THE EFFECT OF PERMAFROST THAW ON MERUCRY- AND METHANE-CYCLING MICROBES AND THEIR POTENTIAL INTERACTIONS

by

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Abstract

In this study, I investigated potential interactions between methane and mercury cycles in boreal forest soils. Additionally, I examined the changes in these cycles relative to shifts in soil moisture along an environmental soil moisture gradient. This investigation is pertinent due to the escalating rate of permafrost thaw driven by climate change in Arctic and subarctic ecosystems. Permafrost thaw leads to increased soil moisture, fostering favorable conditions for anaerobic microbial processes such as mercury methylation, methanogenesis, and anaerobic methanotrophy. Microbial mercury methylation creates monomethylmercury, a neurotoxin that accumulates in aquatic food webs. Methane cycling results in the production of greenhouse gases that can create a climate-warming feedback loop. In this study, I explored the mercury and methane cycles and analyzed the microbial communities involved in these cycles along an environmental soil moisture gradient. Microbial communities were analyzed by quantifying the relative abundance of taxonomic groups and by quantifying functional genes associated with mercury methylation, methanogenesis, and anaerobic methanotrophy. The relationship between soil water content and functional gene quantities was not significant. However, my findings did reveal a significant relationship between relative beta diversity and gravimetric water content along the environmental soil moisture gradient. The functional potential was predicted by quantifying net methane and net monomethylmercury production through incubations designed to measure total production in completely saturated, anoxic conditions I found that total mercury increases as soil moisture increases, methane efflux increases as soil moisture increases, and carbon dioxide efflux increases as soil moisture increases. This suggests that the activity of the mercury and methane cycles may change as permafrost continues to thaw and soil moisture content increases. In a changing climate, continuing to monitor these cycles in Alaska is pertinent due to its robust fishing industry, indigenous communities, subsistence fishing practices.

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Chapter 1: General Introduction

The Impacts of Climate Change on Boreal Soil Anaerobes

1.1 Introduction

As the climate continues to change, Arctic and subarctic environments are experiencing disproportionate impacts (Leddy, 2020). These changes are manifesting in various ways, including increased wildfire frequency, permafrost thaw, glacial melt, and higher soil moisture levels. Permafrost thaw, in particular, is leading to elevated soil moisture levels, creating anoxic soil conditions that promote anaerobic microbial activity (Lawrence et al., 2015). Among the microbes thriving in these conditions are those participating in the methane (CH4) and mercury (Hg) cycles. The accelerated rate of permafrost thaw exposes previously inaccessible organic matter (OM), providing a carbon and energy source for microbes involved in CH4 production (Knoblauch, 2021). Permafrost thaw also releases stored inorganic Hg that may be methylated by microbes, which has implications for the release of monomethylmercury (methylmercury or MMHg), into watersheds (Tarbier, 2021). Both of these processes have an impact on the environment and these cycles may be interacting with one another. Certain microbes that break down CH4 and those that methylate inorganic Hg both use sulfate as an electron acceptor (Timmers, 2016; Luo, 2023). However, sulfate limitation has been observed among sulfate- reducing bacteria (SRB) in boreal peatlands. (Vile, 2003). These microbes could be competing for sulfate, so the cycles may be changing in response to each other as well as the climate.

Permafrost, defined as soil that remains frozen for extended periods ranging from two to 700,000 years, is particularly susceptible to the effects of climate change (Froese, 2008; Dobinski, 2011). Permafrost thaw and wildfire activity are increasing as a result of the changing climate. While wildfires are a natural part of the boreal ecosystem, recent years have seen a rise in both size and frequency (Kato, 2020). The frequency of wildfires is influenced by climate, ignition sources, and available fuel. Consequently, the increasing climate variability is contributing to a larger annual burn area (Kasischke, 2006; Wang, 2015;

Yoshikawa et al., 2002). This escalation in wildfire activity has resulted in notable changes in soil OM and moisture content, often leading to near-surface permafrost thaw (Michaelides et al., 2019; Yoshikawa et al., 2002). The warming climate is accelerating permafrost thaw through a combination of increased wildfire activity and general warming trends. This is supported by the fact that Northern latitudes are experiencing more pronounced temperature increases, with Arctic land warming at twice the rate of other regions. Continued warming is expected to exacerbate these issues, leading to further permafrost thaw, glacial melt, and the loss of summer Arctic sea ice (IPCC, 2021).

Areas with discontinuous permafrost, such as Interior Alaska, are particularly vulnerable to the impacts of climate change due to the rapid thawing of thin permafrost layers. Discontinuous permafrost refers to sections of permafrost encompassed by boundaries that are often at the phase equilibrium temperature, just slightly below 0°C. Even minor warming can disrupt these delicate balances, triggering thawing at the surface of the discontinuous permafrost (Osterkamp, 2005). This increased surface exposure can lead to permafrost thaw rates of up to 0.04 meters per year (Ostercamp, 2005). Permafrost thaw in Interior Alaska often creates highly moist soil conditions, especially in regions with ice-rich soils. When permafrost thaws near the soil surface, a frozen layer can remain below, impeding moisture drainage (O'Donnell, 2012). Consequently, this leads to waterlogged, boggy conditions and the formation of hydric soils. Hydric soils are saturated with water, forming under conditions of permafrost thaw and flooding, often creating anoxic environments that promote anaerobic microbial processes (NRCS, 2024; Vasilas and Vasilas, 2013).

An increase in soil moisture can drive anaerobic processes, such as CH4 cycling and Hg methylation. Permafrost acts as a vast reservoir of OM, and when thawed, anaerobic methanogens can decompose this OM, yielding carbon dioxide (CO_2) and CH4 (Walz, 2017). CH4 produced through methanogenesis can subsequently be oxidized by anaerobic methanotrophs, leading to additional CO_2 release (Conrad, 2007). Microbial degradation of OM plays a crucial role in altering greenhouse gas emission rates and influencing climate warming. With a changing climate and increased permafrost thaw, there is a further escalation in greenhouse gas production and potentially Hg methylation. Permafrost serves as a reservoir

of Hg (Schuster et al., 2018; Chételat et al., 2022; St. Pierre et al., 2018), and some anaerobic microbes can methylate inorganic Hg to form MMHg, which bioaccumulates and biomagnifies within food webs. MMHg is a potent neurotoxin, posing risks to the physiological health of both wildlife and humans (Wu, 2024). The release of Hg due to permafrost thaw is particularly concerning due to the potential for MMHg production in boreal environments.

1.1.1 Methane Cycle

Soil moisture and OM play pivotal roles in shaping the CH4 cycle within boreal soils. Boreal topsoil is a reservoir of OM (Deluca and Boisvenue, 2012), encompassing organic carbon and essential nutrients like nitrogen and phosphorus incorporated into organic molecules. Labile carbon, a component of OM easily degraded by microbes over short periods (Mueller et al., 2015; Lim et al., 2022), becomes accessible for microbial metabolism upon permafrost thawing, fueling processes such as CH4 production (methanogenesis) and the aerobic or anaerobic oxidation of CH4 (methanotrophy) to produce CO_2 (Mackelprang et al., 2011).

CH4, a potent greenhouse gas, exhibits over 28 times the heat-trapping efficiency of CO_2 (US EPA, 2023). Methanogens, deriving their energy through anaerobic respiration, are responsible for CH4 production (Kotsyurbenko et al., 2004) and belong to the anaerobic archaea group, particularly Euryarchaeota (Liu & Whitman, 2008). Once methanogens produce CH4, methanotrophs can mitigate some of the CH4 entering the atmosphere by oxidizing CH4 to form CO_2, a less potent greenhouse gas (He et al. 2012; Nazaries et al., 2013). Methanotrophs, bacteria that use CH4 as their carbon and energy source, function aerobically or anaerobically (Yun, 2010), potentially offsetting some CH4 emissions from thawing permafrost by 20-60% (Singleton et al., 2018). Furthermore, soil conditions in high moisture areas that have experienced permafrost thaw are primarily anoxic, allowing for concurrent methanogenesis and methanotrophy. Since methanogenesis is an anaerobic process and methanotrophy can occur anaerobically through sulfate reduction, likely, both of these

processes are actively occurring and potentially altering Arctic and subarctic soils following permafrost thaw (Meulepas et al., 2009).

1.1.2 Mercury cycle

Soil moisture dynamics also play a significant role in the Hg cycle, particularly in response to changing climate conditions. Hg, a naturally occurring element, ranks among the top ten chemicals of major health concern according to the World Health Organization (Mercury and Health, 2017). Relevant forms of Hg involved in Hg cycling include elemental mercury (Hg^0), inorganic Hg (Hg(II)), and MMHg. The current release of Hg^0 into the environment is predominantly driven by human activities such as mining, coal combustion, industrial processes, and waste incineration, while historically emitted from volcanic events and wildfires (Streets, 2017). Hg^0 is volatile, so when released into the environment it disperses into the air and slowly oxidizes to Hg(II) (Lindqvis, 1985; AMAP, 2021). Hg(II) is less volatile than Hg^0, and it accumulates in the soil via dry or wet deposition (Cooke, 2020). Additionally, Hg^0 is less volatile in colder temperatures due to the decrease in vapor pressure (Gustin, 1997). Consequently, there has been an accumulation of Hg in soils near the poles over the last few millennia. Specifically, the total amount of Hg in global vegetation, the ocean, the atmosphere, and all other soils combined is far less than the amount of Hg stored in Arctic and subarctic permafrost (Schuster, 2018). Boreal soils in interior Alaska (Bonanza Creek LTER Caribou-Poker Creek Research Watershed) vary in their total Hg concentrations (16-152 ppb dry weight). During permafrost thaw, previously deposited Hg stored in the frozen soil can be released into the environment, becoming more accessible to microbial processes in the newly thawed soil.

In thawed soil, microbes can create MMHg, an organic neurotoxin that can bioaccumulate and biomagnify within food webs (Dorea, 2008; Córdoba-Tovar, 2022). Methylation, the production of MMHg, is primarily anaerobic and biologically mediated. SRB and other bacteria carrying the *hgcAB* gene pair are crucial in biotic methylation (Bravo & Cosio, 2020). This gene pair serves as a biomarker for Hg methylation (Parks, 2013; Gilmour, 2018). Microbial activity can lead to methylation or demethylation of Hg, with demethylation

occurring either aerobically (reductive demethylation) or anaerobically (oxidative demethylation), mediated by different microbial processes (Barkay and Gu, 2021). Despite extensive research on reductive demethylation, oxidative demethylation remains poorly understood, with SRB proposed as the primary group of organisms responsible. (Barkay & Gu, 2022). Limited research has been conducted on Hg cycling in boreal wetlands (Huang et al., 2022). Still, existing studies indicate that water content and nutrient availability significantly influence the methylating community (Xu et al., 2019). Hence, it is important to understand changes in the Hg cycle in anaerobic soils and to improve understanding of the microbes controlling these changes.

Biogeochemical alterations impacting net MMHg levels could reverberate through the food web in Alaska. MMHg accumulates in aquatic food webs and is more toxic than Hg^0 (Hsu- Kim et al., 2013). Given that fish consumption constitutes a primary pathway for human Hg exposure (Hong, 2012) and Alaska residents have high fish consumption rates, understanding and monitoring Hg dynamics is valuable.

1.2 Cycle Interactions

The Hg and CH4 cycles could be changing in response to each other in boreal soils. Drivers of microbial community assembly and interactions in the boreal include habitat conditions, available resources, and electron acceptors. As climate change alters habitat conditions, partial permafrost thaw induced by warming and wildfires leads to water-saturated topsoil (Aiken et al., 2012; Walvoord & Kurylyk, 2016), creating favorable anaerobic conditions for microorganisms such as methanogens, anaerobic methanotrophs, and Hg methylators.

The success of these microbial groups can also depend on available resources. For instance, Hg methylation requires the presence of Hg, electron acceptors, and OM (Hsu-Kim et al., 2013). Preliminary data indicate higher total Hg concentrations in high moisture sites as opposed to low moisture sites in Interior Alaska. Likewise, the availability of OM plays an essential role in Hg flux in topsoils (Meili, 1991). While boreal topsoils typically boast abundant soil organic carbon (Wickland, 2008), electron acceptors might be the primary

limiting factor in Hg flux in the boreal soils. Sulfate is widely acknowledged as the predominant electron acceptor for Hg methylation (Bravo & Cosio, 2020; Compeau & Bartha, 1985; Hu, et al., 2020).

Resources required for anaerobic methanotrophy include CH4 and sulfate (Meulepas et al., 2009; Hinrichs & Boetius, 2002). In a hydric, anoxic boreal environment, methanotrophs could compete with Hg methylating bacteria for sulfate. Given the shared use of sulfate as an electron acceptor, these cycles might mutually influence each other (Figure 1.1).

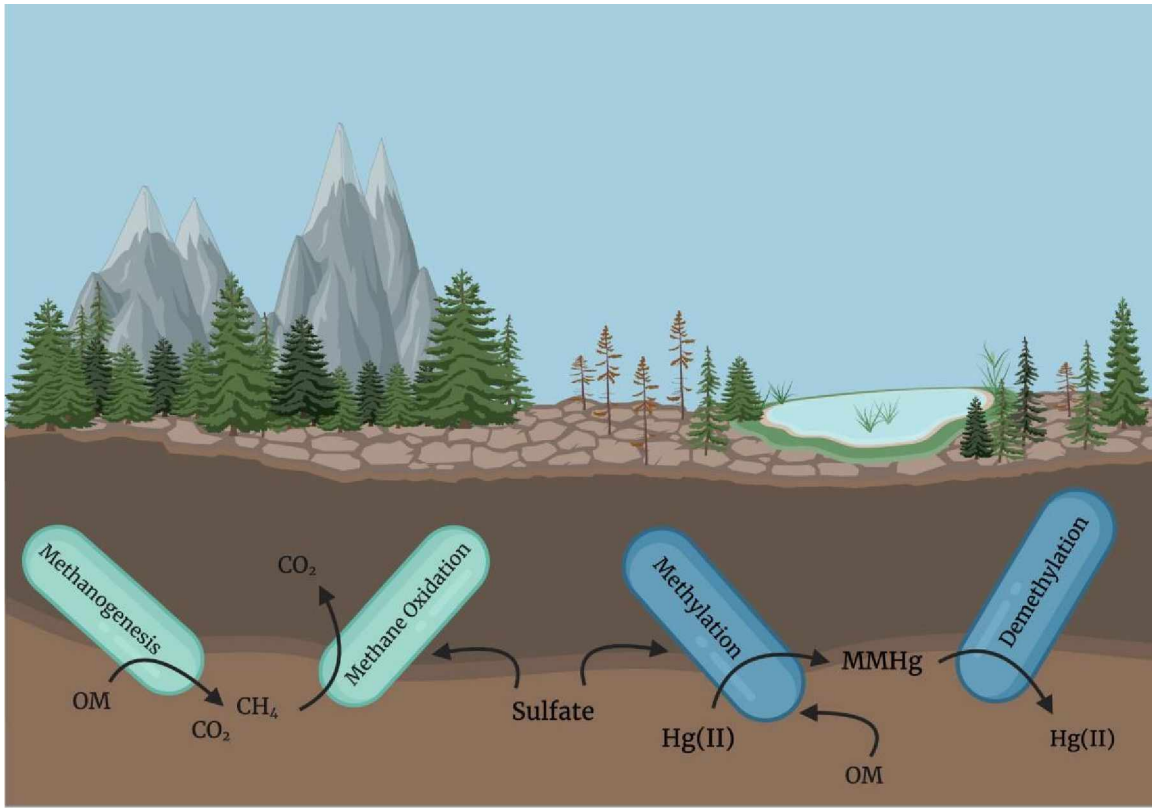

Figure 1.1. Methane and Mercury Cycle Interactions: Methanogenesis, anaerobic methanotrophy, and mercury (Hg) methylation all occur in anaerobic conditions. Methane cycling microbes are shown in light blue and Hg cycling microbes are shown in dark blue. Methanogens consume organic matter (OM) and produce methane (CH4). Then, anaerobic methanotrophs consume some of the CH4, producing carbon dioxide (CO_2). Hg methylating microbes create monomethylmercury (MMHg) from inorganic mercury (Hg (II)). Anaerobic methanotrophs and Hg-methylating microbes both use sulfate as an electron acceptor and may compete for this resource.

1.3 Functional potential of Hg and CH4 cycles

To investigate the influence of soil moisture on the Hg and CH4 cycles and the potential interactions between them, I explored the rates and microbial community functional potential for each cycle. In Chapter 2, I address the possible correlation and biogeochemical mechanisms between CH4 efflux and total Hg content in boreal soils. Specifically, I explored the mercury and methane cycles in permafrost thaw sites. Since Hg is released during permafrost thaw and is likely to bind to carbon in recently thawed soils, it is likely to accumulate in highly saturated, lowland soils. Additionally, methanogenesis occurs in anoxic, high moisture soils, and is likely to use carbon released during permafrost thaw as an energy source. For these reasons, I hypothesized that due to shared environmental optima (soil moisture and carbon content), net CH4 efflux and total Hg content would correlate spatially across boreal landscapes. Additionally, I hypothesized that due to the shared role of sulfate as an electron acceptor, there would be competition between Hg methylation and anaerobic methanotrophy. Thus, I predicted that the strength of the positive correlation between net Hg methylation and net CH4 efflux would be reduced under reduced sulfate limitation. This expectation stems from the fact that Hg methylation, methanogenesis, and anaerobic CH4 oxidation predominantly occur in anoxic soils, potentially at higher rates in saturated environments than in drier soils (Zhou, 2022; Angel, 2012; Segarra, 2015). Consequently, regions with elevated water content or anoxic conditions may exhibit increased net CH4 efflux and MMHg production.

In Chapter 2, I determined the biogeochemical potential for methanogenesis and methanotrophy at sites along a soil moisture gradient due to permafrost thaw disturbance. I also performed incubations with known additions of isotopically labeled inorganic Hg(II) (202) and MMHg (198) to quantify net MMHg production in soils collected along this gradient. I quantified total Hg, carbon, and nitrogen content and measured CH4 potentials using bottle incubations. My findings revealed a significant relationship between soil moisture and total Hg content and a significant relationship between soil moisture and CH4 efflux. This suggests a potential covariance between the CH4 and Hg cycles across a moisture gradient driven by shared environmental factors such as soil moisture and carbon availability.

However, we cannot draw conclusions about changes in MMHg production without further measurements of net MMHg production and speciation in the boreal soil samples (analyses are ongoing). Furthermore, I observed a decrease in net CH4 efflux when sulfate was added to the incubation, indicating that reducing competition for sulfate may increase the activity of anaerobic methanotrophs, thereby decreasing net CH4 efflux. This suggests possible interactions between the CH4 and Hg cycles under anoxic conditions.

1.4 Microbial community composition

My hypothesis posits that boreal forest sites with high soil moisture or saturated conditions, resulting in anoxic environments, will harbor distinct microbial communities with higher abundances of organisms and genes involved in methanogenesis, anaerobic methanotrophy, and Hg methylation compared to drier/unsaturated upland sites. This expectation stems from these anaerobic processes predominantly occurring in saturated soils, leading to higher quantities of relevant organisms and functional genes in such environments. Given their spatial coexistence, these cycles may interact with each other, considering their shared inputs and outputs.

Microbial community and functional gene analyses offer valuable insights into the influence of soil moisture on the microbes driving the Hg and CH4 cycles. In Chapter 3, I address how spatial patterns in microbial community composition and the abundance of microbial functional groups correlate with changes in soil moisture, total Hg concentration, and net CH4 production. I hypothesized that boreal soils with high soil moisture or saturated conditions, resulting in anoxic environments, will harbor distinct microbial communities with higher abundances of organisms and genes involved in methanogenesis, anaerobic methanotrophy, and Hg methylation compared to drier/unsaturated upland sites. This expectation stems from these anaerobic processes predominantly occurring in saturated soils, leading to higher quantities of relevant organisms and functional genes in such environments.

The Hg and CH4 cycles share inputs, outputs, and electron acceptors. For instance, methanogenesis generates methane, serving as an input for methanotrophy. Additionally, both biogeochemical cycles utilize sulfate as an electron acceptor, indicating potential competition or mutual response between them. To explore this connection, Chapter 3 assessed the diversity of

microbial functional groups and quantified the abundance of genes involved in methanogenesis (*mcrA*), methanotrophy (*pmoA*), and Hg methylation (*hgcAB*). I characterized microbial communities by function in areas affected by permafrost thaw and wildfire using 16S rRNA gene sequencing, targeted functional gene sequencing, and quantitative PCR, enabling comprehensive characterization of microbiomes, functional groups, and functional gene abundance. My findings revealed a significant relationship between gravimetric water content and relative beta diversity. The relationship between functional gene quantity and gravimetric water content was not significant. However, I found a significant relationship between soil moisture and the relative abundance of taxonomic groups capable of methylation, methanogenesis, and methanotrophy.

As the Earth's climate continues to warm, leading to the thawing of permafrost and an increase in soil moisture, studying anaerobic soils becomes increasingly important. Elevated soil saturation can enhance the production of MMHg, known for its biomagnification in aquatic food webs (Bravo & Cosio, 2020). This issue is particularly significant in Alaska, given its common fish consumption, robust fishing industry, Indigenous communities, and reliance on subsistence fishing practices. Moreover, besides the risks associated with MMHg, an increase in CH4 efflux has the potential to influence global climate patterns. Subsequent warming could further exacerbate permafrost thawing, raise soil moisture levels, and perpetuate changes in both the CH4 and Hg cycles. Therefore, comprehensive research into these processes is crucial for understanding the potential environmental and health implications stemming from their interactions.

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Chapter 2: Microbial Potential for Mercury Methylation and Methane Efflux Across a Soil Moisture Gradient

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2.1 Abstract

As climate change accelerates permafrost thaw in interior Alaska, soils are becoming increasingly saturated, creating new anoxic environments where soil anaerobes thrive. Among these anaerobes are key players in controlling the methane and mercury cycles, including methanogens, anaerobic methanotrophs, and mercury methylators. As the mercury cycle results in the production of methylmercury, a neurotoxin, and the methane cycle results in the production of greenhouse gases, an increase in anaerobic activity could have an impact on the environment and the health of Alaskans. In this study, I explored the functional potential of these cycles in Alaskan boreal soils along a soil moisture gradient. I found a strong linear relationship between total mercury content and soil gravimetric water content. This is likely a result of the topography and hydrology of the area. The high mercury-carbon affinity is pertinent in permafrost thaw areas due to high carbon content and high moisture. I also found significant relationships between carbon dioxide efflux and soil moisture, as well as methane efflux and soil moisture, strongly indicating that higher soil moisture levels will lead to increased greenhouse gas production in boreal soils.

2.2 Introduction

Mercury (Hg) is one of the top ten chemicals of major health concern, according to the World Health Organization (Mercury and Health, 2017). Hg cycling requires several forms of Hg including elemental mercury (Hg^0), inorganic Hg (Hg(II)), and monomethylmercury (MMHg). Hg^0 is a naturally occurring element and is completely inorganic (Lidqvist, 1985; Si, 2018)). Mobilization of Hg to terrestrial environments has been elevated through human activities such as mining, coal combustion, industrial processes, and waste incineration, and is naturally emitted from volcanic events and wildfires (Streets, 2017). As Hg^0 is volatile, it can

disperse into the air following natural and anthropogenic emissions. Volatilized Hg^0 oxidizes in the air creating Hg(II) (AMAP, 2021). As Hg(II) is less volatile than Hg^0, it becomes deposited more readily (Cooke, 2020). Additionally, as temperatures drop, the volatility of Hg^0 decreases because of reduced vapor pressure, resulting in larger quantities of deposition in Arctic environments (Gustin, 1997). This increased accumulation of Hg deposition in Artic carbon-rich soils has the potential to impact rates of Hg cycling (AMAP, 2021). For example, in water-saturated, anoxic conditions, inorganic Hg can be biologically transformed into MMHg, a potent neurotoxin known to biomagnify in aquatic food webs (Bravo & Cosio, 2020). Research on this process is needed to understand the potential environmental health impacts of Hg methylation in high-latitude environments where Hg concentrations are elevated.

Likewise, research is needed to understand how increasing methane production is impacting Arctic and subarctic ecosystems. Methane (CH4) is a potent greenhouse gas with a warming capacity over 25 times that of carbon dioxide (CO_2), and anaerobic microbes biologically produce CH4 as they metabolize organic matter (OM) in the soil, sediments, and water (Conrad, 2007). While this is a naturally occurring process, the rate of CH4 production is increasing due to climate change; an increase in the rate of CH4 production could add to the issue of climate warming and create a climate warming feedback loop (Bischoff, 2013). Additionally, climate change is disproportionately impacting high-latitude ecosystems and may be impacting the Hg and CH4 cycles in very similar ways. In recent years, the changing climate has altered soil moisture in boreal ecosystems (Jorgenson et al., 2022). Soil warming, resulting in permafrost thaw and increased soil moisture, often increases the activity of anaerobic organisms in the soil (Natali, 2015). As such, soil moisture is a major driver of anaerobic biogeochemical cycles such as the Hg and CH4 cycles (Maietta, 2020; Zhou, 2022).

Climate change is impacting the Hg cycle in interior Alaska. A previous study on Hg export from the Yukon River Basin observed that permafrost is a large reservoir of Hg with a significant correlation between Hg export and spring season watershed discharge (Schuster, 2011). This suggests that as the climate warms and permafrost thaws, Hg will continue to be released into the soil and water system in Interior Alaska. In high-carbon sediments, such as boreal soils, total mercury (THg) can be an indicator of MMHg potential (Araujo, 2017; Li,

2024). Higher THg concentrations in these saturated carbon-rich soils provide more inorganic Hg available for potential methylation by anaerobic microbes (Myrbo, 2017). Specifically, anaerobic microorganisms contribute to this process by adding a methyl group to Hg through a detoxification mechanism (Trevors, 1986; Bravo and Cosio, 2020). Consequently, this process results in the release of MMHg into the environment. Both the Hg and the CH4 cycles are changing in response to the climate and, as a result, impacting the surrounding environment. Additionally, a warming climate can influence the rate of methylation. Notably, methylation rates have been observed to increase with temperature, up to 40°C. This temperature dependence is attributed to the enzymatic activity of the *hgcAB* gene, which is responsible for Hg methylation (Date, 2019). Another study in interior Alaskan fens observed Hg methylation rates were highest near a groundwater input when measured along a groundwater gradient (Roth, 2021). This finding further reinforces the idea that climate change is impacting the rate of Hg methylation in interior Alaskan soils. However, the broader implications of changes in the Hg cycle on other microbial processes are not fully understood, especially considering that the majority of freshwater methylation research has been conducted in the porewaters of lakes and rivers. Consequently, our knowledge regarding changes in methylation within soil environments remains limited.

Climate change exerts a profound impact on the CH4 cycle, particularly evident in the transformation of subarctic soils from a carbon sink to a carbon source due to thawing permafrost (Belshe, 2013). This shift has significant implications for the CH4 cycle, which thrives with increased soil moisture and OM. Boreal soils serve as reservoirs for both OM and Hg and as permafrost thaws, these materials are liberated, becoming available for microbial metabolism (Coolen, 2011; Fahnestock, 2019). Globally, permafrost contains a substantial store of 1,460-1,600 Pg carbon in OM (Schuur, 2019). Within the CH4 cycle, OM serves as a vital carbon and energy source for methanogens, leading to CH4 production (Conrad, 2020). Subsequently, methanotrophs use a portion of the produced CH4 as a carbon and energy source, generating CO_2 (Gal'chenko et al., 2001). Despite this process, boreal ecosystems continue to exhibit persistent CH4 fluxes (Euskirchen, 2024). The availability of OM plays a pivotal role in influencing the rate of CH4 cycling, as evidenced by previous findings indicating active layer depth and soil moisture as primary drivers of net

CH_4 efflux (Arndt, 2019). As climate warming progresses and permafrost degradation intensifies, it is anticipated that both active layer depth and soil moisture in the boreal forest will increase, leading to amplified net greenhouse gas efflux. Moreover, factors beyond soil moisture, such as the relationship between the CH_4 cycle and other microbial cycles, as well as the availability of electron acceptors, can further influence anaerobic biogeochemical processes (Bravo and Cosio, 2020). Therefore, understanding these dynamics is crucial for predicting and mitigating the consequences of climate-induced changes on the CH_4 cycle and associated greenhouse gas emissions.

As climate change progresses, CH_4 and Hg cycling will likely continue to fluctuate. To predict future rates of greenhouse gas production and MMHg release in boreal soils, it is important to explore the rates of these processes and their relationship to soil moisture changes. This study aimed to determine the biogeochemical potential for methanogenesis, methanotrophy, and Hg cycling across an environmental soil moisture gradient and pinpoint the mechanism of a possible interaction between the two cycles. Measuring the rates of these cycles provides information on the biogeochemical potential of Hg methylation and net CH_4 efflux. While methylation rate data will not be completed in the timeline of this study, it is in progress and will be added to the overall study retroactively. Thus, in this study, I explored the possible correlation and biogeochemical mechanisms between net CH_4 efflux and total Hg content in boreal topsoil. Since methanogenesis occurs anaerobically and Hg is released during permafrost thaw, both total Hg and CH_4 efflux are likely to be high in carbon-rich and moisture-rich soils. For this reason, I hypothesized that net CH_4 production and total Hg content would covary spatially across a soil moisture gradient. Additionally, I hypothesized that there would be competition between Hg methylation and anaerobic methanotrophy due to the shared role of sulfate as an electron acceptor. I explored the biogeochemical potential for CH_4 efflux and Hg cycling at sites along a soil moisture gradient via bottle incubations and total Hg analysis. In addition, an independent incubation experiment with known biogeochemical inhibitors was used to determine the interaction mechanism. For instance, inhibition of methylation could alter net CH_4 efflux if there is competition for sulfate. Finally, I assessed total Hg, carbon, and nitrogen concentrations and quantified CH_4 efflux potentials.

2.3 Methods

2.3.1 Soil Core Collection

Soils were sampled from plots in the Bonanza Creek Long Term Ecological Research (LTER) Regional Site Network (Table 2.1, Figure 2.1). In 2021, samples were collected at 27 sites (see Appendix A), which were used to identify focal sites for additional sampling. Between June and July, samples were collected from 6 sites in 2023, and two sites in 2022 (BFY7 and UP4C). From each site, a soil core measuring 15 cm in depth and 5 cm in diameter was extracted from each of three randomly selected quadrants (Figure 2.2). Soil cores were transported on ice and immediately homogenized using a 2 mm sieve on the day of collection. The samples were then split into three groups based on the intended downstream analysis: biogeochemical function (stored at 4°C), soil properties (stored at 20°C), and molecular analysis (stored at -80°C).

Site	Dryness	Permafrost	FIR	Tree Species	Elevation	Slope	Aspect	GWC
UP4A	Subxeric	No	Mature	Black Spruce	472.44	13.07	303.52	0.34
UP4B	Subxeric	No	Mature	Black Spruce	381.0	16.63	340.32	0.33
UP4C	Subhydric	No	Mature	Black Spruce	234.09	4.42	115.23	0.51
BFY4	Subxeric/mesic	Yes	Young/High	Birch	259.08	11.05	257.48	0.45
BFY7	Mesic/subhydric	Yes	Young/Low	Black Spruce	243.84	5.76	259.53	0.40
BCEFM2	Subhydric	Yes	Mature	Black Spruce	122.22	0	-1	0.77

Table 2.1. Site Characteristics: Soil samples were taken from six sites within the Bonanza Creek Long Term Ecological Research (LTER) Regional Site Network in the summers of 2021, 2022, and 2023. The Bonanza Creek LTER Regional Site Network has maintained long-term monitoring of climate and vegetation at "core" sites since the 1980s, providing background data on sites, including dryness, permafrost presence, fire intensity recovery (FIR), tree species, elevation, and aspect.

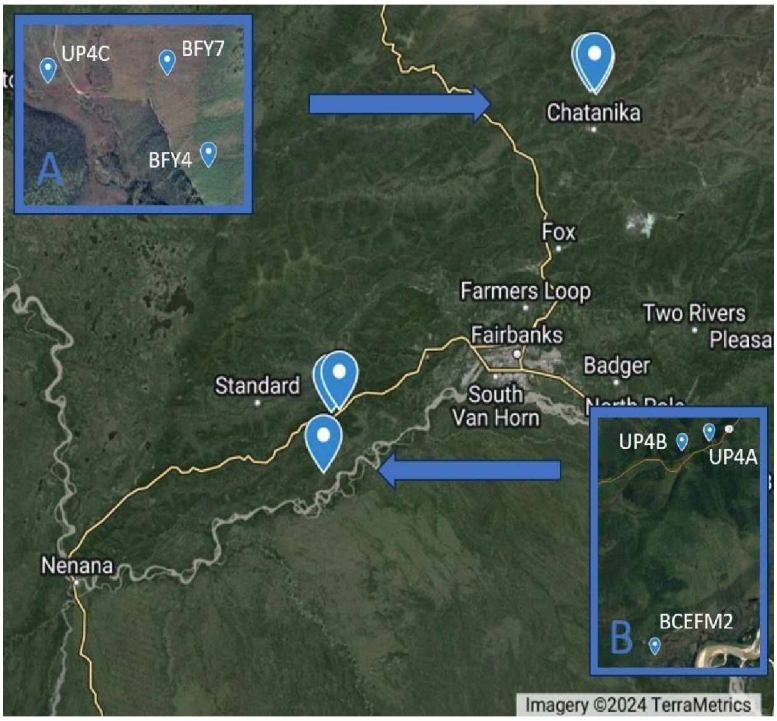

Figure 2.1. Site Map: Soil samples were taken from six sites within the Bonanza Creek Long Term Ecological Research (LTER) Network in 2021, 2022, and 2023. Three sites are 20 miles north of Fairbanks, AK in the Caribou Poker Creek Research Watershed (Inset A), and three sites are 13 miles southwest of Fairbanks in the Bonanza Creek Experimental Forest (Inset B).

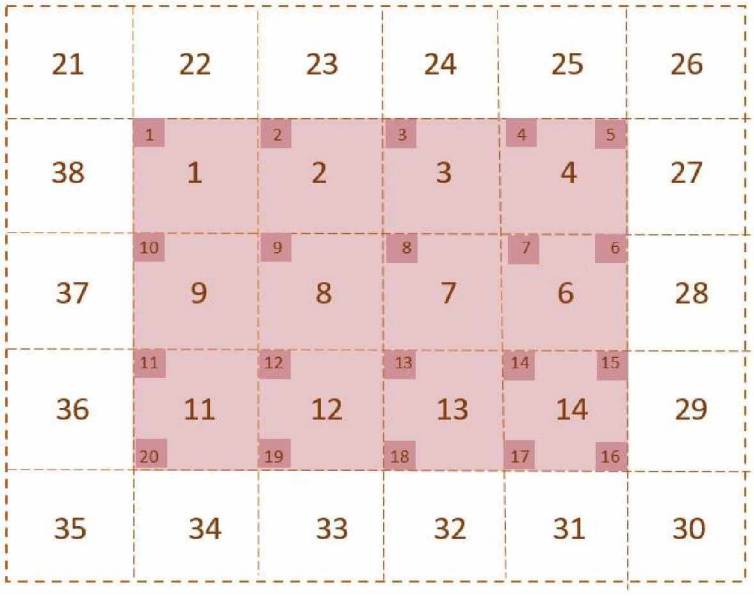

Figure 2.2. Site Quadrants: In the Bonanza Creek LTER regional site network, each "core" site is organized in a grid of quadrants, allowing for random sampling within each site. Quadrants grids consist of 12 quadrants shown in red (1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 14) that are 10 m by 10 m.

2.3.2 Soil Properties

Soils were analyzed for pH and gravimetric water content (GWC). To calculate GWC, 510 grams of soil were weighed, placed in a drying oven at 105°C for 48 hours, and weighed again after drying. GWC was quantified using the formula: (Mass (T0) - Mass (Tf))/ Mass (T0). To determine soil pH, I made a slurry with 10 g of soil and 20 ml of DI water. I let the mixture settle for 30 minutes and measured pH with a Mettler Toledo Seven Compact pH/ion meter.

2.3.3 Total Mercury Concentrations

THg concentration in each homogenized, dried soil sample was measured in duplicate using direct Hg analyzers DMA-80 (Milestone) and MA-3000 (Nippon) according to USEPA Method 7473. Quality assurance and control included the analysis of certified reference materials MESS-3 (marine sediment; NRC Canada) and NIST-2702 (inorganics in marine sediment) and blank analytical vessels. The average percent recovery of MESS-3 was 92.7% +/- 0.00945% (n=12). The average percent recovery of NIST-2702 was 98.4%+/- 0.0475% (n=10). The theoretical method of detection limit, calculated as 3 times the standard deviation of 6 blank analytical vessels, was 0.00567 ng Hg for the MA-3000 (6 blanks) and 0.0821 ng for the DMA-80 (12 blanks).

2.3.4 Net Greenhouse Gas Efflux

Net CO_2 and CH4 effluxes were measured for each soil sample using bottle incubations. For each sample, four incubations were established in 60-mL sterile glass bottles and sealed with a butyl stopper. Each bottle contained 5 g of soil and 5 mL of sterile nanopore water. The soil was mixed with water to establish a completely saturated environment in each bottle and used as a measure of the functional potential of CO_2 and CH_4 efflux under anoxic, optimal conditions. Additionally, half of the incubations were flushed with ultra-high purity (UHP) N_2 to create a completely anoxic environment. Bottles with no additional water or N_2 were used as controls. All bottles were incubated in the dark at room temperature (21 ºC) for one week. After incubation, duplicate 1-mL headspace samples were injected into a LI-COR Trace Gas Analyzer (LI-7810; LI-COR Environmental, Lincoln, NE) using a UHP N_2 carrier. CO_2 and CH_4 concentrations were

recorded simultaneously for each injection. Raw data were processed using custom R-scripts and the net efflux was calculated as the integrated area under the curve of each sample peak.

2.3.5 Inhibition Experiments

Biogeochemical competition between Hg-methylating bacteria and anaerobic CH4 oxidizers was estimated for each soil sample using bottle inhibitions with known inhibitors. For each sample, 5 g of soil was added to a sterile 60-mL bottle and flushed with UHP N2 to create an anoxic environment. Bottles were sealed with butyl stoppers. Bottles were assigned to one of three treatments: cysteine, serving as a Hg methylation inhibitor (Schaefer, 2011); 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), added to inhibit anaerobic CH4 oxidation (Steinsdóttir, 2022); or sulfate, introduced to mitigate competition for electron acceptors among anaerobes (Schnakenberg, 2021). Each reagent was added at 100 µL per bottle, with the following concentrations: PTIO at 58.3 mg/50 mL, Cysteine at 15.15 mg/50 mL, and Sulfate at 4.2 g/100 mL. All bottles were incubated in the dark at room temperature (21 ºC) for one week. After incubation, net CO_2 and CH4 efflux were measured as above to determine if the inhibition of anaerobic methanotrophy or methylation resulted in a change in the net CO_2 and CH4 efflux.

2.3.6 Statistical Analyses

I used nested mixed-effects models to analyze the results of our functional potential incubations and inhibition experiments. For each site, I nested quadrants within the site. I used independent models to test the effect of soil moisture (measured as gravimetric water content) on THg concentration, net CH4 efflux, and net CO_2 efflux. For the inhibition experiment, I tested for the effect of inhibition treatment on the net CH4 efflux while controlling for soil moisture as a covariate. All statistical calculations were performed in R (version 4.3.2) using packages lme4 (1.1-35), DescTools (0.99.53), Tidyverse (2.0.0), and ggplot2 (3.4.4).

2.4 Results

2.4.1 Total Mercury Concentrations

I found a strong linear relationship between soil moisture and THg content in boreal forest soils sampled from 2021-2023 (Figure. 2.3) ($F_{1,124} = 3.28$, $p < 0.001$). Across sites, dry weight THg

concentrations increased by an average of 9.8 ppm for every 10% increase in GWC. THg concentrations were highest in lowland areas with soil moisture greater than 50%.

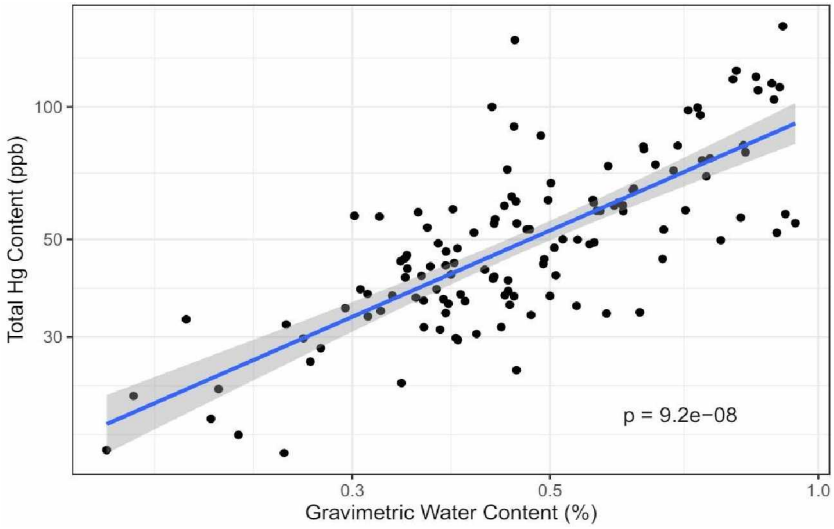

Figure 2.3: Correlation of Gravimetric Water Content on Total Hg: Soil samples with higher water content showed higher concentrations of Total Hg (THg; ng/g or ppb dry weight). THg was quantified using dried soil, and gravimetric water content was quantified as mass water per mass dry soil from the original sample.

2.4.2 Net Greenhouse Gas Flux

I found a significant relationship between soil moisture and greenhouse gas efflux. The addition of moisture significantly increased the net efflux of CO_2 (Figure 2.4) ($F_{1,175} = 0.388$, p = 0.04). The relationship between saturation and mean CH4 efflux was not significant. Additionally, the lowland, high moisture site (UP4C) showed higher net CH4 efflux than the upland site (BFY7); both methanogenesis and methanotrophy occur in high-moisture soils, and overall flux increases when soils are saturated. I also found a significant relationship between soil moisture and net CH4 efflux (Figure 2.5) ($F_{1,13} = 0.265$, p = 0.03). Across sites, CH4 efflux increased 55 ppb for every 10% increase in gravimetric water content, during a one-week incubation. As expected, the anoxic high-moisture conditions promoted anaerobic metabolic processes such as anaerobic methanogenesis. I found a significant relationship between soil moisture and net CO_2 efflux (Figure 2.6) ($F_{1,13} = 0.229$, p = 0.003); however, this relationship was more complex because methanotrophy can occur in oxic or anoxic soils; the highest efflux was found in sites with intermediate soil moisture (Figure 2.6) ($F_{1,13} = 0.229$, p = 0.03).

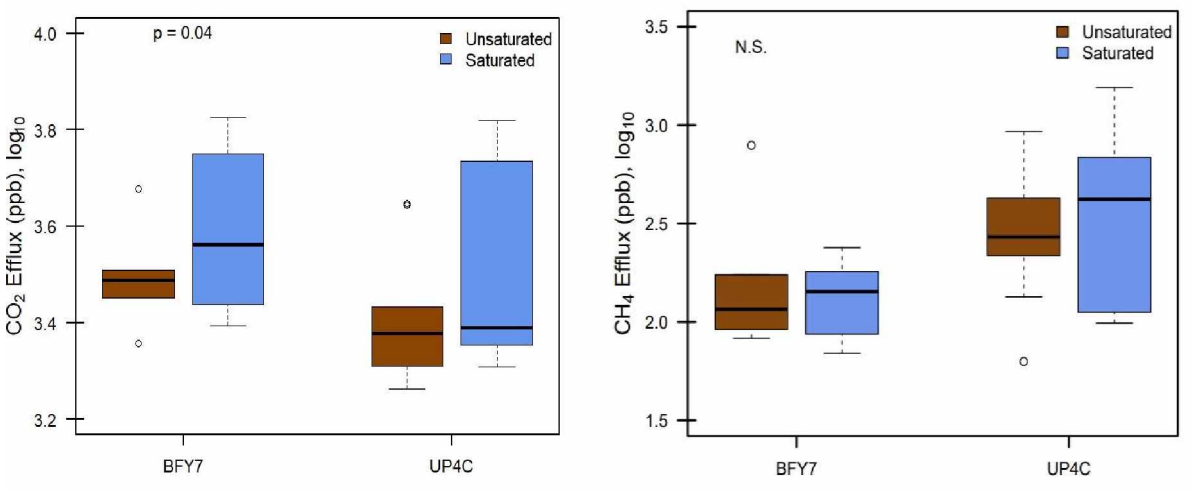

Figure 2.4: Impact of Saturation on Net Efflux. Soil samples with additional water (saturated) showed higher net CO_2 efflux compared to those with environmental soil moisture content (unsaturated). Additionally, a high moisture site (UP4C) shows higher net CH4 efflux than an upland, drier site. Saturated samples contained equal parts soil and ultrapure sterile water, while unsaturated samples contained only soil. All samples were incubated in the dark for 1 week.

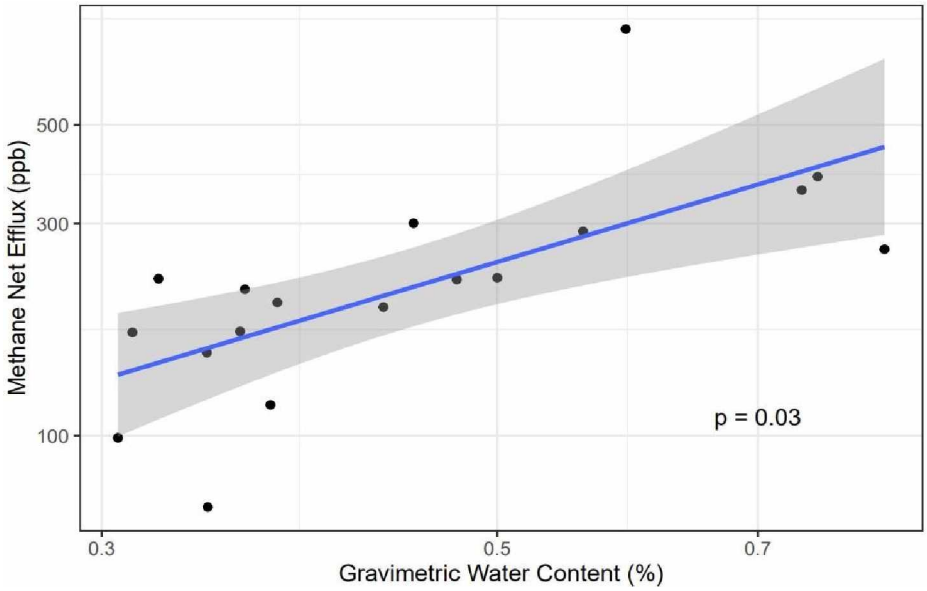

Figure 2.5. Impact of Gravimetric Water Content on Methane Efflux: Soil samples with higher water content showed higher net CH4 efflux potential. Methane efflux potential was measured by incubating in a saturated environment containing equal parts soil and ultrapure sterile water. Bottles were flushed with N2 to create a completely anoxic environment.

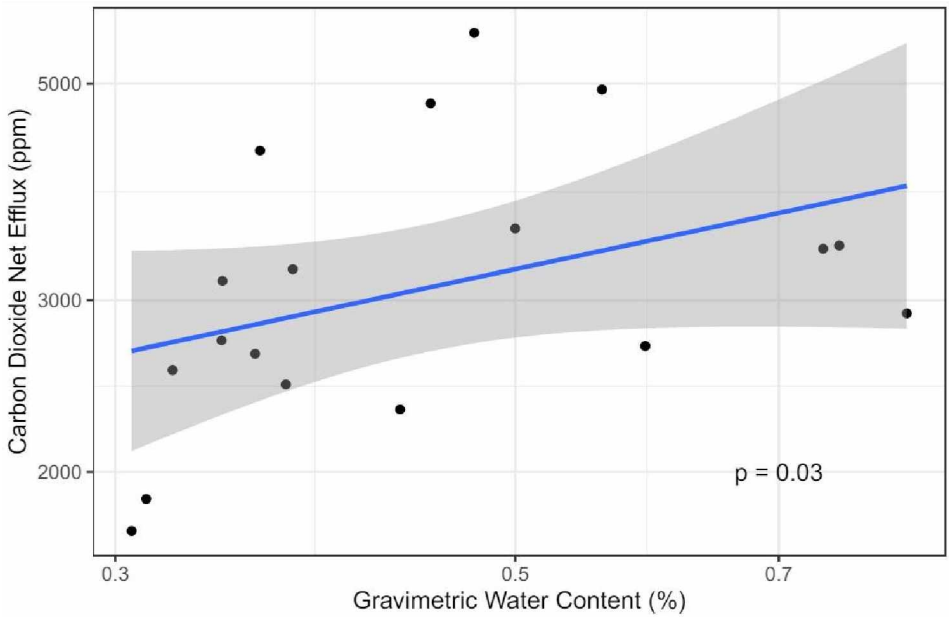

Figure 2.6. Impact of Gravimetric Water Content on Carbon Dioxide Efflux: Soil samples with intermediate water content demonstrated higher net CO_2 efflux potential. Efflux potential was measured by mixing equal parts soil and sterile ultrapure water to create a fully saturated environment. Bottles were flushed with N2 to create a completely anoxic environment.

2.4.3 Inhibition Experiment

I found that known inhibitors (cysteine, PTIO) did not have a significant effect on net CH4 efflux from each site (Figure 2.7) ($F_{1,139} = 0.139$ N.S.). However, there was an overall increase in efflux as moisture increased. In the high moisture site, BCEFM2, the addition of sulfate decreased CH4 efflux. By decreasing competition for electron acceptors in anoxic conditions, there was an increase in anaerobic methanotrophy.

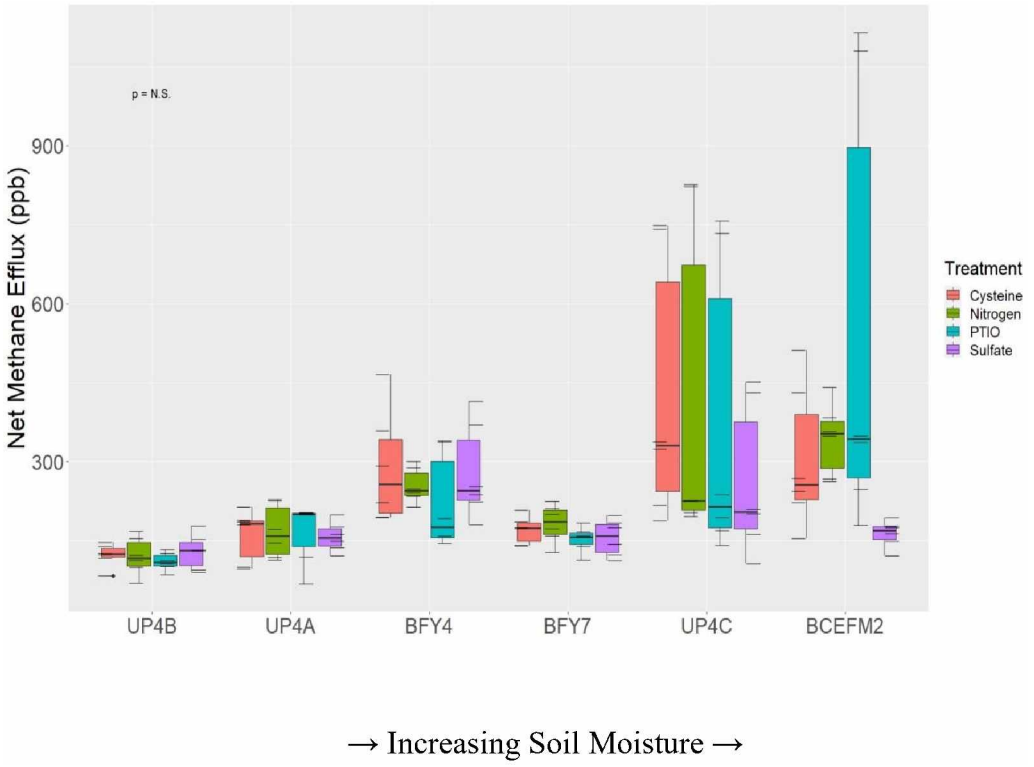

Figure 2.7. Inhibitions Impacting Methane Efflux: For each site, 5 soils were saturated, and each was placed in a treatment group (Cysteine, PTIO, Sulfate, Nitrogen, Air). After a one-week incubation, headspace was sampled in duplicate. Soil samples from sites with higher soil moisture showed higher net CH4 efflux potential, and sulfate decreased net CH4 efflux in saturated sites. Cysteine was used to inhibit Hg methylation, PTIO was used to inhibit anaerobic methanotrophy, and sulfate was added to reduce competition for electron acceptors.

2.5 Discussion

In this study, I explored the Hg and CH4 cycles by quantifying total Hg across a soil moisture gradient and measuring the net efflux of CO_2 and CH4 across the same gradient. Additionally, I tested the effect of known inhibitors on the net efflux of CO_2 and CH4 to identify a mechanism of interaction between cycles. I predicted that CO_2 and CH4 efflux would increase along a soil moisture gradient due to anoxic conditions favoring anaerobic processes such as methanogenesis and anaerobic methanotrophy. I also predicted that Hg methylators and anaerobic methanotrophs are competing for sulfate as an election acceptor. Thus, the addition of sulfate would result in an increase in the activity of Hg methylators and methanotrophs, observed through an increase in methylation potential and a decrease in net CH4 efflux.

The functional potential of the Hg and CH4 cycles increased as soil moisture increased. Additionally, I saw a positive linear relationship between GWC and THg across boreal soils. This provides some evidence that CH4 efflux and methylation might covary due to their shared environmental optima of high moisture anoxic conditions. While I cannot determine the biogeochemical potential of Hg methylation without complete net methylation data, some inferences can be made based on THg concentrations. In boreal soils, THg accumulates in lowland/saturated, carbon-rich (See appendix C), lowtemperature soils due to runoff, carbon affinity, and a decrease in vapor pressure (Giesler, 2017; Gustin, 1997). Since the majority of atmospheric deposition of Hg settles in OM near the soil surface, Hg is easily transported by water (Wang, 2013). The elevated THg content in lowland, anoxic soils shows that more inorganic Hg is available for methylation within saturated soils.

As soil moisture increases, $CO_2$and CH4 efflux increases. Methanotrophy, which generates CO_2 and can operate under both oxic and anoxic conditions, is predicted to produce CO_2 constantly in both aerobic and anaerobic soils. However, methanogenesis, which solely occurs under anoxic conditions, yields both CO_2 and CH4, and is likely responsible for the observed higher values of CO_2 and CH4 in anaerobic soils. Previous research has also found lowland soils to show elevated CH4 efflux and the potential for upland, dry soils to be CH4 sinks. (Moosavi, 1997, Moosavi, 1996, Sturtevant, 2012). This also supports the hypothesis that net CH4 efflux and methylation are likely to correlate, given their preference for high-moisture anoxic conditions.

Cysteine (methylation inhibitor), PTIO (methanotrophy inhibitor), and sulfate (shared electron acceptor) were used to pinpoint the mechanism of cycle interaction. These treatments did not significantly impact net CH4 efflux. However, in high moisture conditions, sulfate decreased CH4 efflux and PTIO increased CH4 efflux. An increase when treated with PTIO was expected since an inhibition in methanotrophy will increase total CH4 due to the lack of CH4 oxidation carried out by methanotrophs. The decrease in CH4 efflux when treated with sulfate also provides some support for this hypothesis. Because anaerobic methanotrophs and Hg methylators use sulfate as an electron acceptor, adding sulfate can relieve competition for limited electron acceptors. As a result, elevated sulfate concentrations increase anaerobic methanotrophy. This increase in CH4 oxidation results in an overall

decrease in CH4 efflux since a large amount of the available CH4 is being broken down by sulfate-reducing methanotrophs. A previous study observed similar results, with a 50% decrease in CH4 efflux, due to increased activity of sulfate-reducing methanotrophs when sulfate was increased (Wegener, 2009). Cysteine had less of an impact on CH4 efflux than the other reagents. This could be because cysteine is a sulfur-containing carbon and energy source for some microorganisms (Qi, 2014). An increase in the activity of the microbial community could increase the resulting CH4 efflux.

This study indicates that CH4 efflux and THg content covary along an increasing soil moisture gradient, as both cycles are driven by anaerobes. Competition for sulfate as an electron acceptor is the primary mechanism driving interaction, between the CH4 and Hg cycles supporting our hypothesis. Further research is needed to fully elucidate the mechanisms behind these cycle interactions. Measuring the net production of MMHg in these soils would enhance our understanding of how the Hg cycle is impacted by permafrost thaw, increased soil moisture, and THg content. Moreover, the Hg cycle can affect Arctic and subarctic aquatic food webs and communities reliant on subsistence fishing, while the CH_4 cycle influences greenhouse gas production. Therefore, further research on the mechanisms controlling these cycles is pertinent to protect the environment and human health.

Code and Data Availability:

All code is available on GitHub (MuscarellaLab/BNZ-anaerobe). Environmental data is archived with the Bonanza Creek Long-Term Ecological Research Network

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Chapter 3: Impacts of Soil Moisture on Functional Gene Abundance and Community Composition

Citation: Olson, R., Muscarella, M., Barst, B., Leigh, MB., Pender, J., Maura, G. (2024). *Impacts of Soil Moisture on Functional Gene Abundance and Community Composition*.

3.1 Abstract

As permafrost thaw intensifies in interior Alaska due to climate change, saturated soil conditions are becoming prevalent. These newly waterlogged environments result in the flourishing of anaerobic microorganisms. Among these anaerobes are methanogens, anaerobic methanotrophs, and mercury methylators. Since methylmercury, a neurotoxin, is microbially mediated and the methane cycle produces greenhouse gases, heightened anaerobic activity could impact the environment and the health of Alaskans. This study delves into the microbial community composition and functional gene abundance in Alaskan boreal soils across a gradient of soil moisture. Our findings reveal that there is no significant relationship between soil gravimetric water content and functional gene quantity. However, there was a significant relationship between beta diversity and gravimetric water content.

3.2 Introduction

Monomethlymercury (MMHg) is a neurotoxin and can bioaccumulate and biomagnify in aquatic food webs (Foster, 2012; Fitzgerald, 1991). Anaerobic microbes can release MMHg in anoxic, high-moisture soil conditions, specifically in high-latitude ecosystems experiencing unprecedented permafrost thaw rates (Tarbier, 2021; Xu 2019). Research on the microbes controlling this process is crucial in understanding and predicting the potential environmental and food-system impacts of mercury (Hg) methylation in Arctic and subarctic environments. Likewise, research is needed to understand the impacts of climate change on microbes controlling the methane cycle. Methane (CH4) is a potent greenhouse gas produced by methanogenic microbes that break down organic matter (OM) in the soil (Conrad, 2007). In high-latitude ecosystems, permafrost thaw releases available OM, increasing the activity of methanogens and thus, the production of CH4. If climate change continues to increase the rate of CH4 production, it may create a climate-warming feedback loop (Bischoff, 2013).

Understanding the microbial communities controlling the Hg and CH4 cycles will allow us to predict future environmental health and food-system conditions.

Climate change is disproportionately impacting high-latitude ecosystems and may be impacting the Hg and CH4 cycles in very similar ways. Climate warming is resulting in increased rates of permafrost thaw, often increasing soil moisture. An increase in anoxic conditions increases the activity of anaerobic microbial processes involved in the Hg and CH4 cycles. To explore changes in these cycles, relevant microbial groups were analyzed in hydric boreal soils. Anaerobes are not easily cultured, so it is more common to study their abundance, distribution, and biodiversity through molecular techniques. In addition to microbial community composition and structure, functional gene analysis can reveal the functional potential of the microbial community under ideal conditions.

To understand the potential capability of the microbial community, I explored the relative abundance of functional groups involved in methanogenesis and methanotrophy, along with their functional genes. Since water-saturated, anoxic soils promote the microbial breakdown of OM, the activity of microbes involved in the CH4 cycle is likely to increase in saturated soils (Christiansen, 2017). These microbes include methanogens and methanotrophs. Of 83 well-known species of methanogens, 61 species are hydrogenotrophic (oxidize H2 and reduce CO_2), 20 species use methyl compounds (methanol, methylamines, or dimethylsulfide), and nine species are acetoclastic (oxidize acetate and reduce CO_2) (Garcia et al., 2000). All of these species are known to live in anoxic conditions, but hydrogenotrophic methanogens are most commonly found in peat bogs (Kotsyurbenko et al., 2004). A common biomarker gene for methanogenesis is *mcrA*. This gene encodes for a subunit of the enzyme methyl-coenzyme M reductase (MCR) (Juottonen et al., 2006). Anaerobic oxidation of CH4 is coupled with sulfate reduction in anaerobic methanotrophs (Bhattarai, 2019). Anaerobic methanotrophs are phylogenetically related to some methanogens, specifically the orders Methanosarcinales and Methanomicrobiales (Meulepas et al., 2009.). A common biomarker for the oxidation of CH4 is *pmoA*. This gene encodes for methane monooxygenase, an enzyme that catalyzes the transformation of CH4 to methanol. There are two forms of methane oxygenase: pMMO and sMMO (McDonald et al., 2008). pMMO is found in nearly all methanotrophs, while sMMO is only present in some

strains. A three-gene operon, pmoCAB, codes for pMMO which is membrane-bound, while sMMO is cytoplasmic (McDonald et al., 2008; Theisen et al., 2005). Quantifying these biomarker genes may provide insight into the impact of soil moisture on community function.

To understand the functional potential of microbial communities, we can analyze the relative abundance of microbes involved in Hg methylation and their functional genes. A common biomarker for methylation is *hgcAB* (Parks, 2013). Both *hgcA* and *hgcB* provide methyl groups required for Hg^{2+} methylation while encoding different proteins (Podar et al., 2015.). The gene *hgcA* induces the biosynthesis of the folate branch of the acetyl-CoA pathway by encoding a corrinoid protein. The gene *hgcB* encodes a ferredoxin-like protein, which may be an electron donor to *hgcA* (Bravo & Cosio, 2020). There are many unidentified methylating microbes because *hgcAB* strains are rare thus they have not been well identified in 16S rRNA databases (Bravo & Cosio, 2020). Microbes also anaerobically mediate the demethylation of MMHg through oxidative demethylation (OD). The known biomarker for demethylation is the gene pair *merAB*. MMHg is transformed into CH4 and Hg(0) when the mer system (*merA* and *merB*) degrades MMHg. (Barkay & Gu, 2022). Exploring these organisms and their biomarker genes will enable us to understand the functional potential of the microbial community to produce MMHg and its impact on food webs.

In this study, I addressed how microbial community composition, and the relative abundance of microbial functional groups vary along an environmental soil moisture gradient. I hypothesized that sites with high soil moisture will have distinct microbial communities and higher abundances of organisms and genes capable of methanogenesis, anaerobic methanotrophy, and Hg methylation because water-saturated sites result in anoxic conditions. I used 16S rRNA gene sequencing to measure relative abundance, richness, community composition, and diversity in microbial communities across a soil moisture gradient. Quantitative PCR (qPCR) was used to measure functional gene abundance for each process: methanogenesis (*mcrA*), methanotrophy (*pmoA*), and Hg methylation (*hgcAB*), along the same moisture gradient. This will allow us to address

how microbial community composition and the relative abundance of microbial functional groups vary across a soil moisture gradient in boreal soils.

3.3 Methods

3.3.1 Soil Core Collection

Soils were sampled from plots in the Bonanza Creek LTER Regional Site Network (Table 3.1) (Figure 3.1). In 2021, samples were collected at 27 sites (see Appendix A), which were used to identify focal sites for additional sampling. Between June and July, samples were collected from six sites in 2023 and two sites in 2022 (BFY7 and UP4C). At each site, a 15 cm-deep, 5 cm-diameter soil core was collected from each of three randomly selected quadrants (Figure 3.2). Soil cores were transported on ice and immediately homogenized using a 2 mm sieve on the day of collection. The samples were then split into three groups based on the intended downstream analysis: biogeochemical function (stored at 4°C), soil properties (stored at -20°C), and molecular analysis (stored at -80°C).

Site	Dryness	Permafrost	FIR	Tree Species	Elevation	Slope	Aspect	GWC
UP4A	Subxeric	No	Mature	Black Spruce	472.44	13.07	303.52	0.34
UP4B	Subxeric	No	Mature	Black Spruce	381.0	16.63	340.32	0.33
UP4C	Subhydric	No	Mature	Black Spruce	234.09	4.42	115.23	0.51
BFY4	Subxeric/mesic	Yes	Young/High	Birch	259.08	11.05	257.48	0.45
BFY7	Mesic/subhydric	Yes	Young/Low	Black Spruce	243.84	5.76	259.53	0.40
BCEFM2	Subhydric	Yes	Mature	Black Spruce	122.22	0	-1	0.77

Table 3.1. Site Characteristics: Soil samples were taken from six sites within the Bonanza Creek Long Term Ecological Research (LTER) Network in the summers of 2021, 2022, and 2023. Site characteristics are listed above.

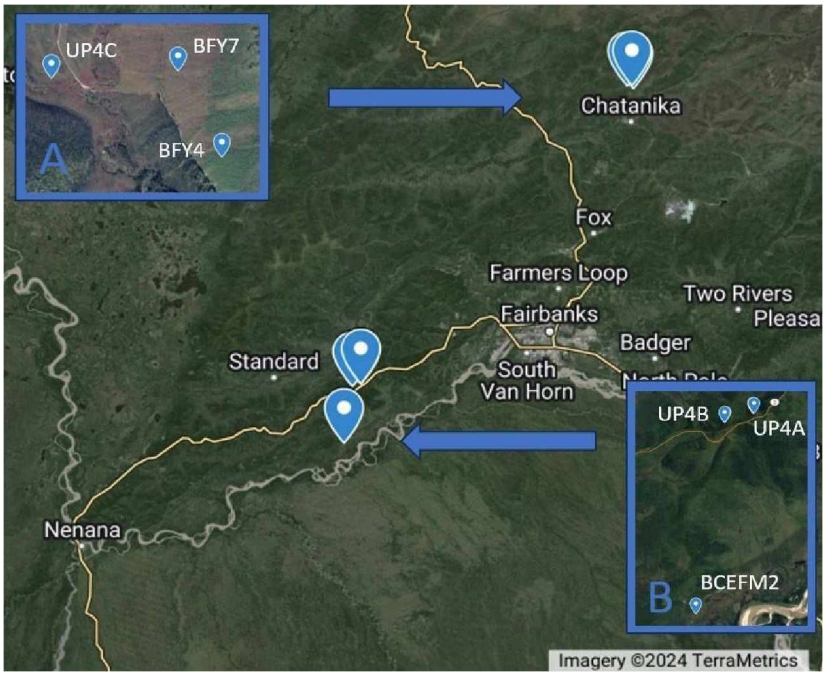

Figure 3.1. Site Map: Soil samples were taken from six sites within the Bonanza Creek Long Term Ecological Research (LTER) Network in 2021, 2022, and 2023. Three sites are 20 miles north of Fairbanks, AK in the Caribou Poker Creek Research Watershed (Inset A), and three sites are 13 miles southwest of Fairbanks in the Bonanza Creek Experimental Forest (Inset B).

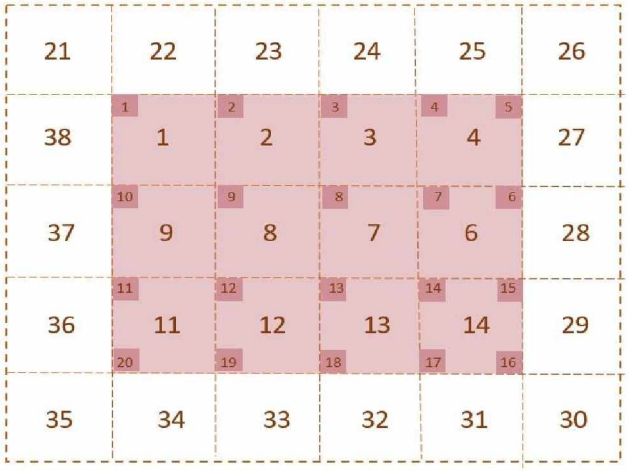

Figure 3.2. Site Quadrants: In the Bonanza Creek LTER regional site network, each "core" site is organized in a grid of quadrants allowing for random sampling within each site. Quadrants grids consist of 12 quadrants shown in red (1, 2, 3, 4, 6, 7, 8, 9, 11, 12, 13, 14) that are 10 m by 10 m.

3.3.2 Soil Properties

From Soils were analyzed for pH, gravimetric water content (GWC) total carbon, and nitrogen. Carbon and nitrogen content was obtained using continuous-flow isotope ratio mass spectrometry (CFIRMS). This method utilizes a Thermo Scientific Flash 2000 elemental analyzer and Thermo Scientific Conflo IV interfaced with a Thermo Scientific DeltaV^{Plus} Mass Spectrometer (Bremen, Germany). Stable isotope ratios were reported in delta notation as parts per thousand (%) deviation from the international standards VPDB (carbon) and Air (nitrogen). Typically, instrument precision is <0.2 %. Analyses were performed by the Alaska Stable Isotope Facility at the University of Alaska Fairbanks's Water & Environmental Research Center. To calculate GWC, soils were weighed, placed in a drying oven for 48 hours, and weighed again after drying. To quantify GWC, 5-10 g soil was dried for 48 hours at 105 °C. GWC was calculated as Mass (T0) - Mass (Tf) / Mass (T0). To determine soil pH, I made a slurry with 10 g of soil and 20 ml of DI water. I let the mixture settle for 30 minutes and measured pH with a Mettler Toledo Sevencompact pH/ion meter.

3.3.3 DNA Extraction and Community Composition

From each soil core, the soil microbiome was analyzed using 16S rRNA amplicon sequencing. Briefly, I extracted DNA from 0.25 g soil using the Qiagen PowerLyser DNA extraction kit according to the manufacturer's guidelines. Extracted DNA was quantified fluorometrically using a Qubit and the high-sensitivity dsDNA assay kit (Invitrogen). The 16S rRNA gene was amplified using the EMP 515F and EMP 926R primers according to the Earth Microbiome Project (EMP) standard protocols (Parada et al., 2016; Caporaso et al. 2012, Appendix B), and each sample was assigned a unique barcoded forward primer. All samples were amplified in triplicate and pooled. Negative controls were used for each barcoded primer. Amplicons were purified using Sera-Mag select magnetic beads (Cytiva) and quantified using the Quant-iT high-sensitivity dsDNA assay kit (Invitrogen). Individual barcoded samples were then mixed at equal molar concentrations (20 ng /sample). Amplicon sequencing was performed using the Illumina MiSeq platform in the UAF Institute of Arctic Biology Genomics Core Lab (see Appendix B).

Raw sequences were quality-checked, aligned, and binned into operational taxonomic units (OTUs) using the mothur platform (Schloss et al., 2009; Westcott & Schloss, 2017). Briefly, forward and reverse reads were aligned into congits, and quality filtered using the default parameters. Sequences with ambiguous bases or improper alignments were then removed. Sequences were aligned to the full-length Silva 16S reference database (release 132). Sequences were then checked for alignment quality and poorly aligned sequences were removed. Unique sequences were then denoised (Huse et al, 2010) using the pre-cluster command, and chimeric sequences were removed using the Vsearch algorithm (Schloss et al, 2009). Sequences were then classified based on the Silva taxonomy, and contaminants were removed (including chloroplasts, mitochondria, eukaryotic, and sequences with unknown domains) (Table 3.2). Sequences were then clustered into 97% similarity OTUs using the optiClust algorithm (Westcott and Schloss, 2017)). The resulting OTU table was used to determine the within-and between-site microbial diversity. To explore the diversity within sites (i.e., alpha diversity), I compared the taxonomic identity of each OTU based on the Silva database taxonomy. I also calculated OTU richness as the number of unique OTUs observed and community diversity using the
Shannon Index (Shannon, 1948; Ortiz-Burgos, 2016). Shannon's Index was used because it takes into account both the number of OTUs present (i.e., richness) and the equitability of their abundance (i.e., evenness). For all measures of alpha diversity, I rarefied each community based on sequence coverage. To explore the diversity between (i.e., beta diversity), I calculated the Bray-Curtis dissimilarity between sites based on the relative abundances of OTUs in each site. I then visualized Bray-Curtis dissimilarity using Principal Coordinates Analysis (PCoA) and used the envfit() function in the vegan R-package to determine environmental drivers of community composition across sites.

3.3.4 Functional Gene Quantities

To quantify functional gene abundance, I used qPCR to measure the relative abundance of genes critical for methanogenesis (*mcrA*), methanotrophy (*pmoA*), and Hg methylation (*hgcAB*). For each, a standard was generated by cloning a locally amplified gene onto a plasmid (pCR 2.1 TOPO TA Cloning Kit, Invitrogen). Plasmids were sequenced

to confirm proper construction and quantified to generate a standard curve. Following the preparation of standards, each functional gene was amplified using the PowerUP SYBR Green RT-Master Mix Kit (Applied Biosystems) on a real-time PCR thermocycler (Anyk, qTower). All reactions were done in quadruplicate.

Statistical Analyses: I used a nested mixed-effects regression with the sample quadrant nested within the sample site. In our regression models, I used gravimetric water content as the independent variable, and qPCR replicates were the dependent variable. Statistical calculations were done in R studio and used packages lme4 (1.1-35), DescTools (0.99.53), tidyverse (2.0.0), and ggplot2 (3.4.4). Community composition was analyzed using pCoA via the cmdscale function in the vegan package (2.6-4).

3.4 Results

3.4.1 Community Composition

Environmental soil moisture content was a highly significant driver of community composition. By visualizing Bray-Curtis dissimilarity using Principal Coordinates Analysis, I observed variation in community assembly based on soil moisture (Figure 3.3). Hygric and subhygric sites had higher beta diversity than drier, subxeric sites. Furthermore, I found a significant positive linear relationship between GWC and relative beta diversity (Figure 3.3,) ($F_{1,81}$ = 2.6297, p = 0.004).

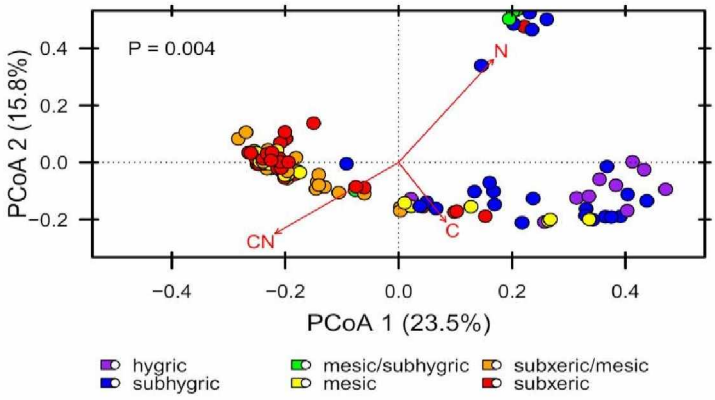

Figure 3.3. 16S rRNA Principal Coordinates Analysis: Water content is a significant predictor of relative diversity. Relative diversity was determined with 16S rRNA sequencing. Soil moisture was quantified as mass water per mass dry soil from the original sample (Hygric = high moisture, mesic = medium moisture, and xeric = low moisture)

3.4.2 Relative Abundance of Taxonomic Groups

While I have demonstrated that soil moisture is a significant predictor of community composition and diversity (Figure 3.3), I have further explored which taxonomic groups are in saturated soils. The relative abundance of Hg-methylating taxonomic groups increased as soil moisture increased (Figure 3.4). Specifically, the majority of Hg methylators belong to groups: Desulfobacterota, Bacteroidota, Actinobacteriota, and Proteobacteria. All of these methylating groups increased as soil moisture increased, while non-methylating groups remained constant (Figure 3.4). I also found that methanogenic taxonomic groups increased in relative abundance as soil moisture increased (Figure 3.5). The majority of the increase in the relative abundance of methanogens is due to an increase in Euroarchaeota and Halobacteriota as soil saturation increased. I also found an increase in methanotrophic taxonomic groups as soil moisture increased (Figure 3.6). Proteobacteria were almost entirely responsible for the increase in relative taxonomic abundance as soil saturation increased. Additionally, there was an increase the in Shannon diversity of observed taxonomic units (OTUs) as gravimetric water content increased (Figure 3.7) ($F_{1, 81} = 2.6297$, p =0.004). Saturated, lowland soils had more diverse communities, in comparison with dry, upland soils. Anoxic conditions allowed for the development of diverse microbial communities that included Hg methylators, methanogens, and methanotrophs.

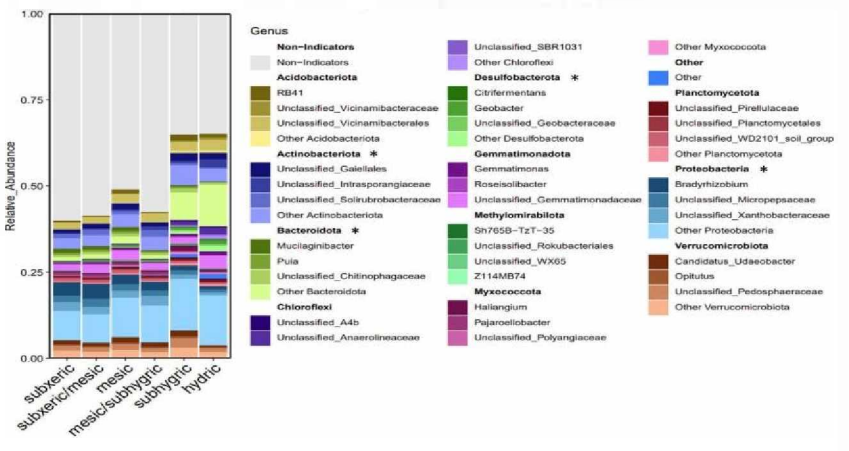

Figure 3.4. Relative Abundance of Methylating Taxa: High-moisture soils have higher relative abundances of methylating microbes. Relative abundance was determined with 16S rRNA sequencing. Soil moisture was quantified as mass water per mass dry soil from the original sample (subxeric = extremely dry, mesic = moderate moisture, hygric = saturated).

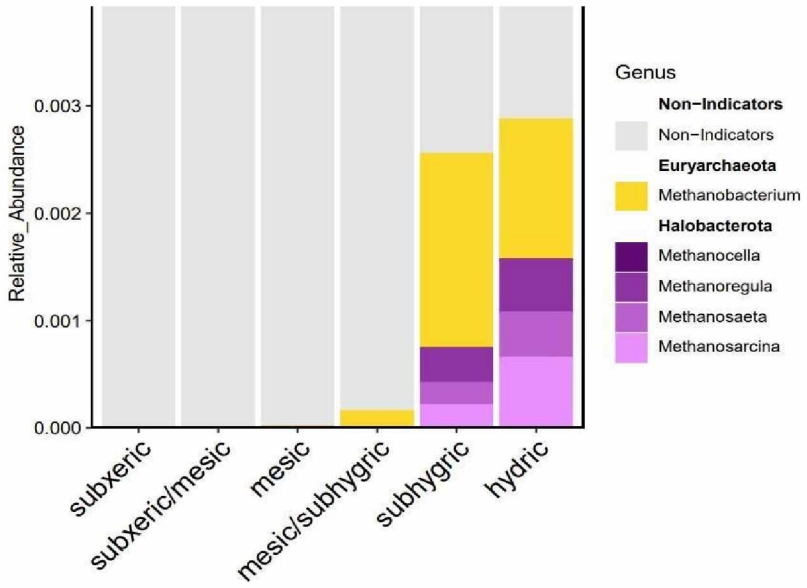

Figure 3.5. Relative Abundance of Methanogenic Taxa: High-moisture soils have higher relative abundances of methanogenic microbes. Relative abundance was determined with 16S rRNA sequencing. Soil moisture was quantified as mass water per mass dry soil from the original sample (subxeric = extremely dry, mesic = moderate moisture, hygric = saturated).

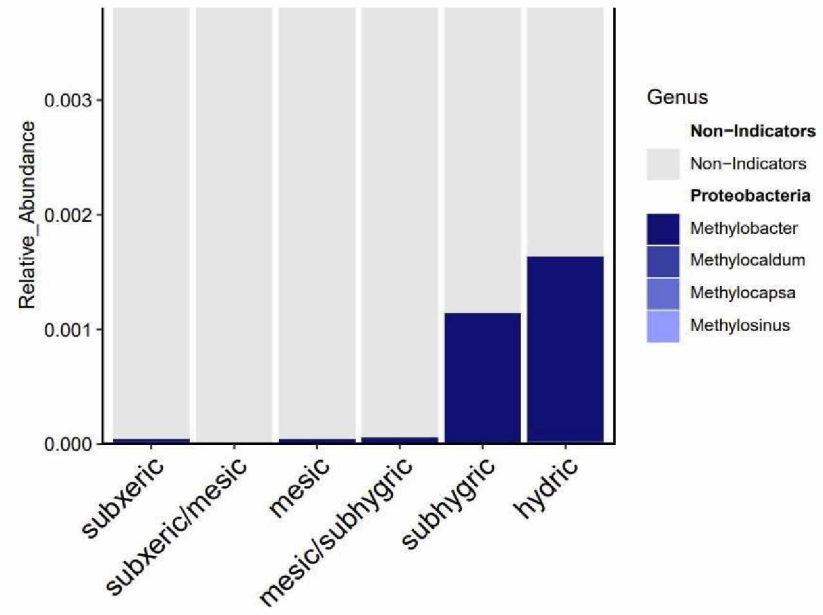

Figure 3.6. Relative Abundance of Methanotrophic Taxa: High-moisture soils have higher relative abundances of methanotrophic microbes. Relative abundance was determined with 16S rRNA sequencing. Soil moisture was quantified as mass water per mass dry soil from the original sample (subxeric = extremely dry, mesic = moderate moisture, hygric = saturated).

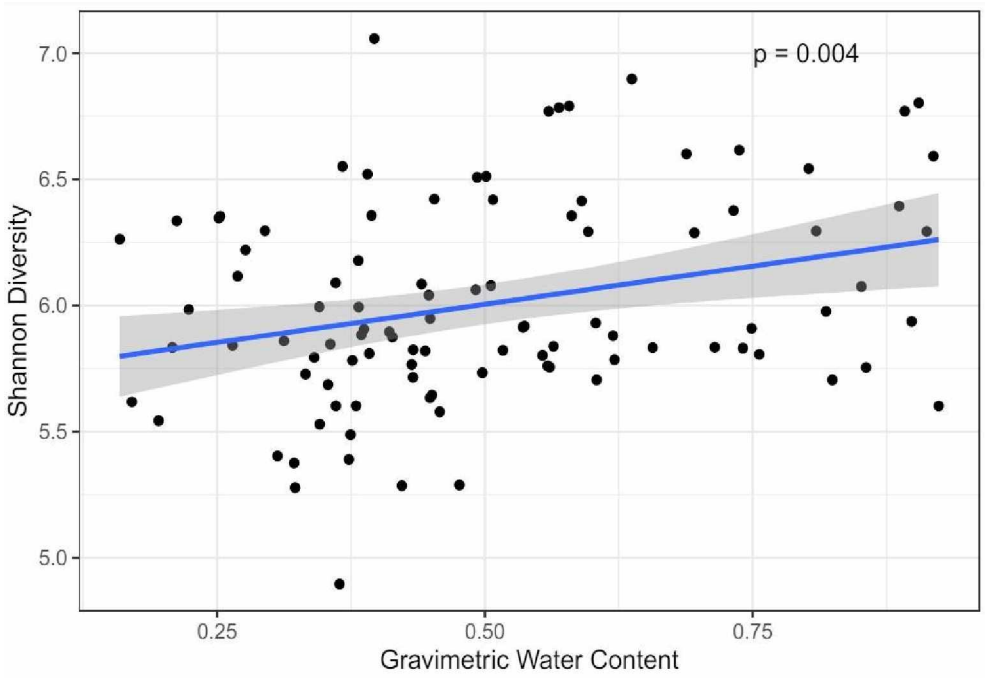

Figure 3.7. Impact of Gravimetric Water Content on Diversity: High moisture soils showed more diverse microbial communities. Shannon's Index was used to portray community richness and evenness. Gravimetric water content was quantified as mass water per mass dry soil.

3.4.3 Functional Gene Quantities

From Gravimetric water content did not impact functional gene concentration. The relationship between *hgcA* concentration and soil moisture content was not significant (Figure 3.8) ($F_{1,13}$ = 0.0006, N.S.). I also did not find a significant relationship between the concentration of *mcrA* and gravimetric water content (Figure 3.9) ($F_{1,13}$ = 0.102, N. S.). Additionally, there was not a significant relationship between *pmoA* replicate concentration and gravimetric water content (Figure 3.10) ($F_{1,13}$ = 0.116, N.S.). There were several samples below the detection limit of the qTOWER and overall, low DNA concentrations from all samples.

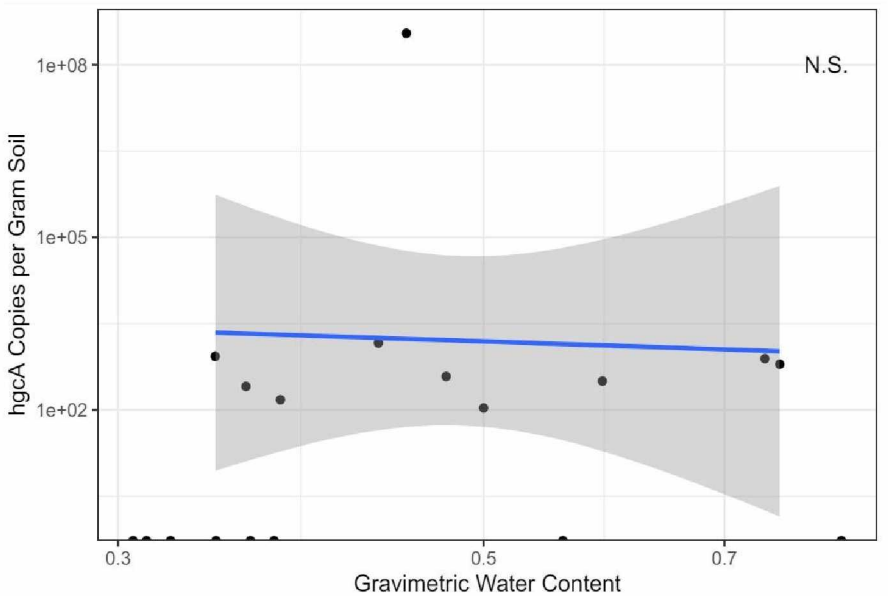

Figure 3.8. Impact of Gravimetric Water Content on *hgcA* Concentration: Soil moisture did not affect *hgcA* concentration. The *hgcA* gene is a biomarker for Hg methylation. Gravimetric water content was quantified as mass water per mass dry soil.

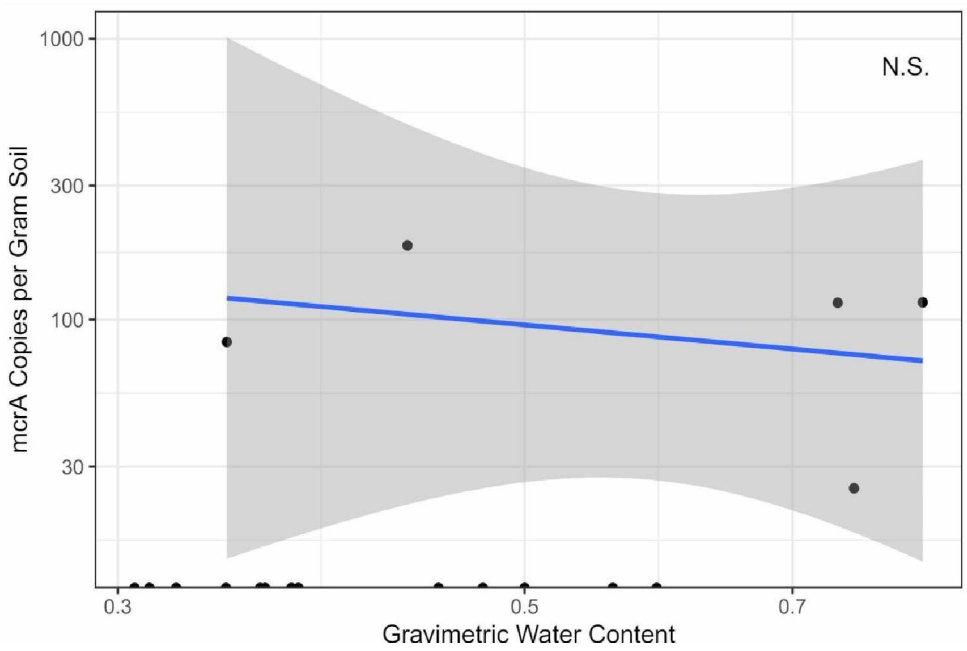

Figure 3.9. Impact of Gravimetric Water Content on *mcrA* Concentration: Soil moisture did not affect *mcrA* concentration. The *mcrA* gene is a biomarker for methanogenesis. Gravimetric water content was quantified as mass water per mass dry soil.

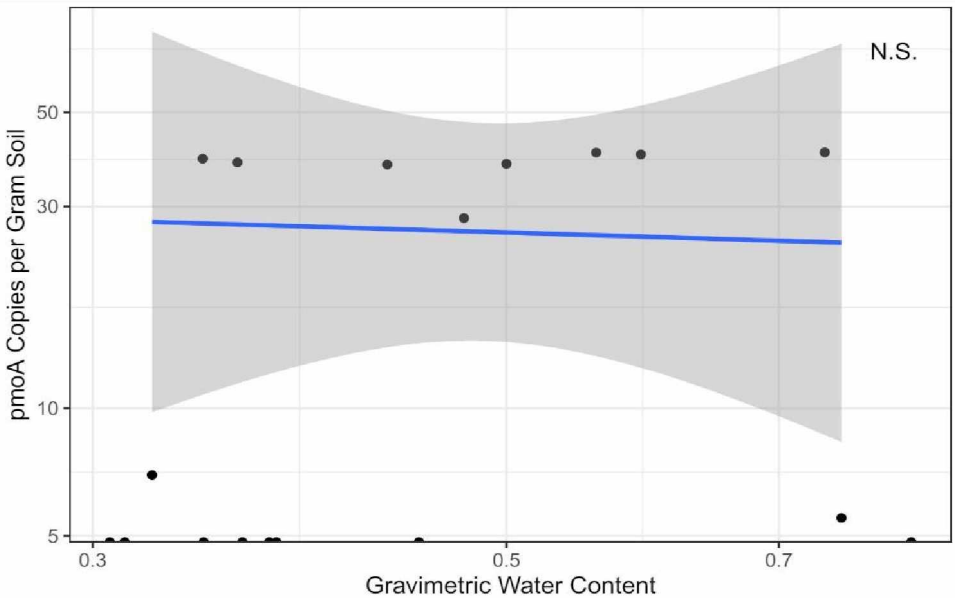

Figure 3.10. Impact of Gravimetric Water Content on *pmoA* Concentration: Soil moisture had no effect on *pmoA* concentration. The *pmoA* gene is a biomarker for methanotrophy. Gravimetric water content was quantified as mass water per mass dry soil.

3.5 Discussion

From In this study, I explored microbial community composition and function across a soil moisture gradient through 16S rRNA sequencing and by quantifying functional genes (*hgcA, mcrA, pmoA)* with qPCR. I hypothesized that saturated, anoxic soils, will have distinct microbial communities and higher abundances of genes and organisms involved in the Hg and CH4 cycles relative to drier upland soils. I predicted that high moisture sites would have a higher relative abundance of functional genes required for Hg methylation (*hgcA*), methanogenesis (*mcrA*), and methanotrophy (*pmoA*). I also predicted that high moisture sites would have a higher relative abundance of taxonomic groups involved in the Hg and CH4 cycles.

Soil moisture was a significant driver of community composition and relative diversity. This was predicted since anaerobes thrive in anoxic, saturated soils, while aerobic microbes require drier soils. Additionally, I found an increase in C:N in drier, upland soils, suggesting possible nutrient limitation due to a decrease in nitrogen. A previous study found that soil moisture is a strong predictor of bacterial community composition, while the C:N ratio was a main predictor of archaeal community composition (Li, 2017). This aligns with our hypothesis that due

to anoxic conditions, soils with high moisture will have distinct microbial communities and higher abundances of organisms and genes capable of methanogenesis, anaerobic methanotrophy, and Hg methylation.

Increased soil moisture supports anaerobic taxonomic groups related to the Hg and CH4 cycles. Taxonomic groups known to methylate Hg, including Proteobacteria, Bacteriodota, and Disulfobacteria (Xu, 2019), are more abundant in high soil moisture. This is likely because these organisms are anaerobic, so high moisture conditions are favorable. Likewise, taxonomic groups known to be methanogens, including Euryarcheota, Methanocella, Methanosaeta, and Methanosartcina (Bomberg, 2011), are more abundant in saturated soils. Furthermore, taxonomic groups known to be anaerobic methanotrophs, such as Methylobacter (Peltoniemi, 2016) increase in abundance in high-moisture soils. This supports our hypothesis that, due to anoxic conditions, boreal forest sites with high soil moisture will have increased relative abundances of organisms capable of methanogenesis, anaerobic methanotrophy, and Hg methylation.

Functional gene abundance does not vary in response to site conditions. Soil moisture content does not impact the abundance of functional genes for Hg methylation (*hgcA*), methanogenesis (*mcrA*), and methanotrophy (*pmoA*). For *pmoA*, this is likely because this process can occur in anaerobically or aerobically. Moreover, qPCR quantifies the abundance of functional genes present, so some of these genes may be present without being active. It is likely also that the qPCR results from this study would be more accurate with higher DNA concentrations because several values were below the detection limit, a common issue with quantifying functional genes (Rocca et al., 2015). In previous research, DNA concentrations from 50 to 500 ng/µl were successfully quantified (Christensen, 2016), but the concentrations from this study were often under 20 ng/µl.

Since saturated sites result in anoxic conditions, boreal forest sites with high soil moisture have distinct microbial communities and higher abundances of organisms capable of methanogenesis, methanotrophy, and Hg methylation. Across sites, saturated soils enhanced anaerobic processes, and likely are anoxic or have more anoxic microsites. However, the hypothesis that there will be a higher abundance of functional genes for these processes in high moisture sites was not supported. Using meta-transcriptomes or higher

DNA concentrations for qPCR in future research will help address this knowledge gap. Understanding the functional genes behind these cycles will be an integral part of predicting greenhouse gas efflux and MMHg production in Arctic and subarctic regions that are disproportionately impacted by climate change.

Code and Data Availability:

All code is available on GitHub (MuscarellaLab/BNZ-anaerobe). Environmental data is archived with the Bonanza Creek Long-Term Ecological Research Network and sequence data is on the NCBI sequence read archive.

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Chapter 4: General Conclusions

4.1 Introduction

Through this study, I aimed to determine the impact of soil moisture on the mercury (Hg) and methane (CH4) cycles and identify possible interactions between cycles. In Chapter 2, I analyzed the functional potential and biogeochemical mechanisms between CH4 efflux and total Hg content in boreal soils. I hypothesized that CH4 efflux and total Hg content would covary across boreal soils since methanogens require anoxic environments and Hg readily binds to carbon in high moisture soils such as permafrost thaw sites. Additionally, I hypothesized there would be interaction between Hg methylation and anaerobic methanotrophy, due to competition for sulfate as an electron acceptor. To determine the biogeochemical potential for CH4 efflux and Hg methylation at sites along a soil moisture gradient, total Hg was quantified and CH4 potentials were measured using bottle incubations. An additional incubation used inhibitors to pinpoint a mechanism of cycle interaction.

In Chapter 3, I examined how microbial community composition, and the abundance of microbial functional genes respond to fluctuations in soil moisture. I hypothesized that high moisture soils would have unique microbial communities and higher relative abundances of organisms and genes capable of methanogenesis, anaerobic methanotrophy, and Hg methylation, compared to dry or upland sites. Given their similar preferences for anoxic environmental conditions, I anticipated these microbes would thrive and be more active in oxygen-deprived environments. To define microbial communities by function in regions affected by permafrost thaw and wildfire, I employed a combination of 16S rRNA gene sequencing, targeted functional gene sequencing, and quantitative PCR (qPCR) to analyze microbiomes, functional groups, and the abundance of functional genes. I assessed the diversity of microbial functional groups and quantified the abundance of genes associated with methanogenesis (*mcrA*), methanotrophy (*pmoA*), and Hg methylation *(hgcA*).

4.2 Findings

Through our analysis of functional potential and community composition, our findings indicate that the rate of Hg cycling may change in response to soil moisture. I found a significant linear relationship between total Hg content and gravimetric water content (GWC). This supported our hypothesis that net CH4 efflux and methylation will covary due to their shared environmental optima of high moisture anoxic conditions. There was no significant correlation between soil moisture content and the abundance of the functional gene (*hgcA*). However, a significant relationship was observed between soil moisture and relative diversity. The absence of significance in functional gene abundance is likely attributable to low DNA concentrations, as our data indicate the presence of the taxonomic groups, which increase in relative abundance with soil moisture.

In our examination of functional potential and community composition, I observed that the rate of CH4 cycling may change in response to changes in soil moisture. Specifically, I identified a significant correlation between net CH4 efflux and GWC. This finding supports our hypothesis that the functional dynamics of the CH4 and Hg cycles would co-vary along a moisture gradient due to shared environmental preferences. While no significant correlation emerged between soil moisture content and the abundance of functional genes related to methanogenesis and methanotrophy (*mcrA* and *pmoA*), our hypothesis garnered partial support. Notably, our data revealed a positive linear relationship between the abundance of methanogenic and methanotrophic functional groups and soil moisture levels. This reinforces our hypothesis that high moisture boreal soils increase the relative abundances of organisms capable of methanogenesis, anaerobic methanotrophy, and Hg methylation due to anoxic conditions.

Our analysis of functional potential and community composition suggests an intricate interaction between the Hg and CH4 cycles. As both the taxonomic abundance and functional potential of these cycles increase with rising soil water content, it's plausible that the microbes involved in these processes live in proximity to each other. Apart from their shared preference for anoxic conditions, these microbes also rely on some of the same electron acceptors, notably sulfate. I hypothesized that competition for sulfate serves as the mechanism for this cycle interaction. Supporting this hypothesis, I observed a decrease in net CH4 efflux when sulfate was

introduced during incubation at our highest moisture site. This implies that alleviating competition for sulfate enhanced the activity of anaerobic methanotrophs, consequently reducing net CH4 efflux.

4.3 Implications

The interior Alaskan boreal region is experiencing amplified impacts of climate change, ranging from permafrost thaw to wildfire. The extensive permafrost surface area in areas with discontinuous permafrost exacerbates these impacts (Ostercamp, 2005). Permafrost thaw in these regions often leads to elevated soil moisture levels and the formation of anoxic soils. Particularly in regions with abundant ice-rich soils, permafrost can thaw near the surface while a frozen layer persists below, resulting in poorly drained, waterlogged conditions (O'Donnell, 2012). These anoxic conditions, brought about by permafrost thaw, foster anaerobic microbial processes (NRCS, 2024, Vasilas and Vasilas, 2013). Our findings support this assertion, revealing significant relationships between soil moisture levels and the taxonomic abundance of anaerobes, as well as between soil moisture levels and functional potential. These findings suggest that the increase in soil moisture induced by climate change will likely impact future CH4 efflux and Hg methylation, with potential implications for human health and the environment.

An observed rise in soil moisture levels within boreal soils has the potential to exert significant impacts on human health, food security, and climate dynamics. With the escalation of water-saturated, anoxic conditions, there is a heightened likelihood of increased production of monomethylmercury (MMHg), a potent neurotoxin known for its biomagnification within aquatic food webs (Bravo & Cosio, 2020). This is particularly concerning in high-latitude environments where Hg concentrations are already elevated. Moreover, given that fish consumption constitutes a primary pathway for human Hg exposure (Hong, 2012), this issue holds particular significance in Alaska, given its substantial fishing industry, indigenous communities, and reliance on subsistence fishing practices. In addition to the threats posed by MMHg, an uptick in CH4 efflux bears the potential to impact global climate patterns. As both CH4 and CO_2 are recognized greenhouse gases, a change in CH4 cycle activity could precipitate a positive climate feedback loop, further exacerbating warming trends (Bischoff, 2013).

Subsequent warming may intensify permafrost thaw, elevate soil moisture levels, and perpetuate alterations in both the CH4 and Hg cycles. Therefore, thorough research on these processes is essential to grasp the potential environmental and health implications arising from their interactions.

To comprehensively anticipate the health and environmental implications of climate change on the Hg and CH4 cycles in Alaska, further research is essential. Recommended approaches include measuring rates of Hg methylation using spiked isotopes, quantifying the merA gene (responsible for demethylation), and employing quantitative PCR with higher DNA concentrations. Additionally, using metatranscriptomes may offer a more precise method of assessing community activity compared to functional gene abundance alone. The evident impact of climate change and soil moisture on the Hg and CH4 cycles endorses the need for continued investigation. As climate patterns evolve and permafrost thaws, ongoing research into the emissions of these cycles is critical for understanding their effects on food security, climate dynamics, and the health of Alaskans.

4.4 References

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Appendix A: Extended Site Data

Site Characteristics: Soil samples were taken from 27 sites within the Bonanza Creek Long Term Ecological Research (LTER) Network in the summers of 2021. Site characteristics are listed above.

Site	Slope	Aspect	Fire recovery, intensity	Permafrost	Dryness
BCEFM2	0.00	-1.00	mature	yes	subhygric
BFY1	3.22	45.00	young, med	no	subxeric
BFY4	11.05	257.48	young, high	yes	subxeric/mesic
BFY6	0.00	-1.00	young, med	no	mesic
BFY7	5.75	259.53	young, low	yes	mesic/subhygric
BFY10	13.14	318.31	young, med	no	subxeric/mesic
BFY11	14.47	246.27	young, high	yes	subxeric
BFY12	16.32	249.90	young, med	yes	subxeric
BFY13	6.62	157.34	young, high	yes	subxeric/mesic
BTL	64.92	147.82	mature	no	hydric
GSI1	0.53	24.28	intermediate	no	mesic
GSI2	0.00	-1.00	intermediate	yes	subhygric
GSI3	3.59	274.62	intermediate	no	mesic
GSM1	4.51	162.15	mature	yes	subhygric
GSM2	2.81	114.65	mature	yes	subxeric

GSM3	0.00	-1.00	mature	no	subhygric
GSM4	17.62	17.97	mature	no	subxeric/mesic
GSM5	10.47	302.94	mature	yes	subxeric
MDI1	3.54	75.26	intermediate	no	subxeric/mesic
MDI2	10.37	59.07	intermediate	no	subxeric
MDI3	4.90	62.13	intermediate	no	subxeric/mesic
MDI4	6.81	207.56	intermediate	no	subxeric
MDI5	7.63	332.30	intermediate	no	mesic
MDM1	1.94	42.56	mature	yes	mesic
UP4A	13.07	303.52	mature	no	subxeric
UP4B	16.63	340.32	mature	no	subxeric
UP4C	4.42	115.22	mature	no	subhygric

Appendix B: Amplicon Sequencing Details

Primer Sequences:

Forward (515F):5′- GTGYCAGCMGCCGCGGTAA-3′
Reverse (926R): 5′-CCGYCAATTYMTTTRAGTTT-3′
Primers were designed to include the Illumina adapters, (5' adapters on forward primer, 3' adapter on reverse primer) 12 base pair Golay Barcode on reverse primer, and sequence linker (GT on forward, CC on reverse). (Caporaso, 2012; Parada, 2015)

PCR Conditions: Concentrations are given per 50 µL reaction. Components are from the PowerUP SYBR Green RT-Master Mix Kit (Applied Biosystems)

Component	**Stock Concentration**	**Final Concentration**	**µL per 50 µL reaction**
Platinum 2X Master Mix	2X	2X	25
926 Reverse Primer	5 µM	5 µM	1
GC Enhancer			10
Molecular Grade Water			8
Added to each individual reaction			
515 Forward Primer	10 µM	10 µM	1
Template		2-10ng	5

Thermocycler Conditions: All samples were amplified on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific) with the conditions listed below.

Temperature (^{0}C)	**Time (seconds)**	**Cycles**
95	180	
95	45	-
50	30	30
72	90	-
72	600	
4	Hold	

Appendix C: Soil Carbon Content