INTERACTIONS BETWEEN NITROGEN FIXATION AND METHANE CYCLING IN BOREAL PEAT BOGS

A Thesis
Presented to
The Academic Faculty

By

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In Partial Fulfillment
of the Requirements for the Degree
Master’s of Science in the
School of Earth and Atmospheric Sciences

Georgia Institute of Technology
May 2015

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INTERACTIONS BETWEEN NITROGEN FIXATION AND METHANE CYCLING IN BOREAL PEAT BOGS

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DEDICATION

To My Family
ACKNOWLEDGEMENTS

I would first and foremost like to thank my advisor Dr. Jennifer B. Glass for sharing with me her knowledge, time and advisement. She provided growth during my graduate studies while showing enthusiasm towards teaching others. I would also like to thank my committee members, Dr. Joel E. Kostka and Dr. Christopher W. Schadt for not only the insight they provided along the way, but also for the positive energy they displayed working in the field. I would also like to thank the rest of the members of the SPRUCE team for allowing me to take part in a project bigger than what I had imagined. All of this would not have been possible without them. I would like to give a huge thank you to Sarah Weber who shared her time and knowledge with me on the use of a gas chromatograph. I ran to her office many times for help and she was always there to lend a helping hand. I would also like to thank Dr. John C. Gaby for his insightful mentoring in the lab. I would like to thank undergrad Helena Ochoa for being my left hand during incubation setups. Her hard work and persistence will take her very far. I would also like to thank undergrad Damian Horton for assistance with incubations as well as always keeping a positive attitude.

I would like to thank my family for always reminding me what comes first in my life. I would also like to thank Joanne Easterling Hearn for the support and encouragement along the way. I would also like to thank Tyler Gunzenhauser and Buster for being by my side throughout my studies and providing friendship, love and coffee.
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<td>ANOVA</td>
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<td>ARA</td>
<td>Acetylene Reduction Assay</td>
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<td>BNF</td>
<td>Biological Nitrogen Fixation</td>
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<tr>
<td>CEC</td>
<td>Cation Exchange Capacity</td>
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<tr>
<td>nifH</td>
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SUMMARY

Microbial nitrogen (N$_2$) fixation supplies important nitrogen inputs to boreal peatlands, extremely oligotrophic ecosystems dominated by Sphagnum mosses. In this study, we coupled major and trace nutrient analyses and rate measurements to characterize interactions between N$_2$ fixation and CH$_4$ cycling at the S1 peat bog in Marcell Experimental Forest and the Zim bog (Minnesota, USA). Total dissolved inorganic nitrogen (NO$_3^-$+NO$_2^-$+NH$_4^+$) and phosphate were both consistently < 2 µM in the porewater of surface peat, indicating severe nutrient limitation. While dissolved Fe was fairly abundant (18-35 µM), Mo, V and Cu were scarce (2-40 nM), suggesting that alternative metalloenzymes containing Fe in place of other metals may be favored. Rates of diazotrophy measured by both $^{15}$N$_2$ incorporation and the acetylene (C$_2$H$_2$) reduction assay (ARA) were 7-fold higher under anoxic vs. oxic incubations conducted at both 4°C and 25°C. No significant difference in N$_2$ fixation rates measured by either method was observed with or without the amendment of 1% CH$_4$ at 25 °C; however, a significant inhibitory effect by methane was seen at 4°C in material from the S1 bog hollows. Anoxic $^{15}$N$_2$ incorporation was 3-4x higher in treatments lacking acetylene, suggesting that the ARA likely underestimates N$_2$ fixation by inhibiting diazotrophs sensitive to C$_2$H$_2$. Aerobic methanotrophy was also inhibited by 1% C$_2$H$_2$ when incubated under oxic conditions. No observations for the production of ethane (C$_2$H$_6$) were detected during the ARA, a biomarker for alternative nitrogenase activity. Major differences in ARA rates were observed to vary locally within microhabitats and between two bogs. In June 2014, peat sampled from hollows incubated under anoxic conditions showed the highest ARA rates (94.9 ± 11.0 nmol C$_2$H$_4$ g$^{-1}$ moss dry mass hr$^{-1}$), while the lowest rates were observed in
hummock samples incubated under oxic conditions (5.1 ± 0.8 nmol C$_2$H$_4$ g$^{-1}$ moss dry mass hr$^{-1}$) in the S1 bog (T3 site). Observed rates have the potential to be a function of oxygen concentrations and or water content. ARA rates in all microcosm treatments were significantly lower at Zim bog compared to the S1 bog. The developed conversion factor between the regression of $^{15}$N$_2$ and ARA in this study was 3.9 and agrees with the theoretical conversion factor as well as previous studies of soils and forest mosses.
CHAPTER 1: INTRODUCTION

1.1 Motivation

Northern peatlands cover only 3% of the Earth’s surface but are estimated to store a third of the organic soil carbon (Gorham 1991). These regions act as important carbon (C) sinks; however, perturbations such as increasing global temperature can shift these regions to a major atmospheric C-source (Dorrepaal et al 2009, Gorham 1991). Higher rates of primary production compared to decomposition in peatlands have contributed to current global climate by storage of CO$_2$ from the atmosphere (Frolking and Roulet 2007, Gorham 1991). Currently, wetlands emit approximately 196 Tg yr$^{-1}$ of methane (CH$_4$) with 46 Tg CH$_4$-C yr$^{-1}$ originating from peatlands, a greenhouse gas with a global warming potential approximately 30 times that of CO$_2$ (Pachauri et al 2014).

Although peatlands contribute approximately a quarter of global wetlands CH$_4$ emissions, they have the ability to capture a portion of this CH$_4$ before it is released to the atmosphere (Kirschke et al 2013, Raghoebarsing et al 2005). With northern latitudes predicted to experience the greatest temperature impacts under global climate change scenarios, the question arises as to whether peatlands will become a net source of C to the atmosphere or if they will have the ability to self regulate and continue to act as a sink (Dise 2009, Stocker et al 2013). Analysis of peatland geochemistry coupled to microbial activity will be important to determine environmental factors driving community structure and subsequent carbon storage capacity.

In this thesis, the remainder of Chapter 1 will discuss the importance of Sphagnum moss to C sequestration in peatlands, and the microbial processes of C and nitrogen (N)
cycling that regulate biogeochemical cycling in these regions. Macro- and micro-nutrient availability in these regions will also be discussed, and finally, methods to measure nitrogen fixation will be described. Chapter 2 will discuss the methodology and statistical analysis utilized in this thesis. Chapter 3 will discuss the results of the work and Chapter 4 will be a discussion of the results, concluding with the main findings and future studies.

1.2 Influence of *Sphagnum* on Carbon Storage

Peatlands are dominated by species of *Sphagnum* mosses, non-vascular plants, estimated to contribute approximately 15-20% of the total organic matter storage in peatlands (Clymo and Hayward 1982, Rydin and Jeglum 2006). *Sphagnum* spp. create a specialized niche by expelling protons in exchange for cations and exuding phenolic compounds, therefore acidifying the environment due to their high cation exchange capacity (CEC; Clymo 1963). Phenolic acids alter the biogeochemistry of peatlands due to their capacity to bind important nutrients and metals, while acidifying porewater and consequently repressing heterotrophic respiration (Clymo 1963, Rydin et al 2006, Rydin and Jeglum 2006). The specialized niche then allows peatlands to become a carbon sink by decreasing organic matter decomposition (Clymo and Hayward 1982, Gorham 1991).

Recently, increased attention has focused on the processes that mediate C and N cycles in *Sphagnum* spp. Methanotrophic bacteria that oxidize methane associate closely with the tissue of the moss host, specifically dead hyaline cells (Larmola et al 2010, Raghoebarsing et al 2005b). Similarly, diazotrophic bacteria that conduct biological nitrogen fixation (BNF) were also found to occur in close association with the host *Sphagnum* in classic studies of the subarctic Storladen mire peatlands in Sweden (Basilier et al 1978,
Granhall and Selander 1973, Rosswall and Granhall 1980). Recently, more evidence has emerged for this close association (Berg et al 2013, Bragina et al 2011). Alphaproteobacterial isolates from peatlands have been shown to oxidize methane and fix nitrogen simultaneously (Dedysh 2011). Furthermore, members of the class Methanomicrobia were shown to couple methanogenesis, the microbial production of methane, to diazotrophy (Deppenmeier et al 2002, Galagan et al 2002).

Previous studies of ombrotrophic peatlands, those that receive nutrients from atmospheric deposition, reported that BNF likely contributes a large portion of peatland nitrogen accumulation (Vile et al 2014, Weston et al 2014). Based on primer-based molecular studies in peatlands, active diazotrophy has also been attributed to organisms that are methanotrophic (Bragina et al 2013, Liebner and Svenning 2013b, Tveit et al 2014, Vile et al 2014). For example, cultured isolates from the families of Beijerinckiaceae and Methylocystaceae have been found to fix N\textsubscript{2} as well as oxidize CH\textsubscript{4} (Dedysh 2011). Aerobic methanotrophy, with a potential role for anaerobic methanotrophy, allows peatlands to act as a methane filter (Smemo and Yavitt 2007, Smemo and Yavitt 2011), while nitrogen limitation induces diazotrophy, thereby linking the carbon and nitrogen cycles.

1.3 Carbon and Nitrogen Cycling

Methanogenesis occurs at depths below the water table in the catotelm, the dead organic layer of peat, via acetoclastic and hydrogenotrophic pathways (Galand et al 2002, Lin et al 2014a). Boreal peatlands are predominated by acetoclastic methanogens of the Methanosarcinales (Cadillo-Quiroz et al 2006, Galand et al 2005, Galand et al 2003, Kotsyurbenko et al 2004, Lin et al 2014b), while hydrogenotrophic methanogens of the Methanobacteriales, Methanomicrobiales, and Methanosarcinales were abundant at depth in
fens (Tveit et al 2012). In ombrotrophic peatlands, *Methanocella* and *Methanoplanus* were the dominant hydrogenotrophic methanogen genera with a smaller portion of acetoclastic *Methanoseta* and *Methanosarcina* where the percentage of total Euryarchaeota decreased with depth from ~20% to ~10% (Lin et al 2014a). Methane produced within the catotelm then diffuse upward to the active peat layer, the acrotelm, where a portion may be oxidized to CO₂ by aerobic methanotrophs. This has been coined as “the methane filter” (Matthews and Fung 1987).

Methanotrophs use CH₄ as an energy and/or carbon source and are found in two forms: Type I (Gammaproteobacteria) and Type II (Alphaproteobacteria). The distinction between Type I and Type II is found in the C-assimilation pathway. Type I uses the ribulose monophosphate pathway while the Type II uses the serine cycle (Auman et al 2001, Le Mer and Roger 2001). Another distinction found in methanotrophs is their affinity for methane. Methanotrophs classified as “high affinity oxidizers” prefer to take up methane at concentrations <12 ppm, concentrations of which are closer to atmospheric conditions. Methanotrophs classified as “low affinity oxidizers” consume methane more efficiently at higher methane concentrations (>40 ppm; (Le Mer and Roger 2001). The most prevalent aerobic methanotrophs in peat bogs generally belong to the Type II group predominated by Alphaproteobacteria while Gammaproteobacteria in the Type I group are also important (Chen et al 2008a, Chen et al 2008b, Dedysh 2009, Kip et al 2011a, Kip et al 2011b, Le Mer and Roger 2001, Tveit et al 2012).

The initial step in methane oxidation is mediated by two forms of enzymes. The first is the copper-containing particulate methane monooxygenase (pMMO) that was previously thought to be found in all methanotrophs (Crombie and Murrell 2014, Kip et al 2011b,
Semrau et al 2010). More recently, a second iron-containing soluble methane monooxygenase (sMMO) was found in the periplasm of some methanotrophs, which may be favored in copper-limited environments (Semrau et al 2010). However, the contribution of pMMO vs. sMMO pathways to methane oxidation is unknown in peatlands (Crombie and Murrell 2014, Liebner and Svenning 2013b). Facultative methanotrophs such as Methyloccella spp. demonstrate the ability to oxidize other carbon sources such as acetate, pyruvate, succinate, malate and ethanol while pMMO-containing members of the Methylocapsa and Methylocystis genera (both of which are diazo- and methano-trophs) have been shown to oxidize only acetate and ethanol (Crombie and Murrell 2014, Dedysh 2011, Liebner and Svenning 2013a). Oxidized methane may serve as a carbon source for Sphagnum C-fixation or may be emitted to the atmosphere (Raghoebarsing et al 2005a).

Using isotopic analyses, it has been shown that Sphagnum spp. contain intracellular Type II methanotrophic bacteria that can provide up to 10-15% of the CO₂ used as a C-source for moss growth (Kip et al 2010b, Raghoebarsing et al 2005b). Furthermore, uncultured Alphaproteobacteria affiliated with Methyloccella and Methylocapsa colonize the hyaline cells of Sphagnum spp. (Kip et al 2010a, Kip et al 2011b, Raghoebarsing et al 2005a). Methanotrophy and diazotrophy thus appear to be interconnected, and it is speculated that N₂ fixation allows for the transfer of fixed nitrogen to the Sphagnum spp., although microorganisms involved in this process have not been elucidated (Berg et al 2013, Larmola et al 2014b).

1.4 Macronutrient and Metal Availability

In ombrotrophic peatlands, those that receive their nutrients from atmospheric deposition, the macro- and micro-nutrients such as phosphorous, nitrogen and trace metals
are commonly scarce and potentially bio-limiting to microorganisms (Aerts et al. 1992, Basiliko and Yavitt 2001, Damman 1978, Leppänen et al. 2014, Vile et al. 2014). These limitations can then have potential significant impacts on the primary productivity rates in peatlands (Aerts et al. 1992, Berg et al. 2013). The formation of peatlands occurs through slow glacial melt and retreat contributing to the macro- and micro-nutrient profiles that are dependent on the drainage pattern and underlying bedrock mineralogy. Subarctic peatlands are characterized by successional gradients ranging from nutrient groundwater-fed minerotrophic fens to nutrient-poor ombrotrophic bogs, that reflect large heterogeneities in peatland nutrient limitation (Aerts et al. 1992, Basiliko and Yavitt 2001, Damman 1978, Leppänen et al. 2014, Vile et al. 2014). However, it must be noted that the individual nutrients added during the aforementioned field studies are not the sole driving factors to peatland production, and a multifactorial approach must be taken.

Northern peatlands formed during the last ice age left behind waterlogged and nutrient-poor peats overlain by layers of minerals and clay deposits (Glaser 1987, Ponnamperuma 1972, Rydin and Jeglum 2006). Specifically, the bedrock of Northern Minnesota peatlands are primarily composed of clayey deposits originating from limestone, dolomite and shale parent materials (Heinselman 1970). With the glacial retreat, the Pleistocene glaciers abraded portions of quartz (SiO₂), feldspar (KAlSi₃O₈—CaAl₂Si₂O₈—NaAlSi₃O₈), calcite (CaCO₃) and dolomite (CaMg(CO₃)₂), the variations which in turn influenced the concentrations of trace metals necessary for certain biological processes (Hill and Siegel 1991). Additionally, trace metals adsorb to organic deposits under waterlogged, anoxic and acidic (pH 3–7) peatland conditions (Rydin and Jeglum 2006) leading to low micronutrient concentrations (Bertine 1972, Wichard et al. 2009).


Typical molybdenum (Mo), copper (Cu) and vanadium (V) concentrations for sedimentary rocks are <3 mg kg\(^{-1}\), <75 mg kg\(^{-1}\) and <200 mg kg\(^{-1}\) respectively (Alloway and Alloway 1995). These three trace metals are of interest as they are required for diazotrophy and methanotrophy (Glass et al 2012, Semrau et al 2010). The uptake of Mo is important to the production of metalloenzymes involved in electron transfers within the cells, such as the nitrogenase enzyme that catalyzes \(N_2\) reduction to \(NH_3\) (Mendel 2005, Wang 2012). The nitrogenase metalloenzyme is made of an ATP-dependent iron (Fe) protein, which is the site for electron transfer, and a molybdenum-iron (MoFe) protein that is the catalytic site for the reduction of \(N_2\) (Dixon 2004). In some common bacteria of Mo-limited environments, two other forms of nitrogenase can be favored by some diazotrophs. These isoforms contain vanadium (V) or iron (Fe) as opposed to Mo as their central reactive metal, yet they are not as efficient in the conversion process as the molybdenum form. (Eady 1996; Dixon 2004; Walmsley 1991).

1.5 Potential Nitrogen Fixation Rates and \(^{15}N_2\) Stable Isotope Probing

Diazotrophs have the potential to provide fixed nitrogen to N-limited ecosystems such as ombrotrophic peatlands. Diazotrophs can be symbiotic, associative or free living, and both archaeal and bacterial diazotrophs exist (Dixon 2004, Canfield 2010). Microorganisms often control the rates of nitrogen cycling and in turn the productivity of an ecosystem. For example, in *Sphagnum* dominated bogs, diazotrophic cyanobacteria are predicted to transfer approximately 35% of fixed nitrogen to their *Sphagnum* host; however, this process has not been fully elucidated (Berg et al 2013). The coupling of nitrogen and carbon cycling is especially important in peatland environments, in that they have outsized impacts on global soil carbon storage.
The acetylene reduction assay (ARA) has been used as a proxy for N\textsubscript{2} fixation for decades (Dilworth 1966, Schöllhorn and Burris 1967). ARA allowed the study of N\textsubscript{2} fixation in peatlands to begin and progress from studies involved in Swedish peatlands (Basilier et al 1978, Basilier 1980, Granhall and Selander 1973, Rosswall and Granhall 1980). However, ARA suffers from potential biases due to the fact that acetylene inhibits pMMO (Dalton and Whittenbury 1976, Stirling and Dalton 1977, Whittenbury et al 1970). To correct for this potential bias, \textsuperscript{15}N\textsubscript{2} tracer methods are used in conjunction with ARA to calculate a conversion factor between the two techniques (Basilier 1980, Bellenger et al 2014b, Larmola et al 2014a, Vile et al 2014).

1.6 Objective

In my thesis research, the objective was to determine rates and controls of nitrogen fixation and the coupling of the N cycle with the C cycle within Sphagnum spp. samples originating from peatlands of northern Minnesota. My three main hypotheses were: (1) N\textsubscript{2} fixation may provide a significant portion of the fixed nitrogen to the S1 bog with a low atmospheric nitrogen deposition rate of 0.38 g m\textsuperscript{-2} y\textsuperscript{-1} (Sebestyen et al 2011); (2) microbial processes are limited by low trace metal concentrations in both ombrotrophic S1 and Zim bogs (3) incubations amended with methane would enhance N\textsubscript{2} fixation and ARA since organisms playing both diazotrophic and methanotrophic roles make up a large fraction of the nitrogen fixers (Larmola et al 2014a, Vile et al 2014).

To test these hypotheses, I coupled macronutrient and trace metal concentrations to potential rate measurements of nitrogen fixation under varying headspace, temperature, light, and methane treatments for the S1 peat bog in Marcell Experimental Forest as well as the Zim bog located 80 km southeast (Minnesota, USA). Total dissolved nitrogen (NO\textsubscript{3}\textsuperscript{-} + NO\textsubscript{2}\textsuperscript{-} + ...
NH₄⁺, phosphate and trace metal nutrient (Fe, Cu, V and Mo) concentrations were analyzed in peat porewaters using colorimetric assays and inductively coupled plasma mass spectrometry (ICP-MS), respectively. Potential N₂ fixation rates were measured by ARA and \(^{15}\)N₂ incorporation to generate conversion factors specific to the S1 bog. Variations in headspace treatments were performed on samples of the top 10 cm of peat to characterize how N₂ fixation rates vary with oxygen content, methane addition and temperature at a range of spatial and temporal scales.
CHAPTER 2: METHODS

2.1 Study Sites and Sampling Procedure

Samples were collected from the black spruce-\textit{Sphagnum} spp. S1 peat bog at Marcell Experimental Forest (MEF), the site of the DOE \textit{Spruce and Peatland Responses Under Climatic and Environment Change} (SPRUCE) experiment, in northern Minnesota, USA (47°30.476’ N; 93°27.162’ W). Samples from the Zim bog located approximately 80 km southeast from the S1 bog were also collected (47°10.8’ N; 92°42.6’ W). The S1 bog and Zim bog differ greatly in methane flux rates with S1 bog producing approximately 200 mg m\(^{-2}\) d\(^{-1}\); and Zim bog producing only 3 mg m\(^{-2}\) d\(^{-1}\) in July of 2013 (Keller & Bridgham, unpublished data). These differences may reflect a range of ombrotrophic versus minerotrophic conditions as the S1 bog is more acidic (pH \textasciitilde 4) and receives water inputs primarily from precipitation, whereas the Zim bog is less acidic and more connected to the regional groundwater table (Kolka et al 2011). Three locations were sampled along the S1 bog transect 3 at near (S1T3N), middle (S1T3M) and far (S1T3F) sites approximately equidistant from the west to east along the transect. Additionally, samples from the north-western portion of the S1 bog (S1T3) were collected. Surface (0-10 cm depth) peat was collected from lower elevation hollows dominated by \textit{Sphagnum fallax} and high elevation hummocks dominated by \textit{Sphagnum magellanicum}. Peat cores were sampled from hollows where the water level reached the surface of the \textit{Sphagnum} layer. Samples for nutrients and potential rate measurements were collected in September 2013, April 2014, June 2014 and September 2014.
2.2 Macronutrient and Metal Concentrations

Peat porewater was collected from the S1 and Zim bogs to measure macronutrient concentration using piezometers from 0, 10, 25, 50, 75, 150 and 200 cm depth. Piezometers were purged and allowed to recharge the same day as collection, and porewater was pumped to the surface, filtered through sterile 0.2 µm polyethersulfone membrane filters and stored frozen at -20°C until analysis. Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) were analyzed using the spectrophotometric assay as described by García-Robledo et al (2014). Ammonium (NH$_4^+$) concentrations were determined with the indophenol blue colorimetric assay described by Strickland and Parsons (1972). Phosphate concentrations were measured with the molybdate-antimony ascorbic acid colorimetric assay (Murphy and Riley 1962).

Porewater was collected from S1 bog peat cores from surface, 0-30, 30-50, and 100-150 cm depths to determine dissolved micronutrient concentrations. All plastics used for micronutrient sampling were acid-washed prior to sampling. Porewater was collected by filtration through 0.15 µm Rhizon soil samplers (Rhizosphere Research Products, Wageningen, The Netherlands), previously acid washed by pumping through 10 mL of 1 N HCl followed by a rinsing with 18.2 MOhm-cm water (~100 mL/filter) until the pH returned to neutrality. Samples were acidified to 0.32 M HNO$_3$ (Fisher Optima) and analyzed using a Thermo ELEMENT2 HR-ICP-MS (National High Magnetic Field Laboratory, Florida State University, USA). Initial analyses resulted in frequent clogging of the nebulizer, likely due to abundant dissolved organic carbon. Therefore, samples were diluted 1:10 to minimize interruptions from nebulizer clogs. Concentrations were quantified with 7-point external calibration using standards prepared in 0.32 N HNO$_3$ from a multi-element standard mix (High Purity Standards, Charleston, SC, USA).
In order to measure low concentrations we avoided sample dilution by digesting samples before ICP-MS analysis. Briefly, 1 mL porewater samples were digested for 36 hours at 250°C in a trace metal clean polypropylene exhausted laminar flow hood with 1 mL of 16 N NHO₃ (Ultrex II, JT Baker, Center Valley, PA) and 100 µL of 30% H₂O₂ (Ultrex II, JT Baker, Center Valley, PA) in acid-washed Teflon vials. Samples were then evaporated to near dryness, resuspended in 0.32 N HNO₃, and analyzed by ELEMENT2 HR-ICP-MS along with parallel blank solutions. A possible contamination was observed in the Mo and V samples of September 2014.

2.3 Potential Rate Measurements

The acetylene reduction assay (ARA) was used as a proxy for N₂ fixation rates in the S1 and Zim bogs. Samples of bulk peat (including both living Sphagnum spp. and surrounding dead material) were collected from each sampling site at S1 bog (S1T3N, S1T3M, S1T3F) and stored at 4°C until the start of incubations in the laboratory (~3-6 weeks later). Additionally, samples from the Zim bog were collected at three random equidistant locations for a spatial comparison to the S1 site. Tests showed no significant effect of storage time on potential rate measurements (data not shown). Samples from 0-10 cm depth were gently homogenized so as not to rupture the live moss while samples from 10-30 cm depth were fully homogenized. For each sample, 5 g of bulk material were placed in 70 mL glass serum bottles, stoppered with black butyl stoppers (Geo-Microbial Technologies, Ochelata, OK, pre-boiled 3x with 1 N NaOH), and sealed with an aluminum crimp seal. Headspace was either room air for oxic treatments or mixed gas for anoxic treatments (80% N₂ and 20% CO₂) with or without amendments of 1% C₂H₂ or 1% CH₄. Controls that were not amended with C₂H₂ did not produce ethylene (C₂H₄). September 2013 and September 2014 samples
were incubated for one week in the light and dark at 25°C. Additionally, September 2013, April 2014, June 2014, and September 2014 samples were incubated in the light at 25°C while the April 2014 and June 2014 samples were incubated for one week in the light at 4°C. A gas chromatograph with a flame ionization detector equipped with a HayeSep N column (SRI Instruments, Torrance, CA, USA) was used to quantify CH₄, C₂H₂ and C₂H₄. Samples were taken daily until C₂H₄ production was linear (~7 days). The samples were then placed in 15 mL cryovials, frozen in liquid N₂, and stored at -80 °C for future RNA and DNA qPCR and sequencing analysis.

In addition to ARA, N₂ fixation rates in the September 2014 samples were quantified with ¹⁵N₂ labeling. Incubations were set up as described above and supplemented with 7 mL of 98% ¹⁵N₂ bringing the final ¹⁵N₂ headspace to 10% in the serum bottles (Cambridge Isotope Laboratories, Tewksbury, MA, USA; (Vile et al 2014). After 7 days, samples were dried at 80°C, homogenized into a fine powder, and analyzed by Isotope Ratio Mass Spectrometry at UC Berkeley.

2.4 JMP Statistics

JMP 12 statistical software was used to analyze ARA and ¹⁵N₂ incorporation rates due to month, headspace treatments, methane amendments, water content, and interactive effects. A one-way and two-way analysis of variance (ANOVA) was used to determine statistical difference between treatments and interactive effects, respectively. Linear regression was used to determine a correlation between water content and ethylene production. The conversion factor of acetylene reduction to ¹⁵N₂ fixation rates was determined by the slope of linear regression (Bellenger et al 2014b, Hardy et al 1973, Leppänen et al 2013).
CHAPTER 3: RESULTS

3.1 Macronutrient and Metal Concentrations

Total dissolved NH$_4^+$, NO$_3^-$, NO$_2^-$ and PO$_4^{3-}$ were all consistently <2 µM in surface peat for the S1T3 bog (Figure 1). Concentrations of NH$_4^+$ increased with depth up to 42 µM at 200 cm (June 2014; Figure 1a). Concentrations of NO$_3^-$ decreased with depth to 75 cm, whereupon concentrations remained <0.05 µM (the detection limit; Figure 1b). Contrastingly, NO$_2^-$ concentrations were generally constant with depth (0.6 µM to < detection limit of 0.05 µM; Figure 1c). Surface concentrations of PO$_4^{3-}$ were <2 µM at all depth levels with only one point at 150 cm measuring PO$_4^{3-}$ concentrations of 2 µM in June 2014 (Figure 1d).

Dissolved Fe was more abundant (18-35 µM) than Mo, V and Cu (2-40 nM; Figure 2d). Molybdenum concentrations were <1 nM (the detection limit) in surface peat and highest (7 nM) in June 2014 at 125 cm (Figure 2a). Vanadium concentrations (5-21 nM) consistently exceeded Mo concentrations (Figure 2b). Highest Cu concentrations (38 nM) were measured in September 2014 in the 0-10 cm depth and lowest concentrations (3 nM) were observed at 125 m depth (Figure 2c). Copper concentrations showed no significant difference at the surface between the depths of June 2014 and September 2014 (5-38 nM; $P > 0.05$). Fe concentrations ranged from 18-35 µM for June and September 2014 with no significant difference measured between the two months ($P > 0.05$), and showed a slight decrease with depth to 5-30 nM at 120 cm depth.
Figure 1 Macronutrient profiles for the S1T3 bog for June and September 2014.
Figure 2 Micronutrient profiles for S1T3 bog for June and September 2014. Hollow samples are shown in circles and hummock samples are shown in diamonds.
3.2 Potential Rate Measurements

In S1T3 hollows, potential rates of N$_2$ fixation measured by ARA were dependent on headspace treatment and moisture level. ARA was greatest in anoxic treatments for all four sampling dates ($P=0.008$) with significantly higher rates in June than the three other months ($P<0.02$). Oxic treatments showed no significant difference among the months sampled where rates were consistently measured between 5-12 nmol C$_2$H$_4$ produced g$^{-1}$ moss dry mass h$^{-1}$ (Figure 3).

There was no significant effect of 1% CH$_4$ addition on N$_2$ fixation rates in either oxic or anoxic treatments with ARA (Figure 3). The potential for inhibitory affect of C$_2$H$_2$ on CH$_4$ oxidation was tested by measuring rates of CH$_4$ oxidation with and without 1% C$_2$H$_2$ in oxic treatments. Methane oxidation was inhibited by 1% C$_2$H$_2$ after two days of incubation (Figure 4). No significant difference in CH$_4$ oxidation rates (25-30 µmol g$^{-1}$ moss dry mass h$^{-1}$) were measured between day 1-2 of incubations (Figure 5).

A correlation between water content and ARA rates was observed in the anoxic hollows ($R^2=0.7$, $P < 0.001$). The oxic treatments showed no significant correlation between water content and ARA ($R^2=0.1$, $P=0.3$; Figure 6).
Figure 3 Temporal variations of ARA rates in the surface peat of the S1 bog incubated in the light at 25°C. Greatest rates for ARA were observed in the June 2014 samples (* $P \leq 0.05$). Greater ARA rates were observed in the anoxic treatments versus oxic treatments (** $P \leq 0.01$).
**Figure 4** Acetylene inhibition of methane oxidation showed methane oxidation was inhibited by day 2 in oxic incubations incubated in triplicate with surface peat sampled in September 2014 from the S1T3M bog at 25°C in the light.
Figure 5 Methane Oxidation Rates calculated between day 1 and day 3 of incubations (25°C in the light) incubated in triplicate with surface peat sampled in September 2014 from the S1T3M bog.
A positive correlation was observed between water content and ARA rates in the hollows incubated at 25°C in the light under anoxic conditions. Samples were collected from September 2013, April 2014, June 2014, and September 2014 from the S1T3 bog.
3.3 Seasonal and Spatial variation

Potential nitrogen fixation rates were compared at the high mid-summer CH$_4$ flux S1 bog (July: ~200 mg m$^{-2}$ d$^{-1}$; Keller & Bridgham, unpublished data) vs. the low CH$_4$ flux Zim bog (July: ~3 mg m$^{-2}$ d$^{-1}$; Keller & Bridgham, unpublished data). In April 2014, Zim bog showed 93% ($P = 0.02$) and 75% ($P \leq 0.01$) lower ARA rates compared to the S1 site in both the anoxic and oxic treatments respectively (Figure 7a). Similarly, in June 2014, Zim bog displayed 98% and 91% lower ARA rates than the S1 bog in oxic and anoxic treatments, respectively ($P < 0.006$; Figure 7b). Significantly higher ARA was measured in April and June for the anoxic treatments without methane ($P = 0.02$). Methane amendments had no significant effect on ARA in the Zim bog, similar the observations from the S1 bog (Figure 8).
Figure 7. Observed ARA rates for the S1 bog in (a) April 2014 and (b) June 2014 were significantly greater than the Zim bog in both anoxic ($P \leq 0.05$) and oxic ($P \leq 0.01$).
Figure 8. Zim Bog ARA rates in (a) April and (b) June of 2014 showed no significant difference between month or treatments.
Microhabitat variations for ARA rates in the S1T3 bog in June 2014 were significantly greater ($P \leq 0.01$) in the anoxic hollows than anoxic hummocks.

**Figure 9.**
Microhabitat ARA rates in the S1T3 bog were significantly higher in the anoxic hollows than hummocks \((P = 0.009; \text{Figure 9})\). In hummocks, no significant difference in ARA rates was measured between the anoxic and oxic treatments. A microhabitat comparison was also performed in the Zim bog and no significant difference was measured between anoxic and oxic treatments in the hummocks and hollows.
Figure 10. ARA rates for Zim bog in June 2014 was significantly greater in the oxic hummocks than the oxic hollows (* $P \leq 0.05$). No significant difference was observed between all treatments in the hollows while no significant difference was observed between all treatments in the hummocks.
Overall, ethylene production was detected in all four months sampled with the exception of 30 out of 177 samples tested. No ethylene was produced in control samples containing peat only in both oxic and anoxic treatments.

3.4 Temperature variations

Temperature effects were tested in the S1T3 bog at 4°C and 25°C in the light for the months of April and June of 2014 (Figure 11). A significant difference was observed in the April 2014 anoxic treatments between the samples with and without CH4 at 4°C ($P = 0.008$) (Figure 11a). The April 2014 anoxic treatments were significantly greater by 11 times between between 4°C and 25°C ($P = 0.02$) (Figure 11a). A significant difference was observed between the rates of April and June the anoxic and oxic treatments at 4°C ($P = 0.005$) and ($P = 0.04$) respectively (Figure 11).

A microhabitat comparison of the S1T3 bog showed significant temperature effects for the hummocks in June 2014 at 4°C. No significant difference was observed across the hummock rates at 25°C; however, rates at 4°C were significantly lower in the oxic treatments ($P = 0.005$).
Figure 11. Temperature variations for ARA rates in the S1 bog from (a) April 2014 and (b) June 2014.
Figure 12. Temperature variations of ARA in the hummocks of the S1 bog in June 2014 at 25 °C and 4 °C showed no significance among treatments at both temperatures with the exception of the oxic treatment ($P \leq 0.01$). There while significantly greater rates in the hummocks at 4 °C in the anoxic treatments compared to the oxic treatments ($P \leq 0.01$).
3.5 $^{15}\text{N}_2$ Incorporation

The labeled $^{15}\text{N}_2$ experiments showed similar trends to ARA results; however, $^{15}\text{N}_2$ incorporation in $\text{C}_2\text{H}_2$-amended samples was 2-4x lower than samples lacking $\text{C}_2\text{H}_2$ (Figure 13a,b). No significant difference was seen for $\text{CH}_4$-treated samples. There was a significant difference between the anoxic and oxic treatments ($P < 0.0001$).
Figure 13 Comparison of (a) ARA rates to (b) $^{15}\text{N}_2$ rates from the S1NW bog in September 2014. Significantly greater $^{15}\text{N}_2$ rates were observed in the anoxic treatments compared to the oxic treatments ($P \leq 0.0001$)
CHAPTER 4: DISCUSSION

4.1 Macro and Micronutrient Concentrations

The extremely low levels of dissolved nitrogen (<2 µM in surface peat) as well as the potential rates of nitrogen fixation measured in this study from S1 and Zim bogs are consistent with previous findings that N$_2$ fixation associated with *Sphagnum* is an important process to supplement low atmospheric nitrogen deposition in peat bogs (Berg et al 2013, Leppänen et al 2014, Vile et al 2014). The low levels of N in the S1 and Zim bog can be compared to the higher concentrations found in a peat bog in the Netherlands where total N-concentrations were > 60 µM L$^{-1}$ (Kip et al 2011b). Interestingly, no significant difference between the dissolved nitrogen and PO$_4^{3-}$ concentrations was measured between the months of July 2014 and September 2014 in the S1T3 site; however, potential rates were significantly lower in September 2014, suggesting that macronutrient limitation was not the sole controlling factor.

Nitrogen amendment studies in the past have shown that deposition of atmospheric nitrogen in some regions stimulates primary production; conversely, other amendment studies did not find increased primary productivity, leading to the hypothesis that another nutrient or nutrients are limiting (Larmola et al 2014a, Limpens et al 2006, Urban and Eisenreich 1988a, Vile et al 2014). Dissolved PO$_4^{3-}$ was <2 µM in the top 25 cm and increased with depth. The low dissolved PO$_4^{3-}$ concentrations at the surface suggest nutrient limitation or rapid uptake of PO$_4^{3-}$ by plants and or microorganisms. Low levels of PO$_4^{3-}$ (<2 µM) have been observed in bogs in the Netherlands, suggesting P could be an important limiting macronutrient after N (Kip et al 2011b). Total P concentrations have been found to be greater in the top 10 cm of peat (36.8 mg kg$^{-1}$) than at depth (19.6 mg kg$^{-1}$), consistent
with rapid uptake of available P such as the $\text{PO}_4^{3-}$ at the surface (Lin et al 2014a). In the top 10 cm, orthophosphate monoester, which adsorbs strongly to soils under acidic conditions, was the dominant form of P (Hawkesd et al 1984, Lin et al 2014a, Turner et al 2002).

Throughout the peat porewater profiles, dissolved Fe was approximately one order of magnitude more abundant than Mo and V (Figure 2), suggesting that alternative metalloenzymes containing Fe in place of Mo or V may be favored (Bellenger et al 2014a, Crombie and Murrell 2014). X-ray Absorption Near Edge Structure (XANES) and Extended X-ray Absorption Spectroscopy (EXAFS) studies show that tannic acids complex molybdate strongly and therefore may be the most abundant Mo sink in soils (Wichard et al 2009). Molybdenum adsorption to organics occurs over a broader pH range (~5-9) than adsorption to Fe-oxides and other minerals, therefore the Mo-organic complexes may allow Mo to be bioavailable but not detected in the collected dissolved peat porewater from this study (Bellenger et al 2014b, Goldberg et al 1996, Wichard et al 2009). This is in agreement with Bertine (1972) where the sorption of Mo to peat occurred at a rate of approximately 12% Mo adsorbed per pH unit decrease with a maximum adsorption at pH 1. An alternative hypothesis to the differences in ARA rates for the months of June 2014 and September 2014 could be the differences in Mo concentrations (4 nM and <1 nM respectively) suggesting that Mo might be a limiting micronutrient in the S1 bog, at least seasonally, similar to other findings in freshwater systems (Glass et al 2012). These low Mo and V concentrations suggested that alternative nitrogenase usage might possibly be favored; however, no detectable production of ethane, a biomarker for alternative nitrogenase activity, was observed (Dilworth et al 1987, Eady 1996). Recent metagenomic data has shown evidence for genetic potential of alternative nitrogenases in the S1T3 bog, however,
metatranscriptomics showed no alternative nitrogenase gene transcription (Warren et al. in prep).

4.2 Headspace Treatment Effects

Incubations to measure ARA rates in the dark for the top 0-10 cm of *Sphagnum* and peat showed no detectable acetylene reduction. Similarly, peat at depths of 10-30 cm showed no detectable acetylene reduction when incubated in the light or dark. All rates were thus taken from the top 0-10 cm of *Sphagnum* and peat incubated in the light. The general temporal trend for ARA rates by month is positively correlated with water content and a significant interaction effect was observed between headspace treatment and moisture content (*P*<0.0001) in the S1T3 hollows. In the past, it was shown that water content was not a driving factor in N$_2$ fixation in peatlands (Granhall and Selander 1973, Rosswall and Granhall 1980); however, our study shows that increased water content leads to elevated N$_2$ fixation in anoxic headspace treatments (0 – 170 nmol g$^{-1}$ dry moss h$^{-1}$). This is consistent with recent findings that higher rates of nitrogen fixation in water-submerged samples compared to samples incubated in air; however, the finding could be a function of an interaction between water content and oxygen tension similar to this study (Leppänen et al 2014). Water table levels may be relevant to the transport of diazotrophic microorganisms in the anoxic porewater similar to what has been seen for methanotroph transport (Larmola et al 2010). Interestingly, the rates of N$_2$ fixation in the S1 bog were greater by up to 16 x in the hollows than in an oligotrophic fen in a Finnish peatland (Larmola et al 2014a). Although the ARA rates were measured to be greatest in the anoxic treatments, the concentration of O$_2$ during the incubations released by the *Sphagnum* were not measured, so that low concentrations of O$_2$ may be a factor in N$_2$ fixation rates and will be tested in the future.
The nitrogenase enzyme is inhibited by oxygen (Wong and Burris 1972) possibly explaining the lower rates of ARA in the oxic treatments. Some diazotrophs have the ability to compartmentalize the nitrogenase enzyme allowing for N₂ fixation to occur in some oxic environments (Berman-Frank et al 2003, Dixon and Kahn 2004). Methanotrophs and cyanobacteria have been found to inhabit host *Sphagnum*, so it is possible that the host environment can protect a diazotroph from oxygen while receiving fixed forms of nitrogen and carbon (Larmola et al 2010, Raghoebarsing et al 2005b, Vile et al 2014).

Methanotrophs have been found to be abundant amongst the diazotrophs in surface peat; therefore, CH₄ addition was hypothesized to stimulate ARA rates and ¹⁵N₂ incorporation (Larmola et al 2014a, Vile et al 2014). Contrasted to this expectation, CH₄-amended treatments had no effect on potential N₂ fixation rates measured by both methods at 25°C; however, the addition of acetylene halted CH₄ oxidation by day two of the incubation (Figure 4). Because acetylene is a potent inhibitor of methane monooxygenase enzymes that catalyze the first step in methane oxidation (Stirling and Dalton 1977), it is possible that this inhibition subsequently led to the inhibition of N₂ fixation by methanotrophs (Figure 3 and Figure 4). To test the effect of acetylene on CH₄ oxidation, labeled ¹⁵N₂ incubations were tested side-by-side ARA incubations. The results showed consistent levels of ARA activity, further evidence that CH₄ does not enhance diazotrophy (Figure 13). This finding is similar to other studies on N₂ fixation in an ombrotrophic bog (Leppänen et al 2014); whereas CH₄ has been found a to stimulate N₂ fixation in systems nearer fen along the ombrotrophic/minerotrophic gradient (Larmola et al 2014a).
4.3 Spatial Variation Effects

Peatland micro-topography in the S1 bog consists of waterlogged depressions (hollows) and raised areas (hummocks) forming microhabitat variations. The hollows and hummocks in the S1 bog are dominated by the two types of moss: *Sphagnum fallax* and *Sphagnum magellanicum*, respectively. Hummocks were subjected to the same treatments as hollows, but interestingly, no significant difference was measured between anoxic and oxic treatments. The ARA rates in the hummocks ranged from 0-8 nmol g\(^{-1}\) dry moss dry weight hr\(^{-1}\) (Figure 9) suggesting different nitrogen requirements along microhabitats with differing elevations. Lower levels of N\(_2\) fixation in hummocks versus hollows have been observed in a Finnish peatland where N\(_2\) fixation rates were 2-7x lower in hummocks (Larmola et al 2014a). *Sphagnum magellanicum* is found to be the dominant moss species in extremely nutrient-limited bogs (Daniels and Eddy 1990), so the difference in ARA could be species dependent or due to other factors such as nutrient limitation, water content or differing microbiome.

Macronutrients were not collected from the hummocks due to low water content; however, sufficient porewater was collected to determine the soluble metal concentrations. Interestingly, the hummocks showed lower concentrations compared to hollows for all metals with the exception of one site for Cu (Figure 2). This suggests a possible trace metal limitation to N\(_2\) fixation in the hummocks.

Another hypothesis to explain the difference in ARA rates in the microhabitat comparison is water content. Although the water content for the S1T3 hummocks differed from the hollows (96% and 90%, respectively), no significant difference was found between the ARA rates of the hummocks and hollows incubated under oxic treatments, suggesting a
possible community shift of diazotrophs during the development of a hummock. The addition of water to the hummocks was not tested in this study, but might be useful for future studies to rule out or confirm water content as a driver for N\textsubscript{2} fixation.

### 4.4 Acetylene Reduction Rates: Temperature variation

Peatlands show major temperature variations within and during the warm summer and the frozen winter (Rydin and Jeglum 2006). It was hypothesized that incubations would show a significant decrease in ARA rates at the lower temperatures. Enzymatic reactions have been found to occur with a lower activation energy (E\textsubscript{a}) in psychrophilic diazotrophs suggesting a community shift in N\textsubscript{2} fixation (Pandey et al 2004). Conversely, ARA rates increased in the hollows between the months of April and June 2014 whereas no significant difference in ARA rates between 4°C and 25°C was measured (Figure 11a,b).

Interestingly, the hollow material incubated under anoxic conditions at 4°C showed significantly greater ARA rates in treatments without 1% CH\textsubscript{4} amendments compared to those with 1% CH\textsubscript{4} (Figure 11b). The oxic treatments showed a similar pattern. It could be hypothesized that the community shift towards psychrophiles is inhibited by CH\textsubscript{4} additions. This trend was not seen in the April 2014 hollow rates incubated at 4°C; however the combined ARA rates were low (0.9 ± 0.3 nmol g\textsuperscript{-1} dry moss dry weight hr\textsuperscript{-1}).

### 4.5 \textsuperscript{15}N\textsubscript{2} incorporation

Labeled \textsuperscript{15}N\textsubscript{2} experiments showed similar trends to ARA. The addition of acetylene lowered \textsuperscript{15}N\textsubscript{2} incorporation 2-4x, suggesting diazotrophs sensitive to acetylene such as methanogens and methanotrophs were compromised. A conversion factor of 3.9 was determined for the ratio of \textsuperscript{15}N\textsubscript{2} incorporation to ARA in the September 2014 hollow samples from the S1NW bog. These findings can be compared to the theoretical conversion factor of
3.2 determined by the electrons required per reaction of N\textsubscript{2} fixation to ARA (Bellenger et al 2014a). Previous studies have measured conversion factors between 3.0 and 4.0 for soils (Hardy et al 1973) and forest mosses (Leppänen et al 2013). \textsuperscript{15}N\textsubscript{2} fixation rates in a Canadian peatland were approximately 1.8-3.4 g N m\textsuperscript{-2} yr\textsuperscript{-2} (Vile et al 2014) and are within the range of \textsuperscript{15}N\textsubscript{2} fixation rates in this study of 0.2-3.6 g N m\textsuperscript{-2} yr\textsuperscript{-2} suggesting similarities among bogs. Other boreal peatland studies have observed ARA and \textsuperscript{15}N\textsubscript{2} rates 0.05-2.9 g N m\textsuperscript{-2} yr\textsuperscript{-1} within the rates found in this study (Hemond 1983, Larmola et al 2014a, Markham 2009, Schwintzer 1983, Urban and Eisenreich 1988b).
CHAPTER 5: CONCLUSIONS

5.1 Conclusions

This thesis showed that anoxic conditions in the surface peat hollows of the S1 bog led to highest levels of ARA (0-165 nmol C₂H₄ produced g⁻¹ moss dry mass h⁻¹). The Zim bog showed overall lower rates of potential nitrogen fixation. Furthermore, we found variation in N₂ fixation potential in our study to respond to the interactive effect of anoxic and increased water content. Notably, CH₄ additions did not have an effect on potential rates of N₂ fixation nor on ¹⁵N₂ incorporation at 25°C; however, CH₄ additions did have significant effects on potential rates at 4°C, suggesting a possible community shift with temperature. High variability was observed in the measurements of N₂ fixation across micro (hummock versus hollow) macro (S1 versus Zim) scales and seasonal scales (Spring thru Fall), thus an in-depth spatial and seasonal analysis should be considered. Due to the complexity of the system, multiple factors contributing simultaneously to N₂ fixation rates will have to be determined, and incorporated into terrestrial ecosystem carbon cycling models. In the future the SPRUCE site will have available samples to understand the potential effects of increased temperatures on N₂ fixation in peatlands. This leads to the important question of whether methanotrophy will be decoupled from N₂ fixation in warming treatments.

5.2 Future Work

Future work will include in situ measurements of N₂ fixation by ARA and ¹⁵N₂ incorporation in the S1 bog coupled to nutrient profiling. Shifts in community composition of incubations will be determined using primer-based sequencing of the 16S DNA and rRNA genes as well as metagenomic and metatranscriptomic nifH diversity from in situ samples. All samples in this work were frozen in liquid nitrogen and stored at –80°C so that DNA and
RNA extractions and subsequent sequencing and analyzed. The future goal of this work is to understand the cycling of nitrogen and carbon in peat bogs through the processes of nitrogen fixation, methanotrophy, and methanogenesis.
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