

Uncovering the peatland root microbiome: Environmental factors shaping microbial community structure

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**Uncovering the peatland root microbiome:
Environmental factors shaping microbial community
structure**

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Introduction

The rising concern of climate change and its anticipated effects across the globe has prompted the scientific community to gain a better understanding of this impending crisis. Special consideration has been focused on peatlands, a freshwater ecosystem characterized by the accumulation of deep organic matter (i.e. peat) up to several meters belowground (Turunen, 2003; Beilman et al., 2008). Peatlands form when the sequestration of photosynthetically fixed carbon belowground exceeds its decomposition. This typically occurs in northern climate zones prone to consistently cool temperatures and waterlogged environmental features. While peatlands make up only 3% of global surface area, accumulated peat stores nearly a third of all terrestrial carbon (Maltby and Immirzi, 1993). The response of this major carbon repository to environmental stressors, such as warming or atmospheric carbon dioxide enrichment, has yet to be fully understood (Gorham, 1995). Historically, northern peatlands have operated as a net carbon sink, but recent climate change simulations have indicated a shift to a greater production of methane and carbon dioxide in these environments, potentially putting this classification at risk (Hopple et al., 2020).

The cycling of substrates, such as carbon, through an environment is largely controlled by microbial metabolism. Fundamental features of northern peatlands shape the resident microbial community, and in turn control the extent of microbial decomposition that occurs belowground. These features are typically categorized by three peat layers according to soil depth: the primarily oxic region which is closest to the surface (acrotelm), the periodically oxic/anoxic (mesotelm), and the deepest layer that is completely anoxic (catotelm). The availability of oxygen plays a large role in the types of microbial decomposition that can occur within these different regions. In its absence, alternative electron acceptors, such as SO_4^{2-} , NO_3^-

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, and Fe(III), are used to break down complex molecules. However, because many peatlands are classified as ombrotrophic environments in which all nutrient inputs are dependent on precipitation, they are typically limited in nutrients and alternative electron acceptors. Microbial metabolism is further constrained by the low pH and the persistently low temperatures typical of peatlands. Due to the vastness of the carbon repository and its heterogeneity in plant species composition and soil chemistry, a variety of microbial metabolic guilds exist in peatlands.

Fungal decomposition is believed to thrive in the oxic acrotelm due to the ample availability of plant litter and plant-associated exudates. The hydrolytic exo-enzymes produced by fungi and certain types of bacteria break down complex carbons to produce metabolic intermediates that can be utilized by higher trophic levels (Berman et al., 2000). In the mesotelm, where oxygen availability is more limited, fermentation takes place and is important for its role in the mineralization of organic matter in peatlands. This represents a critical step in the decomposition of complex organic matter into intermediates that serve as methanogenic substrates (Drake et al., 2009). Another critical component of these two zones is the fact that they typically constitute the rooting zone of vascular plants in peatlands. This rooting zone can further shape the belowground microbial community by recruiting microbial guilds that form tight associations with the roots and surrounding rhizosphere.

The relationship between plants and their root-associated microbial partners is widely regarded as a mutualistic relationship that promotes plant health and microbial growth. Bacterial assemblages with plants are known to play a role in pathogen suppression and provide key nutrients to the plant roots (Berendsen et al., 2012). In turn, plants recruit host-specific root microbiomes through rhizodeposition of complex plant products, the production of oxygen through the roots in anoxic soils, and the recruitment of genotype-dependent endophytic

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assemblages (Bulgarelli et al., 2013). This relationship is important to peatland ecosystems considering the impact of harsh conditions of low pH, low nutrient availability, and generally low temperatures to both plants and microbes.

Below the water table depth, the anoxic catotelm stores ancient peat deposits in a completely water-logged environment (Blodau, 2002). Microbes active in this zone depend entirely on anaerobic metabolism to survive. A key component of this zone is the role of methanogenesis as one of the terminal steps of anaerobic decomposition. The production of methane, an extremely potent greenhouse gas, makes it an important component relevant to future climate change.

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Another important characteristic of peatlands is the composition of the surface into distinct hummock-hollow microtopography (Nungesser, 2003). This type of microtopography is formed through physiological features such as water content, differing rates of decomposition, nutrient availability, vascular and non-vascular plant species distribution and productivity. Hummocks represent elevated and more aerated mounds of vegetation, while hollows are much more waterlogged regions present at lower elevations (Nungesser, 2003). An important feature of peatland surfaces also includes the domination of Spruce trees (*Picea mariana*), Larch trees (*Larix laricina*), and a variety of shrubs in plant vascular vegetation. These features play a role in determining nutrient availability in the root microbiome through plant-specific rhizodeposition and the release of oxygen from roots.

Rising global temperatures and elevated levels of atmospheric carbon dioxide may cause peatlands to release this carbon repository to the atmosphere as either methane or carbon dioxide, which would further exacerbate the current greenhouse effects (Gorham, 1995). The ambivalence revolving around this ecosystem as either a carbon source or sink with the onset of

drastic climate changes may be resolved using a whole-ecosystem prediction experiment (Hanson et al., 2017).

The Spruce and Peatland Responses Under Changing Environments (SPRUCE) project, led by the Oak Ridge National Laboratory, represents an innovative approach to examine the effects of climate change on peatland ecosystems. The project site is located in the S1-Bog of the Marcell Experimental Forest (MEF) near Grand Rapids, Minnesota and hosts 12 m enclosures that artificially warm the entire ecosystem from the deep peat to surrounding air in increasing temperature increments. These enclosures also have the ability to raise the level of atmospheric carbon dioxide to 500 ppm above ambient concentrations, to further simulate the effects of climate change on the environment. The response of peatlands to these perturbations, and the effects on their substantial carbon repository, are largely orchestrated by the microorganisms present in the belowground environment.

Thorough characterization of the root-soil interface is important to climate change considerations because it is thought to house a hotspot of microbial activity belowground (Reinhold-Hurek et al., 2015). Plant roots exude photosynthetically fixed carbon substrates that stimulate the activity of surrounding microbes. These root-associated microbes are believed to drive an increased release of greenhouse gases through organic matter decomposition of both old and new peat carbon deposits (Fan et al., 2013). As plants experience warmer temperatures and increased availability of carbon dioxide, they are expected to increase production of these carbon exudates, potentially causing an elevated generation of greenhouse gasses in the peatland environment and fueling a process known as “priming” (see Literature Review) (Walker et al., 2016).

This study will examine the effects of simulated whole-ecosystem warming and elevated levels of carbon dioxide on the microbial communities within the root-soil microbiomes of vascular plants in peatlands. Root samples obtained from the SPRUCE site in Minnesota will be used to carefully characterize the microbial communities in the root tissue and the surrounding rhizosphere. The microbial communities can then be examined for any effects of whole ecosystem warming and the enrichment of carbon dioxide in the atmosphere, creating a glimpse into the future of peatlands and the associated effects of climate change drivers. This study marks an important step on a path to a greater understanding of peatland root microbiomes and climate driven effects on these microbial communities.

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Literature Review

Peatland ecosystems represent an at-risk environment to the main drivers of climate change, including increased temperatures and elevated levels of carbon dioxide in the atmosphere. Despite the major role of these ecosystems in the global carbon cycle, the predicted response of peatlands to future climate change is only just beginning to be understood. A key area of current research revolves around gaining a deeper understanding of the ecological role of plant microbial partners in peatland environments.

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One of the more recent foci of research in peatland ecosystems has been the characterization of microbial communities within plant microbiomes and their correlation with specific environmental features. Extensive research has gone into elucidation of the *Sphagnum* moss microbiome; a plant which is a major contributor to the development of peat in this ecosystem (Kostka et al., 2016). There exists a high degree of specificity among *Sphagnum*

species and their bacterial endophytic communities, which has been shown to be even stronger than geographic association (Opelt et al., 2007).

However, much remains to be explored and resolved on other types of microbial associations. Fungal assemblages have been shown to relate to vegetative succession and the chemical composition of organic litter (Artz et al., 2007; Trinder et al., 2008). In addition, fungal and bacterial community profiles consistently vary vertically with depth (Williams and Crawford, 1993; Morales et al., 2006; Lin et al., 2014a&b). However, Morales et al. (2006) found no significant correlations of bacterial communities with site chemistry, vegetation composition, or geographical characteristics in 24 peatland bog sites. In contrast, Yan et al. (2008) found that different vegetation types support functionally distinct rhizosphere communities based upon substrate-based respiration assays. This finding is likely due to a combination of effects including the density and distribution of plant roots, the chemical composition of specific plant species rhizoexudates, and interactions between the root and microbes as mutualistic partners (De Deyn et al., 2008). This project examines the effects of microtopography, microbiome compartment, and vascular plant type in relation to fungal and bacterial root microbiome assemblages.

Current research on peatlands has focused on future climate change predictions and the stability of the vast carbon storage within the ecosystem. However, the complex and multifaceted relationship between the production of greenhouse gasses (such as carbon dioxide and methane) by microbes and peatland warming remains understudied. This is especially true when applied to microbial assemblages and plant symbionts. Hines et al. (2008) observed a definitive increase in microbial methanogenesis with greater vascular plant coverage replacing *Sphagnum* moss, which is a common response of peatlands to warming and drying out of the

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bog. In order to rectify this knowledge gap, Oak Ridge National Laboratory has devised an innovative whole-ecosystem warming approach to model future climate change effects in peatlands.

The Spruce and Peatland Responses Under Changing Environments (SPRUCE) project, led by the Oak Ridge National Laboratory, leads the way in climate manipulation experiments (Hanson et al., 2017). The researchers have developed a whole-ecosystem warming experiment, including both deep-peat warming and atmospheric heating in order to simulate comprehensive temperature increases. This detailed design has provided many research groups the opportunity to devise projects that can examine the intricate effects of climate change on peatlands.

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Due to the great interest in the stability of C storage in peatlands, several groundbreaking studies at the SPRUCE site have undertaken addressing this question. Wilson et al. (2016) demonstrated that although deep-peat warming increases CH₄ production, it only affected the surface peat processes and not the stability of the deeper, anoxic layer of peat known as the catotelm. However, this assessment seems have been made before the effects of warming could be fully observed. After five years of warming and two years of elevated CO₂ treatments, Hopple et al. (2020) measured continued exponential release of methane from the surface peat as well as a positive response of CH₄ production in the deep peat soil layers. This means that more ancient carbon sources are now being degraded, creating a positive feedback response than can further exacerbate warming of the planet.

A potential explanation for the degradation of ancient peat sources during extended warming stems all the way from microbial root assemblages with the process of 'priming'. Priming refers to the degradation of previously stable organic matter following the introduction of labile C on account of the associated changes in microbial biomass, community structure, and

metabolic activity (Aertz, 2009). Recent experiments conducted by Oak Ridge National Laboratory have shown that this process may be occurring during prolonged warming and catalyzing a shift in the availability of certain nutrients as warming increases in these ecosystems. The presence of plant-available nutrients was shown to increase with warming (Iverson, unpublished data). As temperatures increase and atmospheric carbon dioxide climbs, plants increase their metabolic activity and release more photosynthetically fixed carbon, rhizoexudates, from their roots. Priming occurs as the rhizodeposition of these organic substances by roots increases the microbial activity in the rhizosphere through the primary use of newly available carbon sources, and the subsequent intensive microbial utilization of more recalcitrant nutrients from the soil organic matter (Kuzyakov, 2002). In peatland ecosystems, the recalcitrant organic matter is made up of ancient and vast peat deposits, putting the stability of these in question with increased warming. A thorough characterization of the root microbiomes associated with peatland plants may give insight into the potential effects on community structure and diversity that may indicate the occurrence of priming.

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This study will utilize the SPRUCE peatland site to obtain root samples from a range of artificially warmed ecosystems from +0°C to +9°C above ambient temperatures, as well as those subject to elevated levels of atmospheric CO₂. The bacterial and fungal communities will be examined for correlation with environmental parameters (microtopography type, root microbiome compartment, and plant type) as well as significant community structuring changes with warming and elevated carbon dioxide.

Methodology

The goal of this project is to characterize the bacterial and fungal microbial communities present in the roots of vascular plants in peatland bogs, an environment significant for its role in

the global carbon cycle. The methodology outlined below is based upon the protocol by Simmons et al., 2018.

Sample Collection

The sample roots in this experiment originated from the Spruce and Peatland Responses Under Changing Environments (SPRUCE) site managed by Oak Ridge National Laboratory in the S1 Bog of the Marcell Experimental Forest in Grand Rapids, Minnesota. The site contains whole-ecosystem warming chambers elevated to +0, +2.25, +4.5, +6.75, and +9 degrees Celsius in a regression-based design measured from the ambient atmospheric temperature. This site houses a second replication of all the heated chamber treatments with the addition of atmospheric CO₂ levels that have been elevated to 800-900ppm, approximately 500 ppm above ambient concentrations. Two plots consisting of only the structural base of the enclosures were built as negative controls to account for any chamber induced effects. In-growth cores consisting of autoclaved peat wrapped in plastic mesh netting were incubated in each of these enclosures for six months before retrieval. This setup was used to ensure that all of the fine roots contained within the cores were grown under the treatment conditions. The cores were extracted from the ground using aseptic technique in October 2017 and stored at -20°C at Oak Ridge National Laboratory. They were then transported from Oak Ridge National Lab to Georgia Institute of Technology on blue ice in July 2019 and stored at -20°C until processing.

Separation of the Root Endosphere and Soil Rhizosphere Each in-growth core was thawed overnight in a 4°C cold room and homogenized in a sterile plastic bag when sufficiently defrosted. Individual roots greater than 0.5 cm in length were carefully picked out of the soil and sorted as either originating from a tree or shrub plant with the closely-associated soil still attached. All root sorting was performed at 4°C. The sorted roots were suspended in 20mL of

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filter-sterilized (0.2 µm pore size) Epiphyte Removal Buffer (6.75 g of KH₂PO₄, 8.75 g of K₂HPO₄, and 1 mL of Triton X-100, to 1 L of sterile water). The samples were agitated via sonication for 10 min and washed three times with sterile water to remove any surface soil. At the end of this process, the clean roots and the detached soil were processed separately as endosphere root tissue and rhizosphere soil, respectively. The cleaned roots were ground into powder using 3 washes of liquid nitrogen in a mortar and pestle to ensure cell lysis in the DNA extraction protocol. The rhizosphere suspensions were centrifuged at 8000 x g for 10 minutes at 4 °C to form a distinct peat pellet at the bottom of the tube. Any supernatant was discarded before storing both the rhizosphere soil and endosphere fractions at -80°C until the DNA extraction process.

DNA Extraction and PCR Amplification

Genomic DNA was extracted from 0.10 g-0.25 g of source material using a series of cell lysing and product cleaning steps documented in the Qiagen PowerSoil DNA Extraction kit. All of the rhizosphere samples used 0.25 g in the DNA extraction while the endospheric samples ranged from 0.10 g - 0.25 g depending on the amount of source material available. The concentration of extracted DNA was measured using the Qubit high-sensitivity benchtop fluorometer. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA region of the prokaryotic DNA for taxonomic classification using the primer set 806F and 515R (Caporaso et al., 2012). Two Peptide Nucleic Acid (PNA) clamps were used: the anti-mitochondrial PNA (mPNA) 5'-GGCAAGTGTCTTCGGA-3' and the anti-plastid PNA (pPNA) 5'-GGCTCAACCCTGGACAG-3' were ordered from PNA Bio. The lyophilized PNA was resuspended in sterile water to a concentration of 100 µM (Lundberg et al., 2013). Each DNA sample was PCR amplified in duplicate using 0.5µl of Bovine Serum Albumin (BSA;

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20mg/mL), 0.19µl of mitochondrial PNA (100µM), 0.19µl of chloroplast PNA (100µM), 5µl of F primer (1µM), 5µl of R primer (1µM), 2.5µl of 10X Buffer (Thermo Scientific DreamTaq Buffer), 2.5µl of dNTPs, 0.125µl of Taq (Thermo Scientific DreamTaq DNA Polymerase), 3.995µl of molecular grade water, and 5µl of sample genomic DNA per PCR sample reaction.

The thermocycler conditions for the 16S rRNA gene primer reactions were 98°C for 180s, followed by 30 cycles of 98°C for 45s (denaturing), 78°C for 10s (PNA annealing), 50°C for 60s (primer annealing), and 72°C for 90s (extension), then 10min at 72°C followed by a hold at 4°C.

A separate PCR reaction was used to amplify the ITS2 region of the fungal DNA using the ITS86F and ITS4 primer set (Vancov & Keen, 2009). Each DNA sample was amplified in duplicate using 0.4µl of BSA (20mg/mL), 0.08µl of F primer (100µM), 0.08µl of R primer (100µM), 10µl of Qiagen Taq Hot Start Master Mix, 4.44µl of molecular grade water, and 5µl of sample genomic DNA per PCR sample reaction. Thermocycler conditions were 95°C for 5 min, 30 cycles of 94°C for 45s (denaturing), 55°C for 45s (primer annealing), and 72°C for 120s (extension), then 7 min at 72°C followed by a hold at 4°C. The amplicons of each primer type were screened for size and contamination using agarose gel electrophoresis.

Illumina Sequencing and Data Analysis

The amplified DNA fragments, or amplicons, were delivered to the Sequencing Core at Georgia Institute of Technology to be further purified, pooled in equimolar concentrations, and sequenced on an Illumina MiSeq Platform. The sequencing results included in this analysis are from various rhizosphere and endosphere samples taken from the ambient negative control plots with and without PNA clamps to ensure their efficacy. Specifically, these include 15 samples (made up of 6 root endosphere samples, 7 rhizosphere soil samples, a negative control for DNA extraction kit contamination, and a sample of autoclaved peat) both with and without PNA

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clamps. The sequencing run was repeated due to low quality results in the first run. As a result, the data presented are the combined duplicates of two MiSeq runs, giving a total of 60 samples in the final analysis.

All subsequent work proceeded in the bioinformatic analysis of the sequencing results from these two runs using Rstudio. During the analysis, low quality DNA reads were removed and taxonomy was assigned to visualize the bacterial and fungal community compositions within the different treatment plots. These processes were performed using the DADA2 package and standard pipeline (Callahan et al., 2016) in Rstudio. In the first run, F reads were truncated at 150bp and R reads at 120bp, while the higher quality results of the second run were truncated at 140bp and 160bp. The filterandTrim function of DADA2 was used to ensure reads were truncated and of sufficient quality. For each run, a unique error model was constructed in DADA2. The derepFatsq function was used to dereplicate the reads and used to infer the sequence variants in each sample. Forward and reverse reads were paired using the mergePairs function. Sequences were filtered for length within the expected length of 251-255bp. Finally, chimeras were removed using removeBimeraDenovo function. Taxonomy was assigned to the reads within mothur using the Silva SSU Ref v132 (Quast et al., 2013; Yilmaz et al., 2014). The phangorn package was used to create the phylogenetic tree. The resulting data was merged into a phyloseq object for further downstream analysis using the phyloseq package (McMurdie et al., 2013). Any reads outside of the bacterial domain were removed from further analysis. The phyloseq object was further processed using the decontam program to remove sequences that were significantly associated with the negative controls of autoclaved peat and DNA extraction kit contamination. Any remaining mitochondrial and chloroplast reads were removed bioinformatically and the read abundances were normalized using the cumulative sum scaling

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(CSS) normalization available in the metagenomeSeq Bioconductor package (Paulson et al., 2013).

Using different metrics of diversity, I examined how several environmental features affected the microbial community composition of peatland root microbiomes. The primary environmental factors examined in this analysis are the rhizosphere soil versus the endosphere root tissue, hummock versus hollow microtopography, and association with a tree or shrub type root. In this project, I used the Shannon Diversity test to examine whether alpha diversity varied with these factors. I also employed a weighted UNI-FRAC metric to determine the beta diversity of the sampled root microbiomes. I also used a Kruskal-Wallis Abundance Test to determine which specific microbial taxonomic groups were statistically different between environmental factors.

Statistical Analysis

The Mantel permutation test was used to determine whether the association between an environmental parameter and community composition can be statistically correlated using two distance matrices. I used a Bray-Curtis dissimilarity test to create a distance matrix for the taxa abundance among samples. Euclidean distances were used to create unique distance matrices for the delegation as either a hummock or hollow, rhizosphere or endosphere, and root species type. The Mantel test used the abundance distance matrix and each environmental parameter distance matrix to determine any statistical correlation with $p < 0.05$ performed using the vegan package in R.

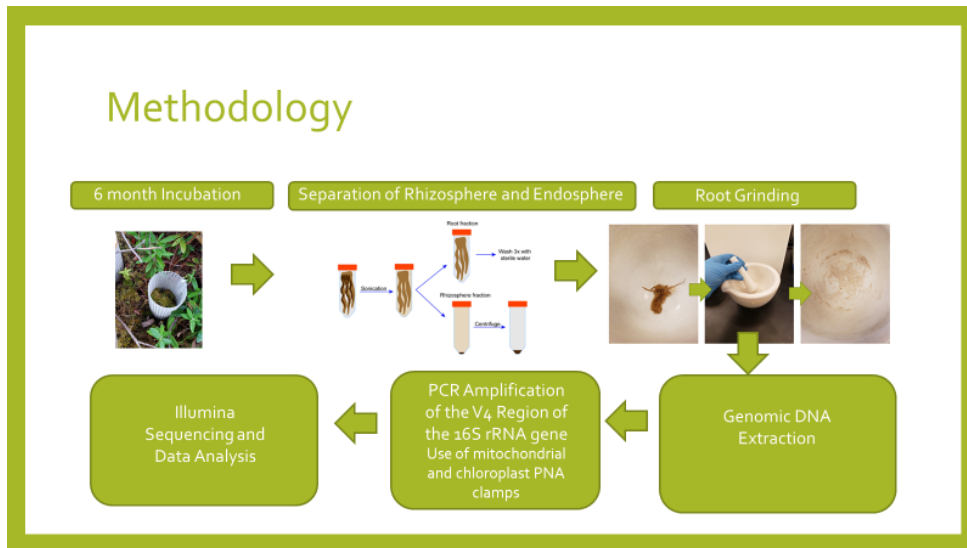


Figure 1: An illustration of the methodology employed in this project to characterize the root microbial communities of peatland plants.

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Results

The goal of this project was to fully characterize the microbial community of the root endosphere and rhizosphere in peatland plants. This begins with implementing a successful protocol to separate the root tissue (endosphere) from the surrounding soil (rhizosphere). The methodology must also isolate bacterial genomic DNA as well as fungal genomic DNA and successfully amplify the ITS2 and 16S V4 regions as amplicons in the polymerase chain reaction (PCR). In the 16S rRNA gene amplifications, the use of PNA clamps must be verified in order to prevent the amplification and subsequent sequencing of mitochondrial and chloroplast genes in the root tissue. After the methodology is confirmed to complete the intended purpose of successfully characterizing a microbial community using a small subset of samples, the same

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methodology will be applied to all samples. At this point, the only available results are those of the methodology optimization using samples that were not subject to the warming and elevated temperature treatments. The entire set of samples will undergo the same process and will feature the same data analysis protocol.

Successful Application of Peptide Nucleic Acid (PNA) Clamps

The PNA clamps were implemented into the PCR protocol during the 16S rRNA gene amplification to prevent the overwhelming amplification of mitochondrial and chloroplast amplicons that are abundant in the plant root tissue from ancient endosymbiosis. In Figure 2 below, the abundance of these genes is illustrated in the root and soil samples with and without PNA clamps. The success of the PNA clamps is predicated as a reduction in the abundance of mitochondrial and chloroplast genes in the samples with added PNA. A dramatic reduction in the abundance of chloroplast genes was observed with the use of PNA clamps in the endosphere samples (Figure 2). The abundance of chloroplast reads within the samples ranged from 0 % to 55 %, while mitochondrial reads were more restricted within the range of 0 % to 22 %. Within the soil samples, no significant differences were found between samples with and without PNA clamps, indicating that few mitochondrial and chloroplast genes were present.

Taxonomic Diversity of the Root Microbiome

An important step in characterizing a microbial community using 16S rRNA genes is determining whether there is a significant difference in the species diversity within each habitat. In microbial ecology, communities can be compared using a taxonomic bar plot that showcases the relative abundance of each taxonomic group between samples. In this study, the taxonomic groups of microorganisms were shown to be remarkably similar between the root endosphere and the soil rhizosphere at first glance (Figure 3). In order to determine whether these root

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compartments house different microbial communities, a clustering of samples by beta diversity is needed and a Mantel test will determine the statistical significance of the environmental parameters.

Beta Diversity of the Root Microbiome

In order to compare the diversity of microbial species between each habitat, metrics of beta diversity are often employed to illustrate how similar or different certain microbial communities are. Using a weighted UNI-FRAC distance method, the beta diversity of the different microbial communities were displayed in an PCoA plot (Figure 4). In this type of plot, the closer two microbial communities are to each other, the more similar in species composition they are. In this project, we are hoping to define what environmental parameters influence the composition of these communities and what taxonomic groups are responsible for their differentiation. Microbial communities clustered by their presence in either the root endosphere or the soil rhizosphere (49% Axis 1). Clustering was also observed according to peatland microtopography (23.3% Axis 2), by hummock and hollow environmental features. However, clustering along the PCoA axis does not necessarily indicate that the effect of these features is statistically significant.

The Mantel permutation test is used to determine whether the association between an environmental parameter and the community composition can be statistically correlated. The correlation between the environmental parameter, hummock or hollow, and the taxonomic abundance is not strongly related, although remains statistically significant (Mantel statistic R: 0.3628, p-value: 1e-04). The species Bray-Curtis dissimilarity matrix and the root species (Tree/shrub) designation produces a weaker association (Mantel statistic R: 0.1314, p-value:

0.0099). The strongest environmental parameter association with the species abundance is with the rhizosphere and endosphere compartment (Mantel statistic R: 0.645, p-value: 1e-04).

Variation of Specific Microbial Taxonomic Groups with Environmental Features

The significance of different microbial groups between the environmental parameters of the 16S rRNA gene dataset was determined using a Kruskal-Wallis Abundance Test. The top 50 amplicon sequence variants (ASVs) with $p < 0.05$ varying between microbiome compartment, microtopography, and root species type are displayed in Figures 5-10 by the Phylum and Family taxonomic levels. The most statistically significant differentially abundant ASVs between the endosphere and rhizosphere originate from a total of seven phyla. Acidobacteria, Bacteroidetes, Planctomycetes, and Verrucomicrobia have a greater representation within the rhizosphere than the endospheric root tissue (Figure 5). On the other hand, Proteobacteria represents between 20% and 50% of the relative abundance within the endosphere compared to less than 20% of any soil samples. The distinction between the hummock and hollow microbiomes looks to stem from the complete presence or absence of specific microbial taxa, rather than a differential abundance (Figure 7). Several phyla present in the hummock samples and absent from hollows includes Actinobacteria, Dependentiae, Planctomycetes, Bacteroidetes, and Verrucomicrobia.

Figures:

Commented [CP26]: Because these R values are so low, I would say that these are weak associations, although they are statistically significant. The strongest effect is due to the compartment (root vs. endosphere).

Commented [CP27]: Your figures look good, but I would include some sentences in the text about which groups jump out at you as being significantly different. You don't have to go over all of them, just a subset, particularly those that have higher relative abundances. For instance, in Figure 6, the Burkholderiaceae have relative abundances of about 15-20% in the roots, but only 5% in the rhizosphere! That's a big difference.

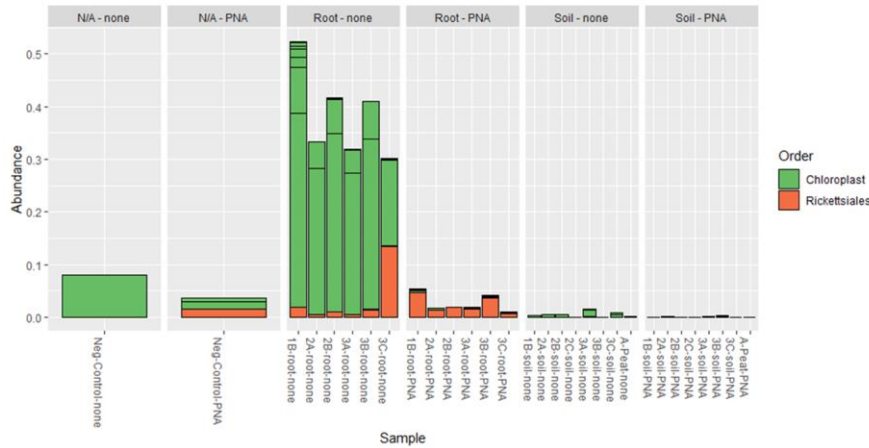


Figure 2: Abundance plot of the presence of chloroplast and mitochondrial genes in the sequenced samples with and without peptide nucleic acid (PNA) clamps

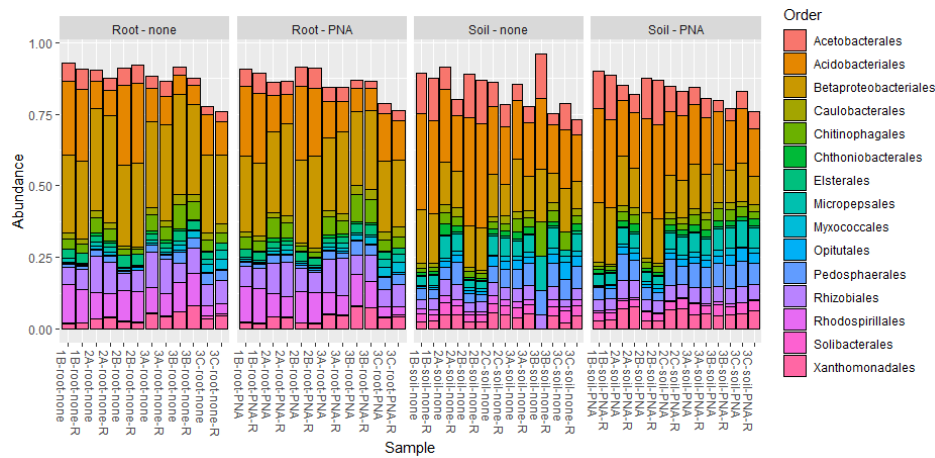


Figure 3: Histogram depicting the relative abundance of microbial communities associated with the root/soil microbiome at the order level including the presence/absence of Peptide Nucleic Acids clamps (PNAs).

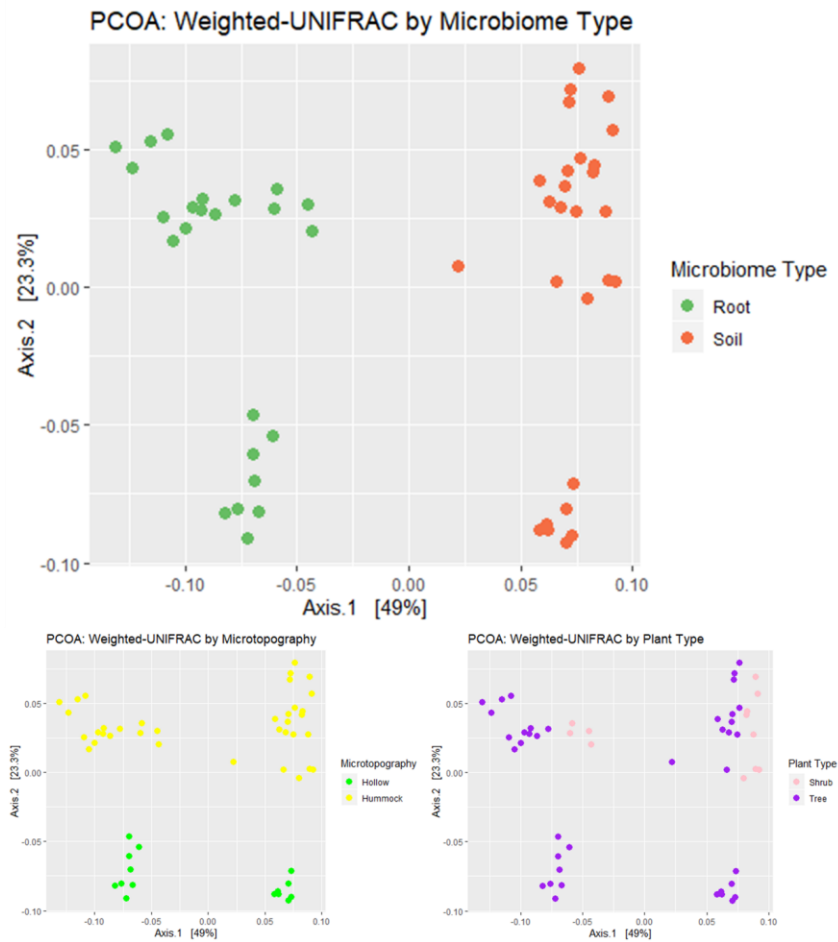


Figure 4: Beta-diversity plots (PCoA) showing the clustering of microbial communities. The uppermost plot shows differences in community composition between the endosphere and rhizosphere compartments. The lower plots show differences between hummocks and hollows (left) as well as shrubs and trees (right).

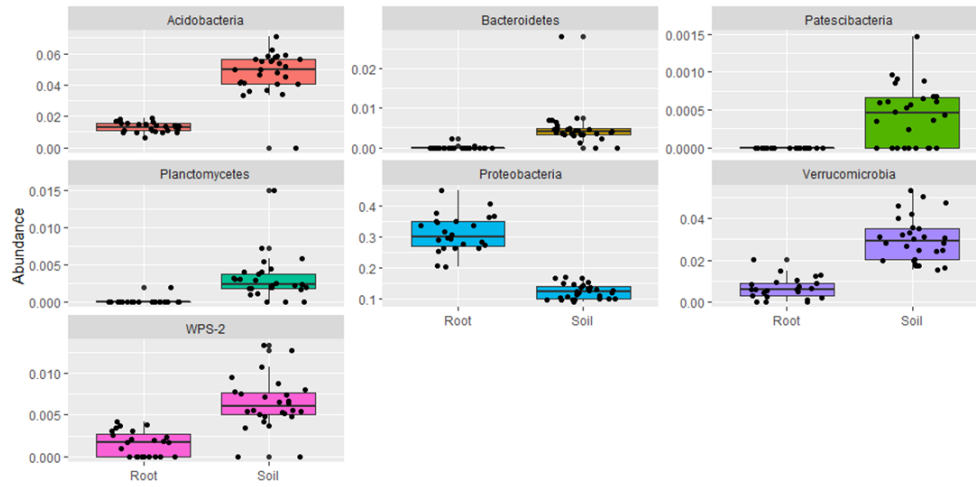


Figure 5: Box plots illustrating the seven phyla of the top 50 ASVs that are significantly different between the rhizosphere and endosphere samples in a Kruskal-Wallis abundance test ($p < 0.05$).

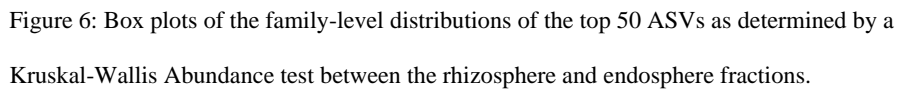


Figure 7: Box plots of the Phylum level distributions of the top 50 ASVs that significantly differ between the hummock and hollow microtopographies.

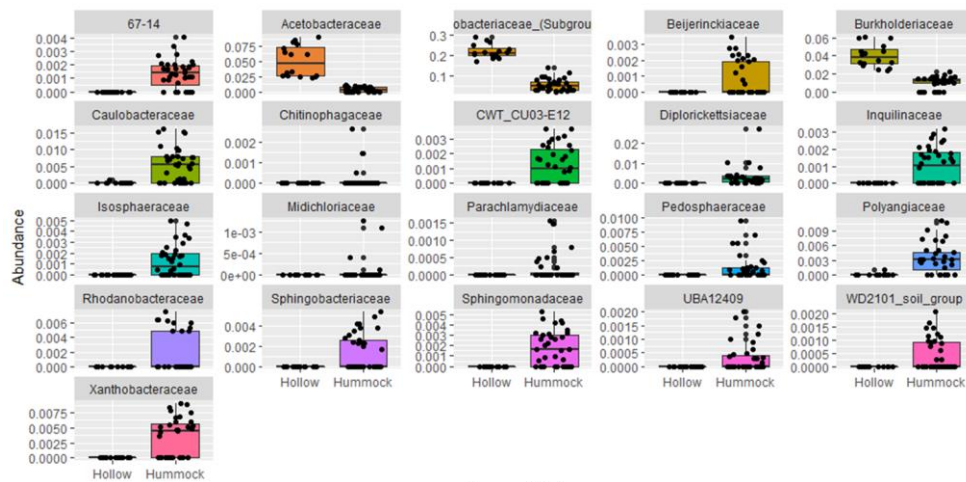


Figure 8: Box plots of the taxonomic families that differ between the hummock and hollow samples within the top 50 ASVs determined to be significantly different by a Kruskal-Wallis Abundance Test.

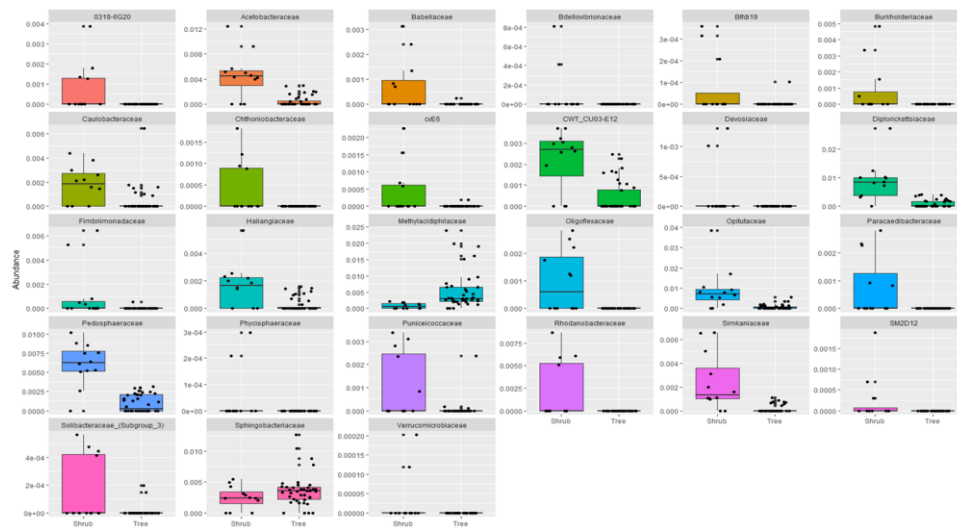


Figure 9: Boxplot showcasing the taxonomic phylum of the top 50 ASVs significant different between the root types (tree versus shrub).

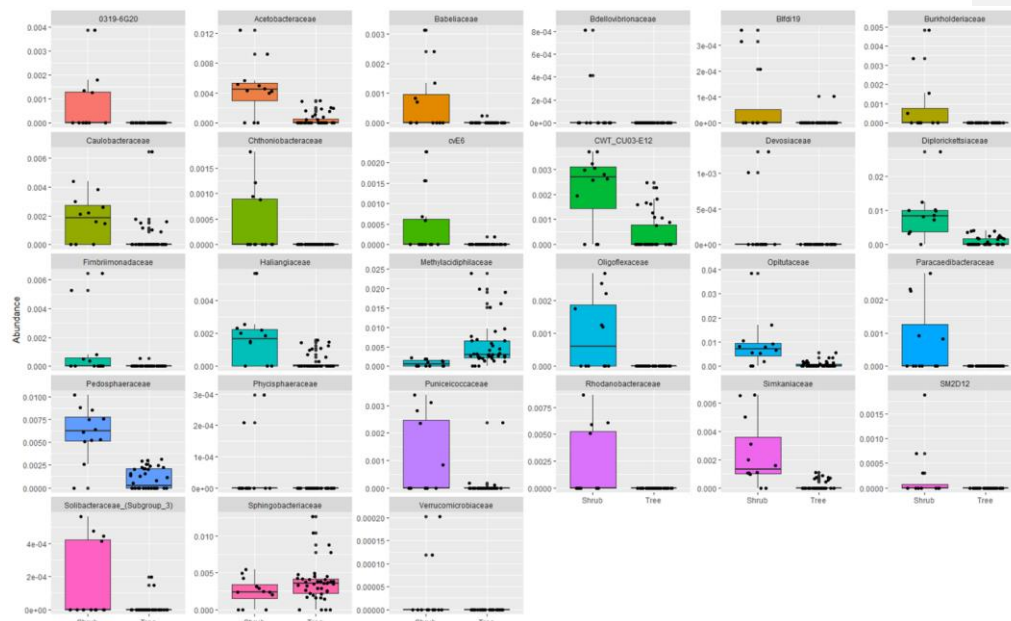


Figure 10: Boxplots of the most significant families in the top 50 ASVs that differ between the association with tree or shrub roots.

Conclusions

The ultimate objective of this project is to fully characterize the microbial community of the root endosphere and rhizosphere in peatland plants. To work towards this objective, I have developed and successfully implemented a protocol to sort plant roots from ingrowth cores, separate roots from the surrounding rhizosphere, and amplify extracted DNA that is of sufficient quality for next-generation sequencing (NGS). The subsequent analysis of the sequenced 16S rRNA gene amplicons for a limited set of samples demonstrates that the methodology is ready to be applied to the entire dataset. The addition of PNA clamps prior to PCR amplification dramatically reduced the amount of mitochondrial and chloroplast genes present in the amplicon dataset (Figure 2). The rhizosphere and endosphere samples differed significantly, indicating that the different compartments are colonized by distinct microbial communities (Figure 3). The communities were also affected by the peatland microtopography, as seen by differences in community composition between the hummocks and hollows.

Comparisons of beta diversity revealed that the composition of root microbial communities is strongly shaped by specific environmental factors. The distinction of a tree or a shrub root seems to make a less important contribution to the community structuring indicating that the different types of plants present within the peatland harbor similar microbial assemblages.

The increased abundance of certain microbial taxonomic groups potentially reflects the changing presence of functional abilities between environmental features. Comparisons of the

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root microbiome compartments reveal a greater abundance of taxa in the Verrucomicrobia, Acidobacteria, and Planctomycetes phyla within the rhizosphere when compared to the endosphere. Acidobacteria represents a common phylum within peatland soils, but is commonly noted for its exclusion from the root endosphere and its difficulty to isolate using culture dependent studies and, as a result has a lesser understood metabolism (Bulgarelli, 2013).

Members of the phylum Planctomycetes are common inhabitants of boreal peatlands and have been found to have hydrolytic capabilities via metatranscriptomics, suggesting an important role in degrading plant-derived carbon including those originating from rhizodeposition (Dedysh and Ivanova, 2019). Certain members of the phyla Verrucomicrobia have been found to be capable of performing methanotrophy in extremely acidic environments (Dunfield et al., 2007). The endosphere compartment was dominated by members of the Proteobacteria, an incredibly diverse phylum commonly found in soil environments that encompasses a diverse range of metabolisms (Kersters et al., 2006).

The microtopography of peatlands also plays a role in shaping the makeup of the root microbiome. Hummocks typically represent a more aerobic environment with a greater abundance of vascular plants compared to peat moss. A greater presence of Actinobacteria in hummocks could signify more hydrolytic enzymes with the increased vascular vegetation (Dedysh et al., 2006; and Rheims and Stackebrandt, 1999). However, vegetation species type seems to play a smaller role in shaping the root microbiome. None of the phyla mentioned above varied between shrub and tree roots. The phyla that did vary were generally composed of lesser abundant taxa, suggesting that the species-specific characteristics of rhizodeposition of complex plant polymers may play a relatively smaller role in shaping the root microbiome in peatlands. The microbial species diversity and potential functional capabilities of the root microbiome in

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peatlands is indicative of the potential effects of priming in these environments and is critical to understanding the stability of the vast carbon repository that defines peatland ecosystems.

Future Directions

The future of this project is predicated on the completion of the entire methodology outlined above and the production of the same type of results using the entire sample set. This translates to the sequencing of 16S rRNA genes for microbial communities from the root and soil samples subjected to artificial warming and elevated levels of carbon dioxide. This project is also looking to characterize the entire fungal community by using the ITS2 primer set in addition to the bacterial community. This primer set has already been tested on DNA extracts from root and soil samples, demonstrating successful extraction and amplification of fungal DNA using the described methods. Both of these sequencing datasets will give detailed information on the taxonomic classification of the fungal and bacterial communities, but limited insight into the function of these microbes. The inclusion of RT-qPCR of genes involved in methane cycling, such as the *mcrA* gene, would yield quantitative results on the subset of the microbial community that is capable of producing methane (Hallam et al., 2003). This information could be utilized to make inferences about the presence of methanogenic microbes in peatland root microbiomes under the influence of climate change drivers.

Works Cited

- Artz, R. R. (2009). Microbial community structure and carbon substrate use in northern peatlands. *Carbon Cycling in Northern Peatlands*, 111-129
- Artz, R. R. E., I. C. Anderson, S. J. Chapman, A. Hagn, M. Schlöter, J. M. Potts, and C. D. Campbell (2007). Fungal diversity and community composition change in response to vegetational succession during natural regeneration of cut-over peatlands, *Microb. Ecol.*, 54, 508–522
- Beilman, D. W., D. H. Vitt, J. S. Bhatti, and S. Forest (2008), Peat carbon stocks in the southern Mackenzie River Basin, uncertainties revealed in a high-resolution case study, *Global Change Biol.*, 14, 1221–1232
- Berendsen, R. L., Pieterse, C. M., & Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends in plant science*, 17(8), 478-486
- Bergman, I., P. Lundberg, C. M. Preston, and M. Nilsson (2000), Degradation of C-13-U-glucose in *Sphagnum majus* litter: Responses to redox, pH, and temperature, *Soil Sci. Soc. Am. J.*, 64, 1368–1381
- Blodau, C. (2002), Carbon cycling in peatlands—A review of processes and controls, *Environ. Rev.*, 10, 111–134.
- Bulgarelli, D., Schlaeppi, K., Spaepen, S., Van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annual review of plant biology*, 64, 807-838.
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nature methods*, 13(7), 581.
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., ... & Gormley, N. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*, 6(8), 1621-1624.
- De Deyn, G. B., J. H. C. Cornelissen, and R. D. Bardgett (2008), Plant functional traits and soil carbon sequestration in contrasting biomes, *Ecol. Lett.*, 11, 516–531
- Dedysh, S. N., & Ivanova, A. A. (2019). Planctomycetes in boreal and subarctic wetlands: diversity patterns and potential ecological functions. *FEMS microbiology ecology*, 95(2).

Drake, H. L., Horn, M. A., & Wüst, P. K. (2009). Intermediary ecosystem metabolism as a main driver of methanogenesis in acidic wetland soil. *Environmental Microbiology Reports*, 1(5), 307-318.

Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B., Hou, S., ... & Wang, J. (2007). Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. *Nature*, 450(7171), 879-882.

Fan, Z., Jastrow, J. D., Liang, C., Matamala, R., & Miller, R. M. (2013). Priming effects in boreal black spruce forest soils: quantitative evaluation and sensitivity analysis. *PloS one*, 8(10), e77880.

Gorham, E. (1995), The biogeochemistry of northern peatlands and its possible responses to global warming, *Biotic Feedbacks in the Global Climatic System*, edited by G. M. Woodwell, and F. T. Mackenzie, pp. 169–187, Oxford Univ. Press, New York.

Hallam, S. J., Girguis, P. R., Preston, C. M., Richardson, P. M., & DeLong, E. F. (2003). Identification of methyl coenzyme M reductase A (mcrA) genes associated with methane-oxidizing archaea. *Appl. Environ. Microbiol.*, 69(9), 5483-5491.

Hanson, P. J., Riggs, J. S., Nettles IV, W. R., Phillips, J. R., Krassovski, M. B., Hook, L. A., ... & Warren, J. M. (2017). Attaining whole-ecosystem warming using air and deep-soil heating methods with an elevated CO₂ atmosphere. *Biogeosciences (Online)*, 14(4).

Hines, M. E., Duddleston, K. N., Rooney-Varga, J. N., Fields, D., & Chanton, J. P. (2008). Uncoupling of acetate degradation from methane formation in Alaskan wetlands: connections to vegetation distribution. *Global Biogeochemical Cycles*, 22(2).

Hopple, A. M., Wilson, R. M., Kolton, M., Zalman, C. A., Chanton, J. P., Kostka, J., Hanson P. J., Keller, J. K., & Bridgman, S. D. (2020) Massive peatland carbon banks vulnerable to rising temperatures. Unpublished Manuscript

Kerstens, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., & Stackebrandt, E. R. K. O. (2006). Introduction to the Proteobacteria. *The prokaryotes*, 5(3), 3-37.

Koelbener, A., Ström, L., Edwards, P. J., & Venterink, H. O. (2010). Plant species from mesotrophic wetlands cause relatively high methane emissions from peat soil. *Plant and Soil*, 326(1-2), 147-158.

Kolton, M., Marks, A., Wilson, R., Chanton, J. P., & Kostka, J. E. (2019). Impact of warming on greenhouse gas production and microbial diversity in anoxic peat from a Sphagnum-dominated bog (Grand Rapids, Minnesota, USA): A laboratory incubation study. *Frontiers in microbiology*, 10, 870.

- Kostka, J. E., Weston, D. J., Glass, J. B., Lilleskov, E. A., Shaw, A. J., & Turetsky, M. R. (2016). The Sphagnum microbiome: new insights from an ancient plant lineage. *New Phytologist*, 211(1), 57-64.
- Kuzyakov, Y. (2002). Factors affecting rhizosphere priming effects. *Journal of Plant Nutrition and Soil Science*, 165(4), 382-396.
- Lin, X., Tfaily, M. M., Steinweg, J. M., Chanton, P., Esson, K., Yang, Z. K., ... & Kostka, J. E. (2014a). Microbial community stratification linked to utilization of carbohydrates and phosphorus limitation in a boreal peatland at Marcell Experimental Forest, Minnesota, USA. *Appl. Environ. Microbiol.*, 80(11), 3518-3530.
- Lin, X., Tfaily, M. M., Green, S. J., Steinweg, J. M., Chanton, P., Invittaya, A., ... & Kostka, J. E. (2014b). Microbial metabolic potential for carbon degradation and nutrient (nitrogen and phosphorus) acquisition in an ombrotrophic peatland. *Appl. Environ. Microbiol.*, 80(11), 3531-3540.
- Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., & Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. *Nature methods*, 10(10), 999.
- Maltby, E., and P. Immirzi (1993), Carbon dynamics in peatlands and other wetland soils, regional and global perspectives, *Chemosphere*, 27, 999–1023.
- McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one*, 8(4).
- Moore, T. R., & Dalva, M. (1993). The influence of temperature and water table position on carbon dioxide and methane emissions from laboratory columns of peatland soils. *Journal of Soil Science*, 44(4), 651-664.
- Morales, S. E., P. J. Mouser, N. Ward, S. P. Hudman, N. J. Gotelli, D. S. Ross, and T. A. Lewis (2006), Comparison of bacterial communities in New England Sphagnum bogs using terminal restriction fragment length polymorphism (T-RFLP), *Microb. Ecol.*, 52, 34–44
- Opelt, K., C. Berg, S. Schonmann, L. Eberl, and G. Berg (2007), High specificity but contrasting biodiversity of Sphagnum-associated bacterial and plant communities in bog ecosystems independent of the geographical region, *ISME J.*, 1, 502–516
- Paulson, J. N., Pop, M., & Bravo, H. C. (2013). metagenomeSeq: Statistical analysis for sparse high-throughput sequencing. *Bioconductor package*, 1(0), 191.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, 41(D1), D590-D596.

Reinhold-Hurek, B., B nger, W., Burbano, C. S., Sabale, M., & Hurek, T. (2015). Roots shaping their microbiome: global hotspots for microbial activity. *Annual review of phytopathology*, 53, 403-424.

Rheims, H., & Stackebrandt, E. (1999). Application of nested polymerase chain reaction for the detection of as yet uncultured organisms of the class Actinobacteria in environmental samples. *Environmental microbiology*, 1(2), 137-143.

Simmons, Tuesday, et al. "Exploring the root microbiome: extracting bacterial community data from the soil, rhizosphere, and root endosphere." *JoVE (Journal of Visualized Experiments)* 135 (2018): e57561.

Turunen, J. (2003), Past and present carbon accumulation in undisturbed boreal and subarctic mires, a review, *Suo*, 54, 15–28

Trinder, C. J., D. Johnson, and R. R. E. Artz (2008), Interactions among fungal community structure, litter decomposition and depth of water table in a cutover peatland, *FEMS Microbiol. Ecol.*, 64, 433–448

Vancov, T., & Keen, B. (2009). Amplification of soil fungal community DNA using the ITS86F and ITS4 primers. *FEMS microbiology letters*, 296(1), 91-96.

Walker, T. N., Garnett, M. H., Ward, S. E., Oakley, S., Bardgett, R. D., & Ostle, N. J. (2016). Vascular plants promote ancient peatland carbon loss with climate warming. *Global change biology*, 22(5), 1880-1889.

Williams, R. T., and R. L. Crawford (1993), Microbial diversity in Minnesota peatlands, *Microbiol. Ecol.*, 9, 201–214

Wilson, R. M., Hopple, A. M., Tfaily, M. M., Sebestyen, S. D., Schadt, C. W., Pfeifer-Meister, L., ... & Kolka, R. K. (2016). Stability of peatland carbon to rising temperatures. *Nature Communications*, 7(1), 1-10.

Yan, W., R. R. E. Artz, and D. Johnson (2008), Species-specific effects of plant colonising cut-over peatlands on patterns of carbon source utilisation by soil microorganisms, *Soil Biol. Biochem.*, 40, 544–549.

Yilmaz, P., Parfrey, L. W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., ... & Gl ckner, F. O. (2014). The SILVA and “all-species living tree project (LTP)” taxonomic frameworks. *Nucleic acids research*, 42(D1), D643-D648.