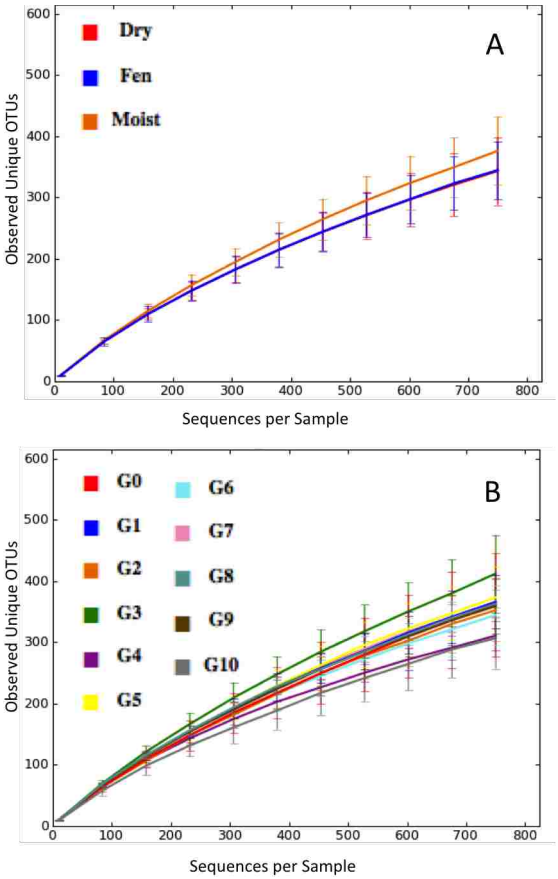

Figure 2. Rarefaction curves of the unique OTUs observed in dry, fen, and moist active layer permafrost (A) and in each sampling point along the gradient (B). Error bars represent that standard deviation.

α-Diversity *of the Microbial Communities*

There is no difference in the α-diversity of the dry (5-25% volume moisture), moist (25-55% volume moisture), and fen active layer permafrost (greater than 55% volume moisture) (Fig. 2A) Additionally, the individual sample sites along the gradient all have similar α-diversity (Fig. 2B). Figure 2 also illustrates that the coverage depth of 750 16S rRNA reads per sample is of adequate depth and allowed for complete sampling of these active layer samples. This is indicted by the leveling off of the rarefaction curves.

Microbial Community Structure

Actinobacteria (25-37%), Proteobacteria (35-48%), Acidobacteria (3-6%), and Chloroflexi (5-8%) were some of the most prominent phyla detected in the active layer (Fig. 3). However, shifts in phyla can be noted between the soil moisture classifications.

Proteobacteria increased along the moisture gradient from dry to fen, but were slightly higher in the moist samples compared to the fen samples. In the dry active layer permafrost Proteobacteria make up 35.03% of the community, where as in the moist and fen active layer the total community consists of 47.87 and 44.66% Proteobacteria, respectively. Additionally, Firmicutes and Bacteroidetes increase in relative abundance along the moisture gradient. In the dry active layer permafrost Firmicutes make up 1.84% of the community and increase to 3.05 and 4.44% in the moist and fen active layer. The relative abundance of Bacteroidetes in the dry active layer was 1.67% and increased to 2.86 and 3.02% in the moist and fen active layer samples.

Where as, Actinobacteria, Acidobacteria, Chloroflexi, and Planctomycetes generally decreased along the gradient. The dry active layer contained 37.46% Actinobacteria, the moist active layer contained 25.10%, and the fen active layer contained 27.43% Actinobacteria. Acidobacteria made up 5.41% of the dry active layer, 6.15% of the moist, and 3.16% of the fen active layer. Chloroflexi ranged from 7.82% in the total dry active layer community, to 8.64% in the moist active layer, and declined to 5.66% in the fen active layer. The relative abundance of Planctomycetes was highest in the dry active layer at 3.29%, decreased to 2.26% in the moist active layer, and had the lowest abundance (1.77%) in the fen area.

The Cyanobacteria phylum was very low in abundance in the moist active layer samples, and had an average relative abundance of about 0.7%. The abundance was higher in the dry (2.29%) and fen area active layers (2.33%).

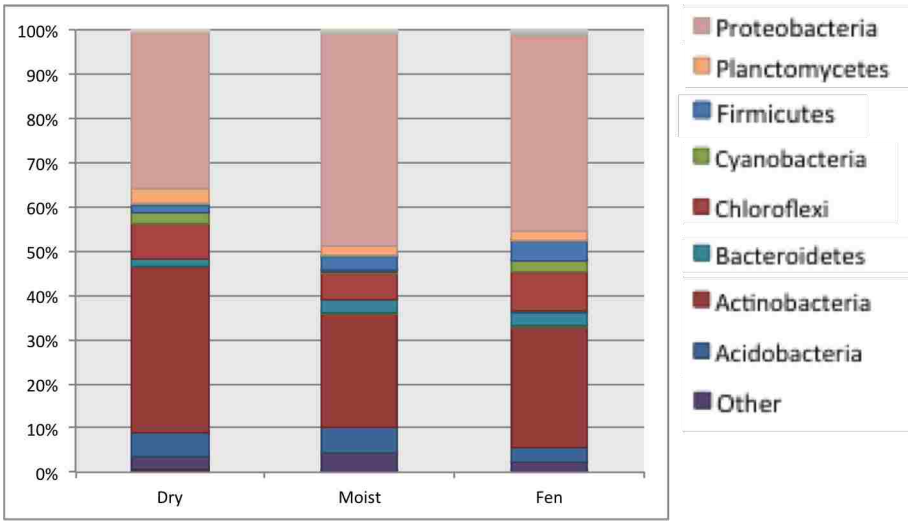

Figure 3. Shifts in phyla between dry, moist, and fen active layer permafrost. The major phyla seen in this data set are noted in the key to the right of the graph. Other Phyla were detected but were in low abundance. The grouping denoted as "Other," were phyla that were not identifiable.

Beta-Diversity of the Microbial Communities

β-diversity metrics were used to further examine the differences in microbial communities observed along the moisture gradient. Principal coordinates analysis based on weighted UniFrac scores revealed that soil moisture was a strong determining factor in the separation of these samples (Fig. 4A, ADONIS, $p= 0.001$, $R^2= 0.227$, permutations =999). For the most part, the dry and moist samples cluster close together, where as the fen samples cluster apart from the other two (Fig. 4A).

Additionally, a principal coordinates analysis based on these same weighted UniFrac scores revealed that methane flux measured in July, the closest flux

measurements obtained to sample collection, was also a strong determining factor in the separation of these samples (Fig. 4B, ADONIS, p=0.001, R^2= 0.143, permutations=999). The samples from sites with a high net positive methane flux cluster close together and the samples collected from sites exhibiting methane oxidation cluster together (Fig. 4B). The lower effect size (R^2) is greater when analyzing the samples based on soil moisture, indicating that soil moisture may be a stronger determining factor of sample clustering than methane flux.

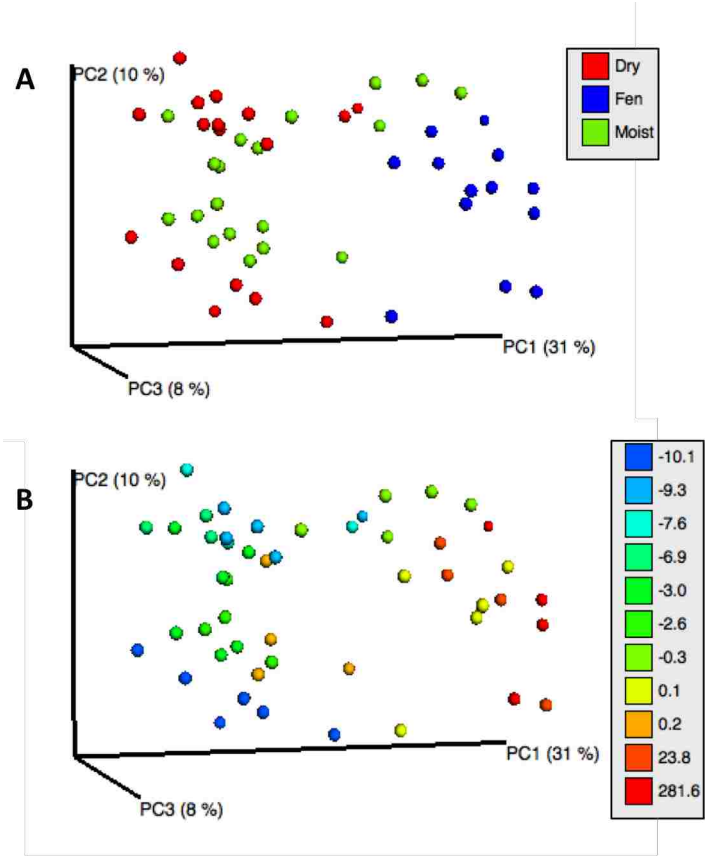

Figure 4. Principal coordinate analysis based off of weighted UniFrac scores. The top panel shows samples colored by soil moisture classification (A) and the bottom panel shows samples colored by methane flux measurements obtained close to soil sample collection time (B). Each point on the graph represents an individual active layer sample.

Active Layer Depth

There was no significant difference between the β-diversity of the microbial communities of the samples collected between 2-4 cm and of the samples collected between 8-10 cm, so both depths were included in all other analyses (ADONIS, P= 0.3, R^2 = 0.0226, permutations=999).

The Methane Cycling Community

It is clear that the relative abundance of microorganisms involved in the methane cycle increased in the fen areas (Fig. 5). General methylotrophs were detected in the dry, moist, and fen active layer samples, but the most were detected in the fen samples. The most prominent methylotrophs in all soil moisture types belonged to the genera *Methylibium* and *Hyphomicrobium* (Table 2). Additionally, type II methanotrophs were detected in all soil moisture classifications. The most common families of type II methanotrophs included Methylobacteriacea and Methylocystaceae. Type I methanotrophs and methanogens were exclusively detected in the fen active layer samples. The most common type I methanotrophs were the *Methylocaldum* and *Crenothrix* genera (Table 2). The NC10 phyla, a phylum of nitrite-dependent anaerobic methanotrophs (N-DAMO), were also only detected in the fen samples (Fig. 5).

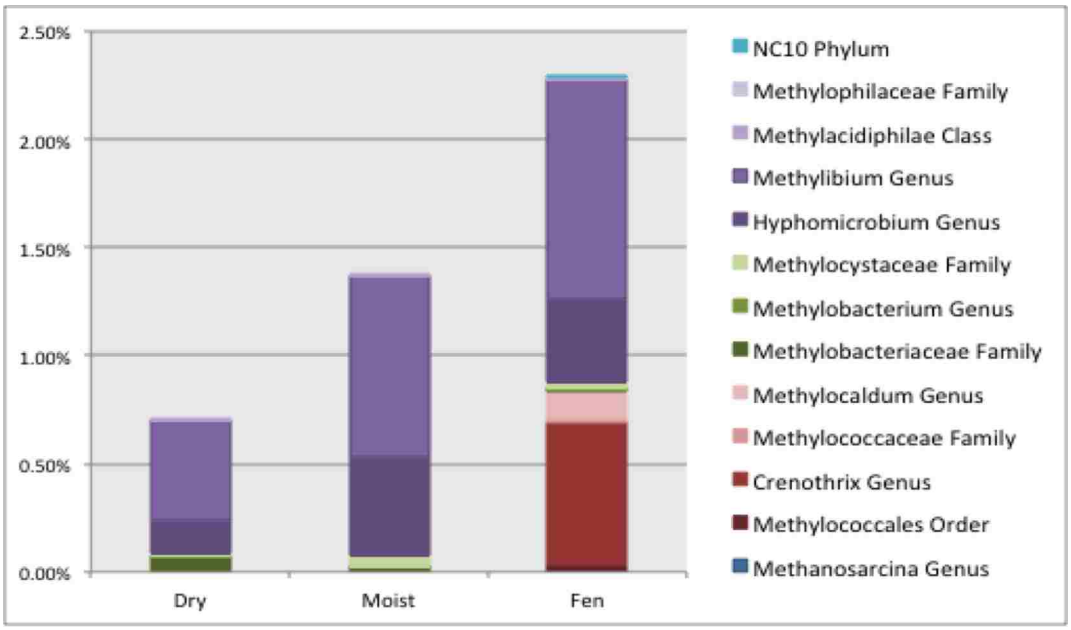

Figure 5. The methane cycling community observed in dry, moist, and fen active layer permafrost. Methylotrophs are colored in shades of purple, type II methanotrophs are colored in shades of green, type I methanotrophs are colored in shades of red, anaerobic methanotrophs are colored in teal, and methanogens are colored in blue. If OTUs were not identifiable to the genus level, the families, or class are listed.

Table 2. The classification of Methane cycling communities detected in dry, moist, and fen active layer permafrost samples and the relative abundance of each.

Metabolic Classification	Taxa	Dry	Moist	Fen
Anaerobic Methanotrophs	NC10 Phylum	0.0000%	0.0000%	0.0083%
Other Methylotrophs	Hyphomicrobium Genus	0.1526%	0.4541%	0.3832%
	Methylibium Genus	0.4598%	0.8400%	1.0192%
	Methylacidiphilae Class	0.0038%	0.0078%	0.0041%
	Methylophilaceae Family	0.0000%	0.0000%	0.0041%
Type II Methanotrophs	Methylobacteriaceae Family	0.0725%	0.0249%	0.0124%
	Methylobacterium Genus	0.0057%	0.0028%	0.0041%
	Methylocystaceae Family	0.0114%	0.0497%	0.0249%
Type I Methanotrophs	Methylococcales Order	0.0000%	0.0000%	0.0331%
	Crenothrix Genus	0.0000%	0.0000%	0.6608%
	Methylococcaceae Family	0.0000%	0.0000%	0.0124%
	Methylocaldum Genus	0.0000%	0.0000%	0.1264%
Methanogens	Methanosarcina Genus	0.0000%	0.0000%	0.0021%

DISCUSSION

This study reinforces that fen areas have a net positive methane flux whereas drier areas exhibit a net negative methane flux but also that each area has a distinct microbial community structure. Additionally, this study found that a larger relative amount of methanotrophic and methylotrophic organisms are observed in these same fen areas where high amounts of methane emissions are observed. This solidifies the fact that the methane cycle is complex and methane production and oxidation are not linearly related. Further more, this study reveals that soil moisture is a strong predictive measure of what type of microbial community will be found in the active layer of permafrost. As well as, that the community within the moist active layer appears to be more similar to the dry active layer than the fen community.

In general, the typical phyla of bacteria that have been observed in permafrost and active layers in other areas of the Arctic are detected in the active layer in Zackenberg. Acidobacteria, Actinobacteria, Bacteroidetes, and Proteobacteria tend to be the major

phyla detected in both active layer and permafrost samples in the arctic (21, 24, 42, 43). The proportions of the phyla help to distinguish the dry, moist, and fen active layer samples. These shifts in phyla are useful in distinguishing these environments, but this data still leaves much uncertain about the functional aspects of these communities. Looking at more specific assignments of the taxa reveals possible functional differences in the communities found in the dry and fen areas.

Interestingly, this study found that the abundance of methanotrophs was highest where a net positive methane flux had been measured in the fen areas. If the relationship of methane cycling organisms was linear, one might expect to see an increase in methanogens and a decrease in methanotrophs in the areas of net positive methane flux. This data speaks to the complex nature of the methane cycle and the non-linear relationship between methane synthesis and oxidation. It is likely that the increased presence of methane in the fen areas allows for more methanotrophs and methylotrophs to persist in this area because more precursors for the methanotrophic pathway are available. Type I methanotrophs are the most commonly detected methane oxidizing bacteria in the Arctic (16, 22), however this could be a byproduct of studies favoring fen sampling sites. This study shows that type II methanotrophs were detected across all soil moisture types and are likely ubiquitous in Arctic permafrost.

Additionally, type I methanotrophs are exclusively found in the fen areas. Generally, type I methanotrophs are classified as high affinity methane oxidizers meaning that they do not require high amounts of methane to be in the environment. However, the genus *Crenothrix* has been found to only persist in areas of high methane concentration (44). This genus is even used as a biological indicator of methane production in drinking

wells (44). This organism could be used similarly in permafrost to gauge potential areas of concern. *Crenothrix* may be an ideal organism to use as an indicator for areas of permafrost that could shift from negative to positive methane emissions as methanogens may persist below detection levels and still produce significant amounts of methane.

A previous study at this same field site indicated that this area was largely a methane sink and would likely continue to be a sink well into the future (36). This study noted that most of this area of Greenland is made up of dry tundra permafrost. Taking into account that the authors found that methane oxidation increases with temperature, it seems likely this area will continue to be a methane sink well into the future (36). However, it is not clear what the long-term effects of thawing permafrost will have on these ecosystems. For instance, as permafrost continues to thaw and precipitation increases in the Arctic, active layer permafrost may shift from dry to moist. These authors also found that moist areas continued to be a methane sink (36), and so these dry tundra permafrost areas in Eastern Greenland would need to shift into saturated fen areas to become significant sources of methane.

It is both fascinating and helpful for future projections that the findings presented here largely agree with the study described above. The abundance of microbial community members, particularly organisms involved in the methane cycle is consistent with observed methane flux measurements from this study and the other studies done in Zackenberg. This data is supportive of the prediction regarding Zackenberg largely remaining a methane sink. It is clear that the moist active layer microbial community is more similar to the dry active layer community. This may further solidify the idea that moist active layer permafrost is not likely to be a source of methane production. The dry

tundra that dominates Zackenberg would need to become saturated with water and resemble a fen before it could become a methane source. Additionally, if the methanogens are not present and waiting in the substrate, it seems unlikely that there was historical positive flux and their absence will not support increased positive flux into the future unless there are shifts in the community.

Another unique finding of this study was the detection of the NC10 phylum in active layer permafrost samples. NC10 is the phylum that contains nitrite-dependent anaerobic methane oxidation (N-DAMO) and *Methylomirabilis oxyfera* is the only cultured organism from this phylum (45). N-DAMO has been an overlooked methane sink in many environments. This process has only recently gained attention in temperate wetlands (45). This is the first study to detect organisms capable of N-DAMO in permafrost and the organisms were exclusively detected in the fen areas. This makes sense, as this is a strictly anaerobic process. The detection of these organisms reveals an additional and previously unknown methane sink in permafrost. This may be an important process to further investigate as it creates an important link between the carbon and nitrogen cycle in permafrost and will have implications for climate change. Generally, as nitrite increases, methane oxidation and carbon dioxide emissions will increase. If climate change impacts the nitrogen cycle and nitrogen no longer becomes a limiting nutrient, N-DAMO may increase in permafrost further decreasing the likeliness of the potential of permafrost becoming a significant source of methane emissions.

Conclusions

Because most historical research on permafrost has focused on fen areas, what is known about permafrost microbial communities is restricted to this type of permafrost. A much broader depiction of the microbial communities in permafrost allows for a better understanding of what a microbial community looks like in a methane sink versus a methane source and how that knowledge can be applied to methane emission predictions. This study works to fill this gap in knowledge and provides a baseline for what microbial communities may be found across different soil moistures. Additionally, this study shows that the communities found in moist active layer are similar to that of dry active layer samples, bolstering previous findings indicating that the Zackenberg area is largely a methane sink and will continue to be.

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CHAPTER FOUR

As the Seasons Turn: Impacts of *in situ* Variables and Seasonality on Microbial Community Structure and Function in the Permafrost Active Layer

ABSTRACT

This study seeks to describe the succession of microbial communities throughout the brief Arctic growth season and to examine the impacts of climate change scenarios on active layer microbial communities *in situ.* The inherent nature of climate change combines many variables impacting this permafrost ecosystem in a dynamic way. Due to these complexities, it is best to create a manipulated field setting to track microbial communities *in situ* rather than under lab conditions. This work examined microbial communities in June, July, and August (i.e. the Arctic growth season), and under increased snow accumulation and soil warming manipulations *in situ*. Further, the goal of this study was to integrate microbially focused questions with process level-measurements in the field. High throughput sequencing of 16S rRNA transcripts and genes were used to examine the structure and inferred activity of the microbial communities under these various conditions. Additionally, quantitative PCR was used to measure the abundances of key functional genes including: nitrogenase (*nifH)*, nitrous oxide reductase (*nosZ)*, nitrite reductase (*nirS)*, methanol dehydrogenase (*mxaF)*, and methyl-coenzyme M reductase (*mcrA).* Methane cycling organisms were found to peak in activity in July when methane oxidation was highest. The nitrogen fixation community peaked in July, and the activity of this group was seen to increase in both July and August as indicated by the copy numbers of *nifH* in the soil. Despite the low abundance of

denitrifiers, functional trends were still clear. *NosZ* peaked in July and August and *nirS* peaked in July when nitrate levels were at their lowest. It seems that the methane cycling organisms have a shorter window of activity than the N-cyclers, which largely remained active in August. The results of this study confirmed that one year of snow accumulation and soil warming impacted the structure of the microbial community and indicated that microbial communities are susceptible to changing environmental conditions. Further, it was also clear that both microbial community structure and function exhibited fluctuations in conjunction with seasonal trends.

INTRODUCTION

Arctic environments have been found to be much more sensitive to changes in climate and are predicted to warm much more rapidly than any other environment on the planet (1). Climate changes may cause this once frozen carbon pool to become a readily available and highly labile carbon source for bacteria to metabolize. Increased air temperatures in the Arctic may induce a shift from Arctic tundra serving as a carbon sink to becoming a carbon source (2). Seasonal thawing of the topmost portion of permafrost, called the 'active layer', is normal. However, due to increased air temperatures from climate change, the thawed active layer is growing deeper and deeper every year (3, 4). In a twelve-year study in northeast Greenland, the active layer was found to be increasing at a rate greater than one centimeter per year (5).

Large amounts of carbon are stored in the layers of this soil that remain frozen year long. It has even been estimated that 50% of the global belowground carbon pool may be contained in permafrost (6). As more carbon substrates become available to microbes as

permafrost continues to thaw, more microbes are actively metabolizing carbon producing greenhouse gases such as carbon dioxide and methane as byproducts. These gases could potentially further contribute to warming air temperatures, creating a positive feedback loop, thereby exacerbating the effect of climate change. In order to understand the repercussions of these positive feedback loops, additional studies need to investigate microbial activities in this and other changing permafrost ecosystems.

There are many environmental variables that will be impacted by climate change including air temperature, precipitation, and soil moisture. In addition to these variables, more long-term environmental trends will be impacted such as the general seasonal dynamics of the Arctic. For example, climate change and warmer air temperatures could mean a longer growth season. It has been found that winter warming exceeds summer warming in the Arctic (7). This may lead to earlier snowmelt, earlier active layer thaw, and an earlier spring in the Arctic. Further, current models predict that vegetation patterns could shift extensively as climate change progresses and seasonal trends shift (8). Perhaps the biggest mystery is how microbial community structure and activity will contribute to and respond to changes in these environmental parameters.

Global nutrient cycles including the carbon, nitrogen, and phosphorus cycles are all tightly intertwined, each impacting the other. However, the nitrogen and phosphorus cycles are not always considered in climate change models (9). Additionally, no current models integrate relevant microbial activities into predictive measures, yet microbes are the key regulators of nutrient cycles in most environments including permafrost. The relationship of microbial community activity and greenhouse gas production is still largely unknown (10). Examples of how microbially regulated nutrient cycles mediate net

greenhouse gas fluxes include the production of nitrous oxide by denitrifiers in the final steps of the nitrogen cycle and how the net production of methane is dependent on the balance between methanogens and methanotrophs. Additionally, methanotrophic activity is known to be a major sink for methane in permafrost (11). Microbial activities are among the main factors driving fluxes of gases from permafrost and if accurate predictions concerning gas emissions from these environments are to be made, a better understanding of microbial community structure, susceptibility to climate change effects, and what these mean as far as their functional activities (i.e. gas production) are required. Developing a better understanding of how these nutrient cycles vary with seasonal dynamics in the Arctic may be the first step.

Seasonal dynamics impact nitrogen (N) cycling in temperate regions (12, 13) and so it seems likely that similar seasonal effects occur in the Arctic, but over a more condensed time frame. The Arctic has been described as N limited (14) and so microbial responses controlling the N cycle may reveal important trends that hold implications for long-term shifts in these ecosystems. It is unknown what will happen if the Arctic region is no longer N limited. For example, more readily available nitrogen may allow more shrub growth in the Arctic or may increase rates of denitrification, leading to greater nitrous oxide emissions. Additionally, understanding nitrogen fixation activity under differing conditions has important implications for total N concentrations in soil and the amount of N available to plants and other soil organisms.

Further, reduction of nitrogen oxides by bacteria results in the production of nitric oxide (NO) and nitrous oxide (N_2O). Denitrification accounts for approximately 83% of the nitrous oxide produced after a thawing event and is the main source of N_2O emissions

(15, 16). This may be in part due to the extreme diversity and numerical abundance of bacteria involved in denitrification (17). Studying which denitrifiers are present in a system and how active they are may have direct implications for nitrous oxide emissions from thawing permafrost. In agricultural soil, nitrous oxide emissions have been linked to denitrifying gene abundances (18). Freezing and thawing in soils may lead to an increase in substrate availability and thereby stimulate denitrification activity (16). This pattern is further exemplified in temperate soils where there tends to be a large positive flux of nitrous oxide after spring thaws (19). It was found that freeze-thaw cycles did not impact microbial biomass, but did impact microbial community structure resulting a ten fold increase in *nirS* gene abundance after soil thawing (16).

Furthermore, it has been shown in temperate soils that bacterial abundance increased in warm plots with elevated CO_2 and decreased in warm plots with ambient CO_2 levels (20). This same study found that changes in precipitation impacted the relative abundance of Acidobacteria and Proteobacteria (20). Given these trends in temperate regions, it is likely that microbial communities in permafrost will also be sensitive to environmental shifts such as thawing of permafrost, increased precipitation, and soil warming. Specifically, an increase in denitrification may be expected as soon as the active layer begins thawing.

Field studies have shown that seasonal dynamics impact gross N pools (12). Clear shifts in concentrations of ammonia, nitrates, and other nitrogen sources in the soil likely suggest shifts in microbial N cyclers in these soils. Other studies that have focused on specific genes, such as *nirS* (13), have also shown evidence of microbial shifts with seasons. Additionally, it has been shown that nitrogen cycling phyla and functional

groups shift with seasonal changes in temperate forest soils (21). However, in another study, soil communities were found to be less variable with time than any other environment tested including microbial communities in air, streams, marine systems, and microbiomes associated with plants and humans (22). This suggests resilience in soil community structure throughout seasonal shifts.

Shifts in microbial metabolism have been observed in Arctic soils transitioning from 2 to 0.5°C (23), however, there is an incomplete understanding of how microbially mediated nutrient cycles change with the relatively quick seasonal shifts in the Arctic. PLFA analysis revealed seasonal trends in microbial communities in mid alpine environments with a strong shift in community composition after snow melt (24). Further, there is evidence for high rates of microbial turnover and shifts in microbial community composition during spring snow melt in alpine ecosystems (25, 26).

As previously mentioned, the nutrient/biogeochemical cycles that microbes mediate are all tightly related. Predicting net fluxes of gasses will not be as simple as measuring the abundances of denitrifiers or methanogens. The net flux of methane depends on the balance of activity between methanogens and methanotrophs (including those methylotrophs that can utilize methane) (27). This balance is impacted by many environmental variables and in most research reports to date there is not a linear relationship between the two (28, 29). Many studies have found that ammonia and ammonium can inhibit methanotrophs from oxidizing methane (30-34). This is likely due to the similar size of methane and ammonia (33). Similarities in physiology promote competition between methane and ammonia oxidizing bacteria (35). This phenomenon begins to illustrate the interconnectedness of nutrient cycles, and in this case the

connections between methane and nitrogen cycling. Nitrite-dependent anaerobic methane oxidation (N-DAMO) may be an additional connection between nitrogen and methane cycling. N-DAMO is a possible methane sink in wetlands, and has only been recently studied in this ecosystem (36). This mode of methane oxidation has not been previously detected in permafrost. The addition of nitrate as fertilizer may initiate the N-DAMO metabolic process and increase carbon dioxide emissions while decreasing methane emissions (36). It is clear that understanding shifts in nitrogen cycling in the environment may hold implications for the amount of carbon dioxide and methane that are emitted from thawing permafrost. Identifying taxa, their functional groups, and key functional genes will help to disentangle these nutrient cycles.

Recent studies of a variety of permafrost environments have revealed the major phyla present, which are fairly consistent with those found in temperate soils. Acidobacteria, Actinobacteria, Bacteroidetes, and Proteobacteria tend to be the major phyla detected in both active layer and constantly-frozen permafrost samples (37-40). Yet, the taxonomic portion of most studies to date does not go much further than describing which taxa are present in a sample, and only at a coarse level – primarily at the phylum level. Better determination of permafrost microbial community phylogenetic composition, but at a much higher resolution, and the ability to monitor how communities change in response to certain variables would make for much more revealing studies.

Interesting results have risen from these earlier works including that the active layer is slightly more diverse than the permafrost (37, 38). Interestingly, after two days of thaw, permafrost microbial communities start to resemble the active layer communities by exhibiting increases in diversity (41). This data substantiates predictions concerning

thawing of permafrost impacting microbial community structure. It has also been found that carbon cyclers and nitrogen cyclers shift in response to permafrost thaw (40). An early metagenomic study of permafrost compared active layer and frozen permafrost and found that type I methanotrophs were common in both layers, and that *nifH* was abundant in both layers (42). Another study investigating different depths of permafrost and found *nifH* to be the most abundant functional gene at all depths (up to 10^8 copies /g wet soil) and negatively correlated with depth (43). This study also found *mcrA* copies ranged from 10^1 - 10^7 copies/g wet soil and distributed randomly (43). Overall, the integration of functional gene analyses is sparse and when they have been implemented the analyses do not reveal consistent patterns or major trends. Until now, most previous studies rely on small sample size, likely due to the inherent difficulties of working in the Arctic, and this may impact the conclusions drawn from microbial community structure and function analyses.

The current study seeks to build upon earlier permafrost studies in order to monitor successional changes in microbial communities throughout the brief Arctic growth season. The inherent nature of climate change combines many climate factors impacting this permafrost ecosystem in a dynamic way. For example, both increased air temperature and increased rain events in the Arctic will have varying effects on the soil moisture of the active layer. Due to these complexities, it makes sense to develop a strategy for experiments that utilize manipulated field conditions to control the multiple variables encountered under ambient conditions. By controlling for complex environmental variables, it becomes feasible to monitor and compare the structures of microbial communities in the context of seasonal shifts under likely climate change

scenarios including increased snow cover and soil warming. Another goal of this work was to frame and interpret data these microbially-focused questions in the context of process level measurements. For example, soil moisture and nutrient level measurements may help to confirm patterns observed in microbial and molecular data. Specifically, this body of work addresses the following questions: 1) How does one year of a suite of *in situ* variable treatments impact microbial community structure and function? and 2) How does seasonal succession impact microbial community structure and function in active layer permafrost?

Under laboratory conditions, bacteria are generally considered to respond quickly to change due to their rapid generation times. Based on this expectation, it might be that one-year of exposure to *in situ* variables may impact the structure of microbial communities. Conversely, one year is not a long time for an environmental variable manipulation particularly in this permafrost environment in Greenland with it's greatly foreshortened growing season (i.e. one month each of spring summer and fall, followed by a 9-month long winter). Based on previous work in temperate environments, it was expected that microbial communities would vary with season in the Arctic. Thus, it seemed reasonable to expect that the combination of snow melt and normal active layer thaw would impact both microbial community structure and function. If seasonal variations can be detected in permafrost microbial communities, then it could be assumed that, as seasonal trends shift in the arctic due to climate change, normal microbial community seasonal changes too would shift, impacting nutrient cycling and thus greenhouse gas emissions from permafrost. Predicting the responses of microbial communities to changing climatic patterns in the Arctic may be challenging given the

constraints of logistics, short growing season, cold mean annual temperatures and other factors. However, working to understand these changes will prove invaluable to climate change predictions as microbes are the key controllers of greenhouse gas emissions and even weather itself. Net flux data have a limited predictive capacity and thus understanding microbial community roles and responses under climate change scenarios will help to reveal the overall potential of the ecosystem.

METHODS

Sample Site and Sample Collection

To assess the relative contributions of seasonal variation, snow accumulation, and increased soil temperatures to microbial community compositional shifts in permafrost, a field site containing snow fences and open-top soil-warming chambers was established in 2012 on Disko Island, Greenland. Six replicate snow fence blocks were established, each with control (C), warmed with open top chamber (W), shrub removal (R), and shrub removal and warmed with

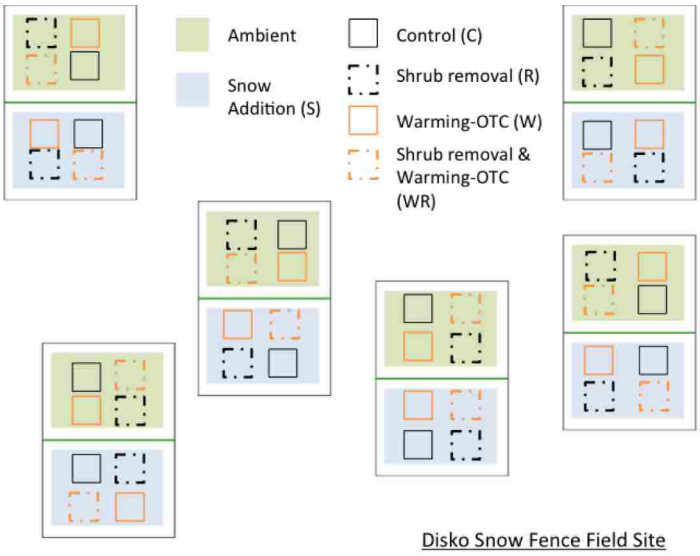

Figure 1. The six replicate blocks set up on Disko Island. A snow fence was built through the middle of each block and on either side of the fence were four treatment plots including the control (C), shrub removal (R), warming (W), and shrub removal plus warming (SWR).

open top chamber sites (WR) on either side of the snow fences. The fences lead to enhanced accumulation of snow on the downwind side of the fence. Thus, there are four treatments in each block with ambient snow accumulation and four with enhanced snow accumulation leading to later thaw in spring and presumably a shorter growing season. Samples were collected from the permafrost active layer in June, July, and late August of 2013.

A 10 cm long and 5 cm in diameter custom metal core was used to collect active layer samples. A rubber mallet was used to drive the core into the active layer. One core was collected from each plot in each snow fence block (Fig. 1) for a total of 48 plots at each sampling time, with the exception of the June sampling where 1.SC, 1.SR, 1.SW, 2.SC, 5.SC, 5.SR, and 5.SW (each number designates Block number.Treatment) samples were not collected due to snow cover still persisting. Each 10 cm core was divided in half (0-5 cm and 5-10 cm) and each section was placed in a separate sterile plastic bag. All core holes were refilled with active layer permafrost from outside the experimental area to minimize potential impact of changes in soil conditions due to open holes in the plot. Samples were transported back to the Arctic Research Station <http://arktiskstation.ku.dk/english/about/> the same day of collection. The samples were held in a refrigerator for up to one day before further processing. Any visible plant roots were carefully removed from samples for other analyses prior to nucleic acid extractions.

Soil Moisture Content and Nutrient Measurements

Approximately 5-10 g of sorted fresh soil was weighed out and dried in an oven at 70°C for a minimum of 48 h to determine soil moisture content. Approximately 10 g of

fresh, sorted soil was extracted with 50 ml cold water for soil nutrient measurements. The soil extracts were filtered through Whatman GF-D filters (Sigma-Aldrich, St. Louis, MO, US) and frozen until analysis of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), ammonium (NH4+) content, nitrogen (NO3-) content, and phosphorous (PO43-) content. Further details on soil extract measurements are provided in (44) and (45).

Methane Flux measurements

The *in situ* methane flux measurements were performed by using opaque polycarbonate closed-static chambers placed on water sealed frames permanently installed in the soil. The air in the headspace of the chambers was circulated in a closed loop to a DLT-100 Fast Methane Analyzer (Los Gatos Research, Mountain View, CA, US) at a flow rate of approximately 0.3 L min-1. A temperature sensor was mounted in the lid of the chambers (107 temperature probe; Campbell Scientific, Leicestershire, UK) to measure the air temperature inside and outside the chamber. The concentrations of CH4 were measured during 15 minutes period with 10 seconds sampling frequency. All the data were recorded into a Campbell Scientific data logger (CR1000). Rates of CH4 uptake were calculated by fitting a second order polynomial function to the changes in gas concentrations in the headspace of the chamber over an 11 minute period. The slope of the regressions was accepted by $P \leq 0.05$ and $R2 \geq 0.85$.

Nucleic Acid Extraction and Quantification

Both DNA and RNA were extracted for deep 16S rRNA gene sequencing to assess overall microbial community composition (16S gene abundance in metagenomic

DNA) and the most active members (16S transcripts in the metatranscriptome), respectively. Two aliquots of each 0-5 cm core portion were weighed out to approximately 0.25 g each, the wet weight was recorded, and one aliquot of sediment was placed into a bead tube for DNA extraction. DNA extraction was performed with the MOBIO PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) at the Arctic Research Station. DNA samples were transported in liquid nitrogen back to Copenhagen for further analysis. The 2.SWR DNA sample was compromised by tube breakage during transport and was not used for further analyses.

The other 0.25 g aliquot was placed into a microcentrifuge tube, immersed in liquid nitrogen, and transported back to the GEUS laboratory in Copenhagen for RNA extraction. RNA was extracted using the MOBIO PowerMicrobiome RNA Isolation Kit. The optional DNA isolation step was also performed, however, this DNA was not used in any of the subsequent analyses because it was determined to be of insufficient amount and quality. The manufacturer's protocol was followed as specified with the exception of the addition of 500 µl instead of 100 µl of phenol:choloroform:isoamyl alcohol pH6.5-8.0 (included with kit) at the first step.

Reverse Transcriptase PCR

The MOBIO RTS DNase kit was used to remove genomic DNA from the RNA by mixing 17 µl of the RNA extract with 2 µl of the 10X RTS DNase Buffer and 2 µl of the RTS DNase. The RT PCR master mix was made up as follows: 1.0 µl random hexamer primer (10 pmol), 1 µl dNTP (10mM), 0.5 µl DEPC treated water, 4 µl 5X reaction buffer, 0.5 µl Ribolock RNase inhibitor, and 1 µl RevertAid premium reverse

transcriptase (all components from Thermo Fisher Scientific, Waltham, MA, US) for each reaction. Eight µl of the master mix was added to 12 µl of DNase treated RNA. All steps were performed on ice prior to reverse transcription. The reverse transcriptase incubation conditions were as follows: 25 °C for 10 minutes, 50 °C for 30 min, 85 °C for 5 min. All samples were stored at -80 °C until further use.

Quantitative PCR

Quantitative PCR and the development of positive controls for the targeted genes for nitrogenase gene (*nifH)*, nitrous oxide reductase gene (*nosZ)*, nitrite reductase gene (*nirS)*, methanol dehydrogenase gene (*mxaF)*, and the methyl-coenzyme M reductase gene (*mcrA)* primer sets have been previously described (18, 46). The assigned functions of these genes are further described in Fig. 2. Quantitative PCR was used to amplify these genes of interest in the extracted metagenomic DNA. All RNA extracts were in too low in concentration to use for quantitative PCR. Quantitative PCR reactions (20 µl) were performed in triplicate for each sample. The reaction mixture included 10 pmol of each forward and reverse primer, 20 µg of bovine serum albumin, and 12.5 µl of iTaq Universal SYBR Green Supermix (BIO-RAD, Hercules, CA, US). The *nifH* gene was amplified using an initial denaturation step of 15 min at 95°C, followed by 10 cycles of touchdown consisting of denaturation (1 min at 96°C), primer annealing (1 min starting at 65°C and lowering 1.5°C per cycle), and primer extension (1 min at 72°C), followed by 30 cycles of amplification consisting of denaturation (1 min at 96°C), primer annealing (1 min at 50°C), and primer extension (1 min at 72°C), with a final extension step of 10 min at 72°C. The *nirS* and *nosZ* genes were amplified using an initial

denaturation step of 15 min at 95°C; followed by 6 cycles of touchdown consisting of denaturation (15 s at 96°C), primer annealing (30 s starting at 63°C and lowering 1°C per cycle), and primer extension (15 s at 72°C); followed by 35 cycles of amplification consisting of denaturation (15 s at 96°C), primer annealing (30 s at 58°C), and primer extension (15 s at 72°C). The *mxaF* gene was amplified using an initial denaturation step of 15 min at 95°C; followed by 40 cycles consisting of denaturation (1 min at 96°C), primer annealing (1 min at 66°C), and primer extension (1 min at 72°C). The *mcrA* gene was amplified using an initial denaturation step of 15 min at 95°C, followed by 45 cycles of amplification consisting of denaturation (40 s at 96°C), primer annealing (1.5 min at 55°C), and primer extension (2 min at 72°C), and a final extension step of 10 min at 72°C.

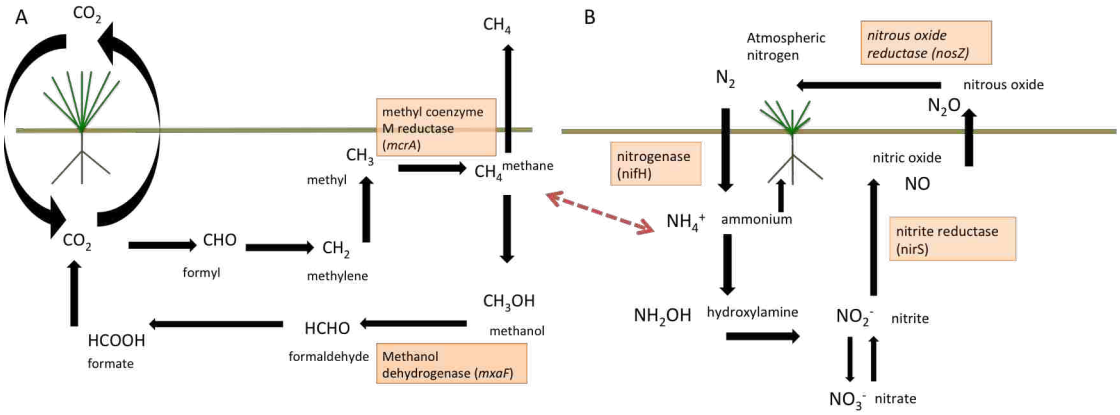

Figure 2. The general methane cycle (A) and nitrogen cycle (B) with enzymes of interest in this study indicated in orange boxes. The dashed red arrow shows one point of connectedness between these two nutrient cycles.

Barcoded PCR and Sequencing

Dual-labeled 16S 515F/806R primers with 4-6 base tag (515F: GTGCCAGCMGCCGCGGTAA, and 806R: GGACTACHVGGGTWTCTAAT (47))

were used to target the V4 and V5 regions of the bacterial ribosome. A 30 µl PCR reaction was set up as follows: 6 µl Buffer (PCR Biosystems(PCRBIO) London, UK), 3 µl BSA, 0.6 µl dNTP (10mM), 3 µl primer mix (10 pmol mix of each dual labeled primers), 0.3 µl PCRBIO HIFI Polymerase (PCR Biosystems(PCRBIO) London, UK), 15.6 µl PCR H_2O, and 1.5 µl DNA or cDNA template. This 30 µl reaction was split into 3 independent 10 µl reactions in separate tubes. The cycling conditions included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation (95°C for 15s), primer annealing (50°C for 20s), and extension (72°C for 20s), and a final extension step (72°C for 5 min).

The three replicate reactions were combined and purified with HighPrepPCR (MAGBIO, Gaithersburg, MD, US) according to the manufacturer protocol. DNA and cDNA concentrations were measured with a Qubit (Qubit, London, UK). One µg of amplicon DNA was used for one ligation reaction using the MiSeq Ligation protocol. Illumina adaptors were ligated on the PCR product using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, US). The Illumina MiSeq 2x250 base pair paired-end platform at the National High-throughput DNA Sequencing Centre (Copenhagen, Denmark) was used for sequencing.

Sequence Analyses and Statistical Analyses

Fastq-join software < https://code.google.com/p/ea-utils/wiki/FastqJoin> was used to join the paired reads with an 8% maximum difference in the joined regions and a minimum overlap of 6 base pairs allowed, however, the average overlap was 80 bp. Reads that were not paired were not used in further analyses. The Qiime pipeline version

1.8 (48) was used for general sequence quality control and downstream analyses. The sequences were demultiplexed and operational taxonomic units (OTUs) were clustered at the 97% similarity level using *de novo* OTU picking. The identified OTUs were classified to the genus level with the RDP classifier (49). A phylogenetic tree was generated using the Pynast aligner within the Qiime pipeline. Paired-end sequence reads from all samples (both DNA and RNA data sets) were normalized (a.k.a. "rarefied") to 2420 sequences per sample (the lowest number for any sample) for a more robust bioinformatic analysis. To visualize β-diversity (microbial community similarity between samples), weighted UniFrac scores were determined and used for a principal coordinates analysis (PCoA). Significance in the clustering was determined with an Adonis test done in Qiime through the Vegan package in R (50).

Linear Discriminant Analysis (LDA) was employed with leave-one-out cross-validation to assess whether microbial community composition varies as a function of season, treatment, or both. Inside each fold of the cross validation, a J3 floating search algorithm was used to identify genera that discriminate between groups in the data. The purpose of the cross-validation is to check classifier performance on unknown data. Statistical information representing the genera chosen inside of cross-validation is analyzed in a box and whisker plot and the results used to generate a 3D Pareto frontier, wherein the best-performing scenarios (there can be multiple scenarios that perform equally well) are plotted on the frontier. The more often a genus was selected as being important for discrimination during the cross-validation process, the more likely it was ultimately used for visualizing the results. LDA was also used for the final visualizations of these patterns in the data.

RESULTS

Sample Collection

Samples were collected in June, July, and August. The sampling time in June occurred immediately after the general snow-melt for the area. Some snow was still present on the snow accumulation side of the snow fence, and so these samples could not be collected in June. These included 1.SC, 1.SR, 1.SW, 2.SC, 5.SC, 5.SR, and 5.SW, so there was no RNA or DNA from these samples. It should also be noted that the RNA sample (2.SC) from July and the 2.SWR DNA sample from August were lost due to breakage during transport back to Copenhagen.

Samples collected in June represent the onset of spring in Greenland, those from July represent the peak of the growth season, while samples collected in August represent the onset of fall. These seasonal trends based on observational data including the snow-melt period and active layer thaw occurring during the sample collection in June, the abundant growth of shrubs and warmer air temperatures in July, and finally the change in foliage color and drop in air temperature during sample collection in August.

Nucleic Acid Extraction and RT-PCR

Due to previous difficulties with the co-extracted DNA (see Chapter 3), a separate DNA extraction was performed using a more optimal protocol and yielded substantial amounts of metagenomic DNA for downstream use. All DNase controls from the RNA extraction did not produce 16S rRNA amplicons when PCR amplified with the generally conserved (so-called ‘universal’) primers, indicating that the RNA extractions were not contaminated with DNA and were appropriate for sequencing and further analyses.

Sequencing

After general quality control and joining paired-ends, the DNA data ranged from 2,421 to 22,549 sequences per sample. The RNA data set ranged from 5,539 to 37, 985 sequences per sample. For β-diversity analyses, the data sets were subsampled to 2,420 sequences per sample. For the LDA analyses, the data sets were not normalized (rarefied).

β-*diversity and Community Structure*

To analyze how significantly season and treatment impact the β-diversity of microbial communities, distance matrices were generated from weighted UniFrac scores and the significance in the separation of the data was tested with the Adonis test in R. Season (i.e. sampling time) was found to be a significant determinant of separation in the DNA data set and accounted for 15.5% of the variability (Adonis, P= 0.001; R^2= 0.155). Additionally, treatment also explained variation in the data, but had both a higher P value and a lower effect size (R^2) (Adonis, P= 0.011; R^2= 0.079). Treatment accounted for only 7.9% of the variability in the data set. These same patterns were observed for the RNA data set. Both season (Adonis, P= 0.002; R^2= 0.033) and treatment (Adonis, P= 0.02; R^2= 0.066) were found to significantly explain separation in the RNA data set, however the effect sizes were low. Season explained only about 3.3% of the variation in the RNA data set, while treatment explained 6.6% of the variation. Overall, these Adonis results indicated significant impacts of season and treatments separating the data, but low effect

size, creating motivation to examine the data in more meaningful ways in order to confirm these observed patterns.

Linear Discriminant Analysis and Community Structure

Linear discriminant analysis (LDA) and cross validation (CV) confirmed patterns in the data due to season and the warming and snow accumulation treatments. The LDAs for the seasonal effects had CV values of 66.91 and 59.85% for the DNA and RNA data sets respectively. Since three classes were being tested (June, July, and August), there is a 33% chance that the algorithm would randomly assigned a sample to the right class during cross validation. Both CV values were substantially higher than this probability, indicating a meaningful pattern. The clusters in both the DNA and RNA data sets progressed from June to July to August, mirroring the natural progression of spring, summer, and fall. The algorithm was successful in finding patterns by detecting the genera (or features) that contribute most to discriminating patterns in the data. For the DNA data set, it was found that 5 different genera were the best at differentiating patterns in data including *Conexibacter*, *Mucilaginibacter*, *Sediminibacterium,* and two additional unclassifiable genera. It was found that 8 genera were most influential in creating patterns due to season in the RNA data set including *Gemmata, Singulisphaera, Solitalea, Labrys, Streptomyces, Burkholderia* and two unclassifiable genera. Both the sizes and positions of clusters and the CV values indicate that the DNA data separate more clearly than do the RNA data (Fig. 3).

The treatment data, including all treatments of warming, snow addition, shrub removal, and controls, were also analyzed with the three sampling timepoints together.

The algorithm did not detect patterns when all treatments were included, but when the shrub removal plots were excluded from the data set, the LDA-CV analysis revealed patterns in the data. The LDAs for the treatment effects had cross validations of 44.78% and 43.28% for DNA and RNA respectively. There is a 25% chance of the algorithm randomly assigning an unknown sample to the correct group because there were four groups being examined (control (C), warming (W), snow-side control (SC), snow-side warming (SW)). Both CV values were much greater than 25% supporting the visualized patterns in the data. In the by-treatment DNA plot, the control clusters centrally with the treatments separating from it radially (Fig. 3). Additionally, the snow-side variables clustered closer together. In the RNA data set, the snow-side warming plots are most variable, whereas control, warming, and snow-control overlap more. It was shown that 29 different genera were useful in distinguishing patterns in the data. Four of the 29 key genera were not identifiable and the other genera included: *Streptacidiphilus, Acidovorax, Streptomyces, Paenibacillus, Aminobacter, Pirellula, Planctomyces, Flavisolibacter, Humicoccus, Schlesnaria, Herbaspirillum, Cystobacter, Steroidobacter, Pedomicrobium, Beijerinckia, Gemmata, Caldilinia, Rhodanobacter, Rhodoplanes, Phenylobacterium, Actinoallomrus, Burkholderia, Duganella, Gemmatimonas*, and *Leifsonia*. Five genera were shown to be useful in distinguishing patterns associated with treatment in the RNA data set including *Streptacidiphilis, Chitinophaga, Virgisporangium, Pedobacter,* and *Nocardioides*. Similar to the LDAs generated to explore seasonal trends, the LDAs created to display treatment patterns showed less separation for the RNA data set, especially in the treatment-based plot (Fig. 3).

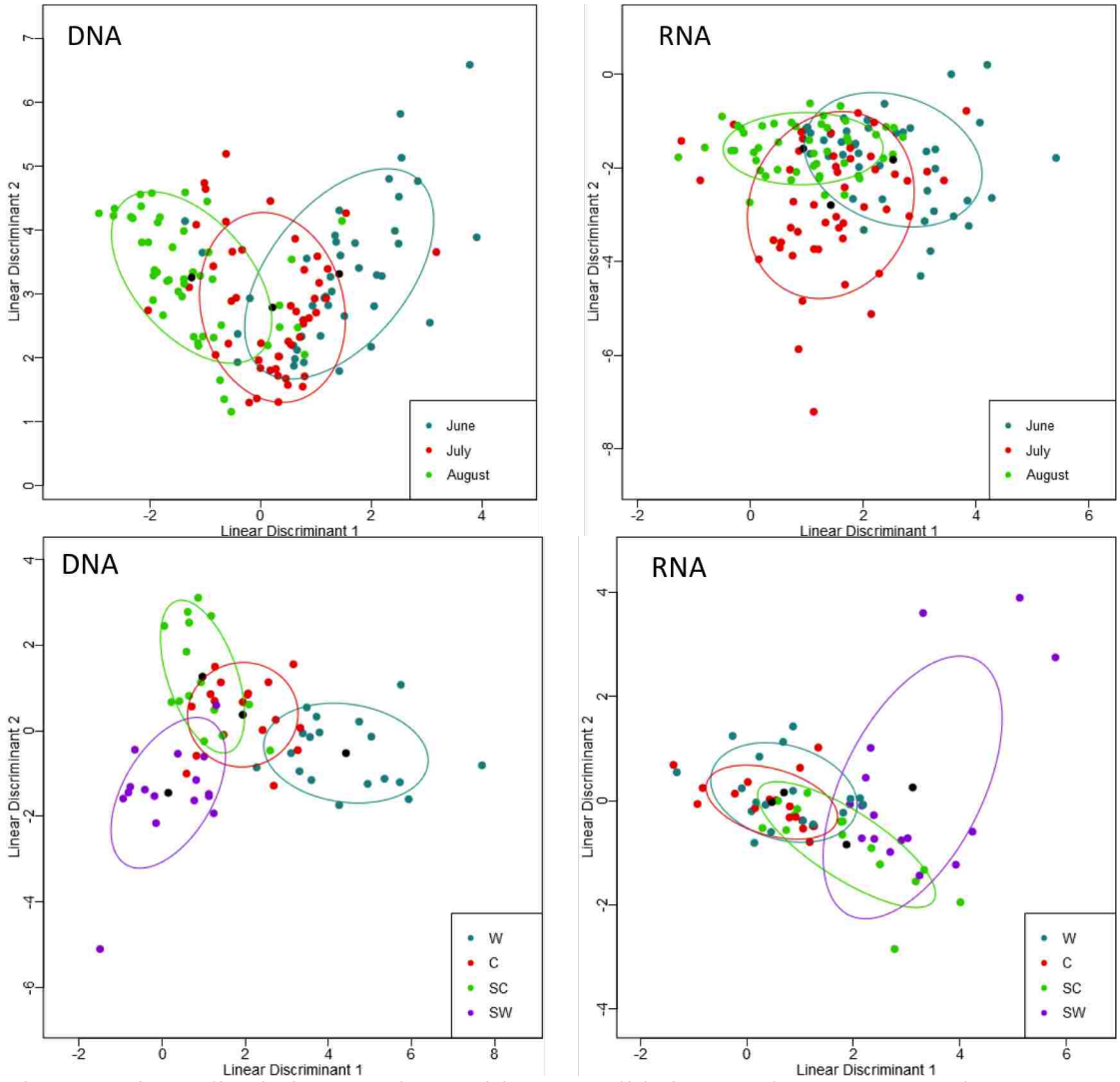

Figure 3. Linear discriminant analyses with cross validations to observe patterns due to seasonal trends (top) and treatments (bottom). The LDAs displaying treatment data include the treatments warmed (W), controls (C), snow side control (SC), and snow side warming (SW) from all time points. The LDAs for the seasonal effects had cross validations of 66.91% and 59.85% for the DNA and RNA respectively. The LDAs for the treatment effects had cross validation of 44.78% and 43.28% for DNA and RNA respectively.

Community Function – Methane Cycle

Metatranscriptomic RNA was used as template for both *mcrA* and *nifH* Q-PCR amplification, but the specific mRNA template concentrations were too low for any amplification to occur. Thus, the metatranscriptomic RNA was not used as template for any subsequent RNA reactions and no data is presented using mRNA for qPCR.

Metagenomic DNA was used as template in qPCR reactions to measure the abundance of the genes of interest (Fig. 4). The copy numbers of the *mxaF* gene ranged from lower than 5 copies per mg wet soil to about 22 copies per mg wet soil. The copy numbers of *mxaF* were highest in July across the six replicate blocks and most treatments (Fig. 4, panel A). The methane flux measurements were all negative (Fig. 4, panel A), indicating that this site is largely a methane sink. Further, it was found that the average methane oxidation (indicated by net negative flux) across all blocks was highest in July (-0.305 mg CH4 /m^2/ hr) compared to June (-0.155 mg CH4 /m^2/ hr) and August (-0.055 mg CH4 /m^2/ hr).

The methylotrophs (capable of metabolizing methane and other 1-carbon compounds) detected in the metagenomic DNA based on classification of 16S rRNA gene amplicons) data set included *Methylocella, Hyphomicrobium, Methylobacterium, Methylocystaceae* family, *Methylibium,* and the *Methylophilaceae* family. Based on this approach, the total methylotroph abundance in the total community DNA pool ranged from less than 0.5 to about 2% of the total community (Fig.4, panel C). The methylotrophs detected in the total community RNA data set included *Methylocella, Hyphomicrobium, Methylobacterium, Methylocystis, Methylosinus, Mehylocystaceae* family, *Methylibium, Methylophilaceae* family, and *Methylovirgula.* Total methylotroph counts ranged from less than 0.1 to 0.9% of the total community (Fig. 4, panel D). Thus, the indicated relative abundance of methylotrophs was lower in the RNA data set compared to DNA data set. No distinct seasonal trend was noted in the abundance of methylotrophs. However, block two had a consistently low abundance of methylotrophs in both the DNA and RNA data set (Fig. 4, panels C and D).

The copy numbers of the *mcrA* gene ranged from lower than 5 to nearly 90 copies per mg wet soil (Fig. 4, panel B). In general, copy numbers of the *mcrA* gene were highest in most treatments in July. Methanogenesis is closely linked to soil moisture (SM), and so SM is plotted with this data. SM was highest in June across all blocks (106.1 – 158.6 g water/g dry soil). SM was lowest in plot 2 in July and August, with measurements of 77.2 and 71.5 g H_2O/g dry soil, respectively (Fig. 4, panel B).

For the most part, *mxaF* gene copy numbers peak in July (Fig. 4, panel A). By contrast, the absolute values (flux) were lowest in July. Gene copy numbers for *mcrA* also peak in July (Fig. 4, panel B). Soil moisture content is plotted on the secondary axis and was highest in June. The error bars in panels A and B represent standard deviation between the three replicate qPCR reactions.

Figure 4. Seasonal trends in *mxaF* gene copy number (A), *mcrA* gene copy number (B), and functional groups of methylotrophs identified in the DNA data set (C) and RNA data set (D). The average rate of methane oxidation plotted on the secondary axis in (A).

Community Function - Nitrogen Cycle

Generally, *nifH* was more abundant than any other N cycling genes measured. There were very low copy numbers in June (the average copy number in June ranged from 12.4 to 144.0 per g wet soil), with a huge increase (100-1000 fold) in most samples in July (up to 8.0×10^3 copies per mg wet soil) and August (up to 5.3×10^3 copies per mg wet soil) (Fig. 5, panel A). Further, dissolved ammonia levels peaked in July (0.856 – 4.668 μg/g soil).

Based on 16S amplicon-based sequencing, the nitrogen fixers detected in the DNA data set included *Cyanobacteria, Clostridium, Beijerinckia, Bradyrhizobium, Rhodopseudomonas, Mesorhizobium,* and *Rhizobium* (Fig. 5, panel B). The total relative abundance of N fixers ranged from approximately 1-5% in the DNA sequence data set. It was found that most treatment blocks had their peak in N fixer community abundance in August (Fig. 5, panel B). The N fixers detected in the RNA-based data set included *Cyanobacteria, Clostridium, Beijerinckia, Bradyrhizobium, Rhodopseudomonas, Mesorhizobium, Rhizobium,* and *Azotobacter* (Fig. 5, panel C). Total N fixers ranged from approximately 1-5% of the total community (Fig. 5, panel B). A relatively similar abundance of total N fixer community was found compared to DNA data set (Fig. 5, panel C).

Copy numbers of the *nirS* gene were lowest in June (1.15 – 9.93 copies /mg wet soil) with a large increase in July (1.68 to 14.8 copies /mg wet soil) and August (1.08 to 6.14 copies/mg wet soil) (Fig. 6, panel A). The *nirS* gene copy numbers were highest in most treatments in July. Dissolved nitrate levels were lowest in July (Fig. 6, panel A). Additionally, the dissolved nitrate levels were lowest in July across all blocks. The *nosZ*

copy numbers were very low in June (ranged from $1.47 \times 10^1 \pm 0.99$ to $2.37 \times 10^2 \pm 9.04$) with a large increase (100 fold increase in most samples) in July ($9.12 \times 10^1 \pm 7.59$ to $3.38 \times 10^3 \pm 6.81 \times 10^2$) and August (4.92 ± 0.75 to $2.13 \times 10^3 \pm 5.76 \times 10^2$) (Fig. 6, panel B).

The nitrifiers (N fixers) detected in both the DNA- and RNA-based data sets included *Nitrobacter* and *Nitrospira.* The nitrifiers were present at a very low abundance and ranged from 0.01% to just under 0.14% of the total community in the DNA data set (Fig. 6, panel C). Similarly, these organisms were also present at a low relative abundance in the RNA data set (0.01 to 0.1%) (Fig. 6, panel D). Denitrifiers detected in the DNA data set included *Micrococcus, Thiobacillus, Nitrosospira, Pseudomonas, Hyphomicrobium,* and *Flavobacterium.* The relative abundance of the denitrifier population ranged from 0.01 to 2.3% of the total community based on the DNA based analysis (Fig. 6, panel E). In the RNA-based data set, *Micrococcus, Nitrosospira Pseudomonas, Hyphomicrobium,* and *Flavobacterium* were found. The abundance range of denitrifiers in the RNA-based data set (0.01 to 1.3%) was slightly lower than that of the DNA data set Fig. 6, panel F). Block two had relatively low denitrifier abundance compared to all other blocks in both the DNA- and RNA-based data sets.

Figure 5. Seasonal trends in *nifH* gene copy number (A) and functional groups of nitrogen fixers identified in the DNA data set (B) and RNA data set (C). For the most part *nifH* peaks in July and August. The average of dissolved ammonia per block is plotted on the secondary axis in (A) is lowest in June and highest in July.

Figure 6. Seasonal trends in *nirS* gene copy number (A), *nosZ* gene copy number (B), functional groups of nitrifiers identified in the DNA data set (C) and the RNA data set (D), and functional groups of denitrifiers identified in the DNA data set (E) and the RNA data set (F). For the most part, *nirS* peaked in July and *nosZ* peaked in July and August. The average dissolved nitrate concentrations per block are plotted on the secondary axis in panel A and were lowest in July.

DISCUSSION

The results of this study indicated that microbial communities were susceptible to changing environmental conditions, specifically those that mimic climate change scenarios. Further, it was also clear that both microbial community structure and function exhibited fluctuations in conjunction with seasonal fluctuations. Methane cycling organisms peaked in function in July, correlating to the process level measurements of methane oxidation that also peaked in July. The nitrogen fixation community peaked in July, and the activity of this group was seen to increase in July and August as indicated by the copy numbers of *nifH*. Denitrifiers were in relatively low abundance, but the functional trends were still apparent. *NosZ* peaked in July and August and *nirS* peaked in July when nitrate levels were measured to be the lowest throughout the growth season. It seems that the methane cycling organisms have a shorter window of activity than the N cyclers who largely remain active into August.

The variations in weighted UniFrac scores revealed significant impacts from treatments, however because of the low effect sizes in both the DNA and RNA data set this result was not completely convincing. The LDA with cross validation did not detect significant separation in data when considering all treatments; however, separation was detected between the control, snow control, warming, and snow warming. As expected, the control plot serves as a "baseline" community from which the other communities diverge when exposed to certain treatments. There were no obvious indications of treatment effects on community function based off of functional groupings of taxa or Q-PCR results. The results of this study do confirm that one year of snow accumulation and soil warming impact the structure of the microbial community.

Many scientists assume that all microbes have a quick generation time, as this is how microbial growth appears under lab conditions. However, microbial replication in the natural environment may be a different story. Considering that most of the microbial biomass of soil consists of dormant cells (51), it likely takes soil microorganisms time to adapt to new conditions in their natural environments. The *in situ* variables in this study, snow accumulation, warming, and shrub removal, had only been implemented one year prior to sample collection. Detection of treatment effects on microbial communities was surprising. The fact that the snow accumulation and warming did not impact the microbial community structure as much as seasonal trends is less surprising. Continual and long lasting variables may impact microbial communities more strongly.

There is a lot of evidence supporting microbial community succession with the changes in season in temperate regions. However, there is a lack of evidence supporting these trends in the Arctic. It was predicted that season would impact both community structure and function. The seasonal trends offer a long-term or legacy effect on this ecosystem, as these transitions from winter through summer have been occurring for thousands of years in this area. It seemed likely that the legacy effects of seasonal changes would impact permafrost microbial communities as they do to communities within temperate soils. The variations in weighted UniFrac scores tested with Adonis as well as the LDA-CV analysis revealed strong patterns in the microbial community data due to seasonal trends. The variation in microbial community data was better described by season than by treatment effects.

Most methylotrophs detected were in the Alphaproteobacteria group. Further, the most commonly detected methanotrophs were Type II methanotrophs.

Methylobacterium, Methylocystaceae, and *Methylocella* are all type II methanotrophs (52, 53) and *Methylosinus* is an obligate aerobic type II methane oxidizer (54). Type II methanotrophs have a lower affinity for methane, and so persist in areas with high concentrations of methane. Type I has previously been said to be most abundant in the Arctic (28, 55). This trend may be misguided as fen or wetland areas are studied most frequently when investigating methane emissions. It is still surprising that Type II methanotrophs made up the bulk of the methanotrophs in an area that is largely a methane sink and likely supports a low concentration of methane within the active layer. Despite this, Type II seem to be abundant in this dry arctic tundra active layer and so there must be some methane percolating to the surface allowing for these organisms to persist.

The general methylotroph functional group was not affected by season or by treatments. However, clear seasonal shifts were observed when analyzing the corresponding Q-PCR data. Both *mxaF* and *mcrA* peak in July. Initially, it may seem odd that both a gene involved in methanotrophy and one involved in methanogenesis peak at the same time. This indicates that methanogens may have a larger amount of substrate available in July and so are most active at this time. Yet, the general activity of methanotrophs must be greater at this site as net methane oxidation is recorded throughout the area. The fact that the peak in methane cycling activity mirrors the peak in methane oxidation indicates that methane cyclers are most active in July and during summer conditions. However, it is interesting that an earlier peak in *mcrA* was not observed in June, as soil moisture was highest in June. It is known that soil moisture is a key regulator of methanogenesis, even more so than soil temperature (56) and so it was

predicted that *mcrA* may have higher copy numbers right after snow melt. It may be that micro sites maintain anoxic conditions for methanogens throughout the growth season. It is also likely that most methanogens persist much deeper in the active layer.

Copy number of *mcrA* ranged from lower than 5 to nearly 90 copies per mg wet soil. A previous permafrost study found *mcrA* copies ranged from 10^{1-7} copies/g wet soil (43). This area in the arctic may have less methanogenesis activity than other areas, but the abundance and presence of *mcrA* emphasizes the fact that this permafrost has the potential to become a methane source. The organisms that would be responsible for generating methane are present and active in this permafrost. The lower copy number of *mxaF* compared to *mcrA* is not too surprising as many genes other than *mxaF* are involved in the process of methanotrophy and methylotrophy, where as *mcrA* is one of the most common methanogenesis genes.

In general the nitrogen cycle accelerates in July and in to August. *NirS* clearly peaks in July and *nifH* and *nosZ* have a large increase from June to July and August. *NifH* was highest in July and August. Ammonia concentrations were highest in July, reflecting a peak in organic waste. The higher levels of ammonia in July did not seem to impact methane oxidation in July. Further, the nitrogen fixer population peaks in august as indicated by the DNA data set. Similar to our findings, a previous permafrost study found *nifH* to be the most abundant functional gene (43). This study found that *nifH* copy numbers were around 10^8 copies /g wet soil. Where as, the data presented here revealed maximum copy numbers in July to be around 8.0×10^3 copies per mg wet soil. In general, it appears that functional genes are overall lower than those detected in other permafrost

studies and those measured in temperate soils. Lower microbial activity levels may be characteristic of dry Arctic tundra.

A clear peak occurred in *nirS* during July and at this same time nitrate levels were the lowest. This indicates that most of the nitrate is be utilized and denitrified in July. The highest copy number (14.8 copies /mg wet soil) of *nirS* occurred in July. A study in a temperate grassland found *nirS* copy numbers to range from 7000 gene copies/ng of DNA to 10,000 gene copies/ng DNA (16). Clearly, permafrost may exhibit lower activity than soils collected in warmer areas. Additionally, the peak in denitrifier activity, as indicated by the abundance of *nirS,* agrees with previous findings. Studies in other environments have also shown that freeze thaw cycles impact denitrification. One study detected a ten fold increase in nirS after soil thawing (16). Further, temperate soils have been found to show similar trends where a large flux of nitrous oxide follows spring thaws (19). The final step of denitrification, indicated by copies of *nosZ*, was highest in July and August. A relatively low abundance of nitrifiers were detected, which is typical in soils and is the main reason why no Q-PCR was done to monitor nitrification activity. This study confirms the low abundance of nitrifiers in soil ecosystems. There were no general patterns in nitrifier and denitrifier taxa functional groups throughout the growing season.

There are inherent issues with setting up studies in remote locations. Obtaining enough replicate samples can be a challenge. Each replicate block was several meters apart, making it illogical to use these blocks as replicates for microbiological data. This is supported by differing trends in the blocks. For example, block two had consistently lower abundance of methylotrophs and denitrifiers compared to the other blocks.

Additionally, no general patterns were seen in the Q-PCR data across treatments in the replicate blocks. Due to these variations, the blocks were not averaged as replicates for microbial data. Only one sample was collected from each treatment, making statistical tests of significance difficult. In future studies, at least three replicate soil samples should be obtained from each treatment.

A consistent disparity between DNA and RNA existed throughout this study. In general, the RNA data set did not show as strong of trends or patterns in both the LDA-CV community structure analysis as well as in the functional group analysis. This is due to a multitude of factors. One issue is the inherent problem of with working with RNA from soil. Nucleic acids can become tightly bound to clay and humic acids in the soils making both DNA and RNA difficult to extract and RNA is readily degraded by DNases in the soil and DNases that may be encountered during extraction (57). As mentioned previously, many microorganisms may not be actively dividing in this Arctic ecosystem. Many of the microbes in soil are in a dormant state. This means that transcription and thus RNA concentrations are expected to be lower. If this is true then a much more subtle shift in 16S rRNA would be expected after an environmental change. Indeed, less RNA was extracted from these samples than DNA, but this may be due to the difficulties in extracting RNA.

This study reveals the difficulties in disentangling nutrient cycles. For example, relying on process level methane measurements obscures the fact that methanogens are still active in the environment. Relying solely on gas fluxes or nutrient pools will not portray the complete picture of the nutrient cycling occurring. Additionally, microbes are notorious for conserving function, meaning some taxa do the same thing. Relying only on

taxonomical data would also make it difficult to observe shifts in function. This study works to combine process level measurements with both taxa assignments and functional gene abundances to begin to disentangle these complex and intertwined nutrient cycles.

Given this data, it can be concluded that seasonal succession is strongly apparent in both structure and function of microbial communities. Methane cycling peaked in July and nitrogen cycling was accelerated during July and August. Nutrient cycling peaks after snowmelt and when soil temperatures are likely rising. Snow accumulation and soil warming impacted microbial community structure, but no impact in function was detected. It is predicted that these patterns in structure would amplify and functional trends would appear as these treatments are in place longer. The microbial data emphasizes the potential of the environment and illustrates that methanogens are already present and active in this site; they just need conditions to switch that favor their activity over methanotrophs. Gas flux data cannot reveal this potential.

Having the capabilities to predict what conditions support more methanogens or higher methanogen activity that methanotrophs will be an important factor in future methane emissions predictions. It will become more and more important to understand the "tipping point" of methanogens. Under what conditions will the activity of methanogens become high enough to out weigh the activity of methanotrophs? Changes in functional groups may go unnoticed when relying on 16S rRNA gene assignments and so using both 16S and amplification of specific functional genes will be necessary in future work. Employing 16S rRNA data will help to solidify overall general patterns observed in a sample site. After determining what gross patterns exist in community

structure, more specific tests can be designed to determine meaningful fluctuations in community function.

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CHAPTER FIVE

The Real World: Understanding the Communities and Functions of Microorganisms in their Natural Environments

"*I see the question of biological organization taking two prominent directions today. The first is the evolution of (proteinaceous) cellular organization…The second major direction involves the nature of the global ecosystem. This is both a very practical (imminent) and a very basic problem, involving biological organization on a level over and above the cellular/organismal. Bacteria are the major organisms on this planet – in numbers, in total mass, in importance to the global balances. Thus it is microbial ecology that matters most; it is microbial ecology that is most intimately and importantly connected to the earth's exterior. And it is microbial ecology that is most in need of development, both in terms of facts needed to understand it, and in terms of the framework in which to interpret them*." Carl Woese, 2005 (2)

ABSTRACT

"*Coli*-centric" microbiology describes the general trend of assuming characteristics of *Escherichia coli* and similar organisms to the entire and highly diverse branch of bacteria. This chapter outlines the rise of *coli*-centric microbiology and how it resonates through current "Omics" era microbial ecology. The most common and potentially most problematic extrapolation from *coli*-centric microbiology may be the expectation that the same levels of activity and rates of replication we've grown to expect from *E. coli* under controlled laboratory conditions in the laboratory apply to the myriad taxa that comprise microbial communities in natural environments. Microorganisms survive in the natural environment by incorporating many life strategies including the formation of spores, cysts, other dormant cells, and biofilms. These inferences create broad gaps in knowledge in microbiology including an accurate understanding of the scale at which to study microbes, what the normal state of life is like for microorganisms in the natural environment, understanding the complexities of nutrient cycles, and how to accurately measure or study these organisms with DNA and RNA based studies. This

summary chapter addresses how the body of work presented in this thesis seeks to fill these broad gaps as well as tackle specific gaps in knowledge relating to Arctic ecosystems including the lack of integration of microbial data with climate models, what organisms persist in the Greenland Ice Sheet, seasonal variation of microbial communities in active layer permafrost, and how microbial communities respond to climate change variables.

THE RISE OF COLI CENTRIC MICROBIOLOGY

The "Golden Age" of microbiology spanned from 1857 to 1914 and includes major discoveries and progressions made in the field of microbiology. During the Golden Age major milestones included Pasteur's studies and discoveries of fermentation and pasteurization from 1857-1864, Koch's Germ Theory of disease linking microbes to disease in 1876, Koch's development of techniques to isolate pure cultures in 1881, the development of antimicrobial drugs, Escherich's discovery of *Escherichia coli* in 1884, as well as the development of the petri dish by Petri in 1887, and many more. Towards the end of the Golden Age many more discoveries in disease and infectious bacteria were made including *Clostridium tetani, Shigella dysenteria,* Syphilis, and *Trypanosoma cruzi*. Other notable discoveries include Beijerinck's discovery of nitrogen fixation in 1901. Winogradsky, deemed the father of microbial ecology, was also a contributor during this exciting time of microbiology making advancements in understanding the sulfur cycle and the nitrogen cycle. This era was key in the development of practical applications of microbiology and involved advancements in microbial cultivation, understanding

diseases, nutrient cycles, and applying all of these concepts to industrial purposes (For a review on the history of microbiology see (3)).

Unfortunately, a division between microbiological sub-disciplines may have arisen just after the First Golden Age and gave rise to this *coli*-centric microbiology. Winogradsky and Beijerinck were pioneering environmental microbiologists during the First Golden Age, but their work and the work following theirs was heavily involved in biochemistry and created separation in this field from medical microbiology. The Second Golden age of microbiology ranged from 1928 to 1983 and introduced bacterial genetics and nucleic acid sequencing and is largely responsible for the focus on specific organisms such as *E. coli*. Due to its easy cultivation and quick generation time, *E. coli* became a canonical model organism for studying genetics. The first free living organism to have its complete genome sequenced was *H. influenza* in 1995 (4) but *E. coli* was soon to follow with it's genome being completed in 1997 (5). In fact, *E. coli* may be one of the organisms we know most about on this planet (6). The work of Woese and Fox in the late 1970s incorporated new taxonomic techniques based on the analysis of the ribosomal gene and restructured the tree of life to include the then new domain of archaea (7). Unfortunately, it took several years for this new tree of life to become widely accepted. Interests in medical microbiology may have been the driver and motivator for enhancing sequencing technologies, but Woese paved the way for this data to be integrated in other sectors of microbiology. Despite his work, a skewed focus remained during this Second Golden Age of microbiology. Carl Woese described this issue eloquently in 2005,

> *While one cannot deny the tremendous advances that molecular biology (and genetics) wrought, one can and should decry the price biology paid for them. A*

holistic perspective was effectively banished from biology. The cell was reduced to merely the sum of its parts – the cell as a whole became no more than a shadowy backdrop for the molecular drama (8).

Yet, by the 1990s the application of Woese's ribosomal gene analysis technique, spear headed by Norman Pace, opened up the world of sequencing and identifying microbes from the natural environment. The dichotomy between lab and the environment is no new concept. The phrase, "the great plate-count anomaly," was coined in the mid 1980s (9), however, this idea was gaining attention nearly twenty years prior (10). With the methods available at the time, it was deemed nearly impossible to determine precise counts of bacteria in soil and thus estimations were heavily relied upon (10). The basis of these observations was the inability to culture as many different bacteria as were visible via microscopy. The extent of this anomaly has only been magnified with current sequencing efforts, based on the 16S rRNA gene, revealing the extreme microbial diversity in ecosystems such as soil. As we now enter this new era of microbiology, the "Omics" era, there is a much larger appreciation for the importance of understanding microbes and microbial communities in their natural environments for both medical and environmental purposes. Yet, this "*coli*-centric" microbiology still echoes throughout microbial ecology research.

An early example includes the review by Tempest and Neijssel (11) that argues because *E. coli* has such a fast generation time, dividing almost every 20 minutes, and the fact that we do not observe microbial overgrowth in most natural environments is due to nutrient limitations in these environments. It is not accurate to assume that all bacteria are replicating at a similar rate to *E. coli*. A more recent example (12) employs this same

assumption to justify the hypothesis that microbes are expected to have quick response times in the natural environment because they divide so rapidly. Indeed, there are examples of microorganisms responding quickly in natural environments. For example, cyanobacteria seem to quickly respond and follow soil wetting in dry desert soil (13). However, there are many different microbial life strategies used to survive in the natural environment including the formation of spores, cysts, and biofilms. Bacteria may be the most diverse branch on the tree of life (14), making the frequent *E.coli* comparisons rather inappropriate. One of the largest mistakes current microbial ecologists may be making is the assumption that the cells are active and replicating at all. *Coli*-centric microbiology leads to broad gaps in knowledge including the appropriate scale at which to study microbes, what the normal state of life is like for microorganisms in the natural environment, understanding the complexities of nutrient cycles, and how to accurately measure or study these organisms with DNA and RNA based studies.

SCALE

Soil may be one of the most heterogeneous environments on earth (1). In one gram of soil a spectrum of environments exists spanning from aerobic areas created by pockets of air to anoxic and water saturated areas created by pockets of water (Fig. 1). Additional variables include varying texture of sediment. For example, that same gram of soil may contain clay, silt, and sand particles. There is extreme variability in microenvironments in soil (10). The average bacterium is approximately 1×10^7 smaller than an elephant, and an elephant is 1×10^7 smaller than our planet (6, 12). Given these proportions, it seems that the microbial world works on a molecular scale (15). The

complexity of the natural environment and the scale at which bacteria live make both reproducing these conditions in the lab improbable and the scope at which to obtain replicate samples difficult.

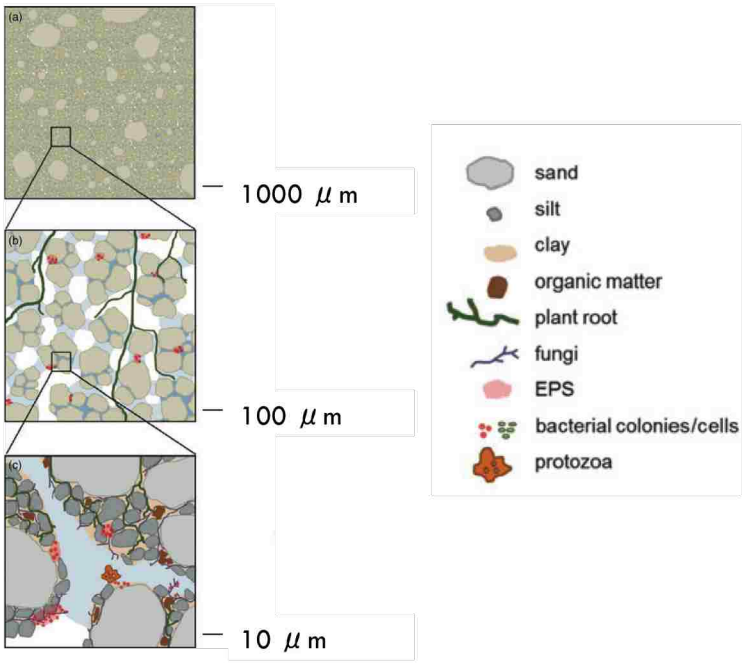

Figure 1. This image is reproduced from Vos, et al., 2013 (1) and depicts the degree of complexity of soil on a small scale.

LIFE STRATEGIES

Additionally, the most normal or frequent stage of microbial growth is not well understood (16). Dormant stages are widely recognized in organisms that form spores and cysts, but there may be dormant stages in other bacteria, generally referred to as vegetative cells (16-18). These cells are viable but not replicating and so are neither dead nor highly active. The term vegetative gives the impression that these cells are completely inactive, however, house-keeping genes for maintenance are likely being actively transcribed. Due to this, these cells will be referred to generally as dormant cells. Many of these dormant cells may just take an extremely long time to replicate. In both culture techniques and current sequencing techniques slowly replicating organisms may be overlooked. It may be that the DNA from a slowly replicating organism is in a lower concentration than that of a more actively dividing organism and so may not have a high signal in current 16S data sets. Dormancy of many microbial organisms has been proposed in aquatic systems (19). The

average generation time for aquatic bacteria has been estimated to be around 53 hours and to range between 20 – 200 hours (20). A doubling time of 210 hours was calculated for marine bacteria living at 5 °C at a depth of 5500 m (21). This is frequently referred to as, "life in the slow lane."

Since the 1960s it has been suggested that many bacteria in soil are dormant and that more appreciation is required for the fact that not all cells initiate growth at the same rate (10). The majority of microbial biomass in soils likely consists of dormant cells during most of the year (22). Azotobacter cells maintained in dry soils have been found to persist and survive in a cyst-like state for over 10 years (23). Substrate availability in soils may determine the abundance of dormant cells, with recent additions of substrate increasing the number of active cells (24). It seems likely that dormancy cycles may mirror seasonal trends in the environment.

It is not surprising that many bacteria do not replicate under lab conditions, as the variation in environmental conditions and microbial life cycles are not replicated in the lab. Lab media often contains 100 times the amount of carbon per liter than what would be present in the natural environment (25). Further, lab incubation temperatures frequently differ than environmental temperatures (26). Even Sergei Winogradsky in the late 1800s had an appreciation for the complex relationship between the environment and the microbe and wondered whether if the nitrification processes he disentangled in lab proceeded in the same way in natural soil ecosystems (27). Biofilms are another strategy for microbial growth and survival in many natural environments (28). Microbes are cultivated in the lab and are examined as individual cells in a culture, however, in their natural ecosystem they may survive as biofilms (29, 30). There are many examples of

wild bacterial strains growing differently than the same cultivated lab strain (30). Again assuming that all cells are replicating at similar rates, and rates similar to those of *E. coli*, may be one of the largest misconceptions in microbial ecology of the "Omic Era."

NUTRIENT CYCLES

Syntrophy, the cross feeding between microbial organisms, provides another example of the difficulties in replicating or understanding microbial life in lab studies. Sometimes this is referred to as obligately mutualistic metabolism or community metabolism. A by-product from one organism is an energy source for another, and those relationships can be hard to replicate in lab. These relationships involve the transfer of reducing agents, organic, sulfurous, and nitrogenous compounds (31). Relying on newer nucleic acid sequencing may also not reveal these associations. A classic example involves a methanogen reducing methane to hydrogen and carbon dioxide, a sulfate-reducing bacteria then uses the hydrogen and produces hydrogen sulfide. The entire process of the methane cycle has been described as a syntrophic process, as each step of the cycle involves a conversion of a compound required by an organism involved in the next step (32, 33).

This concept of syntrophy relates to the interconnectedness of the nutrient cycles. As discussed in previous chapters, the nutrient cycles are heavily intertwined and disentangling them may be tricky. It is not surprising that populations of microbes have metabolic interactions with other populations of microbes. In terms of methane cycling, work in wetlands and rice paddies has investigated the impacts and interactions between this cycle and the nitrogen cycle. Many studies have found ammonia and ammonium can

inhibit methanotrophs from oxidizing methane (34-38). Additionally, high organic N input, in the form of fertilizer, increased methane output from rice paddies (39, 40). Clearly the progression of the N cycle has direct impacts on the cycling of methane. Drawing back on the issues of scale and soil heterogeneity, it has been found that rates of denitrification increase in pockets of soil that have high concentrations of organic carbon (41). This concept builds on the difficulties of understanding natural microbial communities. Clearly, to understand the function of the community, all populations need to be considered as their metabolisms may be connected. This would make replicating a natural system in lab difficult.

Further, the flexibility of the microbial community as a whole in terms of terminal electron acceptors increases the complexities of understanding these organisms in nature. Although oxygen may be the most electropositive electron acceptor, many other electropositive electron acceptors are available and utilized by microbes in nature including iron, nitrate, and nitrite. Nitrate is a common electron acceptor used in anaerobic respiration during denitrification. However, other terminal electron acceptors are electronegative. Methanogens that couple carbon dioxide and methane in carbonate respiration are utilizing electronegative terminal electron acceptors. Organisms reducing more electronegative terminal electron acceptors are not gaining as much energy as organisms reducing more on electropositive terminal electron acceptors. This may impact doubling times. For example, the carbonate respiring methanogens may have a slower doubling time than an organisms utilizing aerobic respiration. Facultative anaerobes may have varying doubling times as their energy yields may vary depending on which terminal electron acceptors are available in the environment. And so, in order to

completely understand a microbial community structure and function not only do we need to identify these organisms, but we also need to work to understand their metabolisms and physiologies in natural environments.

COMMUNITY RESPONSES - SHORT TERM VS LONG TERM

Understanding who makes up a community of microorganisms has been largely tackled by the use of ribosomal sequencing. However, understanding the activity of this community and how a community responds to external variables is a continuing battle. In general, a short-term response in a microbial community may involve a shift in which genes are transcribed, but a long-term shift may involve actual restructuring of the microbial community. It is commonly accepted that the 16S rDNA of a microbial community represents the total community or community potential and the 16S rRNA represents the most active members. If understanding the function of the community is desired, then analyzing 16S rRNA may prove to be more useful. If the misconception of all bacteria responding quickly to environmental changes is held as true, then a large shift in 16S RNA may be expected. However, if it is accepted that many microorganisms in soil have an extremely slow doubling time or are generally dormant, then a much more subtle shift in 16S rRNA would be expected after an environmental change.

RNA is useful in determining metabolically active members in the community because the amount of rRNA produced by a cell correlates to the growth and activity of the bacterium (42). Unfortunately there are many issues involved in working with RNA. RNA is more susceptible to degradation by nucleases than DNA (43), RNases are present in the soil and may be encountered during the extraction process, mRNA has a short half

life and is used immediately as template for protein synthesis, and finally humic substances in soils make extraction of both DNA and RNA difficult (44). Illustrating the difficulties of RNA extraction, nearly all environmental studies suggest lower yields of RNA than DNA from any given sample despite the fact that bacterial cells generally contain more RNA than DNA at any given time (45). Another issue involves the large abundance of dormant cells in soils. Dormant cells make up a significant pool of cell biomass and thus nucleic acid abundance. However, there may be a lower concentration of RNA being contributed to the total RNA pool because dormant cells are less active and so likely have low rates of transcription. Sporulating *B. subtilis* cells are known to restrict transcription of certain proteins when sporulating (46) and so this likely holds true for other organisms entering different types of dormant states. Dormant cells were found to require 2-3 times less carbon for survival than metabolically active cells (47) illustrating lower levels of metabolism in dormant stages than active stages. Dormant cells are likely transcribing housekeeping and maintenance related genes and so compared to an active cell, dormant cells would contribute less RNA to the total RNA pool.

GAPS IN KNOWLEDGE IN ARCTIC MICROBIOLOGY

There are certain challenges associated with living in a cold Environment. Scientists that study deep ocean sediment and ocean water columns have often described their work as "life in the slow lane" as the organisms tend to live "very slowly" (48). These broad gaps in knowledge that persist in general microbial ecology are also present if not amplified when examining cold environments. Not only do you have the issues of

scale, disentangling the nutrient cycles, but also the amount of dormant cells is likely larger in colder environments. Detecting any shift or response in the community is like detecting signal in a largely static background. This means that short-term manipulative experiments are less revealing given life in the slow lane. In conjunction with these broad conceptual issues with microbial ecology, there are specific gaps in knowledge associated with Arctic microbiology that this thesis addresses including: i) the lack of integration of microbial data with climate models; ii) what organisms persist in the Greenland Ice Sheet; iii) what effects does the compressed seasonality in Arctic environments have on microbial communities in active layer permafrost; and iv) how do microbial communities affect and respond to climate change variables? The body of work presented in this thesis also ties into greater themes that remain a challenge in microbial ecology including disentangling the major nutrient cycles and drawing robust conclusions from DNA and RNA based data sets under the context of life strategies occurring in soils.

INTEGRATING MICROBIAL DATA WITH CLIMATE CHANGE MODELS

Microbes are the key regulators of nutrient cycles and so it makes no sense leaving microbial data out of climate change models. While there is a long way between the data presented in this thesis to the actual integration into models, the work presented here provides initial steps to starting this process. Microbial data may help to reveal active "hot spots" for specific greenhouse gases, and more importantly reveal potential and future hot spots of greenhouse gas emissions. *Crenothrix* was a type I methanotroph that was the most abundant methanotroph genus in the fen samples from Chapter Three. This genus is known to only persist in areas of high methane concentration and is already

used as a methane indicator in wells (49). Further, both chapter three and four illustrate the previously unknown ubiquity of type II methanotrophs in permafrost active layer, whereas type I methanotrophs are exclusively found in fen areas. The proportions of these two types of methanotrophs might be used to gauge areas of concern and potential methane emissions. The data presented in chapter three also confirms previous predictions of Zackenberg remaining a methane sink. The data clearly shows that moist active layer is more similar to dry active layer communities, illustrating how the permafrost in this area would have to completely collapse and form wetlands in order to become a likely methane source. Moist active layer permafrost is not likely to support the community necessary for net positive methane emissions. These general trends observed in microbial data is helpful in supporting previous predictions and further the data can be used to find specific genera or groups that may help to indicate potential of greenhouse gas emissions.

COMMUNITY STRUCTURE OF THE GREENLAND ICE SHEET

The microbial community structure in bare ice from the Greenland Ice Sheet is still largely unknown. Most of the current investigative microbiology work on the ice sheet involves sampling snow or sampling cryoconite holes. However, establishing a library of known organisms in bare ice is key to understanding what organisms will inoculate downstream environments as the ice sheet continues to melt. In order to predict the future productivity and health of these downstream ecosystems as well as the carbon budget of the GrIS, it is necessary to know what organisms will be introduced as ice continues to melt. Melt water running from the ablation zone eventually running into the

ocean will transport dust, nutrients, and microorganisms. Based on unique OTUs and alpha diversity indices, this body of work found that bare ice is fairly similar to snow and cryoconite microbial communities on the GrIS. The major phyla detected across all samples included Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, and Proteobacteria. Alphaproteobacteria were a large member of the core communities, specifically, the genus *Bradyrhizobium*. This genus and Cyanobacteria contain many known nitrogen fixers, and the inoculation of these organisms into new ecosystems could alter the nitrogen cycle in the downstream environments. Potential fish pathogens were also detected in the samples.

Surprising results from this data set reveled extreme heterogeneity in ice samples. Even on a relatively small scale, 40 meters as opposed to 70 km (50), the structure of the communities found in the replicates and sample sites indicate heterogeneity in microbial communities of supraglacial bare ice. This heterogeneity is likely due to the scattered deposition of Aeolian dust or aerosolized soils. The patchiness of these microbial communities emphasizes the need for careful sample collection.

SEASONAL VARIATION OF ACTIVE LAYER MICROBIAL COMMUNITIES

General seasonal patterns may begin to shift in the arctic. For instance, as air temperature continues to rise during the Arctic winters, spring may arrive earlier along with earlier snow and glacial melt. This may spark earlier microbial activity. Climate changes may progress to such an extent that the active layer may not freeze over the winter. This would allow for higher levels of microbial activity year round. It seems probable that pulses of soil moisture and organic material largely contribute to microbial

activity in permafrost. Working to understand how these microbial communities shift in both structure and function with typical seasonal trends will help to predict how these patterns will change with climate change.

From this data, it is clear that both microbial community structure and function exhibit trends of seasonal succession. Methane cycling organisms peaked in activity in July when methane oxidation was also measurably highest. The nitrogen fixation community and activity peaked in July. Despite the low abundances of denitrifiers, functional trends were still clear. Denitrification activity showed a large increase from June to July and this level of activity persisted through August. It seems that the methane cycling organisms have a shorter window of activity than the N cyclers who largely remain active in August. Studying trends in seasonal variations allows the encompassing of the entire assemblage of variables that impact microbial communities most such as soil moisture, temperature, and organic matter. This is the first study to truly map out microbial community dynamics in *in situ* active layer permafrost.

MICROBIAL RESPONSES TO CLIMATE CHANGE VARIABLES

A few studies exist that examine how microbial communities shift when permafrost is thawed in lab conditions, but as has become obvious throughout this chapter - lab conditions are very different from environmental conditions. The research described in Chapter three described analysis of microbial communities under varying soil moistures including dry, moist, and fen tundra areas. The Zackenberg study determined that dry active layer (soil moisture < 25%) microbial communities and moist active layer (soil moisture 25-50%) communities are more similar to each other than to

the fen (soil moisture > 50%) microbial communities. Both type I and II methanotrophs were detected in these samples, however, type I methanotrophs were exclusively found in the fen samples. These areas of high soil moisture were also the only areas with net positive methane flux. This just reiterates that microbial community structure is different under different moisture conditions.

The results of Chapter four indicate that microbial communities are susceptible to changing environmental conditions, specifically those that mimic climate change scenarios such as increased soil warming and snow accumulation. From this data it appeared that the control plot served as a sort of "baseline" community from which the other communities diverge when exposed to certain treatments. There were no obvious indications of treatment effects on community function based on functional groupings of taxa or Q-PCR results. The results of this study did confirm that one year of snow accumulation and soil warming was sufficient to impact the structure of the permafrost active layer microbial community.

DISENTANGLING NUTRIENT CYCLES

Other than filling these specific gaps in knowledge, this body of work addresses more broad issues that still persist in microbial ecology that were previously discussed including the complexity of nutrient cycles and issues with examining both DNA and RNA data sets. This study found that a larger relative amount of methanotrophic and methylotrophic organisms were present in the same fen areas where high amounts of methane emissions were observed. Additionally, at the Disko field site it was found that both methanogenic and methylotrophic activity peaked when methane oxidation was

highest. If the relationship of methane cycling organisms was linear, one might expect to see more methanogens than methanotrophs in an area exhibiting net positive methane flux. Similarly, one might expect to see more methanotrophs than methanogens in areas exhibiting high levels of methane oxidation. However, the trend is that peak process level measurements correlate to a peak in general methane cycling meaning a peak in both methanogens and methanotrophs whenever the peak net methane flux is observed. The methane cycle is complex and methane production and oxidation were not linearly related.

When attempting to determine the potential of methane cycling in an environment, or the relative contributions of producers and consumers, it is clear that process-level measurements can only reveal the sum of its parts. Relying on process-level methane measurements obscures the fact that methanogens are still active in the environment. Relying solely on gas fluxes or nutrient pools will not portray the complete picture of the different steps within a nutrient cycle. However, issues would also arise if studies were to only rely on 16S rRNA taxa classifications. Microbes are known for exhibiting conservation of function across different phylogenetic lineages, a phenomenon often termed "functional redundancy". For example, methanotrophs and methylotrophs are made up of a diverse group of microorganisms all capable of doing the same thing. Further some microorganisms are capable of performing a multitude of functions. One example is that many nitrogen fixers are also denitrifiers. Relying only on taxonomic data would make it difficult to observe shifts in function. This study works to combine process level measurements with both taxa assignments and functional gene abundances to begin to disentangle the complex and intertwined nutrient cycles.

DNA vs RNA

The *in situ* variables in this study, snow accumulation, warming, and shrub removal, had only been implemented one year prior to sample collection. As a result, detection of treatment effects on microbial communities was somewhat surprising in what might be perceived to be an example of a community that is inactive much of the year. Continual and long lasting variables may impact microbial communities more strongly and so it was not surprising that seasonal variations impacted community structure and function more strongly than the *in situ* variables.

In general, the RNA data set did not show data trends or patterns in either the LDA-CV community structure analysis or in the functional group analysis that were as strong or compelling as those observed for DNA-based analyses. The bulk of microbial biomass in soils is known to be dormant cells and it may be that the ratio of dormant to active cells increases in polar environments. This means that transcription and thus RNA concentrations are expected to be lower than DNA concentrations in permafrost and active layer samples. Under such conditions, a subtle shift in 16S rRNA would be expected after an immediate environmental change. Spore formers and dormant cells may take many years to begin to show bulk changes due to short-term shifts in the environment. Samples likely need to be collected repeatedly over the years to come to show more magnified shifts due to these *in situ* variables. Further, the data presented herein illustrate how both RNA and DNA show trends associated with changes in season, but the trends are more strongly reflected in the DNA data set. This again, reflects the idea that less RNA is expected in these soils because the organisms are largely dormant and so any pattern would be more prominent in DNA. This study at Disko Greenland,

because it incorporates both DNA and RNA, is one of the only current microbial ecology investigations in Arctic permafrost to address these issues in understanding and accounting for cell dormancy.

FUTURE DIRECTIONS

This work continues to emphasize broad gaps in microbial ecology as it portrays issues with scale and replication in soil samples. This concept should be taken into consideration in future studies. Six treatment blocks were established in Disko to serve as replicates for process-level measurements. The data presented herein suggest that these are not reliable replicates for molecular soil community analyses. There were no consistent patterns in microbial data in treatments between the replicate blocks. Further, some blocks showed consistently lower abundances of functional groups and functional genes. Only one sample was collected from each treatment established in the block in Chapter four. It is generally deemed necessary to collect 3 - 5 replicate soil samples from each sample point in any study to serve as replicates for community structure and functional analyses and my findings only reiterate this necessity. Of course, the extreme heterogeneity of these Arctic soils may prove that even these smaller-scale replicates exhibit high variance.

The extreme heterogeneity in the ice samples also reflects the challenges and difficulty of obtaining replicates and determining what constitutes a replicate in what can be considered extreme environments with quite limited access for sampling. In this study, replicate cores were collected from multiple sites, however they proved to be dissimilar suggested that their spacing exceeded the “repeat interval” for the microbial

communities being sampled. The idea of replication and the appropriate scale at which to achieve replication will vary from environment to environment. It may be that collecting a larger core of ice, allowing the core to melt, and then dividing the melt water into triplicates for nucleic acid extraction may be the best way to analyze these low-biomass, heterogeneously distributed communities.

Unfortunately, there are numerous inherent challenges when setting up studies in remote locations, which of necessity, impact experimental design. For example, optimal replication during sample collection may not be a reasonable option for a number of practical reasons including plot sizes and layouts that don't support extensive sampling, the need to minimize site perturbation, and the number of total samples that can be analyzed where multiple and often expensive analyses are involved. For example, in many of the studies presented here only one core could be collected from each sample location as the collection of more cores could impact gas flux recordings. Collecting samples from extreme environments such as the Greenland Ice Sheet may be a difficult task as sampling season, site access and the necessity of sharing time across multiple investigations is often a limiting factor. Additionally, storing and transporting unprocessed environmental samples from far afield introduces elements of risk ranging from evaporation of dry ice needed to maintain sample integrity to problems passing through customs to get the samples to a distant laboratory for analysis. This was seen in chapter 4 where two samples were compromised during transport to the laboratory for processing prior to analysis. These samples were transported on liquid nitrogen between Greenland and Copenhagen. These samples had to be carried from Disko Island on to a ferry to Ilulissat, Greenland. From here the samples were flown to Kangerlussuaq,

Greenland and finally to Copenhagen, Denmark. This was expensive and only feasible because of the relaxed rules of Air Greenland. Nucleic acids were freeze-dried in Copenhagen and carried back to the US on dry ice. This dry ice did not last the entirety of the journey from Copenhagen to Missoula; however, because the samples were dried down the integrity should have remained intact. The samples discussed in Chapter three were shipped from Copenhagen and were retained in the US customs for a week. Thankfully, these samples were also dried-down.

The bulk of my thesis research was undertaken to support the importance of integrating process level measurements, community structure analysis, and community functional analysis in the realm of climate change studies in Greenland. Each type of data set comes with certain limitations and so integrating data from multiple independent approaches can strengthen data interpretation and the conclusions drawn. For example, changes in functional groups may go unnoticed when relying on 16S rRNA gene assignments due to conservation/redundancy of function across phylogenetic groups and so using both 16S phylogenetic approaches and amplification of specific functional genes will continue to be necessary in future work. Still, integrating 16S rRNA-based data is still necessary to detect overall patterns observed in a sample site. After determining what gross patterns exist in community structure, more specific analyses can be designed to determine meaningful fluctuations in community function. Given the immensely important roles and responses of microbial communities in primary biogeochemical cycles on a global scale, microbial gene and taxon abundance combined with process-level measurements will better inform climate change models. It will become increasingly important to understand the "tipping point" of methanogens so that we can

understand what conditions promote certain steps in the methane cycle and predict potential shifts in methane emissions. The environment of the Arctic is going to continue to change and it is necessary to understand what environmental conditions promote greater methanogen activity versus methanotroph activity.

Continuing to study how seasonal variations impact microbial communities is also an important element for future work in climate change microbial ecology. The ability to study microbial communities *in situ* allows for the incorporation of all variables at play. It has become clear that it is difficult to pin point one variable driving variation in microbial community structure and function. Multiple variables are at play, and many of these are similarly important, so collecting samples repeatedly throughout the season may be a necessary way to cope with the issue of seasonal drivers that might overwhelm more subtle, but equally important, factors in climate change scenarios.

As originally predicted, soil moisture proved to be an important driver of methane cycling. However, the observed seasonal trends likely involved fluctuations in parameters other than pulses of soil moisture. Pulses in organic matter are likely important, and, further, it may be that there are oscillations in the activity and identity of dormant versus active cells at various times and locations. A large gap in knowledge that persists in microbial ecology is the lack of consideration of different life cycle strategies when relying on primarily molecular data. The notion of dormant and active cells, and oscillations between those states, needs to be better incorporated in future work.

One approach to mitigate the issues related to microbial dormancy in natural environments would be to rely more on RNA-based studies. Although, RNA extraction from environmental samples is difficult and prone to both contamination and degradation,

it seems reasonable to believe that extraction methodologies, and hence the data obtained from downstream analyses will continue to improve. Until RNA extractions are optimized, it seems more robust to perform analyses like those described herein with both DNA and RNA. It is also necessary to understand which taxa are dormant versus active and under which conditions to truly understand microbial community dynamics. Inferring the aforementioned *coli*-centric biases into microbial ecology research and its interpretation likely masks the true interactions and activities that actually occur in natural environments in the Arctic and elsewhere. Future studies should work towards understanding these pulses of dormant and active cells. Determining whether dormant cells persisting in ice become active at certain times and in downstream environments will be very important in understanding potential changes in ecosystem processes *in situ* as well as after transport of ice-melt to downstream environments. As climate change progresses, the observed pulses of net microbial activity will indefinitely impact nutrient cycling in Arctic environments.

The opening quote from Carl Woese describes the importance of developing a framework from which to make interpretations from microbial ecology findings in terms of global ecosystem processes. The immense numbers and total biomass of microbes on this planet make these organisms key drivers of global cycles. It is necessary to understand both the variations in environment on the small scale, the scale at which microorganisms persist, and how these small-scale variations reflect the broad scope of biogeochemical cycles. Shifts in nutrients and soil moisture will directly impact which microbes make a living and are active at this local scale. And the activities at the local scale will drive bulk trends in biogeochemical cycles. The body of work here represents

initial steps in developing a framework that links microbial activity *in situ* to biogeochemical cycles. Further development of this framework will prove to be important in future climate change research allowing for a more complete and accurate understanding of climate change scenarios.

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