UNDERSTANDING ANAEROBIC CARBON CYCLING IN TROPICAL AND BOREAL WETLAND ECOSYSTEMS

by

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DISSERTATION ABSTRACT

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Title: Understanding Anaerobic Carbon Cycling in Tropical and Boreal Wetland Ecosystems

Understanding methane (CH_4) cycling dynamics is of paramount importance because CH_4 has 45 times the sustained-flux global warming potential of carbon dioxide (CO_2) and is currently the second most important anthropogenic greenhouse gas. Wetland ecosystems emit one-third of total global CH_4 emissions, making them the single largest natural CH_4 source and placing them among the most important terrestrial ecosystems in the global carbon (C) cycle. Wetlands in tropical and boreal regions are drivers of recent inter-annual variation in atmospheric CH_4 concentrations because they play vital roles in the global CH_4 cycle by storing vast amounts of C (~31% of total soil C in boreal peatlands) and generating a significant proportion of total global wetland CH_4 emissions (47-89% in tropical wetlands). However, despite the recognized importance of these ecosystems, tropical wetlands have received limited study concerning CH_4 flux and, although boreal wetlands have been more thoroughly studied, significant questions remain surrounding the biogeochemical controls over CH_4 dynamics in these systems.

My dissertation addresses these concerns using a combination of in situ field measurements and controlled laboratory incubations across field sites in equatorial Gabon, Africa and at an experimentally-manipulated (surface and deep warming and atmospheric CO_2 enrichment) peatland in northern Minnesota. Specifically, my research

provides novel information about the rates and abiotic and biotic controls over methanogenesis and methanotrophy in tropical African wetland and upland habitats (Chapter II). This chapter paired functional datasets with corresponding measurements of microbial community composition, using a holistic research approach that provided unique ecological insights into tropical ecosystem CH_4 cycling. In northern Minnesota, I investigated the C source fueling anaerobic C mineralization across a variety of boreal peatlands, as well as if methanogenesis was limited by labile C availability at depth (Chapter III). Finally, my dissertation includes novel results on the response of boreal peatland CH_4 and CO_2 production, as well as anaerobic oxidation of CH_4 (AOM), to deep peat heating (Chapter IV; does not include AOM) and whole-ecosystem warming with atmospheric CO_2 enrichment (Chapter V), expanding our mechanistic understanding of how climate-driven variables affect peatland C mineralization.

This dissertation includes previously published and unpublished coauthored material.

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For Tyler,

My partner in adventure and

The light in my life that will never die.

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CHAPTER I

INTRODUCTION

The Global CH_4 Budget

Methane (CH_4) is a trace, greenhouse gas (GHG) that plays a critical role in the chemistry of Earth's atmosphere. The atmospheric concentration of CH_4 has increased by over 150% since pre-industrial times, making it responsible for about 20% of human-induced radiative forcing and the second most important anthropogenic GHG after CO_2 (Myhre et al., 2013). A relatively recent, multi-decadal analysis of changes in atmospheric CH_4 levels found that, after a period of stabilization in the early 2000s, CH_4 levels have begun rising again (Kirschke et al., 2013).

Traditionally, the potency of GHGs has been described by their global warming potential (GWP), a common metric for normalizing the radiative forcing of GHGs to CO_2 equivalents based on their respective atmospheric lifetimes, radiative characteristics, and cycling. However, it has recently been demonstrated that this methodology may provide misleading conclusions concerning the impact of ecosystems on climate because it assesses radiative forcing due to a one-time pulse of a GHG into the atmosphere, when, in reality, GHG fluxes are sustained over time. Neubauer and Megonigal (2015) developed a new metric – the sustained-flux global warming potential (SGWP) – which specifically models GHG emissions as persistent events. Using this improved approach, it was determined that CH_4 has 45 times the SGWP of CO_2 over a 100-year time frame (Neubauer and Megonigal, 2015); therefore, small changes in its atmospheric concentration have large implications for future climate (Myhre et al., 2013).

The global atmospheric CH_4 budget comprises numerous terrestrial and aquatic surface sources that are primarily balanced by a single atmospheric sink. Methane emissions are grouped into three broad categories that can be differentiated using their unique isotopic signatures (Monteil et al., 2011): biogenic, thermogenic, and pyrogenic. Biogenic CH_4 is generated under anaerobic conditions by methanogens (CH_4-producing microorganisms) in environments such as wetlands and rice paddies, digestive systems of ruminants and termites, and landfills. Fossil fuels represent thermogenic CH_4 sources, which have been formed via geologic processes over millions of years. Thermogenic CH_4 can be emitted into the atmosphere through either the burning of coal, oil, and natural gas or through natural terrestrial and/or marine seeps. Finally, pyrogenic CH_4 is formed from the incomplete combustion of organic material during wildfires. Ninety percent of the CH_4 emitted into the atmosphere is oxidized by hydroxyl radicals in the troposphere, representing the largest sink in the global CH_4 budget (Cicerone & Oremland, 1988). Methanotrophic (CH_4-consuming microorganisms) bacteria in aerated soils are the second largest atmospheric CH_4 sink, oxidizing roughly 4% of global CH_4 emissions (Zhuang et al., 2004; Curry, 2007).

"Top-down" and "bottom-up" approaches are the primary methodologies for estimating global CH_4 emissions and their geographic distribution. Top-down estimates emerged in the 1970s with the ability to directly measure atmospheric CH_4 concentrations and reached global coverage by the 1980s (Blake et al., 1982). Currently, atmospheric CH_4 concentrations are determined using discrete air samples collected regularly or continuously at the surface (e.g., Cunnold et al., 2002) or in the troposphere (e.g., Schuck et al., 2012) and remotely sensed measurements of atmospheric CH_4 columns obtained

from the surface or space (e.g., Griffith et al., 2011). Regional estimates of CH_4 emissions are then determined using an 'inverse' Bayesian statistical approach that incorporates prior information on the spatial distribution of CH_4 sources and sinks, as well as atmospheric transport models (Bridgham et al., 2013). While top-down methodologies act as an important, empirically-derived constraint on regional CH_4 sources, they are limited by several factors, including the sampling network density (Dlugokencky et al., 2009), the accuracy of *a priori* estimates used in Bayesian modelling (Neef et al., 2010), and a lack of data necessary to accurately resolve sources (Spahni et al., 2011). Additionally, this approach provides no mechanistic understanding of the abiotic or biotic drivers controlling CH_4 emissions from specific sources.

Conversely, bottom-up methodologies scale CH_4 fluxes acquired with empirical ground-based or model-derived techniques over a given area, providing site-specific estimates, as well as information on local process drivers. Ground-based measurements are typically collected using chambers or eddy-flux towers; however, these data are extremely spatially variable and underrepresented in many global regions, such as the tropics. Model-derived estimates are similarly limited by their ability to accurately capture CH_4 dynamics, which can vary significantly based on the system of interest (Bridgham et al., 2013). A recent, multi-decadal synthesis of bottom-up and top-down methodologies found that bottom-up techniques yielded total global emissions of 678 Tg CH_4 yr^{-1} in the 2000s, while top-down studies reported approximately 20% lower emissions of only 548 Tg CH_4 yr^{-1} during this period (Kirschke et al., 2013). The discrepancy between these two values was attributed to the higher global source estimates for wetland, freshwater, and geologic sources derived from bottom-up techniques.

Overall, natural wetlands had the largest absolute uncertainty of any of the emission categories, with a range of 107 Tg CH_4 yr^{-1} using bottom-up approaches (Kirschke et al., 2013; Bridgham et al., 2013; Melton et al., 2014), highlighting both the importance of these systems in the global CH_4 budget, as well as the difficulty associated with the accurate determination of their contribution to worldwide CH_4 emissions.

The Role of Wetlands in the Global CH_4 Budget

Wetland ecosystems emit about a third of total global CH_4 emissions, making them the single largest natural CH_4 source and placing them among the most important terrestrial ecosystems in the global carbon (C) cycle (Bridgham et al., 2013). Anaerobic conditions promote slow decomposition rates of organic matter, accumulation of soil C, and production of CH_4. Methane emissions from natural sources have been shown to be partially controlled by changes in climate from past glacial-interglacial cycles (Blunier et al., 1995; Loulergue et al., 2008) and, moreover, large recent inter-annual variability in atmospheric CH_4 levels may be driven by climate effects on wetland CH_4 emissions (Kirschke et al., 2013; Melton et al., 2014). The close coupling between climate and wetland CH_4 emissions generates justifiable concern that wetland ecosystems will act as a positive feedback to anthropogenic-driven climate change.

Despite the significant inconsistencies that exist between top-down and bottom-up techniques, both methodologies highlight the importance of tropical and boreal wetlands as drivers of recent multi-year changes in atmospheric CH_4 concentrations (Bousquet et al., 2006 and 2011; Bloom et al., 2010; Kirschke et al., 2013; Melton et al., 2014). Geographically, about 56% of global wetlands are located in tropical and subtropical humid regions (Melton et al., 2013). Recent studies estimate that 47-89% (median 73%)

of global wetland CH_4 emissions originate in the tropics (Bridgham et al., 2013); therefore, this region plays a critical role in the global CH_4 cycle. While extensive research has been conducted in temperate, boreal, and arctic zones to understand biogeochemical and microbial controls on wetland CH_4 emissions, much less research has been conducted across tropical regions (Bridgham et al., 2013; Kirschke et al., 2013; Melton et al., 2014). Organic-soil wetlands (peatlands) dominate boreal regions and, conversely, mineral-soil wetlands are the most abundant wetland type in tropical areas. Soils are the foundation and a key controller of ecosystem function; thus, these two wetland types have distinctly different sets of ecosystem controls (Spahni et al., 2011) and responses to climate (Bloom et al., 2010; Hodson et al., 2011). It is unlikely that the anaerobic C mineralization relationships and CH_4 cycling dynamics observed in other, more heavily researched, ecosystems will transfer to equatorial regions given their distinct differences in climate and soil type. Thus, net and gross ecosystem CO_2 and CH_4 fluxes, as well as drivers of biogeochemical and microbial relationships remain unknown throughout the tropics.

Although boreal wetlands have been more thoroughly studied, significant questions remain surrounding the biogeochemical controls over CH_4 dynamics in these systems and their response to changing climate. The vast majority of global wetland C is stored in northern boreal peatland soils, which, by definition, have extensive soil C accumulation (≥40 cm) (Yu, 2012). Thus, despite covering <3% of the Earth's surface, peatlands contain one-third of total global soil C and are responsible for approximately 10% of global CH_4 flux (Bridgham et al., 2013). Additionally, most peatlands occur above 40°N latitude, where the largest relative temperature changes are projected to

occur under current climate models and, therefore, are perceived to be particularly susceptible to climate change (Kirtman et al., 2013). Changes in temperature and hydrology throughout the boreal zone have the capacity to diminish the role of these ecosystems as C sinks by triggering the return of currently stored organic C to the atmosphere as CO_2 and/or CH_4, thereby amplifying the impacts of a changing climate. It is currently unknown whether or not a significant fraction of the large soil C pool in peatlands will be respired as CH_4 in future climates, creating a pressing problem in global change biogeochemistry and modelling (Bridgham et al., 1995; Limpens et al., 2008; Frolking et al., 2011; Yu, 2012; Bridgham et al., 2013).

To fully understand the difficulties associated with the accurate prediction of wetland CH_4 emissions, it is necessary to comprehend the complex set of processes underlying CH_4 cycling, as well as their unique abiotic and biotic controls. In the following section, I provide a brief review of current knowledge on this topic.

A Primer on Methanogenesis and Methanotrophy

Energy yield is a core difference between aerobic and anaerobic metabolism. Under aerobic conditions, the complete oxidation of glucose to CO_2 and water (H_2O) generates approximately 2,900 kJ mol^{-1} and can be performed by a single organism. Conversely, only roughly 400 kJ mol^{-1} are obtained from the oxidation of glucose in methanogenic environments and no single organism can complete this process alone (Megonigal et al., 2004). Instead, the mineralization of organic C to CO_2 under anaerobic conditions is a multistep process performed by a diverse microbial consortium, with each group conserving a proportion of the total energy yield. Thus, anaerobic microorganisms are adapted to conserve quantities of energy near the theoretical minimum for metabolism

(20 kJ mol^{-1}) and are often dependent upon other anaerobic taxa for their metabolic substrates.

During anaerobic decomposition, high-molecular-weight carbohydrates are broken down through three major processes: respiration, fermentation, and methanogenesis. Anaerobic respiration utilizes alternative terminal electron acceptors (TEAs), such as NO_3, Fe (III), and SO_4^{2-}, during the final stages of the electron transport chain and generates CO_2 as an end product. Fermentation occurs when organic matter simultaneously acts as the electron donor and acceptor in anaerobic respiration, forming various low-molecular-weight acids, alcohols, and H_2 that can be exploited by other anaerobes. Finally, methanogens perform the terminal step in the anaerobic mineralization of organic C: methanogenesis. Methanogens are Archaea that can be divided into at least three functional groups: hydrogenotrophs (which use CO_2 and H_2 to produce CH_4), acetoclasts (which use acetate to produce CH_4), and methylotrophic methanogens (which use methanol, methyl-amides, or methyl-sulfides to produce CH_4) (Costa & Leigh, 2014). Hydrogenotrophic and acetoclastic methanogenesis are considered to be the most important pathways of CH_4 production as other substrates have never been shown to be responsible for more than 5% of CH_4 production (Segers, 1998); although the potential for methylotrophic methanogenesis was recently demonstrated across a variety of *Sphagnum*-dominated peatlands (Zalman et al., 2018). Additionally, although hydrogenotrophy is more common among methanogen taxa, acetoclastic methanogenesis is thought to be responsible for approximately two-thirds of biogenic CH_4 production globally (Conrad, 1999). These two methanogenic pathways are mediated by distinct methanogen groups with unique environmental controls, but their

actual rates have rarely been measured under realistic conditions in wetlands (Moore et al., 2011; Bridgham et al., 2013).

Methane production is the result of a complex suite of microbial activities that include both syntrophic interactions and competition for key substrates, with these relationships further modified by the physiochemical environment. Main controllers over this process in wetlands include O_2 concentration (Roulet and Moore, 1995; Sundh et al., 1995), the amount and quality of organic matter (Christensen et al., 2003), the availability of alternative TEAs (Megonigal et al., 2004), and soil pH (Garcia et al., 2000) and temperature (Yvon-Durocher et al. 2014). Water table position is often a strong predictor of CH_4 emissions as a higher water table both increases the potential for CH_4 production by increasing the soil anaerobic zone and simultaneously decreases the potential for aerobic CH_4 oxidation (Roulet and Moore, 1995; Sundh et al., 1995).

Methanogens are dependent upon fermentative microorganisms to produce their simple substrates and must compete for these substrates with other microorganisms that use respiratory pathways with more thermodynamically favorable TEAs (i.e. NO_3^-, Fe(III), SO_4^{-2}, and even humic substances). Hence in most ecosystems, CH_4 production rates are very low until these more favorable TEAs have been consumed (Megonigal et al., 2004). For example, methanogenesis rarely occurs in surficial soils of saline systems because of the abundance of SO_4^{-2}, which allows sulfate-reducing bacteria to out-compete methanogens for C substrates. The fact that methanogenesis is often inhibited by alternative TEAs also provides evidence of competition for fermentation products and, thus, widespread C limitation of the process. Indeed, many studies have linked CH_4 production to the quantity and quality of organic compounds (Christensen et al., 2003).

Finally, as with most chemical reactions, the rate of an enzyme-catalyzed reaction, such as methanogenesis, increases as the temperature is raised. Typically, a 10 °C temperature increase will approximately double the activity of most enzymes (Petrucci and Harwood, 1989). A recent meta-analysis has shown that the seasonal variation in CH_4 emissions from a wide range of ecosystems exhibit an average temperature dependence similar to that of CH_4 production derived from pure cultures of methanogens and anaerobic microbial communities (Yvon-Durocher et al. 2014). Furthermore, this study also found the CO_2:CH_4 ratio to decrease rapidly with increasing temperature, suggesting that CH_4 production has a higher sensitivity to changes in temperature than other respiratory processes.

Following its production, CH_4 is then transported out of the anaerobic zone either through diffusion, ebullition, or vegetation-mediated transfer in vascular plants. In diffusive transport, CH_4 is oxidized in the aerobic region of the soil, which has been shown to consume 40 and 70% of gross CH_4 production on average (Megonigal et al., 2004). Aerobic CH_4 oxidation is carried out by obligate methanotrophic bacteria (Hanson & Hanson, 1996) that convert CH_4 gas to CO_2. This process occurs across non-flooded areas, as well as oxic horizons or microsites within the soil profile, and is limited by the diffusion rate of its required substrates (O_2 and CH_4) (Sundh et al., 1995). Most aerobic methanotrophs are members of the Proteobacteria or Verrucomicrobia, with the latter more recently described and less studied. The Proteobacteria methanotrophs are divided into two distinct classes: Gammaproteobacteria (a.k.a. Type I methanotrophs) and Alphaproteobacteria (a.k.a. Type II methanotrophs) (Hanson & Hanson, 1996). These two types of methanotrophs have distinct characteristics and ecological traits (Ho et al.,

2013; Knief, 2015), including differences in activity, growth rate, stress tolerance, and affinity for CH_4. Gammaproteobacteria methanotrophs tend to have higher activity and growth rates under optimal (i.e. high CH_4) conditions but decline more quickly under stress (such as desiccation, low resource levels, or declining O_2). Alphaproteobacteria methanotrophs tend to have lower rates of activity and growth, but can persist under stressful conditions, including low CH_4 levels.

Aerobic methanotrophy is not only the best-studied process of wetland CH_4 consumption, but has historically been assumed to be the *only* pathway of CH_4 oxidation in freshwater ecosystems. In sulfate-rich marine environments, anaerobic oxidation of CH_4 (AOM) is an important process, consuming as much as 90% of the CH_4 produced (Hinrichs & Boetius, 2002; Reeburgh, 2007) through a reaction exclusively coupled to SO_4^{-2} reduction (Boetius et al 2000; Orphan et al., 2001; Michaelis et al., 2002). Freshwater ecosystems generally have low concentrations of SO_4^{-2} and, thus, AOM was thought to be negligible in these systems. However, recent studies have reported AOM driven through other TEAs, such as NO_3^- (Hu et al., 2014) and Fe (III) (Crowe et al., 2011), and suggest that this process is widespread in freshwater wetlands where it has the potential to consume as much as 50% of the CH_4 produced (Segarra et al., 2015). Despite these recent advances in our understanding of AOM, global rates remain poorly constrained (1.6-49 Tg CH_4 yr^{-1} in peatlands; Gupta et al., 2013) and the principal drivers of the process (Gupta et al., 2013) and potential impacts on ongoing environmental change remain unknown.

Dissertation Research

The overall objectives of my dissertation are to (1) provide critical knowledge about the rates of CH_4 cycling processes across a variety of equatorial African habitats, as well as the microbial and biogeochemical controls over these processes, and (2) to expand our mechanistic understanding of how substrate- and climate-driven variables in northern peatlands affect anaerobic C mineralization and CH_4 dynamics. Chapter II is entitled "Microbial community attributes drive methane-cycling dynamics across Congo Basin upland and wetland ecosystems" and is co-authored by Kyle M. Meyer, Brendan J. M. Bohannan, and Scott D. Bridgham. In this study, we conducted an ecosystem-scale investigation of the abiotic and biotic controls over tropical CH_4 cycling processes across a variety of central African Gabonese ecosystems using a combination of in-situ field measurements and laboratory incubations with paired biogeochemical and microbial community analyses. Our specific goals were (1) to quantify in-situ Gabonese ecosystem CH_4 fluxes, (2) to determine how abiotic variables contribute to variation in CH_4 flux across ecosystem types, and (3) to assess the ability of physiochemical measurements and microbial community attributes to predict Gabonese ecosystem CH_4 production and consumption and methanogenic pathways.

Chapter III is entitled "Does dissolved organic matter or solid peat fuel anaerobic respiration in peatlands?" and is co-authored by Laurel Pfeifer-Meister, Cassandra A. Zalman, Jason K. Keller, Malak M. Tfaily, Rachel M. Wilson, Jeffrey P. Chanton, and Scott D. Bridgham. In this study, we manipulated available C sources under laboratory conditions to empirically determine the primary C source – solid-phase peat or dissolved organic matter (DOM) – fueling anaerobic respiration at surface and deep depth increments within two bogs and a poor fen in northern Minnesota. We investigated (1)

whether DOM or solid-phase peat fuels peatland anaerobic respiration, (2) whether this varies in two bogs and a poor fen in northern Minnesota, and (3) if methanogenesis deep in the peatland profile is limited by the availability of surface-derived DOM. Based on radiocarbon profiles of C sources and products of respiration (Chanton et al., 2008), we hypothesized that DOM would act as a primary driver of anaerobic respiration in peatlands across depths, but that the influence of DOM would be less in the bogs. We also expected that CH_4 production deep in the peatland profile would be stimulated by surface-derived DOM addition, and that the stimulatory response would be greater in fen vs. bog peatlands because of the greater lability of fen DOM.

Chapter IV is entitled "Stability of peatland carbon to rising temperatures" and is published in *Nature Communications* (2016). This publication is co-authored by Rachel M. Wilson, Malak M. Tfaily, Steven D. Sebestyen, Chris W. Schadt, Laurel Pfeifer-Meister, Cassandra A. Zalman, Karis J. McFarlane, Joel E. Kostka, Max Kolton, Randy K. Kolka, Laurel A. Kluber, Jason K. Keller, Tom P. Guilderson, Natalie A. Griffiths, Jeffrey P. Chanton, Scott D. Bridgham, and Paul J. Hanson. We assessed how northern peatland ecosystems respond to a changing climate in collaboration with the Spruce and Peatland Responses Under Changing Environments (SPRUCE) project, a regression-based, ecosystem-scale climate manipulation experiment. Our specific objective was to determine if the slow decomposition of deep peat C was due to kinetic constrains, which would result in parallel increases in CH_4 and CO_2 production rates as global temperatures increase. To address this objective, we combined multiple lines of evidence, including in situ greenhouse gas fluxes, laboratory incubations, in situ analyses of ^{14}C and dissolved

gasses, and microbial community structure and metabolic potential, to evaluate the response of a northern Minnesota peatland following 13 months of deep-peat heating.

Chapter V is entitled "Rising temperatures increase peatland methane production and anaerobic oxidation throughout the entire soil profile" and is co-authored with Kaitlin Brunik, Laurel Pfeifer-Meister, Jason K. Keller, Glenn Woerndle, Cassandra A. Zalman, Paul Hanson, and Scott D. Bridgham. This chapter builds off the research conducted in Chapter IV by examining the response of peatland anaerobic CH_4 cycling to whole-ecosystem warming (WEW) and elevated atmospheric CO_2 (eCO_2) concentrations using controlled laboratory incubations, completed under near-in-situ conditions, of peat samples collected from surface (30 cm) to deep (200 cm) depth increments. Specifically, we investigated changes in peatland CH_4 production, CO_2:CH_4 ratios, and AOM throughout the entire peatland profile following 14 months of WEW and initial responses to eCO_2 ($\leq$ 4 months). We hypothesized that (1) CH_4 production rates would positively respond to increasing temperatures in surficial soil horizons, but that deeper soil layers would be unaffected by temperature increases during WEW. We expected that (2) eCO_2 would further stimulate surface rates of methanogenesis by increasing methanogenic substrate availability through heightened rates of plant root exudation. Taken together, we hypothesized that these effects would (3) decrease surface CO_2:CH_4 ratios, but that those of deeper soil layers would remain constant. Finally, we anticipated that (4) AOM would occur in surficial soil layers where organic TEAs could be periodically re-oxidized by water-table fluctuations.

Chapter VI summaries the results of the preceding chapters (II-V) and discusses implications for future research.

CHAPTER II

MICROBIAL COMMUNITY ATTRIBUTES DRIVE METHANE-CYCLING DYNAMICS ACROSS CONGO BASIN UPLAND AND WETLAND ECOSYSTEMS

Contributions

This chapter is co-authored by myself, Kyle M. Meyer, Brendan J. M. Bohannan, and Scott D. Bridgham. Kyle M. Meyer and I are co-first authors on this paper. Biogeochemical data collection, analysis, and interpretation were performed by myself, while microbial community data collection, analysis, and interpretation were completed by Kyle M. Meyer. All four co-authors contributed to the experimental design and field work of this study. Brendan J. M. Bohannan and Scott D. Bridgham filled the advisory roles on this project and provided text edits.

Introduction

Methane is 45 times more effective in retaining heat in the atmosphere over a 100-year time frame relative to carbon dioxide (CO_2) (Neubauer & Megonigal, 2015); therefore, small changes in its atmospheric concentration have large implications for future climate (Myhre et al., 2013). The atmospheric concentration of CH_4 has increased by over 150% since pre-industrial times, making it responsible for about 20% of human-induced radiative forcing and the second most important anthropogenic greenhouse gas after CO_2 (Myhre et al., 2013). A recent, multi-decadal analysis of changes in atmospheric CH_4 levels found that, after a period of stabilization in the early 2000s, CH_4

levels have begun rising again, partially due to increased emissions from wetlands (Kirschke et al., 2013).

Wetlands are responsible for about one-third of global CH_4 emissions (500 to 600 Tg CH_4 yr^{-1}) and have the largest uncertainty of any CH_4 emission source (Bridgham et al., 2013; Kirschke et al., 2013). Wetlands tend to show a bimodal distribution worldwide, with the largest concentrations occurring in the tropics and boreal/arctic areas. Tropical wetlands are responsible for 47 to 89% of global CH_4 emissions, with equatorial Africa, the Amazon Basin, and Southeast Asia being particular emission "hot spots" (Kirschke et al., 2013). Additionally, although CH_4 emission increases have recently been observed in boreal and arctic regions, the largest increases have occurred throughout tropical areas (Spahni et al., 2011). While extensive research has been conducted in temperate, boreal, and arctic zones to understand biogeochemical and microbial controls on wetland CH_4 emissions, little research has been conducted across tropical regions (Bridgham et al., 2013; Kirschke et al., 2013; Melton et al., 2014). It is unlikely that the anaerobic carbon (C) mineralization relationships and CH_4 cycling dynamics observed in other, more heavily researched, ecosystems will transfer to equatorial regions given the distinct differences in climate and soil type (Spahni et al., 2011). Additionally, these different environments also likely select for dissimilar microbial traits relevant to CH_4 cycling. Thus, net and gross tropical ecosystem CH_4 fluxes, as well as their biogeochemical and microbial drivers and interactions, remain largely unknown.

Ecosystem CH_4 emissions are regulated by a complex set of controls over two primary, counteracting processes: CH_4 production (methanogenesis) and aerobic CH_4

consumption (methanotrophy). Both processes are mediated by distinct microbial communities whose structure, function, and activity are tightly coupled to the physiochemical characteristics of their environment (Tfaily et al., 2014; Kotiaho et al., 2010; Cadillo-Quiroz et al., 2006; Teh et al., 2005; Galand et al., 2003; Segers, 1998). Methanogens are Archaea that can be divided into at least three functional groups: hydrogenotrophs (which use CO_2 and H_2 to produce CH_4), acetoclasts (which use acetate to produce CH_4), and methylotrophic methanogens (which use methanol, methyl-amides, or methyl-sulfides to produce CH_4) (Costa & Leigh, 2014). Hydrogenotrophic and acetoclastic methanogenesis are considered to be the most important pathways of CH_4 production as other substrates have never been shown to be responsible for more than 5% of CH_4 production (Segers, 1998). Additionally, although hydrogenotrophy is more common among methanogen taxa, acetoclastic methanogenesis is thought to be responsible for approximately two-thirds of biogenic CH_4 production globally (Conrad, 1999). These two methanogenic pathways are mediated by distinct methanogen groups with unique environmental controls, but their actual rates have rarely been measured under realistic conditions in wetlands (Moore et al. 2011, Bridgham et al. 2013).

Aerobic CH_4 oxidation is carried out by obligate methanotrophic bacteria (Hanson & Hanson, 1996) that convert CH_4 to CO_2. This process occurs across non-flooded areas, as well as oxic horizons or microsites within the soil profile, and is limited by the diffusion rate of its required substrates (O_2 and CH_4) (Sundh et al., 1995). Most aerobic methanotrophs are members of the Proteobacteria or Verrucomicrobia, with the latter more recently described and less studied. The Proteobacteria methanotrophs are divided into two distinct classes: Gammaproteobacteria (a.k.a. Type I methanotrophs) and

Alphaproteobacteria (a.k.a. Type II methanotrophs) (Hanson & Hanson, 1996). These two types of methanotrophs have distinct characteristics and ecological traits (Ho et al., 2013; Knief, 2015), including differences in activity, growth rate, stress tolerance, and affinity for CH_4. For example, Gammaproteobacteria methanotrophs tend to have higher activity and growth rates under optimal (e.g., high CH_4) conditions, but decline more quickly under stress (such as desiccation, low resource levels, or declining O_2). Conversely, alphaproteobacteria methanotrophs tend to have lower rates of activity and growth, but can persist under stressful conditions, including low CH_4 levels.

Individual ecosystem processes such as methanogenesis and methanotrophy face microbial community-level constraints that may act as important predictors of process rates. One commonly observed form of limitation is driven by numerical constraints (i.e. abundance-limitation), whereby the process can only proceed as quickly as the total number of microorganisms in the environment capable of carrying out that process. This has been shown for both methanogenesis (Ma et al., 2012) and methanotrophy (Freitag & Prosser, 2009), where a positive relationship occurred between functional group abundance and process rates. Additionally, distinguishing between active and inactive individuals may be especially important when considering that most soil organisms are in an inactive state at any given time (Lennon and Jones, 2011). Therefore, even when there may be more than enough microorganisms with the potential to carry out a process, the rate of that process could still be hindered by the level of activity exhibited by those microbes, and, thus, determining the total number of active individuals involved in a process should help us further refine our predictive capability of that process (as shown in Freitag et al., 2010).

The composition (i.e. membership) of a community can impose additional constraints on important ecosystem processes (e.g., Nazaries et al., 2013). This level of control on process rates involves the suites of traits (i.e. organismal characteristics involved in growth, survival, or reproduction) exhibited by community members. Even closely related methanogen/methanotroph taxa can differ in traits, such as substrate affinity, substrate preference, disturbance tolerance, or competitive ability (Ho et al., 2013), suggesting that communities differing in composition will likely differ in the rates by which they perform ecosystem processes.

Lastly, microbial functional group diversity may also play a role in predicting ecosystem processes. The abilities to produce and consume CH_4 are both highly phylogenetically conserved (Martiny et al., 2013), suggesting that relatively few taxa possess the ability to carry out these processes. This implies that there are likely lower levels of functional redundancy in these groups, which could influence the rate, as well as the resilience or stability of this process through time, a trend that has been demonstrated experimentally in methanotroph communities (Schnyder et al., 2018). Thus, an important step towards better predicting ecosystem functions, such as methanogenesis and methanotrophy, is to elucidate and quantify the attributes of a community that may impose constraints on these process rates.

While there has been extensive work focusing on the drivers of CH_4 cycling from high latitude zones, there has been considerably less in tropical areas. This is especially true for tropical Africa, which has been the focus of few studies (Delmas et al., 1992; Tathy et al., 1992; MacDonald et al., 1998; MacDonald et al., 1999; Prieme & Christensen, 1999; Werner et al., 2007), despite its substantial contribution to global CH_4

emissions (Kirschke et al., 2013). To address this knowledge gap, we conducted an ecosystem-scale investigation of the abiotic and biotic controls over tropical CH_4 cycling processes across a variety of central African Gabonese ecosystems using a combination of in-situ field measurements and laboratory incubations with paired biogeochemical and microbial community analyses. Our specific goals were (1) to quantify in-situ Gabonese ecosystem CH_4 fluxes, (2) to determine how abiotic variables contribute to variation in CH_4 flux across ecosystem types, and (3) to assess the ability of physiochemical measurements and microbial community attributes to predict Gabonese ecosystem CH_4 production and consumption and methanogenic pathways.

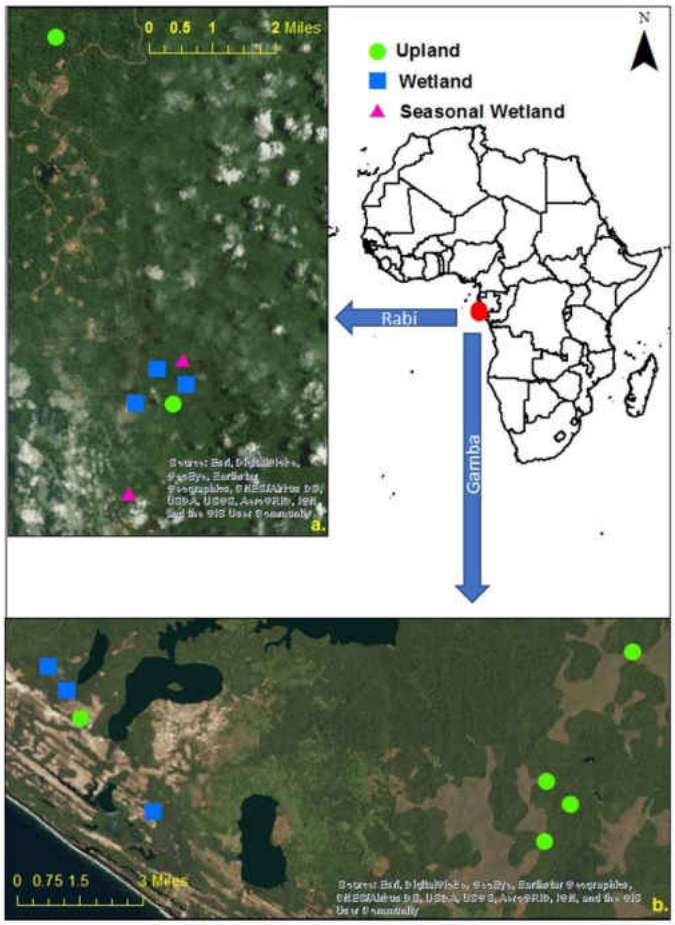


Figure 2.1. Aerial view of Gabonese study site locations in **(a)** Rabi and **(b)** Gamba. Upland sites are denoted with green circles, wetlands sites are shown with blue squares, and seasonal wetlands are represented as pink triangles.

Methods

Site selection and sampling: We investigated abiotic and biotic controls over ecosystem CH_4 cycling dynamics along a wetland to upland gradient in the equatorial African nation

of Gabon. The southwestern (SW) region of Gabon, along the Atlantic coastline, is composed of a matrix of natural tropical ecosystems, including wetlands, riverine systems, swamps, mangroves, forests, grasslands, and lagoons. We conducted an ecosystem-scale survey of CH_4 cycling dynamics throughout two locations, Gamba and Rabi, in SW Gabon in October and November 2014 (Figure 2.1). We selected a diverse assortment of 15 study sites, including 2 organic-soil wetlands, 4 mineral-soil wetlands, 2 seasonally inundated mineral-soil forests, 2 upland grasslands (1 with termite mounds), 3 upland forests, a 1-year old plantation, and 1 abandoned plantation, for biogeochemical and microbial analysis. Field sampling took place at the beginning of the rainy season and, thus, all upland and seasonal wetland sites were dry, with water tables ≥ 40 cm below the surface, while wetland water table positions were, on average, 10 cm below the surface. Seasonal wetlands were identified using established field identification criterion, such as tree water-marks and redoximorphic soil characteristics.

In-situ CH_4 flux: Transects of six static chambers were established at each site to measure ecosystem CH_4 flux. Due to the remote location of our field sites, we used a light-weight chamber design by retro-fitting 8 L opaque, plastic buckets (bottom diameter = 22 cm, top diameter = 21 cm) with 20-mm sampling ports, 15-mm ventilation ports, internal electric fans (120 x 120 mm, 12V, Allied Electronics, Fort Worth, Texas), and beveled edges. The chambers were installed at seven-meter intervals and 2.5 cm into the soil to ensure a gas-tight seal. After installation, we opened the ventilation port for 40 minutes to allow the internal chamber headspace to come to equilibrium with the external atmosphere. Upland site chambers were spiked with 5 ppmv CH_4/mL headspace to

measure rates of in-situ CH_4 oxidation, while wetland site chambers received no additional CH_4. Then, 13-mL gas samples were pulled from the chamber headspace at 0, 5, 10, 20, and 40 minutes and stored in pre-evacuated 12 mL Exetainers (LABCO Ltd.; www.labco.uk.com). Upon return to the University of Oregon (UO), CH_4 concentrations were determined with an SRI 8610C gas chromatograph (GC; Torrance, CA) equipped with a methanizer and flame ionization detector. Flux rates were determined by the accumulation of CH_4 over time, following adjustment for volume and temperature. Only CH_4 fluxes with $r^2 \geq 0.63$ were included in subsequent analyses. One upland grassland site was not spiked with CH_4 prior to sample collection and we were unable to measure changes in CH_4 concentration because they were below our detection limits. Additionally, the thick root layer of one forested upland site resulted in a non-gas-tight chamber seal and prevented us from reporting flux estimates for this site. Therefore, flux estimates are given for 13 sites, while subsequent laboratory experiments encompass all 15 sites.

Potential CH_4 oxidation: We collected three intact soil cores from above the water table at the beginning, middle, and end of each field transect. The samples were stored in PVC tubes (diameter = 5 cm, height = 8 cm) to maintain soil structure (aerobic and anaerobic microsites) and kept at in-situ air temperatures (~28 °C) until the end of the sampling trip (~3 weeks). Upon return to UO, each PVC core was placed into a gas-tight Mason jar that was retro-fitted with a headspace sampling port and incubated at 28 °C in the dark. Rates of aerobic CH_4 oxidation were determined under initially low (5 ppmv CH_4 in headspace) and high (1000 ppmv CH_4 headspace) CH_4 concentrations by injecting 1 cm^3 of the

headspace into the GC at 0.33, 3, 6, and 9 hours. The same soil cores were used to determine CH_4 oxidation rates under low and high CH_4 concentrations in quick succession, with low CH_4 oxidation rates measured first to enhance our analysis sensitivity. We applied a pseudo-first-order exponential decay function to our measurements to determine the rate constant (k, units = d^{-1}; i.e. $dCH_4/dt = k[CH_4]$) of the exponential decrease in CH_4 at low initial CH_4 concentrations. The high initial CH_4 concentration of 1000 ppmv should exceed the maximum capacity of the pMMO enzymes (Baani and Liesack, 2008), meaning we will have achieved substrate saturation, so we used a pseudo-zero-order linear equation to determine maximum velocity rates of CH_4 consumption (V_{max}, units = μmol CH_4 cm^{-3} d^{-1}) in these samples. Final rates of CH_4 oxidation represent the average of three replicates from each site.

Potential CH_4 and CO_2 production and methanogenic pathways: Across all wetland sites (n = 6), we collected three soil samples from 0 – 10 cm below the water table at the beginning, middle, and end of each field transect. The samples were topped with site water and tightly sealed in 50 mL centrifuge tubes to ensure an anaerobic environment, stored at air temperature (~28 °C) to reflect near in-situ conditions, and shipped to UO at the end of the sampling trip (~3 weeks). Anaerobic incubations commenced at field temperatures within two days of returning to the USA. In a glove box filled with a N_2 atmosphere (<5% H_2 in the presence of palladium catalyst; Coy Laboratory Products, Grass Lake, Michigan), approximately 10 g of wet weight soil were added to 120 mL serum bottles and mixed with 10 mL of deoxygenated, deionized water. Sample bottles were then flushed with N_2 for 15 minutes to begin the incubation. Headspace samples

were analyzed over the course of three days (0, 1, 2, and 3 days) for CH_4 and CO_2 simultaneously by GC. Total CH_4 and CO_2 were calculated using Henry's Law, adjusting for solubility, temperature, and pH (Bridgham &Ye, 2013). Methane and CO_2 production rates were calculated using the linear accumulation ($r^2 > 0.90$ in all cases) of gasses through time. Additionally, a $^{14}CO_2$ tracer method (Keller & Bridgham, 2007; Ye et al., 2012) was used to measure hydrogenotrophic methanogenesis over three days with a gas chromatograph fitted with a radioactive gas proportional counter (IN/US Systems Inc., Pinebrook, NJ), with rates of acetoclastic methanogenesis determined by difference from total CH_4 production. Rates of CH_4 and CO_2 production, as well as methanogenic pathways, represent the average of three replicates per site.

Soil physical and chemical analysis: We measured a suite of abiotic variables across all sites to assess their relative ability to predict CH_4 flux and potential CH_4 production and oxidation. At wetland sites, we recorded pH (Oakton WP pHTestr 10, The Lab Depot, Dawsonville, GA), water table position, and soil temperature 5 cm below the surface from within each field chamber (n = 6). Total % N and organic C and moisture content, as well as soil texture, were determined in the laboratory using soil cores (n = 3 per depth) collected from 0 – 8 cm above and 0 – 10 cm below the water table at the beginning, middle, and end of each field transect. Total % N and organic C were measured on a Costech ECS 4010 Elemental Analyzer (Valencia, CA), with each sample analyzed in duplicate. Moisture content was measured by the change in weight of a soil sub-sample following 48 hours of drying at 60 °C. Finally, material from the three soil

cores was combined and homogenized for texture analysis using the hydrometer method (Gavlak et al., 2003), with 5% sodium hexametaphosphate as the dispersing solution.

At seasonal wetland and upland sites, we measured the following abiotic variables: soil temperature, pH, moisture content, total % N and organic C, and soil texture. Soil temperature 5 cm below the surface was recorded from within each field chamber (n = 6), while all other abiotic variables were determined in the laboratory from soil cores collected 0 – 8 cm below the surface at the beginning, middle, and end of each transect. Total % N and organic C, moisture content, and soil texture were measured as described above. Soil pH was determined from a 1:1 soil to deionized water solution, with a Denver Instrument ultrabasic pH meter (Bohemia, NY).

Soil RNA/DNA co-extraction and sequencing: At the beginning of anaerobic and the end of aerobic laboratory incubations, a subset of each soil sample was collected and preserved with Lifegaurd for microbial community analysis. We collected soil samples from the beginning of anaerobic incubations due to the addition of a radioisotope tracer and at the end of aerobic incubations because we did not want to disturb the intact soil cores. Soil DNA and RNA were co-extracted using MoBio's Powersoil RNA Isolation Kit with the DNA Elution Accessory Kit (MoBio, California, USA) following manufacturer's instructions. RNA was reverse transcribed to cDNA using Superscript III first-strand reverse transcriptase and random hexamer primers (Life Technologies, USA). Extractions were quantified using Qubit (Life Technologies, USA). All known methanogens express an isozyme of methyl-coenzyme M reductase; the gene encoding the α subunit of this enzyme (*mcrA*) is commonly used as a genetic marker for the

detection, enumeration, and classification of methanogens (Luton et al., 2002). We amplified two gene targets in sample DNA and cDNA: 1) the V4 region of the 16S SSU rRNA gene using the primers 515F and 806R (Caporaso et al., 2011), and 2) the *mcrA* gene using the mlas and mcrA-rev primer combination (Steinberg & Regan, 2008). Sequencing libraries were prepped using a dual-indexing approach (Kozich et al., 2013; Fadrosh et al., 2014). In short, each PCR reaction was performed using 12.5 µl NEBNext Q5 Hot Start HiFi PCR master mix (New England Biolabs, USA), 11.5 µl gene-specific primer mix (1.09 µM of each primer), and 1 µl template (DNA or cDNA). A sample subset was used to find 1) the optimal primer annealing temperature, and 2) the minimum number of cycles for adequate target amplification. For the 16S rRNA gene target this was 61° C and 20 cycles, for mcrA this was 67° C and 30 and 25 cycles for cDNA and DNA, respectively. The final reaction conditions were: 98° C and 30 seconds (initialization), 98° C and 10 seconds (denaturation), gene-specific annealing step for 20 seconds (see above), and 72° C for 20 seconds (final extension). Reactions were followed by magnetic bead purification using 20 µl Mag-Bind RxnPure Plus isolation beads (Omega Bio-Tek, USA). Reactions were quantified using Qubit (Life Technologies, USA), then were pooled together at equimolar concentrations. Final pooled amplicon libraries were sequenced using the Illumina Miseq (300PE) platform at the Oregon State University Center for Genome Research and Biocomputing facility.

Bioinformatic processing: Paired end reads were joined, then demultiplexed in QIIME (Caporaso et al., 2010) before quality filtering. Primers were removed using a custom script. UPARSE was used to quality filter and truncate sequences (Edgar, 2013).

Sequences were retained only if they had an identical duplicate in the database. Operational taxonomic units (OTUs) were clustered *de novo* at 97% similarity using USEARCH (Edgar, 2010). OTUs were checked for chimeras using the gold database in USEARCH. We used a custom script to format the UCLUST output for input into QIIME. To assign taxonomy, we used the repset from UPARSE in QIIME using greengenes version 13_5 (RDP classifier algorithm).

The 16S community data were divided for previously reported methanogen and methanotroph taxa. Methanogen and methanotrophs community matrices were rarefied 100 times each, to avoid problems associated with unequal sampling extent.

Quantitative PCR: We quantified the abundance of methanogens in the laboratory soil samples used to determine rates of CH_4 production using qPCR of the mcrA gene with the mlas-mcrA-rev primer combination (Steinberg & Regan, 2008). Samples were run on an ABI StepOnePlus thermocycler (ABI, USA), using Kapa SYBR reagents (Kapa Biosystems, USA) according to manufacturer recommendations. For each sample, 8 ng DNA was used, and the following amplification conditions were applied following optimization: 98° C for 10 minutes, 98° C for 15 seconds, 55.6° C for 15 seconds, and 72° C for 60 seconds. A melt curve analysis was performed to verify target amplification. We used a similar approach to quantify methanotroph abundance and transcriptional activity by targeting pmoA in the sample DNA and cDNA, respectively, using the A189 – mb661 primer combination (Bourne et al., 2001). Reactions were performed on a Bio-Rad CFX96 real-time qPCR instrument, using SsoAdvanced Universal SYBR Green supermix (Bio-Rad, USA) following manufacturer instructions. For each sample, 2 ng of

template were used with the following reaction conditions: 98° C for 10 minutes, 98° C for 15 seconds, 55.6° C for 15 seconds, and 72°C for 60 seconds. All samples were amplified in triplicate. In both cases, sample amplification was compared to standard positive control to quantify total gene (or transcript) copy number. In the case of mcrA, the positive control was a mcrA plasmid and in the case of pmoA we used purified DNA from strain *Methylococcus capsulatus* (ATCC 33009D-5). We used LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009) to process amplification data, which allows for the calculation of individual PCR efficiencies. We tested whether individual PCR efficiencies differed among habitat types. This was not the case, so gene copy was calculated using the average PCR efficiency of all reactions. Finally, gene copy (or transcript) number was normalized to total ng DNA (or cDNA) used in the reaction.

Statistical analysis: All statistical analyses were completed using R Statistical Software. Data were tested for normality and log-transformed where the transformation resulted in an improvement in the overall distribution. Transformed values were then used in subsequent analyses; however, non-transformed data are shown in figures to convey actual process rates. For all analyses, seasonal mineral-soil wetland sites were grouped with upland sites as they were dry with deep ($\geq$ -40 cm) water tables at the time of sampling (and likely had been for some months prior during the dry season).

One-way analysis of variance (ANOVA) was used to determine whether CH_4 fluxes varied across wetland and upland sites, followed by Tukey's HSD post-hoc tests ($p < 0.05$) to investigate pairwise differences when appropriate. Stepwise multiple linear regression with Akaike Information Criterion (AIC) as the model selection condition was

used to assess the ability of abiotic variables to predict wetland and upland CH_4 flux. Abiotic variables collected for all wetland flux chambers included pH, water table position, soil temperature 5 cm below the soil surface, and soil texture (sand, silt, and clay content (%)). Additionally, total N and organic C (%) were also measured for a subset of the wetland flux chambers using the collected soil cores (n = 3). Thus, multiple linear regression analyses were run twice with the full and subset CH_4 flux data sets for wetland sites. Conversely, only a subset of the CH_4 flux values were used for this analysis in upland sites because the abiotic predictor variables could only be measured from the collected soil cores. For upland sites, pH, soil temperature 5 cm below the surface, soil texture (sand, silt, and clay content (%)), moisture content, and total % N and organic C were included as predictor variables. After removing predictor variables with correlations ≥ 0.7, model analyses included the following abiotic factors: (1) full wetland CH_4 flux data set = soil pH and temperature, water table position, and soil clay and silt content, (2) subset CH_4 flux data set = soil pH and temperature, water table position, organic C content, (3) upland CH_4 flux data set = soil pH and temperature, organic C content, and soil silt and sand content.

We employed a similar statistical approach to investigate site-specific differences and potential ecosystem process predictor variables from our laboratory incubation data. One-way ANOVA was again used to investigate whether the exponential decay constant k (d^{-1}) and V_{max} values (μmol CH_4 g $soil^{-1}$ d^{-1}) measured from CH_4 oxidation laboratory experiments differed between wetland and upland sites, as well as whether CH_4 production rates, CO_2:CH_4 ratios, and methanogenic pathway dominances measured from anaerobic laboratory incubations varied across wetland sites. If sites differed

significantly, Tukey's HSD post-hoc tests ($p < 0.05$) were used to find pairwise differences. Stepwise multiple linear regression with AIC as the model selection criteria was used to determine the ability of abiotic variables to predict rates of CH_4 oxidation under low (k) and high (V_{max}) initial CH_4 concentrations in wetland and upland sites and rates of CH_4 production in wetland sites. For all analyses using anaerobic and aerobic laboratory incubations, pH, soil texture (sand, silt, and clay content (%)), moisture content (%), and total % N and organic C were included as predictor variables. Water table position was also included as a predictor variable for analyses using anaerobic incubation data. Following the removal of predictor variables with correlations ≥ 0.7, model analyses included these abiotic factors: (1, 2) wetland low- and high-affinity CH_4 oxidation = soil pH and temperature, and organic C content, (3) upland low-affinity CH_4 oxidation = soil pH, temperature, sand content, and moisture content (4) upland high-affinity CH_4 oxidation = soil pH, temperature, sand content, and organic C content, (5) wetland CH_4 production = soil pH, temperature, organic C content, and water table position.

We tested whether four main groups of community attributes were related to CH_4 cycling dynamics: 1) the abundance of functional groups (i.e. methanogen and methanotroph) -or their relative abundance in the prokaryotic community, 2) the diversity of methanogen/methanotroph communities (using species richness and Shannon diversity), 3) the composition of methanogen/methanotroph communities (using the relative abundance of individual taxa or broader taxonomic groups, (e.g., genera, or families), and 4) the activity of methanogens/methanotrophs, by inferring communities via RNA and using the aforementioned approaches, or in the case of methanotrophy, by

quantifying pmoA transcriptional level using qPCR. Community attributes were correlated with methane dynamics (i.e. CH_4 production, pathway predominance, CO_2:CH_4 production ratio, low-affinity CH_4 oxidation, or high-affinity CH_4 oxidation) using a linear model assuming a Gaussian distribution. In the case of large numbers of variables (e.g., the relative abundance of many different taxa), we corrected for multiple comparisons by multiplying the p-value of each correlation by the total number of comparisons (i.e. the total number of taxa being tested).

Results

Soil physical and chemical analysis: Soil pH exhibited a wide range of variation (3.6-6.1) across upland and wetland sites, while soil temperature was relatively consistent (26.1 – 31.0 ℃) among sites (Table 2.1). Mineral soils were composed predominantly of sand (~86%), with little silt (~12%) and clay (~2%) content, resulting in the low N (~0.4%) and moderate organic C (~7%) concentrations typical of tropical ecosystems.

Table 2.1. Characterization of the physical and chemical variables measured across all sites. Averages with standard errors shown, as well as the number of replicates for each analysis. WT = water table; T = temperature; MC_a = moisture content above the water table.

Ecosystem Type	pH	WT (± cm)	Soil T (°C)	% Total N	% Organic C	% Clay	% Sand	% Silt	MC_a (%)
Open Peatland	3.8 ± 0.02 (n=4)	-11.3 ± 3.1 (n=4)	29.6 ± 0.3 (n=4)	1.6 ± 0.01 (n=3)	39.6 ± 0.9 (n=3)	0	0	0	89.2 ± 0.9 (n=3)
Forested Peatland	3.6 ± 0.1 (n=3)	-22.9 ± 3.9 (n=3)	26.6 ± 0.1 (n=3)	1.2 ± 0.1 (n=2)	45.7 ± 1.6 (n=2)	0	0	0	74.7 ± 3.1 (n=3)
Forested MSW	6.1 ± 0.1 (n=5)	-6.9 ± 1.5 (n=5)	26.9 ± 0.2 (n=5)	1.3 ± 0.7 (n=2)	24.7 ± 16.0 (n=2)	1.2	95.1	3.7	60.1 ± 17.9 (n=3)
Forested MSW	5.0 ± 0.1 (n=4)	-1.7 ± 7.2 (n=4)	26.1 ± 0.1 (n=4)	0.4 ± 0.2 (n=2)	5.5 ± 2.8 (n=2)	3.2	84.2	12.6	49.7 ± 3.9 (n=3)

Forested MSW	4.7 ± 0.3 (n=5)	-5.1 ± 3.4 (n=5)	26.9 ± 0.2 (n=5)	0.5 ± 0.4 (n=2)	9.9 ± 9.3 (n=2)	2.3	80.1	17.6	49.1 ± 13.3 (n=3)
Forested MSW	4.8 ± 0.1 (n=6)	-10.9 ± 5.3 (n=6)	27.0 ± 0.1 (n=6)	0.2 ± 0.04 (n=3)	5.1 ± 2.5 (n=3)	1.2	75.3	23.5	31.9 ± 10.4 (n=3)
Seasonal MSW	4.2 ± 0.1 (n=3)	N/A	26.4 ± 0.1 (n=6)	0.3 ± 0.05 (n=3)	3.6 ± 0.7 (n=3)	1.5	79.6	18.9	22.9 ± 2.1 (n=3)
Seasonal MSW	4.1 ± 0.1 (n=3)	N/A	26.7 ± 0.1 (n=6)	1.2 ± 0.2 (n=3)	23.6 ± 4.2 (n=3)	1.7	98.3	0	53.5 ± 3.7 (n=3)
Upland Grassland	4.3 ± 0.03 (n=3)	N/A	29.6 ± 0.11 (n=6)	0.1 ± 0.07 (n=3)	3.7 ± 2.9 (n=3)	0.4	99.2	0.4	1.7 ± 0.4 (n=3)
Upland Grassland (with termite mounds)	4.7 ± 0.2 (n=3)	N/A	28.0 ± 0.03 (n=6)	0.04 ± 0.01 (n=3)	0.8 ± 0.1 (n=3)	1.1	98.1	0.8	4.6 ± 0.5 (n=3)
Upland Forest	3.9 ± 0.03 (n=3)	N/A	26.5 ± 0.3 (n=5)	0.2 ± 0.02 (n=3)	2.1 ± 0.2 (n=3)	3.6	84.2	12.2	11.1 ± 1.2 (n=3)
Upland Forest	4.1 ± 0.1 (n=3)	N/A	26.7 ± 0.1 (n=6)	0.2 ± 0.01 (n=3)	2.0 ± 0.3 (n=3)	4.1	66.9	29.0	12.9 ± 0.1 (n=3)
Upland Forest	4.0 ± 0.06 (n=3)	N/A	27.0 ± 0.1 (n=6)	0.4 ± 0.2 (n=3)	7.3 ± 4.0 (n=3)	0	89.3	10.7	23.5 ± 7.3 (n=3)
Upland Plantation	4.0 ± 0.1 (n=3)	N/A	31.0 ± 0.3 (n=5)	0.1 ± 0.04 (n=3)	1.3 ± 0.5 (n=3)	8.1	66.0	25.9	12.1 ± 1.2 (n=3)
Upland Ab. Plantation	4.1 ± 0.03 (n=3)	N/A	27.0 ± 0.3 (n=6)	0.1 ± 0.03 (n=3)	1.8 ± 0.6 (n=3)	3.3	95.9	0.8	6.2 ± 1.6 (n=3)

In-situ CH_4 flux: Gabonese wetland CH_4 fluxes were extremely variable both across and within sites (Figure 2.2a), particularly in mineral-soil wetlands. We observed net consumption rates of up to 6.3 μmol CH_4 m^2 d^{-1} and net emission rates of as much as 121.6 μmol CH_4 m^2 d^{-1}, with an average flux of 16.1 ± 31.4 μmol CH_4 m^2 d^{-1}. Given this high variability, CH_4 fluxes across wetland sites did not differ significantly ($F = 0.57$, $p = 0.72$). Water table position was the best predictor of wetland CH_4 flux with both the full

(F = 6.89, p = 0.015) and subset (F = 2.69, p = 0.127) data sets, explaining 18% and 12% of its variance, respectively.

Gabonese upland CH_4 fluxes were less variable than those observed in wetland sites with an average net consumption rate of 2.6 ± 1.7 μmol CH_4 m^2 d^{-1} (Figure 2.2b). Only one chamber installed in a seasonal wetland (out of 40 flux chambers total) showed net emission of 3.3 μmol CH_4 m^2 d^{-1} CH_4. Differences in CH_4 fluxes across upland sites were marginally significant (F = 1.96, p = 0.101) and driven by a difference between the fluxes of a seasonal mineral soil wetland and a plantation (p = 0.049). Soil pH and % organic C were the most predictive abiotic variables of upland CH_4 flux (F = 3.98, p = 0.037), explaining 23% of its variation.

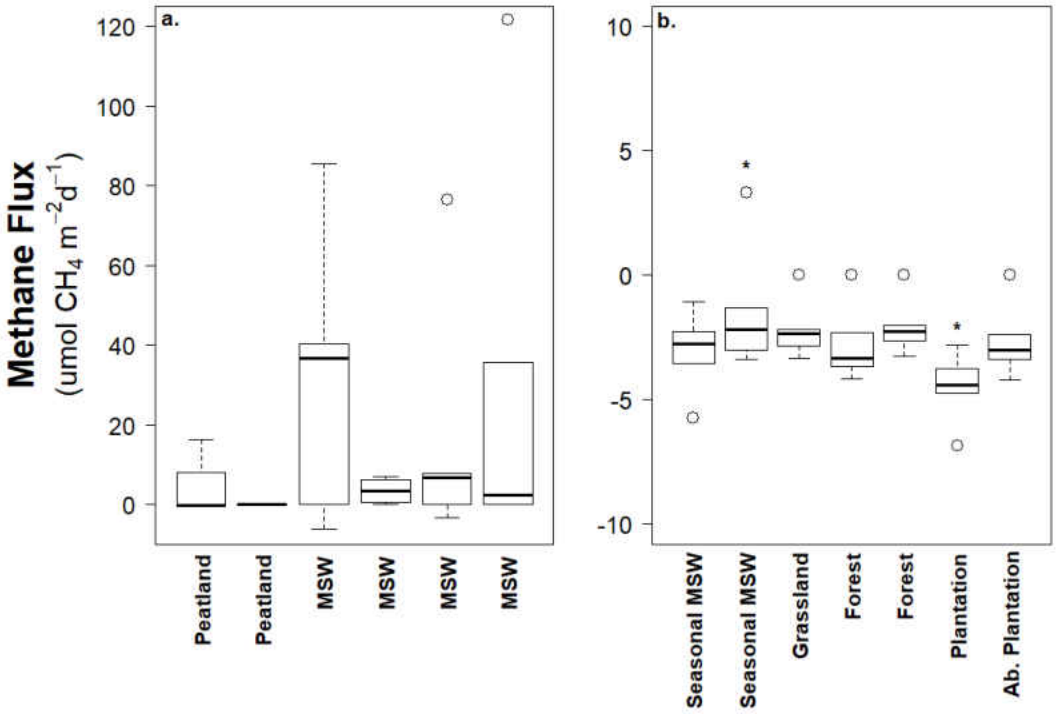


Figure 2.2. Methane fluxes measured across a variety of Gabonese (**a**) wetland and (**b**) upland ecosystems in October 2014. Data from individual chambers (n ≤ 6 per site) are displayed to emphasize intra-site variability, particularly in mineral-soil wetlands. Wetland flux rates did not vary significantly across sites, while marginally significant differences in flux rates were found across upland sites (p = 0.101). Significant differences (p < 0.05) between upland sites are denoted with *. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values (excluding outliers). Outliers are shown with open circles. MSW = mineral soil wetland; Seasonal MSW = seasonally inundated mineral soil wetland; Ab. Plantation = Abandoned Plantation.

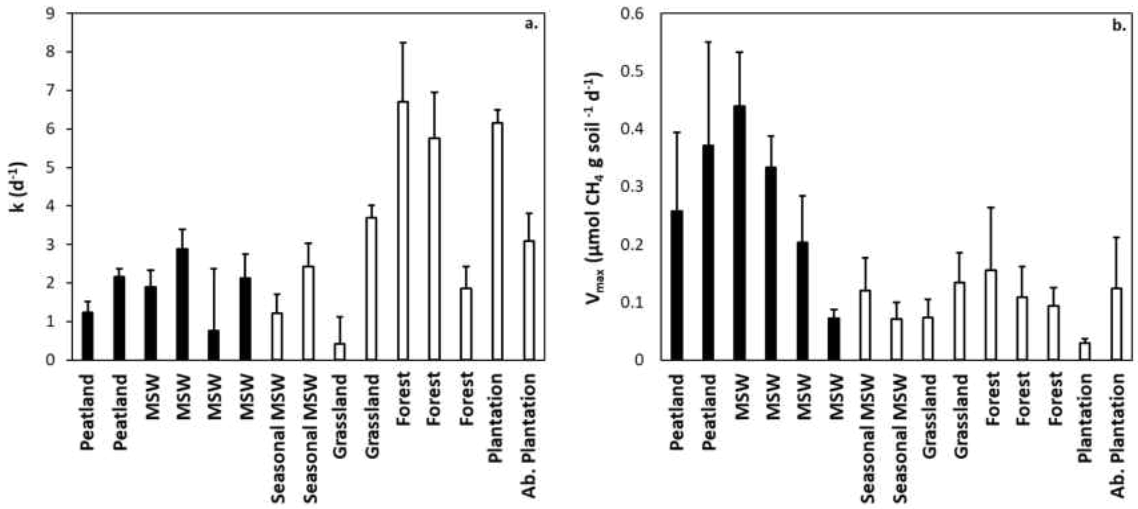


Figure 2.3. (a) Rate constant (k) for CH_4 oxidation rates at low (5 ppmv) initial CH_4 concentrations and **(b)** maximum velocity rate (V_{max}) at high (1,000 ppmv) initial CH_4 concentrations from aerobic laboratory incubations with intact Gabonese soil cores in October 2014 (n = 3). Standard error bars are shown. Wetland sites are shown in black and upland sites are shown in white. Note the different y-axes. MSW = mineral soil wetland; Seasonal MSW = seasonally inundated mineral soil wetland; Ab. Plantation = Abandoned Plantation.

Potential CH_4 oxidation:

High affinity CH_4 oxidation: During aerobic laboratory incubations, rates of CH_4 consumption under initially low CH_4 concentrations (5 ppmv CH_4) were higher in upland sites relative to wetland sites ($F = 13.77$, $p < 0.01$; Figure 2.3a). Upland site oxidation rate constants were 4.2 ± 2.3 d^{-1}, while wetland site rate constants were, on average, 133% lower, with values of 1.8 ± 1.3 d^{-1}. In upland sites, % sand and moisture content were the most predictive abiotic variables of k ($F = 3.57$, $p = 0.049$) and explained 20% of the variation. Conversely, none of the abiotic variables measured predicted wetland CH_4 oxidation under low CH_4 concentrations. Across all sites, the best explanatory variable for k was the relative abundance of a single OTU (*Methylovirgula sp.*) in the RNA-inferred community (Adj. $R^2 = 0.45$, $p < 0.001$). The relative abundance of methanotrophs in the RNA-inferred community was negatively correlated with k (Adj. R^2

= 0.12, p < 0.05, Figure 2.4a). Additionally, we observed a similar inverse relationship between uptake rate and diversity of methanotrophs in both RNA- and DNA- inferred communities (Adj. $R^2 = 0.16$, $p < 0.05$, Adj. $R^2 = 0.15$, $p < 0.01$, respectively, Figure 2.4b).

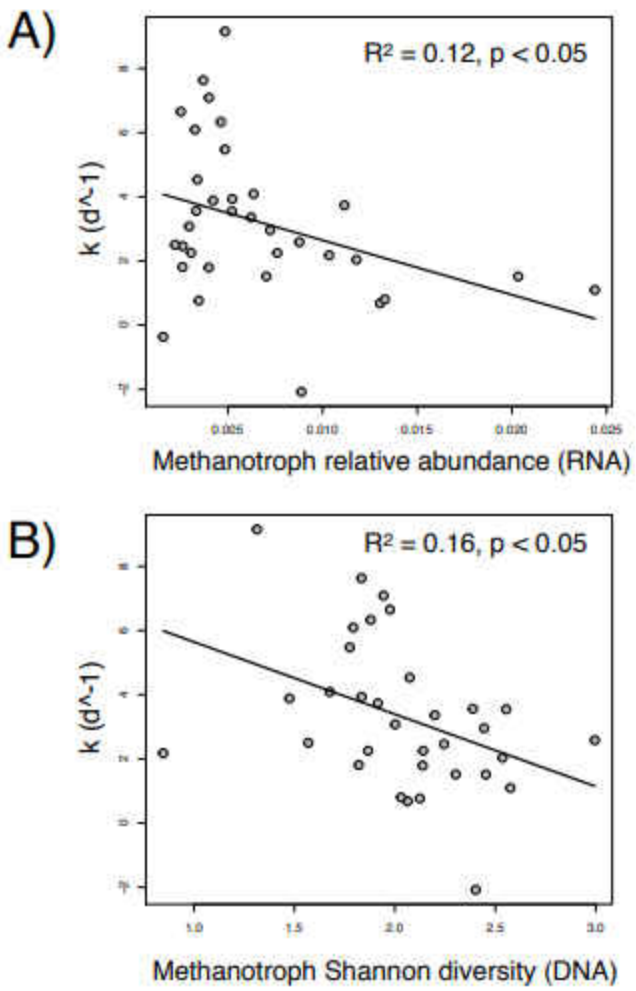


Figure 2.4. The negative relationship between k and **(a)** the relative abundance of methanotrophs in the RNA-inferred 16S prokaryotic community and **(b)** the Shannon diversity of the DNA-inferred methanotroph community.

Low affinity CH_4 oxidation: During aerobic laboratory incubations under initially high CH_4 concentrations (1,000 ppmv CH_4), wetland sites consumed roughly 200% more CH_4 than upland sites ($F = 5.83$, $p = 0.002$; Figure 2.3b). Rates of CH_4 consumption, measured as V_{max}, in wetland sites were 0.3 ± 0.05 µmol CH_4 g $soil^{-1}$ d^{-1} and those of uplands were 0.1 ± 0.02 µmol CH_4 g $soil^{-1}$ d^{-1}. Together, pH and total % organic C explained 15% of the variation in wetland V_{max} values ($F = 2.52$, $p = 0.11$), whereas none of the abiotic variables measured predicted upland V_{max} values.

Potential CH_4 production and methanogenic pathways: Wetland CH_4 production rates varied across sites ($F = 3.11$, $p = 0.05$) and ranged from 0.1 – 6.7 µmol CH_4 g $soil^{-1}$ d^{-1},

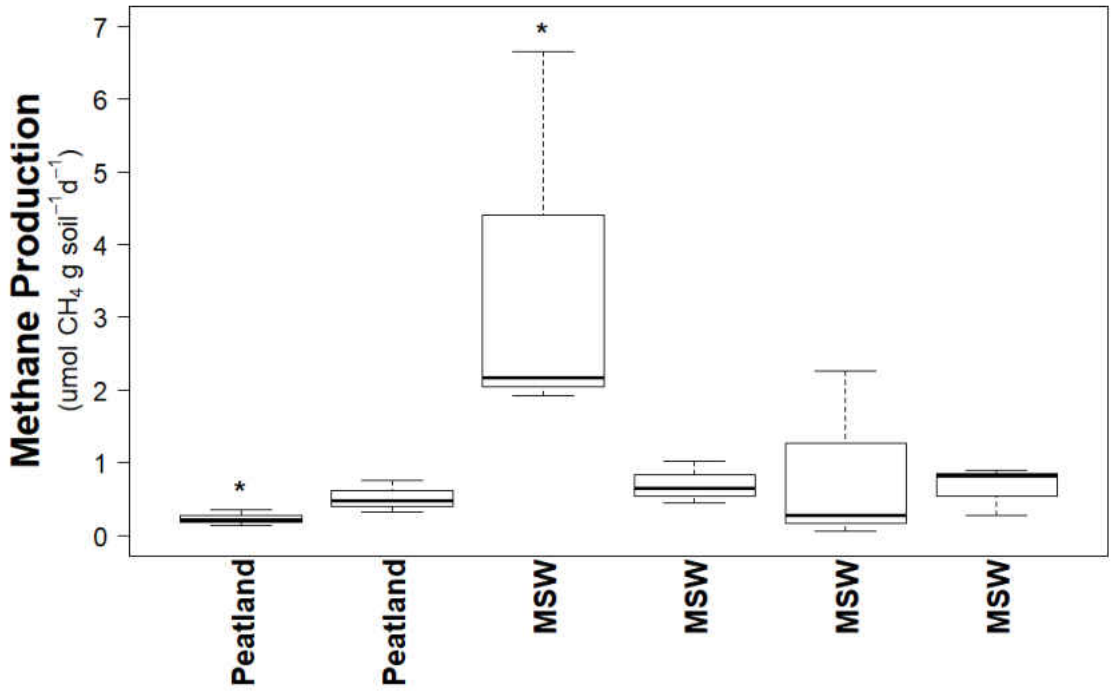


Figure 2.5. Gabonese wetland CH_4 production rates measured from anaerobic laboratory incubations conducted at in situ air temperatures (28 °C) in October 2014 (n = 3). Significant differences ($p < 0.05$) are denoted with *. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values. MSW = mineral soil wetland.

with an average rate of 1.1 ± 1.5 µmol CH_4 g $soil^{-1}$ d^{-1} (Figure 2.5). Mineral-soil wetlands produced 120% more CH_4, on average, than peatlands; however, this difference was only marginally significant ($p = 0.07$). Of the abiotic variables measured, soil pH and moisture content explained 35% of the variation in wetland CH_4 production rates ($F = 5.56$, $p = 0.02$). Additionally, a number of microbial community attributes were also significantly correlated with potential wetland CH_4 production. These included mcrA gene abundance (Figure 2.6a; derived from qPCR, Adj. $R^2 = 0.62$, $p < 0.01$), the relative abundance of *Methanoregula sp.* (a hydrogenotrophic methanogen) in the RNA- and DNA- inferred communities (Adj. $R^2 = 0.75$, $p < 0.001$, Adj. $R^2 = 0.56$, $p < 0.001$, respectively), and the relative abundance of *Methanothrix sp.* (an acetoclastic methanogen) in the RNA- and DNA- inferred communities (Adj. $R^2 = 0.73$, $p < 0.001$, Adj. $R^2 = 0.86$, $p < 0.001$,

respectively). Neither *Methanoregula sp.* nor *Methanothrix sp.* were particularly abundant members of the methanogen community, ranking 46th and 38th most abundant, respectively. We performed stepwise model building using AIC to identify the most explanatory combination of abiotic and biotic variables. This analysis identified *mcrA* gene abundance (Figure 2.6a) plus the relative abundance of *Methanoregula sp.* (in the DNA-inferred community) to be the most explanatory combination of variables (Adj. R^2=0.98, $p < 0.001$). However, these correlations were strongly driven by two higher samples on both axes.

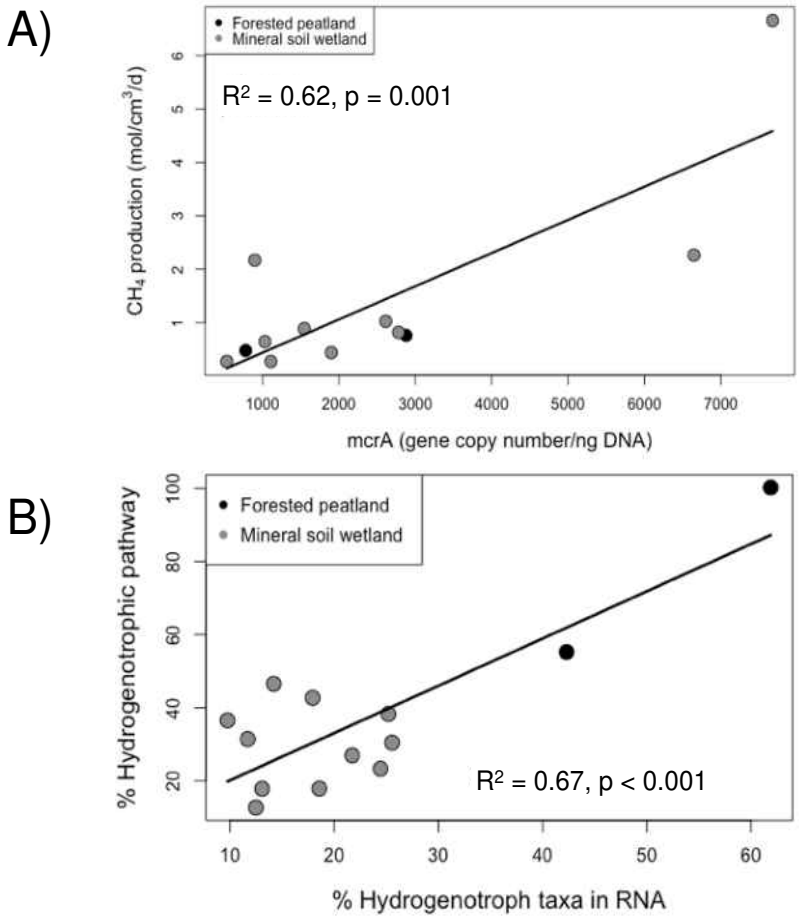


Figure 2.6. The positive relationship between CH_4 production rate and *mcrA* gene copy number **(a)** and between percent hydrogenotrophic pathway predominance and relative abundance of hydrogenotrophic taxa in the RNA-inferred community **(b)**.

The dominant methanogenic pathway varied across wetland soil types ($F = 23.22$, $p < 0.001$), with peatland CH_4 production dominated by hydrogenotrophic methanogenesis and mineral-soil wetland CH_4 production dominated by acetoclastic

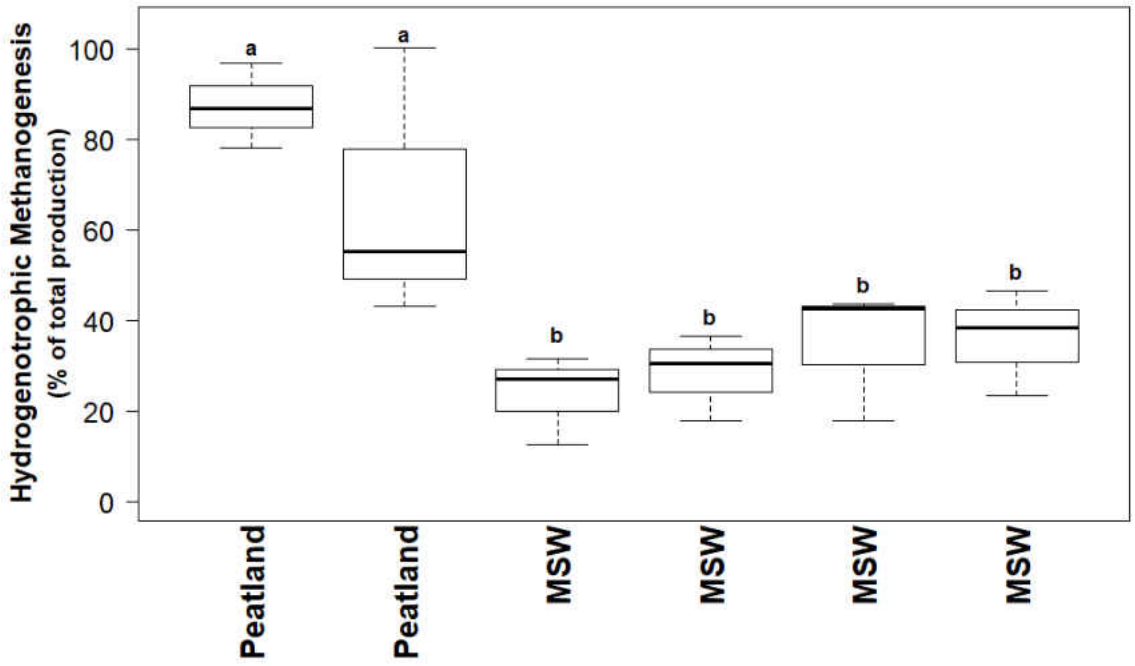


Figure 2.7. Proportion of hydrogenotrophic methanogenesis measured as a percent of total CH_4 production from anaerobic laboratory incubations of Gabonese wetland soil samples (n = 3) in October 2014. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values. Lower case letters represent significant differences ($p < 0.05$). MSW = mineral soil wetland.

methanogenesis (Figure 2.7). In peatlands, hydrogenotrophic methanogenesis was responsible for 55 – 100% of total CH_4 production and averaged 76.7 ± 23.0 % of total CH_4 production. Conversely, hydrogenotrophic methanogenesis was responsible for only 30.7 ± 11.2 % of total CH_4 production in mineral-soil wetlands, ranging from 12.7 – 46.6% of total production. The percentage of methanogens using hydrogenotrophy as their predominant methanogenic pathway in the RNA-inferred community was positively correlated with the percent predominance of the hydrogenotrophic pathway across all sites, driven by the difference between mineral-soil wetlands and peatlands (Figure 2.6b; Adj. $R^2 = 0.67$, $p < 0.001$). The best single predictor of pathway predominance, however, was the relative abundance of the hydrogenotrophic genus *Methanobacterium* in the RNA-inferred community (Adj. $R^2 = 0.77$, $p < 0.001$).

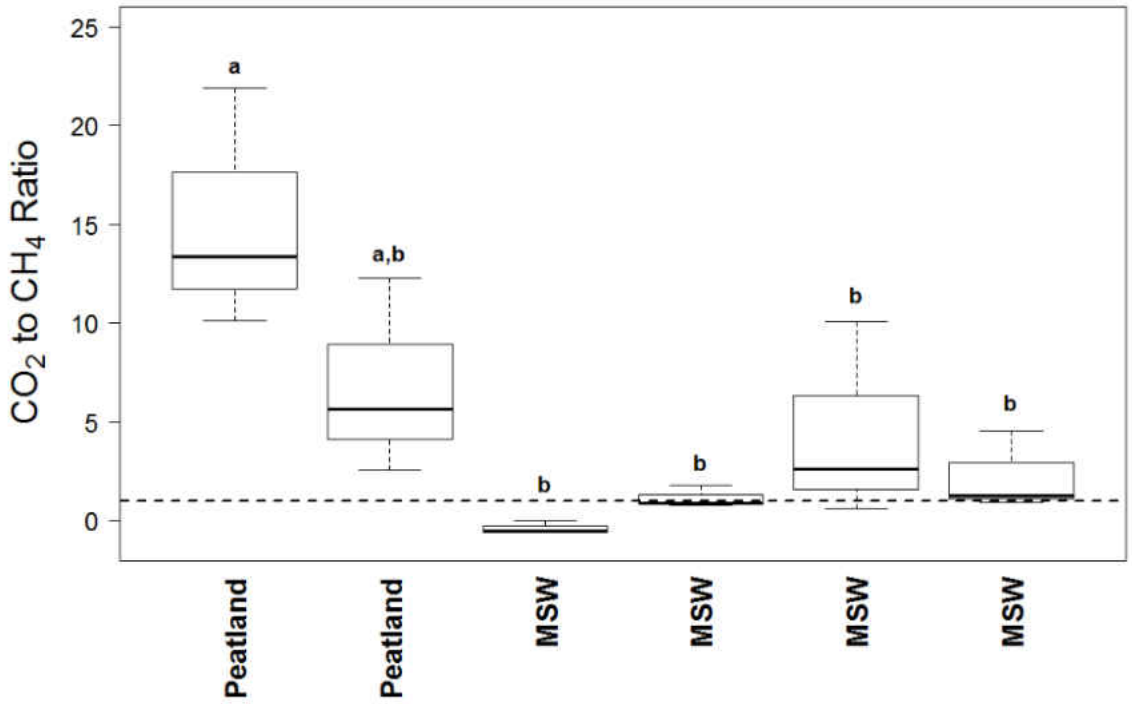


Figure 2.8. Gabonese wetland CO_2:CH_4 production ratios measured from anaerobic laboratory incubations done at in situ air temperatures (28 °C) in October 2014 (n = 3). The dotted line represents a CO_2:CH_4 ratio of 1. Significant differences ($p < 0.05$) are denoted with lower case letters. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values. MSW = mineral soil wetland.

Ratios of CO_2:CH_4 production were higher in peatlands relative to mineral-soil wetlands ($F = 6.24$, $p = 0.004$; Figure 2.8). The majority of mineral-soil wetland CO_2:CH_4 ratios were at or below 1. The CO_2:CH_4 ratio was positively correlated with the percent hydrogenotrophic pathway predominance (Adj. $R^2 = 0.87$, $p < 0.001$), indicating that samples with higher levels of hydrogenotrophic production tended to produce less CH_4, relative to CO_2. The relative abundance of the hydrogenotrophic genus *Methanobacterium* in the RNA-inferred community was strongly correlated with the CO_2:CH_4 production levels (Adj. $R^2 = 0.75$, $p < 0.001$).

Discussion

Despite continued work to improve global wetland CH_4 models, a recent comparison of current efforts determined that there is a lack of observational studies with appropriate mechanistic data to vet CH_4 models against and singled out the need for more mechanistic research in the equatorial zone (Melton et al., 2014). Africa has been the focus of particularly few studies (Delmas et al., 1992; Tathy et al., 1992; MacDonald et al., 1998; MacDonald et al., 1999; Prieme & Christensen, 1999; Werner et al., 2007), which largely measured only CH_4 and CO_2 fluxes, providing no mechanistic data. Here, we help alleviate this knowledge gap by demonstrating that a combination of microbial community attributes provided greater explanatory power over methanotrophy and methanogenesis rate potentials across a variety of Gabonese uplands and wetlands rather than a suite of abiotic factors. CH_4 production, in particular, was highly predicted by the abundance of a few non-dominant methanogen taxa. Additionally, although our in-situ CH_4 measurements represent a single point in time, we provide evidence of the high intra- and inter-site variability of Gabonese wetland CH_4 emissions, as well as the general uptake of CH_4 from upland ecosystems. Taken together, our work contributes unique insights into how microbial community structure imposes constraints on CH_4 cycling, as well as an impression of landscape-scale variation in in-situ CH_4 fluxes across a variety of equatorial habitats.

In-situ CH_4 flux: Gabonese wetland CH_4 emissions varied by an order of magnitude across sites, with this difference largely driven by high variation in mineral-soil wetlands (Figure 2.2a). Conversely, upland ecosystems exhibited much less intra- and inter-site variation and generally consumed CH_4 (Figure 2.2b). For both ecosystem types, abiotic

factors only accounted for a modest amount of the variation in CH_4 flux. For example, water table position, a variable of known importance in the regulation of higher latitude wetland CH_4 emissions (Updegraff et al., 2001; Turetsky et al., 2008; Moore et al., 2011), explained only 18% of the variation in Gabonese wetland CH_4 flux. Similarly, soil pH and total % organic C content explained roughly 20% of the variation in upland CH_4 flux. These results suggest that variables beyond the typically measured abiotic factors, such as microbial community structure, may play a role in controlling Gabonese CH_4 emissions. However, we must acknowledge that our CH_4 flux measurements represent a single snap-shot in time and, thus, we cannot address how abiotic seasonal factors affect Gabonese wetland CH_4 fluxes or how they vary at inter-annual timescales. While temperatures are relatively constant in this area of Gabon, it does experience two distinct rainy seasons. Despite these caveats, our CH_4 flux estimates provide an in-situ context for our gross laboratory process analyses and demonstrate the minimal predictive power of abiotic variables in this instance.

Potential CH_4 oxidation: Upland microbial communities were more effective at oxidizing CH_4 at near-atmospheric concentrations relative to those of wetland ecosystems. Rates of CH_4 oxidation under low CH_4 concentrations (initial 5 ppmv CH_4) were 133% greater in Gabonese uplands relative to wetlands (Figure 2.3a). The single best predictor of high-affinity CH_4 oxidation across all sites was the activity level of an Alphaproteobacteria methylotroph, *Methylovirgula sp*, which explained 45% of the variance in this metric. This taxon is not a known methanotroph, but rather a methylotroph (Dedysh, 2016). This correlation could either represent cross-feeding, whereby this taxon receives substrate

from another community member that is consuming CH_4, or it could be that this taxon is indeed capable of utilizing CH_4 as a substrate, but has not yet been reported to do so. While none of the abiotic variables explained the variation of this process at wetland sites, % sand and moisture content explained 20% of the variation high-affinity CH_4 oxidation in upland sites. Previous studies have also observed effects of soil texture and moisture content on rates of CH_4 oxidation, as these two parameters control the rates of O_2 and CH_4 diffusion to methanotrophic microbial communities (Saari et al., 1997; Teh et al., 2005). However, neither of these variables significantly affected upland CH_4 fluxes, possibly because of the more controlled nature of the laboratory incubations which isolated a defined depth of the soil column.

Interestingly, we saw an inverse relationship between methanotroph relative abundance in the RNA-inferred community and high-affinity CH_4 uptake rates (Figure 2.4a). This same inverse relationship was also observed for methanotroph community diversity (Figure 2.4b), suggesting that smaller populations of methanotrophs with fewer taxa consume low concentrations of CH_4 at a higher rate. The ability to complete this process is likely much more narrow than low-affinity CH_4 oxidation and, thus, would be performed by a smaller number of taxa (Knief, 2015). Methanotrophs in upland soils are largely dependent on low concentrations of atmospheric CH_4 diffusing into the soil, leading to extreme substrate limitation despite possessing efficient CH_4 oxidation enzymes, likely leading to lower abundance and diversity.

We found the opposite trend in aerobic laboratory incubations at initially high CH_4 concentrations (1,000 ppmv CH_4): maximum rates of Gabonese wetland CH_4 consumption were 200% greater than those of upland ecosystems (Figure 2.3b). Thus,

maximum rates of CH_4 oxidation were found in environments that experienced higher rates of CH_4 production and flux. Soil pH and total % organic C were the best predictors of this process in wetland sites (explaining 15% of the variation), while no abiotic variables were effective predictors of low-affinity CH_4 oxidation in upland ecosystems. Methanotrophs have been shown to have an optimum pH of 6-7 (Dunfield et al., 1993) and, likewise, rates of wetland CH_4 oxidation increased with rising pH in this study. Additionally, the growth and activity of methanotrophs has also been observed to be stimulated by increased mineral N availability (Bodelier et al., 2004). In our study, there was a strong positive correlation between total N and organic C content, which both increased with increasing low-affinity CH_4 oxidation. It is likely that higher nutrient availability in some Gabonese soils enhanced the activity and growth of microbial taxa, including low-affinity methanotrophs. However, as we found direct measures of microbial community composition, abundance, activity, and diversity to be strong predictors of CH_4 oxidation under initially low concentrations, we hypothesize that these biotic variables will be better predictors of this process at initially high CH_4 concentrations, relative to abiotic factors, and plan to investigate these relationships in the future.

Potential CH_4 and CO_2 production and methanogenic pathways: Similar to methanotrophy, abiotic variables explained less variation in methanogenesis rates than microbial community attributes. For instance, we found that mcrA gene abundance plus the relative abundance of a single methanogen taxon (*Methanoregula sp.*) had exceptionally high explanatory power across our wetland sites (Adj. $R^2 = 0.98$). An

important caveat to this finding is that *Methanoregula sp*. was only abundant in the sites with high CH_4 production, and had very low abundance, or was absent, in most other samples. Nevertheless, the combination of overall gene abundance and composition information could represent two separate community-level constraints that limit CH_4 production across our wetland sites: numeric constraints and trait-based constraints. In other words, the rates by which CH_4 is produced seem to be limited by the total number of microorganisms capable of producing CH_4, as well as the presence of a single member of the community that may have very high activity under certain conditions.

Conversely, soil pH and moisture content explained much less of the variation in Gabonese wetland CH_4 production (35% of variation). Methanogenesis is known to be very sensitive to acidity (Segers, 1998) and, accordingly, increased with increasing pH; however, we also observed substantial CH_4 production in a Gabonese peatland with a pH of 3.3. Additionally, although all samples were incubated under 1:1 soil to deionized water conditions, CH_4 production rates were positively correlated with increasing moisture content, indicating that the activity of either the methanogens and/or the microbial consortia controlling the anaerobic oxidation of CH_4 may be mediated by soil moisture conditions.

Consistent with other studies (Updegraff et al., 1995; Bridgham et al., 1998), we saw a strong separation of methanogenesis pathway predominance across peatland and mineral-soil wetlands (Figure 2.7). In particular, we see that in peatlands the hydrogenotrophic pathway tended to predominate, whereas in mineral-soil wetlands, the acetoclastic pathway was dominant. Increased prevalence of the acetoclastic pathway, along with coincident shifts in microbial community composition and activity, have been

coupled to increased CH_4 emissions from melting permafrost peatlands (McCalley et al. 2014), indicating that the response of total peatland CH_4 production to climate change is tightly linked to the relative importance of the two CH_4 pathways and microbial community dynamics. Such results underpin the importance of better understanding the mechanisms underlying changes in pathway predominance.

We found that by assessing the structure of the putatively active fraction of the communities in our study, we could predict methanogenesis pathway predominance. Simply by inferring communities from RNA, then categorizing methanogens based on their previously-reported ability to use hydrogenotrophic vs. acetoclastic pathways, we could predict the degree to which CH_4 has been produced via a given pathway (Figure 2.6b; Adj. $R^2 = 0.67$). Moreover, the RNA relative abundance of a single group of methanogens (genus *Methanobacterium)* even more precisely predicted pathway predominance (Adj. $R^2 = 0.77$). Furthermore, the relative abundance of this genus in the active community was also a strong predictor of CO_2:CH_4 production ratios (Adj. $R^2 = 0.75$), indicating that the relative activity levels of this genus may be useful for predicting both pathway predominance, as well as terminal C mineralization ratios. A parallel relationship has been reported in thawing permafrost, where the DNA-inferred relative abundance of a single OTU (*M. stordalmirensis*) was a key predictor of shifts in methanogenic pathway dominance, which in turn predicted the amount of C emitted as CH_4 and as CO_2 (McCalley et al., 2014). In our study, we observed CO_2:CH_4 ratios that approached or were at one, indicating extremely efficient methanogenesis (Figure 2.8). These results have large implications as the CO_2:CH_4 ratio is an ecologically relevant

ratio representing the flow of C through anaerobic pathways and, ultimately, the impact ecosystem emissions will have on future climate.

Overall, abiotic variables tended to be less predictive of gross processes of wetland and upland Gabonese CH_4 cycling, relative to biotic variables. While we acknowledge that by bringing soil cores into a controlled laboratory setting, we are likely downplaying certain environmental factors that could be important (e.g., temperature fluctuations or rainfall events), the majority of our environmental variables were independent from our incubation conditions (e.g., %C, %N, pH, soil texture). Furthermore, field CH_4 fluxes were also poorly predicted by abiotic variables in comparison to most other studies. Thus, our findings suggest that CH_4 dynamics across these tropical ecosystems appear to be strongly regulated by microbial community attributes, such as abundance, diversity, activity, and composition. Whether our findings extrapolate to other tropical systems remains untested.

Much of the variation of CH_4 dynamics in higher latitude systems is driven by seasonal fluctuations in temperature and water table depth (Updegraff et al., 2001; Turetsky et al., 2008; Olefeldt et al., 2017). It is possible that tropical CH_4 dynamics may not be as influenced by external conditions as higher latitude ecosystems due to the constancy of temperatures in the tropics. For example, methanogen population growth rates are slow because all the methanogenic pathways have very low thermodynamic yields that often barely above the threshold for growth under in-situ conditions (Conrad, 1999; Megonigal et al., 2004). Thus, methanogen populations are expected to recover slowly from unfavorable environmental conditions, such as freeze-thaw events. Therefore, the constancy of temperatures in tropical regions, as well as high rates of C

input, may lead to large active methanogen communities and unique taxa with high rates of activity, capable of producing low CO_2:CH_4 ratios during anaerobic heterotrophic respiration. Although our study does not directly test this, it is possible that biotic controls on CH_4 dynamics are stronger in tropical areas than in higher latitude regions and future work could attempt to address this hypothesis by comparing drivers of CH_4 cycling across latitudes and gradients of environmental perturbation.

Conclusions

Microbial community structure and ecosystem function are notoriously difficult to connect. Methane-cycling is an ideal C-cycle process to study this connection because the microorganisms that produce and consume CH_4 are phylogenetically constrained and relatively well studied. Tropical wetlands emit more than half of the global wetland CH_4 flux; yet, there have been comparatively fewer studies on controls over tropical wetland methane flux relative to boreal and temperate wetlands. Our study addressed this knowledge gap by (1) estimating rates of CH_4 flux, production, consumption, and methanogenic pathway dominances in a variety of equatorial African habitats and (2) by using a suite of physiochemical and microbial community attributes to determine the relative ability of abiotic and biotic variables to predict these processes across ecosystem types. Here, we show that a combination of microbial community attributes, including composition, abundance, activity, and diversity, are better predictors of CH_4 production, consumption, methanogenic pathway, and CO_2:CH_4 ratios relative to a standard set of physiochemical parameters. Of particular interest was that the relative abundance of *Methanobacterium sp.* in the active community explained 77 and 75% of the variation in

methanogenic pathway dominance and CO_2:CH_4 ratios, respectively. Methanogenic pathway predominance and CO_2:CH_4 ratios are important ecological indicators that provide insight into the underlying mechanisms and efficiency of ecosystem CH_4 cycling. Thus, our research highlights the central role of microbial ecology in controlling ecosystem-scale processes, as well as the potential benefits of incorporating microbial dynamics into terrestrial CH_4 modelling efforts.

Bridge to Chapter III

Wetlands in tropical and boreal regions are globally important ecosystems due to their influence on the CH_4 cycle. Despite this acknowledgement, little is known about the fundamental rates and controls over gross and net tropical wetland CH_4 emissions and, although boreal wetlands have been more thoroughly studied, significant questions remain surrounding the biogeochemical controls over CH_4 dynamics in these systems. Chapter II addressed this knowledge gap by providing novel data on the rates of CH_4 flux, production, consumption, and methanogenic pathway dominances in a variety of tropical Gabonese habitats and demonstrating that variation in many of these processes is better explained by microbial community attributes relative that of abiotic factors. In Chapter III, we continue to investigate the biogeochemical controls over anaerobic C cycling processes in wetlands; however, we transition to a study conducted across a variety of boreal peatlands. These ecosystems are also of critical importance to the global CH_4 cycle and examining the C sources fueling methanogenesis in these systems will enhance our mechanistic understanding of peatland CH_4 cycling.

CHAPTER III

DOES DISSOLVED ORGANIC MATTER OR SOLID PEAT FUEL ANAEROBIC RESPIRATION IN PEATLANDS?

Contributions

This chapter is co-authored by myself, Laurel Pfeifer-Meister, Cassandra A. Zalman, Jason K. Keller, Malak M. Tfaily, Rachel M. Wilson, Jeffrey P. Chanton, and Scott D. Bridgham. I was responsible for the data collection, analysis, and writing of this manuscript. Laurel Pfeier-Meister, Scott D. Bridgham, Jason K. Keller, and myself designed this experiment. Malak M. Tfaily, Rachel M. Wilson, and Jeffrey P. Chanton provided the DOC and SUVA measurements. All co-authors gave text edits.

Introduction

Peatlands are among the most important terrestrial ecosystems largely due to their significant influence on the global carbon (C) cycle. In these systems, imbalances between net primary production and slow decomposition under cold, wet conditions have led to the accumulation of about one-third of the world's soil C (Gorham, 1991; Bridgham et al., 2006) since the Last Glacial Maximum (Yu et al., 2010). Northern peatlands alone store half as much C (473-621 Pg C; Yu et al., 2010) in soil as the atmosphere stores as carbon dioxide (CO_2) (829 Pg C; Ciais et al., 2013). However, despite their immense C storage capacity, peatlands are also responsible for roughly 13% of global emissions of methane (CH_4) (Kirschke et al., 2013), a potent greenhouse gas with 45-times the sustained-flux global warming potential of CO_2 over a 100-year time frame (Neubauer and Megonigal, 2015). Given the key role these ecosystems play as

both global C stores and CH_4 sources, it is imperative to gain a comprehensive understanding of peatland C cycling to elucidate current and future biosphere-climate feedbacks and mechanisms.

While solid-phase soil organic matter represents the largest C stock in peatlands, the much smaller pool of dissolved organic matter (DOM) is a key control of microbial activity (Bragazza et al., 2013) and may be particularly important in regulating the production of CH_4 in peatlands (Pastor et al., 2003; Chanton et al., 2008). Climate change could have large effects on the quality and concentration of DOM, and thus microbial activity, in peatlands. For example, changes in precipitation will affect DOM retention and transport through the peat column, both of which affect respiration of CH_4 and CO_2 (Pastor et al., 2003; Glaser et al., 2016). Additionally, studies have shown that peatland plant communities undergoing climate warming and/or drying experience decreases in the abundance of *Sphagnum* spp. and lichens and increases in the abundance of sedges and shrubs (Weltzin et al., 2000; Walker et al., 2006; Bragazza et al., 2013; McCalley et al., 2014), which may affect the type of DOM available, as well as the respiration of greenhouse gases (Corbett et al., 2013b; Wang et al., 2015). Despite the considerable attention peatlands have received, our understanding of the role of DOM in anaerobic C respiration is incomplete, limiting our ability to accurately predict climate forcing from peatlands (Bridgham et al., 2013a).

Peatlands are typically classified along a hydrogeomorphic gradient of differing groundwater or surface-water inputs that influences a suite of factors, including water-table dynamics, topography, water chemistry, peat characteristics, and plant community composition. Minerotrophic fens maintain some level of groundwater connectivity and

mineral-soil influence in an otherwise organic soil matrix, with porewater pH generally higher than that of bogs (CCELC, 1987). Fens with more minerotrophic features, such as higher soil pH and basic cation content, are often termed "rich", while those with soil chemistries more similar to bogs are denoted as "poor". Rich fens also tend to have higher nitrogen and lower phosphorus availability than that of poor fens (Kolka et al., 2016). Fen plant communities are dominated by varying proportions of graminoids, forbs, shrubs, and *Sphagnum* mosses depending on the degree of minerotrophy and hydroperiod. Conversely, extensive peat accumulation in ombrotrophic bogs, typically derived from *Sphagnum* moss, isolates the surface peat and vegetation from groundwater influence (CCELC, 1987). As a result, bog porewater pH is much lower than that of fens, with values generally $\leq$ 4.0 (CCELC, 1987; Kolka et al., 2016). Finally, bog plant communities typically do not contain large abundances of graminoid and forb species, but instead have greater coverage of woody shrubs and trees.

Several studies have characterized the source, reactivity, and transport of DOM with respect to vegetation and solid-phase peat along ombrotrophic-minerotrophic peatland gradients (Charman et al., 1994; Chanton et al., 1995; Corbett et al., 2013a). Radiocarbon data replicated across multiple northern peatlands from Alaska to Minnesota have linked changes in vegetation composition among bogs and fens to corresponding changes in the source of C driving heterotrophic anaerobic respiration (Chanton et al., 2008). It has been suggested that modern (recently fixed plant photosynthate) DOM is driving anaerobic respiration throughout the peat column in fens, while solid-phase peat appears to have a comparable influence over these processes in *Sphagnum*- and woody-dominated bogs. Moreover, fen DOM is generally more labile than bog DOM (Chanton

et al., 2008; Corbett et al., 2013a). For example, in a comparison of bog and fen DOM chemistry in northern Minnesota, Tfaily et al. (2013) found that the majority (>80%) of surface and deep (3 m) DOM in bogs had the same chemical composition, while a considerable number of new compounds were observed in the deep fen horizon relative to surface samples, indicating greater processing of fen DOM and overall labile C limitation of decomposition at depth in both systems. Solute transport mechanisms may also affect DOM chemistry and quantity, and hence anaerobic C mineralization. For example, decadal increases in precipitation flushed surface-derived DOM deep within the peat column of bogs and fens in northern Minnesota, enriching labile C compounds and increasing the methanogenic potential at depths where microbial metabolism was likely previously limited by labile C availability (Glaser et al., 2016). The observed differences in C sources and reactivity suggest that these systems may respond to climate change very differently. These findings are also important in evaluating the stability of peat buried deep in the peatland profile (Wilson and Hopple et al., 2016). If the bulk of heterotrophic respiration in deep subsurface peat is supported by surface production conveyed to depth by DOM, then the C stored in these deeper layers is more stable than evidence for respiration rates would indicate.

Here, we investigate (i) whether DOM or solid-phase peat fuels peatland anaerobic respiration, (ii) whether this varies in two bogs and a poor fen in northern Minnesota, and (iii) if methanogenesis deep in the peatland profile is limited by the availability of surface-derived DOM. Based on radiocarbon profiles of C sources and products of respiration (Chanton et al., 2008), we hypothesized that DOM would act as a primary driver of anaerobic respiration in peatlands across depths, but that the influence

of DOM would be less in the bogs. We also expected that CH_4 production deep in the peatland profile would be stimulated by surface-derived DOM addition, and that the stimulatory response would be greater in fen vs. bog peatlands because of the greater lability of fen DOM. While there is a body of literature consistent with our hypothesis (Chanton et al., 1995; Chanton et al., 2008; Corbett et al., 2013a; Tfaily et al., 2014; Glasar et al., 2016; Wilson and Hopple et al., 2016), these studies are primarily field observations. In this study, we conducted a direct test of the hypotheses by conducting a series of controlled incubation studies.

Methods

Study Sites – We examined the extent to which DOM or solid-phase peat fuels anaerobic respiration in three *Sphagnum*-dominated peatlands, two bogs and one poor fen, in northern Minnesota. The site characteristics of Bog Lake (BL) Fen, S1 Bog, and Zim Bog have previously been described in Medvedeff et al. (2015). BL Fen (N 47º30.304'; W 93º29.339') and S1 Bog (N 47º30.388'; W 93º27.256') are located within the U.S. Forest Service's Marcell Experimental Forest. S1 Bog is the site for the whole-ecosystem warming and CO_2-enrichment experiment, Spruce and Peatland Responses Under Changing Environments (SPRUCE) (Krassovski et al., 2015; Wilson and Hopple et al., 2016; Hanson et al., 2017). Zim Bog (N 47º10.745'; W 92º42.877') is ~95 km to the southeast of the other sites, within a large peatland complex that has developed in the glacial Lake Upham basin.

BL Fen is a poor fen with a soil pH of ~4.2 and a water table that is typically fairly close to the surface (-7.0 cm in July 2013 at the time of sampling; Kolka et al.,

2011). It is a relatively open *Sphagnum* "lawn" with a plant community dominated by *S. papillosum* (also *S. capillifolium* and *S. magellanicum*), sedges (*Carex* spp., *Eriophorum chamissonis*), and ericaceous shrubs (*Chamaedaphne calyculata*, *Vaccinium macrocarpun*). S1 Bog and Zim Bog are both black spruce (*Picea mariana*) bogs with soil pH values $\leq$ 4.0, but the trees in S1 Bog are taller (5-8 m tall) and denser despite having undergone experimental strip cutting in 1969 and 1970 (Kolka et al., 2011). All sites have an average peat depth of 2 - 3 m.

Experimental Design and Sampling Protocol – In June 2013, we completed a pilot experiment to investigate whether DOM or solid-phase peat is the primary driver of heterotrophic respiration in peatlands. Peat and porewater samples were collected from 25-50, 75-100, 150-200 cm depths at each site. Peat cores were extracted in triplicate using a Russian corer, stored on ice, and frozen within three days after transport to the University of Oregon. Two months later, we thawed the peat samples, homogenized replicate cores, and removed visible roots from the samples in an aerobic environment. Porewater samples were collected with a peristaltic pump from 1.25 cm-diameter PVC piezometers installed at 25, 75, and 150 cm depth increments below the hollow surface. They were then immediately frozen on dry ice and stored frozen in 500 mL polycarbonate bottles. Porewater samples were thawed, filtered through 0.7 µm glass-fiber filters (Whatman) to remove particulate C upon return to the laboratory, and re-frozen until we initiated anaerobic incubations.

In July 2013, additional porewater samples were taken (using the above collection method) at 10, 25, 50, 75, 100, 150, and 200 cm depth increments across all three sites

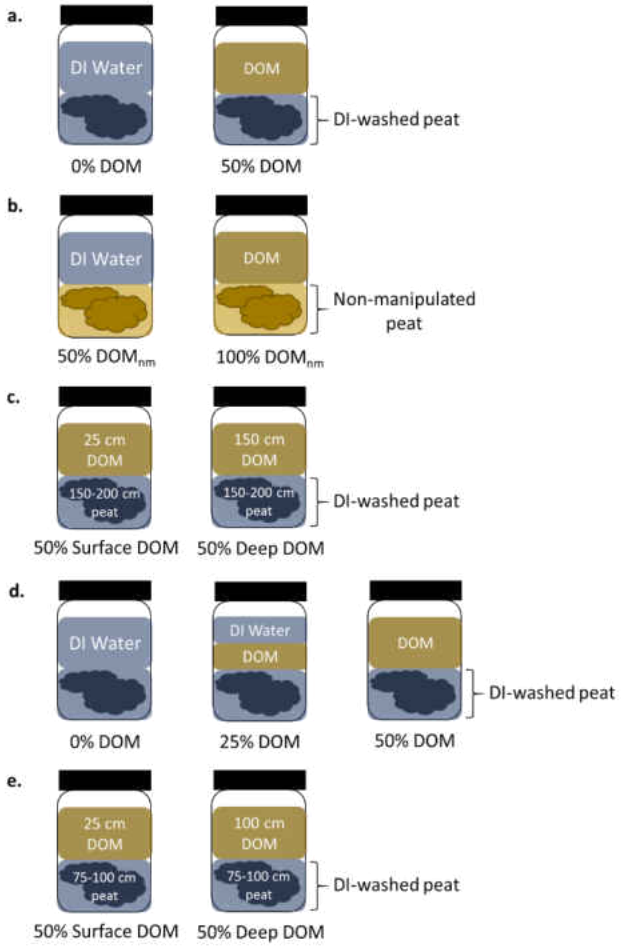


Figure 3.1. Illustrative schematic of our experimental design that manipulates available C sources during anaerobic laboratory incubations of peat samples collected from two bogs and a poor fen in northern Minnesota. In our June 2013 pilot experiment, we compared CH_4 and CO_2 production rates of **(a)** DI-washed peat samples with liquid phases containing 0 and 50% DOM, **(b)** non-manipulated peat samples with aqueous phases of 50 and 100% DOM, and **(c)** deep DI-washed peat samples incubated with either surface (25 cm) or deep (150 cm) DOM. Incubations were done with peat and porewater samples collected from 25-50, 75-100, and150-200 cm depth increments and included three experimental replicates. Peat samples were frozen for two months prior to analysis. In July 2014, we further explored the effects of DOM on CH_4 and CO_2 production rates in anaerobic incubations of **(d)** DI-washed peat samples with liquid phases containing 0, 25, and 50% DOM and **(e)** deep DI-washed peat samples incubated with surface (25 cm) or deep (100 cm) DOM. In July 2014, incubations were completed with peat and porewater samples collected at 25-50 and 75-100 cm depths increments and included five experimental replicates. Fresh, non-frozen peat samples were used in this analysis. DI = deionized water; DOM = dissolved organic matter.

for in-situ dissolved organic carbon (DOC) analysis. The concentration of DOC was measured by high-temperature catalytic oxidation using a Shimadzu Total Organic Carbon analyzer equipped with a non-dispersive infrared detector. Samples were analyzed in triplicate with a coefficient of variance < 2%. As an index of DOC aromaticity or humification, we measured sample specific UV absorbance (SUVA) at 254 nm and then divided the absorbance by DOC concentration (Weishaar et al., 2003). The UV absorbance was measured with a Cary Varian 100 dual beam UV/Vis spectrometer using a 10 mm Suprasil cuvette. Purified Milli-Q system water was used to blank-correct sample spectra.

We used a variety of experimental treatments to manipulate available C sources under laboratory conditions in an effort to establish the primary substrate controlling anaerobic respiration across peatlands and depths. To address our principal hypothesis – that DOM acts as a primary driver of heterotrophic anaerobic respiration in peatlands, we compared the anaerobic CH_4 and CO_2 production rates in incubations with liquid phases composed of either 0 or 50% DOM (Figure 3.1a). This was accomplished by first removing the majority of DOM from field-moist peat samples (protocol described below) and then creating a slurry with either deionized water (0% DOM) or piezometer-extracted porewater (50% DOM) from the same site and depth. The latter treatment is referred to as 50% DOM because manipulated peat samples were saturated with deionized water following DOM removal; thus, half of the liquid phase was deionized water and the other half was piezometer-extracted porewater. We also tested this hypothesis at DOM concentrations above 50% in surface peat by adding either deionized water or surface porewater from the same site to non-manipulated (i.e., non-washed) surface peat samples (50 and 100% DOM_{nm}; nm = non-manipulated peat) (Figure 3.1b). These treatments are denoted as 50 and 100% DOM_{nm} because the 100% DOM_{nm} contains double the concentration of surface-derived DOM.

We found no difference between surface anaerobic respiration rates from incubations using peat- or piezometer-extracted DOM (peat-extracted: $CH_4 = 0.14 \pm 0.05$ μmol C g $soil^{-1}$ d^{-1}, $CO_2 = 2.9 \pm 0.19$ μmol C g $soil^{-1}$ d^{-1}; piezometer-extracted: $CH_4 = 0.15 \pm 0.03$ μmol C g $soil^{-1}$ d^{-1}, $CO_2 = 2.6 \pm 0.21$ μmol C g $soil^{-1}$ d^{-1}). Peat-extracted DOM was acquired through vacuum filtration using 0.7 μm glass-fiber filters (Whatman) and piezometer-extracted DOM was collected as previously described. The lack of

difference indicates that the two DOM sources supported comparable rates of microbial respiration, thereby justifying the use of piezometer-extracted DOM in our incubation experiments.

To test our secondary hypothesis – that methanogenesis deep within the peatland profile is limited by the availability of high quality surface-derived DOM, we added surface-derived DOM in incubations of deep (150-200 cm) peat from each site and compared rates of CH_4 and CO_2 production between treatments. We again removed the majority of DOM from field-moist peat samples collected from 150-200 cm within the peatland profile and then created a slurry with either surface (25 cm; 50% Surface DOM) or deep (150 cm; 50% Deep DOM) piezometer-extracted porewater from the same site (Figure 3.1c). As previously stated, these treatments are referred to as 50% DOM treatments because the manipulated peat samples are saturated with deionized water following DOM removal and, therefore, contain aqueous phases composed of equal parts deionized water and porewater. If methanogenesis deep within the profile was limited by DOM quantity and/or chemistry, then CH_4 production rates should be higher with surface-derived DOM vs. deep-derived DOM. This assumes that surface-derived DOM has greater C lability and concentration than deep-derived DOM based on previous studies (see Introduction), but our experimental design does not eliminate potential effects of other surface-derived DOM properties.

Based on intriguing, but marginally significant findings (see Results) from our pilot study in June 2013 suggesting that DOM is an important driver of surface CH_4 production, we further investigated the influence of C source on peatland anaerobic respiration in July 2014 using a slightly modified experimental design and increased

replication from three to five replicates to enhance our statistical power. All three sites were included in this set of experiments, and we compared the CH_4 and CO_2 production rates of peat samples with aqueous phases containing 0, 25 (a new treatment), and 50% DOM. We focused on surface (25-50 cm) and deep (75-100 cm) peat depth increments with porewater samples collected at 25 and 100 cm (Figure 3.1d). Peat and porewater sample collection and storage followed the same procedures used in June 2013, except that peat was refrigerated, not frozen, prior to laboratory analyses and incubations commenced within two weeks of field collection, not two months.

We also continued to examine our hypothesis that deep methanogenesis is limited by surface-derived DOM by repeating an experiment from June 2013 with experimental replicates increased from three to five. In this experiment, we removed the majority of DOM from deep (75-100 cm) peat samples and then added either surface (25 cm; 50% Surface DOM) or deep (100 cm; 50% Deep DOM) porewater from the same site and compared rates of CH_4 and CO_2 production between the two treatments (Figure 3.1e).

DOM Removal – After homogenizing the three (2013) or five (2014) replicate peat cores collected at each site and depth, samples were separated into two treatments. The majority of DOM was removed from one portion, while the other was set aside for our non-manipulated peat treatments. To remove DOM, we washed peat samples held in mesh nylon stockings with multiple rinses of deionized water over 24-72 hours. Ultraviolet absorbance at 320 nm was used to monitor the loss of DOM from sample leachate over the course of the washing using the DOC (mg C L^{-1}) and A_{320} regression equation established by Pastor et al. (2003) for northern Minnesota bogs and fens,

including Zim Bog. Initial DOC concentrations ranged from 29.7-61.0 mg C L^{-1} for surface (25-50 cm) peat, 31.4-70.2 mg C L^{-1} for intermediate (75-100 cm) peat, and 18.0-38.9 mg C L^{-1} for deep (75-100 cm) peat. All samples were washed until DOC concentrations at A_{320} were no longer detectible. Absorbance measurements were determined on a Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) at room temperature at a 10 mm path length with nanopure water serving as a blank.

When using frozen peat samples in June 2013, we observed a significant washing-associated disturbance effect between the 50% DOM and 50% DOM_{nm} that reduced surface CH_4 production by 56% ($p < 0.05$), but did not affect methanogenesis in deeper peat or CO_2 production (Supplemental Table 1). No washing-associated disturbance effect was observed when using fresh, non-frozen peat samples (Supplemental Table 1).

Anaerobic Respiration – For all experiments, approximately 8 g of wet-weight peat were added to 120 mL serum bottles and flushed with N_2 for 15 minutes to create anaerobic conditions. All samples were then pre-incubated at 18°C for three weeks to reduce terminal electron acceptors as the peat samples had been exposed to aerobic conditions for a considerable amount of time. After the pre-incubation period, peat samples were moved into a glove box filled with a N_2 atmosphere (<5% H_2 in the presence of a palladium catalyst; Coy Laboratory Products, Grass Lake, Michigan) to maintain anaerobic conditions. Peat samples were brought to field moisture levels with either deoxygenated porewater or deionized water. The sample pH was adjusted to *in situ* pH using small aliquots of either 0.5 M HCl or 0.1 M NaOH. Sample bottles were then flushed with N_2 for 15 minutes to begin the incubation. Headspace samples were

analyzed over the course of two weeks (days 2, 4, 7, and 13) for CH_4 and CO_2 simultaneously using an SRI gas chromatograph equipped with a methanizer and flame ionization detector. Total CH_4 and CO_2 were calculated using Henry's Law, adjusting for solubility, temperature, and pH (Bridgham and Ye, 2013b). Methane and CO_2 production rates were calculated using the linear accumulation ($r^2 > 0.90$ in all cases) of gasses through time.

Statistical Analyses – Two-way Analysis of Variance (ANOVA) was used to determine the effects of site and treatment (as fixed effects) on CH_4 and CO_2 production rates between experimental treatments within statistically different depth increments using SPSS Statistics version 22. If no significant interaction was observed, we combined sites and analyzed differences among experimental treatments using Tukey's HSD tests ($p < 0.05$). Two-way ANOVA was also used to test for the effects of site and depth on DOC and SUVA measurements. Data were tested for normality and log-transformed where the transformation resulted in a significant improvement in overall distribution.

Results

In-situ DOC concentration and aromaticity – In July 2013, in-situ porewater DOC concentrations changed significantly with depth ($p < 0.001$), but this difference depended on the site ($p < 0.001$). DOC concentrations decreased with depth at BL Fen and S1 Bog; however, there was little change in DOC concentration throughout the peat profile at Zim Bog (Figure 3.2a). Dissolved organic carbon concentrations ranged from 36.9-53.4 mg C L^{-1} at BL Fen, 49.7-84.3 mg C L^{-1} at S1 Bog, and 53.5-61.1 mg C L^{-1} at Zim Bog. In the

surface (10-50 cm), BL Fen and Zim Bog had similar DOC concentrations (~55 mg C L^{-1}), while those of S1 Bog were approximately 33% higher (~80 mg C L^{-1}). However, at deeper depths (150-200 cm), Zim Bog exhibited the highest DOC concentrations (53 mg C L^{-1}), followed by S1 Bog and BL Fen with 46 and 38 mg C L^{-1}, respectively.

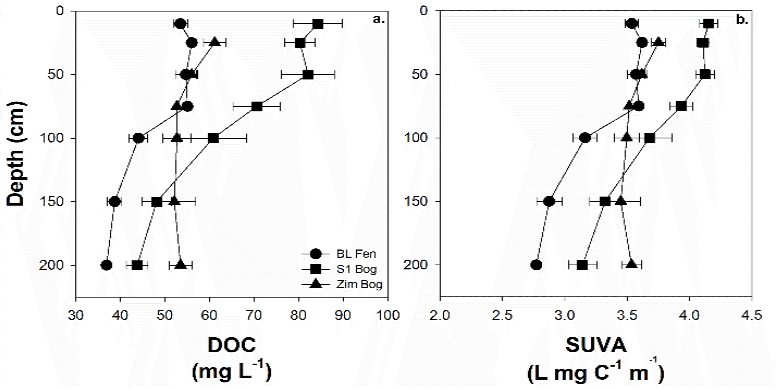


Figure 3.2. Dissolved organic carbon (DOC; **a**) and specific UV absorbance (SUVA; **b**) of porewater samples collected from multiple depth increments (10-200 cm) across three northern Minnesota peatlands in July 2013.

Additionally, SUVA measurements showed systematic decreases in DOC aromaticity with depth across all sites ($p < 0.001$); however, this decrease was much more muted at Zim Bog (range = 3.5-3.8 L mg C^{-1} m^{-1}) relative to BL Fen and S1 Bog (Figure 3.2b). SUVA values ranged from 2.8-3.6 L mg C^{-1} m^{-1} and 3.1-4.2 L mg C^{-1} m^{-1} at BL Fen and S1 Bog, respectively. In surface peat (10-50 cm), S1 Bog had the highest aromaticity measurements (~4 L mg C^{-1} m^{-1}), while BL Fen and Zim Bog both had lower SUVA values of approximately 3.5 L mg C^{-1} m^{-1}. At depths below 100 cm, Zim Bog had the highest DOC aromaticity (3.5 L mg C^{-1} m^{-1}), followed by S1 Bog (3.2 L mg C^{-1} m^{-1}) and BL Fen (2.8 L mg C^{-1} m^{-1}).

Carbon source fueling anaerobic respiration - In our June 2013 pilot study, both CH_4 and CO_2 production decreased with depth in the peat column ($p < 0.01$, Supplemental Table 2). The interaction between site and treatment was marginally significant ($p =$

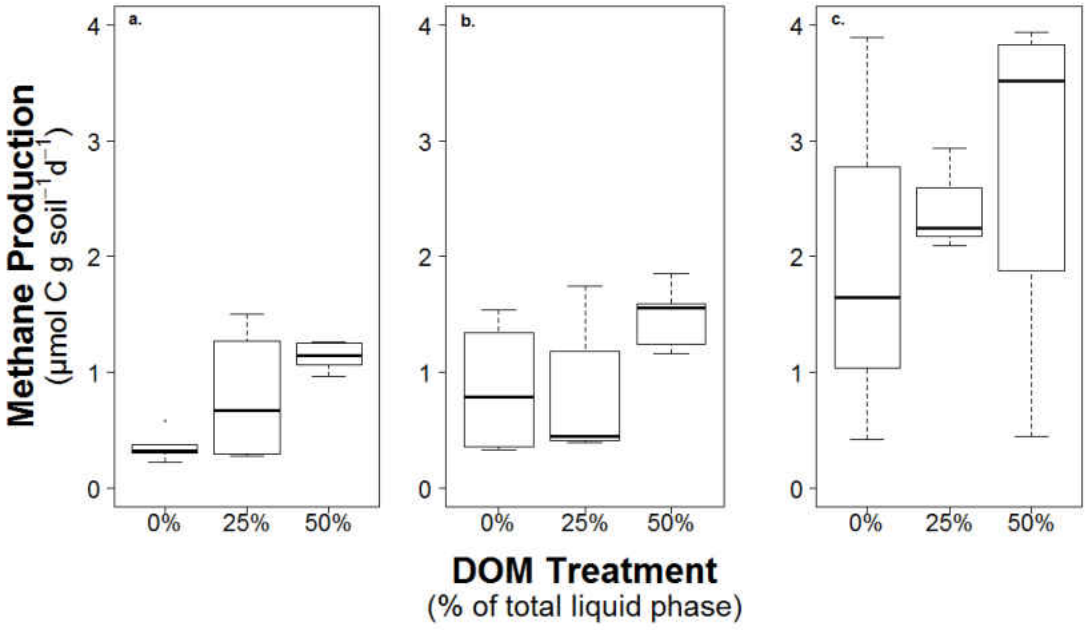


Figure 3.3. Methane production in peat samples collected at 25-50 cm in July 2014 from BL Fen **(a)**, S1 Bog **(b)**, and Zim Bog **(c)**. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values (excluding outliers). Outliers are shown with open circles. DOM content significantly increased CH_4 production across all sites ($p < 0.001$).

0.094), but obvious differences among sites were not evident; therefore, we analyzed CH_4 and CO_2 production responses to DOM removal and addition across all sites. Surface (25-50 cm) CH_4 production was 53% lower in incubations containing 0% DOM than in those with 50% DOM across all peatland types ($p \leq 0.07$). However, we observed no significant DOM treatment effects on CH_4 production at any other depth, and there were no treatment effects on CO_2 production (Supplemental Table 2, $p \geq 0.05$). Additionally, surface CH_4 and CO_2 production rates did not increase when DOM concentrations were doubled from 50% to 100% in non-manipulated peat samples (50% DOM_{nm}: $CH_4 = 0.34 \pm 0.07$ µmol C g soil^{-1} d^{-1} and $CO_2 = 2.3 \pm 0.13$ µmol C g soil^{-1} d^{-1}; 100% DOM_{nm}: $CH_4 = 0.36 \pm 0.07$ µmol C g soil^{-1} d^{-1} and $CO_2 = 2.7 \pm 0.21$ µmol C g soil^{-1} d^{-1}).

In our more replicated study from July 2014, there were highly significant site and DOM treatment effects in surface peat (site: $p < 0.01$; treatment: $p < 0.001$), but treatment

effects did not differ among sites ($p = 0.981$) for CH_4 production (Figure 3.3). Across all sites, average rates of CH_4 production increased from 0.93 ± 0.28 µmol C g soil^{-1} d^{-1} in the 0% DOM treatment to 1.21 ± 0.26 and 1.75 ± 0.29 µmol C g soil^{-1} d^{-1} in the 25 and 50% DOM treatments, respectively, representing 30 and 88% increases in surface methanogenesis with increasing DOM content. Regardless of treatment, there was no measurable CH_4 production in anaerobic incubations of deep (75-100 cm) peat in July 2014. Consistent with June 2013 results, DOM treatment, site, and their interaction had no significant effects on surface (Figure 3.4) or deep (Figure 3.5) CO_2 production.

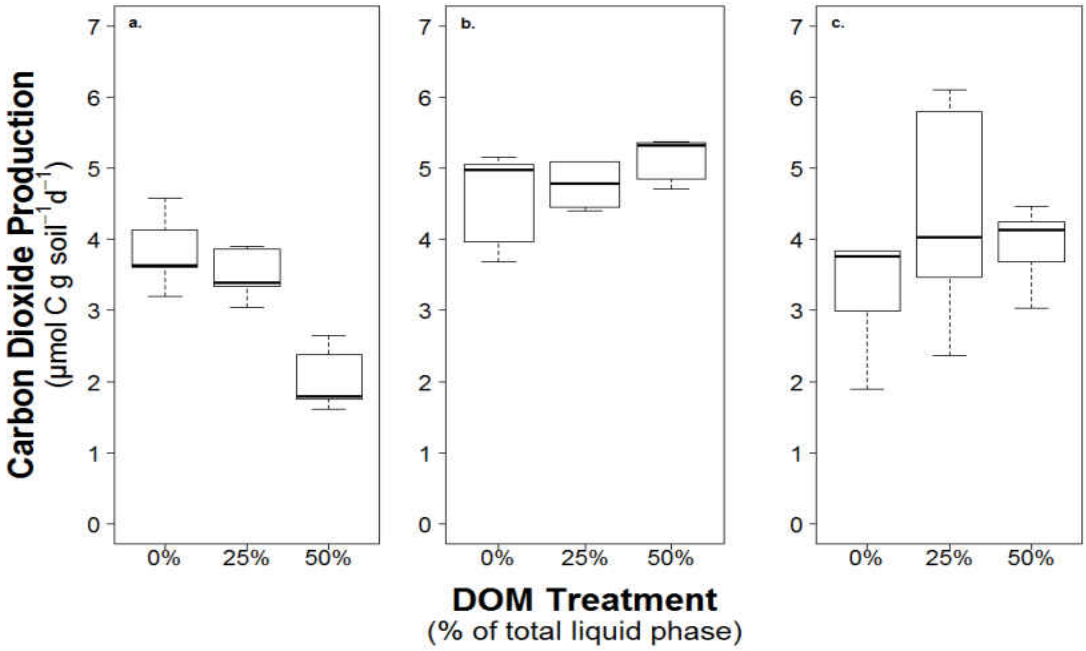


Figure 3.4. Carbon dioxide production in peat samples collected at 25-50 cm in July 2014 from BL Fen **(a)**, S1 Bog **(b)**, and Zim Bog **(c)**. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values (excluding outliers). Outliers are shown with open circles. No significant differences were observed within treatments or across sites.

Increasing surface-derived labile carbon availability – In our June 2013 pilot study, deep peat samples incubated with surface porewater from the same site did not show increases in their rates of CH_4 production (50% Deep DOM: $CH_4 = 3.1 \pm 0.013$ nmol C g soil^{-1} d^{-1}

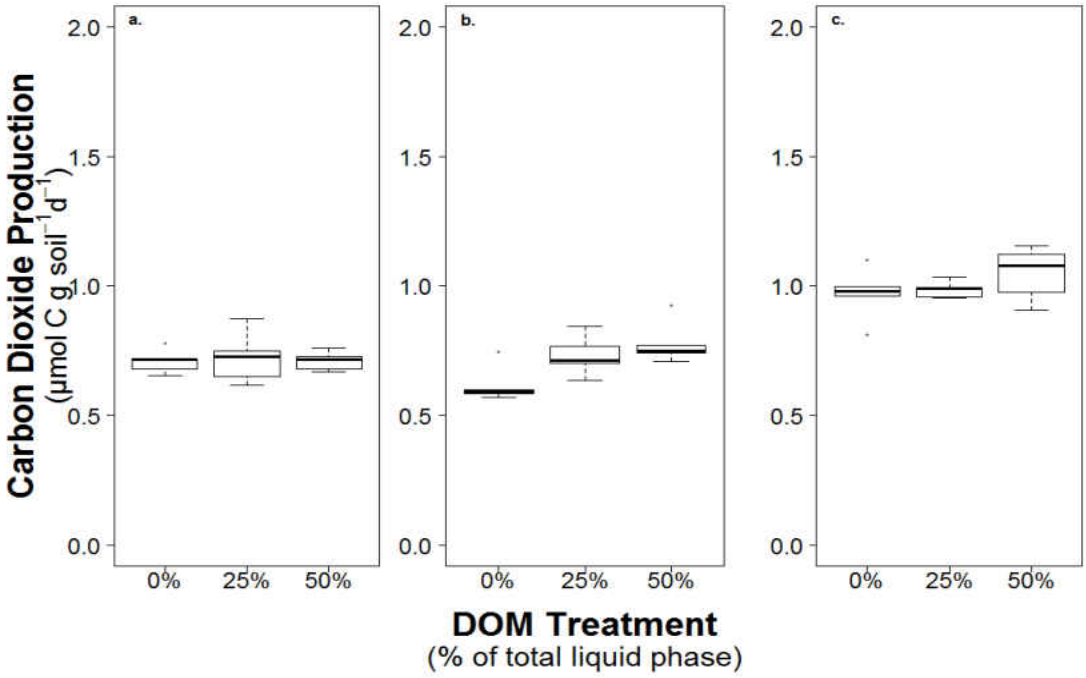


Figure 3.5. Carbon dioxide production in peat samples collected at 75-100 cm in July 2014 from BL Fen **(a)**, S1 Bog **(b)**, and Zim Bog **(c)**. The thick black line represents the median value, the box edges denote the upper and lower 25% quartiles, and the whiskers show the maximum and minimum values (excluding outliers). Outliers are shown with open circles. No significant differences were observed within treatments or across sites.

and CO_2 = 0.85 ± 0.043 µmol C g soil^{-1} d^{-1}; 50% Surface DOM: CH_4 = 3.0 ± 0.099 nmol C g soil^{-1} d^{-1} and CO_2 = 0.88 ± 0.075 µmol C g soil^{-1} d^{-1}). When we repeated this experiment with increased replication in 2014, we found no measurable CH_4 production in anaerobic incubation of deep (75-100 cm) peat samples that were combined with surface (25 cm) or deep (100 cm) porewater. Throughout this incubation, sample CH_4 concentrations were often at or below our methodological detection limits (~0.25 ppmv CH_4).

Discussion

In-situ DOC concentration and aromaticity – DOC concentration and aromaticity decreased with depth in BL Fen and S1 Bog, suggesting that these peatlands contain

reactive DOC that is utilized by microbial communities. Conversely, the lack of change in DOC concentration and aromaticity at Zim Bog indicates that this C source is fairly non-reactive. Previous studies in northern Minnesota peatlands, including S1 Bog, have shown that decreases in DOC aromaticity with depth are often associated with the formation of new compounds with low O/C and high H/C elemental ratios, indicating humification of peat and its utilization as a microbial C source (Tfaily et al., 2013; Tfaily et al., 2014). The DOC concentration and SUVA values reported here are in line with previous studies and highlight the differences in DOC quantity and chemistry across peatland types and with depth (Pastor et al., 2003; Leifeld et al., 2012; Corbett et al., 2013a; Tfaily et al., 2014; Wilson and Hopple et al., 2016).

Carbon source fueling anaerobic respiration – Our experimental, laboratory-based study confirms the importance of DOM as a primary driver of methanogenesis by demonstrating that increasing DOM content significantly increased surface CH_4 production rates across all peatland types. However, we observed no effect of increasing DOM content on rates of CH_4 production when DOM was doubled from 50 to 100% in non-manipulated surface peat samples, suggesting a saturating function of DOM past 50% concentrations and indicating that some other unknown factor limits surface methanogenesis. Perhaps a longer incubation time allowing methanogen population growth would have resulted in a positive response at higher DOM concentrations. This result is consistent with field studies of radiocarbon ages of CH_4, CO_2, DOM, and peat, which suggest that anaerobic respiration in peatlands is fueled by DOM (Chanton et al., 1995; Chanton et al., 2008; Corbett et al., 2013a; Tfaily et al., 2014; Hoyt et al., 2014;

Wilson and Hopple et al., 2016). Furthermore, a mesocosm study that incorporated warming and water-table manipulations in a Minnesota bog (monoliths were from Zim Bog) and fen found that greater DOM retention was correlated with a large increase in CH_4 and dark CO_2 flux (Pastor et al., 2003).

Overall, our results suggest that the response of methanogenesis to changes in DOM content is consistent across peatland type, contrary to our expectations. However, site-specific analyses provide weak evidence that fen CH_4 production may respond more strongly to increasing DOM than that of bogs. For example, when examined within each site in July 2014, the influence of DOM on CH_4 production was only statistically significant in BL Fen ($p < 0.05$), where methanogenesis increased by 54 and 68% as DOM concentration increased from 0 to 25 and 50%, respectively (Figure 3.3a). There was a stepwise increase in mean CH_4 production with increasing DOM addition in Zim Bog and the highest mean CH_4 production occurred in the 50% DOM treatment in S1 Bog (Figure 3.3b and c), but large variation within treatments made these effects nonsignificant.

DOM chemical quality has been cited as a main factor for controlling its use as a C substrate for microbial respiration in peatlands (e.g., Glaser et al., 2016) and was found to vary across the three peatlands included in this study (Figure 3.2b). Fen DOM has a lower molecular weight, is 3-12 times more photo-reactive, and has lower C/N ratios than that of bog DOM and, furthermore, fen DOM aromaticity decreases with depth, but aromaticity remains constant in bogs (Leifeld et al., 2012; Corbett et al., 2013a; Tfaily et al., 2013; Tfaily et al., 2014). All of these properties indicate that fen DOM is more labile and reactive than bog DOM, leading to its preferential utilization as a C source in fens

relative to bogs. However, our results provide only weak evidence supporting this hypothesis, possibly because our washing process likely removed soluble compounds, such as nutrients, that could be important in controlling microbial respiration. Nevertheless, further study is necessary to better understand how different peatland types respond to changes in DOM availability and chemistry.

Field studies employing radiocarbon analyses have also found that the age of CO_2 is very similar to that of DOM in fens and intermediate between the ages of DOM and solid-phase peat in bogs –demonstrating the importance of DOM as a driver of CO_2 production in addition to CH_4 production. In contrast, we found no influence of DOM manipulation on CO_2 production rates at any site or depth in either experiment. The radiocarbon field studies were observing the net effect of production and transport pathways of gasses within the peat profile that are likely temporally and spatially variable, but are measured at a single (or a few at most) point in time, which may provide different dynamics than observed in our laboratory study.

Nonetheless, it is intriguing that methanogens in our study responded to the DOM treatments in surface peat while the CO_2-producing microbial consortia did not. This study emphasizes the sensitivity of methanogenesis to differences in DOM availability and chemistry, which may be substantially altered under current and future climate change. Most peatlands occur above 40 °N latitude, where the largest relative temperature changes are projected to occur under current climate models and, therefore, are perceived to be particularly susceptible to climate change (Kirtman et al., 2013). Climate warming has been shown to decrease the abundance of *Sphagnum* spp. and lichens and increase the abundance of sedges and shrubs in peatland plant communities, which could alter organic

matter reactivity and humification rates. For example, Medvedeff et al. (2015) found that *Sphagnum*-derived leachates, collected from the three peatlands used in our study, played a role in regulating peatland CH_4 production and that the various *Sphagnum*-derived extracts produced responses that differed based on peatland type. Similarly, our results indicate that DOM is a major driver of peatland methanogenesis and that this process is particularly sensitive to differences in DOM availability; however, DOM measurements are not widely quantified nor incorporated into ecosystem process models or climate forcing predictions (Bridgham et al., 2013a).

Increasing surface-derived labile carbon availability

While we provide evidence of the importance of DOM as a substrate for CH_4 production in surface peat, we were unable to document any effect of DOM manipulation on rates of CH_4 production in deeper peat. Although both fen and bog DOM at depth are relatively young in age compared to the solid peat matrix, only deep fen DOM has been shown to have lower lability and reactivity than surface DOM, while bog DOM exhibits similar C chemistry throughout the peat profile (D'Andrilli et al., 2010; Tfaily et al., 2013; Corbett et al., 2013a,b; Tfaily et al., 2014). Numerous studies have documented similar low CH_4 production rates in deep peat (Galand et al., 2003; Cadillo-Quiroz et al., 2006; Kotiaho et al., 2010), and we recently found that even one year of *in situ* warming of deep peat did not increase the low rates of methanogenesis in S1 Bog (Wilson and Hopple et al., 2016). While radiocarbon data show that most anaerobic mineralization at depth is derived from relatively young DOM (Chanton et al., 2008), CH_4 production is apparently proximally inhibited by unknown factors beyond temperature and substrate

availability. We have observed a large decrease in gene and transcript number for the α-subunit of the methyl coenzyme M reductase (*mcrA*), a functional gene common to all methanogens, from a peak at 20 cm depth in these sites (Zalman et al., in review), suggesting lower methanogen abundance and activity with increasing peat depth. It is likely that the methanogen population was unable to respond to increased labile DOM availability over the two-week incubation.

Conclusions

Traditionally, peat C mineralization is quantified on a per solid-gram basis and is used in this form to determine ecosystem respiration rates. However, peatlands have two forms of C reservoirs (as do other wetland ecosystems): solid-phase organic matter (peat) and DOM. This DOM pool is not widely incorporated into ecosystem process models or climate forcing predictions because peat has been presumed to be the major contributor of CH_4 and CO_2 emissions since it is the primary form of C storage (Bridgham et al., 2013a). However, our study confirms the importance of DOM as a primary driver of methanogenesis by demonstrating that increasing DOM content significantly increased surface CH_4 production rates across a variety of peatland types. These findings generally support the conclusions of recent radiocarbon studies which suggest that anaerobic respiration in peatlands is fueled by DOM (Chanton et al., 2008; Corbett et al., 2013a; Hoyt et al., 2014). Additionally, the lack of response of CO_2 production to DOM manipulation highlights the sensitivity of CH_4 production to change in DOM quantity and quality, which are likely to occur under future climate change scenarios and emphasizes the importance of continued study into the controls of heterotrophic peatland respiration.

Finally, we also show that increasing surface-derived DOM content in incubations of deep peat does not stimulate deep methanogenesis, suggesting that this process is limited by additional factors beyond labile C limitation.

Bridge to Chapter IV

Predicting the fate of stored C and future CH_4 emissions from peatlands is particularly challenging due to the mechanistic complexity of CH_4 cycling in these systems. We can begin to tackle this issue by addressing a deceptively basic question – what is the C source fueling CH_4 production in peatlands? Traditionally, solid peat has been presumed to be the major contributor of peatland CH_4 emissions since it is the primary form of C storage; however, Chapter III demonstrates the importance of DOM as a driver of surface methanogenesis across a variety of boreal peatlands, in-line with recent radiocarbon studies. Yet, this variable is not currently incorporated in any ecosystem process models. In addition to re-parameterizing our Earth system models to incorporate DOM as a primary methanogenic substrate, it is imperative that we also expand our mechanistic understanding of peatland CH_4 cycling in the context of environmental change. As the majority of peatland C is stored at depth, it is particularly important to understand the variables controlling deep peatland methanogenesis - as Chapter III showed, it is limited by factors beyond simple labile C availability, so what exactly is limiting methanogenesis at depth? How will peatland responses to environmental change vary throughout the entire soil profile? In Chapter IV, I address these questions in collaboration with the Spruce and Peatland Responses to Under Changing Environments (SPRUCE) experiment, a regression-based, ecosystem-scale

climate manipulation experiment. This chapter integrates measures of in situ greenhouse gas flux, laboratory incubations, in situ analyses of ^{14}C and dissolved gasses and microbial community structure and metabolic potential throughout the soil profile to assess the response of a boreal peatland to 13 months of deep-peat heating.

CHAPTER IV

STABILITY OF PEATLAND CARBON TO RISING TEMPERATURES

From Wilson, R. M. and Hopple, A. M., Tfaily, M. M., Sebestyen, S. D., Schadt, C. W., Pfeifer-Meister, L., Medvedeff, C., McFarlane, K. J., Kostka, J. E., Kolton, M., Kolka, R. K., Kluber, L. A., Keller, J. K., Guilderson, T. P., Griffiths, N. A., Chanton, J. P., Bridgham, S. D., & Hanson, J. P. (2016). Stability of peatland carbon to rising temperatures. *Nature communications*, **7**, 13723.

Contributions

R. M. Wilson and A. M. Hopple wrote the manuscript with contributions from all co-authors. A. M. Hopple, J. K. Keller, S. D. Bridgham, and C. Medvedeff designed the incubation experiments. A. M. Hopple conducted the incubations with C. Medvedeff and analyzed resultant data with S. D. Bridgham and J. K. Keller. R. M. Wilson, J. P. Chanton, K. J. McFarlane, and T. P. Guilderson collected and analyzed radiocarbon samples. C. Medvedeff, R. K. Kolka, L. Pfeifer-Meister, C. W. Schadt, L. A. Kluber, J. E. Kostka, J. K. Keller, and M. Kolton assisted in coring events. S. D. Sebestyen and N. A. Griffiths collected and analyzed DOC samples. P. Hanson collected and analyzed flux measurements, and manages all field activities with R. K. Kolka. J. E. Kostka, M. Kolton, C. W. Schadt, and L. A. Kluber designed, collected, and analyzed microbial community data. M. Kolton and J. E. Kostka designed the enzyme activity experiments, with M. Kolton conducting the analyses.

Introduction

Peatlands store a globally significant fraction of the world's carbon (C) in deep recalcitrant peat[1] which, if destabilized, could result in catastrophic positive feedbacks to climate warming. However, all soil warming experiments exploring the response of peatland C banks to climate forcing to date have utilized limited surface warming techniques (generally +1°C), ignoring the effects on deeper buried layers[2, 3]. Thus, despite the established significance of peatlands in the global C cycle, their response to future climate change remains poorly constrained[4, 5], because under long-term warming, deep soil temperatures will increase in parallel with atmospheric temperatures[6, 7, 8, 9]. The large reservoirs of C at depth (well over a meter) mean that this largely ignored C fraction could play a significant—though as yet unquantified— role in future climate change.

To address this gap, the Spruce and Peatland Responses Under Climatic and Environmental Change (SPRUCE; http://mnspruce.ornl.gov) experiment is assessing how northern peatland ecosystems react to a changing climate with a regression-based, ecosystem-scale climate manipulation that incorporates deep peat heating to a depth of 2 m[10]. The SPRUCE experiment is located at the S1 bog within the Marcell Experimental Forest (Minnesota, USA) [11]. Ultimately, the SPRUCE experiment will include both above- and below-ground warming, as well as ambient and elevated air CO_2 concentrations in a multifactorial experimental design. However, belowground deep peat heating (DPH) was initiated first and is the sole treatment reported here. DPH represents a novel experiment that provides the first field-scale examination of the response of deep C and associated heterotrophic microbial communities to warming. From June 2014 through August 2015, DPH treatments to > 2 m depth were established within ten, 12 m

diameter plots (0, +2.25, +4.5, +6.75 and +9 °C, relative to ambient, in duplicate, see Extended Data Figure 4.1 for schematic of the site) within the S1 bog, following the approach described in Hanson et al.[6]. Briefly, low-wattage, 3 m long belowground heaters were installed equidistant around the circumference and beneath each treatment plot to heat the soil to the desired temperature differential[10]. Target temperature differentials were achieved at 2 m depth by September 2014 (Extended Data Figure 4.2a). The absence of air warming during this phase of the experiment resulted in heat loss at the surface, creating less separation among temperature treatment plots in the shallow peat relative to the 2 m depths (Extended Data Figure 4.2b). Deep peat is expected to warm naturally, in parallel with surface warming, due to the propagation of heat downwards into the peat column. However, to achieve this effect in a tractable timescale for experimentation, active heating of the peat at depth is required[7]. While the highest climate trajectories project temperature increases up to +8.3°C (±1.9°C) in the Arctic between 2081 and 2100[12], the +9°C treatment employed in this study is an upper limit on what can be expected under the most extreme scenarios. We employ this treatment to explore threshold response surfaces to temperature change (*e.g.*, Epping et al. [13]) and because the multiple treatment effects above the median +2°C temperature projection allow for non-linear curve response fitting. During the DPH experiment, we measured water table depth in each plot (30-min measurement frequency), and did not observe—nor did we expect—any changes in water table elevation that was attributable to the deep peat warming treatment. Water tables were usually within 20 cm of the mean hollow surface and fluctuated over an approximately 40 cm range due to rainfall, snowmelt inputs, near-surface lateral flow, and evapotranspiration, but with no apparent effect of

DPH. In addition, water table dynamics inside experimental plots mirrored those measured in ambient reference plots and the surrounding bog.

Results and Discussion

Once the experimental plots reached target temperature differentials, CH_4 flux increased exponentially with deep soil temperature (Figure 4.1a) despite reduced warming at the surface due to energy losses. CH_4 flux was dramatically reduced by snow and ice cover during the winter (Figure 4.1b) and the temperature response was maximal during the peak growing season (Figure 4.1c). Net ecosystem respiration—as measured by dark CO_2 flux—was not correlated with deep soil temperature during any measurement time (Extended Data Figure 4.3).

Consistent with these field emission results, when peat was incubated anaerobically within 1°C of *in situ* temperatures, CH_4 production in surface peat (20-30 cm below the hollow surface) increased with temperature

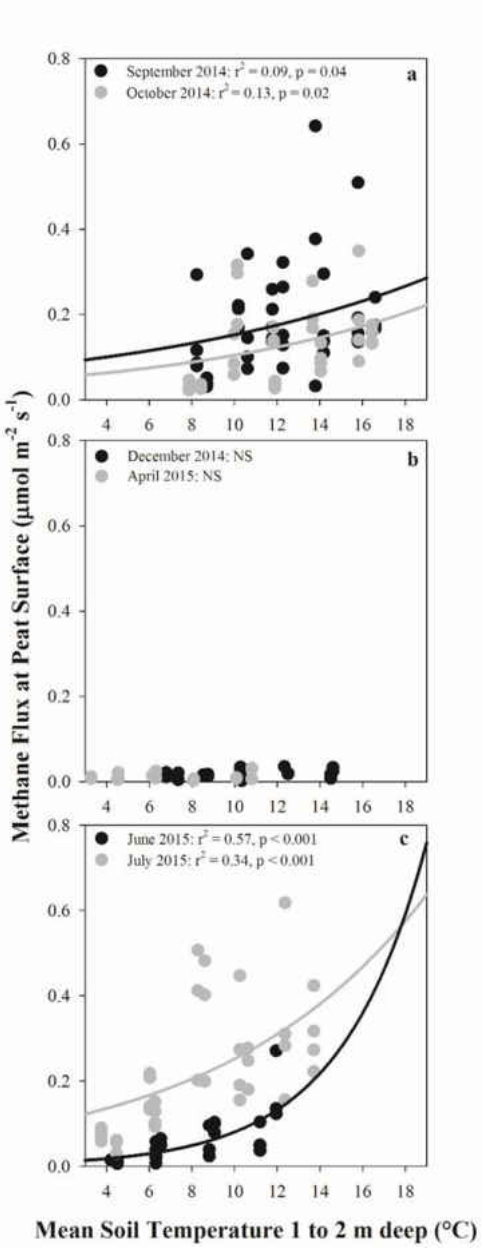


Figure 4.1. Seasonal CH_4 flux vs. *in situ* temperatures from 1.2 m diameter collars during **(a)** fall 2014, **(b)** winter 2015, and **(c)** summer 2015. Black and gray dots distinguish between daily averages for two different sampling times during each season. Significant correlations between flux and temperature are indicated on the graphs by exponential regressions.

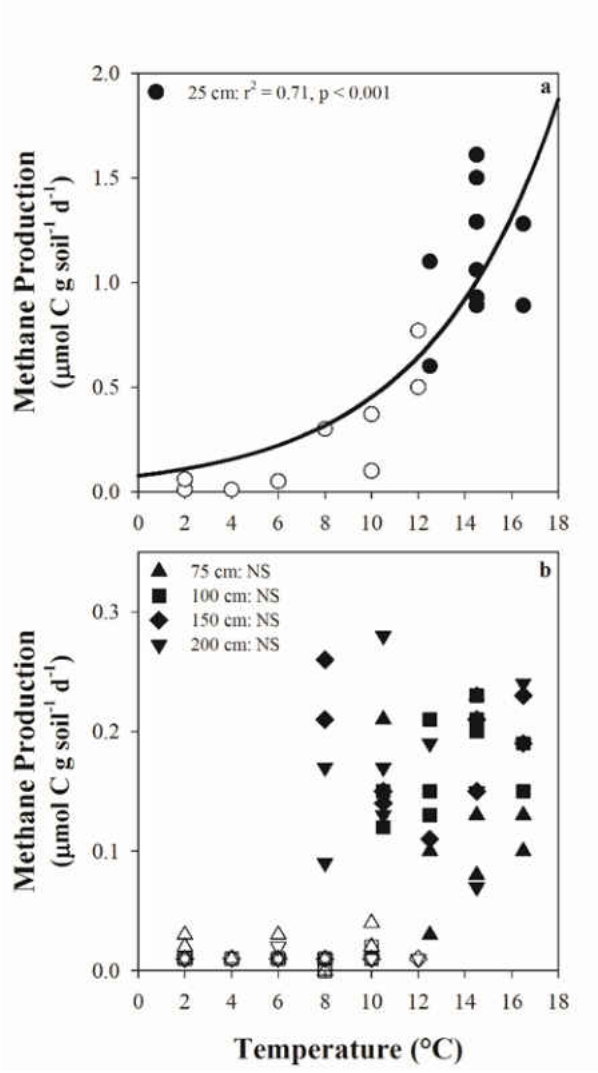


Figure 4.2 Temperature response of CH_4 production from surface **(a)** and deep **(b)** peat samples that were anaerobically incubated within 1ºC of *in situ* temperatures after approximately 4 (closed symbols, September 2014) and 13 (open symbols, June 2015) months of deep peat warming. Temperatures reflected *in situ* temperatures at time of collection. The temperature response of deep peat **(b)** for each season was analyzed separately due to a distinct bimodal distribution. NS = not significant.

($p < 0.001$) (Figure 4.2a). This layer is within the acrotelm[14], but was consistently anaerobic at the time of sampling. Surface peat had greater CH_4 production rates than peat from deeper depths except at the coldest experimental treatment temperatures. Importantly, no relationship between temperature and CH_4 production was observed in incubations of peat from deeper depths ($p \geq 0.97$, Figure 4.2b), implying that the increased CH_4 emissions observed in the field were largely driven by surface peat warming. *In situ* microbial data support this conclusion. The majority of Archaea in the shallow peat were methanogens whose relative abundance declined with depth, in concert with methanogenesis rates (Extended Data Figure 4.4). Correspondingly, the functional methanogen gene methyl coenzyme A reductase (mcrA) was more than ten-times higher at depths 20-40 cm than at depths below 50 cm (Extended Data Figure 4.5d). No temperature effect was observed on the relative abundance of methanogens or

Archaea (Extended Data Figures 4.4 and 5), suggesting that factors other than temperature are limiting decomposition in the deep peat.

CO_2 production in the surface peat incubations was greater than that at other depths (GLM, $p < 0.001$), and increased with temperature ($p < 0.001$; Extended Data Figure 4.6a), reflecting only the response of heterotrophic processes to temperature since photosynthetic and aerobic processes were excluded by the incubation design. These results differ from the field, where dark CO_2 flux did not correlate with temperature treatment, possibly because autotrophic processes were excluded in the incubations, or because CO_2 production in the field was greatest at depths shallower than 20 cm. No consistent response of CO_2 production to temperature was observed in incubations of deeper peat (Extended Data

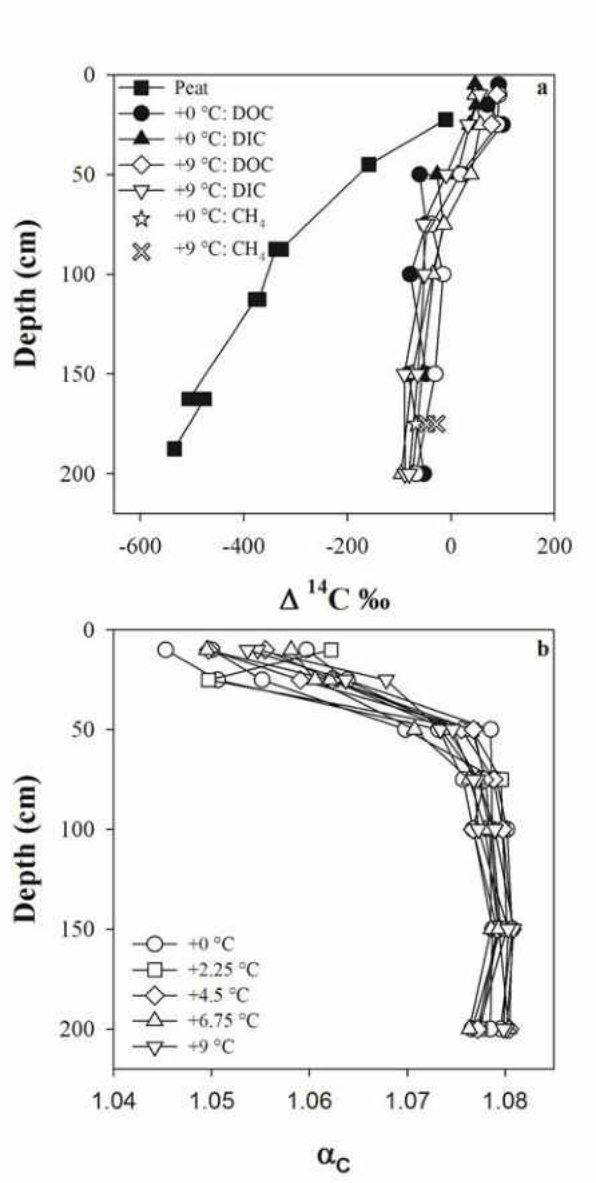


Figure 4.3. Isotopic composition of respiration products and substrates prior to and during DPH. Depth profiles of ^{14}C for solid peat, dissolved organic carbon (DOC), CH_4, and dissolved CO_2 (DIC) **(a)**. In panel **(a)** closed symbols represent values from control plots prior to DPH when no treatment (i.e. +0°C) was applied, open symbols represent values from +9°C treatment plots during DPH (June 2015). *Note the age difference between solid peat and all DOC and DIC values.* Difference in stable carbon isotopic ($\delta^{13}C$) composition between DIC and methane (α_C = [($\delta^{13}CO_2$ + 1000)/($\delta^{13}CH_4$ +1000)]) with depth during DPH (June 2015) **(b)**.

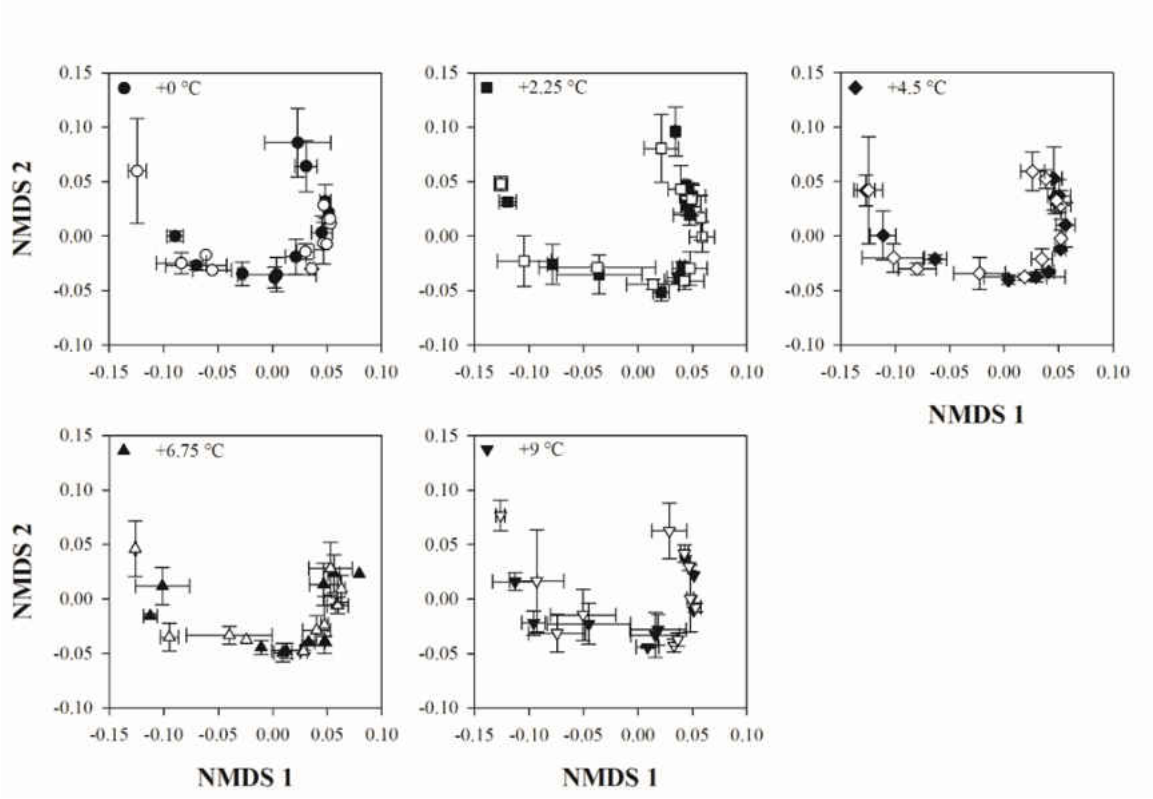


Figure 4.4. Characterization of *in situ* microbial community structure by non-metric multidimensional scaling (NMDS) indicates no significant effect of temperature or time on community diversity or composition. Values represent each plot/depth within each temperature treatment plot from pre-DPH (2014, closed symbols) and 13 months post-initiation of the DPH (2015, open symbols) experiment. Final sequence data were normalized by cumulative sum scaling (CSS), and beta diversity indices were estimated based on Bray–Curtis and weighted as well as unweighted Unifrac distances. Significant differences in beta diversity were analyzed by a PERMANOVA test on weighted Unifrac distance metrics with 1000 permutations followed by Bonferroni correction of p-values.

Figure 4.6b). The CO_2:CH_4 ratio was negatively correlated with temperature in the surface ($p \leq 0.01$, Extended Data Figure 4.7), indicating that anaerobic respiration may become increasingly methanogenic with warming in agreement with results from previous incubation studies[15, 16].

Enclosure water table positions did not fluctuate due to DPH, indicating that the ecosystem responses we observed were driven solely by warmer temperatures. However, direct warming of surface peat is expected to lead to a decrease in water table depth, increasing O_2 availability throughout this soil horizon. Greater O_2 availability will

enhance decomposition and aerobic CH_4 oxidation, likely resulting in an overall reduction of CH_4 emissions[17].

Replicate anaerobic incubations from each treatment/depth were conducted at 20°C to determine if any "legacy effects" following 13 months of temperature treatment persisted after removing the direct effects of temperature. In agreement with the incubations at *in situ* temperatures, we observed greater CH_4 and CO_2 production in surface peat relative to that of deeper peat. Although CH_4 and CO_2 production rates were higher at 20°C than in the incubations at *in situ* temperatures, there was no correlation between production rates and the initial treatment temperature (Extended Data Figure 4.8). The lack of a legacy warming effect in the surface peat after the first 13 months suggests that the warming treatment did not have a lasting effect—relevant to CO_2 and CH_4 production rates—on the microbial community or the peat itself.

To further verify the role of surficial processes in the field CH_4 flux response, we compared the natural abundance $\Delta^{14}C$ of the CO_2 (DIC) and CH_4 dissolved in peat porewater with the dissolved organic carbon (DOC) and solid peat. DOC at S1 bog is younger than the peat at all depths[14], indicating that it is largely derived from recent photosynthate as opposed to the progressively older solid phase peat at depth (Figure 4.3a). Increasing temperatures are likely to stimulate photosynthesis rates and increase root exudation of organic C available for decomposition[18]. The young age of the DOC and the lack of a temperature effect on DOC concentrations (Extended Data Figure 4.9) show that there was not significant leaching of ancient catotelm C into the dissolved pool after 13 months of warming. Thus, we could use DOC and the peat as endmembers to differentiate the source of organic matter fueling heterotrophic respiration—either recent

photosynthate or ancient catotelm peat[19, 20]. In all plots and depths, the $\Delta^{14}C$ indicate that CH_4 and DIC were relatively young, ^{14}C-enriched relative to the peat, and indistinguishable from the $\Delta^{14}C$ of the DOC (Figure 4.3a). These results are consistent with respiration fueled by younger surface-derived C sources, rather than by degradation of ancient catotelm C[19, 20]. DIC and dissolved CH_4 concentrations were also stable across all treatments (not shown).

The difference in stable C isotope values of DIC and CH_4, represented by α_C $[(\delta^{13}CO_2 + 1000)/(\delta^{13}CH_4 + 1000)]$, identifies shifts in the dominant methanogenic pathway because hydrogenotrophic methanogens fractionate C more than acetoclastic methanogens[21]. The $|\ |_C$ increased with depth in all treatment plots consistent with a shift from acetoclasty in the shallow (<50 cm) to hydrogenotrophy at depth. The magnitude of the isotopic shift as well as the depth at which the shift occurred was similar across temperature treatments (Figure 4.3b), suggesting that DPH did not significantly influence the depth distribution of dominant CH_4 production pathways. This finding is contrary to what Dorrepaal et al.[22] and McCalley et al.[23] found following warming-induced permafrost thaw in peatlands and suggests that the response of heterotrophic respiration to climatic warming may differ between peatlands with different cryogenic histories, mineral contents, microbial population dynamics, and plant community compositions[24]. In particular, our results contrast those of permafrost peatlands exposed to thaw. In permafrost settings—particularly syngenetic permafrost—the organic matter is frozen at a partially decomposed state, that is, decomposition is suspended preserving labile material. As permafrost thaws, that labile material becomes available enhancing decomposition rates. In contrast, non -permafrost peatlands only experience seasonal

freezing in surface peat, leading to millennia of slow decomposition of deep peat. In the case of S1 bog over at least the time frame of this study, the result is temperature insensitivity of the decomposition of recalcitrant deep peat.

Microbial community data corroborate findings from laboratory incubations and porewater chemistry. Based on 12 million gene sequences retrieved from 220 samples, microbial community composition and diversity were similar across all temperature treatments and between years (Figure 4.4, Extended Data Figure 4.10). Both C decomposition and microbial community structure exhibited strong vertical stratification, similar to pre-treatment findings[25]. The majority of microbial populations (~70%) were taxonomically affiliated with *Proteobacteria* and *Acidobacteria* (Extended Data Figure 4.11), and microbial diversity decreased with depth (not shown). Members of the *Alphaproteobacteria* and *Acidobacteria,* classes—which in peat contain abundant aerobic heterotrophs[26]—decreased in relative abundance with depth, while putative anaerobes (*e.g,. Deltaproteobacteria* and TM1) increased in the catotelm (Extended Data Figures 4.12 and 4.13). One year after treatment initiation, quantitative PCR of the 16S and 18S rRNA genes also showed decreasing overall bacterial, archaeal and fungal abundance with depth but no significant response to temperature (Extended Data Figure 4.5).

Degradation of recalcitrant, lignin-like compounds that are abundant in peatland soils is mediated by the activity of extracellular oxidative enzymes, namely phenol oxidases and peroxidases[27, 28]. In agreement with sequence-based results, the metabolic potential of microbial communities, as determined by enzyme activity potentials, was consistent across temperature treatments after 13 months of heating (Extended Data Figures 4.14 and 4.15). A clear vertical stratification in phenol oxidase activity occurred

with peat depth likely reflecting differences in dissolved O_2 availability. While the highest phenol oxidase activity occurred in shallow peat (0-30 cm), the highest phenol peroxidase activity occurred in the catotelm (>30 cm) (Extended Data Figure 4.15).

Deep peat heating up to 9°C above ambient failed to stimulate catotelm C decomposition in this ombrotrophic bog within the first 13 months of this experiment. It should be noted that the lack of response reported here may be specific to ombrotrophic bogs and does not necessarily reflect the expected or observed response from peatland habitats such as fens or permafrost peatlands. While there is evidence of kinetic control on surface peat decomposition in our experiment, non-kinetic factors—such as chemical recalcitrance[14]—appear to be controlling the decomposition of deep C at S1 bog. Tfaily et al.[14] report a marked decrease in the o-alkyl C content of catotelm peat relative to acrotelm peat at S1 bog, indicating intensive decomposition of carbohydrates. Previous studies have linked o-alkyl C content to peat reactivity[29, 30] and have observed clear decreases in o-alkyl peat content from northern peatlands to tropical peatlands (S. Hodgkins, *pers. comm.*, 2016). Thus, we hypothesize that the lack of reactivity of SPRUCE deep peat was due to the low o-alkyl C content of the soil organic matter. Therefore, future warming will likely have little effect on the conversion of catotelm C to CO_2 and CH_4. However, catotelm peat recalcitrance is a relative term. We have shown that catotelm peat is recalcitrant with respect to temperature under its present conditions—water saturated, with fermentation and methanogenesis as the dominant organic matter decomposition processes.

Other climate-induced perturbations to the ecosystem—changes in water-table depth, increased plant productivity, belowground exudation of labile plant compounds, or

changes in plant communities—could have cascading effects on peatland C dynamics. For example, lowering of the water table due to increased evapotranspiration could increase O_2 availability providing the necessary conditions for degradation of recalcitrant phenolic compounds in the catotelm, which have been proposed to protect the global C bank in deep peat through inhibition of microbial heterotrophy according to the "enzyme latch" hypothesis[31]. However, recent studies have shown that temperature, water-table depth, and perhaps even nutrient availability may control the strength of the enzyme latch and that therefore the response of phenolic compound degradation to climate drivers may be more complicated than originally hypothesized[32, 33]. While peat decomposition was enhanced in incubations of surface peat, our results provide evidence that C decomposition in deep anaerobic peat is not kinetically constrained; therefore, peat decomposition is most likely thermodynamically limited by the absence of suitable electron acceptors.

However, even if global warming-induced increases in CH_4 production are confined to surface processes in ombrotrophic bogs, this could still represent a substantial natural feedback to anthropogenic climate forcing. Specifically, the exponential increase in CH_4 flux observed in the field plots coupled with the decrease in CO_2:CH_4 ratios in the surface peat incubations is troubling given that CH_4 has a sustained global warming potential (SGWP) 45-times that of CO_2 on a 100-year timescale[34]. Further, these surface responses were underestimated due to energy loss at the surface that muted the warming treatment in surface peat. With surface warming, it is likely that the surficial response will be even greater. Thus, even if warming stimulates plant biomass production and enhances soil C sequestration it is unlikely these effects will completely offset the

increases in CH_4 flux on this time scale. However we must temper our interpretation because the observed surface response may be a transient perturbation effect as has been seen in other climate manipulation experiments[35, 36, 37]. In addition, increased frequency and duration of low water table elevations and flow along near-surface lateral flowpaths are most likely to affect surface peat, which may exacerbate or mitigate the responses that we observed[38, 39]. For example, even with a temperature increase, a lowered water table could reduce CH_4 production, enhance oxidation and result in lowered CH_4 emissions. In peatlands feedbacks exist among plant communities, water table dynamics, and physical properties of the peat resulting in a tight coupling between C and water cycling[35,40] that allows the system to self-regulate, resisting gradual environmental change until a catastrophic tipping point is reached and the system shifts towards a new steady-state[13,35]. For example, *Sphagnum* and vascular plants, respectively, alter environmental conditions such as light and nutrient availability, water table depth, temperature, and pH[13, 35]. The long-term SPRUCE experiment will enable us to examine whole-ecosystem warming, enhanced atmospheric CO_2 and water table feedbacks to these treatments, allowing us to clarify the internal mechanisms that control C cycling in a bog over a decade-long manipulative climate change study.

Methods

Site description: The SPRUCE experimental site, S1 bog (8.1 ha), is located in northern Minnesota, USA within the Marcell Experimental Forest (MEF: N 47°30.476´; W 93°27.162´). The S1 bog has been the subject of extensive past research and has been described previously[10, 14, 25]. This precipitation-fed, ombrotrophic bog has an average pH

of 4.1 at the surface that increases with depth to an average value of 5.1 at 2 m. Overstory vegetation is dominated by two tree species, Picea mariana (black spruce) and Larix laricina (larch), while the understory is composed mainly of low ericaceous shrubs, such as Rhododendron groenlandicum (Labrador tea) and Chamaedaphne calyculata (leatherleaf), as well as the herbaceous perennials Maianthemum triflorum (three-leaved Solomon's seal) and Eriophorum vaginatum (cottongrass). The bog surface is characterized by hummock and hollow microtopography, with Sphagnum magellanicum colonizing the hummocks and S. angustifolium the hollows. Typically, the hummocks are 10-30 cm higher than the hollows. Plant cover varies only slightly among the plots measured for surface CO_2 and CH_4 efflux. All plots have a nearly uniform cover of Sphagnum over the hummock-hollow complex over which an ericaceous shrub layer is present. During the summer months, limited populations of the forb Maianthemum and some sedges also occupy the plots.

Deep-peat heating: The SPRUCE project involves an ecosystem-scale climate manipulation in the S1 bog. The experimental design includes ten 12 m diameter chambers that are warmed to 5 temperatures (+0, +2.25, + 4.5, +6.75, and +9 °C), with duplicate plots to be subjected to ambient and ~ +500 ppmv CO_2. In the most novel aspect of this experiment, the peat is warmed throughout the peat column to depths of 2 - 3 m[6], providing the first field-scale examination of the responses of deep peat to climate forcing. The open-top chamber design allows surface warming and enhancement of atmospheric CO_2; while sub-surface corrals hydrologically isolate each experimental plot and allow for changes in water table associated with warming and elevated CO_2 to

develop. **However, DPH was the only experimental treatment applied during this study.**

DPH was initiated between June 17 and July 2, 2014 as the electrical systems for each plot became available. Stable target treatment temperature differentials at 2 m deep were achieved in all plots by early September 2014. DPH is accomplished by an array of 3-m vertically installed low wattage (100 W) heating elements housed within plastic coated iron pipes and placed throughout the plots in circles of 48, 12, and 6 heaters at 5.4, 4 and 2 m radii, respectively. A single heater was also installed at the plot center. Exterior heaters in the circle of 48 apply 100 W across the full linear length of the heater, and all interior heaters apply 100 W to the bottom one third of each resistance heater (pipe thread core heaters, Indeeco, St. Louis, MO). DPH within the experimental plots is achieved through proportional-integral-derivative (PID) control of three exterior (the circle of 48 split into alternating thirds) and two interior circuits of the resistance heaters. The reference depth for temperature control is 2 m deep.

Temperature differentials within a treatment pair were typically within 0.5 °C of the target temperatures throughout the measurement period. Temperature variation in the no-energy-added control plots was likely driven by differences in tree canopy cover with greater cover leading to warmer peat temperatures (i.e., less heat loss to the sky). Once deep peat temperature differentials were achieved, they were largely maintained from 1 m to 2 m deep during large seasonal shifts in temperatures (Extended Data Figure 4.2).

Analysis of CH_4 and CO_2 flux: Measurement of CO_2 and CH_4 emissions from the peatland was conducted in 1.2 m diameter permanent collars embedded 10 cm into the

peat. Briefly, collars were covered with an opaque dome under which headspace accumulation techniques were applied. Gas accumulation under the darkened dome was measured with open path CO_2/H_2O (LiCor 7500) and CH_4 analyzers (LiCor 7700). An individual observation lasted only minutes without dramatic changes in temperature, pressure or target gas concentration above the surface of the peat. Seasonal flux measurements were fit against the average temperature from 1 to 2 m below the hollow surface with an exponential regression model using SigmaPlot v 12.3 and significant relationships identified at $p < 0.05$.

Analysis of CH_4 and CO_2 production with anaerobic incubations: Intact soil cores were collected at 20-30, 50-75, 100-125, 125-150, and 175-200 cm depths from each experimental plot in September 2014 and June 2015, after approximately four and thirteen months of DPH, respectively, to discern how rates of CO_2 and CH_4 production varied with depth. All depths were measured relative to the surface of the hollows. To prevent compression of surface peat samples, a serrated knife was used to collect a 10cm-diameter core from the hollow surface to approximately 20cm within the peat profile. A 5cm-diameter Russian corer was subsequently used to extract the remaining samples up to a 2m depth. The soil cores were kept anaerobic, stored on ice, and shipped overnight to the University of Oregon, where incubations commenced immediately within 1°C of in situ temperatures. Samples were slurried with a 1:1 mixture of peat and porewater collected from the same plot and depth. CO_2 and CH_4 production concentrations were determined[42] during the course of the 10 day incubation.

All statistical analyses were conducted using SPSS Statistics version 22. Data were tested for normality and log-transformed where the transformation resulted in a significant improvement in overall distribution. General Linear Model (GLM) analysis was used to investigate the effect of temperature, depth, and the interaction of these two variables on CH_4 and CO_2 production, as well as the CO_2:CH_4 ratio. If significant differences among depths were detected ($p < 0.05$), pairwise comparisons using Tukey's HSD (honest significant difference) test ($p < 0.05$) were conducted. If not significantly different, depths were combined for linear regression analysis. If normally distributed, CH_4 and CO_2 production rates and CO_2:CH_4 ratios were combined across sampling time points and linear or exponential regression was used to determine the temperature response of each process.

Analyses of porewater gas and isotopic composition: Porewater samples were collected in June 2015 for analysis of CH_4 and CO_2 concentrations, $\delta^{13}C$ and ^{14}C using permanently installed piezometers at 25, 50, 75, 100, 150, and 200 cm depths within each experimental plot. Piezometers were covered, but not sealed, when not being actively sampled, the diameter of the piezometers was less than 1 cm which limited oxygen diffusion, and piezometers tubes were pumped dry 24 hours prior to sampling to ensure that the sampled water was not in prolonged contact with the atmosphere prior to sampling. Surface water samples were collected using perforated stainless steel tubes that were inserted into the peat to 10 cm or the top of the water table, whichever was shallowest. Porewater was immediately filtered to 0.7 ||m in the field using Whatman glass-fiber filters, then stored in pre-evacuated glass vials sealed with butyl stoppers.

Phosphoric acid (1 mL of 20%) was added to each sample to preserve for shipment to Florida State University. Samples were analyzed for CH_4 and CO_2 concentrations and stable isotopic composition ($\delta^{13}C$) on a ThermoFinnigan Delta-V Isotope Ratio Mass Spectrometer using the headspace equilibration method with He. Each sample was analyzed twice and the average results for each sample were recorded. Analytical precision was 0.2‰.

Preparation of $\Delta^{14}C$-DOC, $\Delta^{14}C$-DIC and $\Delta^{14}C$-CH_4 and $\Delta^{14}C$-peat samples was done at Florida State University. DOC was freeze dried in combusted 9mm Pyrex glass tubes. Oxidizing agents, cupric oxide, copper shots, and silver, were added and the tubes evacuated and flame sealed on a vacuum line. The sealed tubes were then combusted at 580˚C for 18 hours to convert the organic carbon to CO_2 gas[43]. Following combustion, the produced CO_2 was taken back to the vacuum line, cryogenically purified and sealed into 6mm glass tubing. $\Delta^{14}C$-DIC and $\Delta^{14}C$-CH_4 samples were prepared by He stripping and subsequent combustion (for CH_4 and cryogenic trapping). The 6mm tubes for $\Delta^{14}C$ analysis were sent to National Ocean Sciences Accelerator Mass Spectrometry Facility (NOSAMS) for analysis.

Porewater samples for measurement of total organic carbon (TOC) concentrations were collected every two weeks beginning in late August 2013 and continuing throughout the DPH experiment. These samples were collected from a set of 5-cm internal diameter PVC piezometers installed in each experimental plot. The piezometers had 10-cm screened intervals that opened at depths of 0, 30, 50, 100, 200, and 300 cm. Water was pumped using a peristaltic pump via flexible sections of Salastic and silicon tubing that was attached to a static 0.6 cm internal diameter PVC tube inside each piezometer (a

design that was intended to reduce contamination via intermittent tube insertion into piezometers for sampling). Samples were collected in 250 mL low-density polyethylene (LDPE) bottles that were chilled and transported to the Forestry Sciences Laboratory of the USDA Forest Service. Samples were then refrigerated until analyzed, typically 1 to 4 days after collection for TOC concentration. Total organic carbon concentration was measured on unfiltered water samples using the non-purgeable organic carbon (NPOC) method on a Shimadzu TOC-VCP using Standard Method 5310 B[44] (equivalent to EPA 215.1). The method detection limit was 0.5 mg/L for TOC concentration.

Microbial Community Analyses: Intact soil core samples were collected from 11 depth intervals (0-10, 10-20, 20-30, 30-40, 40-50, 50-75, 75-100, 100-125, 125-150, 150-175 and 175-200 cm) at each of the 10 SPRUCE experimental plots in June 2014 and June 2015, prior to and 13 months into deep peat heating, respectively, to elucidate the microbial community response to warming. Soil samples were frozen immediately and shipped on dry ice to the Georgia Institute of Technology, where they were stored at -80°C until analysis. Total DNA was extracted from homogenized peat samples with the MoBio PowerSoil DNA extraction kit (MoBio, Carlsbad, CA) according to the manufacturer's protocol followed by cleaning with the MoBio PowerClean Pro DNA Cleanup Kit (MoBio, Carlsbad, CA). Abundance of bacterial, archaeal, and fungal populations were determined by quantitative polymerase chain reaction (qPCR) using primers targeted to amplify their respective SSU r-RNA genes[45,46,47,48], and the mcrA gene was targeted to assess the methanogen population[49]. Reactions were performed in triplicate on a CFX96TM Real-Time PCR Detection System (Bio-Rad Laboratories) with

iQ SYBR Green Supermix (Bio-Rad, CA, USA) using previously described standards, and conditions (Extended Data Table 1). DNA extractions were quantified with the Qubit HS assay (Invitrogen) and 20 ng per reaction was applied. The diversity and composition of prokaryotic communities was determined by applying a high-throughput sequencing-based protocol that targets PCR-generated amplicons from V4 variable regions of the 16S rRNA gene using the bacterial primer set 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′)[50]. Amplicons were barcoded with unique 10-base barcodes (Fluidigm Corporation), and sequencing was conducted on an Illumina MiSeq2000 platform at the Research Resources Center (RRC) at the University of Illinois at Chicago following standard protocols[51,52] (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). The generated sequence data are available from the National Center for Biotechnology Information at SRP071256.

Sequence processing and analysis: Initially Illumina-generated 16S rRNA gene sequences were paired with PEAR[53] and primers were trimmed with the software Mothur v1.36.1[54]. Resulting sequences were quality filtered using a Q30 minimum and processed using the standard QIIME 1.9.1 pipeline[46,47]. Sequences were clustered into operational taxonomic units (OTUs) with a threshold of 97% identity. Chimeric sequences, identified by ChimeraSlayer, chloroplast, mitochondria, singletons, unclassified and eukaryotic sequences were removed from the final data. Taxonomies of these high-quality sequences were assigned via the greengenes database using the RDP classifier[55] with a minimum confidence threshold of 50%. Sequences of known

methanogens were extracted from all sequences according to recent methanogen databases [56, 57]. The sequences that did not match any taxonomic Class were also removed. Taxonomic-based alpha diversity was calculated using the total number of phylotypes (richness) and Shannon's diversity index (H′). Faith's phylogenetic diversity (PD) was calculated to assess phylogenetic based alpha diversity. Final sequence data were normalized by cumulative sum scaling (CSS)[58] and beta diversity indices were estimated using Bray–Curtis and weighted as well as unweighted UniFrac distances[59,60]. Significant differences in beta diversity were analyzed by a PERMANOVA test with 1000 permutations followed by Bonferroni correction of P-values. To determine changes in microbial community composition, results from core sections were grouped based on beta-diversity groups (0-10, 10-30, 30-75, 75-200 cm) and significant differences between years (pre- and during heating) were assessed with a Mann-Whitney test.

Enzymatic activities: Enzymatic assays were performed following published microplate protocols[46,61]. Peat suspensions from 7 core sections (0-10, 10-20, 20-30, 30-40, 40-50, 50-75, 75-100 cm) were prepared by homogenizing 2 g of peat in 20 ml of 50 mM acetate buffer (pH 4.0). Peat homogenates were centrifuged for 5 minutes at 5000×g and clear supernatants were used for measurements of phenol oxidase and peroxidase activities. Enzymatic activities were measured by combining 1 mL of clear peat suspension with 1 mL substrate solution (10 mM ATBS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) in 50 mM acetate buffer, pH 4.0). For measurements of peroxidase activity, peat suspensions were diluted 1:20 with 50 mM acetate buffer (pH 4.0) and reaction was initiated by adding 80 µL of 0.3% hydrogen peroxide. Assays were incubated for 12 to

24 h at room temperature. Enzymatic reaction propagation was monitored spectrophotometrically at 420 nm. Maximal reaction rates were calculated from linear reaction stage and expressed as μmol or mmol h^{-1} g^{-1} wet peat for phenol oxidase or peroxidase respectively. Statistically significant differences between years were determined by Student T-test.

Bridge to Chapter V

In Chapter IV, we saw that 13 months of deep-peat heating (DPH) exponentially increased CH_4 emissions from a northern Minnesota peatland. Laboratory incubations conducted across multiple depths, as well as radiocarbon data, determined that this increase was due to increased surface CH_4 production and not decomposition of deep C. Increases in CH_4 flux and surface production occurred despite muted warming of surficial soil horizons during the DPH-*only* phase of the SPRUCE experiment. This raises the questions – will this response remain unchanged or be enhanced with the addition of surface warming (i.e. whole-ecosystem warming)? Or, conversely, is this a transient perturbation effect that will diminish with time as the ecosystem equilibrates to temperature manipulations? Will changes in other ecosystem abiotic factors, such as elevated atmospheric CO_2 concentrations, modify these responses? Chapter V addresses these questions by investigating how CH_4 production and CO_2:CH_4 ratios vary across depths at the SPRUCE site following 14 months of whole-ecosystem warming and 4 months of elevated atmospheric CO_2 concentrations. Additionally, Chapter V expands our knowledge of peatland CH_4 cycling by providing the first-ever measurements of anaerobic oxidation of CH_4 (AOM) throughout an entire soil profile of a peatland experiencing ongoing environmental change. Until recently, this novel microbial process

has received very limited study and its climate-related controls remain poorly understood; thus, this chapter takes an important step forward in enhancing our knowledge of the rates and mechanistic drivers of peatland CH_4 cycling.

CHAPTER V

RISING TEMPERATURES INCREASE PEATLAND METHANE PRODUCTION AND ANAEROBIC OXIDATION THROUGHOUT THE ENTIRE SOIL PROFILE

Contributions

This chapter is co-authored by myself, Kaitlin Brunik, Laurel Pfeifer-Meister, Jason K. Keller, Glenn Woerndle, Cassandra A. Zalman, Paul Hanson, and Scott D. Bridgham. I was responsible for the data collection, analysis, and interpretation, as well as the writing of this manuscript. Scott D. Bridgham, Jason K. Keller, and myself designed the experiments. Scott D. Bridgham and Jason K. Keller served as advisors on this project. Kaitlin Brunik and Glenn Woerndle assisted in sample collection in the field and anaerobic incubations in the laboratory. Laurel Pfeifer-Meister and Cassandra A. Zalman provided input on data analysis and interpretation. Paul Hanson managed all field and SPRUCE-related activities.

Introduction

As global temperatures and atmospheric carbon dioxide (CO_2) concentrations continue to rise, it is critical to examine the responses of natural ecosystems that could generate significant biosphere-climate feedbacks, further exacerbating global climate forcing. Wetland methane (CH_4) emissions have been shown to be partially controlled by changes in climate from past glacial-interglacial cycles (Blunier et al., 1995; Loulergue et al., 2008) and, moreover, large recent inter-annual variability in atmospheric CH_4 levels may be driven by climate effects on wetland CH_4 emissions (Kirschke et al., 2013;

Melton et al., 2013). The vast majority of global wetland carbon (C) is stored in northern boreal peatland soils, which, by definition, have extensive soil C accumulation (≥40 cm) (Yu, 2012). Thus, despite covering <3% of the Earth's surface, peatlands contain one-third of total global soil C and are responsible for approximately 13% of global CH_4 flux (Bridgham et al., 2013a). Additionally, most peatlands occur above 40°N latitude, where the largest relative temperature changes are projected to occur under current climate models and, therefore, are perceived to be particularly susceptible to climate change (Kirtman et al., 2013). Changes in temperature and hydrology have the capacity to diminish the role of peatlands as C sinks by triggering the return of currently stored organic C to the atmosphere as CO_2 and/or CH_4, thereby amplifying the impacts of a changing climate. Additionally, while other field experiments in wetlands have observed positive effects of elevated atmospheric CO_2 (eCO_2) concentrations on CH_4 emissions and production (Dacey et al., 1994; Hutchin et al., 1995; Megonigal & Schlesinger, 1997; Vann & Megonigal, 2003; Cheng et al., 2006) the effects eCO_2 in concert with simultaneous warming on CH_4 cycling have not yet been considered. This is concerning because CH_4 is a potent greenhouse gas with 45 times the sustained-flux global warming potential of CO_2 over a 100 year time frame (Neubauer and Megonigal, 2015); thus, it is critical to examine CO_2:CH_4 ratios resulting from anaerobic mineralization in peatlands. It is currently unknown whether or not a significant fraction of the large soil C pool in peatlands will be respired as CH_4 in future climates, creating a pressing problem in global change biogeochemistry and modelling (Bridgham et al., 1995; Limpens et al., 2008; Frolking et al., 2011; Yu, 2012; Bridgham et al., 2013a).

The accuracy of Earth system model projections hinges on our mechanistic understanding of peatland CH_4 cycling in the context of environmental change. However, despite the extensive study of CH_4 cycling and fluxes in peatlands, emissions are notoriously difficult to predict (Melton et al., 2013). Earth system models conceptualize CH_4 emissions as the balance between CH_4 production in anaerobic zones and aerobic CH_4 consumption in aerobic zones, mediated by CH_4 transport mechanisms from the soil to the atmosphere. However, CH_4 emissions are infrequently explained by the balance between anaerobic CH_4 production and aerobic CH_4 oxidation (Bridgham et al., 2013a), suggesting the possibility of a previously unknown or ignored process(es) involved in peatland CH_4 cycling.

One such process is the anaerobic oxidation of methane (AOM), which is completely ignored for freshwater systems in terrestrial Earth system models. AOM is a dominant process in sulfate-rich marine sediments (Martens & Berner, 1974; Barnes & Goldberg, 1976). Methanotrophic archaea and bacteria have been shown to anaerobically oxidize CH_4 using a range of terminal electron acceptors (TEAs), including sulfate (SO_4^{2-}), ferric iron (Fe^{3+}), and nitrate (NO_3^-) (Valentine, 2002). AOM was thought to be unimportant in peatlands because these systems typically lack high concentrations of oxidized inorganic electron acceptors (Pester, 2012); however, recent studies suggest that this process is widespread in freshwater wetlands (Segarra et al., 2015), but report a wide range (1.6-49 Tg CH_4 yr^{-1}) in the global rate of AOM in peatlands (Gupta et al., 2013). Although this process has not been linked to inorganic TEA availability in peatlands, it has been suggested that organic TEAs may fuel AOM in these organic-rich systems (Keller et al., 2009; Gupta et al., 2013). Thus, the importance of AOM in peatlands is

understudied, and the principal drivers of the process and potential impacts of ongoing environmental change remain unknown.

The Spruce and Peatland Responses Under Changing Environments (SPRUCE) experiment provides an opportunity to explore climate-driven impacts on northern peatland CH_4 cycling under in-situ conditions (Hanson et al., 2017). The SPRUCE project uses a regression-based experimental design to increase air and soil temperatures (+0 to +9 °C above ambient) to a depth of 3 m with and without enhanced atmospheric CO_2 concentrations. All prior soil-warming experiments that have investigated the effects of changing climate on peatland C cycling have used only surface-warming techniques (general +1 °C) and, therefore, have been unable to examine the responses of deeper peat horizons (Chen et al., 2008; Turetsky et al., 2008). Thus, SPRUCE provides the first-ever ecosystem-scale insights of how northern peatland ecosystems respond to a changing climate throughout the majority of the soil profile. Following 13 months of deep-peat heating (DPH) at the SPRUCE site, Wilson and Hopple et al., (2016) observed an exponential increase in CH_4 emissions that was driven by enhanced surface processes but not the mineralization of deep C, despite only muted warming of surface peat in the DPH phase of the experiment. While no temperature response was observed at depth (≥ 75 cm), surface CH_4 production rates increased with rising temperatures, decreasing CO_2:CH_4 ratios in surficial soil horizons (Wilson and Hopple et al., 2016).

In collaboration with the SPRUCE project, we investigated the response of peatland anaerobic CH_4 cycling to whole-ecosystem warming (WEW) and elevated atmospheric CO_2 concentrations (eCO_2) using controlled laboratory incubations at in-situ temperatures of peat samples collected from surface (30 cm) to deep (200 cm) depth

increments. Specifically, we investigated changes in peatland CH_4 production, CO_2:CH_4 ratios, and AOM throughout the entire peatland profile following 14 months of WEW and initial responses to enhanced CO_2 concentrations (≤ 4 months). We hypothesized that (1) CH_4 production rates would continue to positively respond to increasing temperatures in surficial soil horizons, but that deeper soil layers would remain unaffected by temperature increases during WEW, as was observed after 13 months of DPH. We expected that (2) eCO_2 would further stimulate surface rates of methanogenesis by increasing methanogenic substrate availability through heightened rates of plant root exudation. Taken together, we hypothesized that these effects would (3) decrease surface CO_2:CH_4 ratios, but that those of deeper soil layers would remain constant. Finally, we anticipated that (4) AOM would be quantitatively important in surficial soil layers where organic TEAs could be periodically re-oxidized by water-table fluctuations.

Methods

Site description: The SPRUCE experimental site (http://mnspruce.ornl.gov/), S1 bog (8.1 ha), is located in north-central Minnesota, USA within the Marcell Experimental Forest (N 47°.476´; W 93°27.162´). For the past several decades, extensive scientific investigations have been done at this site and include in-depth descriptions of its physiochemical and biotic characteristics (Nichols and Brown, 1980; Urban et al., 1989; Lin et al., 2014; Tfaily et al., 2014; Krassovski et al., 2015; Wilson and Hopple, et al., 2016; Zalman et al., *in press*). This precipitation-fed, ombrotrophic bog has a perched water table with an average pH of 4.1 at the surface which increases with depth to roughly 5.1 at 2 m. The overstory vegetation is primarily dominated by *Picea mariana*

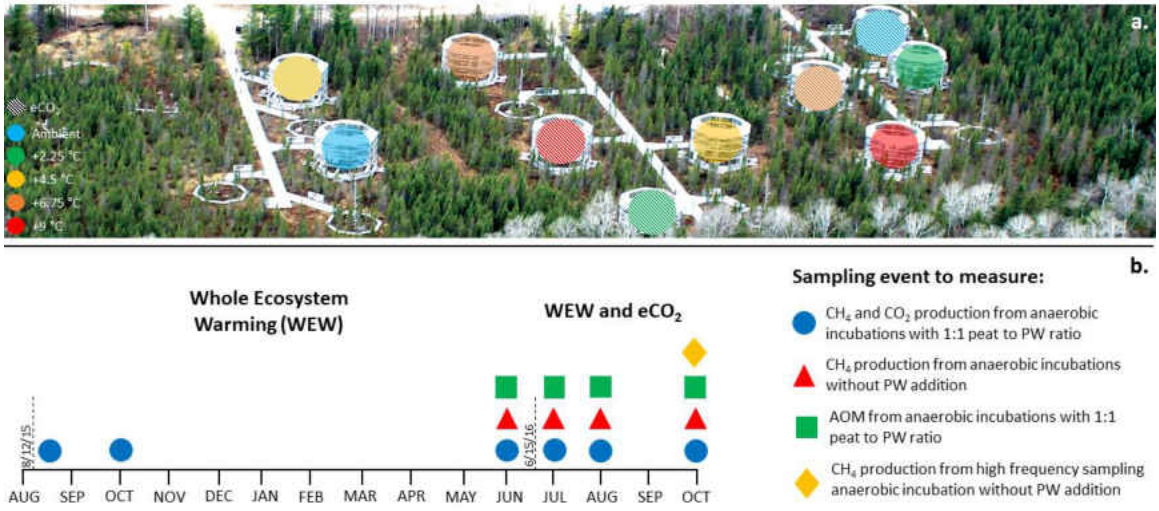


Figure 5.1. Aerial view of the SPRUCE site located in northern Minnesota **(a)**. Experimental enclosures are positioned along three boardwalks that transect the ombrotrophic bog (S1 Bog). Colors denote temperature differentials targeted within each enclosure and hatching identifies those exposed to elevated atmospheric CO_2 concentrations. We collected peat and porewater from multiple depth during six sampling events to discern how climate manipulation affected peatland CH_4 cycling **(b)**. These events occurred following 2 weeks to 14 months of WEW, as shown on the timeline, and samples were used to complete various anaerobic laboratory incubations, as described by the differing symbols. PW = porewater.

(black spruce) and secondarily by *Larix laricina* (larch), while the understory is composed of low ericaceous shrubs, such as *Rhododendron groenlandicum* (Labrador tea) and *Chamaedaphne calyculata* (leatherleaf), and herbaceous perennials, such as *Maianthemum triflorum* (three-leaved Solomon's seal) and *Eriophorum vaginatum* (cottongrass). The bog surface is characterized by hummock and hollow microtopography, with a typical relief of 10 to 30 cm between the tops of the hummocks and the hollows. *Sphagnum magellanicum* generally colonizes the hummocks, while *S. angustifolium* and *S. fallax* cover the hollows. The belowground peat profile and geochemistry are described in Tfaily et al. (2014).

Whole-ecosystem warming and elevated atmospheric CO_2 enrichment: The SPRUCE project is a novel, manipulative experiment designed to address climate-driven questions on an ecosystem-scale and under in-situ conditions over the span of a decade. This study

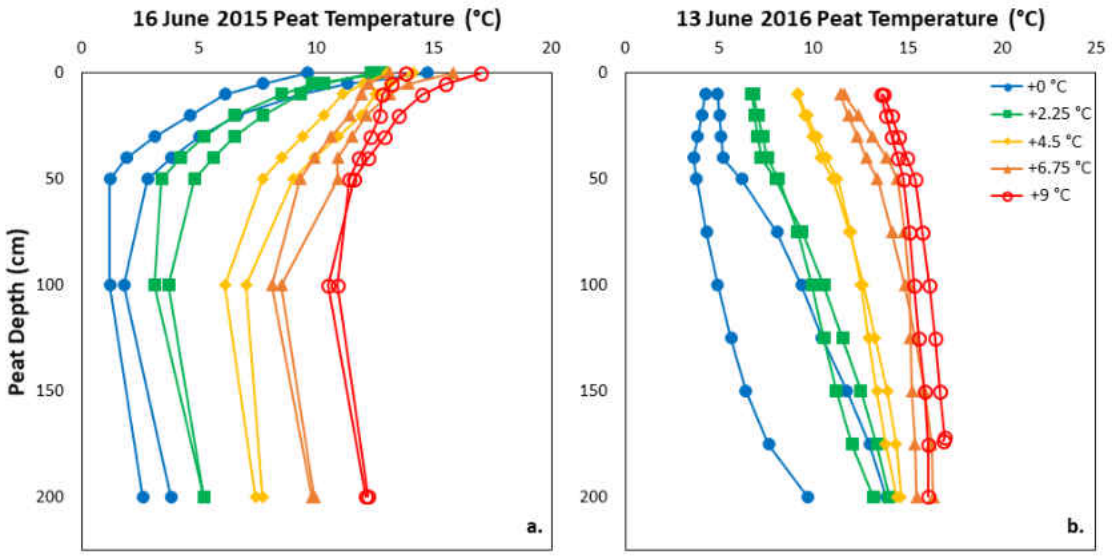


Figure 5.2. Snapshot temperature depth profiles associated with the **(a)** DPH and **(b)** WEW phases of the SPRUCE experiment. Temperature profiles were measured during coring events, which took place 13 and 10 months into DPH and WEW, respectively. Target temperature differentials are denoted with different shape and color combinations. Note muted surface temperature separation during DPH and the consistent 9 °C temperature spread throughout the entire peatland soil profile during WEW.

uses a regression-based experimental design that warms the vegetation and peatland soil profile to 3 m depth within ten 12 m diameter enclosures to five target temperature differentials (+0, +2.25, +4.5, +6.75, and +9°C), with duplicate enclosures subjected to ambient and ~ +500 p.p.m.v. atmospheric CO_2 concentrations (Figure 5.1a). Whole-ecosystem warming (WEW) is achieved within open-topped enclosures (7 m tall by 12.8 m in diameter) by combining air and belowground warming. Air is warmed with propane heaters, whereas belowground warming is attained using low-wattage, 3 m long, belowground concentric rings of heaters (Hanson et al., 2017). The open-top enclosure design allows for surface air warming and enhancement of atmospheric CO_2, while sub-surface corrals hydrologically isolate each experimental enclosure and allow for changes in water-table level associated with climate manipulation to occur.

Whole-ecosystem warming was initiated 12 August 2015, following 14 months of deep peat heating (DPH). During the DPH phase of this experiment, deep-soil

temperature targets were successfully maintained throughout the year following a gradual treatment equilibration period (~3 months); however, the lack of air warming resulted in reduced temperature separation among treatments at the surface (Figure 5.2a; Hanson et al., 2017). After the introduction of air warming (which signaled the start of WEW), we attained 9 °C temperature separation and differentials across treatment enclosures from the tops of the trees to peat depths of at least 2 m (Figure 5.2b). Temperature differentials have largely been maintained thought the WEW period, with some variation observed in surficial peat zones due to rain and snow events (Hanson et al., 2017). Finally, elevated atmospheric CO_2 concentrations were introduced in a subset of the enclosures on 15 June 2016, completing the full set of experimental climatic manipulations planned by the SPRUCE project.

Analysis of CH_4 and CO_2 production and CO_2:CH_4 ratios in anaerobic incubations: Following the same protocol to that was used throughout the DPH experimental phase (Wilson and Hopple, et al., 2016), during six sampling events completed over 14 months of WEW (August 2015 – October 2016), intact soil cores were collected from multiple depths within each experimental enclosure to discern how rates of CH_4 and CO_2 production and CO_2:CH_4 ratios varied with climate treatment and depth (Figure 5.1b). In 2015, soil cores were collected from 20-30, 50-75, 100-125, 125-150, and 175-200 cm depth increments, following 2 weeks and 2 months of WEW (depth increments are denoted with the lower end of their range in figures). We used the same sampling approach in 2016, but collected soil cores at 40-50 cm instead of 125-150 cm to better capture variation in surficial peat horizons. These sampling events took place following

10, 11, 12, and 14 months of WEW, with the last three sampling events also subjected to 1, 2, and 4 months of elevated atmospheric CO_2 concentrations. All depths were measured relative to the surface of the hollows. We began to observe water table drawdowns (~30 – 50 cm below the hollow surface) in 2016 as a result of increased temperatures in experimentally manipulated enclosures (Hanson et al., 2017). We focus here only on depth increments that were anaerobic at the time of sampling. To prevent compression of surface peat samples, a serrated knife was used to collect a 10 cm diameter core from the hollow surface to ~20 cm within the peat profile. A 5 cm diameter Russian corer was subsequently used to extract the remaining samples up to 2 m deep. Soil cores were immediately flushed with nitrogen (N_2) in the field to minimize exposure to aerobic conditions. Additionally, porewater samples were anaerobically collected from 1.25 cm-diameter PVC piezometers at corresponding depth increments (25, 50, 75, 100, 150, and 200 cm below the hollow surface) using a peristaltic pump. Both soil cores and porewater were stored on ice and shipped overnight to the UO.

At the UO, soil samples were incubated within 1 °C of in-situ temperatures within 24 hours of field collection and anaerobic incubations commenced the following day. This rapid turnaround time was intended to generate depth-specific CH_4 and CO_2 production rates that were as representative of in situ conditions as possible (however, note the porewater/headspace caveats described in the discussion section). Samples were slurried with a 1:1 mixture of peat and porewater collected from the same enclosure and depth in a glove box filled with N_2 atmosphere (<5% H_2 in the presence of a palladium catalyst; Coy Laboratory Products, Grass Lake, Michigan) to maintain anaerobic conditions. Sample bottles were then flushed with N_2 for 15 minutes to begin the

incubation. Headspace samples were analyzed over the course of 8 days (days 2, 4, 6, and 8) for CH_4 and CO_2 simultaneously using an SRI gas chromatograph equipped with a methanizer and flame ionization detector. Total CH_4 and CO_2 were calculated using Henry's Law, adjusting for solubility, temperature, and pH (Bridgham and Ye, 2013b). Methane and CO_2 production rates were calculated using the linear accumulation ($r^2 \geq 0.83$ in all cases) of gasses through time.

In 2016, we included a complementary set of anaerobic incubations to test the effects of porewater addition on CH_4 production rates (Figure 5.1b). In these experiments, peat samples were incubated without additional porewater, which approximated field moisture conditions (≥85% water content in all cases), except in the 30 cm increment where the very low bulk density peat made drainage of porewater very difficult to avoid. Finally, we also completed a high frequency sampling event on a subset of samples in October 2016 to capture early (<48 hours) CH_4 production responses during anaerobic incubations (Figure 5.1b). Peat samples were collected from each enclosure (n = 10) at random depths and incubated anaerobically without porewater addition at in situ temperatures. Headspace CH_4 and CO_2 concentrations were measured after 4, 6, 12, 18, and 24 hours and 2, 3, 4, 6, 7, and 9 days.

Because we consistently observed net CH_4 consumption in samples without porewater addition (see results), we verified in bottles with deionized water that we could quantitatively recover added CH_4 and CO_2 over the 8 day sampling period. This was indeed the case, and there was no evidence of gas leakage.

Analysis of Anaerobic Oxidation of Methane in anaerobic incubations: In 2016, we directly measured rates of peatland AOM using a radioisotope tracer technique that involved adding a tritiated CH_4 tracer (C^3H_4) (Valentine et al., 2001) to our previously described anaerobic incubations done at 1:1 peat to porewater ratios and in situ temperatures following 10, 11, 12, and 14 months of WEW (Figure 5.1b). The tracer could not be properly mixed in non-slurried samples, so these were not run. These measurements were done at multiple depths (30-200 cm) within each experimental enclosure.

In the process of AOM, C^3H_4 is oxidized to CO_2 and tritiated water (^3H_2O) is formed as a byproduct (Eqn. 1):

$$C^{3}\mathbf{H}_4 + \text{Electron Acceptor} \rightarrow CO_2 + {}^{3}\mathbf{H}_2O \qquad (1)$$

Thus, AOM can be measured by adding C^3H_4 during an anaerobic incubation, removing the unreacted C^3H_4 after a specified amount of time, and counting the ^3H_2O product as described below. A set of "dead" controls (n = 8) with deionized water were autoclaved twice (45-minute gravity cycle at 121 °C and 14 PSIG; Steris/AMSCO Sterilizer 3011, Sanford, FL). These dead controls served to measure background abiotic isotopic exchange which, if unaccounted for, could falsely inflate rates of AOM. We added 0.2 μCi of C^3H_4 to sample bottles following 2 days of anaerobic incubation to allow time for CH_4 and CO_2 to accumulate in the headspace prior to the tracer addition. Gaseous and aqueous-phase samples were then pulled over the course of 2-3 weeks (2, 4, 8, and 16 days post-tracer addition) to assess changes in CH_4 and CO_2 headspace concentrations with gas chromatography (described above) and ^3H_2O accumulation with liquid scintillation counting. We present 48-hour rates of AOM to provide the most in-situ

estimates; however, we also demonstrate how rates of surface (30 cm) AOM changed during the laboratory incubation.

Unreacted C^3H_4 was removed from aqueous samples by adding 4 M KCl solution to samples and placing them under a strong vacuum (≥ -9 PSIG) for 48 hours. Experimental trails found this to be the most effective process for C^3H_4 removal without losing any aqueous sample to evaporation. Aqueous samples were then combined with 50% ScintiSafe liquid scintillation cocktail, vigorously shaken, and allowed to react overnight. After 24 hours, sample radioactivity was measured using a Beckman LS 9800 Series Liquid Scintillation Counter. Quench was determined for each sample by adding small aliquots of known radioactivity and re-measuring the radioactivity using liquid scintillation counting. We used a similar procedure to determine the dilution of radioactivity levels from the addition 4 M KCl. Final estimates of radioactivity were corrected for quench, source material decay, dilution with 4 M KCl, and background isotopic exchange (based on median radioactivity levels in control samples). Soil dry weight was determined by measuring the change in weight of a soil sub-sample following 48 hours of drying at 60 °C. The amount of CH_4 anaerobically oxidized and the rate of AOM were determined using equation:

$$CH_{4ox} = ({}^3H_2O * CH_{4ave}) / (C^3H_{4added} * wt * t) \quad (2)$$

where CH_{4ox} = the amount of CH_4 that was anaerobically oxidized (µmol CH_4 g^{-1} soil d^{-1}); ${}^3H_2O$ = the total amount of ${}^3H_2O$ radioactivity/sample bottle (µCi); C^3H_{4added} = the amount of tritiated CH_4 added (µCi); CH_{4ave} = the average amount of available CH_4 between the measurement time points (µmol); wt = the dry mass of the soil; and t = incubation time.

Despite extensive experimentation, this technique had very high and variable abiotic isotopic transfer of the tritium on CH_4 molecules to H_2O molecules that we were unable to effectively minimize. Consequently, only the June and July 2016 sampling dates were consistently above background rates, and we focus on these data here as support of concept that AOM was actually occurring in our samples.

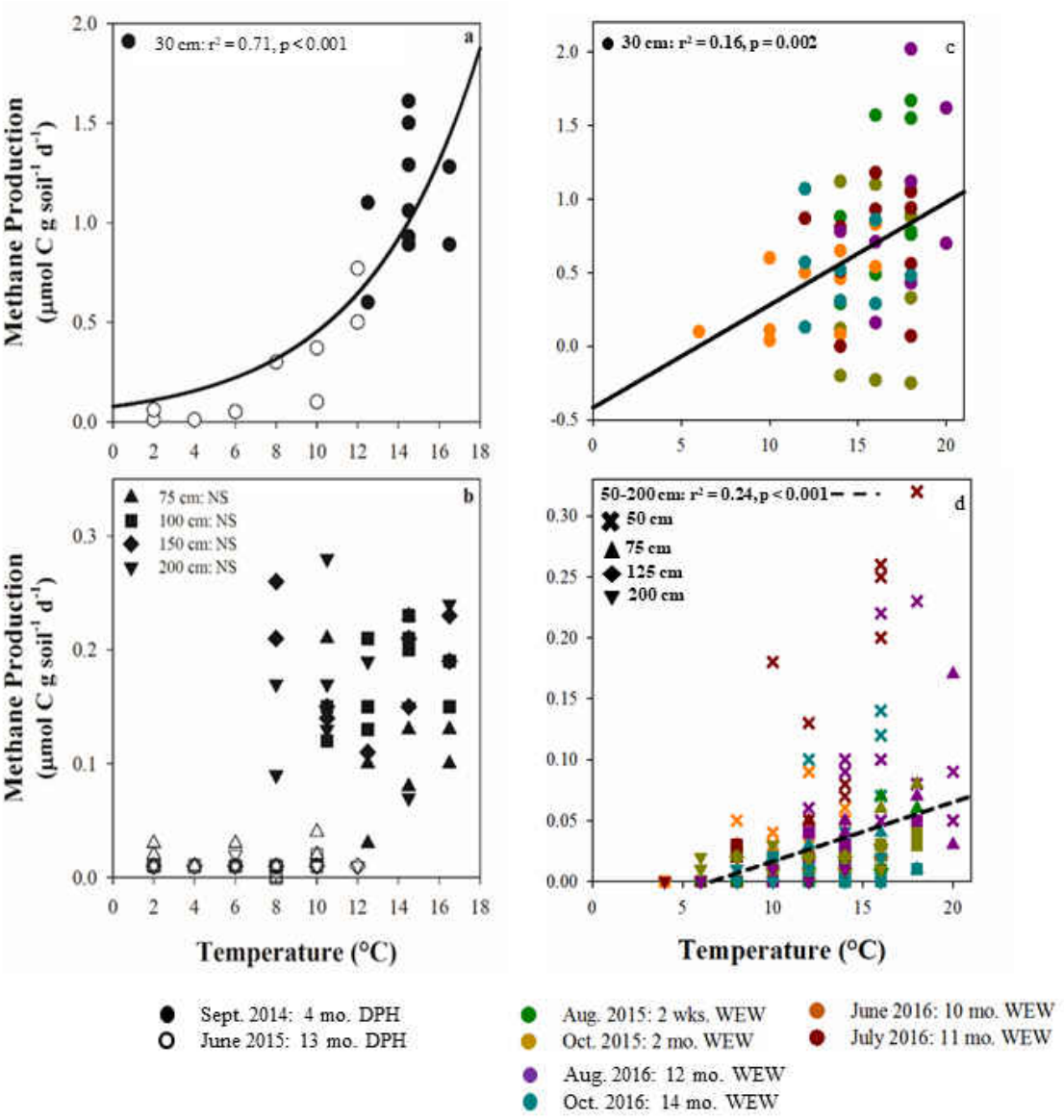


Figure 5.3. Comparison of CH_4 production temperature responses in **(a, c)** surface and **(b, d)** deep peat samples that were anaerobically incubated within 1 °C of in-situ temperatures and at 1:1 peat to porewater ratios during DPH **(a, b)** and WEW **(c, d)**. Different depths are represented by varying shapes and sampling events are denoted by colors. Note the positive temperature response observed at depth (≥ 50 cm) during WEW which was not observed during DPH. Data from the DPH period were taken from Wilson and Hopple et al. (2016).

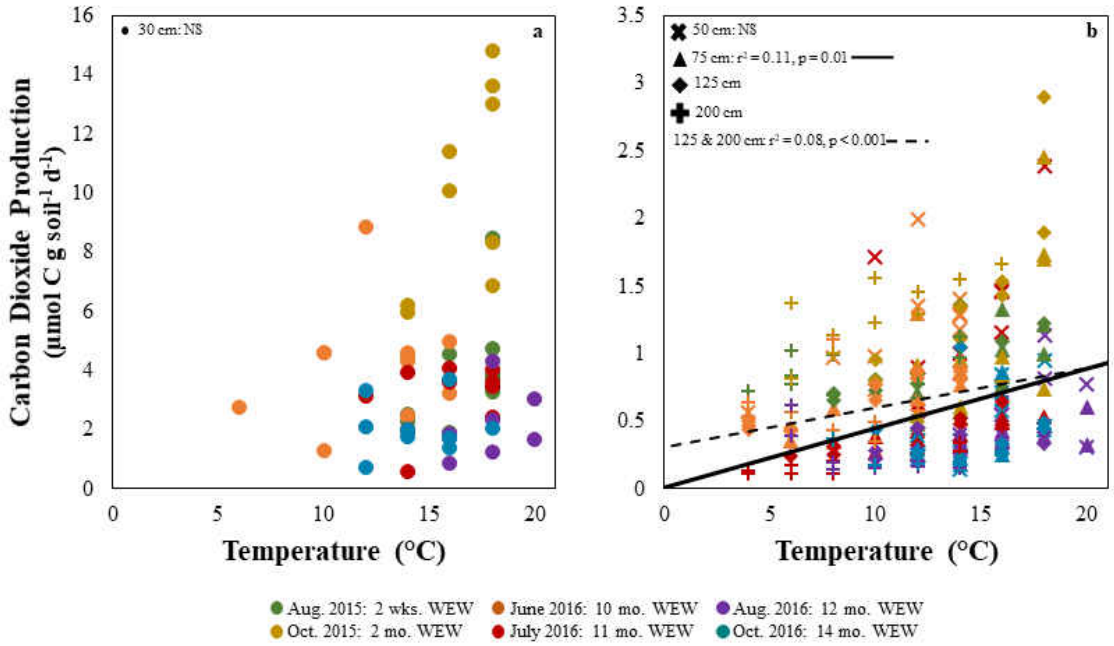


Figure 5.4. Carbon dioxide production temperature responses in **(a)** surface and **(b)** deep peat samples that were anaerobically incubated within 1 °C of in-situ temperatures and at 1:1 peat to porewater ratios during 14 months of WEW. Different depths are represented by varying shapes and sampling events are denoted by colors. NS = not significant.

Statistical analyses: General linear mixed-effect models (GLMM) were used to determine the effects of depth, temperature, elevated CO_2 concentrations, season, and time since WEW initiation on CH_4 and CO_2 production rates, CO_2:CH_4 ratios, and AOM rates. Enclosure was treated as a random effect and all other predictor variables were analyzed as fixed effects. Season was categorized based on time into the growing season and coded as early (June), mid (July and August), or late (September and October). If significant differences among depths were detected ($p < 0.05$), pairwise comparisons using Tukey's honest significant difference test ($p < 0.05$) were conducted. If not significantly different, depths were combined for linear regression analysis. Data were tested for normality and log-transformed where the transformation resulted in an

improvement in overall distribution. All statistical analyses were completed using R 3.2.2 Statistical Software.

Results

CH_4 and CO_2 production and CO_2:CH_4 ratios: Surface (30 cm) CH_4 production rates were much greater than those observed at deeper depths (≥ 50 cm) during anaerobic laboratory incubations of 1:1 peat to porewater mixtures at in-situ temperatures ($p < 0.0001$; Figure 5.3c and d). Rates at 50 cm depth visually appeared higher than deeper depths, but this difference was not statistically significant ($p = 0.62$). There was a strong interaction between temperature and depth ($p < 0.0001$), but rates of methanogenesis across all depths increased with increasing temperatures ($p \leq 0.002$; Figure 5.3c and d). Rates of methanogenesis were lower late into the growing season when compared to early and mid-growing season rates ($p = 0.031$). Additionally, methanogenesis was 27% higher in samples exposed to enhanced atmospheric CO_2 concentrations in the field ($p = 0.11$; eCO_2 CH_4 production = 0.19 ± 0.05 µmol C g peat^{-1} d^{-1}, no eCO_2 CH_4 production = 0.15 ± 0.03 µmol C g peat^{-1} d^{-1}). Time since initiation of WEW did not affect rates of CH_4 production ($p = 0.83$).

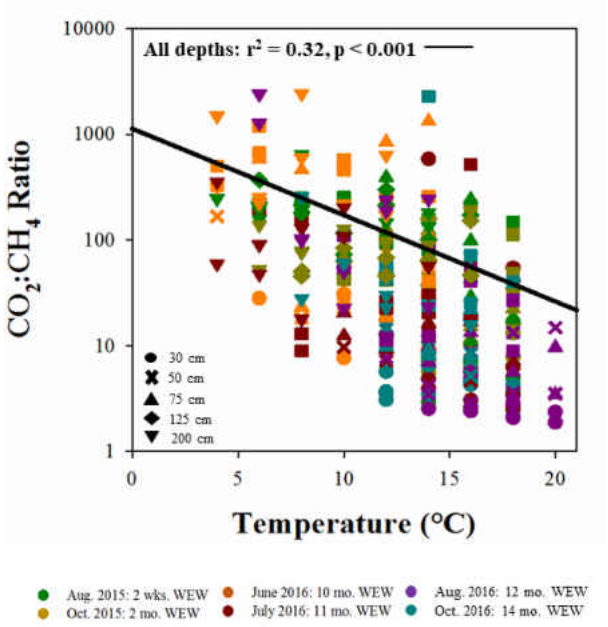


Figure 5.5. CO_2:CH_4 ratios from peat samples that were anaerobically incubated within 1 °C of in-situ temperatures and at 1:1 peat to porewater ratios following 2 weeks to 14 months of WEW. Depth increments are denoted with shapes and sampling events are shown as various colors. Note the log-scale on the y axis.

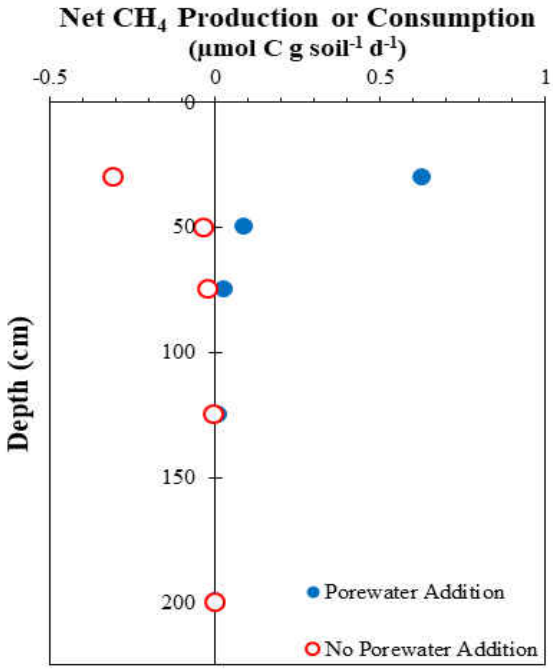


Figure 5.6. Methane production and consumption from peat samples taken from 30-200 cm depths and anaerobically incubated at in-situ temperature with (blue symbols) and without (red symbols) additional porewater. Each point represents average net CH_4 production or consumption of all samples from a specific depth following 10-14 months of WEW. Across all groups, the standard error of the mean was less than 0.07.

In October 2015 and 2016, seven incubation samples showed linear rates of net CH_4 consumption under anaerobic conditions (Figure 5.3c and d). When these net CH_4 consumption samples were removed from the data analysis, CH_4 production rates responded to temperature consistently across all depths ($p = 0.96$), and rates of methanogenesis were highest in mid-growing season and lowest in the early-growing season ($p < 0.0001$). Additionally, rates of net CH_4 production were highest at 30 cm, intermediate at 50 cm, and lowest at depths ≥ 75 cm ($p < 0.0001$).

Rates of CO_2 production decreased with each increasing soil depth ($p < 0.0001$) except the 125 and 200 cm depths were not significantly different ($p = 0.48$). There was a strong interaction between temperature and depth ($p < 0.0001$); only CO_2 production from soil horizons at 75 cm or below positively responded to increasing temperatures ($p \leq 0.01$; Figure 5.4). Surficial CO_2 production was unaffected by WEW (30 cm: $p = 0.15$; 50 cm: $p = 0.79$). Carbon dioxide production decreased from early- to mid-growing season and was the highest late into the growing season ($p < 0.0001$). Additionally, over the course of our six sampling events, CO_2 production appeared to first increase and then

decrease with time since the initiation of WEW ($p < 0.0001$). Ratios of CO_2:CH_4 production increased with depth ($p < 0.0001$) and were negatively correlated with temperature across all depths ($p = 0.0012$; Figure 5.5); however, the effect of temperature was not dependent upon depth ($p = 0.91$).

Surprisingly, lack of porewater addition during anaerobic laboratory incubations resulted in consistent reductions in CH_4 production rates and often led to net CH_4 consumption across all depths ($p < 0.0001$; Figure 5.6). High frequency sampling from a subset of samples in October 2016 consistently revealed that CH_4 is initially produced in anaerobic incubations over the first 48 hours, but is then rapidly consumed (Figure 5.7). Surface (30 cm) net CH_4 consumption rates were greater than those observed at deeper depth ($p < 0.0001$); however, both surface ($p = 0.036$; Figure 5.8a) and deep ($p < 0.0001$; Figure 5.8b) peat horizons positively responded to increasing temperatures. The effect of temperature on net CH_4 consumption was highly dependent upon depth increment ($p < 0.001$). Additionally, samples exposed to enhanced CO_2 concentrations in the field had 80% higher net CH_4 consumption than those that were not ($p = 0.10$; eCO_2 CH_4 consumption = 0.09 ± 0.03 µmol C g peat^{-1} d^{-1}, no eCO_2 CH_4 consumption = 0.05 ± 0.01 µmol C g peat^{-1} d^{-1}). There were no notable

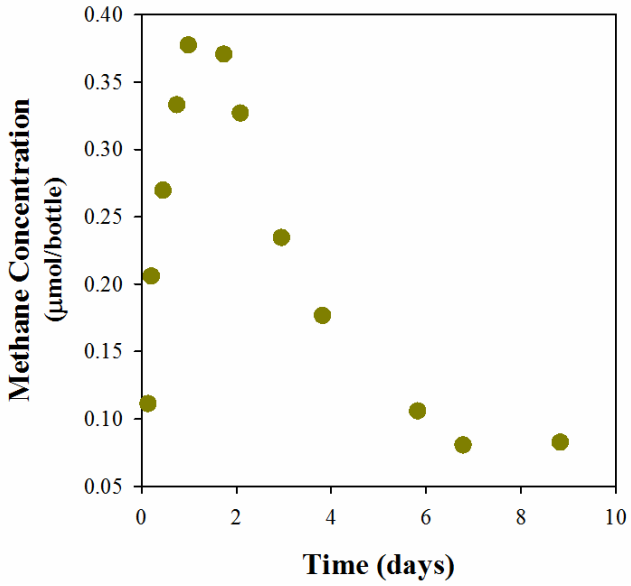


Figure 5.7. A representative sample of frequent measurements of changes in CH_4 concentration in samples without porewater addition (shown +9 °C enclosure at 30 cm) during October 2016. Note that CH_4 was initially produced and was then consumed after approximately 24-48 hours.

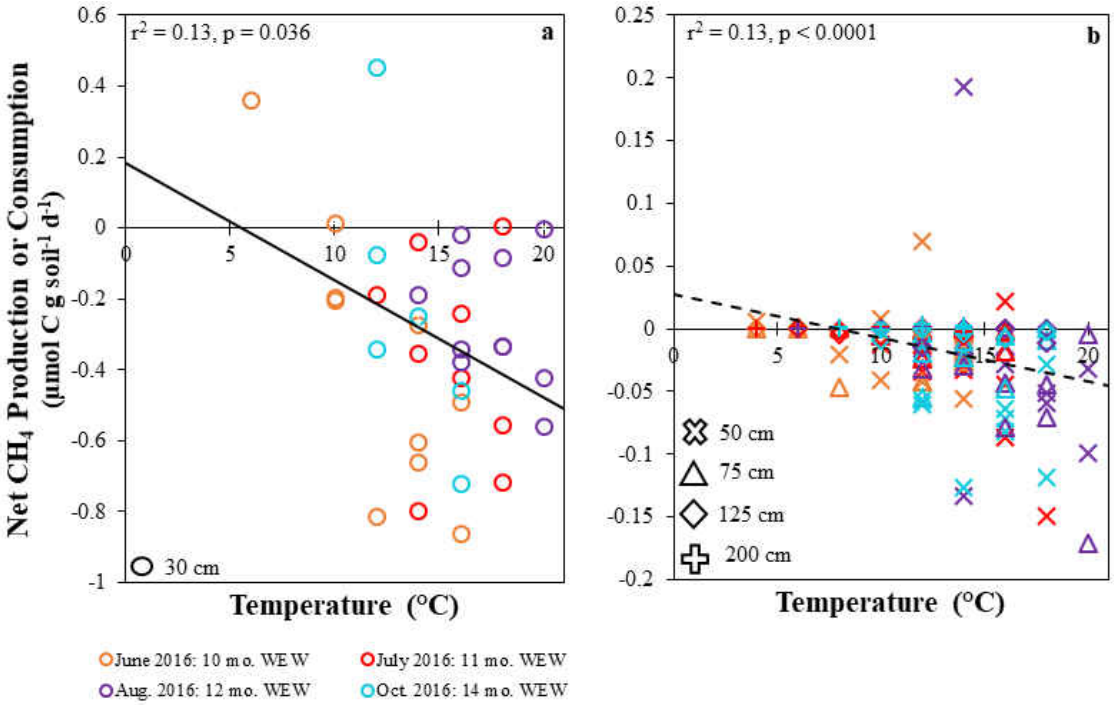


Figure 5.8. Temperature responses of **(a)** surface and **(b)** deep CH_4 production or consumption from peat samples anaerobically incubated at approximately in-situ temperatures and without porewater addition following 10-14 months of WEW. Sampling events are represented as different colors and depth are shown as different shapes.

changes in rates of net CH_4 consumption across season or with time since WEW initiation ($p \geq 0.36$).

Anaerobic oxidation of methane: We observed AOM occurring throughout the entire peatland soil profile in June and July 2016, despite the high rates of abiotic isotopic exchange in the dead controls. Rates of AOM were not affected by season or time since WEW initiation ($p \geq 0.78$) and were thus combined for statistical analyses. Surface (30 and 50 cm) rates of AOM were the highest and sharply decreased with depth ($p < 0.0001$; Figure 5.9a). Additionally, rates of surface CH_4 consumption remained constant over one week ($p = 0.15$; Figure 5.9b), but were not detectable above background levels after two weeks. Rates of AOM positively responded to increasing temperature across all depths (p

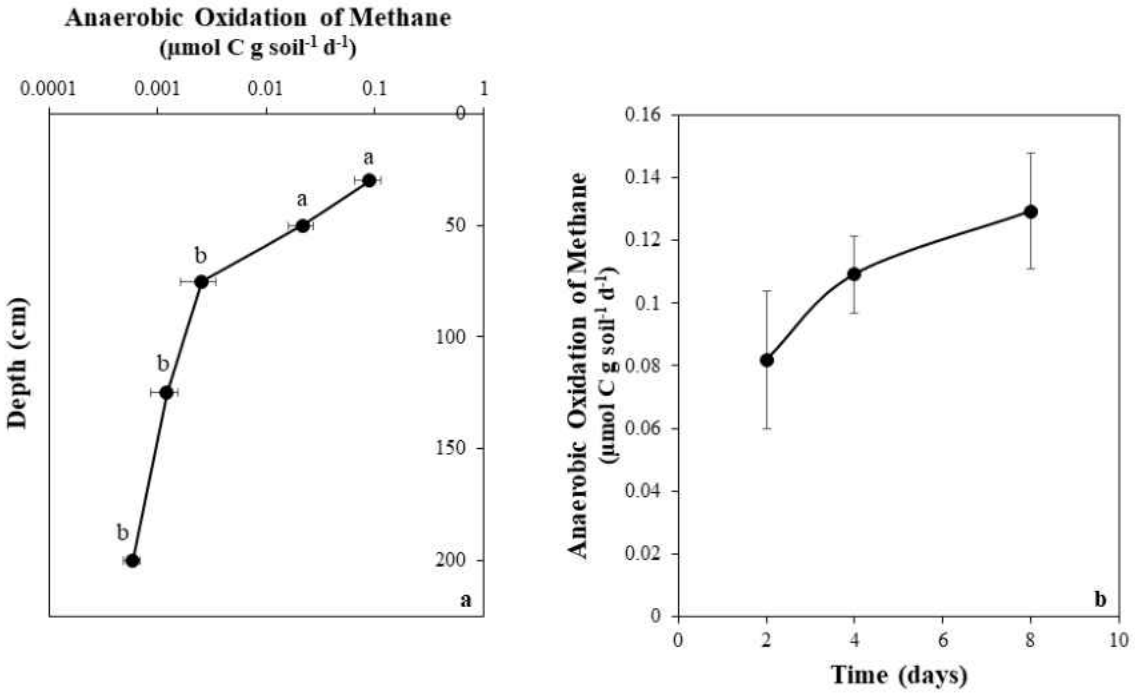


Figure 5.9. Direct measurements of AOM rates with a H^3CH_4 tracer **(a)** throughout the soil profile acquired from samples incubated at 1:1 peat to porewater ratios with a radioisotope tracer and at in-situ temperatures. Changes in the **(b)** rate of surface AOM over incubation time; although there is a gradual increase, no significant differences were observed ($p = 0.15$). AOM was no longer detectible above control background levels after two weeks. Lower case letters represent significant differences ($p < 0.05$) between depth increments. Note the log-scale on the x-axis of panel a.

< 0.01; Figure 5.10a and b) and the response to temperature was not dpendent upon depth ($p = 0.78$). Despite the difficulty with this method, it did indicate that AOM is an important process in S1 bog. Overall, AOM was responsible for consuming 17.2 ± 1.4% of the total amount of CH_4 produced and ranged from 2-74% consumption of total CH_4 production.

Discussion

CH_4 and CO_2 production and CO_2:CH_4 ratios: Here, we present the first-ever evidence of increasing temperatures stimulating rates of methanogenesis throughout the entire soil profile of a non-permafrost boreal peatland (Figure 5.3c and d), as well as rates of deep peatland (≥ 75 cm) CO_2 production (Figure 5.4b). This contrasts with previous results

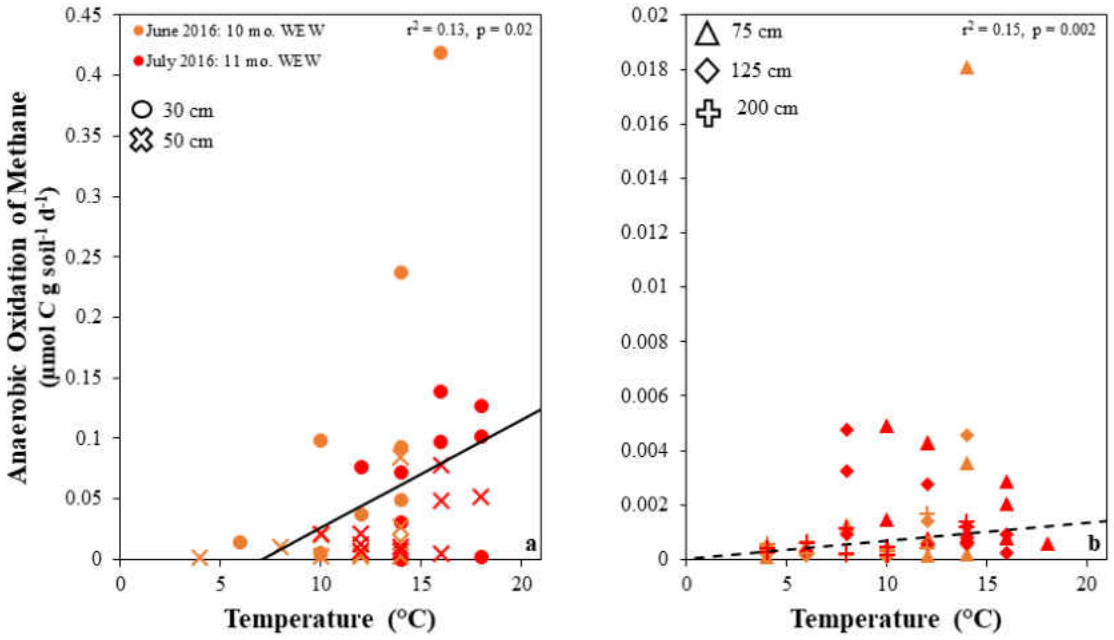


Figure 5.10. Temperature responses of **(a)** surface and **(b)** deep AOM rates observed from peat samples anaerobically incubated at in-situ temperatures and 1:1 peat to porewater ratios following 10 and 11 months of WEW. Sampling events are represented as different colors and depth are shown as different shapes.

from the SPRUCE experiment which showed no temperature effect on CH_4 and CO_2 production in deeper peat following more than one year of deep-peat warming (DPH) (Figure 5.3b; Wilson and Hopple et al., 2016). While surface methanogenesis remained greater than that at depth during whole-ecosystem warming (WEW), the lower 150 cm of the soil profile (a much larger volume) positively responded to increased temperatures during the second year of warming. We hypothesize that this very delayed response is due to the slow growth of methanogen populations given the low thermodynamic yield of methanogenesis (Beer & Blodau, 2007; Thauer et al., 2008; Blodau, 2012). A laboratory experiment supported this hypothesis by showing increased CH_4 production from deep peat in S1 Bog after the addition of a surface microbial inoculum but not with warming and/or the addition of labile substrates (Keller et al., unpublished data). We also show in

Chapter 3 that the addition of surface DOM has no effect on CO_2 and CH_4 production in deep peat.

It should be noted that a positive temperature response at depth does not necessarily indicate enhanced mineralization of ancient (~8,000-10,000-year-old) peatland C. Previous research has shown that almost all heterotrophic respiration in deep peat at S1 Bog is driven by relatively young, surface-derived dissolved organic matter (DOM) with the solid matrix peat remaining essentially unchanged over thousands of years (Wilson and Hopple et al., 2016). However, porewater CO_2, and presumably CH_4, in 2017 was slightly older than DOM in the warmest plots (Chanton and Wilson, unpublished data), suggesting that deep peat has begun to make a contribution to anaerobic respiration, but this took years to be observed.

Interestingly, surface CO_2 production was unaffected by WEW, contrasting with the positive response that was observed following one year of DPH (Wilson and Hopple et al., 2016) and highlighting the higher temperature sensitivity of methanogens compared to other anaerobic microorganisms. This is further demonstrated by decreasing CO_2:CH_4 production ratios observed throughout the entire peatland profile (Figure 5.5), which also been observed in porewater concentrations of these two gasses (Chanton and Wilson, unpublished data), suggesting that this ecosystem is becoming more methanogenic with warming, in-line with results from previous studies (Updegraff et al., 2001; Yvon-Durocher et al., 2014).

Rates of surface (30 cm) methanogenesis were an order of magnitude greater than those observed deeper into the peatland soil profile (≥ 50 cm: Figure 5.3c and d). Numerous studies have documented similar low rates of CH_4 production in deep peatland

soil horizons and have linked this sharp decrease to an array of abiotic and biotic factors (Galand et al., 2003; Cadillo-Quiroz et al., 2006; Kotiaho et al., 2010; Tfaily et al., 2014; Zalman et al., *in press*), highlighting the tight coupling between distinct microbial communities and depth-associated changes in the physiochemical environment. For example, microbial community abundance, composition, and activity have been associated with changing soil pH, O_2 availability, DOM and nitrogen availability, and DOM source and reactivity through depth in a variety of northern peatlands (Galand et al., 2003; Cadillo-Quiroz et al., 2006; Kotiaho et al., 2010; Lin et al., 2012; Wilson and Hopple et al., 2016). Furthermore, in a high-resolution molecular study of soil C mineralization at S1 Bog, Tfaily et al. (2014) attributed low rates of CH_4 production deep in the soil profile to a combination of low lability C sources, the accumulation of inhibitory metabolic end products, and lower population levels of methanogens and/or shifts in the methanogen community with depth. We also previously observed a close correspondence over peat depths between CH_4 production rates and the abundance of the total (via DNA-based techniques) and active (via RNA-based techniques) methanogens in S1 bog and two other nearby peatlands (Zalman et al., *in press*). As discussed above, we have substantial evidense to suggest that long lag in anaerobic microbial respiration response to warmer temperature is due to slow grow of methanogen populations, but these other factors lead to the initial small methanogen population size.

Although we observed direct temperature effects on rates of surface methanogenesis during both DPH (Figure 5.3a) and WEW (Figure 5.3c), temperature explained much less of the variation in CH_4 production during WEW ($r^2 = 0.16$) relative to the DPH phase ($r^2 = 0.71$). Climate-induced perturbations to the ecosystem, such as

changes in water-table depth, increased below-ground exudation of labile plant compounds, or changes plant and/or microbial community composition, may have had cascading ecological effects on peatland CH_4 production (Updegraff et al., 2001; Turetsky et al., 2008), muting the effects of temperature on this process in surface soils. For example, water-table drawdowns observed under WEW (Hanson et al., 2017) likely oxidized organic and inorganic terminal electron acceptors and intermittently decreased the soil anaerobic zone, suppressing rates of surface methanogenesis. However, water-table drawdowns may have also stimulated rates of CH_4 production deeper into the peatland soil profile by increasing root exudations deeper in the peat and vertically re-distributing methanogenic microorganisms and labile C substrates. Substantial changes with warming in the organic chemistry of porewater were observed in 2017 at SPRUCE (Chanton and Wilson, unpublished data). Thus, climate-driven changes in water-table position may have numerous effects on rates of methanogenesis.

Furthermore, as the SPRUCE climate manipulations continue for the next decade, these temperature effects will likely be further modified by the impact of atmospheric CO_2 enrichment on rates of plant community productivity and root exudation. Here, we show a marginally significant, yet suggestive, 27% increase in rates of methanogenesis attributed to enhanced atmospheric CO_2 concentrations. Fertilization with CO_2 has been shown to increase rates of plant root exudation (Dacey et al., 1994; Hutchin et al., 1995; Megonigal & Schlesinger, 1997; Cheng, 1999; McLeod and Long, 1999; Vann & Megonigal, 2003; Cheng et al., 2006), which could increase the availability of C substrates for methanogens. Supporting this explanation, we found a marginally significant ($p = 0.07$) increase in porewater acetate concentrations in the elevated CO_2

treatment during the WEW phase (control = 3.73 ± 2.47 μM acetate-C; eCO_2 = 5.22 ± 7.70 μM acetate-C (mean ± 1 s.e.m.), unpublished data). While these cascading ecological effects likely played a role in modifying the response of CH_4 production to increasing temperatures, our study also identifies AOM as a potentially ubiquitous and important constraint on peatland methanogenesis.

Indirect measurements of anaerobic oxidation of methane: To determine rates of CH_4 production, we mainly used a methodology that is common throughout much of the peatland literature. Briefly, peat samples are incubated at a 1:1 peat to porewater ratio in gas-tight serum bottles with a headspace thoroughly flushed with N_2 to ensure anaerobic conditions. We have generally observed CH_4 production during these experiments; however, we occasionally saw net CH_4 consumption (Figure 5.3c), suggesting that AOM is simultaneously occurring alongside CH_4 production and that this methodology is actually capturing *net* CH_4 production and/or consumption. As discussed below, this method likely minimizes AOM rates and thus more closely approximates gross CH_4 production rates.

We saw further evidence that AOM was occurring when peat samples collected from multiple depths were anaerobically incubated at approximately in-situ moisture conditions (≥ 85% in all cases) and without porewater addition. Using this protocol, we consistently observed reduced rates of CH_4 production and often net CH_4 consumption (Figure 5.6). This trend occurred across all depths and sampling events (n = 4) in which we did not add additional porewater to our anaerobic incubations. We extensively tested for and found no gas leakage from serum bottles during these experiments. Rather, high

frequency sampling of a subset of bottles revealed that CH_4 concentrations initially increased over 48 hours and then quickly decreased over the next several days (Figure 5.7). These results suggest that methanogenesis and AOM are occurring simultaneously and that the occurrence of AOM is driven by the availability of CH_4 as a primary substrate. We hypothesize that porewater addition during anaerobic incubations generally results in net CH_4 production because, as CH_4 is extremely insoluble, the additional porewater inhibits the diffusion of CH_4 from the headspace to the AOM microbial consortia, suppressing AOM. Likewise, we hypothesize that anaerobic incubations done without additional porewater often result in net CH_4 consumption because of the rapid transfer of CH_4 to the microbes performing AOM. The addition of porewater could also serve as a labile C substrate that would be broken down by fermentation reactions to form CO_2, H_2, and acetate to fuel methanogenesis (e.g., Medvedeff et al., 2015). These coupled experiments strongly suggest that the diffusion of CH_4, as dictated by water content and headspace availability, partially controls net CH_4 production and consumption.

Our results also suggest that the many previous studies of CH_4 production in wetlands (including the authors') are highly dependent on their particular incubation conditions, provided AOM is as important as the few previous studies in freshwater wetlands suggest (Smemo and Yavitt, 2007; Gupta et al., 2013; Seggara et al., 2015). In situ wetland porewater often has very high concentrations of CH_4 (typically 0.10 to 1.5 mM at S1 bog, unpublished data), which would rarely be achieved in relatively short-term laboratory incubations that started with headspaces and liquid-phases initially flushed with N_2 or He to remove CH_4. Additionally, the majority of these incubations are done within serum bottles with substantial headspaces where most of the CH_4 that is

produced would reside, further complicating CH_4 transfer dynamics. Thus, it is imperative to gain a better understanding of the controls over and rates of gross AOM and methanogenesis.

Direct measurements of anaerobic oxidation of methane: We used a radioisotope tracer technique to verify high rates of AOM at SPRUCE. We found this previously published method (Valentine et al., 2001) to be highly problematic because of high and variable abiotic isotopic exchange between the tritiated CH_4 tracer and water. Nevertheless, we did observe on two sampling dates that AOM was occurring throughout the entire peatland profile, with the highest rates in surficial soil horizons (Figure 5.9a). This is the first time AOM has been documented in deeper soil layers as previous studies have been limited to the top 40 cm or shallower (Blazewicz et al., 2012; Gupta et al., 2013; Seggara et al., 2015). However, Seggara et al. (2015) also noted that rates of AOM were the greatest in surface samples (0-10 cm) and decreased with depth (up to 40 cm) in three freshwater wetlands (two mineral-soil wetlands and one peatland). We hypothesize that this depth effect is driven by the greater availability of oxidized organic and inorganic TEAs at the surface, relative to deeper soil layers, due to periodic fluctuations in water-table position and release of oxygen from roots. Other studies have also suggested that AOM is driven by TEA availability in freshwater systems (Gupta et al., 2013; Seggara et al., 2015); however, the exact mechanism remains unknown (Gupta et al., 2013). As S1 Bog is an ombrotrophic peatland with very low nutrient inputs and concentrations of inorganic TEAs, such as nitrate and sulfate (Lin et al., 2014), we suggest that humic substances are facilitating AOM in this study. We saw no change in the rate of AOM

over the course of one week; however, radioactivity was no longer detectable above background levels after two weeks (Figure 5.9b), suggesting that we exhausted our supply of TEAs between one and two weeks. We have observed a similar timeframe for the reduction of all peatland organic TEAs in laboratory studies investigating the potential for humic acids to act as electron acceptors in wetland decomposition (Keller, unpublished data).

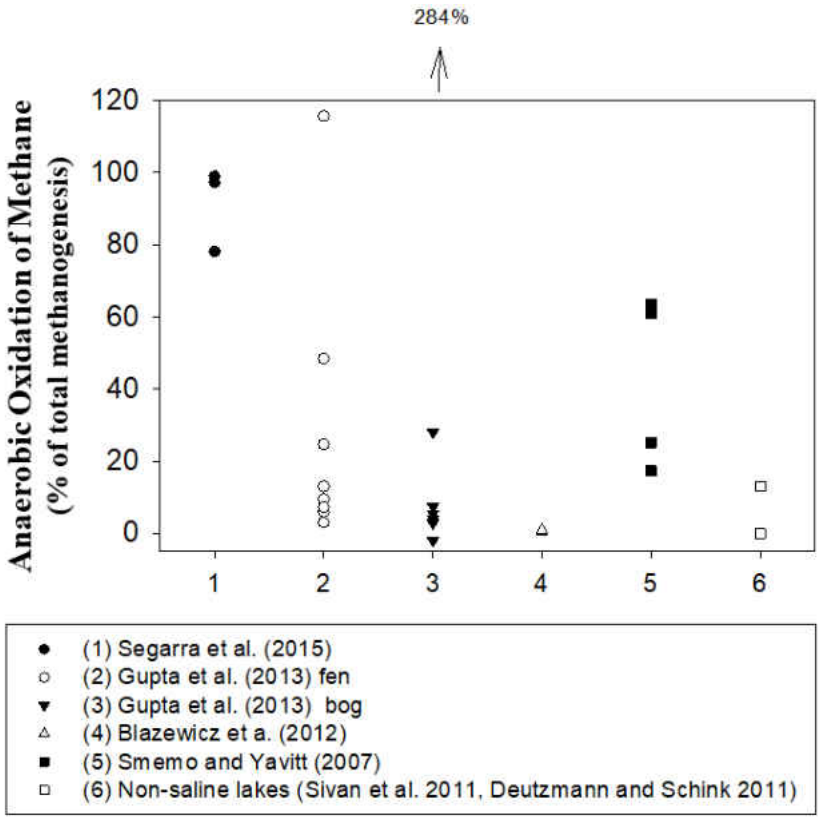


Figure 5.11. A comparison of published estimates of AOM, as a percent of total methanogenesis, observed in freshwater wetlands.

We found that rates of AOM increased with temperature across all depths using both indirect (Figure 5.8) and direct (Figure 5.10) measurement techniques. This contrasts with previous studies which have found no effect of temperature on this process (Gupta et al., 2013; Segarra et al., 2015), although these previous studies did not directly manipulate in-situ temperatures. For example, in a study of 15 North American peatlands that differed in climate, no relationship was found between temperature and rates of AOM (Gupta et al., 2013). These results were corroborated by a similar study that

examined rates of AOM in three freshwater wetlands located along a climate gradient which also found no correlation between climate-driven variables and AOM (Segarra et al., 2015). This result has important implications for understanding the mechanisms underlying anaerobic CH_4 cycling and emissions. For example, although we have shown that peatland methanogenesis will increase with temperature, we also show simultaneous increases in its consumption via AOM under the same conditions, which also would affect the response in the CO_2:CH_4 ratio to temperature that we observed (Figure 5.5). Overall, AOM was responsible for consuming approximately 17% of the total amount of CH_4 produced; however, we observed as much as 74% consumption of total CH_4 production. These percentages are within the wide range of estimates reported throughout the literature (Figure 5.11), which span from 0.27% (Blazewicz et al., 2012) to 284% (Gupta et al., 2013) total consumption, although we suspect the high rates of abiotic isotopic exchange with the method that we used underestimates actual rates of AOM.

If AOM rates are as high as we observed in this study, it sets up a conundrum of how wetlands can maintain high porewater CH_4 concentrations and atmospheric emissions which can only be solved if gross CH_4 production rates are much higher than previously thought. Similarly, the hotly debated question over the past several decades of why many wetlands produce much higher ratios of CO_2:CH_4 under anaerobic conditions than the theoretical ratio of 1:1 when all TEAs are reduced could be explained by high rates of AOM (Conrad, 1989; Bridgham et al., 1998; Yavitt & Seidman-Zager, 2006; Keller & Bridgham, 2007; Wilson et al., 2017). This study represents an important step towards gaining a mechanistic understanding of the climate-driven controls over peatland

AOM. This process is not currently incorporated into Earth system CH_4 models and doing so requires modelling of a much more dynamic system than currently envisioned.

Conclusions

In this study, we show that rising temperatures increased rates of peatland methanogenesis throughout the entire soil profile following 14 months of WEW. Increased CH_4 production drove a decrease in CO_2:CH_4 ratios across all depths, indicating that this ecosystem in becoming more methanogenic as it warms. There is also preliminary evidence of increased CH_4 production with enhanced atmospheric CO_2. The direct effects of temperature on methanogenesis were likely modified by cascading ecological effects, such as changes in water-table position and rates of below-ground root exudation, that could have had contrasting, depth-specific effects on peatland CH_4 production. Additionally, our results emphasized the role of AOM in peatland CH_4 cycling, a historically understudied and often ignored process in freshwater systems. We provide the novel evidence showing that this process occurred throughout the entire peatland soil profile and positively responded to increases in temperature. Our usage of multiple incubation techniques revealed that AOM is likely ubiquitously occurring alongside CH_4 production and that CH_4 production rates measured in previous studies are likely actually net rates of CH_4 production that are highly dependent on the incubation conditions. Anaerobic oxidation of CH_4 is not currently incorporated into Earth system CH_4 models and this may explain why CH_4 emissions are notoriously difficult to predict from CH_4 production and aerobic CH_4 oxidation alone. Continued research efforts in collaboration with projects such as the SPRUCE experiment will enable us to examine

the dynamic response of peatland CH_4 cycling to climate forcing in the long-term, as well as to develop concerted efforts to better parameterize predictive models from empirical, field- and laboratory-based conclusions.

CHAPTER VI

CONCLUSIONS

Wetlands in tropical and boreal regions are among the most important terrestrial ecosystems due to their influence on the global carbon (C) cycle. Anaerobic conditions in these systems promote the sequestration of massive amounts of atmospheric carbon dioxide (CO_2) in soils and vegetation and, as anaerobic mineralization occurs, this C can be released to the atmosphere as the greenhouse gasses CO_2 and/or methane (CH_4) through microbial respiration. Methane is much more effective at trapping heat in the atmosphere relative to CO_2 (Neubauer and Megonigal, 2015) and is currently responsible for approximately 20% of human-induced radiative forcing (Myhre et al., 2013). Thus, as global temperatures continue to rise, it is critical to understand the mechanisms underlying anaerobic C mineralization and CH_4 cycling in natural ecosystems that could generate significant biosphere-climate feedbacks, further accelerating global climate change.

Tropical wetlands emit approximately 47-89% of global CH_4 emissions (Bridgham et al., 2013) and boreal wetlands store roughly one-third of the world's total soil C (Bridgham et al., 2006). However, despite the importance of these ecosystems, tropical wetlands have received limited study concerning CH_4 flux and, although boreal wetlands have been more thoroughly studied, significant questions remain surrounding the biogeochemical controls over CH_4 dynamics in these systems. Therefore, our understanding of the fundamental processes and controls underlying anaerobic C cycling

across ecosystems is incomplete, limiting our ability to accurately predict climate forcing on ecosystem and global scales.

The overall objectives of this dissertation are to help alleviate these knowledge gaps by (1) providing critical knowledge about the rates of and biogeochemical controls over gross and net CH_4 cycling processes across a variety of equatorial African habitats, and (2) expanding our mechanistic understanding of how climate-driven variables in a northern peatland affect anaerobic C mineralization and CH_4 dynamics.

Chapter II investigated the abiotic and biotic controls over ecosystem CH_4 cycling dynamics across a variety of sites located along a wetland to upland gradient in the central African nation of Gabon. Using a landscape-scale sampling approach, we measured CH_4 flux, production, consumption, and methanogenic pathways at each site and used a suite of physiochemical and microbial community attributes to determine the relative ability of abiotic and biotic variables to predict these processes across ecosystem types. In Chapter II, we show that a combination of microbial community attributes, including composition, abundance, activity, and diversity, are better predictors of CH_4 production, consumption, methanogenic pathway, and CO_2:CH_4 ratios relative to a standard set of physiochemical parameters. Of particular interest was that the relative abundance of *Methanobacterium sp.* in the active community explained 77 and 75% of the variation in methanogenic pathway dominance and CO_2:CH_4 ratios, respectively. Methanogenic pathway dominance and CO_2:CH_4 ratios are important ecological indicators that provide insight into the flow of C through ecosystems, as well as the underlying mechanisms controlling ecosystem CH_4 cycling. Thus, our research highlights the central role of microbial ecology in controlling ecosystem-scale processes, as well as

the potential benefits of incorporating microbial dynamics into terrestrial CH_4 modelling efforts.

In Chapters III-V, we transition to the examination of substrate- and climate-driven controls over boreal peatland anaerobic C mineralization and CH_4 cycling. Chapter III begins by asking – is anaerobic C decomposition in peatlands fueled by dissolved organic matter (DOM) or solid-phase peat? Solid-phase soil organic matter has long been assumed to be the primary substrate driving peatland anaerobic respiration as it represents the largest C pool in these systems and, as such, has been used to estimate rates of ecosystem respiration. However, recent radiocarbon data suggest that DOM plays a key, and often dominant, role in fueling heterotrophic respiration across a variety of peatlands (Chanton et al., 2008). In this study, we manipulated available C sources under laboratory conditions to empirically determine the primary C source – solid-phase peat or DOM – fueling anaerobic respiration at surface and deep depth increments within two bogs and a poor fen in northern Minnesota. We found that increasing DOM concentration from 0 to 50% during anaerobic incubations significantly increased rates of surface CH_4 production, but not CO_2 production, indicating that DOM acts as a primary driver of surface methanogenesis in peatlands. Contrary to our expectations, this response was consistent across all three sites despite differences in plant communities and biogeochemical characteristics. However, we observed no effect of DOM availability on CH_4 or CO_2 production at any other depth. The lack of response of CO_2 production to DOM manipulation highlights the sensitivity of surface CH_4 production to changes in the DOM pool quality and quantity, which are likely to occur under future climate change

scenarios. However, CH_4 production in deeper peat appears to be limited by additional factors beyond labile C availability.

Chapter IV delves more deeply into understanding the constraints over anaerobic C mineralization at depth in peatlands and with ongoing environmental change. The vast majority of peatland C is stored at depth in the permanently anoxic zone (Bridgham et al., 2006), where its decomposition may be partially suppressed by low temperatures; yet, all soil warming experiments to date have focused on the response of peatland C degradation to surface warming (Chen et al., 2008; Turetsky et al., 2008). If the slow decomposition of deep peatland C is due to kinetic constraints, then increasing temperatures at depth should cause parallel increases in CO_2 and/or CH_4 production rates. However, it is currently unknown whether the large C reservoirs at depth in peatlands will be released into the atmosphere as CO_2 and/or CH_4, potentially playing a significant, yet unquantified, role in future climate change.

To alleviate this knowledge gap, the Spruce and Peatland Responses Under Changing Environments (SPRUCE) experiment, is assessing how northern peatland ecosystems react to a changing climate using a novel, regression-based, ecosystem-scale climate manipulation that incorporates deep peat heating (DPH) of the soil profile up to a depth of 3 m. In collaboration with this project, we show that 13 months of DPH exponentially increased peatland CH_4 emissions, but not ecosystems respiration of CO_2 (Wilson and Hopple et al., 2016). However, this response was due solely to surface processes and not degradation of deep C. Anaerobic incubations showed that only the top 20-30 cm of peat from experimental plots had higher CH_4 production rates at elevated temperatures. Additionally, radiocarbon analyses demonstrated that CH_4 and CO_2 are

produced primarily from decomposition of surface-derived modern photosynthate, not deep C. Furthermore, differences in microbial community structure, dissolved organic matter concentration, and degradative enzymes activities were driven by depth rather temperature treatment. These results suggested that although surface peat will respond to increasing temperature, the large reservoir of deep C is stable under current anoxic conditions. However, these conclusions were drawn during the DPH-*only* phase of the SPRUCE experiment. This raises the questions – will this response remain unchanged or be enhanced with additional time of surface and deep warming (i.e. whole-ecosystem warming)? Or, conversely, is this a transient perturbation effect that will diminish with time as the ecosystem equilibrates to temperature manipulations? Will changes in other ecosystem abiotic factors, such as elevated atmospheric CO_2 concentrations, modify these responses? What about other processes important to the CH_4 cycle?

Chapter V addresses these questions through continued work with the SPRUCE project, investigating the response of peatland anaerobic CH_4 cycling to whole-ecosystem warming (WEW) and elevated atmospheric CO_2 concentrations (eCO_2) using controlled laboratory incubations, completed under near-in-situ conditions, of peat samples collected from surface (30 cm) to deep (200 cm) depth increments. Specifically, we investigated changes in peatland CH_4 production, CO_2:CH_4 ratios, and AOM throughout the entire peatland profile following 14 months of WEW and initial responses to enhanced CO_2 concentrations ($\leq$ 4 months). In this chapter, we show that rising temperatures increased rates of peatland methanogenesis throughout the entire soil profile following more than a year of WEW. Increased CH_4 production drove a decrease in CO_2:CH_4 ratios across all depths, indicating that this ecosystem in becoming more

methanogenic as it warms. There is also preliminary evidence of increased CH_4 production with enhanced atmospheric CO_2. The direct effects of temperature on methanogenesis were likely modified by cascading ecological effects, such as changes in water-table position and rates of below-ground root exudation, that could have had contrasting, depth-specific effects on peatland CH_4 production.

Additionally, our results emphasized the role of AOM in peatland CH_4 cycling, a historically understudied and often ignored process in freshwater systems. We provide the novel evidence showing that this process occurred throughout the entire peatland soil profile and positively responded to increases in temperature. Our usage of multiple incubation techniques revealed that AOM is likely ubiquitously occurring alongside CH_4 production and that CH_4 production rates measured by typical laboratory techniques are likely actually net rates of CH_4 production that are highly dependent on the incubation conditions. Anaerobic oxidation of CH_4 is not currently incorporated into Earth system CH_4 models and this may explain why CH_4 emissions are notoriously difficult to predict from CH_4 production and aerobic CH_4 oxidation alone. Continued research efforts in collaboration with projects such as the SPRUCE experiment will enable us to examine the dynamic response of peatland CH_4 cycling to climate forcing in the long-term, as well as to develop concerted efforts to better parameterize predictive models from empirical, field- and laboratory-based conclusions.

APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER III

Supplemental Table 3.1. Characterization of the disturbance effect generated for surface CH_4 production from freezing peat samples and DOM removal process across three sites. Significant differences ($p < 0.05$) between treatments are denoted with *.

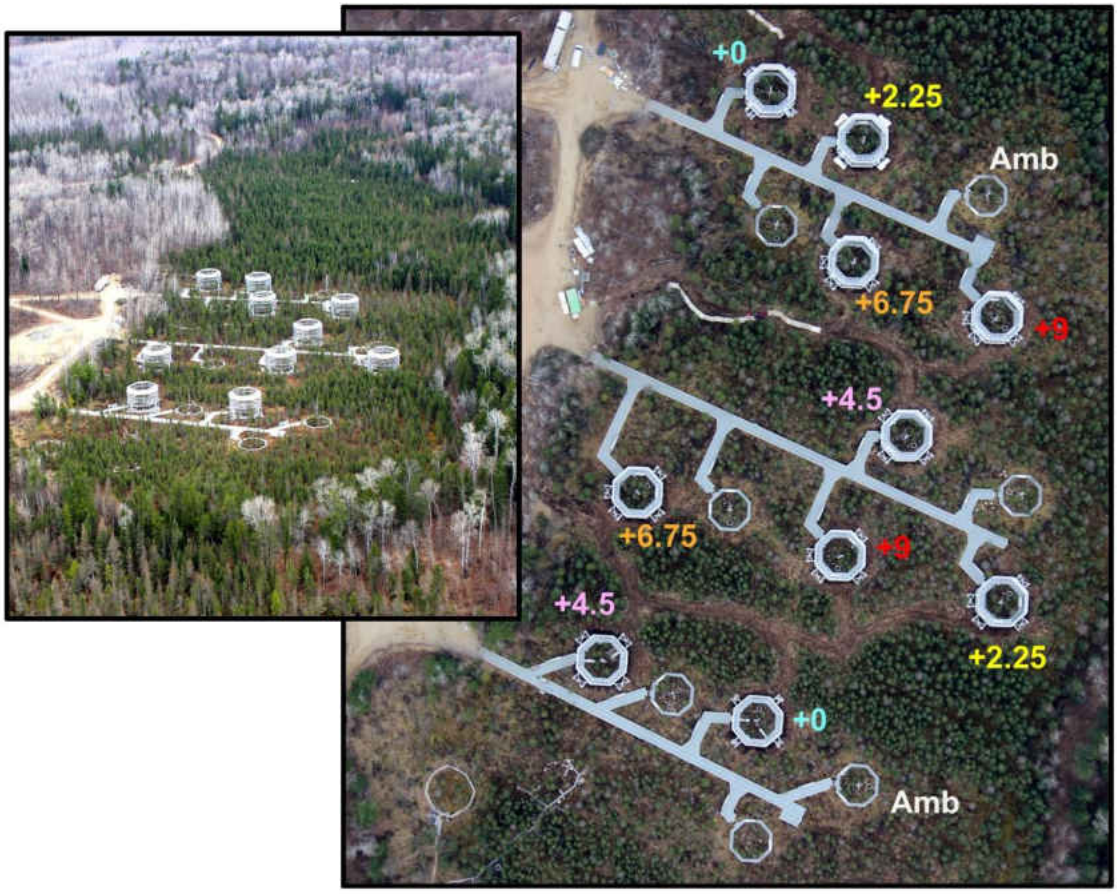
Date	Pre-Treatment	Manipulation	DOM %	CH_4 (µmol C g soil^{-1} d^{-1}) ± SE	CO_2 (µmol C g soil^{-1} d^{-1}) ± SE
June 2013	Frozen 2 mo.	DOM removed and added back.	50	0.15* ± 0.03	2.6 ± 0.21
June 2013	Frozen 2 mo.	Non-manipulated.	50	0.34* ± 0.07	2.3 ± 0.13
July 2014	Fresh	DOM removed and added back.	50	1.7 ± 0.28	3.7 ± 0.36
July 2014	Fresh	Non-manipulated.	50	2.0 ± 0.21	3.3 ± 0.17

Supplemental Table 3.2. Methane and CO_2 production from anaerobic incubations of peatland samples containing either 0 or 50% DOM in June 2013. Data represent the combination of anaerobic respiration rates across three sites. Marginally significant differences ($p < 0.07$) between treatments within each depth are denoted with **^{*m}**.

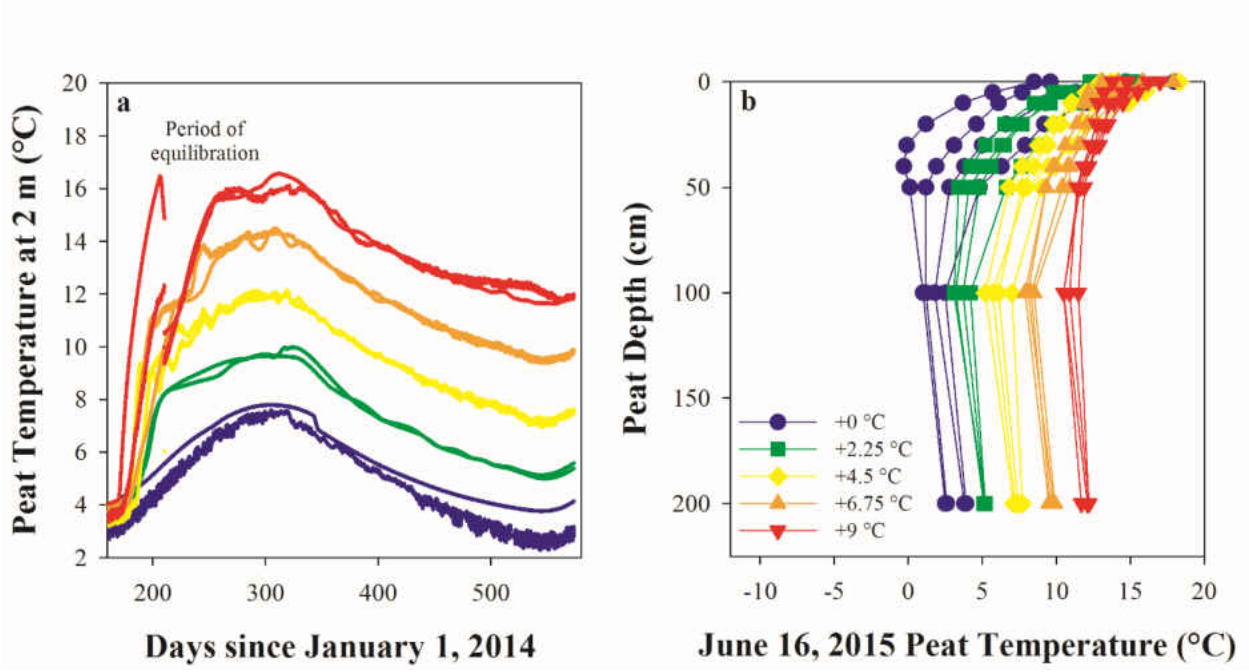
Depth (cm)	DOM %	CH_4 (µmol C g soil^{-1} d^{-1}) ± SE	CO_2 (µmol C g soil^{-1} d^{-1}) ± SE
25-50	0	0.072**^{*m}** ± 0.023	2.8 ± 0.3
25-50	50	0.15**^{*m}** ± 0.033	2.6 ± 0.2
75-100	0	$1.3 \times 10^{-2} \pm 6.1 \times 10^{-3}$	1.4 ± 0.2
75-100	50	$4.3 \times 10^{-3} \pm 7.8 \times 10^{-4}$	1.5 ± 0.1
150-200	0	$2.9 \times 10^{-3} \pm 5.02 \times 10^{-5}$	0.84 ± 0.1
150-200	50	$3.1 \times 10^{-3} \pm 1.3 \times 10^{-4}$	1.1 ± 0.1

APPENDIX B

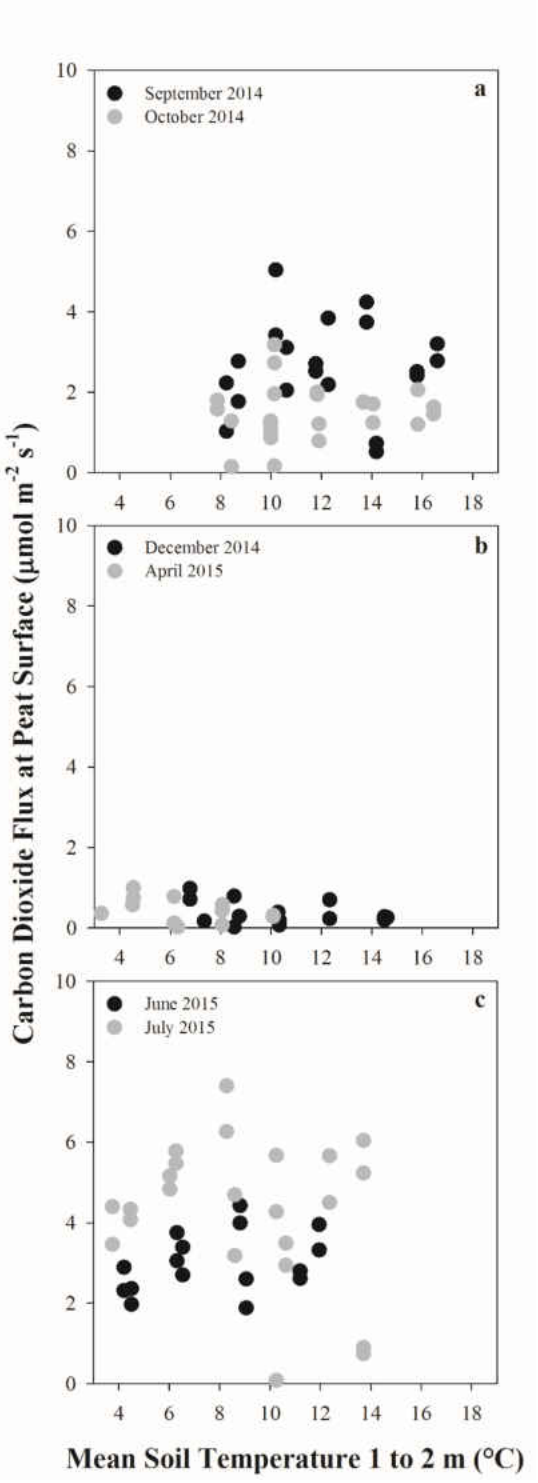
SUPPLEMENTARY INFORMATION FOR CHAPTER IV



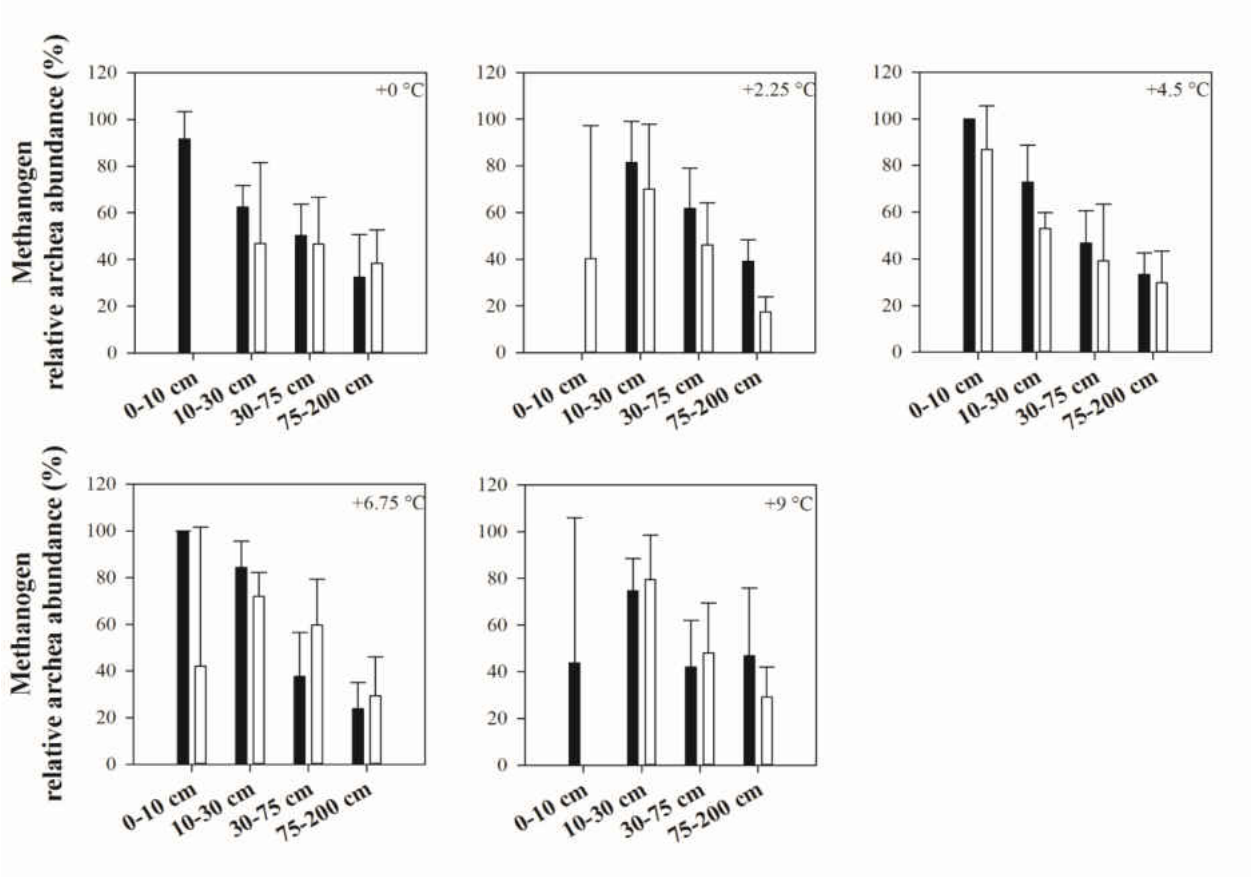
Extended Data Figure 4.1: Schematic of the SPRUCE site, located in northern Minnesota. Three boardwalks transect the site, with experimental treatments plots branching radially off of those boardwalks. Numbers indicate the target temperatures, relative to ambient conditions, established within each enclosure. "Amb" plots indicate that no temperature treatment has been added. Inset shows an aerial overview of the site with the experimental chambers installed in the context of the surrounding bog.



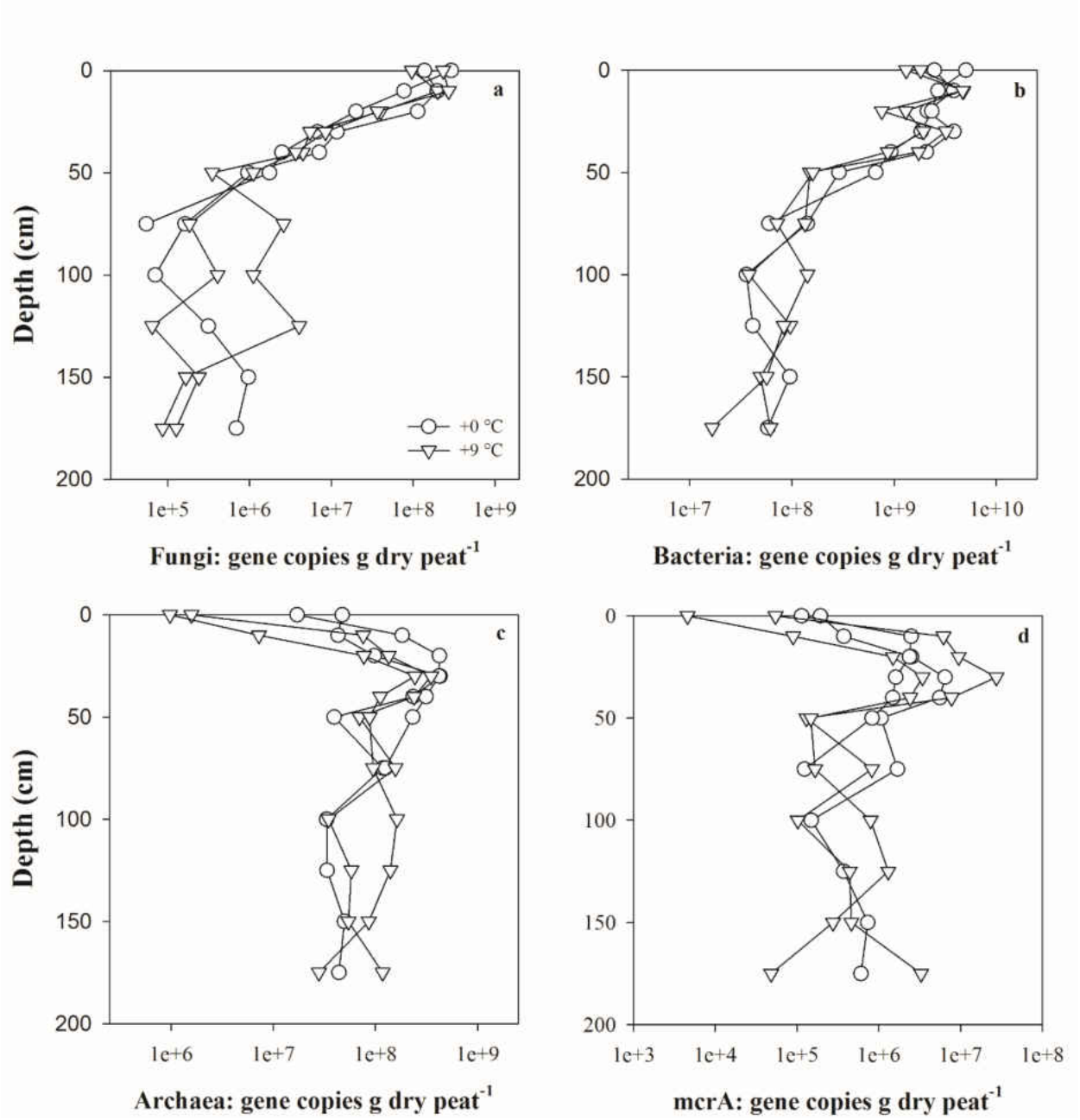
Extended Data Figure 4.2: The seasonal progress of absolute peat temperatures at 2 m below the hollow surface throughout the DPH treatment period **(a)** and the temperature depth profiles associated with the June 16, 2015 coring event **(b)**. This coring event took place 10 months after the deep peat temperature differentials were stable. In the absence of air warming during this phase of the experiment, anticipated energy loss at the surface reduced the separation among treatment temperatures.



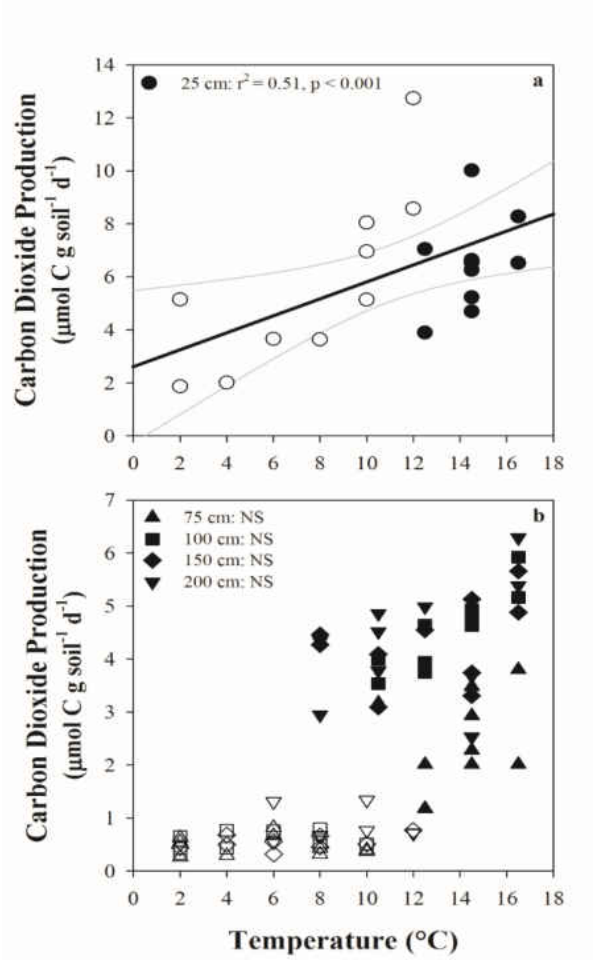
Extended Data Figure 4.3: The seasonal CO_2 flux from 1.2 m diameter collars during fall 2014**(a)**, winter 2015**(b)**, and summer 2015**(c)** across temperature treatments. Black and gray dots distinguish between daily averages for two different sampling points during the season. No significant correlations between CO_2 flux and temperature were observed during these measurement periods.



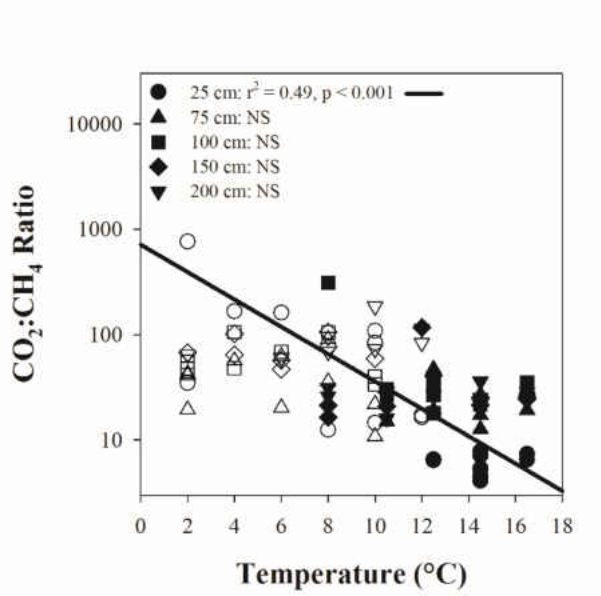
Extended Data Figure 4.4: Depth dependence of known methanogenic Archaeal groups in treatment plots prior to and after exposure to deep peat heating (DPH). Apparent zero abundances (*e.g.,* pre-DPH 0-10cm in the +2.25°C plot) reflect missing data. Abundance of known methanogens gradually decreases with peat depth, while no significant effect of temperature treatment or time on relative abundance of methanogens was observed. Pre-DPH is represented by closed bars and during DPH by open bars.



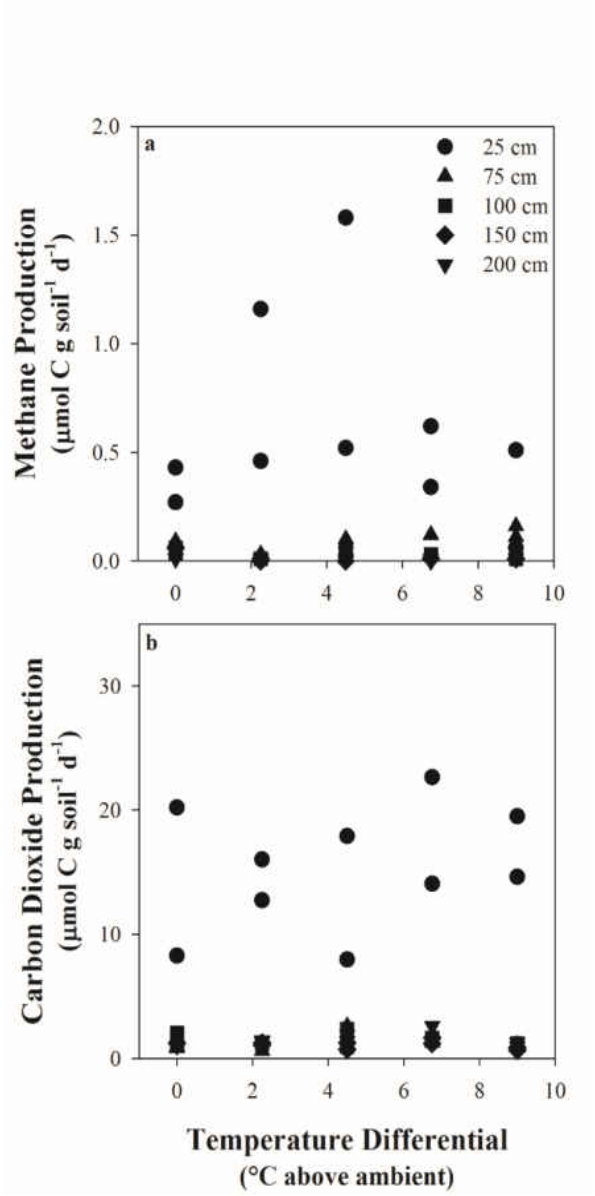
Extended Data Figure 4.5: The abundance of fungal **(a)**, bacterial **(b)**, and archaeal **(c)** populations was determined by quantitative PCR using primers targeted to amplify their respective SSU rRNA genes, and targeting the mcrA gene **(d)** for methanogen populations. After thirteen months of deep peat heating (DPH) treatment, the *in situ* abundance of microbial groups (bacteria, archaea, fungi, and methanogen populations) shows no clear response to temperature, while strong vertical stratification is observed with peat depth. Microbial abundance is expressed for core samples from control ($+0^0$C) and $+9^0$C plots as gene copies per gram dry peat.



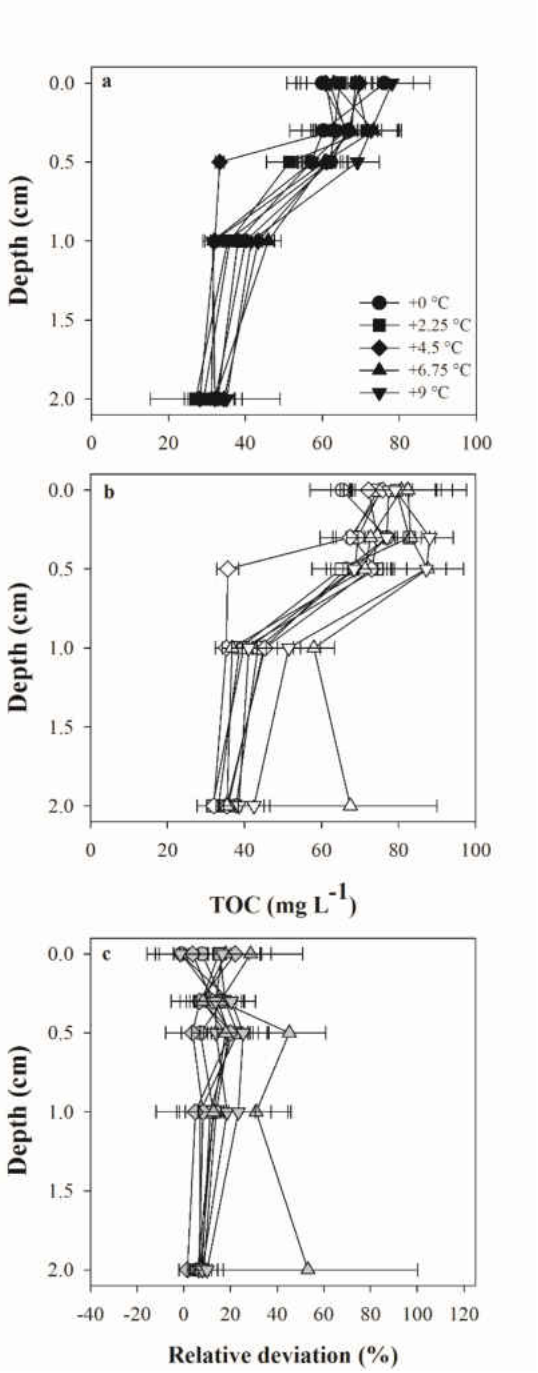
Extended Data Figure 4.6: The temperature response of CO_2 production observed from peat samples taken from 25 cm **(a)** and at depth (75 – 200 cm) **(b)**. Anaerobic incubations were completed within 1ºC of *in situ* temperatures after approximately 4 (closed symbols September 2014) and 13 (open symbols, June 2015) months of DPH. The temperature response at depth was analyzed by season due to a distinct bimodal distribution. NS = not significant.



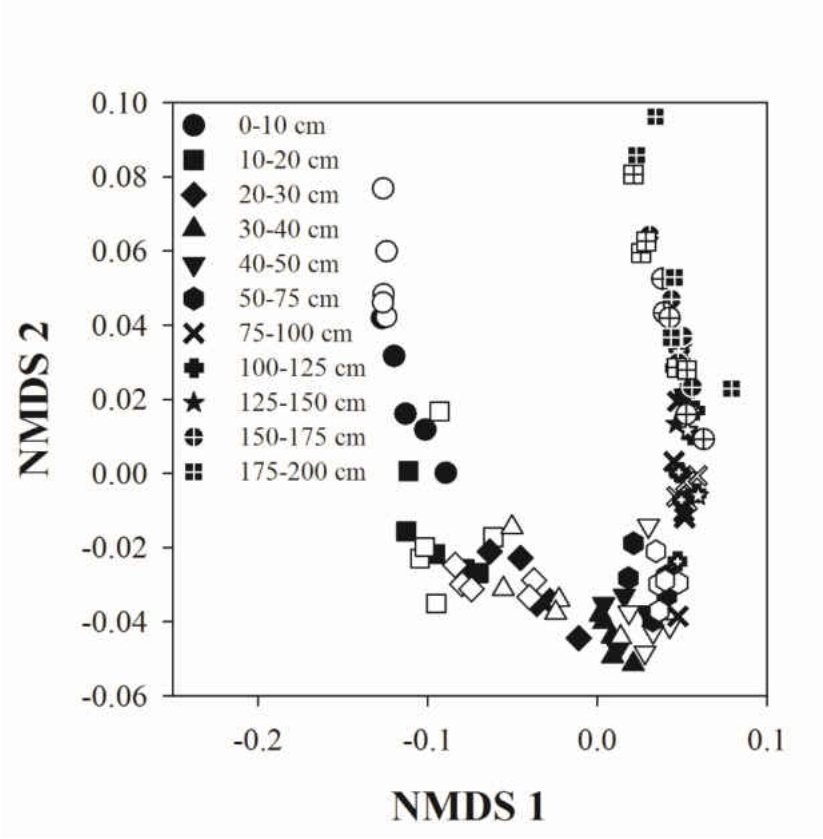
Extended Data Figure 4.7: CO_2:CH_4 ratios determined from incubations. Peat samples were collected from 5 depths and anaerobically incubated within 1ºC of *in situ* temperatures after approximately 4 (closed symbols; September 2014) and 13 (open symbols; June 2015) months of deep peat warming. *Note the log scale.*



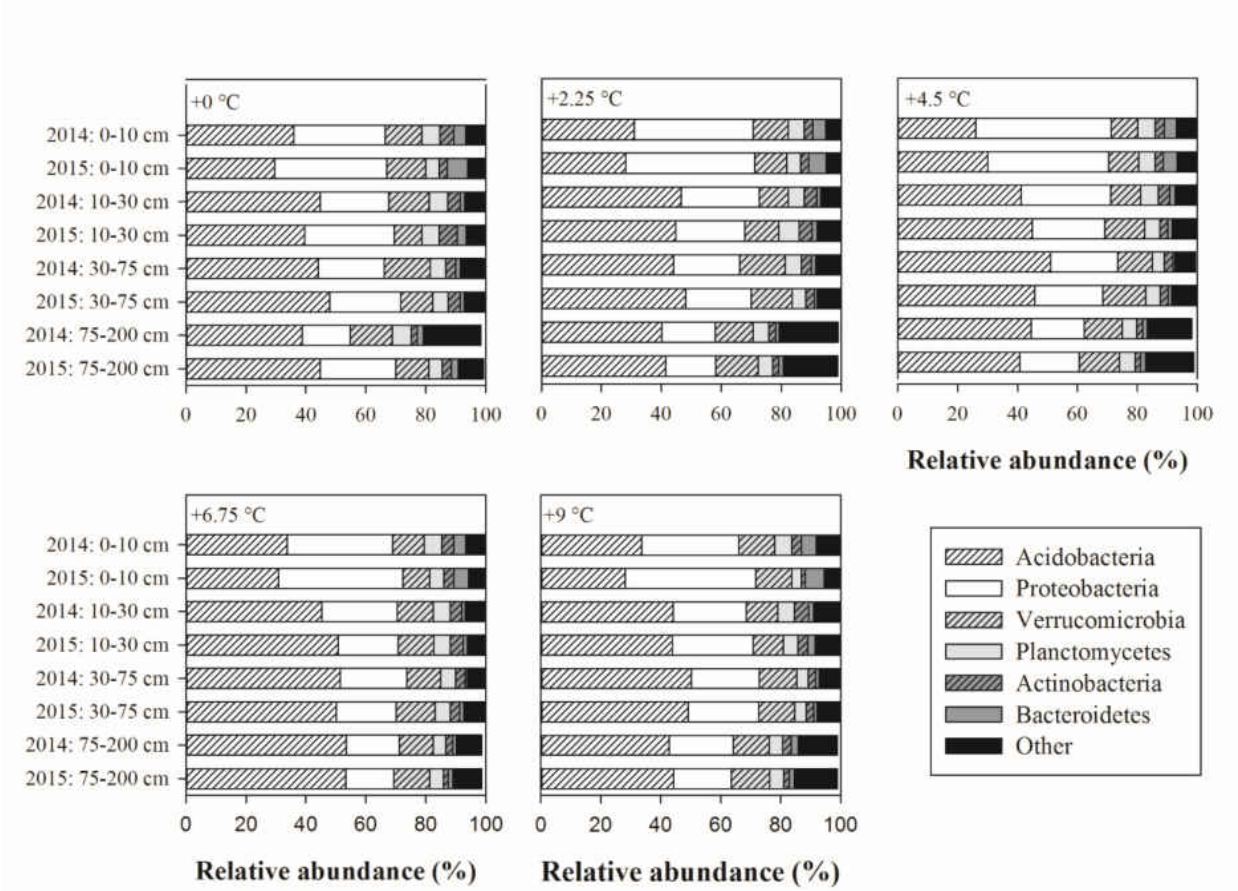
Extended Data Figure 4.8: Test for legacy effects of experimental warming on CH_4 **(a)** and CO_2 **(b)** production from peat samples taken at multiple depths and anaerobically incubated at a common temperature (20°C) after approximately 13 months of DPH. Data are plotted against the temperature treatment from which the peat was collected. *Note the lack of response found across all depths.*



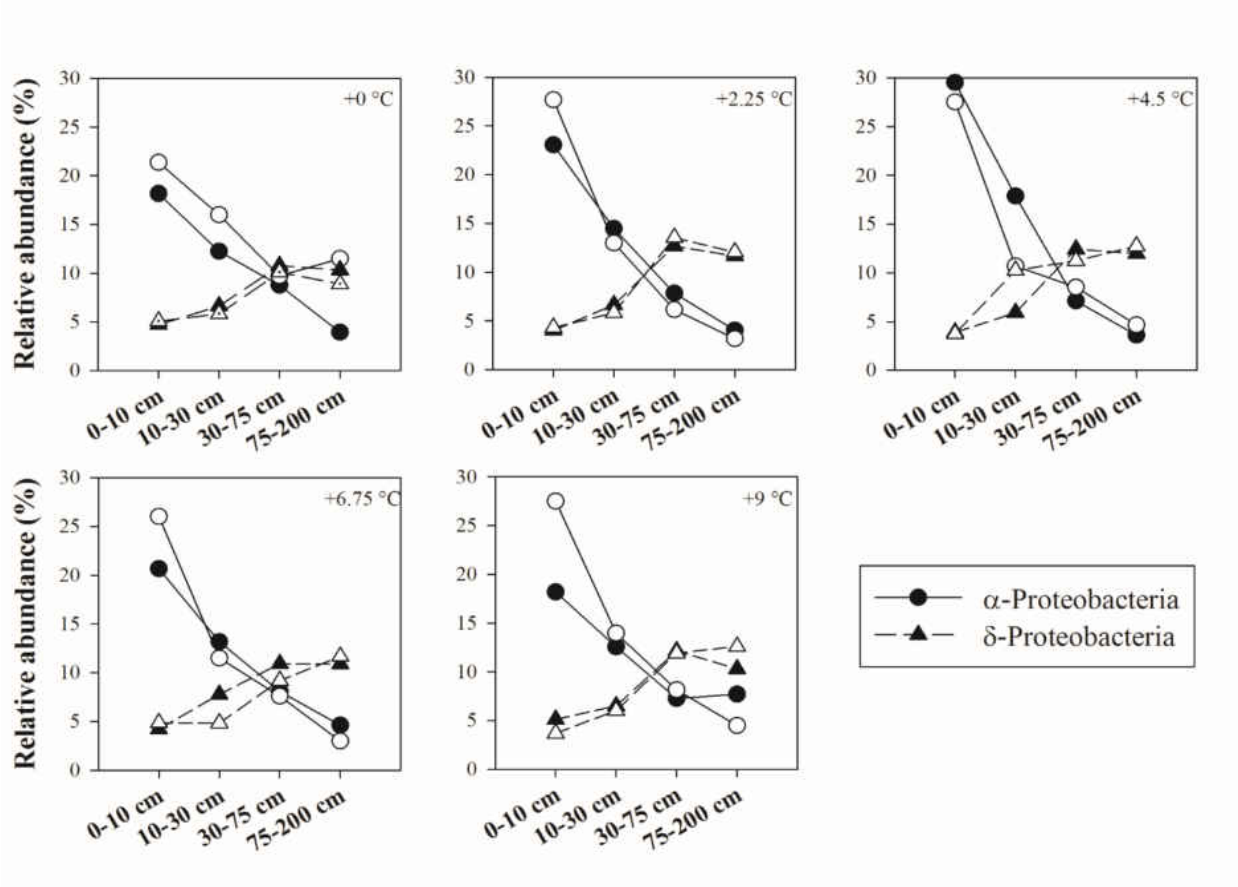
Extended Data Figure 4.9: Total dissolved organic carbon (DOC) concentrations in the peat porewater prior to **(a)** and during deep peat heating **(b)**. The relative deviation of TOC was calculated to account for pre-treatment differences in TOC concentrations across plots. The deviation was calculated by dividing the TOC concentration at a given temperature treatment and a given depth by the mean TOC concentration at that same depth in the two control (0° C) plots prior to DPH **(c)**. Points represent the averages of weekly (pre-DPH) or biweekly (during DPH) sampling and standard deviations of temporal variability are indicated by the whiskers.



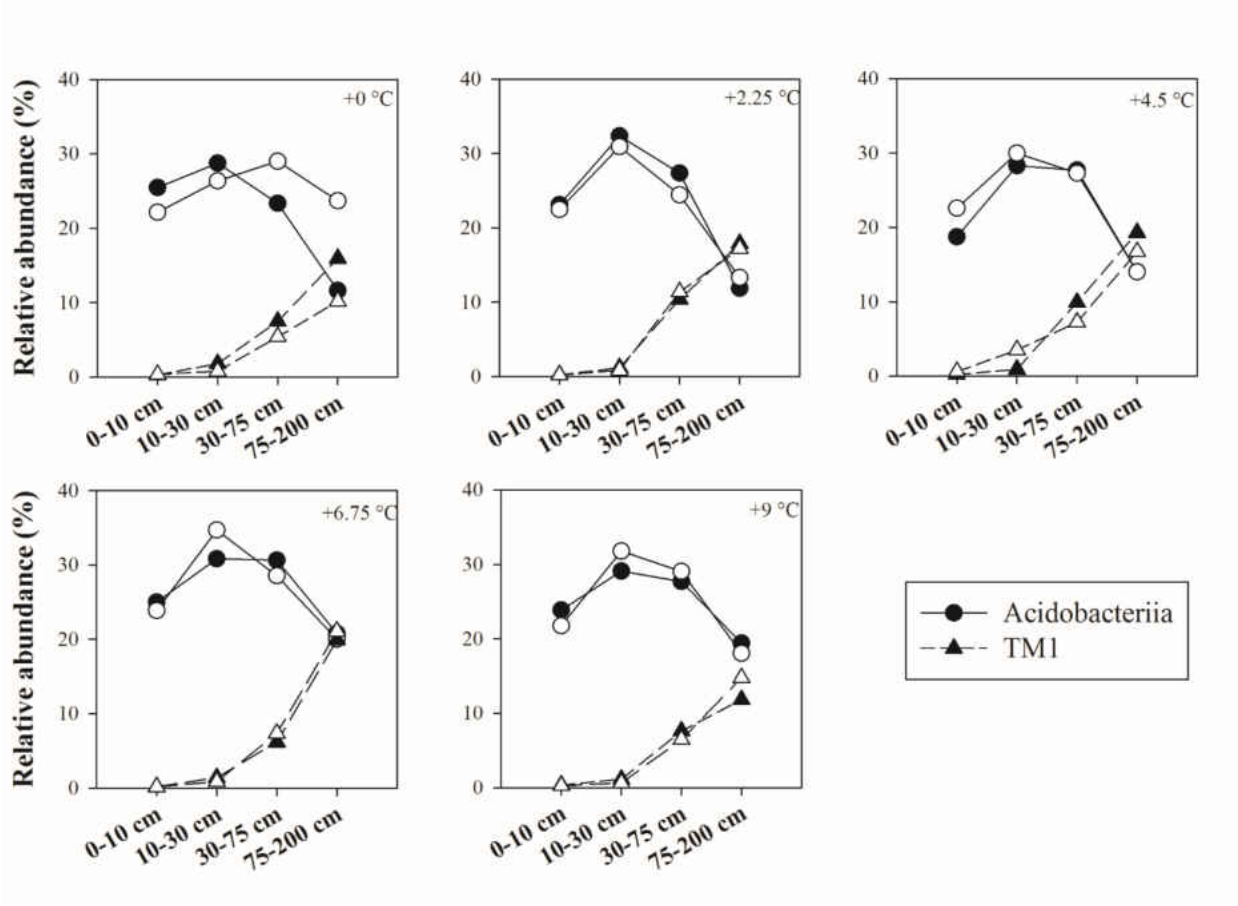
Extended Data Figure 4.10: Depth dependence of soil microbial community structure in SPRUCE site enclosures prior to and after exposure to deep peat heating (DPH). Closed symbols represent pre-DPH samples while open symbols indicate during-DPH conditions. Community structure exhibits strong vertical stratification in the peat column as visualized in a nonparametric multidimensional scaling plot (NMDS). Pairwise community distances were determined using the weighted Unifrac algorithm. A total of 5.35 million of rRNA gene sequences were normalized by cumulative sum scaling (CSS) methods and grouped by depth. As shown in Figure 4, no significant effect of temperature treatment or time is observed on community diversity or composition.



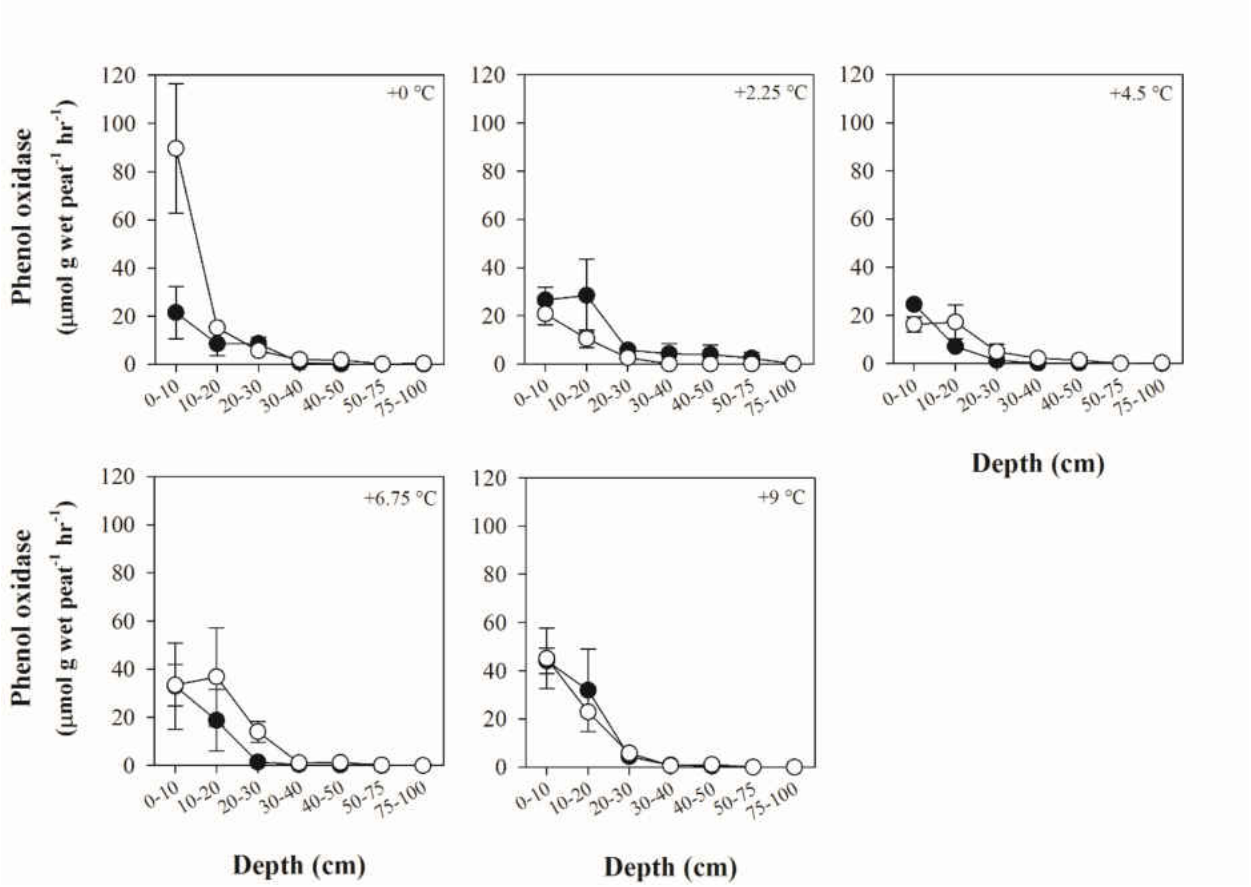
Extended Data Figure 4.11: Depth dependence of soil microbial groups detected at the phylum level (> 1 % divergence in gene sequences) in treatment plots prior to and after exposure to deep peat heating (DPH). Bars are stacked by date such that pre-DPH and during DPH results are proximate. The majority of microbial populations (~70%) are taxonomically affiliated to *Proteobacteria* and *Acidobacteria* phyla. A total of 5.35 million of rRNA gene sequences were assigned to the greengenes database by RDP Classifier at 50% confidence thresholds. Phyla which represented < 1% of relative abundance were not displayed and are summarized as "Other".



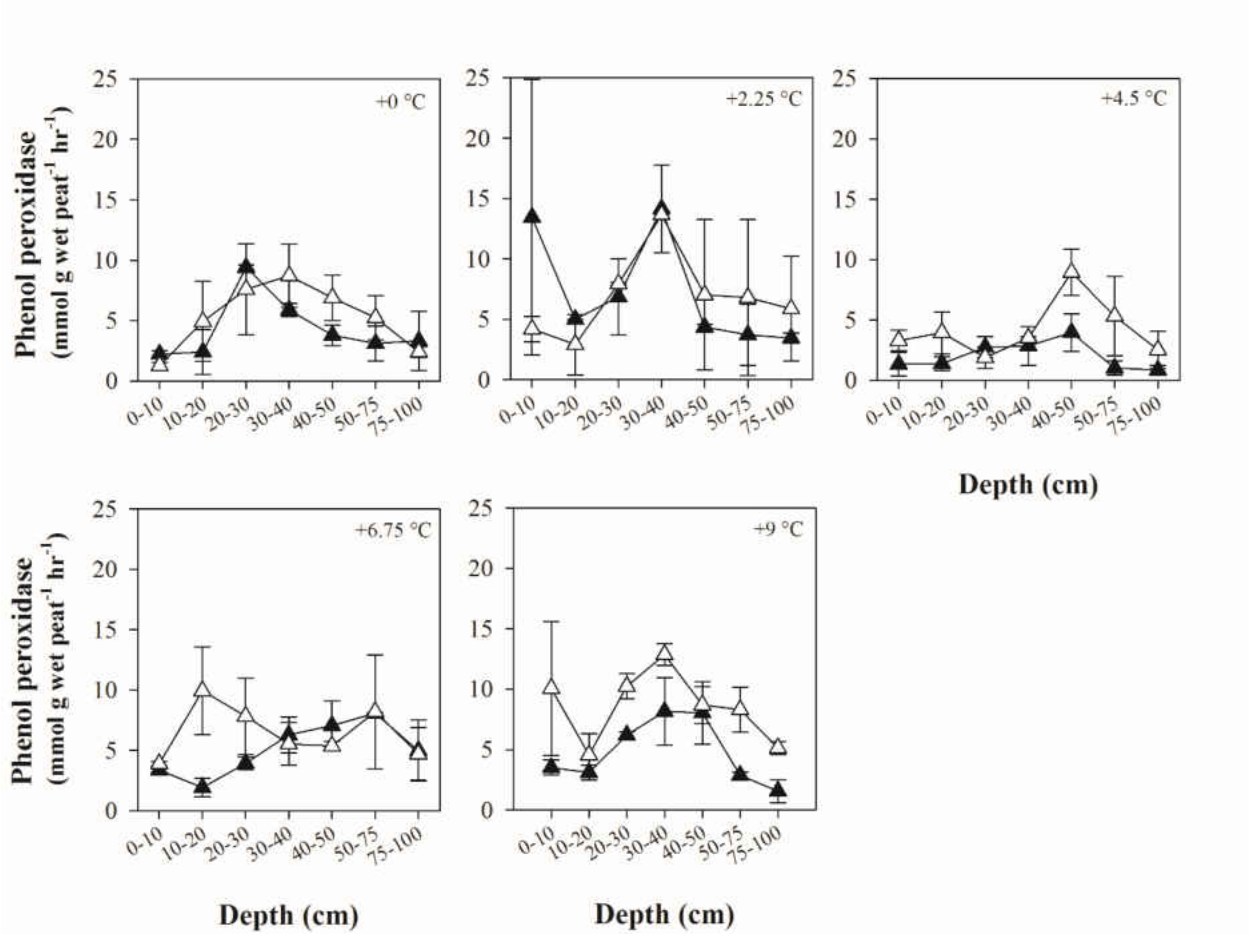
Extended Data Figure 4.12: Depth dependence of soil microbial groups detected at the class level in treatment plots prior to (closed symbols) and after (open symbols) exposure to deep peat heating (DPH). Putative aerobic heterotrophs affiliated with the *Alphaproteobacteria* decreased in average relative abundance with depth, while putative anaerobes in the *Deltaproteobacteria* increase with depth.



Extended Data Figure 4.13: Depth dependence of soil microbial groups affiliated with Class Acidobacteria in treatment plots prior to (closed symbols) and after (open symbols) exposure to deep peat heating (DPH). Putative aerobic heterotrophs affiliated with the *Acidobacteriia* decreased in relative abundance with depth, while putative anaerobes in the *TM1* class increase with depth.



Extended Data Figure 4.14: Potential oxidative enzyme activity (phenol oxidase) in SPRUCE site enclosures prior to (June 2014, closed circles) and after (June 2015, open circles) exposure to deep peat heating (DPH). Temperatures indicated on panels indicate *in situ* temperature treatment. No significant effect of temperature or time on enzymatic activities was observed. Values are the mean of two cores with four technical replicates.



Extended Data Figure 4.15: Potential oxidative enzyme activity (phenol peroxidase) in treatment plots prior to (June 2014, closed circles) and after (June 2015, open circles)—exposure to deep peat heating (DPH). Temperatures indicated on panels indicate *in situ* temperature treatment. No significant effect of temperature or time on enzymatic activities was observed. Values are the mean of two cores with four technical replicates.

Extended Data Table 4.1: Parameters for qPCR analysis of peat microbial communities. Reactions were performed in triplicate on a CFX96TM Real-Time PCR Detection System (Bio-Rad Laboratories) with iQ SYBR Green Supermix (Bio-Rad, CA, USA).

Microbial Group	Gene Target	Primer	Primer Reference	Organism for qPCR Standard
Eubacteria	16S	Eub 338 Eub518	Lane, 1991 Muyzer et al., 1993	*Escherichia coli*
Archaea	16S	915F 1059R	Yu et al., 2005	*Methanococcus maripaludis* S2
Fungi	18S	nu-SSU-1196F nu-SSU-1536R	Borneman & Hartin, 2000	*Saccharomyces cerevisiae*
Methanogens	mcrA	mcrA_F mcrA_R	Luton et al., 2002	*Methanococcus maripaludis* S2

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CHAPTER VI

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