A Rhizosphere-Scale Investigation of Root Effects on Wetland Methane Dynamics

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Abstract

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The largest natural source of CH_4 to the atmosphere is wetlands, which produce 20% to 50% of total global emissions. Vascular plants play a key role regulating wetland CH_4 emissions through multiple mechanisms. They often contain aerenchymatous tissues which act as a diffusive pathway for CH_4 to travel from the anoxic soil to the atmosphere and for O_2 to diffuse into the soil and enable methanotrophy. Plants also exude carbon from their roots which stimulates microbial activity and fuels methanogenesis. This study investigated these mechanisms in a laboratory experiment utilizing rootboxes containing either *Carex aquatilis* plants, silicone tubes that simulated aerenchymatous gas transfer, or only soil as a control. The results are presented in three parts: the output of an isotope mixing model (Chapter 2), a mass spectroscopy investigation of changes caused to chemical composition of soil compounds (Chapter 3), and a metagenomic study of changes to the microbial ecosystem (Chapter 4).

Chapter 2 shows that CH_4 emissions were far greater from planted boxes than from control boxes or simulated plants, indicating that the physical transport pathway of aerenchyma was of little importance when not paired with other effects of plant biology. Plants were exposed to $^{13}CO_2$ at two time-points and subsequent enrichment of root tissue, rhizosphere soil, and emitted CH_4 was used in an isotope mixing model to determine the proportion of plant-derived versus soil-derived carbon supporting methanogenesis. Results showed that carbon exuded by plants was converted to CH_4 but that plants also increased emission of soil-derived carbon relative to the other experimental treatments. This result signifies that plants and root exudates altered the soil chemical environment and microbial community such that microbial utilization of soil carbon was increased (e.g. microbial priming) and/or oxidation of soil-derived CH_4 was decreased (e.g., by microbial competition for oxygen).

Chapter 3 uses FT-ICR-MS analysis of soil compounds to identify the molecular signature of microbial priming in the wetland rhizosphere. The FT-ICR-MS data demonstrated that the root exudates triggered increased processing of both large, energy-rich molecules and small nitrogen-containing molecules, but only in the water-soluble carbon pool. This is evidence for a selective priming effect in which some types of carbon compounds are processed at an increased rate, while others are not.

Chapter 4 examines the total count of microbes in the rhizosphere soil and unplanted bulk soil as well as metagenomic data. Together, these data sets showed that obligate methanotrophs out-compete facultative methanotrophs in the low-oxygen, high methane environment of the wetland rhizosphere. This advantage may come from a higher affinity of the obligates to use what little oxygen there is, or from an ability to conduct anaerobic methane oxidation. The data used in this chapter is extensive, and the end of the chapter identifies potential future research questions.

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Chapter 1. BACKGROUND

1.1 INTRODUCTION

The world is facing an unprecedented challenge in the face of global climate change. Anthropogenic climate change is already claiming at least 150,000 lives each year (World Health Organization 2004), and this toll is projected to worsen as the planet continues to warm(Patz et al. 2005). Leading causes of concern are direct heat-caused conditions, infectious diseases, extreme weather events, and malnutrition (McMichael et al. 2006). Climate change will also be devastating to the environment through extinction of plants and animals (Thomas et al. 2004), increases in wildfires (Westerling et al. 2006), increases in drought (Cook et al. 2004), and loss of coral reefs (Carpenter et al. 2008) among other effects. Addressing this pressing issue requires fully understanding not only the effects of increasing greenhouse gas concentrations, but also the sources.

Methane (CH_4) is a potent greenhouse gas responsible for 15-19% of total greenhouse gas radiative forcings, second only to carbon dioxide (CO2) (Intergovernmental Panel on Climate Change 2014). While measurements of CH_4 concentration in the atmosphere are fairly precise, estimates of critical sources and climate feedbacks are not well constrained. The largest natural source of CH_4 to the atmosphere is wetlands, which produce between 20% and 40% of total (anthropogenic and natural) emissions (Kirschke et al. 2013). These estimates vary widely partly due to significant differences in CH_4 emissions among natural wetlands, which previous research has shown to be partially explained by factors such as plant type (Fritz et al. 2011), season (Wang and Han 2005), climate (Hodson et al. 2011), and soil type (Kayranli et al. 2009). Computer models are required to factor in these effects and generate global estimates, but manage factors such as temperature response (Grant et al. 2015), CH_4 oxidation (Grant 1998; Walter and Heimann 2000) and size and definition of wetland (Bohn et al. 2015) in different ways. Resolving differences in the modelling literature requires an understanding of the system rooted in empirical laboratory and field studies of the physical systems, focusing on key controlling elements such as vascular wetland plants.

As climate change increases global carbon dioxide (CO_2) concentrations and temperatures, there will be major changes to the wetlands of the Boreal region. These constitute over half of global wetland area (Aselmann and Crutzen 1989) and are subject to temperatures rising at a faster than average rate (Intergovernmental Panel on Climate Change 2014). This rising temperature is important because in wetlands that are saturated throughout the year, temperature is the largest controlling factor in CH_4 emission rates (Bloom et al. 2010). Low temperatures currently cause boreal wetlands to both sequester less carbon and emit less CH_4 on a per-area basis than most other types of wetlands (Mitsch et al. 2013). One study found only 25% of wetland CH_4 was emitted from north of 30 latitude (Walter et al. 2001), consistent with other studies that find a majority of wetland CH_4 emitted from tropical regions (Bloom et al. 2010). Boreal wetlands are also predicted to grow due to melting permafrost (Koven et al. 2011), and emit more CH_4 due to increased atmospheric CO_2 concentrations (Megonigal and Schlesinger 1997). Human actions are increasing global temperatures and leading to further permafrost melting, and increasing CO_2 concentrations in the atmosphere. The positive feedback which wetland CH_4 dynamics provides is predicted to enhance the radiative forcing of anthropogenic emissions by 3.5 to 5% (Gedney et al. 2004).

Wetland CH_4 emissions are controlled by three classes of microbes that interact in the soil: methanogens, methanotrophs, and heterotrophs. CH_4 is produced by methanogens, which convert dissolved organic carbon (DOC) into CH_4 in anoxic environments with low oxidation reduction potential (ORP). In oxygenated environments, CH_4 is oxidized into CO_2 by methanotrophs. This transformation is particularly noteworthy because CH_4 has 28 times the 100-year global warming potential of CO_2 (Intergovernmental Panel on Climate Change 2014). Competing with methanotrophs for oxygen and with methanogens for DOC are heterotrophs, which do not directly use or produce CH_4, and anaerobic fermenters. This microbial ecosystem is described in detail in Section 1.4.

Plant growth can influence CH_4 emissions in two ways. First, vascular wetland plants often contain aerenchymatous tissues which can act as a diffusive pathway for CH_4 to travel from the anoxic soil to the atmosphere, or for oxygen to diffuse into the soil surrounding roots (the rhizosphere) creating an oxic zone that precludes methanogenesis and facilitates methanotrophy instead (Fritz et al. 2011). As plants grow larger, they produce more roots and the roots are longer, so more of the soil is connected to the aerenchymatous gas transport pathway. Second, plant roots exude carbon into the rhizosphere and thus can fuel methanogenesis by contributing DOC belowground. Root exudation tends to increase with plant productivity (Weigel et al. 2005). In agreement, higher productivity has been shown to increase CH_4 emissions, suggesting that increased root exudation of carbon is a mechanism for increasing CH_4 emissions (Freeman et al. 2004). DOC and CH_4 are the substrates for heterotrophs and methanotrophs, so increasing both concentrations would likely lead to more growth of these two microbial groups. The ecophysiology of wetland plants is examined further in Section 1.3.

1.2 THE WETLAND SOIL ENVIRONMENT

Wetlands are defined by having water table at or near the soil surface, so the processes being considered are occurring in a saturated environment with limited electron acceptors. Due to the low rates of oxygen diffusion in water and the high amount of organic material degrading, anoxic conditions exist within a few millimeters of the watertable (Moshiri 1993). Immediately below the thin transitional area there is a redox gradient with alternate electron acceptors being used in the order of thermodynamic favorability (White and Reddy 2001). However, due to the lack of vertical mixing in wetlands, these layers are thin and are being recycled as they pass electrons towards the surface, rather than representing an allochthonous source (D'Angelo and Reddy 1994; Keller and Bridgham 2007). Because of this, the atmosphere is the main source of terminal electron acceptors and is the primary factor controlling redox state. Two other minor sources of electron acceptors to be considered are advection of inorganic electron acceptors such as nitrate or sulfate (D'Angelo and Reddy 1994) and degradation of organic material into humic acids, which can act as organic electron acceptors (Keller et al. 2009). Inorganic electron acceptors typically play a minor role in natural freshwater bogs such as those being examined here due to rainwater being the primary water source (Keller and Bridgham 2007). While certain humic substances may be favorable electron acceptors other humic substances act as electron donors, and both are produced through redox reactions from existing organic material (Keller et al. 2009). Therefore, humic acids should be considered as intermediates but are not terminal electron acceptors and do not control the overall oxidation state of the peat. This leaves dissolved oxygen (DO) as the controlling electron acceptor in bogs.

DO has added importance in wetland CH_4 dynamics because it has an inhibitory effect on methanogenesis beyond that of redox alone. In culture, *Methanosarcina* have been found able to perform methanogenesis in redox potentials of up to +400 mV (Fetzer and Conrad 1993). In environmental conditions, microbes able to use more favorable electron acceptors would likely outcompete methanogens for substrates at such high potentials, but the redox conditions themselves are not inhibiting. In soils, methanogenesis increases as redox potential decreases below 0 mV (Yu and Patrick 2003). In peat bogs the redox potential is nearly always less than 100 mV, and usually less than 0 mV (de Mars and Wassen 1999). Methanogenesis becomes inhibited by O_2 at concentrations greater than .005% (Fetzer and Conrad 1993). Therefore, in peat bogs methanogenesis is likely inhibited more by competition for substrates and the presence of O_2 than by redox potential. This is in contrast to upland soils, which can have redox potentials of up to +700 mV in a variety of ecosystems (Miller et al. 2001; Dijk et al. 2004; Yu et al. 2006).

The highly reduced state of bogs is due to the demand for electron acceptors in the breakdown of their extremely high organic content. Boreal peat bogs have been reported to have a loss on ignition (LOI), a common measure of organic content, of over 99% (Lusa et al. 2015). Wetlands store more than seven times as much carbon per surface area as temperate upland soils (Adams et al. 1990; Dixon et al. 1994; Malhi et al. 1999; Eswaran et al. 2000; Lal 2005). However, this carbon is not as readily used by microbes as root exudates (Trinder et al. 2008). While many studies have found that the chemical properties of carbon affect its bioavailability, it is not a simple relationship (Lueking et al. 2000). Differences in bioavailability are specific to the microbial population of interest (Mayer 1995) and can be affected by a number of factors including thermodynamic properties of the carbon compounds (LaRowe and

Van Cappellen 2011), physical accessibility of the carbon (Baldock and Skjemstad 2000), and environmental factors such as pH or ORP (van Bergen et al. 1998). This interplay of factors allows the likelihood of a specific carbon compound to be utilized by a microbial population to vary depending on the circumstance. A particularly interesting case of this is the process of microbial priming, in which the addition of highly bioavailable carbon causes microbes to utilize less bioavailable peat that otherwise would not have been processed (Fontaine et al. 2007).

1.3 PLANT ECOPHYSIOLOGY

1.3.1 *Effects of the Rhizosphere*

Vascular plants have two primary effects on the subsurface environment (Figure 1.1). First, they possess aerenchymatous tissue. This air-filled tissue provides a diffusive pathway for oxygen to travel from the atmosphere to the plant's roots, allowing them to respire in an anoxic environment. The roots and rhizomes can leak(Brix 1997) that oxygen to the surrounding soil creating an oxic zone that precludes methanogenesis and allows bacteria to oxidize existing CH_4 (Armstrong et al. 2006). The aerenchyma can also act as a diffusive pathway for CH_4 to rapidly travel from the soil into the atmosphere, preventing CH_4 from spending a long period of time diffusing through the larger region of oxic groundwater near the watertable (Fritz et al. 2011). Second, vascular plants contribute biologically available carbon in the form of root exudates and leaf litter that is more easily degradable than the *Sphagnum* mosses that otherwise dominate (Kayranli et al. 2009). This carbon is fermented into acetate and hydrogen which fuel methanogenesis.

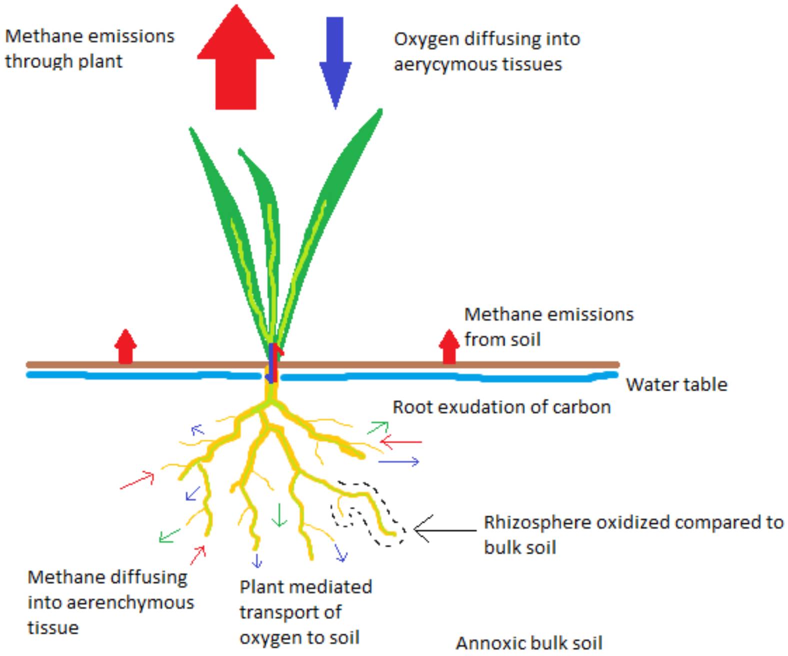

Figure 1.1. Diagram of vascular wetland plant. Red arrows represent CH_4 fluxes, blue arrows represent oxygen fluxes, and green arrows represent root exudation of carbon.

The effects of plants vary considerably depending on a number of variables. Species of plant is important (Fritz et al. 2011) as it affects the depth and density of the root network, root porosity, and stomatal conductance. Season has a major effect, as it affects the plants growth rate and metabolism (Alford et al. 1997). Geographic location matters for three reasons: latitude affects how much sunlight is available, soil composition is dependent on local geology (Kayranli et al. 2009), and it is related to climate. Interannual variability in climate, for example variability stemming from the El Nino cycle, also affects climate. These changes in temperature, moisture, and sunlight availability due to cloud cover can have major effects on how the plants facilitate CH_4 emissions (Hodson et al. 2011). Understanding why these factors have the effects they do requires a mechanistic study of the processes involved.

The second major effect of roots is the addition of bioavailable carbon in the form of rhizodeposits. Rhizodeposition is the flow of carbon from the root to the rhizosphere and can take the form of exudation of DOC, excretion of insoluble organic carbon compounds, death and lysis of root cells, or direct transfer to symbionts (Jones et al. 2009). The exact composition of these various sources has not yet been determined, but the net release of carbon has been found to include a wide variety of molecules including carbohydrates, peptides, lipids, organic acids, and hormones (Jones et al. 2009). During active plant growth in the summer, these rhizodeposits can be major source of DOC (Pinney et al. 2000). Additionally, microbes have been shown to preferentially utilize DOC derived from rhizodeposits over carbon sources derived from degrading peat (Trinder et al. 2008).

1.3.2 *Response to Increased Temperature and Carbon Dioxide*

Plant productivity is a key factor in wetland CH_4 emissions, and one that is likely to be affected in a changing climate. It has long been recognized that plant productivity increases with temperature in the boreal region examined here, although that relationship can be complicated by other limiting factors such as nutrients or for other effects temperature has on plant structure and growth rate (Idso et al. 1987; Kerkhoff et al. 2005; Alexandrov and Yamagata 2007). Increasing atmospheric CO_2 levels is also predicted to have a net positive effect on plant productivity, and also increase root turnover and root exudation (Hungate et al. 1997). The outcome of this is that elevated CO_2 increases CH_4 emissions from wetlands and cycling of bioavailable carbon in the soil (Megonigal and Schlesinger 1997). The increased productivity also increased plant size (Hungate et al. 1997). Larger plants will have more aerenchymatous tissue, and therefore provide a larger diffusive pathway for oxygen into the soil.

1.4 MICROBIAL ECOLOGY

1.4.1 *Overview of Microbial Ecosystem*

The microbes that affect CH_4 emissions fall into four categories: the methanogens responsible for the initial production, which utilize either the acetoclastic or the hydrogenotrophic metabolic pathways; the methanotrophs living in oxic regions consuming CH_4; and heterotrophs that interact with the food-webs of methanotrophs and methanogens in both competitive and syntrophic ways. This food web is summarized in Figure 1.2.

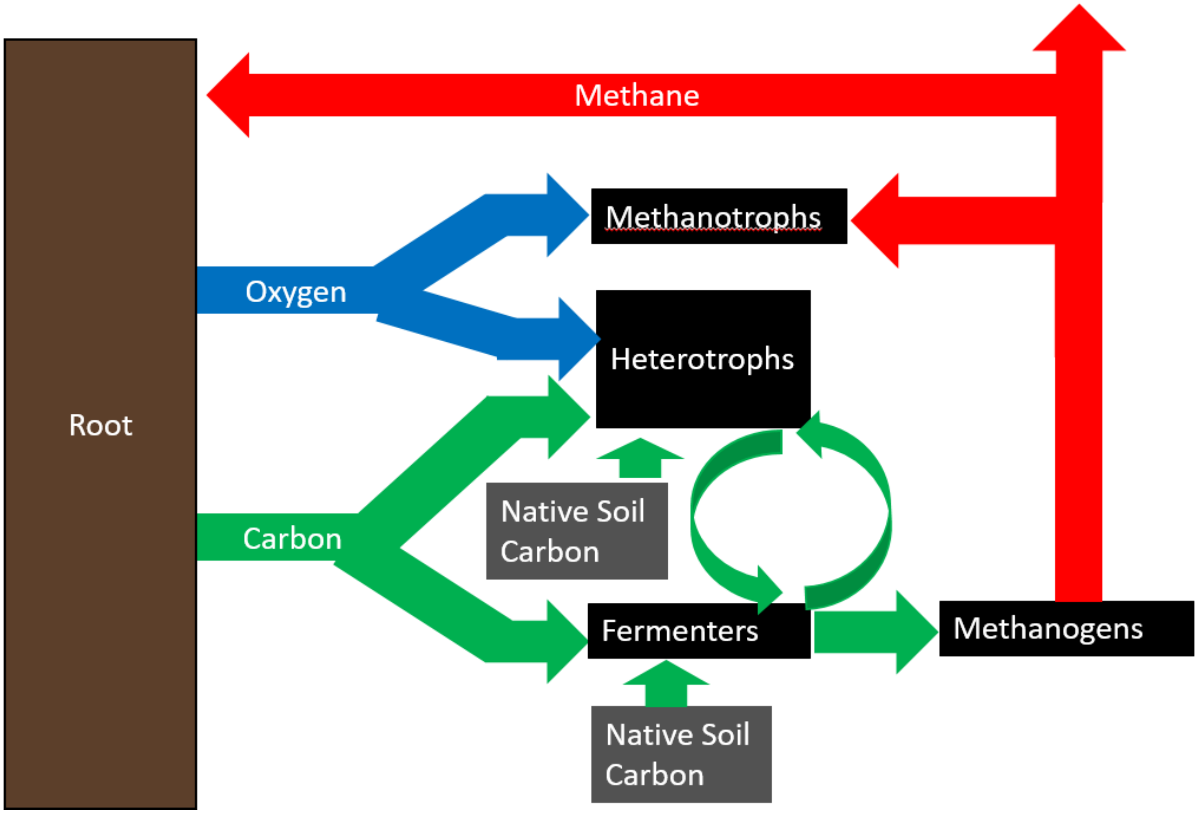

Figure 1.2. Simplified diagram of wetland microbial food web.

1.4.2 *Methanogens – Acetoclasts and Hydrogenotrophs*

Methanogens in wetland systems are obligatory anaerobic Archaea (Garcia et al. 2000). While references to methanogenic bacteria are still found in scientific literature (Pascal et al. 2015; Akob et al. 2015), the organisms they refer to are properly classified as Archaea under widely accepted classifications (Garcia et al. 2000; U et al. 2002).

Because they consume different carbon sources and emit different products, the two primary classes of methanogens must be considered separately in studies of wetland CH_4 dynamics (Kotsyurbenko et al. 2004). Acetoclasts rely on acetic acid to produce CH_4 and carbon dioxide (Equation 1.1), while hydrogenotrophs use hydrogen gas to reduce carbon dioxide (Equation 1.2). There is one genus of Archaea, *Methanosarcina,* capable of both acetoclastic and hydrogenotrophic methanogenesis (De Vrieze et al. 2012). A third methanogenic metabolism is the methylotrophic methanogens: Archaea that convert other one-carbon substances into CH_4 (Garcia et al. 2000). However, acetoclastic and hydrogenotrophic together account for over 95% of methanogenesis in most circumstances (Segers 1998). The type of substrate being degraded is the primary factor determining the relative importance of the two processes (Conrad 1999). CH_4 produced from carbohydrate or cellulose substrate is approximately 2/3 acetoclastic and 1/3 hydrogenotrophic (Conrad 1999; Kotsyurbenko et al. 2004). This is because glucose fermentation typically produces four moles of hydrogen gas and two moles of acetate, which provides substrate for one mole of hydrogenotrophically produced CH_4 and two moles of acetoclastically produced CH_4 (Equation 1.3). There are also metabolic pathways that convert one glucose molecule into three acetate molecules (Equation 4),(Ljungdhal 1986) but the observed acetoclastic to hydrogenotrophic methanogenesis ratio implies that the dominant glucose metabolism pathway produces both acetate and hydrogen gas. While the stoichiometry dictates that hydrogenotrophic methanogenesis only accounts for 1/3 of CH_4 production, it produces five times the energy per mole of CH_4 produced than acetoclastic methanogenesis produces (Jørgensen 2006).

$$(1.1)\ CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$$

$$(1.2)\ CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$

$$(1.3)\ C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ C_2H_4O_2 + 4\ H_2 + 2\ CO_2$$

$$(1.4)\ C_6H_{12}O_6 \rightarrow 3\ C_2H_4O_2$$

1.4.3 *Methanotrophs*

Methanotrophs in wetland systems can be treated as aerobic bacteria (Conrad 2009). Species of both Archaea and Bacteria can carry out anaerobic CH_4 oxidation (Boetius et al. 2000; Ettwig et al. 2008), but are only generally active in sub-aqueous sediments (Boetius et al. 2000) and is not thought to be significant in wetlands (Conrad 2009). This is because anaerobic

CH_4 oxidation requires alternate electron acceptors, which are absent from wetlands for the reasons described above. Most methanotrophs are only capable of using one-carbon compounds (Conrad 2009). However, some facultative methanotrophs have been found in the genera *Methylocella* and *Methylocapsa* which may also use other carbon compounds such as acetate and ethanol (Dedysh et al. 2005; Dunfield et al. 2010). These facultative methanotrophs are widely distributed in the environment, but are especially prevalent in acidic soils, including peatlands (Rahman et al. 2011). An additional complication can come from microbes that partially oxidize CH_4, producing compounds that can be consumed by non-methanotrophic methylotrophs (Hernandez et al. 2015). This type of mutualistic interaction is highly dependent on oxygen concentration (Hernandez et al. 2015), and so is likely to occur sporadically in the rhizosphere as oxygen availability varies.

1.4.4 *Aerobic and Facultative Heterotrophs*

Aerobic heterotrophs, as defined here, are an even more diverse group than methanotrophs, as this term is used to encompass microbes that can oxidize any non-CH_4 organic substrate. Due to this diversity, heterotrophs are usually identified either by taking everything that is neither methanotroph nor methanogen, or by culturing microbes under conditions that facilitate their growth alone (e.g., aerobic culture with acetate as the substrate) (Bodegom et al. 2001). Aerobic heterotrophs typically have higher affinity for oxygen than methanotrophs, but also require higher oxygen concentrations to function (Bodegom et al. 2001). For this reason, they can be expected to dominate closer to the oxygen source while methanotrophs dominate in the zone of intermediate oxidation between the heterotrophs and the anaerobic methanogens.

This relationship is complicated by the existence of facultative heterotrophs: organisms which can use either oxygen or an alternate electron acceptor to drive their metabolism. In other environments and utilizing a variety of substrates it has been found that facultative and anaerobic processes are slower or less effective than the aerobic counterparts (Reddy et al.

1984). This makes theoretical sense, given that oxygen is more thermodynamically favorable than other electron acceptors. The advantage that facultative organisms gain over obligate anaerobes is the ability to withstand changing environmental conditions, such as might occur when the root of an aerenchymatous plant grows into anoxic soil and introduces oxygen. Metabolically it makes little difference whether a reaction is mediated by an obligate or facultative anaerobe, but studies seeking to infer metabolic processes based on genomic data need to account for the uncertainty of facultative microbes.

1.4.5 *Fermenters*

Fermenters, like heterotrophs, are a highly diverse group of bacteria (Juottonen et al. 2005). While they all share the ability to survive without oxygen in acidic environments, within a single bog they may span many phyla and include members which have not been cultivated or fully sequenced (Juottonen et al. 2005). Furthermore, in mires with differing environmental conditions the microbial communities may accomplish the same net metabolism while being composed of completely different microbes (Hunger et al. 2015). Figure 1.3 shows the diversity found across 4 mires examined in a single study, demonstrating this diversity (Hunger et al. 2015). The key features to be examined in studies of methanogenesis are whether the microbial community is producing H_2 and acetate to serve as substrates for methanogenesis, and in what ratio.

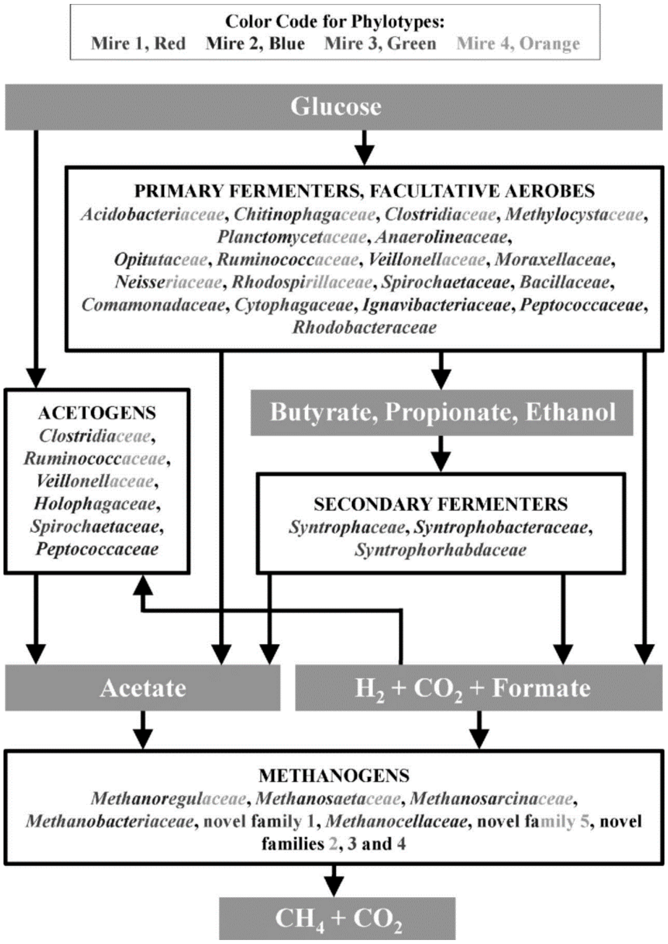

Figure 1.3. Diagram of anaerobic fermenters in four mires from Hunger et al., 2015. Figure is in black and white in original publication, despite “color code”.

1.5 RESEARCH OBJECTIVES

This dissertation advances scientific understanding of boreal wetland CH_4 dynamics by investigating the mechanisms by which plants impact emissions. This is done through three avenues of investigation. First, it will examine how root exudation and aerenchyma interact to impact emissions levels. Second, it will determine what carbon is used in methanogenesis. Finally, it will examine changes in the microbial ecosystem caused by the presence of roots. Chapter two addresses the first and second research topics using optical oxygen measurements, CH_4 flux measurements, and an isotope mixing model which attributes the carbon in CH_4 to either a soil or root source. Chapter three focuses on the second research topic. Building upon the findings of chapter two, it uses high resolution mass spectrometry to describe what types of carbon compounds in the soil are degraded in the presence of roots. Chapter four focuses the microbial ecology investigation on to the methanotrophs, and examines how different methanotrophic metabolisms confer competitive advantage in the environment of the rhizosphere.

Chapter 2. PLANT ROOT EXUDATES INCREASE METHANE EMISSIONS THROUGH DIRECT AND INDIRECT PATHWAYS

2.1 ABSTRACT

The largest natural source of CH_4 to the atmosphere is wetlands, which produce 20% to 50% of total global emissions. Vascular plants play a key role regulating wetland CH_4 emissions through multiple mechanisms. They often contain aerenchymatous tissues which act as a diffusive pathway for CH_4 to travel from the anoxic soil to the atmosphere and for O_2 to diffuse into the soil and enable methanotrophy. Plants also exude carbon from their roots which stimulates microbial activity and fuels methanogenesis. This study investigated these mechanisms in a laboratory experiment utilizing rootboxes containing either *Carex aquatilis* plants, silicone tubes that simulated aerenchymatous gas transfer, or only soil as a control. CH_4 emissions were far greater from planted boxes than from control boxes or simulated plants, indicating that the physical transport pathway of aerenchyma was of little importance when not paired with other effects of plant biology. Plants were exposed to $^{13}CO_2$ at two time-points and subsequent enrichment of root tissue, rhizosphere soil, and emitted CH_4 was used in an isotope mixing model to determine the proportion of plant-derived versus soil-derived carbon supporting methanogenesis. Results showed that carbon exuded by plants was converted to CH_4 but that plants also increased emission of soil-derived carbon relative to the other experimental treatments. This result signifies that plants and root exudates altered the soil chemical environment and microbial community such that microbial utilization of soil carbon was increased (e.g. microbial priming) and/or oxidation of soil-derived CH_4 was decreased (e.g., by microbial competition for oxygen).

2.2 INTRODUCTION

Methane (CH_4) is a potent greenhouse gas responsible for 15-19% of total greenhouse gas radiative forcing, second only to carbon dioxide (CO_2) (Intergovernmental Panel on Climate Change 2014). The largest natural source of CH_4 to the atmosphere is wetlands, which produce between 20% and 50% of total (anthropogenic and natural) emissions (Ciais et al. 2013a). Over half of global wetland area is in the Boreal region (Aselmann and Crutzen 1989) where temperatures are rising at a faster-than-average rate (Intergovernmental Panel on Climate Change 2014), and where wetland CH_4 emissions are increasing (Gedney et al. 2004; Zhang et al. 2017). Wetlands generate CH_4 because the saturated soils are anoxic and the anaerobic microbial metabolic pathways that break down organic carbon terminate in methanogenesis. The amount of CH_4 emitted is controlled by factors such as vegetation type (Fritz et al. 2011), season (Wang and Han 2005), temperature and precipitation (Hodson et al. 2011), and soil types (Kayranli et al. 2009). Understanding how these variables affect wetland CH_4 production and emissions is important because these variables are often sensitive to climate and environmental conditions; as the climate warms and environmental conditions change, the response of these variables could alter wetland CH_4 emissions, creating feedback loops (Gedney et al. 2004; Zhang et al. 2017). One such factor that both influences CH_4 emissions and in turn is influenced by climate warming is the growth of wetland plants (Kayranli et al. 2009). Modelling studies have identified the influence of plants on wetland CH_4 emissions as a prioritized area of needed study, even before climate effects are considered (Riley et al. 2011).

As global CO_2 concentrations and temperatures increase, boreal plants will respond with changed growth patterns and higher productivity (Forkel et al. 2016). Plant growth can influence CH_4 emissions in two ways. First, vascular wetland plants (e.g., sedges, shrubs, grasses) often contain aerenchymatous tissues that facilitate diffusion of gases between the soil and the atmosphere (Armstrong 1971). The hollow aerenchyma run from plant roots up into the leaves, increasing the total surface area of diffusional contact between the atmosphere and soil, and extending that contact area into saturated soil that would otherwise not be exposed to the atmosphere. Aerenchyma allow CH_4 to travel from the anoxic soil to the atmosphere, reducing CH_4 diffusion through the soil system, including through the oxic groundwater layer near the water-table surface and unsaturated soil where oxidation of CH_4 into CO_2 can occur (Shannon and White 1994; Popp et al. 2000; Fritz et al. 2011). The ability of CH_4 to diffuse through aerenchyma and bypass oxic water and unsaturated soil can decrease CH_4 oxidation, but only if the roots themselves are not surrounded by oxic groundwater. The soil surrounding roots (rhizosphere) can become oxygenated because aerenchymatous tissues allow diffusion in both directions, acting as a pathway for oxygen to enter the rhizosphere where it can preclude methanogenesis and facilitate CH_4 oxidation (i.e., methanotrophy) (Fritz et al. 2011). As boreal plants grow larger, which is predicted to occur with increasing CO_2 levels and temperatures (Idso et al. 1987; Jonasson et al. 1996), their root network will grow (Kummerow and Ellis 1984) and connect more of the soil to the aerenchymatous gas transport pathway.

Second, vascular plants contribute carbon to soil in the form of root exudates and leaf litter. It has been hypothesized that root exudates, which are low molecular weight sugars and amino acids, are more readily utilized by microbes than existing soil carbon and thereby fuel microbial activity resulting in methanogenesis (Ström et al. 2003; Ström and Christensen 2007; Picek et al. 2007; Chanton et al. 2008; Kayranli et al. 2009). Methanogens can only use acetate or a combination of H_2 and CO_2 to actually form CH_4, but root exudates may be broken down by other microbes to form the substrates of methanogenesis. Throughout this paper, the eventual conversion of exudate-derived carbon to CH_4 is referred to as being a “direct” effect of exudates, even though the microbial processing takes more than a single step. However, this causation is hard to prove in the complex plant-soil system. Root exudation increases with plant productivity (Weigel et al. 2005) as do CH_4 emissions (Ström et al. 2003), but there are other factors correlated with plant productivity that could influence CH_4 production and emissions, for example increased aerenchyma transport and warmer temperatures (microbial CH_4 production increases with temperature (Yvon-Durocher et al. 2014)). Multiple studies have shown that at least some plant-derived carbon is utilized by microbes and emitted as CH_4(Megonigal et al. 1999; Ström et al. 2003; Trinder et al. 2008; Dorodnikov et al. 2011). However, these studies do not quantify to what extent root exudates increase total emissions, or if they instead replace soil carbon as the carbon source. In one case the portion of emitted

CH_4 that was plant derived was extremely small (Dorodnikov et al. 2011), and in another neither microbial biomass nor metabolic activity was correlated with the rate of root exudation (Trinder et al. 2008). One soil incubation study showed that addition of root-exudate analogs to peat soil increased CH_4 production (Girkin et al. 2018), but this approach cannot replicate the delivery rate and complete carbon composition of root exudates from a living plant, nor can it account for the complex microbial community dynamics that exist in the rhizosphere.

It should be noted that the effects of aerenchyma transport and root exudation do not exist in isolation. For example, if root exudates provide carbon that is more readily used by the microbial community than the native soil carbon, exudates could stimulate increased microbial activity. An increase in aerobic microbial respiration would increase oxygen demand in the rhizosphere, which could decrease methanotrophy depending on total oxygen demand relative to supply from aerenchyma (Segers and Leffelaar 2001) and methanotrophs' affinity for oxygen (Whalen 2005). If the rhizosphere consumes oxygen as quickly as it is delivered, then root exudation could create an anoxic pathway for CH_4 to diffuse to root aerenchyma, where CH_4 would quickly travel to the atmosphere. By decreasing methanotrophy in the rhizosphere, root exudation would allow the aerenchyma to transport more CH_4 than if exudates were not present.

In addition to directly fueling methanogenic pathways, root exudates can also stimulate microbial priming of soil carbon. Priming is when the introduction of carbon in the form of simple sugars and acids can stimulate a microbial population into breaking down soil organic matter. One explanation for priming is the microbial nitrogen mining (N-mining) hypothesis, which assumes that microbes oxidize the carbon-rich but nutrient-poor exudates for energy, but then must process soil organic matter to extract nitrogen (and potentially other nutrients) for biomass production (Craine et al. 2007). From a plant evolutionary perspective, encouraging N-mining with root exudation would be beneficial in nutrient-poor soils (Carvalhais et al. 2011), such as those in peat bogs. Priming has been observed in peat soils using a mass balance approach in soil incubation experiments (Ye et al. 2015) and by analyzing dissolved organic matter characteristics in a boreal peatland field experiment that compared planted plots to plots with plants removed (Robroek et al. 2016). However, other soil incubation experiments have failed to find evidence for priming in laboratory incubations of peat soils (Girkin et al. 2018). While not in peat soils, one field study on a river-bank found evidence that the addition of water-soluble organic carbon decreased processing of solid-phase hydrophobic carbon, which the authors interpreted as contradicting the idea of priming (Graham et al. 2017). However, that study was conducted in a mineral sediment with far lower carbon content than peat soils, so the chemical environment and characteristics of carbon which control bioavailability were different. It is possible that priming occurs only in certain conditions.

Our study was a combined investigation of the multiple mechanisms by which plants enhance CH_4 emissions, utilizing a vascular plant (*Carex aquatilis*) and peat soil typical of the boreal region. *Carex aquatilis* has been studied before and found to increase wetland CH_4 emissions (Schimel 1995). This study used two avenues of investigation. The first approach sought to isolate the effect of aerenchyma transport by comparing soil oxygenation and CH_4 emissions from real *Carex* plants to that from hollow, gas permeable tubes that mimicked the aerenchyma transport capacity of the studied plants. The second approach sought to clarify the extent to which root exudates fuel CH_4 production by tracing the flow of isotopically labeled CO_2 fixed by the *Carex* plants, delivered to the microbial community, and emitted as CH_4. Together, these approaches allowed us to examine the excess CH_4 emitted in the presence of *Carex* and attribute this excess to transport alone, microbial utilization of exudates, and/or indirect effects such as microbial priming and reduced methanotrophy.

2.3 MATERIALS AND METHODS

2.3.1 *Experimental Materials and Conditions*

We grew the wetland sedge *Carex aquatilis* from plugs purchased from a local nursery (Plants of the Wild, Tekoa, WA) for 10 weeks in 5 L (5cm x 20cm x 50cm) rootboxes filled with peat collected from a thermokarst bog in the Bonanza Creek Experimental Forest near Fairbanks, Alaska (Neumann et al. 2015). In addition, there were unplanted control boxes filled only with peat and boxes with silicone tubes inserted into the peat instead of plants (Fig. 2.1).

Silicone-tube boxes are referred to as the “simulated” plant treatment since they were simulating the gas-transfer effects of aerenchymatous plant tissues without adding any root exudate carbon, following the method of (King et al. 1998). The silicone tubes (1.47 mm inner diameter, 0.23 mm wall thickness) had an open top 4 cm above the peat surface and were tied off at the bottom (approximately 20cm below peat surface) to prevent water from entering the tube. This design allowed gases to diffuse through the gas-permeable silicone into and out of the soil. There were four tubes in each box. This number was chosen so that tubes could be spaced widely enough that each tube’s effect could be detected in spatial measurements of oxygen (described under “Oxygen Optode Technology”). Turner et al. (manuscript currently under review) conducted a field experiment using similar simulated plants and optodes in the same thermokarst bog from which peat for this study was collected. Though the aims of that experiment were different, the parallel methodologies allow it to provide useful context for this study’s results.

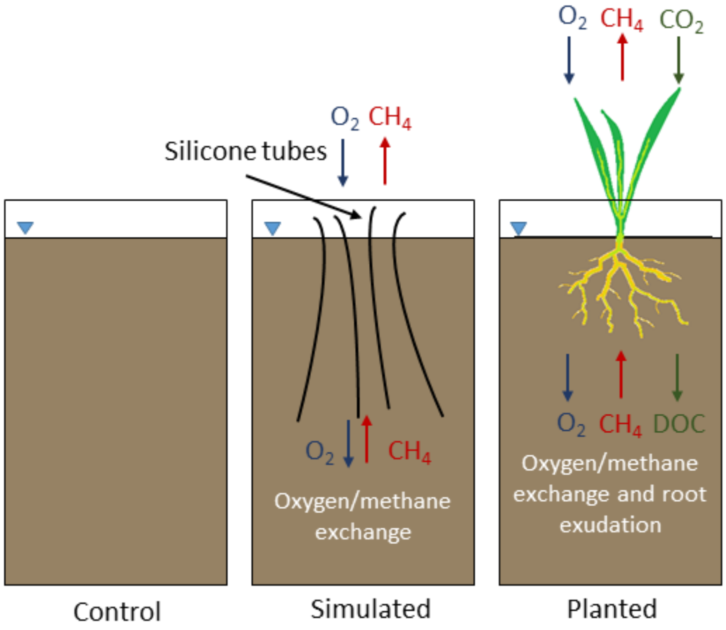

Figure 2.1. Three box types used in experiment. Control boxes had only peat soil. Simulated-plant boxes had soil with silicone tubes that allowed O2 to diffuse down into the soil and CH4 to diffuse up into the atmosphere. Planted boxes had similar ability to transport gas as simulated-plant boxes, but plants additionally performed photosynthesis and released dissolved organic carbon from their roots.

We kept all rootboxes at a 30-degree angle during the experiment to encourage roots to grow along the face of the rootbox, which allowed for sample collection across the rhizosphere and visualization of oxygen concentrations. Oxygen concentrations were measured using an optical oxygen sensor (i.e., an optode) (Larsen et al. 2011) that was applied to the face of a subset of boxes (described under “Oxygen Optode Technology”). Boxes without optodes had opaque front panels instead. Boxes with optodes has opaque covers that were kept on to prevent light from reaching the soil.

We conducted two different experiments. The first experiment consisted of 16 planted boxes that were all outfitted with optodes and focused solely on assessing dissolved oxygen concentrations in the rhizosphere. The second experiment consisted of 11 planted boxes, 3 control boxes, and 3 simulated-plant boxes, and it involved a wider range of measurements aimed at identifying the mechanisms by which plants influence CH_4 emissions. All the boxes with simulated plants were outfitted with optodes in the second experiment.

In both experiments, boxes were watered as needed to keep the peat surface continuously submerged under approximately 1 cm of water. The irrigation solution was de-ionized water with trace amounts of nutrients added to simulate rainwater. The irrigation solution recipe is given in SI table S1.

We kept rhizoboxes in a pair of growth chambers and randomly re-assigned box position within and between the growth chambers twice per week to avoid any effects of spatial variability in growth conditions. Environmental conditions were controlled within the chambers with growth lights and air conditioners set to imitate central Alaska summer growth conditions with 18 hours of daylight, daytime temperatures of 18 °C, and nighttime temperatures of 10 °C. Temperature, relative humidity, and photosynthetically active radiation (PAR) (SI Figures S1.1 and S1.2) were recorded with sensors placed at the height of the tallest plants. During the first experiment, the median temperatures recorded with these sensors were 18.6 °C in one chamber and 20.6 °C in the other. In the second experiment the median temperatures recorded were 20.0 °C in one chamber and 20.5°C in the other. There was a vertical temperature gradient in the growth chambers because the air conditioners were located underneath the rhizoboxes while the sensors were directly under the growth lights.

2.3.2 *Oxygen Optode Technology*

The planar optical oxygen sensors (i.e., optodes) used in this experiment were modified from Larsen et al. (2011). The sensors were made from two fluorescent dyes, an indicator dye and an antenna dye. When the antenna dye was excited by a photon it used part of the energy to fluoresce green and also passed energy to the indicator dye. The indicator dye was reversibly quenched in the presence of oxygen, but when not quenched fluoresced red when excited. The ratio of red to green fluorescence is the foundation of the technology.

We made the optodes by airbrushing a mixture of Pt(II) meso-Tetra(pentafluorophenyl)porphine (the indicator dye) and 10-(2-Benzothiazolyl)-2,3,6,7-tetrahydro-1,1,7,7-tetramethyl-1H,5H,11H-(1)benzopyropyrano(6,7-8-I,j)quinolizin-11-one (the antenna dye) suspended in acetone onto 3/6 inch thick polycarbonate sheets. Once the dye mixture had dried, a graphite coating was applied by pouring a mixture of graphite and silicone dissolved in hexane onto the optode and tilting the optode so that the surface was evenly coated. The graphite coating provides enough opacity to prevent soil and roots behind the optode from interfering with the optical measurements.

We calibrated the optodes by attaching them to boxes filled with water that was bubbled with nitrogen gas and air to create a range of dissolved oxygen (DO) concentrations (as determined by a calibrated oxygen probe (InsiteIG Model 3100)), and photographing the optode as during experimentation. Both calibration and experimental photographs were taken by placing the box in a dark chamber and illuminating it with a blue (447.5 nm wavelength) LED positioned at a 45 degree angle to the optode, and taking a picture with a digital SRL camera (Canon EOS RebelXS) which had its near-IR filter removed and was fitted with a yellow filter (Edmunds Optics, OG-530 Long Pass Filter). The camera was controlled by the Look@RGB software. The red to green ratio (R) in each pixel was used to calculate the oxygen concentration (C) according to Equation 1, using the calibration parameters K_{sv}, R0, and α, as described in Larsen et al. (2011).

$$(1)\ C = \frac{R0-R}{K_{sv} *(R-R0*\alpha)}$$

2.3.3 *Non-Destructive Measurements — Second Experiment*

In addition to optode measurements, in the second experiment plant height and fluxes of CH_4 were measured. CH_4 fluxes were measured using one of two greenhouse gas analyzers (Los Gatos Research Ultraportable Greenhouse Gas Analyzer (LGR) and Picarro G2201-I CRDS) attached to an opaque hood that was strapped over the box of interest. Headspace concentration was recorded by the instrument once per second for 5 to 10 minutes. The LGR recorded only total concentration of CH_4 while the Picarro recorded concentration of CH_4 as well as $\delta^{13}C$. A linear regression was applied to the change in concentration over time to determine the flux rate.

The $\delta^{13}C$ measurements for emitted CH_4 had a non-trivial amount of noise, especially at low concentrations, making it unreliable to use a single starting $\delta^{13}C$ value for CH_4 in the chamber headspace from which to calculate the $\delta^{13}C$ of emitted CH_4. Instead, the isotopic ratio of emitted gas was calculated by taking every possible combination of measurements during the flux and using the difference between the concentrations and $\delta^{13}C$ of CH_4 in the headspace at any two points in time to calculate the $\delta^{13}C$ of emitted CH_4. That method produced tens of thousands of results per flux, from which the median was used.

Flux data was used if the root mean square error (RMSE) of a linear regression was less than 0.5 parts per million CH_4 concentration per hour, which was equal to a flux rate of 3.9 mg m^{-2} d^{-1} from the boxes which had a surface area of 0.01 m^2. Isotope data was used if the RMSE of the flux rate was less than 0.5 ppm, and the R^2 value of the flux rate was greater than 0.9. The additional quality control check was needed for isotope data because at very low mass flux rates the RMSE can be low in absolute terms, but still comprise a significant portion of the total variation. Low flux rates (even those with low R^2 values) needed to be included so as to not bias the data towards large fluxes, but the isotope calculations produced unreliable values when the R^2 was low, regardless of the flux rate; they spanned an extreme range including unrealistically high and low values. All $\delta^{13}C$ results were referenced to Vienna Pee Dee Belemnite and reported in delta notation:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} - 1\right) * 1000$$

Where R_{sample} is the ^{13}C to ^{12}C ratio of the measured sample and $R_{standard}$ is the ^{13}C to ^{12}C ratio of Vienne Pee Dee Belemnite (0.0112372).

2.3.4 *Stable Isotope Labeling and Destructive Sampling — Second Experiment*

In weeks 5 and 10 of the second experiment, 4 randomly selected plants were exposed to $^{13}CO_2$ by placing a clear 6.5 L hood made of extruded acrylic on each rhizobox and injecting 15 mL of 99 atom % $^{13}CO_2$ (Sigma Aldrich) into the headspace every hour for 14 hours each day over a period of five consecutive days. This technique was based on previously described methods (Lu and Conrad 2005). The study which developed this technique demonstrated that the labeling of CH_4 was due to use of carbon that had been photosynthesized (Lu and Conrad 2005). Flux measurements were taken at least once per day per plant during and after both labeling events with a Picarro G2201-I. During each day's 14-hour labelling period fluxes were taken with the clear fluxing hood still in place, immediately before the time for an injection of $^{13}CO_2$. After the entire labelling event was completed, the same fluxing protocol was used as for the routine flux measurements.

Sampled boxes were destructively sampled 3-5 days after isotopic labeling ended. To harvest a box, a spill-guard was attached to the top of the box to prevent water from pouring out, placed the box in an anaerobic glove bag purged three times with nitrogen gas, laid the box on its back and removed the front panel to expose the root-soil system. All samples were taken directly from this exposed surface. For all three box types (planted, control and simulated) root and soil samples were taken at depths of approximately 5 cm, 20 cm, and 35 cm. At each depth, three samples were taken, one in the center and one 6 cm from either edge of the box.

For planted boxes, root sections were cut approximately 8cm in length from each location and placed them in centrifuge tubes filled with phosphate buffered solution (PBS), which were capped and removed from the glove bag. The sealed centrifuge tubes were sonicated with the root samples for 10 minutes then quickly moved the root to a new PBS-filled container. Both containers were then immediately frozen at -20 C. All the soil that fell off the root during sonication was considered rhizosphere soil.

For unplanted control boxes, approximately 1mL cubes of soil were sampled from the 9 standard locations. Samples were immediately placed into 2 mL collection tubes with 1 mL of PBS (to keep the sample storage as similar as possible to the planted box procedure) and placed the tubes on dry ice in the anoxic glovebag. These soil samples were considered ‘bulk soil.’

For simulated-plant boxes, at the 5 and 20cm depths where silicone tubes were present, a thin layer of peat was sampled immediately adjacent to the silicone tubes. The tubes did not extend to the 35 cm depth, so 1mL cubes of bulk soil were sampled at that depth. Samples were immediately placed into phosphate buffered solution (PBS) and stored them on dry ice in the anoxic glove bag.

All samples from all the boxes were then stored at -20 C for up to 33 days before being shipped on dry ice to the Environmental Molecular Sciences Laboratory (EMSL) where they were stored at -80 C until analysis.

2.3.5 *Porewater Collection and Analysis — Second Experiment*

We collected porewater from all boxes during the second week of the second experiment, which were considered an initial time point, and immediately prior to the second labelling event in week 8 from boxes that were not harvested after the first labeling event. Ten to sixteen mL of porewater were collected from a depth of 20cm below peat surface at the center of each box using a syringe attached to PushPoint porewater sampler (MHE Products) with a mesh filter (10μm pore size) over the inlet. Water was filtered (0.2 μm, nylon membrane syringe tip filter) and injected into previously prepared 60 mL serum vials capped with a butyl rubber stopper. Vials were prepared before sampling by evacuating and flushing them with 99.999% nitrogen gas three times, leaving the vials at atmospheric pressure. Vials were pre-acidified with 200 µL of phosphoric acid to minimize microbial activity. After being injected with porewater, they were left for at least one week to equilibrate before 15mL of headspace gas was transferred into a 12mL exetainer vial (Labco) using a syringe and needle.

A Shimadzu GC-FID 2014 was used to measure concentration of CH_4 in the exetainer vial and calculated the porewater concentrations using Henry's Law and the mass of water in the original vial. The GC measurements were calibrated using standards of various concentrations (MESA Specialty Gases & Equipment). The standards purchased from MESA were a 100 ppm CH_4 standard, a 10.2 ppm CH_4 standard, and a 50% CH_4 standard which were diluted with N_2 to form an array of concentrations.

2.3.6 *Isotope Ratio Mass Spectroscopy — Second Experiment*

At EMSL, a Costech Analytical Technologies, Inc. elemental analyzer (EA) coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer (IRMS) was used to perform isotopic analysis on root, rhizosphere and bulk soil samples. All soil samples (both bulk and rhizosphere) were removed from -80 C storage and immediately lyophilized using a VirTis Benchtop K lyophilizer. Twenty-one of twenty-seven root samples were lyophilized in the same manner, but the other 6 root samples were first analyzed at room temperature by laser ablation mass spectroscopy for data to be published in a different manuscript before being lyophilized. The laser ablation removed some mass from the surface of the root, but it was assumed that it was a low enough percentage of the root that the average isotopic composition of the root was unaffected.

After lyophilization, samples were stored at room temperature. Lyophilized rhizosphere samples were sub-sampled into tin capsules (4 by 6 mm, part number 41070, Costech Analytical Technologies, Inc.) using 200-300 µg of sample per replicate with a minimum of triplicate analytical replicates. Lyophilized root samples were cut into 1 to 3 mm thick cross sections using a razor and loaded the cross sections into tin capsules as described above. The EA combustion reactor was loaded with cobaltic oxide and chromium oxide catalyst and maintained at 1,020 °C while the reduction reactor was loaded with copper catalyst and maintained at 650 °C. Two in-house glutamic acid standards were used that were themselves calibrated against USGS 40 and USGS 41 standards ($\delta^{13}C$ = -26.39 ‰ VPBD and 37.63 ‰ VPDB respectively) and applied a two-point, slope intercept correction to the data (Coplen et al. 2006). In addition, an in-house acetanilide standard was used as a check on the isotope measurement accuracy.

2.3.7 *Isotope Mixing Model — Second Experiment*

We used the IRMS and CH_4 flux data to construct a mixing model to determine what portion of the CH_4 from each individual flux measurement was derived from soil carbon and what portion was derived from root exudates (Fig. 2.2). The model consisted of three modules: the exudate age module, the fractionation module, and the mixing module. The exudate age and fractionation modules (described in detail below) generated parameter distributions which were fed into the mixing module for the final calculations that indicated what percentage of each CH_4 flux was derived from soil versus exudate carbon.

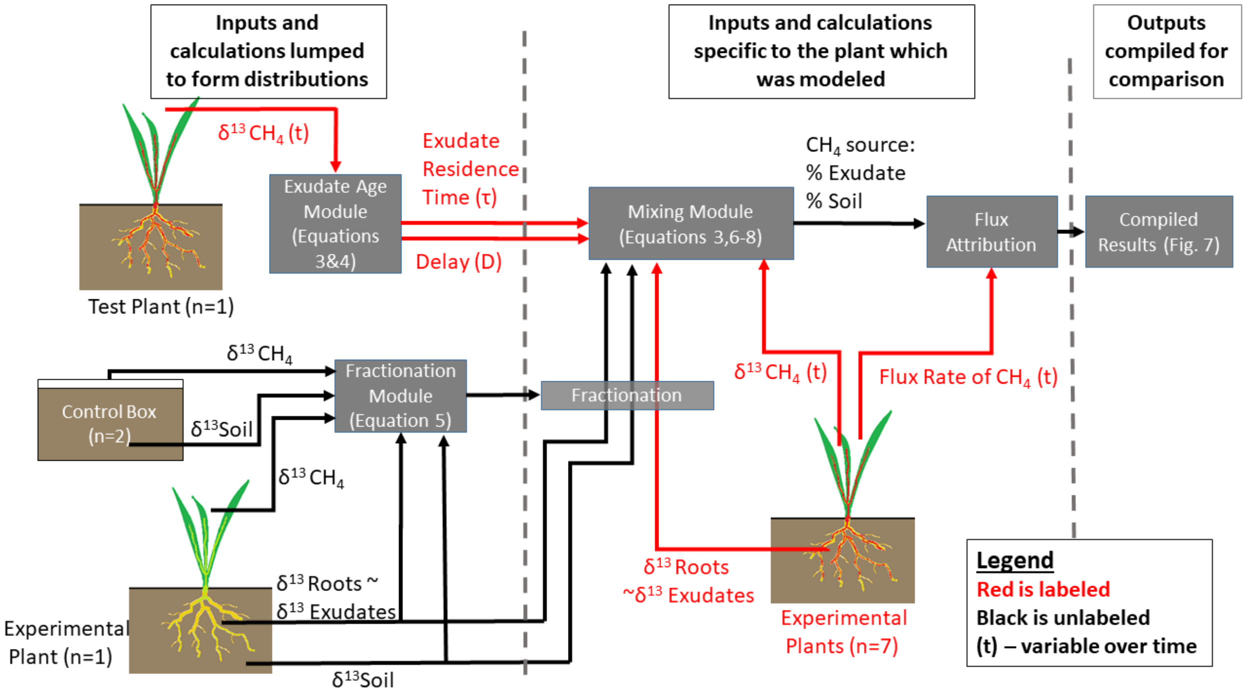

Figure 2.2. The isotope mixing model. In the first section, inputs from multiple boxes were combined to calculate distributions of parameters for the model. Data from a labeled test plant was used to calculate exudate residence times in the exudate age module, while only unlabeled plants and control boxes were used to calculate isotopic fractionation effects. In the second section, each flux was individually modeled using a distribution of parameters generated in the first step using a Monte-Carlo approach. The mixing module used fractionated soil and root data to determine the percentage of each flux that came from soil, labeled exudates, or unlabeled exudates, then in the flux attribution stage those percentages were multiplied by the total amount of CH4 emitted. In the final section of the model all of the fluxes were compiled to establish a range of results, as presented in Fig. 2.7. Any variable given with (t) after it varied over time, while other variables do not.

The exudate age module calculated the portion of total exudates in the rhizosphere that would contain elevated δ^{13}C at specific time points after labeling, given the residence time of exudates in the soil (τ) and the delay (D) between the end of labeling and maximum ^{13}C enrichment of emitted CH_4. The module was run in two separate contexts: once on an isotopically labeled test plant independent of the full model to determine parameter values (as described below), and again on experimental plants using the fitted parameter values. The module modeled exudates in the soil as a continuously stirred tank reactor (CSTR), and assumed the isotopic composition of emitted CH_4 at any time point was linearly correlated with the isotopic composition of exudates in the soil. The isotopic composition of the soil carbon was a constant. This approach required that the ^{13}C content of the exudates did not affect the percentage of CH_4 derived from those exudates. While various factors can affect the degree to which microbes discriminate against ^{13}C *within* a carbon source (Lehmeier et al. 2016), no study was found which showed isotopic enrichment affecting the type of carbon compounds utilized by microbes. Therefore, it was assumed that increasing enrichment of exudates after labeling did not have a measurable impact on the portion of CH_4 emissions that were soil-derived versus exudate-derived.

The general equation for a CSTR is given in Equation 2, and calculates the concentration ($C(t)$) of a species at a given time (t) based on residence time (τ), the initial concentration of the species (C_0) and its concentration after infinite time (C_∞), which is equal to input concentration. In the case of this model, isotopically labeled exudates were defined as a "concentration" of one and unlabeled exudates as a "concentration" of zero, assuming that exudates maintained a consistent enrichment during the period over which they were labeled. Therefore, one can write Equations 3 and 4 to represent the system in which the "concentration" at a given time ($C(t)$ in Equation 2) was the percentage of exudates in the rhizosphere that were labelled ($E_L(t)$). The percentage labeled ($E_L(t)$) was defined in Equation 7 as the portion of total emissions derived from labeled exudates ($P_{LE}(t)$) divided by the portion of total emissions derived from both labeled exudates ($P_{LE}(t)$) and unlabeled exudates (P_{UE}). Because the labelling was a pulse input that ended after five days, the isotopic enrichment of emitted CH_4 reached a maximum and then began to decline at some point after the 5-day long labeling period. Equations 3 and 4 are both modified versions of Equation 2. Equation 3 calculates an input of labeled exudates to a previously unlabeled system and was used prior to the peak, while Equation 4 calculates the loss of labeled exudates from a previously labeled system and was used after the peak. Because the experimental plants were harvested before the peak, only Equation 3 was used on experimental plants. Equation 4 was only used on the test plant to determine parameter values. The delay (D) was the length of time, in days, between when labeling ended (i.e., after the 5-day long labeling period) and when emitted CH_4 reached maximum enrichment. In all cases, time (t) zero was defined as the beginning of labeling. The portion of exudates labeled at the peak (C_{peak}) was calculated using Equation 3 at t equal to length of labeling (five days) plus the delay. C_{peak} was used as the starting percentage of exudates that are labeled for time points beyond the peak (Equation 4).

Table 2.1. Equations used in the isotope mixing model. Variables used: C_0 is concentration at time zero, and no units are assigned since it is not actually used in the model (Equation 2 is a generic form). C(t) is concentration at time t, where t is in days. C_∞is concentration at time infinity. Tau (τ) is residence time of exudates in days. PLE is the percent of carbon used for methanogenesis that is derived from labeled exudates. PUE is the percent of carbon derived from unlabeled exudates. PS is the percent of carbon from soil. F is the fractionation between source carbon and emitted CH4, in units δ13C ‰. MU is the 13C content of CH4 emitted from unlabeled plants, in units δ13C ‰. ML is the enrichment of CH4 emitted from labeled plants, in units δ13C ‰.

Equation #	Modules utilizing	Description	Equation
2	n/a	Generic CSTR	$\mathrm{C(t)} = \mathrm{C}_\infty + (\mathrm{C}_0 - \mathrm{C}_\infty)\mathrm{e}^{-\frac{t}{\tau}}$
3	Exudate age Valid from t=D to t = 5 +D	CSTR pre-peak (modified Eqn. 2) Initial condition: unlabeled exudates = 0 Input: labeled exudates = 1	$\mathrm{E_L(t)} = 1 + (0 - 1)\mathrm{e}^{-(\mathrm{t-D})/\tau}$
4	Exudate age Valid from t = 5+D to t = ∞	CSTR post-peak (modified Eqn. 2) Initial condition: Proportion of labeled exudates calculated in Eqn. 3 at peak (t = 5+D) = $\mathrm{C_{peak}}$ Input: unlabeled exudates = 0	$\mathrm{E_L(t)} = 0 + (\mathrm{C_{peak}} - 0)\mathrm{e}^{-(\mathrm{t-D-5})/\tau}$
5	Fractionation	Definition of fractionation	$F = M_U - S_{UR}$
6	Mixing	$\delta^{13}CH_4$ is linear combination of source δ^{13}	$P_{LE}(t) * [F + S_{LR}] + P_{UE}(t) * [F + S_{UR}] + P_S * [F + S_S] = M_L(t)$

7	Mixing	Ratio of old to new exudates	$\mathrm{E_L(t)} = \frac{\mathrm{P_{LE}(t)}}{\mathrm{P_{LE}}(t) + \mathrm{P_{UE}}(t)}$
8	Mixing	All carbon sources add up to 100%	$P_{LE}(t) + P_{UE}(t) + P_s = 1$

The exudate age module was used in conjunction with data from a test plant to determine what residence times and delay periods should be used as inputs to the model. A variety of residence times and delays were used to generate theoretical enrichment curves, which were graphed against the test plant data (SI Figure S2.3). A likelihood was assigned to each of these curves proportional to the inverse of the sum of squares of the residuals, and those likelihoods were used to generate distributions from which were sampled in the model. The median residence time and delay used are reported in Table 3 along with upper and lower quartiles.

Separate fractionation factors were calculated for planted and control boxes. The fractionation module calculated the apparent isotope fractionation associated with microbial conversion of either soil-derived or root-derived carbon into CH_4 and with subsequent emission of CH_4. The module subtracted the $\delta^{13}C$ of CH_4 emitted from unlabeled plants (M_U, fluxes from all plants prior to labeling were used) from that of unlabeled roots (S_{UR}), and the $\delta^{13}C$ of CH_4 emitted from control boxes and simulated plants from that of unlabeled soil (S_{US}) to calculate the isotopic fractionation of carbon (F). This calculation was done using all emissions from each type of box (planted or control) compared against all source carbon measurements from that type. This was done because only one flux measurement from the unlabeled plant which was harvested passed quality control, so fluxes from other plants prior to their harvest were included. The narrow range of $\delta^{13}C$ for unlabeled fluxes justified this grouping (Fig. 2.5A). A distribution was generated from all fractionation factors calculated, and values used in the model were randomly sampled from that distribution.

The mixing module used output from the fractionation and exudate age modules to solve a system of equations that determined the proportion of emitted CH_4 derived from soil

(P_S), isotopically labeled root exudates ($P_{LE}(t)$), and unlabeled root exudates present in the soil prior to labeling ($P_{UE}(t)$) as a function of time. In Equation 6, the isotopic enrichment of the emitted CH_4 ($M_L(t)$, data from each flux individually) had to be a linear combination of the $\delta^{13}C$ of the three fractionated sources: labeled roots (S_{LR}, data taken from only the box from which the M_L measurement was taken), unlabeled roots (S_{UR}, median of a distribution) and unlabeled soil (S_S, median of a distribution). Medians for S_{UR} and S_S were used instead of a Monte-Carlo sampling approach because the distributions generated were very small and during model development it was determined that simply using the median would reduce the amount of computation needed without influencing the results. Equation 7 established the ratio of the labeled and unlabeled exudates, according to the output of the exudate age module for that time point. Equation 8 made the sum of all carbon sources equal to the full amount of the CH_4 emitted.

The model used 30 randomly selected values from each of the four parameter distributions that were generated (enrichment of exudates (S_{LR}), fractionation factor (F), residence time (τ), and delay (D) between start of labeling and when the label was emitted as $^{13}CH_4$). This resulted in 810,000 results for each of the 21 fluxes used.

2.3.8 *Statistical analysis*

We made all statistical comparisons using either the Wilcoxon rank sum test or the Kruskall-Wallace test, depending on whether it was comparing two groups or more than two groups of data. Both tests are non-parametric methods to determine whether two sets of measurements are likely to be from the same distribution or not.

2.4 RESULTS

All results below are from the second experiment, except for optode images from planted boxes. Oxygen data for planted boxes were collected in the first experiment. Growth chamber conditions, plant height and CH_4 emission data from the first experiment are available in the Online Resources (Figure S2.1 and Tables S2.3 and S2.5).

2.4.1 *Plant Height and Methane Emissions*

Plants grew steadily from a median height of 9.1 cm (n=11) to 32.9 cm (n=6) through the entire period measured (between weeks 1 and 10 of the experiment). Plant height and emissions data for each rootbox are reported in Online Resources tables S4 and S6, respectively.

Planted boxes emitted little CH_4 at first; the median emission flux was less than 25 mg m^{-2} d^{-1} for each of the first four weeks of measurement (Fig. 2.3) ($n \geq 5$ per week). In week five, emissions from planted boxes increased slightly, with a median CH_4 flux rate of 98 mg m^{-2} d^{-1} and one box emitting up to 516 mg m^{-2} d^{-1}. For the remainder of the experiment, planted boxes had high CH_4 emissions with weekly medians ranging from 370 to 580 mg m^{-2} d^{-1} ($n \geq 4$ per week). A Kruskal-Wallace test showed that emissions from planted boxes during the second half of the experiment (weeks six to ten) were significantly greater than those from the first half ($p < 0.05$).

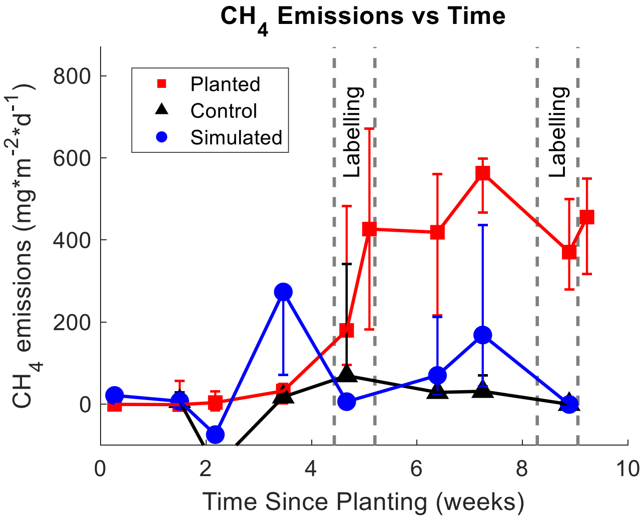

Figure 2.3. Medians for weekly CH_4 emissions from the three experimental treatments with error bars indicating upper and lower quartiles. Vertical dashed lines show days when isotopic labelling began and ended. Isotopic labeling lasted 5 days. Methane emissions from planted boxes increased over the course of the experiment, while those from control boxes and boxes with simulated plants did not increase. The

number of fluxes per week differed because of variability in the number of fluxes that passed quality control and an increase in the number of flux measurements that were made during labeling. For planted boxes, weekly n=2-15, median n=7.5. For control boxes, weekly n=1-3, median n=2.5. For simulated boxes, weekly n=1-3, median n=1.5. Negative fluxes for control and simulated plant treatments after week 2 are each from a single box.

Control boxes emitted little CH_4 throughout the experiment (n=19 measurements), with weekly median emissions never exceeding 70 mg m^{-2} d^{-1}. Boxes with simulated plants typically emitted little CH_4 (n =16), though on three of the nine weeks of measurements they emitted larger than usual amounts, most notably in week 4 when the median flux was 273 mg m^{-2} d^{-1}. The differences between emissions of control boxes and simulated boxes were not significantly different from each other or from the emissions of planted boxes during the first half of the experiment ($p > 0.05$), but emissions from both control boxes and simulated boxes were significantly lower than emissions from planted boxes during the second half of the experiment ($p < 0.05$). These data indicate that emissions were related to plant growth, but not directly correlated; plants switched between a lower-emission state and a higher-emission state quickly (i.e., within two weeks), while their height increased in a nearly linear fashion.

2.4.2 *Optode Imaging*

The planted boxes equipped with optodes during the first experiment never had measurable concentrations of oxygen in the soil. Representative oxygen-concentration images are shown in the two left panels of Fig. 2.4, and all 113 images (16 plants imaged weekly over 9 weeks, with some harvested at intermediate points) are in the Online Resources (Figures S2.4-S2.19). In contrast, the simulated plants in the second experiment had significant amounts of oxygen surrounding the inserted silicone tubes throughout the entire experiment (Fig. 2.4). The 27 simulated plant optode images (three boxes imaged weekly over nine weeks) are also in the Online Resources (Figures S2.20-S2.22).

It is worth acknowleding that neither the gas-transport capacity of the plants or the silicone tubes was directly measured, and so one cannot claim that the simulated plants transported more or less oxygen than the real plants. Rather, what one can say is that the real plants did not allow what oxygen they did transport to build up in the soil.

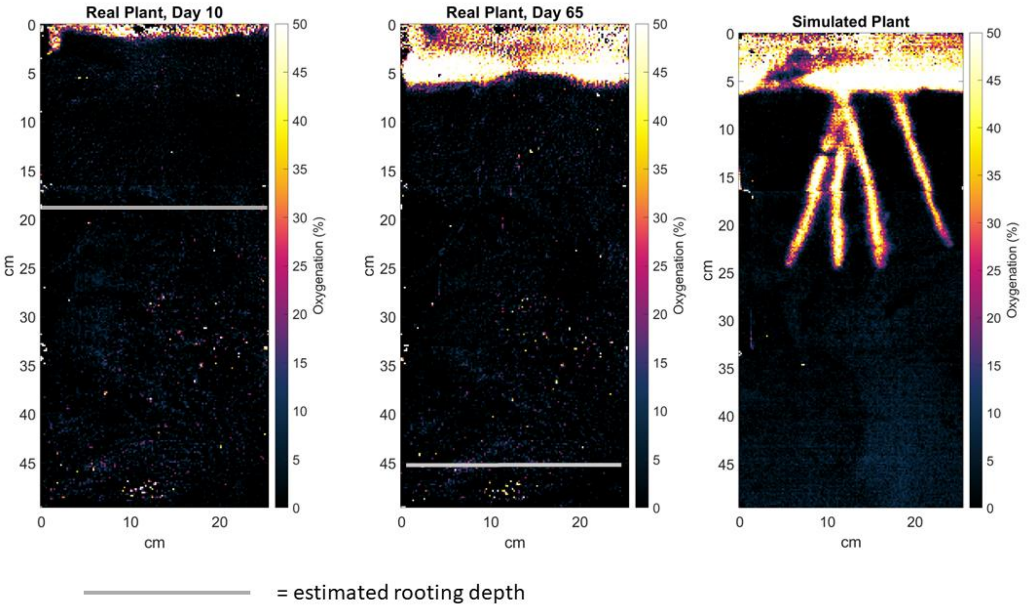

Figure 2.4. Examples of optode data showing that real plants never oxygenated the rhizosphere, while simulated plants had measurable oxygen concentrations surrounding the tubes throughout the experiment.

2.4.3 *Methane, Root, and Rhizosphere Isotope Enrichment*

During both labeling events (weeks 5 and 10 of the experiment), CH_4 emitted from the boxes steadily became more isotopically enriched over a five-day period between the end of labeling and harvest (Fig. 2.5). During the week 5 labeling event fewer flux measurements were made than during the week ten event, and the CH_4 emissions were lower which meant that fewer flux measurements passed the isotope quality control protocol. During the week 10 labeling event, the final CH_4 from each box before harvest had $\delta^{13}C$ greater than +30‰, having increased from a pre-label median of -58‰. This strong enrichment signal shows that plant-derived carbon was converted to CH_4 within days of being photosynthesized.

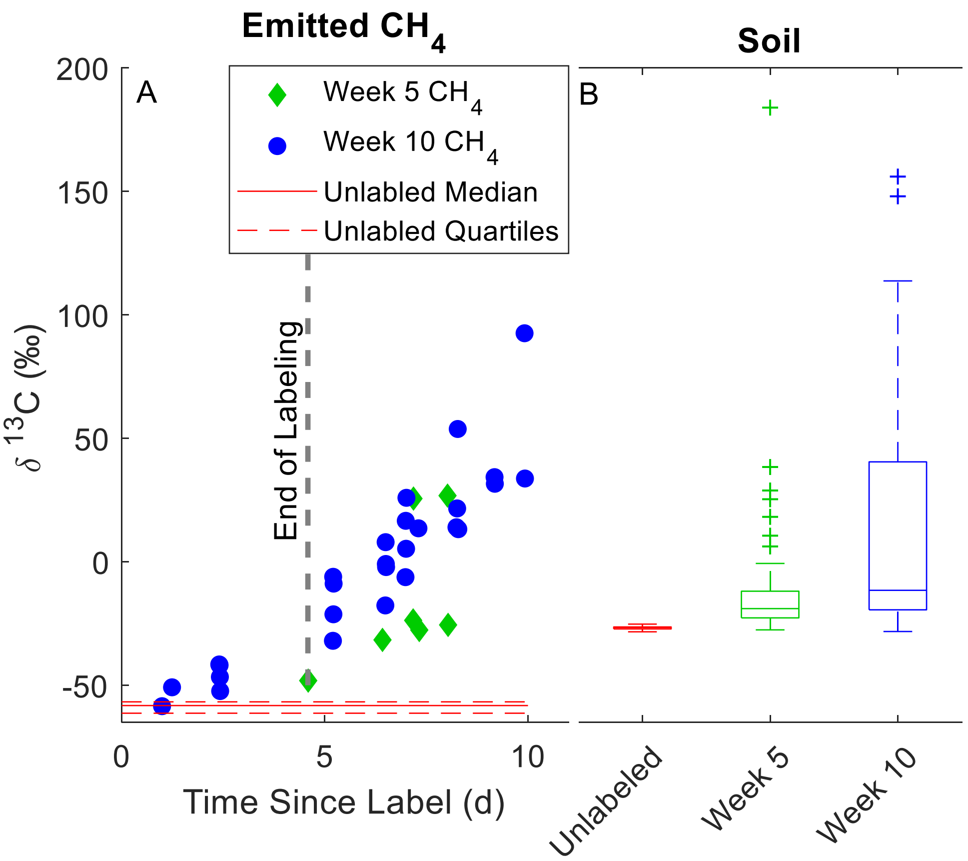

Figure 2.5. Both panels share the y-axis. (A) Isotopic enrichment of CH4 emissions over time since labeling for four planted boxes during each labeling event. Red lines are median and interquartile range of unlabeled emissions. During both labeling events emitted CH4 became substantially enriched. (B) Box and whisker plot of soil carbon isotopic enrichment. The unlabeled soil includes both rhizosphere and bulk soil (n=68) while the soil data from the Week 5 (n=43) and Week 10 (n=69) plant harvests is all rhizosphere soil. The rhizosphere became substantially more enriched after the week 10 harvest.

Roots from both labeling events were highly enriched (Table 2), and there was not a statistically significant difference in root enrichment between the two labeling events ($p > 0.05$), but roots from labeled plants were significantly more enriched than those from unlabeled plants ($p < 0.05$). Unlabeled peat from the control boxes and simulated-plant boxes, and rhizosphere soil from unlabeled plants all had statistically similar isotopic signatures ($p > 0.05$), and thus were treated collectively as 'unlabeled peat.' The unlabeled peat had a significantly lower ^{13}C content ($p < 0.01$) than rhizosphere soil collected after the first and second labeling events, and rhizosphere soil collected after the first labeling events had a significantly lower ^{13}C content ($p < 0.01$) than rhizosphere soil collected after the second labeling event, as shown in Fig. 2.5. The first labeling event enriched carbon in the rhizosphere from -26.6‰ (the median $\delta^{13}C$ of unlabeled peat, mean -26.8‰) to a median of -18.9‰ (mean -8.6‰), while the second labelling event enriched the rhizosphere even more effectively, bringing the median enrichment up to -11.6‰ (mean +30.8‰) (Fig. 2.5B). The means had higher values than the medians for both labeling events because the data were clustered low, with tails skewed high.

Table 2.2. Isotopic composition of roots from unlabeled plants and plants labeled during both events. Roots from both labeling events are significantly different ($p < 0.05$) than those from unlabeled plants, but data from the two labeling events are not different from each other ($p>0.05$).

Isotopic Composition of Roots	Unlabeled	Week 5 Labeling	Week 10 Labeling
Lower Quartile (‰)	-34.9	341.9	104.5
Median (‰)	-33.1	630.4	431.6
Upper Quartile(‰)	-32.4	1178.5	1981.0
Number of samples	9	53	38

2.4.4 *Porewater Dissolved Gases*

None of the treatments had a statistically significant change in dissolved CH_4 concentration between the initial porewater sampling event (week 2) and the final porewater sampling event (week 8) (Table 3). Due to the small sample size for control boxes and boxes with simulated plants (n=3 for each) the statistical power of the Wilcoxon test used was low and it would have been unlikely to detect a small change. Nevertheless, multiplying the largest change in concentration recorded in any box by the volume of the box results in an increase of only 1.6 mg CH_4 over the course of the experiment. If this amount of CH_4 were emitted, it would have increased emission by 3.5 mg m^{-2} d^{-1}, a trivial amount relative to the magnitude of the fluxes recorded. For comparison, control boxes emitted on the order of one to ten mg m^{-2} d^{-1}, while planted boxes emitted over 200 mg m^{-2} d^{-1} during the second half of the experiment (Fig. 2.3).

Given these results, for all boxes one could equate emitted CH_4 with the net generation of CH_4 (production minus oxidation) because a negligible amount of CH_4 was stored within the soil during the experiment.

Table 2.3. Porewater CH_4 concentration changes over the course of the experiment show that there was negligible buildup of CH_4 in the soil in any of the box types.

Box Type	Δ CH_4 Concentration Over Six Weeks (µM)		Increase in Flux if all CH_4 were Emitted (mg m^{-2} d^{-1})		# of boxes
	Range	Median	Range	Median	
Control	-2 to 20	1	-0.3 to 3.5	0.2	3
Simulated	-1 to 10	0	-1.3 to 0.2	-0.7	3
Planted	-14 to 16	6	-2.4 to 2.7	1.1	6

2.4.5 *Isotope Mixing Model*

The results of the fractionation and exudate age modules which produced parameter distributions are shown in the bottom half of Table 4. The fractionation effects in both planted and unplanted boxes were similar, and within the range of values previously reported in the literature. Previously conducted studies have examined the residence time of all root-derived carbon (Gaudinski et al. 2000; Rasse et al. 2005), instead of separating exudate carbon from carbon that was sloughed off of the root itself or part of a dead root. Here, exudates were separated from other types of root-derived carbon. Because of this difference, previous studies have reported values on the time-scale of several years (Gaudinski et al. 2000) as opposed to the timescale of a couple months that were measured for root exudates. Similarly, the delay period is not a variable that other studies have estimated. The residence time of carbon in plants has been measured, but that includes carbon that is incorporated into plant tissues and, as with residence time in the soil, can stretch into years as opposed to the scale of days measured here (Gaudinski et al. 2000).

Table 2.4. Isotopic composition of various carbon pools, along with results of the fractionation module and exudate age module of the mixing model. The fractionation module calculated fractionation factors based on measured $\delta^{13}C$ values in unlabeled boxes. The mixing module calculated the residence times of

exudates in the soil and the delay between the end of labeling and peak enrichment based on data from a test plant. The medians of all values calculated or measured in this study are reported with interquartile ranges in parenthesis. The ranges for the literature values are the full range of values reported in those studies.

Measured Values (‰)	**Control Boxes**	**Number of control boxes/ samples**	**Planted Boxes**	**Number of planted boxes/ samples**	**Literature values**
δ^{13}C of unlabeled soil (S_{US})	-26.7 (-27.1 to -26.5)	3/29	-26.5 (-26.8 to -26.2)	1/6	-28 to -24.5[a]
δ^{13}C of unlabeled roots (S_{UE})	n/a	n/a	-33.1 (-34.9 to -32.4)	1/9	-22 to -34[b]
δ^{13}C of emitted unlabeled methane (M_U)	-58.2 (-61.3 to -56.7)	3/6	-61.0 (-62. 3 to -57.8)	7/12	-70.3 to -54.9[c]
Calculated Values	**Control Boxes and Simulated Plants**	**Number of results for CB & SP**	**Planted Boxes**	**Number of results for planted boxes**	**Literature values**
Net apparent fractionation of CH_4 emission (F) **(‰)**	-31.6 (-34.4 to -29.9)	621	-27.1 (-29.6 to -24.2)	90	-75 to +14[d]
Residence times of exudates in soil (τ) **(days)**	n/a	n/a	61.1 (44.7 to 77.3)	780	n/a
Delay between end of labeling and peak enrichment (D) **(days)**	n/a	n/a	2.6 (1.7 to 3.5)	780	n/a

a. (Chanton et al. 1992; Galand et al. 2010; Krohn et al. 2017)

b. (Bender, 1971; Nielsen et al., 2017; O’Leary, 1988)

c. (Chanton et al. 1992; Popp Trevor J. et al. 1999)

d. (Games et al. 1978; Gelwicks et al. 1994; Botz et al. 1996; Whiticar 1999; Valentine et al. 2004; Londry et al. 2008; Feisthauer et al. 2011; Neumann et al. 2015) Net fractionation calculated by adding fractionation effects of methanogenesis and methanotrophy reported in the various publications cited.

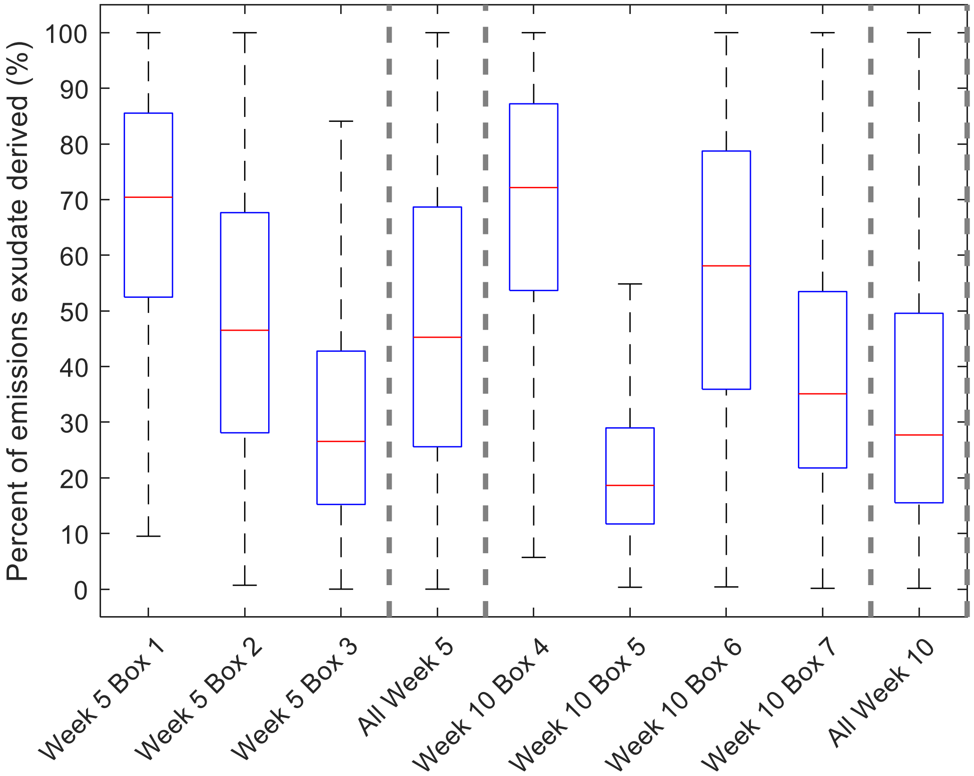

Figure 2.6. Results from isotope mixing model show the percentage of CH4 emitted from each box that was derived from root exudates, with the remainder derived from soil carbon. There was no significant difference between the two labeling events.

Both soil and root exudates were responsible for a large portion of total CH_4 emissions in both the first and second labeling events (Fig. 2.6). Note that only three boxes are presented from the week 5 labeling event. This is because one box harvested during that week did not produce any fluxes which passed isotopic quality control, and therefore could not be used in the model. The median exudate-derived portion of emitted CH_4 from each box ranged from 22 to 68% in the week 5 labeling event with an overall median for that labeling event of 43%. During the week 10 labeling event, the median exudate-derived portion of CH_4 emitted fromeach box ranged from 18 to 69%, with an overall median for the labeling event of 25%. In both cases, all of the CH_4 that was not exudate-derived was soil-derived. In order to compare these values to the amount of CH_4 emitted from simulated plants and control boxes, the percentage of CH_4 derived from each source during each flux was multiplied by the total flux of CH_4. This converted the percentages given in Fig. 2.6 into the flux units shown in Fig. 2.7.

During both events, planted boxes emitted significantly more ($p < 0.05$) soil-derived CH_4 than the simulated plants or control boxes. The median values for soil-derived CH_4 emissions were 359 mg m^{-2} d^{-1} for week five, 423 mg m^{-2} d^{-1} for week ten, 15 mg m^{-2} d^{-1} for simulated-plant boxes, and 25 mg m^{-2} d^{-1} for control boxes. During week 10 the planted boxes emitted significantly more ($p < 0.05$) soil-derived CH_4 than exudate-derived CH_4, but during week 5 the amount of CH_4 emitted from the two carbon sources was not significantly different ($p > 0.05$).

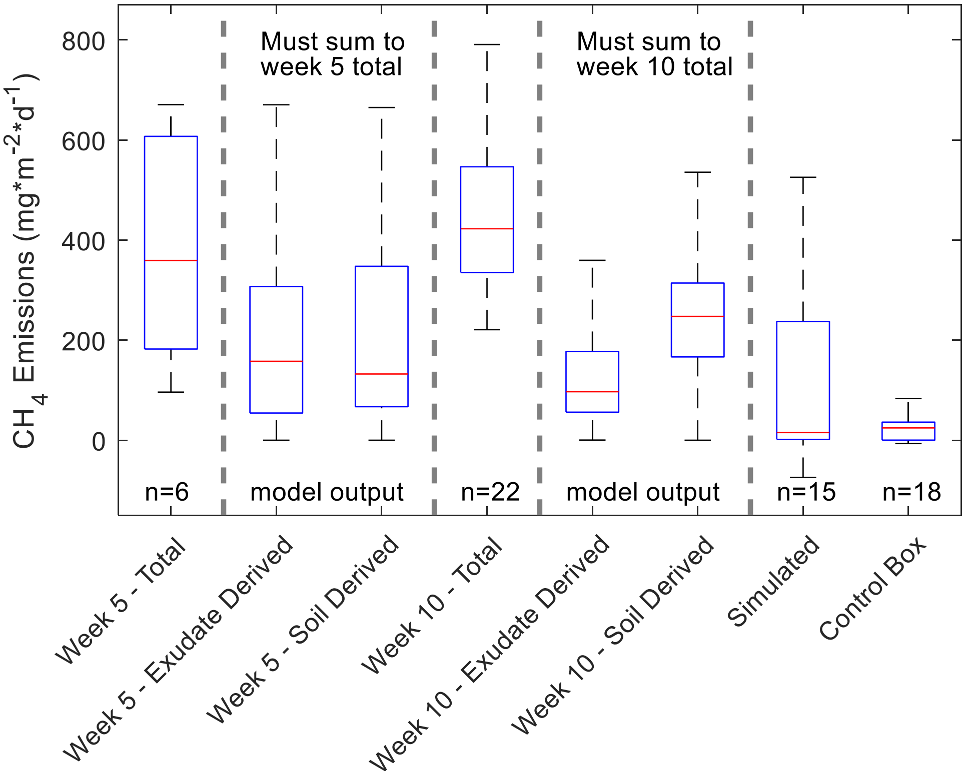

Figure 2.7. Box and whisker plots showing the total measured fluxes from planted boxes (Week 5 – Total and Week 10 – Total), the portion of that total which was calculated to be from each carbon source (Soil Derived and Exudate Derived), and measured emissions from the two unplanted box types (Simulated

Plants and Control Boxes), which were entirely soil-derived. Outliers are not shown. Letters indicate groups that are statistically similar ($p > 0.05$).

2.5 DISCUSSION

2.5.1 *Biological and physical mechanisms for plants to increase CH_4 emission*

The planted boxes' greater emission of CH_4 than control boxes (Fig. 2.3) confirmed that plants can increase CH_4 emissions, as has been shown previously (Shannon and White 1994; Joabsson et al. 1999; Popp et al. 2000; Whalen 2005), but by itself does not explain the mechanism. The use of simulated plants allowed for a direct comparison between a transport-alone scenario, where CH_4 and oxygen are transported without plant use, and real plants, which were biologically active and also transported gases. Gas transport in the simulated plants was clearly extensive, as demonstrated by oxygenation of the soil (Fig. 2.5), but CH_4 emissions were not significantly greater than those from the control boxes (Figs. 2.3 & 2.7). This finding is consistent with what other studies using similar methodology have found. King et al. (King et al. 1998) compared four treatments in a boreal wetland:natural plots with sedges and moss, plots with the sedges removed, plots with the moss removed, and plots with sedges removed and silicone tubes inserted. Turner et al. (under review) similiarly utilized natural, sedge-removal, and simulated-plant plots in a field study. Both experiments found that the natural plots had high CH_4 emissions, while the sedge-removal and simulated-plant plots both had similar, low emissions (King et al. 1998, Turner et al. (under review)). The moss-removal plots in the King et al. (1998) study emitted as much CH_4 as the natural plots, indicating that it is the vascular plants which made the difference. These results reinforce that gas transport alone does not increase emissions.

Plants do more than simply transport gas. They consume some oxygen within their root tissues (Armstrong 1971), and they also release root exudates which can affect oxygen dynamics in the soil by stimulating microbial respiration (Popp et al. 2000; Ding et al. 2004). The real plants in this experiment did not oxygenate the rhizosphere (Fig. 2.4) but they did greatly increase CH_4 emissions relative to the control boxes (Fig 2.4). The fact that the real and simulated plants were different in both regards indicates that the biological effects of the plants were key, and gas transport only increased emissions in conjunction with other root impacts. The importance of biological factors is further supported by the fact that the plants switched between a lower-emission state and a higher-emission state quickly (i.e., within two weeks), while their height increased in a nearly linear fashion. This switch indicates that the emissions increase caused by the plants was not due to an effect that scales linearly with size, such as the gas transport capacity of aerenchyma. Instead, there must have been some change in the plants or rhizosphere which drove the increase in emissions, such as an increase in root exudation of carbon.

Root exudates can increase CH_4 emissions through two effects: either increasing CH_4 production (Ström et al. 2003; Ström and Christensen 2007; Picek et al. 2007; Chanton et al. 2008; Kayranli et al. 2009) or decreasing CH_4 oxidation (Popp et al. 2000). These effects can take place through multiple mechanisms including direct processing of root exudates into substrates for methanogenesis, stimulating the growth of heterotrophic microbes which compete with methanotrophs for electron acceptors such as oxygen, and/or triggering microbial priming which can include changes to the composition and size of the microbial community as well as changes to the soil chemical environment. All three of these mechanisms occurred in this study.

2.5.2 *Root exudate conversion to CH_4*

Direct conversion of root exudates into CH_4 is the most straight-forward mechanism by which exudates can increase CH_4 emissions. The sharp rise in isotopic enrichment of CH_4 after labeling (Fig. 2.5A) showed that root exudates fueled methanogenesis, and the isotope model results (Fig. 2.7) show that exudates were used in conjuction with soil carbon. Exudates did not diminish or replace use of soil carbon. The fact that the two labelling events produced different rhizosphere enrichments (Fig. 2.5B) from similarly enriched roots (Table 2) implies that the amount of exudates being emitted from the roots increased between the two labeling events, consistent with previous research showing root exudation is correlated with plant productivity (Weigel et al. 2005). The use of exudates to ultimately fuel CH_4 production provides an explanation for why CH_4 emissions are often correlated with primary productivity (Whiting and Chanton 1992).

However, the isotope model showed that while the emitted CH_4 was much more isotopically enriched than unlabled emissions, it was not as enriched as it would have been if exudates were the primary carbon source (Fig. 2.7). The model attributed a large portion of emissions from planted boxes to soil-derived carbon. During week 10, more of the CH_4 was soil-derived than exudate-derived. In fact, the model showed that much more soil-derived CH_4 was emitted from planted boxes than from control or simulated boxes, so utilization of root exudates alone cannot fully explain the greater CH_4 emissions from planted boxes relative to the other treatments. There are two potential ways that plants could have increased emissions of soil-derived CH_4 in this experiment. They could have reduced methanotrophy, or they could have stimulated increased production of soil-derived CH_4.

2.5.3 *Plants May Have Reduced Oxidation of Soil-Derived CH_4*

Exudates may have increased emissions by decreasing methanotrophy through increased competition for oxygen (Lenzewski et al. 2018). This effect is a synergy between root exudation and the gas transport effects of aerenchyma. There is significant existing literature showing that plant transport of CH_4 is substantial and can increase total emissions (Shannon and White 1994; Joabsson et al. 1999; Popp et al. 2000; Whalen 2005). Other studies have found that real plants can oxygenate the soil and oxidize CH_4 to CO_2 (Schipper and Reddy 1996; Fritz et al. 2011; Lenzewski et al. 2018), and in some cases soil oxygenation is extensive enough that oxidation reduces CH_4 emissions to zero (Fritz et al. 2011). Gas transport by plants can therefore increase CH_4 oxidation when it creates an oxic zone around roots, or decrease CH_4 oxidation when there is no oxic zone around roots and the aerenchyma allow CH_4 to diffuse from the anoxic rhizosphere directly to the atmosphere. Whether the rhizosphere is oxygenated or not depends on the balance between oxygen supply and demand, which is dictated by the biology of the plant species in question, as well as by carbon bioavailability and microbial ecology of the soil. The lack of rhizosphere oxygen in this system (Fig. 2.4) is consistant with a field study at the site where materials for this experiment were sourced, which utilized field-deployed optodes to study belowground oxygen dynamics (Turner et al., under review), and with previous work showing that *Carex aquatilis* transport gases less than other aerenchymatous sedges (Schimel 1995). Rates of methanotrophy were not directly measured in this study, but the lack of oxygen in the rhizosphere of the real plants and readily available oxygen surrounding tubes of simulated plants indicates that real plants likely reduced methanotrophy relative to simulated plants. Reducing methanotrophy can take multiple forms however. The utilization of oxygen can take place either within roots or in the soil, and competition for oxygen can occur either between microbial species or between metabolisms within facultative methanotrophs.

More oxygen is utilized along the transport pathway in plants than in abiotic tubes because the roots themselves need oxygen for respiration (Armstrong 1971). If this oxygen demand is strong enough relative to the transport capacity of the aerenchyma, the roots could effectively become selective gas-transport pathways where CH_4 can diffuse to the atmosphere but O_2 cannot diffuse to the soil. There is also another mechanism for increasing oxygen utilization: increasing demand in the soil through root exudation of carbon which stimulates aerobic microbial activity (Mueller et al. 2016; Lenzewski et al. 2018). The isotopic enrichment of CH_4 after labeling indicates that microbes did utilize carbon derived from root exudates. While this result indicates anaerobic metabolism, any available oxygen would have allowed micrbes to also aerobically metabolize root exudates, thus competing with methanotrophy for limited oxygen.

Note that this discussion is framed as competition for oxygen between methanotrophy and other metabolisms. While most species of methanotrophs are obligate methanotrophs (Conrad 2009), and therefore would be competing for oxygen against other aerobic microbes, there do exist facultative methanotrophs which can utilize multiple carbon compounds (Dedysh et al. 2005). The list of other carbon compounds which these facultative methanotrophs can utilize is limited (Dedysh et al. 2005; Crombie and Murrell 2014), but when available, the microbes preferentially use the non-methane compounds (Theisen et al. 2005). In the oxygen-limited rhizosphere of this study, it is possible that the addition of root exudates triggered facultative methanotrophs to utilize non-methane substrates and therefore oxidize less CH_4, in addition to the inter-species competition for oxygen which likely occured.

However, the combination of reduced methanotrophy and utilization of exudates was not enough to account for the full increase in CH_4 emissions caused by plants relative to the other treatments. A study at the field site from which peat for this experiment was obtained directly investigated CH_4 oxidation by measuring CH_4 emissions under anaerobic conditions, achieved by flushing the fluxing chamber headspace with N_2 (Turner et al., under review). That study found that a maximum of 36% of produced CH_4 was oxidized prior to emission in plots without vascular plants, regardless of whether they had simulated plants or not (Turner et al., under review). Assuming similar rates of methanotrophy in the unplanted boxes, the simulated plants and control boxes would have produced a median of 31 and 38 mg CH_4 m^{-2} d^{-1} respectively, still an order of magnitude less than the 237 mg m^{-2} d^{-1} of soil-derived CH_4 that was emitted from planted boxes (median of all data). This 88% excess soil-derived CH_4 emitted by planted boxes relative to other treatments could only have been the result of roots triggering increased use of soil carbon.

2.5.4 *Root Effects on Utilization of Soil Carbon*

There are two mechanisms by which roots may enhance anaerobic soil carbon processing: the creation of mixed-redox zones or microbial priming. The mixed-redox mechanism is a result of transitions in either time or space between oxic and anoxic environments (Canfield 1994; Aller 1998; Chanton et al. 2008). In these transitional regions, oxygen may be quickly utilized and so not be present, (as was the case in the rhizosphere in this study (Fig. 2.4)), but still have the effect of raising the overall redox state of the location and facilitating the creation of alternate electron acceptors (Keiluweit et al. 2016). If these additional electron acceptors were used to fully mineralize soil carbon to CO_2 then one would see no increase in CH_4 production. However, it has also been found that in mixed-redox zones, oxygen and other thermodynamically favorable electron acceptors are used to partially oxidize large molecules, and the resulting smaller molecules are further processed anaerobically (Chanton et al. 2008; Corbett et al. 2015). If such partial processing occurred in the rhizosphere of this study, then it could explain some of the increased microbial conversion of soil carbon to CH_4.

However, oxygen visualization around the simulated plants demonstrate that partial processing of soil organic matter was likely only a minor effect in this study. Mixed redox zones could have existed around the oxygenated soil surrounding the simulated plants (Fig. 2.4). The fact that CH_4 emissions from simulated-plant boxes were not significantly greater than control boxes (Figs. 2.3 & 2.7), paired with evidence from the field investigation that rates of methanotrophy were similar between unplanted field plots with and without simulated plants (Turner et al., under review), indicates that any mixed-redox zone surrounding the simulated plants did not substantially increase CH_4 production. Lacking an explanation for why the mixed-redox zones would have been larger or more influential around real roots than around simulated plants, priming is the mechanism that better explains the enhanced CH_4 emissions.

Priming is a broad term encompassing any process by which the addition of a different carbon source (e.g., root exudates or leaf litter) increases microbial utilization of soil carbon. Priming can happen by stimulating an increase in microbial biomass, a change in composition of the microbial community, and/or a change in what metabolisms are active within the microbial community (Craine et al. 2007; Kuzyakov 2010; Ruirui et al. 2014). Priming has been observed in methanogenic systems before, such as one incubation experiment which found that the addition of rice straw to peat soils greatly increased soil-derived CH_4 production (Ye et al. 2015). In this experiment, priming provides a straightforward explanation of why real plants emitted such a large amount of soil-derived CH_4 relative to the simulated plants (Figs. 2.3 & 2.7). Notably, during the week 10 labeling event, more CH_4 was soil-derived than exudate-derived (Figs. 2.6 & 2.7), indicating a notable priming effect. During the week 5 event there was still priming, but the soil-derived and exudate-derived portions were about equal. Based on rhizosphere enrichment data (Fig. 2.5), there was more root exudation happening during week

10. Together, the data indicate that more root exudation led to a greater portion of the total emissions being soil-derived (Figs. 2.6 & 2.7).

If the priming effect is equally powerful in natural systems, it could have profound environmental impacts. A field study in a Boreal peatland, similar to the one upon which this study is based, found that vascular plants contributed to increased microbial processing of soil organic matter, but did not directly link that activity to CH_4 (Robroek et al. 2016). In a laboratory study it was shown that CH_4 was generated from soil carbon that was processed because of plants. Furthermore, because root exudation and wetland CH_4 production are correlated with plant productivity (Whiting and Chanton 1992; Weigel et al. 2005), these findings imply that as plant productivity in the sub-arctic increases with climate change (Forkel et al. 2016), so will the conversion of soil carbon to CH_4. Further research into how priming could act as a positive climate feedback is warranted.

Another area of needed research highlighted by this study is an examination into why there was evidence of priming while other recent studies have failed to find evidence for it (Girkin et al. 2018) or found evidence that root exudates protect soil carbon, which is the opposite of priming (Graham et al. 2017). The data presented here are inadequate to explain why or when priming does or does not occur. In order to elucidate the mechanisms of priming one must understand what microbes are involved, and what types of soil-derived molecules are additionally utilized during priming.

2.6 CONCLUSIONS

In this experiment root exudates drove CH_4 production, but not through direct utilization alone. An isotope mixing model showed that a large portion of the increase in CH_4 emissions caused by plants was fueled by soil-derived carbon. The data indicate root exudates increased the amount of soil-derived CH_4 that was emitted by increasing O_2 demand and thus reducing methanotrophy and/or increasing CH_4 production from soil-derived carbon by stimulating microbial priming. Methanotrophy was not measured directly, but its reduction in planted boxes relative to the simulated plants was supported by the lack of oxygen detected in the rhizosphere. The simulated plants that were used for comparison did not emit significantly more CH_4 than control boxes and did have large oxygenated zones around their roots. The comparison between the real and simulated plants shows that transport of gases alone cannot increase CH_4 emissions without other biological activity. However, the amount of soil-derived CH_4 emitted from the planted boxes was an order of magnitude greater than what was likely ever produced in unplanted boxes, based on prior estimates of methanotrophy. Increased production of soil-derived CH_4 was the only explanation for the unattributed carbon that was emitted from planted boxes, which comprised over half of the total emissions from those boxes. That increase in production is best explained by microbial priming. An order of magnitude increase in conversion of soil carbon to CH_4 was driven by plant growth, which is projected to increase in the boreal region under forecasted climate conditions. The presence of such a large priming effect implies that increased plant productivity could potentially lead to increased conversion of soil carbon to CH_4 on climatically relevant scales.

Chapter 3. MASS SPECTROMETRY SIGNATURE OF MICROBIAL PRIMING IN A BOG RHIZOSPHERE

3.1 ABSTRACT

Wetlands host anaerobic microbes which convert organic carbon into methane (CH_4), a powerful greenhouse gas. Wetland plants can influence which carbon compounds are available for microbial processing by exuding freshly fixed carbon from their roots. This exudation of carbon from plant roots can trigger microbial priming: the process of new carbon stimulating the microbial community into processing more soil carbon than they otherwise would have. This study utilized samples from a previous experiment which demonstrated microbial priming was occurring, and used FT-ICR-MS analysis of soil compounds to identify the molecular signature of microbial priming in the wetland rhizosphere. The FT-ICR-MS data demonstrated that the root exudates triggered increased processing of both large, energy-rich molecules and small nitrogen-containing molecules, but only in the water-soluble carbon pool. This is evidence for a selective priming effect in which some types of carbon compounds are processed at an increased rate, while others are not.

3.2 INTRODUCTION

Wetlands host anaerobic microbes which convert organic carbon into methane (CH_4), a powerful greenhouse gas. Globally, this wetland-generated CH_4 is responsible for approximately a third of total CH_4 emissions (Ciais et al. 2013b), and CH_4 in the atmosphere is responsible for 15-19% of total greenhouse gas radiative forcing (Intergovernmental Panel on Climate Change 2014). This substantial contribution to the greenhouse effect is dynamic and responds to environmental changes; wetland CH_4 emissions are the primary driver of interannual variability in global emissions (Ciais et al. 2013b). The microbial communities that produce CH_4 are sensitive to a variety of variables including temperature (Westermann 1993; Yvon-Durocher et al. 2014), pH (DeLaune et al. 1986; Kotsyurbenko et al. 2007), redox environment (Fetzer and Conrad 1993; Fritz et al. 2011; Boye et al. 2017), and the composition of the organic carbon available to them (Updegraff et al. 1995; Girkin et al. 2018). Wetland plants provide a key avenue of research for understanding CH_4 production in wetlands because they can dynamically influence two important variables: redox environment through gas transport effects and carbon availability through root exudation.

Vascular plants affect soil redox conditions because hollow tissues in their stems and roots, known as aerenchyma, allow oxygen from the atmosphere to enter into the soil surrounding roots (Armstrong 1971; Fritz et al. 2011). The aerenchymatous tissues exist so that the roots are able to respire in the anaerobic, water-logged wetland environment. However, the roots do not necessarily respire all of the oxygen that travels through aerenchyma; some can leak into the soil. In the immediate vicinity of the root (the rhizosphere), this leakage can create an oxic region in which methanotrophy, the oxidation of CH_4, occurs (Fritz et al. 2011). However, if oxygen demand from the root and soil microbial community exceeds that delivered by aerenchyma then oxygen will not build up in the soil (Waldo et al. 2019a). Even when the rhizosphere is anoxic, the redox state may be raised because some of the oxygen from the roots is utilized to create alternate terminal electron acceptors (TEAs) (Frenzel et al. 1999). The presence of TEAs will preclude methanogenesis in the rhizosphere by making more energetically favorable reactions possible. The availability of oxygen and other TEAs can also vary over time, leading to mixed redox zones where molecules are partially processed aerobically, but further processed anaerobically (Chanton et al. 2008). The amount of energy that microbes can gain from oxidizing organic carbon depends on both the TEA used and, in anaerobic environments, the nominal oxidation state of carbon (NOSC) (LaRowe and Van Cappellen 2011; Keiluweit et al. 2016).

The types of carbon molecules present in the soil is an important variable because it can impact the microbial community. It was once thought that the structure of a molecule determined whether it was “labile” or “recalcitrant” to microbial degradation (Sollins et al. 1996; Hedges et al. 2000; Lützow et al. 2006), and authors proposed various means by which the “recalcitrance” could be established from the molecular structure (Lützow et al. 2006; D’Andrilli et al. 2015). Over time, the scientific consensus has shifted to recognize that all carbon compounds are ultimately capable of being microbially processed, albeit at different rates, and that the order of microbial preference can depend on a variety of factors including the composition of the microbial community and the availability of TEAs (Schmidt et al. 2011; Lehmann and Kleber 2015). Nonetheless, authors continue to use the language of “recalcitrance” and “lability”, sometimes acknowledging the changing understanding of these terms (Zhang et al. 2018) and sometimes not (Barcellos et al. 2018). Regardless of the terminology used, many of those papers still provide valuable insights into situations where certain classes of molecules are more or less bioavailable to the microbes present.

The situational bioavailability of carbon compounds can depend on traits of the molecules as well as on environmental factors. One fundamental constraint on microbial use of carbon is the limited pore size of cell walls, which dictates that any molecule that is too large or that is not water soluble must be degraded by exo-enzymes before the microbe can take it up (Benz and Bauer 1988; Fenchel et al. 2012). Among molecules small enough to be brought in through pores, microbes preferentially use water-soluble compounds (Boye et al. 2017; Graham et al. 2017), which makes sense because solutes can be directly used without the need for exo-enzymes. However, microbes have also been shown to preferentially utilize high molecular weight compounds in a variety of environments despite the extra step of extracellular degradation (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018). That microbes benefit from expending the energy required for extracellular degradation indicates that other factors impact microbial preference. Another property that has been studied is aromaticity. More aromatic compounds have historically been thought to be less bioavailable (Lützow et al. 2006), but new evidence indicates that is likely only true in oxic environments, and when oxygen is limiting then thermodynamic limitations dominate and aromaticity is not relevant (Pracht et al. 2018). In those oxygen-limited or highly reduced environments, NOSC can be the most important factor (Keiluweit et al. 2016; Boye et al. 2017).

Wetland plants can influence what carbon compounds are available for microbial processing by exuding freshly fixed carbon from their roots in the form of sugars, carbohydrates, organic acids, amino acids, and a variety of other small, water-soluble compounds (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012). These compounds stimulate the growth of beneficial microbial and fungal communities (Broeckling et al. 2008). Plants also introduce higher molecular weight compounds to the soil through sloughing of root cells and release of mucilage, but these compounds generally are less utilized by the soil microbial community (Bais et al. 2006). Root exudates must provide some benefit to the plant, as they are a carbon cost (Marschner 1974).

One way that root exudation can change the rhizosphere in a way that potentially benefits plants is through microbial priming. Priming is the mechanism by which the addition of a new carbon source to soils stimulates microbes to degrade more of the existing soil carbon than they do in the absence of the new source (Fontaine et al. 2007; Kuzyakov 2010; Ruirui et al. 2014; Ye et al. 2015). Priming can occur because the fresh carbon causes changes to the microbial ecosystem, increasing the demand for carbon compounds, the ability to process them, or both (Fontaine et al. 2004; Kuzyakov 2010; Ye et al. 2015). Priming could benefit plants through nitrogen mining (N-mining), a process by which the addition of nutrient-poor compounds causes microbes to degrade existing soil compounds which contain nitrogen (Craine et al. 2007; Ruirui et al. 2014). Wetland types such as bogs and some fens are often nitrogen limited (Aerts et al. 1992) despite the large amounts of nitrogen bound in very slowly decaying plant matter (Turunen et al. 2004; Moore et al. 2005; Drewer et al. 2010). These wetlands are therefore an environment where it is highly beneficial for plants to be able to access the nitrogen already in the soil, using tactics such as N-mining. However, some recent studies have failed to find evidence for priming in peat soils (Girkin et al. 2018) or have found evidence which the authors interpret as contradicting priming, though in a different soil type (Graham et al. 2017).

In a laboratory experiment using peat from a boreal bog and a typical wetland sedge, *Carex aquatilis*, this study used an isotopic tracer that showed that root exudates stimulated microbial priming (Waldo et al. 2019a). That result was based upon the finding that in the presence of plant roots an order of magnitude more soil-derived CH_4 was emitted than in unplanted boxes, and reduced methanotrophy could only explain part of the increase (Waldo et al. 2019a). Analyzing root, rhizosphere, and unplanted soil samples from that same laboratory experiment using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), this study addresses the question of what soil compounds were additionally processed when priming was active, with a focus on nitrogen containing compounds.

3.3 MATERIALS AND METHODS

3.3.1 *Sample Collection*

This study utilized samples collected from a previously described experiment (Chapter 2). Briefly, *Carex aquatilis*, a common boreal wetland sedge, were grown for ten weeks in rhizoboxes filled with bog peat collected from interior Alaska. The bog site was described in detail by Neumann et al. (2019). Control boxes consisted of peat with no plants. Methane emissions were monitored throughout the experiment. During weeks 5 and 10 of the experiment, 4 plants each were exposed to $^{13}CO_2$ by placing a clear fluxing hood on each rhizobox and injecting $^{13}CO_2$ into the headspace over a period of five consecutive days. This $^{13}CO_2$ was photosynthesized and isotopically labeled the plants. Following labelling, root and soil samples were collected anaerobically, destroying the plants.

For both box types (real plants and control) soil samples were collected from the same locations, but the method of sample collection depended on box type. For planted boxes, roots were sampled simultaneously with the rhizosphere soil. The samples were collected at depths of approximately 5 cm, 20 cm, and 35 cm. At each depth, three samples were taken: one in the center and one six cm from either edge of the box. For planted boxes, root sections were cut from each location. The root sections were sonicated in phosphate-buffered solution (PBS) then moved the root to a new container. All the soil that fell off the root during sonication were considered rhizosphere, as previously described (White et al. 2015) and both roots and rhizosphere were saved for analysis. For unplanted control boxes, cubes of soil were sampled from the 9 standard locations. All samples were stored at -20 °C before shipping them to the Environmental Molecular Sciences Laboratory in Richland, WA, USA, where they were stored at

-80 C until analysis. In total, analysis was done on 73 rhizosphere samples from 9 planted boxes, 43 bulk soil samples from 5 unplanted boxes, and 6 roots from 4 plants.

3.3.2 *Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS)*

FT-ICR-MS was used on extractions of carbon compounds from the soil samples, based on the methods of Tfaily et al. (2015). There were three sequential extractions performed on each sample to extract compounds that dissolve in different solvents: a water extraction for hydrophilic compounds, methanol extraction for compounds of intermediate hydrophobicity, and chloroform extraction for hydrophobic compounds. The extractions were performed in the stated order.

The water extraction was coupled with a solid-phase extraction (SPE) to remove salts from the solution that interfered with FT-ICR-MS readings during method development (Thorsten et al. 2008). To perform the extractions, the samples were thawed at room temperature. Samples were then centrifuged (VWR Symphony 4417) at a rotational centrifugal force of 2720g for 5 minutes and collected the supernant, which contained water-soluble compounds. To extract the remainder of the water-soluble compounds, four cycles of centrifugation were used in which 1 mL of water was added to the sample, shook it for 30 minutes, centrifuged it, and collected the supernant. The combined supernant from the five centrifugations (the initial porewater and four water additions) was acidified to pH 2 using nitric acid. This solution contained all of the water soluble compounds in the sample, in addition to unwanted salts. SPE columns (PPL, 500 mg, Varian Mega Bond Elut, Varian Inc.) were activated with 1 mL of methanol, then had the acidified water samples ran through the column. Each column was then washed three times with 10mM hydrochloric acid and dried by blowing nitrogen gas through the column. Last, the carbon compounds were eluded off the column using 2 mL of methanol.

The methanol extraction was performed on the pellet of peat remaining after the final centrifugation of the water extraction. One mL of methanol was added to each sample, which was then shaken for two hours, centrifuged and had the supernant collected. After the methanol extraction, immediately 1mL of chloroform was added to the remaining peat pellet and let it sit quiescent overnight at 4 °C. The samples were then centrifuged and had the supernant collected as with the previous extractions. Pure chloroform does not run well in the ICR-MS instrument, so after the extraction was completed the chloroform extracts were blended with methanol in a 1:1 ratio so that they could be run directly on the instrument. The water extraction contained water-soluble compounds, the methanol extraction contained compounds on intermediate solubilities, and the chloroform extraction contained compounds which were not soluble in water.

All three extractions were then analyzed on a FT-ICR-MS. A Bruker electrospray ionization source was used in negative ion mode with the following settings: 0.1s ion accumulation time, 200 scans, range of 98-900 m/z, resolution 260k. Negative ion mode was selected because it has been shown to be more effective for ionization of organic molecules (Stenson et al. 2003). The instrument was flushed with a mixture of water and methanol between samples, and methanol blanks were run at the beginning and end of each session to check for carryover from the samples. The instrument was calibrated weekly with a standard solution containing $C_2F_3O_2$, $C_6HF_9N_3O$, $C_{12}HF_{21}N_3O$, $C_{20}H_{18}F_{27}N_3O_8P_3$, and $C_{26}H_{18}F_{39}N_3O_8P_3$. The data was processed using BrukerDaltonik version 4.2 to convert the raw spectra into mass to charge (m/z) ratios, which were then assigned molecular formulas using a Compound Identification Algorithm (Kujawinski and Behn 2006; Minor et al. 2012). Formulas were assigned to peaks with a signal to noise ratio of at least 7, and mass measurement error of less than one ppm. The only atoms allowed to be assigned were the most common isotope for each of C, H, O, N, S, and P, plus ^{13}C. Formulas were only assigned which included at least one each of C, H, and O, which had no more than 100 C, 200 H, 30 O, 20 N, 10 S, and 6 P, and which had at least four O per P.

3.3.3 *Laser ablation electrospray ionization mass spectroscopy (LAESI)*

To analyze compounds present on and in roots, laser ablation electrospray ionization mass spectroscopy (LAESI) was used. Root sections 2-4 cm long were mounted on microscope slides using double-sided tape and the surface of the root was ablated using focused 7-

nanosecond pulses from a mid-IR laser (IR Opolette HE 2731) set to a 2.94 µm wavelength. The ablated material was carried into a Thermo Fischer Velos Orbitrap Pro MS using an ion spray generated from a solution of 50% methanol and 50% water, with a very small amount of acetic acid added for pH control. The spray was dispensed at 500 nL per minute and aligned on-axis to the MS orifice. Both positive ion mode and negative ion mode were used to analyze the ions captured. Roots were sampled using a grid scan in which the surface of the root was ablated in a rectangular grid with between 300 and 400 µm between the centers of each ablation target. All raw spectra were assigned molecular formula using the HMDB 4.0 database (Guo et al. 2017) through the METASPACE platform (Palmer et al. 2017). This system only assigned formulas to m/z ratios which correspond to an identified metabolite in the HMDB 4.0 database.

3.3.4 *Data Organization for Analysis*

We combined FT-ICR-MS formula lists from all extractions of each soil sample to form a single list of compounds extracted from that sample. Due to variability in ionization efficiency, only compound presence, not intensity, was considered in this analysis. Compounds from all the rhizosphere samples were then compiled to form one list of all compounds present in the rhizosphere. A second list was compiled that contained any compound present in an unplanted box. By comparing these lists and removing any compounds which appeared on both, compounds were isolated which were unique to either the rhizosphere or unplanted soil. Compounds were then compared in each extraction of each sample to these master lists of compounds unique to each sample type (rhizosphere or unplanted) to create data-sets of the unique compounds present in each extraction. The lists of compounds in each of the three extractions were combined into a single list of all compounds in that sample. This approach enabled the analysis to focus on the differences between rhizosphere and unplanted soil. All LAESI data from the roots was used, including formula for compounds which were present in either type of soil sample (rhizosphere or unplanted soil). The aim was to identify compounds that differed between rhizosphere and unplanted soil because they shared the same original peat material and therefore many compounds; this rationale does not exist for roots.

3.3.5 *Compound Classification and Bioavailability Metrics*

Compounds were classified according to their molecular formula using two different classification schemes. The first, a frequently used atom-ratio classification scheme (D'Andrilli et al. 2015), involved sorting compounds using only O-to-C and H-to-C ratios, producing classes with properties similar to lipids, lignins, proteins, concentration hydrocarbons, amino-sugars, unsaturated hydrocarbons, carbohydrates, tannins, and "other" undefined compounds. The second was a more recently described multidimensional stoichiometric compound classification (MSCC) scheme that takes into account molecular size and heteroatom (N, S, and P) composition (Rivas-Ubach et al. 2018). The compound classes described in the MSCC are lipids, proteins, amino-sugars, carbohydrates, nucleotides, phytochemicals, and "other" undefined compounds. Classification resulting from both schemes does not indicate the compound is necessarily a lipid, lignin, protein, etc., but rather that the compound has properties similar to that of a lipid, lignin, protein, etc.

Two metrics described chemical properties: aromaticity index (AI)(Koch and Dittmar 2006; Koch and Dittmar 2016) and nominal oxidation state of carbon (NOSC) (LaRowe and Van Cappellen 2011). Both metrics are unitless. Calculations for these metrics are given in Equations 3.1 & 3.2, with the elemental symbols representing the number of atoms of that element present in the molecular formula. AI values calculated below 0 are defined to be zero, because negative aromaticity has no physical meaning (Koch and Dittmar 2006).

$$3.1)\ AI = \frac{1+C-0.5*O-S-0.5*(N+P+H)}{C-0.5*O-S-N-P}$$

$$3.2)\ NOSC = 4 - \frac{4*C+H-3*N-2*O+5*P-2*S}{C}$$

3.3.6 *Microbial Metabolisms and Transformation*

To assess involvement of identified compounds in microbial metabolic pathways the assigned molecular formulae were compared to the KEGG database (release 86.1) to identify metabolic pathways involving the compounds present (Kanehisa and Goto 2000). The full list of all compounds identified by KEGG were used, not just the unique ones as with other analyses. This choice was made because the goal was to assess the full pathway, and some compounds may be present on multiple pathways. Because metabolic pathways can include members of various hydrophobicities, all three extractions were combined for KEGG analysis. Biochemical transformations in the soil were identified using the methodology of Breitling et al. (2006) modified with an updated list of transformations from Graham et al. (2017). This technique finds the difference in mass between all pairwise combinations of mass peaks in the sample and compares them to a list of mass changes caused by known biochemical transformations (SI Table S1). As with the KEGG analysis, because the transformation analysis relies on the relationships between compounds/peaks all data was used and unique peaks were not isolated. If an observed mass difference was within 10^{-4} Da of a transformation on the list then it was counted as a transformation. The total number of transformations was calculated in each extraction, and a principle components analysis (PCA) was performed on the percentage of transformations within each extraction. The PCA was done using the “pca” function in MATLAB (R2018b).

3.3.7 *Statistics*

For the heteroatom and compound classifications, the total count of each classification was analyzed within each sample (all three extractions combined). For metrics that calculate values per compound (AI, NOSC, and atom ratios), statistical tests were performed on the mean values of calculated metrics from individual extractions. Therefore, a reported median value for rhizosphere or unplanted soil is the median of the means of each individual extraction of that type. For all statistical comparisons a mixed-effects ANOVA was used with the box which the sample came from as a random effect variable, and the treatment type as the fixed effect which were tested for significance. The data were not normally distributed, but an ANOVA test was still appropriate because it was applied to the means of samples, and those means can be assumed to be normally distributed. This test was performed using the “fitlme” function in MATLAB (R2018b).

3.4 RESULTS

3.4.1 *Unplanted soil compounds*

Using the traditional classification scheme, the carbon compounds extracted exclusively from the unplanted peat consisted largely of lipid-like and lignin-like compounds, with these two categories comprising a mean of over 50% of compounds (Fig. 3.1). Three quarters of detected compounds did not fall within any of the MSCC classifications (SI Fig. S3.1). The MSCC is intentionally designed to be more precise about class assignments (Rivas-Ubach et al. 2018), but the large number of unclassified compounds demonstrates how, in complex environmental media where compounds degrade and are reprocessed, many molecules fail to fall within the stringent categories.

The compounds in unplanted soil contained many heteroatoms, with only a few outliers having a median of more than 20 percent CHO (SI Figure S3.2). Of the heteroatoms, nitrogen and sulfur were both common: a median of 47% and 51% of combined compounds from all extractions in each sample were N-containing and S-containing, respectively. Phosphorus was less common, but still abundant with a median of 30% of compounds containing it. Graphs showing the ratio of various atoms to carbon can be found in Figure 3.2 (H:C) and SI Figure S3.3 (all other ratios). Bogs are typically nutrient limited (Aerts et al. 1992), and it is important to note that even though these heteroatoms existed they were not necessarily available to plants or microbes, as will be addressed in the discussion below.

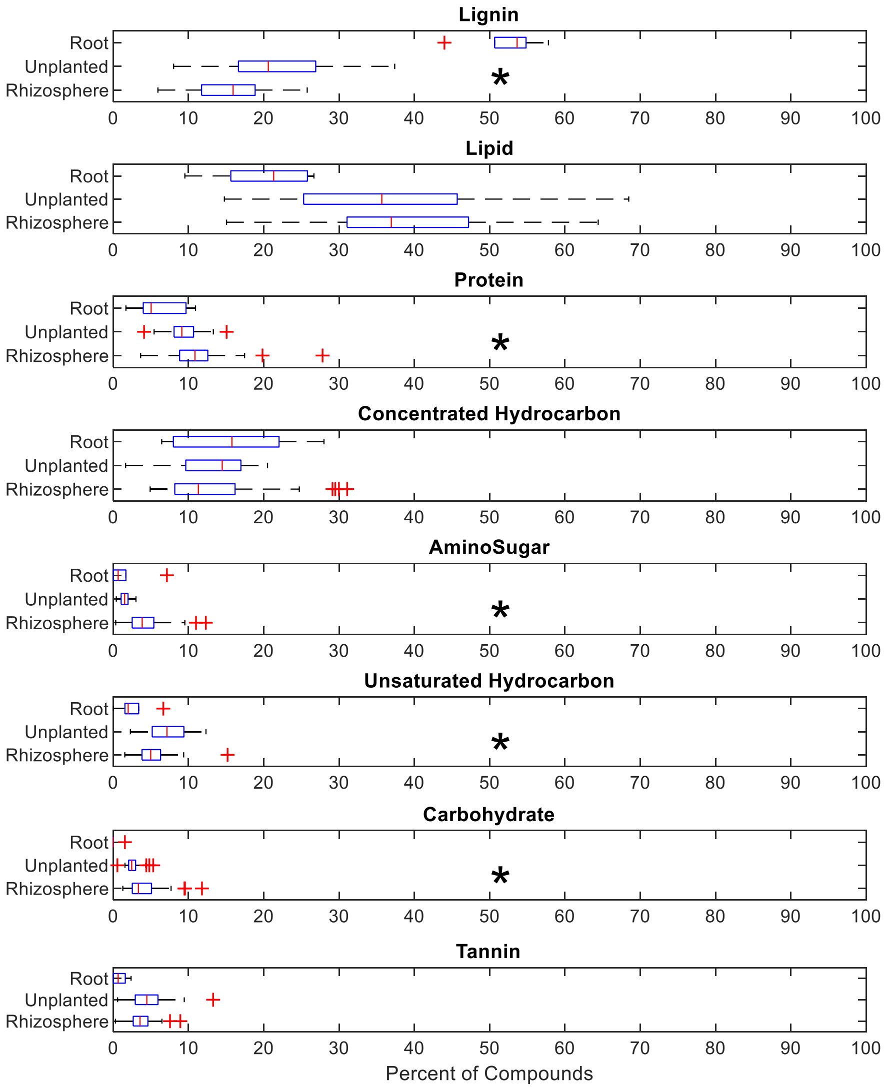

Figure 3.1. Atom-ratio compound classifications for rhizosphere soil, unplanted bulk soil and root surfaces. Boxes show median with upper and lower quartiles, and tails show all data within 2.7σ of the mean. Outliers outside 2.7σ are marked with "+". Rhizosphere n=73, unplanted soil n=40, and roots n=6. The asterisks indicate statistical significance ($p < 0.05$) between the unplanted soil and rhizosphere.

Figure 3.2 contains box and whisker plots of a variety of metrics that have been used in other studies to assess the bioavailability of molecules. The AI in methanol and chloroform extractions were clustered tightly at zero, but in the water extraction it formed a wider distribution at higher values. Higher AI has historically been thought of as being less bioavailable (Lützow et al. 2006), but is now thought to be relevant only in certain environments (Pracht et al. 2018). The H:C ratio had a similar pattern to AI: the water fraction had more compounds with H:C ratios below 1.5, a threshold that has previously been described as a boundary for bioavailability (D'Andrilli et al. 2015), while the methanol and chloroform extractions had higher H:C ratios which would indicate higher bioavailability according to D'Andrilli et al. (2015). The water soluble fraction had higher NOSC than either the methanol or chloroform extractions. Recent research has shown that in anaerobic environments such as this one, microbes preferentially use compounds of higher NOSC because more net energy can be extracted from them (Keiluweit et al. 2016; Boye et al. 2017) but that all compounds can eventually be utilized (Pracht et al. 2018). The median masses in all three extractions were between 500 and 700 Da, which is large enough to be reliably detected by the FT-ICR-MS technique but small enough to pass through the pores in cell walls.

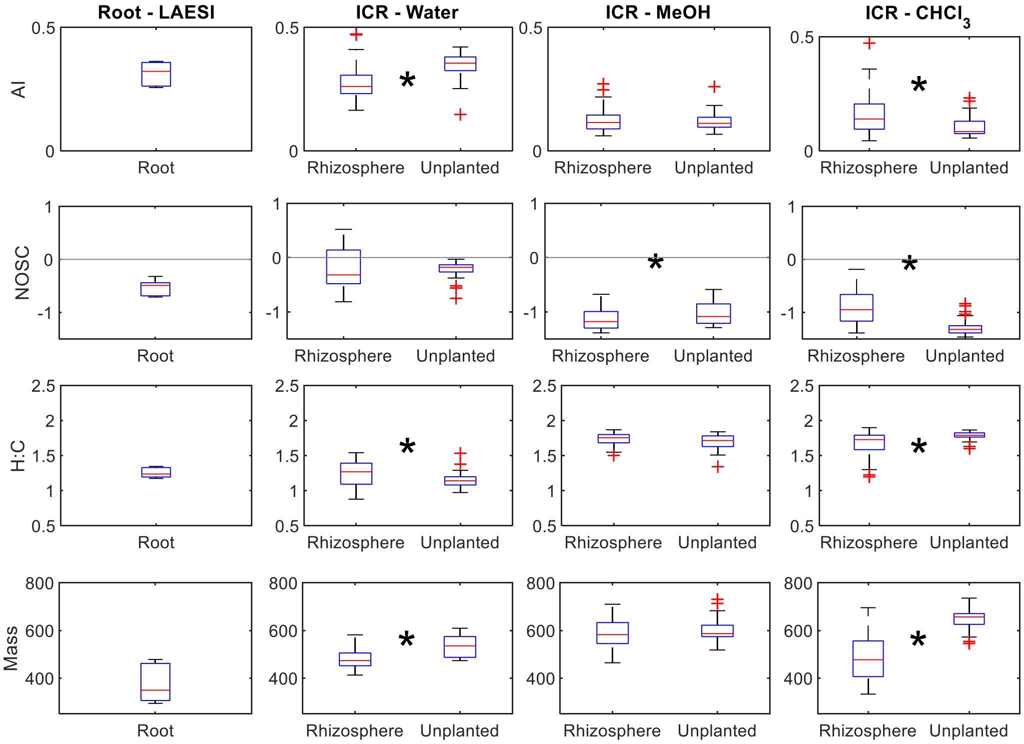

Figure 3.2. Boxplots of a molecular indices. The rows show: aromaticity index (AI), nominal oxidation state of carbon (NOSC), hydrogen to carbon ratio (H:C) and the molecular mass in Daltons. Root-LAESI, roots of plants, measured by LAESI, ICR-Water, FT-ICR-MS water extraction; ICR-MeOH, FT-ICR-MS methanol extraction; ICR-$CHCl_3$, FT-ICR-MS chloroform extraction.*, significant different ($p < 0.05$, mixed-effects ANOVA).

3.4.2 *Root compounds*

The compounds detected on the root surface by LAESI are consistent with fragments of structural lignins comprising the root itself. The traditional compound classification scheme identified over half the compounds as lignin-like, with lipids and concentrated hydrocarbons comprising most of the rest (Fig. 3.1). As with the soil compounds, the majority of root compounds could not be classified with the MSCC (SI Fig. S3.1). Around three quarters of the compounds contained only C, H, and O (SI Fig. S3.2), which is typical of lignins or other structural molecules such as polysaccharides. The AI was high, which is again consistent with structural lignins and polysaccharides that contain many aromatic rings.

Note that these measurements were made of the root material, not the root exudates comprised of amino acids and sugars (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012), which are believed to stimulate microbial activity. However, dead root cells sloughed into the soil may have been be introduced into the rhizosphere.

3.4.3 *Rhizosphere compounds*

Compounds identified as being unique to rhizosphere samples had a similar pattern to those in unplanted soil in terms of AI and H:C ratio (Fig. 3.2), though small but significant ($p < 0.05$) differences did exist in some extractions. The NOSC data showed no difference between rhizosphere and unplanted soil in the water extraction, but the unique compounds in rhizosphere soil had slightly lower NOSC in the methanol extraction than unplanted soil, and slightly higher NOSC in the chloroform extraction. The compound classes were broadly similar, but there were small yet statistically significant ($p < 0.05$) differences in most classes (Fig. 3.1). The compounds unique to the rhizosphere had more proteins, amino sugars, and carbohydrates than unplanted soil, but fewer lignins and unsaturated hydrocarbons (Fig. 3.1). The two soil types had similar amounts of lipids, concentrated hydrocarbons, and tannins. The molecular mass was significantly lower in the water extraction and chloroform extraction ($p < 0.05$), but there was no difference in mass in the methanol extraction. There was no significant difference between the number of compounds extracted from the rhizosphere and bulk soil, either in any of the individual extractions or in the combined list of all compounds (SI Figure S3.4).

Because molecular mass was different between the two treatments in the water extraction and is known to be a key indicator of bioavailability, it is useful to analyze high and low molecular mass compounds separately. Examination of the AI, NOSC, or H:C ratios with either only low molecular mass (<500 Da, SI Figure S3.5 & S3.7) or high molecular mass compounds(>500 Da, SI Figure S3.6 & S3.8) did not reveal any meaningful differences between rhizosphere and unplanted soil. However, small compounds in the rhizosphere had significantly lower N:C ratios than small compounds in unplanted soil did ($p < 0.05$, Fig. 3.3), a difference that did not exist among the large compounds ($p > 0.05$, Fig. 3.3). When examining compound classes by molecular size, the small rhizosphere molecules have significantly ($p < 0.05$) more proteins, amino sugars, and carbohydrates than unplanted soil (SI Figure S3.7), all molecule types that are consistent with root exudates (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012).

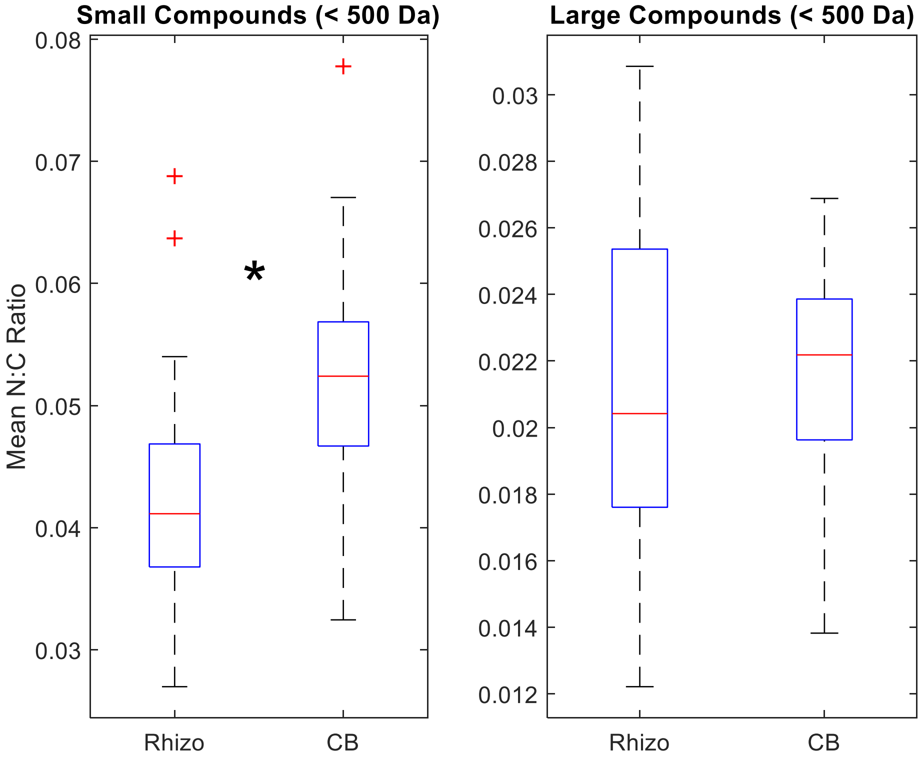

Figure 3.3. Nitrogen to carbon ratios among both large (left) and small (right) compounds. Small compounds in the rhizosphere were depleted in nitrogen relative to small compound in unplanted soil. But no difference in nitrogen content was detected between sample types for large compounds.

3.4.4 *Microbial Transformations*

There were significantly more ($p < 0.05$) microbial transformations in the water extractions than in the methanol or chloroform extractions, but there were no differences ($p > 0.05$) between the number of transformations in rhizosphere or unplanted extractions (Figure 3.4). These results indicate that the compounds in the water fraction were more involved in microbial metabolisms than those in the methanol or chloroform fractions. Similarly, the PCA analysis of the transformation data showed that the first two principle components together explained over 95% of variation and showed that the water extraction was offset from the other two, but there was no difference between rhizosphere and unplanted soil samples (SI Figure S3.9).

KEGG pathway analysis revealed no significant difference ($p > 0.05$) in the number of metabolites or metabolic pathways between rhizosphere and unplanted soil samples. A PCA analysis of the pathways did reveal a statistically significant ($p < 0.05$) difference between rhizosphere and unplanted samples, but the difference was small. The first principle component explained 99.9 % of the variation, and its interquartile range among rhizosphere samples was .050 to .055, while among unplanted soil the interquartile range was .052 to .060. There was no correlation between PC 1 and the flux of CH_4 out of the box ($p > 0.05$, $R^2 = .03$).

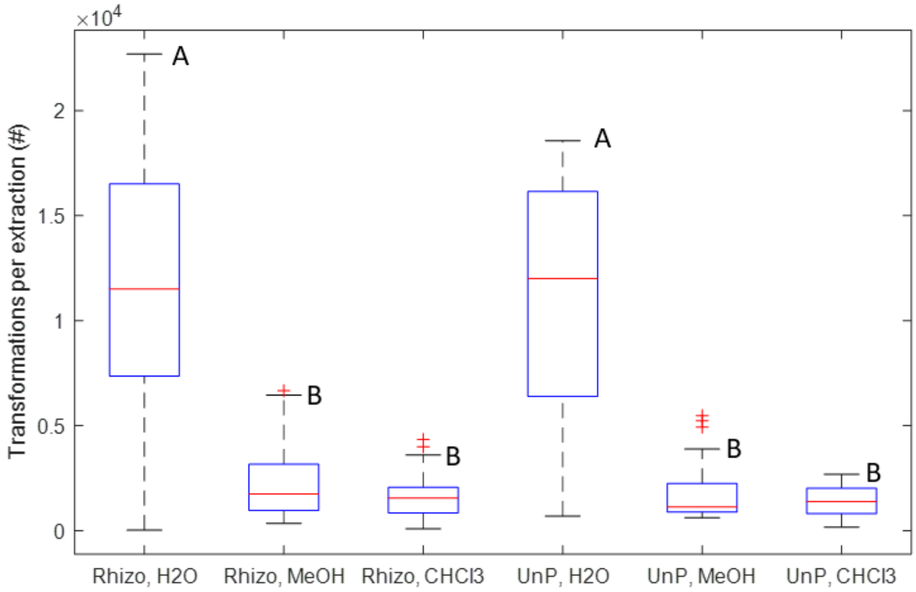

Figure 3.4. Boxplots of the number of microbial transformations in each extraction from both sample types (rhizosphere and unplanted). These data show that more microbial transformations occurred with water-soluble compounds than with methanol or chloroform soluble compounds. However, there is no difference in transformations between rhizosphere and unplanted soil.

3.5 DISCUSSION

3.5.1 *Attribution of Differences between Unplanted Soil and Rhizosphere*

The compounds present in the rhizosphere data represent those that existed in a dynamic system where roots were adding exudates to the soil and microbes were actively processing carbon. Pracht et al., working in a closed incubation system, recently (2018) argued that a compound’s bioavailability in a given environment does not measure whether it can be used or not, but only the order in which microbes will process it. Together, these observations demonstrate that any metric of bioavailability is of little use when applied to a single location at a single time. Because the compounds measured were those that remained after microbial processing, a comparison between different types of samples allowed inference of the compounds that were microbially processed. In this experiment, the compounds that were present in the unplanted soil but were no longer present in the rhizosphere were those which were microbially processed in the presence of roots, and thus the most situationally bioavailable in the context of the rhizosphere.

The carbon compounds in the rhizosphere were different from those in unplanted soil (Figs. 3.1-3.3) due to differences in microbial activity, and due to the input of fresh carbon from roots. Based on other studies utilizing data from this experiment, the rhizosphere housed a more active microbial population due to greater emission of CH_4 than the unplanted soil (Waldo et al. 2019a). However, neither the KEGG database nor microbial transformation analysis (Fig. 3.4) techniques detected this difference, as they have in other studies (Graham et al. 2017). This lack of difference could indicate that these metabolite-based methods are not very sensitive to differences in the population or activity of microbes in two systems that are so similar. Besides increased microbial activity, the rhizosphere samples also had their carbon composition changed by the addition of root exudates. However, some of the sugars and amino acids that comprise root exudates (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012) were too small to be detected consistently using the FT-ICR-MS technique (<100 Da). Additionally, sugars and amino acids are water-soluble and typically have high NOSC values, making them highly bioavailable in anoxic systems (Keiluweit et al. 2016). Because NOSC did not increase in the water-extracted rhizosphere compounds relative to those from unplanted soil, the exudates themselves were used rapidly and did not remain in the soil to be detected. Such preferential use would explain why root exudates have previously been found to increase microbial activity and CH_4 production (Ström et al. 2003; Ström and Christensen 2007; Picek et al. 2007; Chanton et al. 2008; Kayranli et al. 2009). Working from the interpretation that the changes observed were *not* due to the direct detection of root exudates, one can draw conclusions about what kind of microbial processing could explain the observed changes in the rhizosphere.

Molecular mass showed an interesting difference between rhizosphere and unplanted soil (Fig. 3.2). In both water and chloroform extractions, smaller compounds showed up considerably more frequently in the rhizosphere samples than in the unplanted samples (Fig. 3.2), consistent with the findings of Chanton et al. (2008) who found smaller compounds in *Carex* dominated bogs than in those where vascular plants were absent. This shift in molecular size has two possible explanations. First, the sugars and amino acids that comprise root exudates typically have molecular masses of less than 400 Da (Smith 1976; Lugtenberg et al. 1999; Jones et al. 2009; Dommergues 2012), so an increased abundance of these molecules would shift the mean towards lower masses, as observed (Fig. 3.2). However, the above discussion section demonstrated that these root exudates were either quickly utilized by microbes and did not remain in the soil, or were so small that they were not detected at all. An alternative explanation which is more consistent with the rest of this analysis is that the compounds in the rhizosphere were smaller than in the unplanted soil (Fig. 3.2) due to increased microbial use of soil carbon in the rhizosphere. It is well established that microbes break large compounds down into smaller compounds (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018). The observation that this process occurs at a greater rate in the rhizosphere than in the unplanted soil demonstrates that the root exudates triggered a microbial response which increased the processing of soil carbon, i.e., microbial priming.

3.5.2 *Microbial Priming*

Priming is broadly defined as an increase in microbial use of soil carbon due to the addition of a fresh carbon source, but several proposed mechanisms for priming can explain the shift in molecular size in this experiment. The roots could have simply accelerated a microbial process which also occurred in the unplanted soil, which is consistent with the hypothesis that priming is a caused by increased populations of microbes needing more food (Ye et al. 2015). The microbes could have also engaged in N-mining. The N-mining hypothesis predicts that microbes break down molecules that would otherwise be less bioavailable in order to obtain nitrogen (Craine et al. 2007; Ruirui et al. 2014). N-mining could explain why the low molecular mass compounds in the rhizosphere had significantly less ($p < 0.05$) nitrogen than those in unplanted soil (Fig. 3.3). What may have occurred was that microbes utilized large molecules regardless of nitrogen content for energy but only processed small molecules if they contained valuable nitrogen. The molecules that remained and accumulated in the rhizosphere were small, nitrogen-poor compounds that did not meet either of the microbes' needs.

We demonstrated in the previous analysis of the isotopic data from this same experiment that microbial priming was occurring in this system (Waldo et al. 2019a). The FT-ICR-MS data analyzed here advances those findings by demonstrating that the root exudates triggered increased processing of both large, energy-rich (high NOSC) molecules (Fig. 3.2) and small nitrogen-containing molecules (Fig. 3.3). However, this priming did not necessarily occur in all pools of carbon. The NOSC of chloroform extracted compounds was higher in the rhizosphere than in the bulk soil (Fig. 3.2). Because this was an anoxic system, the higher NOSC of remaining rhizosphere compounds implies less microbial processing, because these are the compounds which microbes would use first (Keiluweit et al. 2016; Boye et al. 2017). In contrast, the molecular mass of rhizosphere compounds in the chloroform extraction was lower than that of the unplanted soil compounds (Fig. 3.2), which is consistant with an active microbial population degrading large compounds (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018). Based on conflicting signals from these two metrics (Fig. 3.3) and the lack of difference in the microbial transformation analysis (Fig. 3.4), it is unknown whether root exudates triggered priming in the chloroform-extracted compounds, as they did in the water-extracted compounds. Graham et al. (2017) found that the addition of water-soluble, thermodynamically favorable carbon decreased microbial processing of carbon compounds in the chloroform extraction, referred to as the "physically bound" fraction in that study. Graham et al. (2017) interpreted the preservation of the physically bound compounds as evidence against the priming concept.

While it is unknown whether such preservation occurred in this system, the evidence for priming within the water-soluble compounds indicates that the results of Graham et al. (2017) do not necessarily contradict priming, but instead elucidate how it occurs. Priming is a selective effect which often increases total carbon use, but which may increase *or decrease* the microbial use of specific carbon pools such as water-soluble versus physically bound. This selectivity could be the result of several things. At its most basic level, it could be that priming exacerbates the already-present preference for soluble compounds (Boye et al. 2017; Graham et al. 2017). Alternatively, selective priming could be the result of favoring the growth of one community of microbes over other communities, or the result of encouraging microbes to change their metabolisms and produce a different set of enzymes. Further work is needed to confirm that priming is a selective effect, and to determine why.

3.6 CONCLUSIONS

This study built upon prior analysis of the same experiment, which established that microbial priming occurred and led to increased CH_4 production and emission. The two metrics that were most useful in differentiating between rhizosphere and unplanted soil were size and nitrogen content in water soluble compounds, both traits which were tied to microbial priming in this system. Larger molecules have previously been shown to be preferred by microbes (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018), and these results demonstrated that the addition of root exudates led to increased utilization of those large molecules. This is an example of priming caused by a more active microbial community utilizing more total soil carbon. Small molecules were also utilized at an increased rate in the presence of root exudates, but only if they contained nitrogen, lending support to the N-mining hypothesis (Craine et al. 2007; Ruirui et al. 2014). While this study demonstrated two types of microbial priming occurred, it also found evidence that non-water-soluble compounds may have been protected from degradation, similar to the recent findings of Graham et al. (2017). This selective priming implies a shift in the carbon processing capabilities of the microbial community in the presence of highly preferable compounds. Because boreal bogs tend to be nitrogen-limited environments, this study could imply that such

N-mining may be favorable in natural systems around the world. If verified, that result could imply that substantial amounts of CH_4 emitted to the atmosphere is derived from the priming effect.

Chapter 4. IMPACTS OF METHANOTROPH METABOLISMS ON BOREAL BOG METHANE FLUXES

4.1 INTRODUCTION

Microbial activity in the soil of wetlands is responsible for the emission of more methane (CH_4) to the atmosphere than all other natural sources combined (Ciais et al. 2013a). This flux is influenced by many factors, but in all cases the generation of CH_4 (methanogenesis) and any oxidation of CH_4 (methanotrophy) which may attenuate emissions are microbially mediated. Therefore, when factors like temperature are cited as influencing wetland CH_4 emissions (e.g., Hargreaves and Fowler 1998) they do so by impacting the microbial community either directly, e.g., microbial metabolic rates increase at warmer temperatures, or indirectly by altering the behavior of other environmental factors such as plants, which in turn affect the microbial ecosystem (Gill et al. 2017).

The microbial ecosystem inhabiting wetland soils is comprised of a complex mixture of bacteria and archaea which respond to a host of environmental variables. The majority of species present in wetland soil samples, as in most environments, are uncultured (Ivanova et al. 2016). Community composition can vary greatly based on depth in the soil column (Lipson et al. 2013; Bai et al. 2018), geographic setting of the wetland (Grodnitskaya et al. 2018), and what plants grow in the wetland (Robroek et al. 2015).

Plants impact the wetland microbial community through two primary modes. First, plants exude carbon compounds from their roots which may be more readily processed by microbes than the existing soil carbon (Bais et al. 2006; Girkin et al. 2018). These root exudates can stimulate microbial activity and CH_4 emissions (Ström et al. 2003; Ström and Christensen 2007; Picek et al. 2007; Chanton et al. 2008; Kayranli et al. 2009; Waldo et al. 2019a). While this increase in CH4 emissions is partially driven by the carbon in the exudates being processed into CH_4, the exudates also result in more soil carbon to be converted to CH_4 than would occur in the absence of plants. This phenomenon is known as the microbial priming effect (Waldo et al. 2019a).

The second effect that plants have on the microbial environment is wetland plant roots leak oxygen into the soil from the aerenchyma in their roots (Fritz et al. 2011). This oxygen can be used for methanotrophy (Fritz et al. 2011), but other aerobic metabolisms will compete for the limited oxygen supply (Lenzewski et al. 2018). Even when oxygen is used so quickly that it does not accumulate in the soil (Turner et al. 2019; Waldo et al. 2019a), it can influence microbial communities by facilitating recycling of alternate electron acceptors (Keiluweit et al. 2016), or by creating mixed-redox environments where carbon compounds are partially respired aerobically and partially anaerobically (Chanton et al. 2008). This variety of uses can lead to intense competition for oxygen in the rhizosphere.

The rhizosphere supports two methanotrophic metabolisms other than the traditional model of aerobic obligate methanotrophs. Once considered insignificant in wetlands (Conrad 2009), recent work has shown that anaerobic CH_4 oxidation (AMO) is common in freshwater wetlands (Segarra et al. 2015). However, AMO relies on alternative terminal electron acceptors (TEAs). In freshwater bogs, rain is the primary source of water and nutrients; groundwater is not available to advect TEAs into the wetland. The ultimate destination for electrons is oxygen that diffuses across the air-water boundary, while potential alternative TEAs are recycled by subsurface microbial populations within the bog (Keller and Bridgham 2007). Therefore, atmospheric oxygen still ultimately controls the potential for CH_4 oxidation, even if AMO occurs. Most methanotrophs are only capable of using single-carbon compounds (Conrad 2009). However, some facultative methanotrophs have been found in the genera *Methylocella*, *Methylocapsa,* and *Methylocystis* which may also use other carbon compounds such as acetate and ethanol (Dedysh et al. 2005; Dunfield et al. 2010; Belova et al. 2011; Im et al. 2011; Leng et al. 2015). These facultative methanotrophs are widely distributed in the environment, but are especially prevalent in acidic soils, including peatlands (Rahman et al. 2011).

Plants have great potential to influence the environment for methanotrophs, and by doing so impact the amount of CH_4, a potent greenhouse gas, which is emitted from wetlands.

Determining what metabolisms are favored in the altered soil environment surrounding roots, the rhizosphere, will allow for enhanced understanding of how plants control CH_4 dynamics. In this study, a laboratory experiment is used to compare the microbial communities of planted and unplanted wetland soil to elucidate the factors controlling populations of methanogens and methanotrophs, with special focus on the different types of methanotrophy.

4.2 MATERIALS AND METHODS

This study utilized samples collected during a previous study (Chapter 2), which described the experimental setup in detail. In that experiment, *Carex aquatilis*, a common boreal wetland sedge, were grown for ten weeks in rhizoboxes (48cm tall, 20cm wide, 5cm thick) filled with peat collected from a thermokarst bog in central Alaska. There were also two unplanted box types: control boxes with peat alone, and simulated plants that utilized silicone tubes to transport gases to simulate gaseous exchange without the biochemical effects of roots. Optical oxygen sensors (optodes) were used to measure oxygen concentration around the roots of plants and around the simulated plant roots (Larsen et al. 2011). Methane emissions were monitored throughout the experiment. During weeks 5 and 10 of the experiment, 4 plants were exposed to $^{13}CO_2$ by placing a clear fluxing hood on each rhizobox and injecting $^{13}CO_2$ into the headspace over a period of five consecutive days. This $^{13}CO_2$ was photosynthesized and isotopically labeled the plants. Following labeling, root and soil samples were anaerobically collected. Samples taken for chemical analysis are described in a companion study (Waldo et al. 2019b), and samples taken for DNA analysis and microbe counts are described below.

For all box types (real plants, simulated plants, and control) soil samples were collected from the same locations, but the method of sample collection depended on box type and purpose of sample. In planted boxes, the soil samples collected were of the rhizosphere and the associated roots were also collected. All samples were collected inside a gasbag filled with high-purity nitrogen. Samples were collected at depths of approximately 5 cm, 20 cm, and 35 cm. At each depth, samples were taken from three sites, one in the center and one 6 cm from either edge of the box. At each sample site separate samples were taken for fluorescence microscopy and metagenomics.

4.2.1 *Fluorescence Microscopy*

Fluorescence microscopy was used to count the number of microbes in samples from planted and control boxes, but not in samples from boxes with simulated plants. For planted boxes, root sections were cut from each sampling location. The root sections were sonicated in centrifuge tubes containing 4% paraformaldehyde (PFA) to fix the cells and preserve them intact for analysis, then moved to a new container. All the soil that fell off the root during sonication was considered rhizosphere and saved for analysis (White et al. 2015). The tube containing the rhizosphere was centrifuged and had the PFA solution aspirated then discarded and the sample was stored in a 50/50 mix of ethanol and phosphate buffered solution (PBS). For unplanted control boxes, cubes of soil were sampled from the nine specified locations into 4% PFA, then after at least 15 minutes transferred into the PBS/ethanol mixture. Samples were stored in that mixture initially at -20 C before being shipped on dry ice to another facility where they were stored at -80 C until analysis.

For microbe counting, samples were thawed and either the entire rhizosphere pellet (for plant samples), or an aliquot of bulk soil (for control box samples) that had similar volume to that of a typical rhizosphere pellet was used. To the soil sample (either rhizosphere or bulk), 0.3 to 0.4 g of sterile garnet beads were added along with enough water to bring the total volume up to 1.5 mL. This mixture was vortexed for 45 seconds. Then 98 µL was pipetted into a fresh tube where it was mixed with 2 µL of a 100X Vybrant Green DNA stain. One µL of the stained cell suspension was placed onto a slide and imaged with a 40X NA1.1 water immersion objective lens on a Zeiss LSM 710 inverted confocal fluorescence microscope exciting the dye with a 488 nm laser and measuring fluorescence in the 497-590 nm band. To count the microbes, the images were uploaded into ImageJ (Abramoff et al. 2004; Collins 2007) and the 3D Objects Counter function was used to classify fluorescent objects between 0.5 μm^3 and 3.2 μm^3 as microbes. The combined mass of water and soil in each tube was measured then the soil was dried overnight in an oven. Those measurements were used to calculate the dry mass of soil per volume of water. By dividing the total cell count by the mass of solids in the droplet which was imaged, the mass-normalized cell density was calculated. Any sample which had less than 0.5 mg of soil in the 98 µL aliquot was excluded from analysis.

4.2.2 *DNA Sequencing*

For DNA sequencing, approximately 1 mL of soil was collected from each sample site for all three treatment types. The DNA was extracted from the peat using a MoBio PowerSoil kit, with modifications made to optimize the kit for extractions from peat soils (See Appendix 1). An initial quality check was performed according to the Department of Energy Joint Genome Institute (JGI) "iTag Sample Amplification QC SOP" v. 1.3 (Appendix 2). Briefly, this protocol involved amplifying an aliquot of the DNA sample using PCR, then visualizing the amplified product on an agarose gel along with a set of size standards to ensure that the amplified DNA strands were long enough for sequencing. The extracted and quality controlled DNA was stored at -20 C until transport to JGI for analysis.

We shipped the DNA samples to JGI on dry ice. Once there, the samples were processed to produce one of two sequencing products: iTags or metagenomes. The iTag product is a phylogenetic classification to the genus level using the V4 region of 16S rRNA sequences (Rivers 2016). Sequencing and classification was done using an Illumina MiSeq instrument and the iTagger software (Tremblay et al. 2015). The methods summary produced by JGI is attached as Appendix 3. The metagenomes were sequenced on an Illumina NovaSec instrument. The reads were trimmed and screened using the BBTools software (Bushnell 2015) and read corrected using BFC version R181 (Li 2015). The corrected reads were assembled and mapped using SPAdes assembler 3.11.1 (Nurk et al. 2017) and BBMap version 37.78 (Bushnell 2015), respectively. All analysis of metagenomic data was done through the JGI IMG interface (Markowitz et al. 2012; Chen et al. 2019).

4.2.3 *Statistical Analysis*

The Kruskall-Wallace test was performed using the "kruskalwallis" function in MATLAB (R2018b) to determine whether multiple groups of data were from the same distribution or not. All test for relationships or trends within a dataset were done using a linear regression with the “fitlm” function in MATLAB (R2018b). This function assigns p-values to the regression coefficient using the t-statistic.

4.2.4 *Sequence Data Analysis*

We analyzed the iTag data for the frequency of methanogens and methanotrophs. For methanogens, the classes Methanobacteria and Methanomicrobia were used. For obligate methanotrophs, all members of the family Methylocystaceae, as well as the entire order Methylococcales were included. The iTag data did not include sufficient detail to differentiate facultative methanotrophs of the genera *Methylocapsa* and *Methylocella* from other members of their family, and so metagenomic data was used for facultative methanotroph analysis. The genus *Methylocystis* was counted as facultative methanotrophs.

To determine whether samples had microbes with aerobic or anaerobic metabolisms present metagenomes were used to determine the ratio of genes involved in glycolysis (anaerobic metabolism) to genes involved in the Krebs cycle (aerobic metabolism). For the Krebs cycle, only those genes involved in the first oxidation were used because that limited the number of genes involved and focused the results.

The gene sets were identified through the KEGG Orthology (Kanehisa and Goto 2000; Kanehisa et al. 2016). The gene sets used for the Krebs cycle were K00030 isocitrate dehydrogenase (NAD+) (Kim et al. 1999), K00031 isocitrate dehydrogenase (Camacho et al. 1995; Steen et al. 1997; Ceccarelli et al. 2002), K01647 citrate synthase (Goldenthal et al. 1998), K01681 aconitate hydratase (Varghese et al. 2003), and K01682 aconitate hydratase 2 / 2-methylisocitrate dehydratase (Brock et al. 2002).

The gene sets used for glycolysis were K00134 glyceraldehyde 3-phosphate dehydrogenase (Prüß et al. 1993; Sirover 2011), K00150 glyceraldehyde-3-phosphate dehydrogenase (NAD(P)) (Valverde et al. 1997; Koksharova et al. 1998), K00873 pyruvate kinase (Kenzaburo et al. 1988; Mazurek 2011), K00927 phosphoglycerate kinase (Schurig et al. 1995; Beutler 2007), K01689 enolase (Feo et al. 2000; Marcaida et al. 2006), K01803 triosephosphate isomerase (TIM) (Daar et al. 1986; Schurig et al. 1995), K01834 2,3-bisphosphoglycerate-dependent phosphoglycerate (Johnsen and Schönheit 2007; Davies et al. 2011), K11389 glyceraldehyde-3-phosphate dehydrogenase (ferredoxin) (Mukund and Adams 1995), K12406 pyruvate kinase isozymes R/L, K15633 2,3-bisphosphoglycerate-independent phosphoglycerate (Fraser et al. 1999), K15634 probable phosphoglycerate mutase (Johnsen and Schönheit 2007), and K15635 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Johnsen and Schönheit 2007).

4.3 RESULTS

4.3.1 *Fluorescence Microscopy*

The soil collected from near the roots of plants during week 10 after the start of the experiment had a significantly ($p < 0.05$) higher concentration of microbes than did the unplanted control box soil, which was also harvested during week 10 (Figure 4.1 A). The soil from near the roots of plants harvested in week five of the experiment did not have a significantly different number of microbes from either the week 10 plants or the control boxes. The comparison of the three groups indicates that roots encouraged microbial growth, but that it took time for the increased growth to take effect. However, regressing total microbe count against CH_4 emissions did not yield a statistically significant correlation, though it did have some explanatory power and was on the edge of significance ($p = 0.054$, $R^2 = .39$).

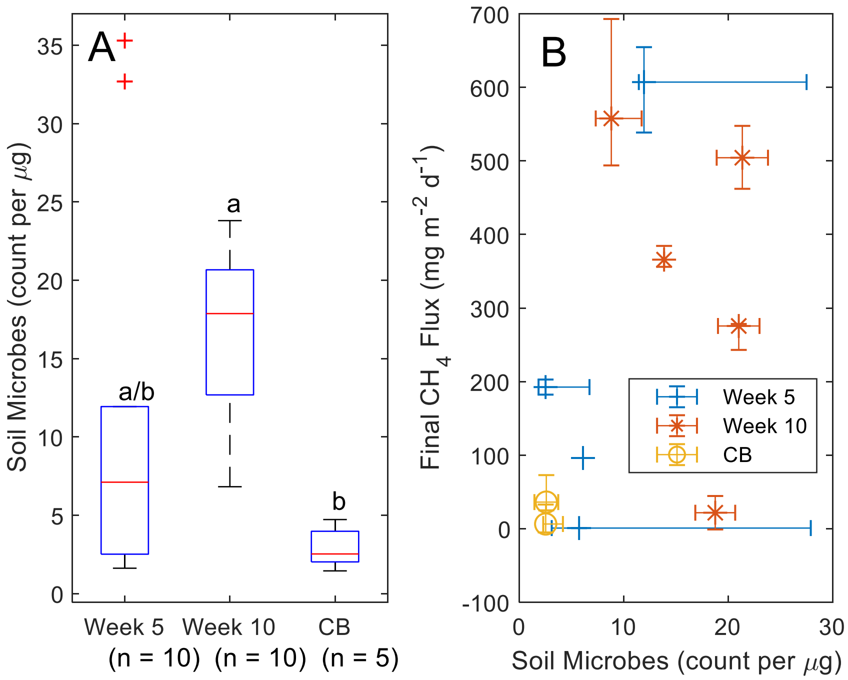

Figure 4.1. A) Microbe counts in soil from planted boxes at week 5 and week 10 compared to unplanted control boxes (CB) at week 10. Data with the same lower case letter were not statistically different (ANOVA, p <0.05) Boxes show median with upper and lower quartiles, and tails show all data within 2.7σ of the mean. B) Methane emissions the week prior to harvest compared to soil microbe counts. Error bars are upper and lower quartiles.

4.3.2 *iTag*

The percentage of microbes that were methanogens was correlated with CH_4 emissions (Fig. 4.2, $R^2 = .39$, $p < 0.05$) as was the percentage of microbes that were methanotrophs, when excluding simulated plants ($R^2 = .77$, $p < 0.01$). Simulated plants were excluded from the linear fit of methanotrophs because in the other three box types (planted boxes from weeks 5 and 10 and control boxes) the oxygen concentration was similarly low, but in simulated boxes the oxygen concentrations were higher so the methanotrophs faced a fundamentally different environment (Fig. 4.2). When combined with the microbe count data to find total microbial numbers, there were positive correlations between CH_4 flux and both methanogen count ($R^2 = .53$, $p < 0.05$) and methanotroph count ($R^2 = .64$, $p < 0.01$). Despite both being correlated with CH4 flux, there was no correlation between methanotroph abundance and methanogen abundance (SI Figure S1).

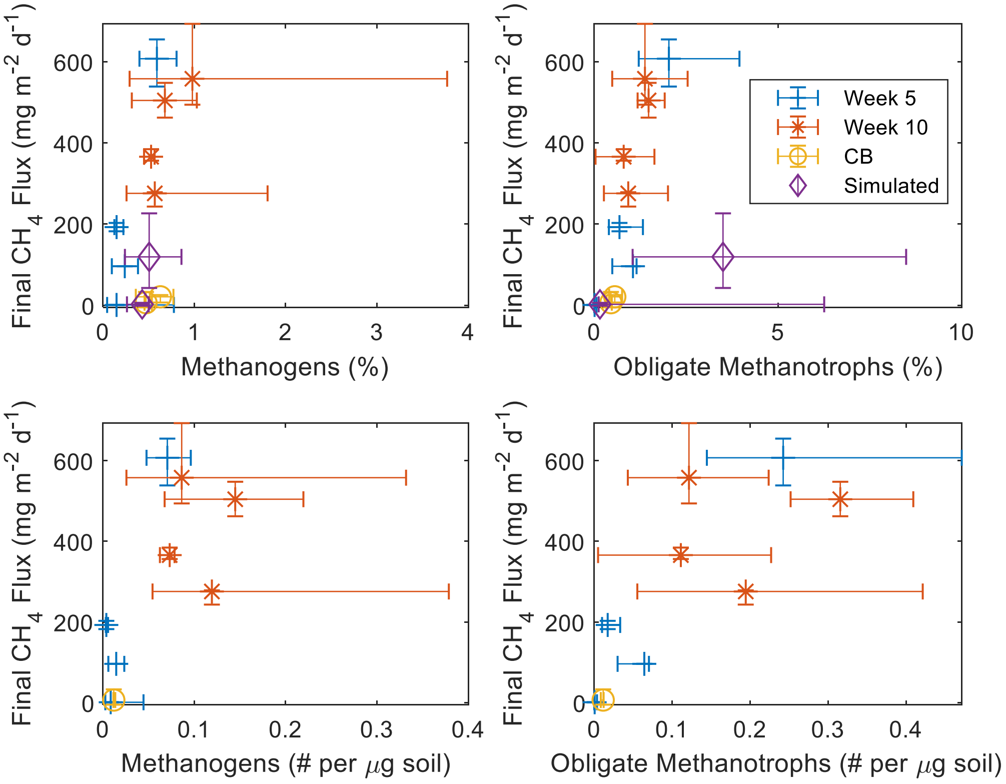

Figure 4.2. CH4 flux versus methanogen abundance (left) and CH4 flux versus methanotroph abundance (right), with abundance measured both by percentage of genes (top) and total microbe count (bottom). Error bars indicate 25th and 75th percentile of the data.

4.3.3 *Metagenomes*

The facultative methanotrophs comprised less than 1% of all samples (Fig. 4.5A). There was no statistically significant ($p > 0.05$) relationship between the flux of CH_4 in the final week before harvest and either the percentage of facultative methanotrophs (Fig. 4.5A) or the number of facultative methanotrophs (Fig. 4.5B). However, the percentage of microbes that were facultative methanotrophs in simulated plant boxes were higher than the other box types, similar to the pattern observed in obligate methanotrophs (Fig. 4.2).

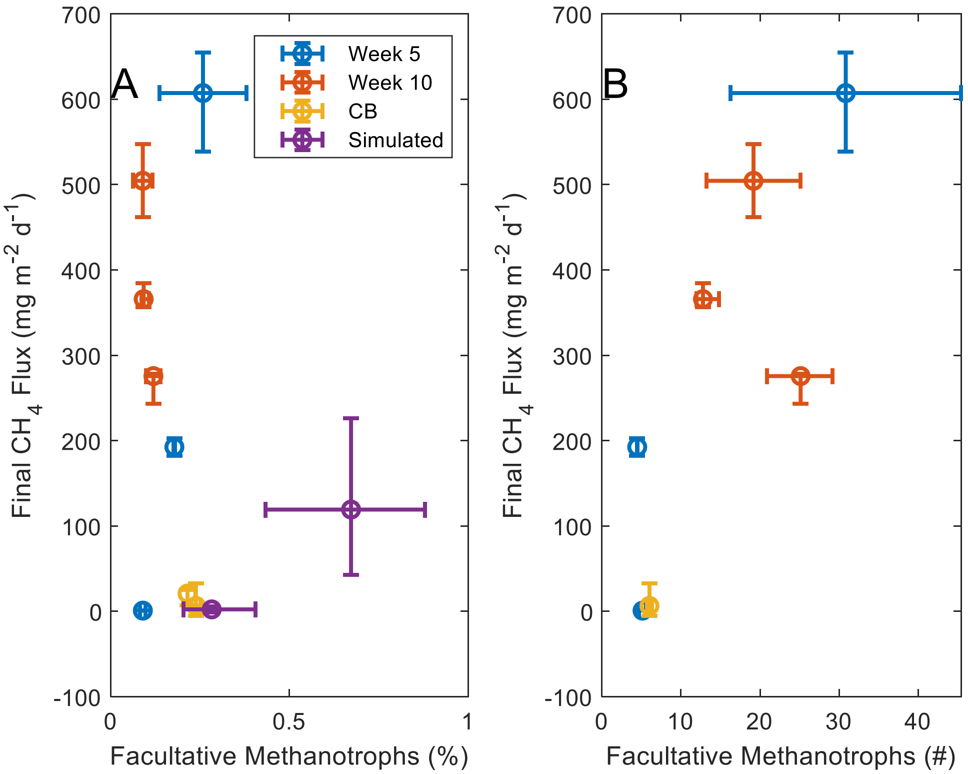

Figure 4.3. CH_4 emissions in the week prior to harvest versus (A) percentage or (B) number of facultative methanotrophs. There was no statistically significant trend ($p > 0.05$) for either.

The ratio of obligate to facultative methanotrophs was significantly higher in week 10 than in simulated boxes, while the other two types had intermediate values that were not significantly different ($p > 0.05$) from any other types (Fig. 4.4A). There was no significant relationship ($p > 0.05$) between the ratio of obligate to facultative methanotrophs and the flux of CH_4 in the final week before harvest (Fig. 4.4B).

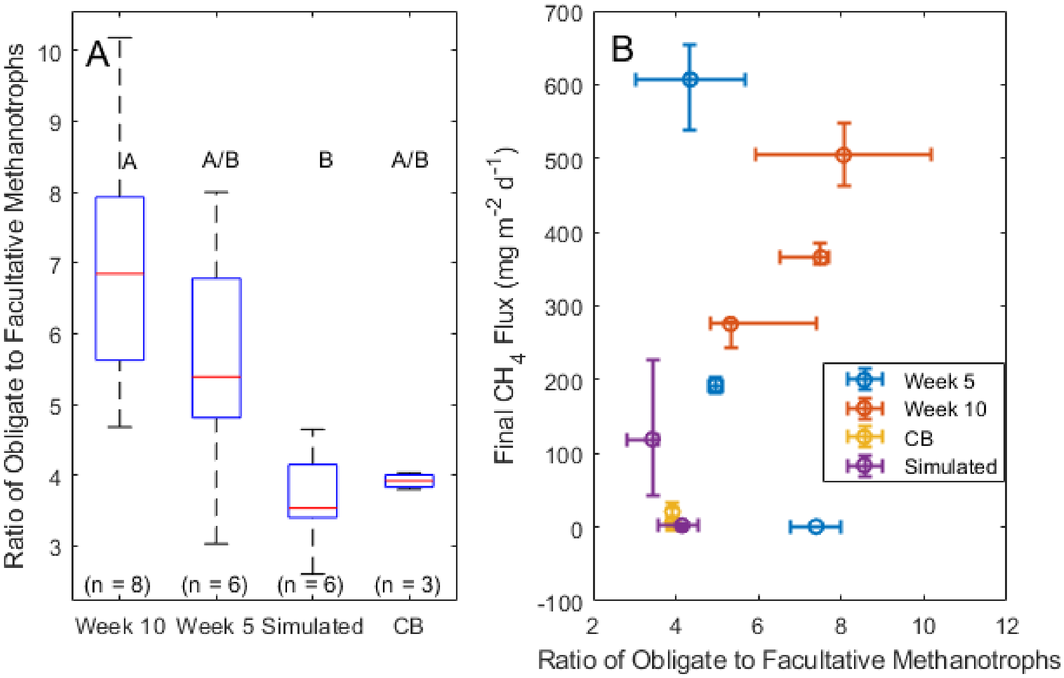

Figure 4.4. (A) Boxplots comparing the ratio of obligate to facultative methanotrophs across sample types. The planted boxes harvested in week 10 had a significantly higher ($p < 0.05$) ratio than simulated boxes, while the other two types had intermediate values that were not significantly different ($p > 0.05$) from any other types. (B) Scatterplot showing the relationship between the ratio of obligate to facultative methanotrophs and the CH4 flux in the final week before harvest. There was no statistically significant relationship ($p > 0.05$).

The ratio of genes involved in glycolysis to those involved in the Krebs Cycle was higher in boxes with higher CH_4 emissions (Fig. 4.5), indicating more anaerobic activity in these boxes.

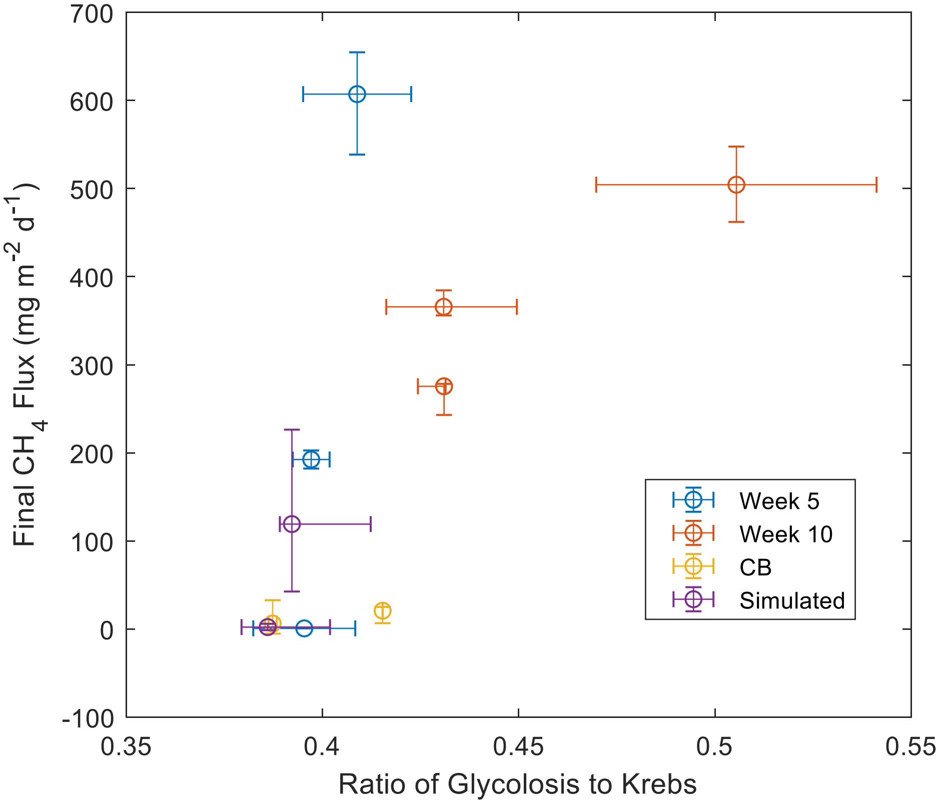

Figure 4.5. CH_4 emissions in the week prior to harvest versus the ratio of genes involved in glycolysis to genes involved in the Krebs cycle. The plot shows that there was a positive but non-linear relationship.

4.4 DISCUSSION

To determine how plant roots impact the potential for the rhizosphere microbial community to influence CH_4 emissions, both total microbial populations and community composition play a role. Finding increased numbers of microbes in the rhizosphere of planted boxes harvested in week 10 (Fig. 4.1) was expected. The first study based on this same experiment found that more root exudates were being added to the soil during week ten than during week five (Waldo et al. 2019a). Given that root exudates fuel microbial metabolism (Ström et al. 2003; Ström and Christensen 2007; Picek et al. 2007; Chanton et al. 2008; Kayranli et al. 2009), the increased root exudation is the most likely explanation for the increased microbial population. However, the lack of significant correlation between microbe count and

CH_4 emissions shows that changes to the composition of the microbial community were more important than its sheer size.

Methanogens and methanotrophs are directly involved in CH_4 dynamics. The correlation between methanogens and CH_4 emissions (Fig. 4.2) is straightforward and unsurprising. The positive relationship between obligate methanotrophs and emissions (Fig. 4.2) tells us more about the system. Obligate methanotrophs rely on both CH_4 and TEAs to function. The samples from planted boxes at both time points and those from control boxes all were from anoxic sites (Waldo et al. 2019a), and the ratio of glycolysis to Krebs Cycle genes from the metagenomic data (Fig. 4.5) indicate the boxes producing the most CH_4 also had the highest prevalence of anaerobes in the microbial community. The increased prevalence of anaerobic metabolisms indicates that oxygen available relative to demand was lower. When demand exceeds supply, competition for oxygen must be more intense. Obligate methanotroph abundance was apparently dependent on the concentration of CH_4, despite the increasing competition for oxygen in those boxes with high CH_4 emissions and highly anaerobic metabolisms. The samples from simulated plants, however, were taken from oxygenated sites (Waldo et al. 2019a). The increased supply of oxygen relieved the competition for oxygen and improved the environment for methanotrophs without increasing CH_4 concentration. That increased supply of oxygen explains why methanotrophs were so abundant in simulated plant boxes, despite the lower fluxes of CH_4.

The apparent success of methanotrophs in environments with highly anaerobic metabolisms has two potential explanations. First, there is likely some oxygen available. While the optodes showed no detectible oxygen in any of the planted boxes (Fig. 2.1), the fact that the ratio of anaerobic to aerobic metabolisms increased with increasing CH_4 emissions indicates that oxygen availability was variable; oxygen was simply used so rapidly in all cases that it did not accumulate enough for the optodes to detect it. Second, the obligate methanotrophs may have been performing AMO. While the soil in this experiment likely had very low concentrations of alternative TEAs, as is generally the case in freshwater bogs (Keller and Bridgham 2007; Conrad 2009), there may actually still be high rates of AMO near oxygen sources where an elevated redox state allows for recycling of low concentrations of TEAs (Keller and Bridgham 2007; Segarra et al. 2015).

Facultative methanotrophs, however, displayed a different pattern than the obligate methanotrophs (Figs. 4.2-4.4). Both types were most common in the simulated-plant boxes where oxygen was most abundant. However, while in the other box types obligate methanotrophs had a significant positive ($p < 0.05$, $R^2 = 0.35$) relationship with CH_4 emissions (Fig. 4.2) whether measured by percentage of genes or by number of microbes, the facultative methanotrophs correlation was not significant ($p > 0.05$) by either percentage or number of cells (Fig. 4.3). These different patterns imply that in low-oxygen environments with high rates of metabolic activity such as the rhizosphere examined in this study (Waldo et al. 2019a) obligate methanotrophs were able to out-compete facultative methanotrophs. This is reinforced by the observation that the obligate to facultative ratio is significantly higher in the rhizosphere from the end of the experiment than it was in unplanted control soil (Fig. 4.4A). Because CH_4 is generated in anoxic environments, being more able to oxidize it at low oxygen levels could confer a strong advantage to the obligate methanotrophs over their facultative competitors. Obligate methanotrophs have been hypothesized to exist because their extreme specialization gives them a competitive advantage over more generalist microbes (Dunfield and Dedysh 2014). The recent finding that facultative methanotrophs are common around natural gas seeps where other molecules they can utilize are abundant (Farhan Ul Haque et al. 2018) supports the notion that facultative methanotrophs are generalists that do well when conditions do not suit the more specialized obligate methanotrophs. The advantage possessed by the obligate anaerobes in this study could have been either an increased affinity for oxygen, allowing them to collect what little was available, or perhaps the ability to perform types of AMO that the facultative methanotrophs could not.

4.5 CONCLUSIONS

The most direct measures of the methanogenic potential of the microbial community behaved as expected: methanogens were positively correlated with CH_4 emissions (Fig. 4.2) and were most common in the boxes with real plants, genetic indicators of oxygen limitation were highest in the boxes with the highest CH_4 emissions (Fig. 4.6), and microbial populations were largest in number when the most root exudates were available (Fig. 4.1). The methanotrophic community demonstrated a competitive difference between obligate and facultative methanotrophs. Both types were most abundant around the simulated plants where oxygen was abundant, but in the rhizosphere and control box soil where oxygen was limited obligate methanotroph abundance was correlated with CH_4 availability (Fig. 4.2), while facultative methanotroph abundance was not (Fig. 4.5). This implies that in low-oxygen, high CH_4 environments the highly specialized obligate methanotrophs were able to out-compete the more generalist facultative methanotrophs through either an increased affinity for oxygen or a greater ability to perform AMO.

4.6 FUTURE RESEARCH

4.6.1 *Objective*

The genetic data presented in this chapter are a rich resource, and there are certainly important conclusions which may be reached beyond the complex methanotrophy hypothesis presented above. While many avenues of future research on these data are possible, a particularly valuable investigation could be made into the question of what microbes are responsible for processing the carbon exuded by plants from the large, complex molecules that come out of the plant into the acetate or H_2 needed by methanogens.

Because it is impractical to assess the role of all the multitude of microbes present in the soil, studies often focus on a key metabolism of interest and aim to identify the microbes which participate in that metabolism. For example, studies of northern wetlands have found which microbes are responsible for degrading litter from *Sphagnum* mosses (Ivanova et al. 2016) or cycling iron as an electron acceptor (Lipson et al. 2013). This approach has its limitations, however. In seeking to understand how plant roots impact CH_4 emissions there are multiple metabolisms that impact emissions including methanogenesis, methanotrophy, anaerobic fermentation of complex molecules into the precursors for methanogenesis, and aerobic respiration which can compete with methanogenesis for limited oxygen. While changes in the relative abundance of methanogens would certainly be relevant, there are too many processes that contribute to the complete system to investigate them all, especially given the uncertainties associated with correlating community composition with function (Wang et al. 2019).

An alternate approach is to begin with multiple treatment types or field sites, identify taxonomic differences, and infer functional differences from there. This was the approach taken by Robroek et al. (2015) in a study which found that removing one or two plant functional types from field plots altered the soil microbial community composition as well as CH_4 production and CH_4 oxidation potentials. However, that study assessed changes to microbial community structure using phospholipid fatty acids and qPCR on a limited number of genes, so they were only able to identify functional changes without being able to precisely identify the taxa involved.

We have made initial attempts to describe the microbial community supporting methanogens using that second approach.

4.6.2 *Initial Methods*

We used two metrics to attempt to identify genera from the iTag data which were relevant to CH_4 emissions. Both metrics were only applied to genera which comprised greater than 2.5% of all iTag reads. One metric was a Kruskall-Wallace non-parametric test to determine if there was a highly significant difference between box types ($p < 0.01$). The other metric was to test for a significant linear correlation with CH_4 emissions ($p < 0.05$) which explained at least 10% of the variation in CH_4 emissions ($R^2 > 0.1$).

4.6.3 *Initial Results*

Table 4.5 shows the list of the 15 most common classes of microbes identified by iTags, averaged across all samples. These classes comprised a mean of 75.8% of genes across all samples.

Table 4.5. List of common classes identified by iTag data.

Class	**Average % of iTags**
d:Bacteria;p:Proteobacteria;c:Alphaproteobacteria	12.3
d:Bacteria;p:Acidobacteria;c:Acidobacteria	10.0
d:Bacteria;p:Proteobacteria;c:Deltaproteobacteria	7.8
d:Bacteria;Other;Other	7.3
d:Bacteria;p:Proteobacteria;c:Betaproteobacteria	6.0
d:Bacteria;p:Verrucomicrobia;c:OPB35_soil_group	6.0
d:Bacteria;p:Proteobacteria;c:Gammaproteobacteria	4.2
d:Bacteria;p:Bacteroidetes;c:Sphingobacteriia	4.1
d:Bacteria;p:Verrucomicrobia;c:Opitutae	4.1
d:Bacteria;p:Actinobacteria;c:Thermoleophilia	3.5
d:Bacteria;p:Chlorobi;c:Ignavibacteria	3.1
d:Bacteria;p:Spirochaetae;c:Spirochaetes	2.2
d:Bacteria;p:Bacteroidetes;Other	1.9
d:Bacteria;p:Actinobacteria;c:Actinobacteria	1.7
d:Bacteria;p:Chloroflexi;c:KD4_96	1.6

Five genera were identified as being both highly significantly different between sample types ($p < 0.01$) and abundant (mean abundance of over 2.5% in at least one sample type). Three of the five genera exhibited a similar pattern in which they were most abundant in week ten, less abundant in week five and in the control boxes, and least abundant in the simulated boxes. These three genera were an unknown genus of the class Actinobacteria, an unknown genus of the class Chloroflexi, and *Opitutus* of the Opitutae class. Different patterns were displayed by the other two genera. Unclassified members of the order Acidobacterialis were most common in control boxes, followed by week five samples, then simulated plants, and were least common in week 10 planted boxes. Finally, an uncultured genus of the class Bacteroidetes was more common in the simulated plant boxes than in any of the anoxic samples.

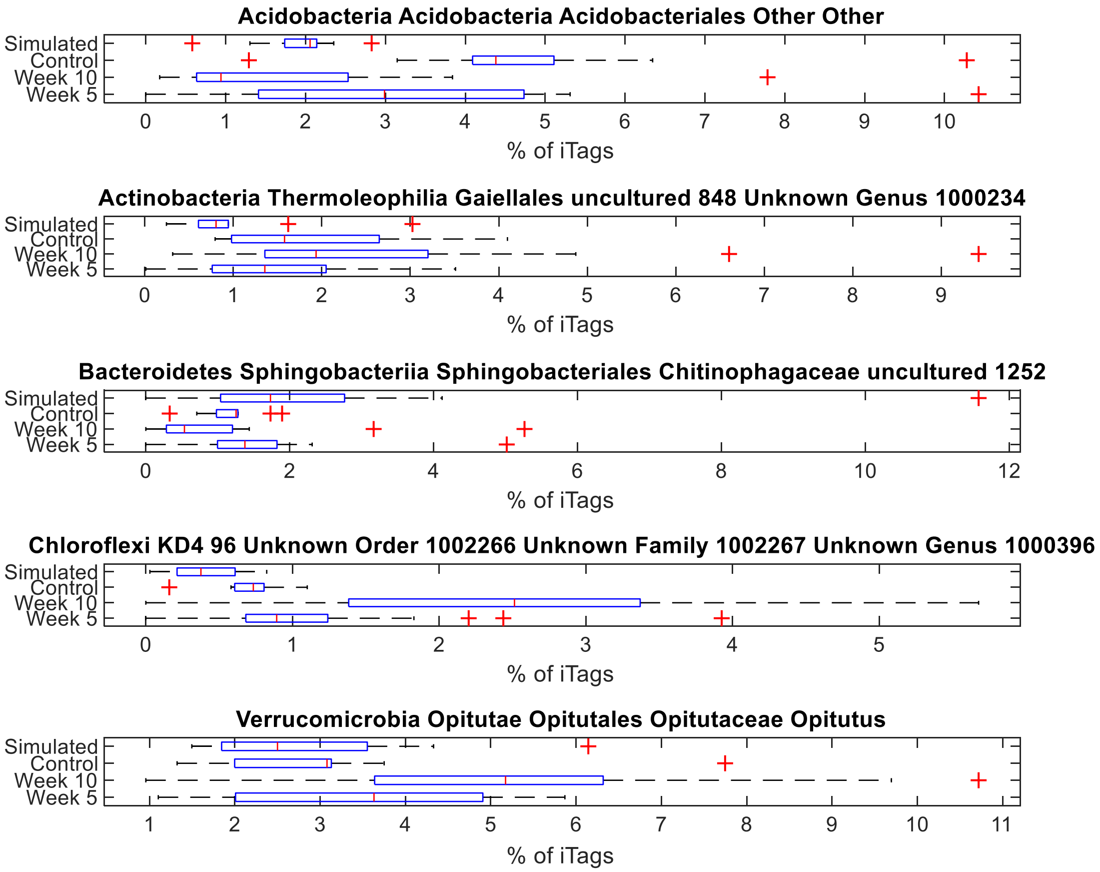

Figure 4.6. The abundance of genera that were both highly significantly different between sample types ($p < 0.01$) and abundant (mean abundance of over 2.5% in at least one sample type). An unknown genus of Actinobacteria, an unknown genus of Chloroflexi, and Opitutus were all most abundant in Week 10. Unclassified Acidobacteria were most common in control boxes, and an uncultured genus of Bacteroidetes were most abundant in simulated plants.

Five genera were identified as being significantly correlated with CH_4 emissions ($p < 0.05$ and $R^2 > 0.1$) and abundant (mean abundance of over 2.5% in at least one sample type). Three of these correlations were positive and two negative. The positively correlated genera were a lumped group of all bacteria that were not classified at any level, an unknown genus of the class Actinobacteria, and *Opitutus* of the Opitutae class. The negative correlations were unclassified members of the Acidobacteria class and an uncultured genus of the class Bacteroidetes. The unclassified Acidobacteria were notable in that they were far less abundant in simulated plants than the correlation predicted.

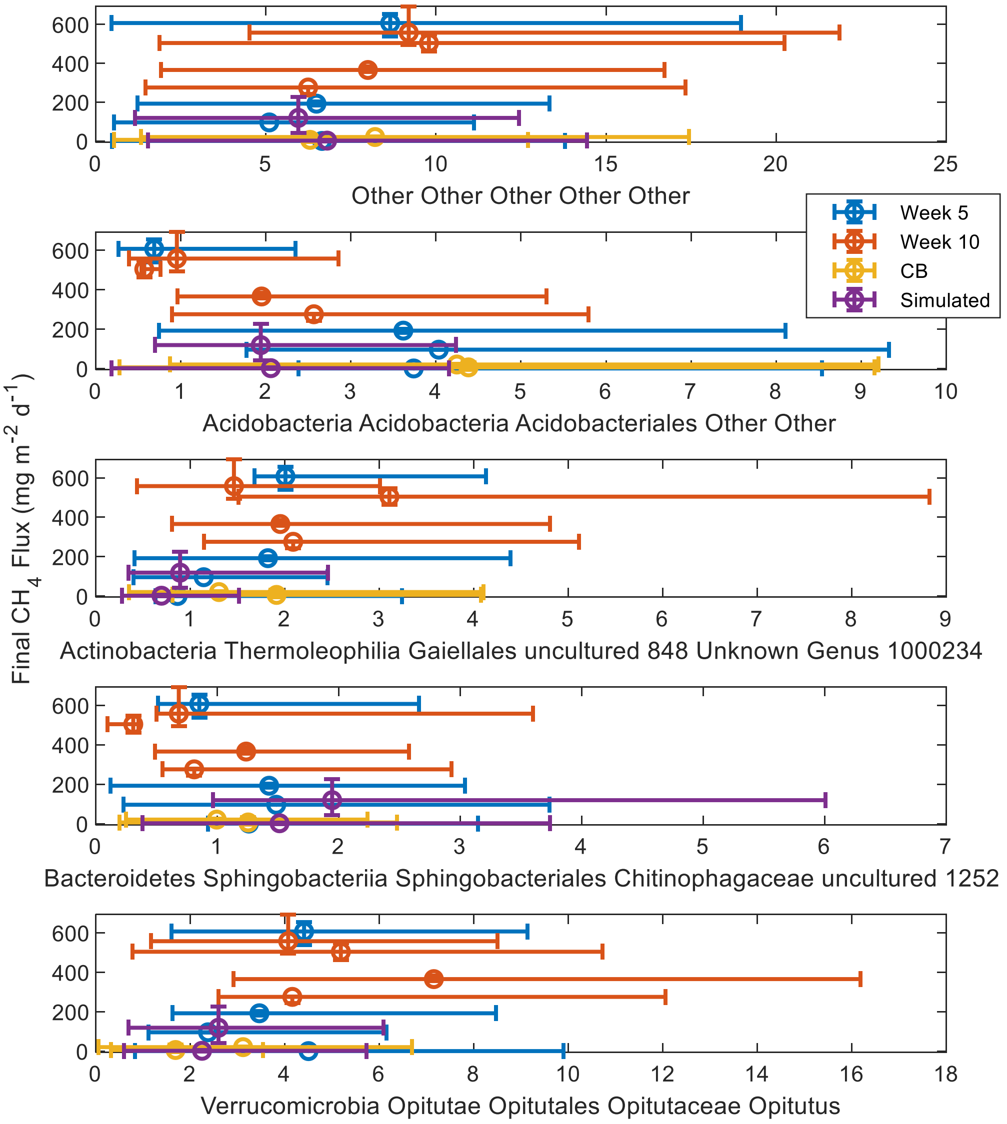

Figure 4.7. The abundance of key genera versus CH4 emissions. Error bars indicate upper and lower quartiles of microbial abundance and of CH4 emissions from the final week before harvest. Positive correlations were found for unclassified bacteria, an unknown genus of Actinobacteria, and Opitutus. Negative correlations were found for unclassified Acidobacteria and an uncultured genus of Bacteroidetes.

The metagenomic data were able to identify with more precision which taxa comprised the *Opitutus* genus and the Acidobacteriales order. All species within *Opitutus* which were identified by metagenomes are listed in Table 4.5 along with their correlations with CH_4

emissions ($p < 0.05$). The only family within Acidobacteriales was Acidobacteriaceae, so all genera within that family are presented. Species present and other additional information are only provided for those genera which had a statistically significant correlation with CH_4 emissions ($p < 0.05$).

Table 4.6. Details from the metagenomes on those taxa identified in the iTag data as being of the highest interest. All data about in the Source and Notes columns are from the IMG database unless otherwise indicated (Markowitz et al. 2012; Chen et al. 2019).

Opitutus Species	**% of genes**	**Taxon ID**	**Correlation with CH_4 emissions**	**Source**	**Notes**
Terrae	45.6	641522643	Positive	Rice Patty Soil	Anaerobic
GAS368	31.2	2690315651	Positive	Forest Soil	Harvard Forest, MA
ER46	19.1	2788500351	Negative	Plant-associated	Aerobic
unclassified	4.2	2603880164, 2687453757	Positive	Insect gut microbiome	Symbiotic
Acidobacteriaceae Genera and species	**% of genes**	**Taxon ID**	**Correlation with CH_4 emissions**	**Source**	**Notes**
unclassified	36.0		None (p>.05)		
Candidatus Koribacter	18.6		None (p>.05)		
Granulicella (5 species)	11.3	multiple	Negative	multiple	Aerobes (Pankratov and Dedysh 2010)
Acidobacterium	8.5		None (p>.05)		
Terracidiphilus	6.8		None (p>.05)		
Silvibacterium	5.4		None (p>.05)		
Terriglobus	5.0		None (p>.05)		
Edaphobacter aggregans, dinghuensis	3.9	2785510734, 2747843220	Negative	Forest Soil (both species)	Aerobe (aggregans)
Acidipila rosea	2.3	2757320406	Negative	Type Strain	Aerobe
Bryocella	2.2		None (p>.05)		

4.6.4 *Discussion of Initial Results*

The other microbes identified as being abundant and important were not directly involved in either the production or oxidation of CH_4. The fact that they were largely uncultured or unclassified makes it harder to identify why they were correlated with CH_4 emissions and/or vary between treatment types. However, examination of the abundance patterns they exhibit and the other information published about these taxa provide hints as to whether each genus was actively involved in a metabolism that led to methanogenesis, or if its abundance was merely correlated due to thriving in similar environments to those microbes which contributed to precursors of methanogenesis.

The *Opitutus* genus within the class Opitutae of the phylum Verrucomicrobia was the only genus in the iTag data which was: 1) significantly correlated with CH_4 emissions (Fig. 4.4) 2) significantly different between the treatment types (Fig. 4.3) and 3) formally classified at the genus level (Chin et al. 2001). Switching from the iTag data to the metagenomes, three known species of *Opitutus* were present in these samples as well as two unclassified genomes. *O. terrae* was the most common *Opitutus* species (Table 2)(Passel et al. 2011). This species is an obligate anaerobe known to ferment monosaccharides and polysaccharides into acetate and propionate (Chin et al. 2001). That metabolism and these data are entirely consistent with a species that utilizes sugars exuded as root exudates and anaerobically ferments them into acetate, which is then used by methanogens. This indicates that it was probably not a coincidence that *Opitutus* were correlated with methanogenesis, and they were in fact a meaningful player in the supporting microbial community.

The only family within Acidobacteriales identified by the metagenomes was the Acidobacteriaceae, indicating that the Acidobacteriales which could not be classified by iTag analysis were likely Acidobacteriaceae. These microbes were most common in the control boxes, least common in planted boxes harvested in week 10, and negatively correlated with CH_4 emissions. Together, these data indicate that Acidobacteriaceae did not gain any advantage from the changes caused by roots, and so were out-competed when other microbes begin to thrive in the presence of root exudates. The Acidobacteriaceae are generally aerobic (Campbell

2014) and the most of the species identified by metagenomics which were correlated with CH_4 emissions were also aerobes (Table 4.6)(Pankratov and Dedysh 2010). While aerobic microbes being negatively correlated with CH_4 emissions (Fig. 4.6) makes sense, a more surprising result was that the iTag data showed Acidobacteriales comprised less than half as much of the total microbial population in the simulated plant boxes as in the control boxes (Fig. 4.3). The only difference between these treatments was the increased oxygen availability in the simulated plant boxes, so aerobic microbes should have, if anything, had higher relative abundance in the simulated plant boxes. It is possible that some of the Acidobacteriaceae in the control boxes were actually anaerobes. If true, this would change the common understanding of the metabolism of this family (Campbell 2014), but this study not have the data here to evaluate this hypothesis. An alternate explanation would be that the Acidobacteriales identified by iTag were not Acidobacteriaceae, but in fact some other family which was not identifiable by the metagenomes.

Three other genera were abundant (mean abundance of over 2.5% in at least one sample type) and either significantly different between box types or significantly correlated with CH_4 emissions (Figs. 4.3 &4.4). There is not adequate data to connect the uncultured members of Actinobacteria or Bacteroidetes to genomes with known metabolisms. Other members of the Chitinophagaceae family, to which the uncultured Bacteroidetes belonged, are facultative anaerobes (Rosenberg 2014). If the same is true of this species, then it would make sense why it did not have significantly different abundances between treatment types, even though they were negatively correlated with CH_4 emissions. Other members of the order Gaiellales, to which the uncultured Actinobacteria belonged, are mild acidophiles and obligate aerobes (Albuquerque et al. 2011). This is likely not true of the uncultured Gaiellales identified here since they were positively correlated with CH_4 emissions (Fig. 4.4) and were more common in control boxes than in simulated plants (Fig. 4.3). The final genus which appeared in at least one of these metrics was of the phylum Chloroflexi, but their taxonomy is unknown at any more detailed level of classification. These microbes were far more common in week 10 boxes than the other types, but given the diversity that exists within Chloroflexi it is not reasonable to speculate why.

Comparing this profile to those previously reported reveals broadly similar composition at the phyla level, with two exceptions. Acidobacteria comprised an average of only 11.1% of these samples, as opposed to 30 to 65% of the profiles reported from samples collected either directly from the field (Lin et al. 2012; Serkebaeva et al. 2013) or from unamended incubation experiments (Pankratov et al. 2011; Ivanova et al. 2016), and Bacteroidetes comprised an average of 10.5% of these samples, while they never exceeded one percent in the other studies examined (Pankratov et al. 2011; Lin et al. 2012; Serkebaeva et al. 2013; Ivanova et al. 2016). However, Pankratov et al. (2011) also conducted incubations of peat with additions of cellulose or cellulose plus nitrogen which resulted in abundances of both Acidobacteria and Bacteroidetes closer to these results. In this study, Acidobacteria were least abundant in the week 10 samples and most abundant in the control boxes, indicating that the root exudates released by the plants led to a similar shift in microbial community composition to the cellulose additions of Pankratov et al. (2011).

4.6.5 *Future Directions*

As demonstrated by the lack of clear and concise conclusions reached in the above discussion, much work remains to be done on this line of research. While these attempts did produce some hints towards the ecological niches of specific genera, they did not lead to a robust description of the community which collectively supports methanogens. Future work could start from these attempts, but also select for genes related to the specific metabolisms known to produce acetate and H_2.

Chapter 5. CONCLUSIONS

As the boreal region changes, understanding the processes controlling CH_4 emissions will have increased importance. Vascular plants play a key role in increasing CH_4 emissions by altering the soil microbial environment. This study examined the mechanisms by which plants cause this increase in emissions, finding roles for both increased production of CH_4 through microbial priming and changes to the methanotrophic community.

The isotopic mixing model showed that the large increase in CH_4 emissions caused by plants in this experiment was driven not only by the direct conversion of root-derived carbon into CH_4, but also by an order-of-magnitude increase in the amount of soil-derived CH_4 emitted (Fig. 2.7). Because reductions in methanotrophy can only account for a small portion of this increase, microbial priming must have occurred here. Microbial priming is an active area of research with recent studies showing evidence both for (Ye et al. 2015; Robroek et al. 2016) and against it (Graham et al. 2017; Girkin et al. 2018) in various environments. Finding such strong evidence for priming in this study will help advance this dynamic debate. Extrapolating this finding to actual wetlands instead of laboratory experiments could imply that a large mass of soil carbon in the boreal region could be destabilized by increasing plant productivity. After all, northern peatlands contain 25% of the world's soil carbon, despite only covering 2% of surface area (Yu 2012). Further work is needed to replicate these findings in a field setting, and modelling should be done to determine how a large priming effect shifts the overall carbon balance of boreal wetlands.

Characterization of the nature of carbon compounds degraded allowed us to explore the root causes of priming. Understanding the mechanisms behind the phenomenon is key to establishing why priming has been observed to occur in some settings, but not others. The signature of priming observed here indicates two previously described mechanisms are both at work. The increased degradation of large compounds (Fig. 3.2) suggested that the microbial community was consuming more carbon than in unprimed peat, likely due to larger microbial population (Ye et al. 2015). This increased population continued to display the well-documented preference for large, energy-rich compounds (Amon and Benner 1996; McArthur and Richardson 2002; Antony et al. 2012; Pracht et al. 2018). However, the observation that small, nitrogen rich compounds were also processed at an increased rate (Fig. 3.3) lends credence to the N-mining hypothesis, which states that microbes were breaking down less energetically favorable compounds in order to access nutrients (Craine et al. 2007; Ruirui et al. 2014). Importantly, evidence for priming in the current study was found predominantly in data from water-soluble compounds. Graham et al. (2017) recently generated much attention for the study's argument against priming based on evidence that non-soluble compounds were preserved following addition of root exudates. The author suggests that the Graham et al (2017) does not necessarily contradict priming, but rather shed light on which pools of carbon are impacted. Just as the scientific understanding of bioavailability has evolved from "labile" and "recalcitrant" into a nuanced understanding that environmental factors dictate microbial preferences, so the conception of priming must shift from a simplistic statement that more carbon is used into a nuanced description of the types of carbon primed or preserved under various conditions.

In addition to priming, decreased methanotrophy may also have increased soil methane emissions. At the outset of the study, the author assumed that the percentage of CH_4 oxidized would be lower in the planted boxes than in the control boxes. The assumption was based on a companion field study (Turner et al. 2019). However, metagenomic data showed that the planted boxes harvested at the end of the experiment – which had the largest microbial populations (Fig. 4.1) and the most anaerobic metabolisms (Fig. 4.5) – also had the highest relative abundance of methanotrophs (Fig. 4.2). This suggested that the increasing availability of CH_4 was more important to methanotrophy than the decreasing availability of O_2, and further supported a role for anaerobic methane oxidation (AMO). While it has long been thought that AMO was unimportant in freshwater wetlands due to a lack of electron acceptors, this study added to recent literature showing that this might be the case (e.g. Segarra et al. 2015). If methanotrophs were highly active in the rhizosphere, then methanotrophy may not even have decreased, which would mean the microbial priming was even stronger than originally assumed. Further complicating the picture of wetland methanotrophy was the finding that in experimental boxes that produced the most CH_4 and were the most anaerobic, obligate methanotrophs outcompeted the facultative methanotrophs (Fig. 4.4). This implied that the extreme specialization of obligate methanotrophs allows them to focus resources on traits that help in low-oxygen, high-methane environments. Analysis of genes coding for AMO or for mechanisms to bind oxygen at low concentrations could elucidate the reason for the obligate methanotrophs' competitive advantage.

The metagenomic dataset has potential to reveal more about the rhizosphere microbial community than just methanotrophs. One area of particular interest would be to identify non-methanogenic microbes which are strongly associated with methanogenesis, and attempt to elucidate the entire carbon processing chain. However, attempts to use different metrics to identify important microbial taxa resulted in divergent and inconclusive results in the current study. While it proved beyond the scope of this dissertation, the potential remains for this data to connect community composition with pre-methanogenic carbon processing.

The core goal of this research has been to understand how plants impact boreal wetland CH_4 emissions. The isotopic mixing model identified microbial priming and decreased methanotrophy as key processes in increasing wetland CH_4 emissions. The carbon composition data allowed us to examine why priming occurred, and the metagenomic dataset showed that methanotrophy may not have been decreased after-all. Together, these three avenues of research have painted a more detailed picture of rhizosphere CH_4 dynamics than previously existed. Two important next steps in that mission will be to confirm that the priming which occurred here is replicable in field studies, and to understand how microbial priming is impacted by elevated CO_2 and temperature. However, the examinations of mechanisms are relevant to other topics as well. A situational understanding of priming could be used to better understand upland soil nutrient dynamics, or be applied to influence agricultural practices. Evidence for greater AMO could also have agricultural implications, as farmers can claim carbon credits for reducing CH_4 emissions from rice paddies. Conclusive evidence for facultative methanotrophy is a fairly recent discovery (Dedysh et al. 2005; Theisen and Murrell 2005), and so any advancement of understanding is likely to be valuable to the biologists seeking to understand it for its own sake. It should be unsurprising that some future research directions depart from the core research question here, given that understanding wetland CH_4 dynamics is an inherently interdisciplinary and multi-scale undertaking.

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SUPPLEMENTARY INFORMATION

Table S1.1, irrigation water

Ion	Concentration (µmol/L)
Ammonium – NH_4^+	1.91
Sodium – Na^+	0.72
Calcium – Ca^+	0.81
Potassium – K^+	0.42
Magnesium – Mg^{+2}	0.2
Nitrate – NO_3^-	2.33
Sulfate – SO_4^{-2}	0.56
Chloride – Cl^-	1.62

Figure S2.1, first experiment growth chamber conditions

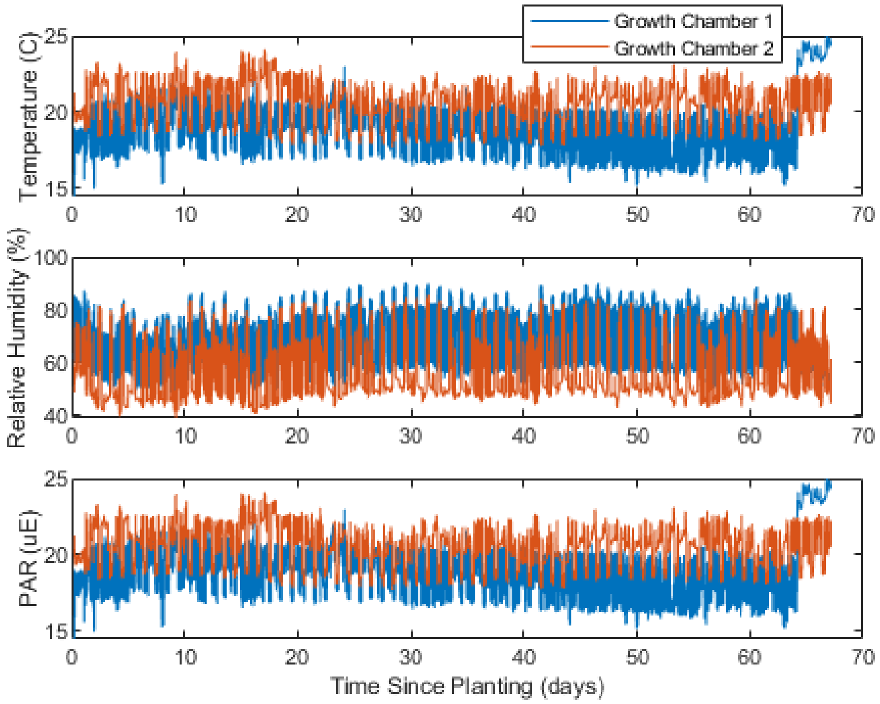


Figure S2.1, temperature, relative humidity, and photosynthetically active radiation (PAR) for both growth chambers during the first experiment. Plants were randomly rearranged weekly within and between chambers. The air conditioners in each chamber were set to daytime temperatures of 18 °C, and nighttime temperatures of 10 °C. There was a vertical temperature gradient in the growth chambers because the air conditioners were located underneath the rhizoboxes while the sensors were directly under the growth lights.

Figure S2.2, Experiment 2 growth chamber conditions

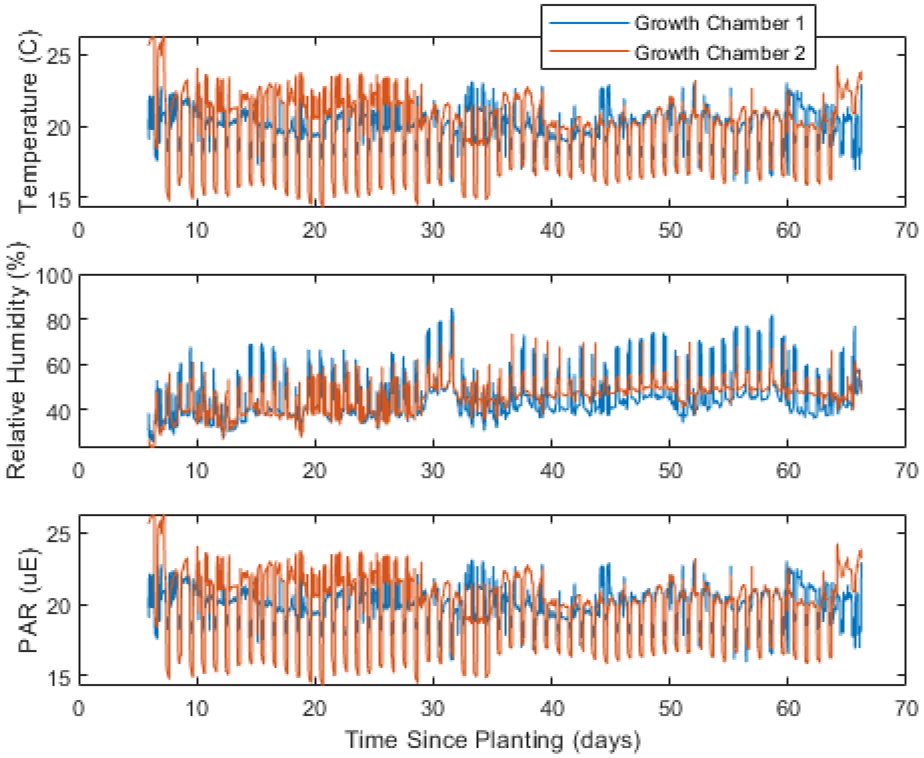


Figure S2.2, temperature, relative humidity, and photosynthetically active radiation (PAR) for both growth chambers during the second experiment. Plants were randomly rearranged weekly within and between chambers. The air conditioners in each chamber were set to daytime temperatures of 18 °C, and nighttime temperatures of 10 °C. There was a vertical temperature gradient in the growth chambers because the air conditioners were located underneath the rhizoboxes while the sensors were directly under the growth lights.

Figure S2.3

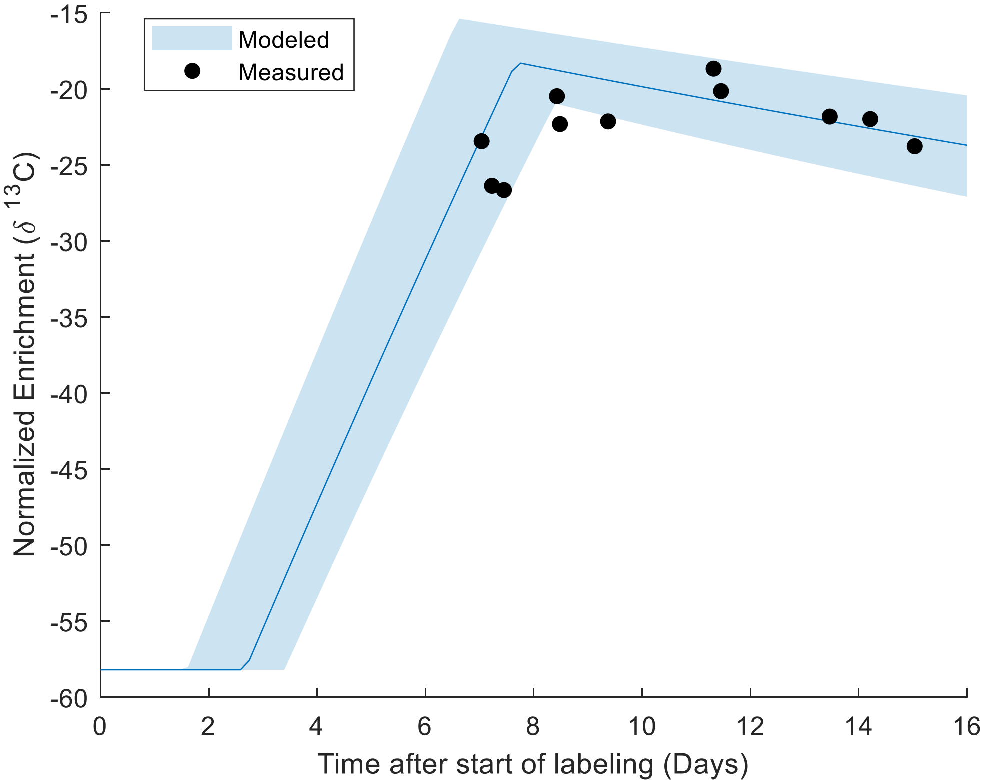


Figure S2.3, comparison of calibration data from a test plant (points) to calculated values based on those data in the exudate age module of the isotope mixing model (Fig. 2 in the main text). The dark line is from the median of the calculated parameter distributions, and the shaded region is the 25th to 75th percentile values. The delay parameter (D) controls the length of the flat line at the low end of the x-axis, and the residence time (τ) controls the curve and steepness of the other two sections of the graph.

Table S2.2, Experiment 1 plant height

Box	Height (cm)								
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
5	14	13.5	16	22	30	29.25	30	30	30
6	18.6	17.6	19.8	24.5	27	26.71	25	27	26
7	22.5	22.3	22	22					
8	17.5	15.2	20.9	25.5	33	36.7	32	33	34
9	24.2	23.1	20.7	25		27.25			
10	21	18.6	17	22					
11	23.5	21.3	21.4	20	29	32.85			
12	16.5	16	14.5	21	27	28.16	33	33	32
13	16	17.1	17	25	28	26.2	30	29	29
14	17	16	15.5	23.5	30	44.89	30	30	30
15	15	15.7	15	24.5	38	34.05	42	43	44
16	16	18.3	18	24.7	31	33	35	32	34
17	17	16	16.5	23	31	26.34	31	32	
19	18.6	18.5	16	20.6	25	33.1	28	28	28
23	20	19.5	22	22	30		30	31	31
24	15.5	13.7	14						

Table S2.3, Experiment 2 plant height

Box	Height (cm)								
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9
1	8.5	13.1	15.6	19.9	21.7				
5	12.5	16.2	16.7	23.3	26.7				
6	8.1	11.2	13.6	16.2	18.6	23.9	26.2	27.1	30.9
7	9.1	15.2	19.1	20.5	24.4				

8	7.4	12.4	15.8	23.9	31.3	33.2	38.4	41.3	46.2
12	6.6	9.5	10.7	17.2	19.9	26.5	32.3	41.2	46.4
14	9.7	13.9	18.2	26.6	24.7	25.2	24.7	26.1	25.9
15	9.2	13.3	20.6	23.8	26.8	27	31.5	31.4	34.5
20	9.7	16.5	24.9	30.7	30.9				
21	11	15	17.8	23.6	26.6	26.9	28.5	29.5	31.1
24	9.1	14	16.1		27.2	30.3	32.3	31.7	32.9

Table S2.4, Experiment 1 plant-by-plant CH_4 fluxes

Includes only those fluxes for which the root mean square error (RMSE) of a linear regression was less than 0.5 parts per million CH_4 concentration per hour. Isotope data were only used if the RMSE of the flux rate was less than 0.5 ppm, and the R^2 value of the flux rate was greater than 0.9. Fluxes listed without isotopic composition were either measured on the LGR instrument which did not measure isotopic composition, or they met the RMSE quality control standard but failed the additional R^2 quality control standard that was used for isotopic data.

Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
5	Planted	2.7	-12.1	
5	Planted	7.0	1.7	
5	Planted	14.7	24.6	
5	Planted	19.9	0.0	
5	Planted	29.8	1084.1	-44.0
5	Planted	42.9	720.5	-56.2
5	Planted	49.8	764.6	-59.6
5	Planted	57.8	805.2	-59.9
5	Planted	62.0	739.1	-62.3
6	Planted	1.8	-0.7	
6	Planted	2.7	-2.4	
6	Planted	8.0	2.8	
6	Planted	14.0	1.0	
6	Planted	19.7	169.9	
6	Planted	29.9	764.4	-54.5
6	Planted	36.8	971.7	-47.7
6	Planted	49.8	713.7	-58.2
6	Planted	57.8	769.8	-56.2
6	Planted	62.0	787.1	-60.4
7	Planted	2.8	-2.7	
7	Planted	7.8	5.9	
7	Planted	13.7	45.2	
7	Planted	19.7	218.4	
7	Planted	27.0	483.2	-59.3
7	Planted	27.2	355.4	-55.9
8	Planted	7.8	6.3	
8	Planted	20.0	0.1	
8	Planted	29.9	305.2	-63.2
8	Planted	36.8	582.8	-61.5

8	Planted	50.7	990.1	-60.7
8	Planted	58.0	642.5	-65.6
Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
9	Planted	1.8	0.2	
9	Planted	2.7	-1.6	
9	Planted	7.8	23.7	
9	Planted	14.8	5.1	
9	Planted	27.1	199.2	-62.3
9	Planted	28.7	232.1	-27.9
10	Planted	3.0	0.3	
10	Planted	7.9	0.3	
10	Planted	14.7	47.6	
10	Planted	27.0	327.5	-57.8
10	Planted	27.7	238.3	-45.8
10	Planted	27.8	319.7	-46.0
11	Planted	2.9	-2.1	
11	Planted	7.0	4.6	
11	Planted	13.9	-0.3	
11	Planted	20.7	163.6	
11	Planted	28.0	496.0	-52.6
11	Planted	28.8	582.9	-48.5
11	Planted	30.0	558.4	-45.5
11	Planted	30.8	515.5	-44.3
11	Planted	31.7	651.5	-44.4
11	Planted	33.7	678.2	-42.0
11	Planted	34.7	667.9	-43.2
11	Planted	35.7	698.0	-43.6
11	Planted	36.7	597.3	-44.2
11	Planted	37.9	649.2	-45.3
11	Planted	39.7	551.8	-46.2
12	Planted	2.7	1.0	
12	Planted	7.9	-0.1	
12	Planted	13.9	1.4	
12	Planted	19.9	47.6	
12	Planted	29.0	73.7	-64.0
12	Planted	58.0	525.7	-56.4
12	Planted	58.9	604.6	-49.5
12	Planted	59.0	426.5	-53.2
13	Planted	3.8	-2.2	

13	Planted	7.7	3.2	
13	Planted	13.7	42.4	
Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
13	Planted	19.9	125.8	
13	Planted	36.9	343.9	-59.3
13	Planted	43.0	700.5	-58.8
13	Planted	62.1	933.0	-57.4
13	Planted	62.7	837.5	-55.4
13	Planted	63.0	854.7	-52.4
13	Planted	63.8	1045.1	-49.1
13	Planted	64.7	949.1	-42.6
14	Planted	3.0	-3.1	
14	Planted	7.8	13.6	
14	Planted	14.7	92.9	
14	Planted	19.9	240.0	
14	Planted	28.9	461.7	-58.7
14	Planted	43.0	647.7	-57.1
14	Planted	49.8	746.5	-58.7
14	Planted	57.9	471.2	-60.7
14	Planted	62.8	917.5	-55.3
14	Planted	63.7	826.1	-47.3
15	Planted	3.8	4.3	
15	Planted	7.7	0.0	
15	Planted	14.7	28.3	
15	Planted	19.9	209.6	
15	Planted	28.9	61.6	-78.0
16	Planted	7.7	6.2	
16	Planted	14.0	-0.1	
16	Planted	19.7	179.3	
16	Planted	29.9	438.8	-60.0
16	Planted	50.9	784.0	-60.7
16	Planted	58.0	1074.6	-50.5
16	Planted	62.1	1119.5	-55.1
16	Planted	62.8	794.4	-56.2
16	Planted	63.8	1028.3	-53.0
16	Planted	64.8	925.1	-51.0
16	Planted	65.0	877.5	-50.4
16	Planted	65.9	1063.1	-47.6
17	Planted	8.0	-5.0	

17	Planted	13.8	36.2	
17	Planted	19.8	106.2	
Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
17	Planted	29.9	370.1	-60.0
17	Planted	36.9	314.0	-58.7
17	Planted	42.9	511.3	-59.3
17	Planted	50.0	15.0	-61.1
19	Planted	7.9	4.0	
19	Planted	13.8	25.7	
19	Planted	19.8	166.8	
19	Planted	27.8	257.6	-52.7
19	Planted	28.1	281.5	-50.6
19	Planted	28.7	223.9	-49.6
19	Planted	30.7	237.0	-43.0
19	Planted	31.7	131.6	-41.9
19	Planted	31.8	143.9	-42.0
19	Planted	33.7	151.7	-41.8
19	Planted	34.8	280.0	-42.1
19	Planted	36.8	417.4	-44.0
19	Planted	42.9	706.4	-32.8
19	Planted	65.7	581.7	-50.8
23	Planted	7.9	13.2	
23	Planted	13.8	44.3	
23	Planted	20.7	105.8	
23	Planted	28.9	382.8	-57.1
23	Planted	59.0	663.2	-63.4
23	Planted	62.2	769.8	-59.4
23	Planted	63.0	562.0	-56.6
23	Planted	63.8	415.6	-48.8
23	Planted	64.9	581.4	-46.2
23	Planted	65.0	646.2	-46.3
23	Planted	65.9	691.4	-44.3
24	Planted	3.8	-2.3	
24	Planted	7.0	7.6	
24	Planted	13.9	0.8	
24	Planted	20.8	18.8	
CB1	Control	1.8	-7.6	
CB1	Control	2.6	-6.9	
CB1	Control	8.0	2.2	

CB1	Control	13.9	8.4	
CB1	Control	19.9	-0.1	
Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
CB1	Control	27.1	39.3	-55.8
CB1	Control	27.8	51.9	-49.1
CB1	Control	28.0	46.6	-62.5
CB1	Control	28.1	37.3	-52.0
CB1	Control	30.8	35.0	-54.4
CB1	Control	31.8	44.4	-51.3
CB1	Control	33.8	29.8	-54.5
CB1	Control	34.8	37.4	-50.8
CB1	Control	37.0	32.9	-50.9
CB1	Control	39.7	21.5	-63.7
CB2	Control	2.9	-9.5	
CB2	Control	7.9	-0.1	
CB2	Control	13.9	0.5	
CB2	Control	29.8	2790.7	-47.2
CB2	Control	36.8	-2.4	
CB3	Control	3.0	-2.0	
CB3	Control	7.8	0.8	
CB3	Control	13.7	3.9	
CB3	Control	20.7	8.3	
CB3	Control	29.0	-9.6	
CB3	Control	29.8	-0.1	
CB3	Control	43.0	-0.1	
CB3	Control	49.9	4.8	
CB4	Control	7.9	-0.8	
CB4	Control	14.0	-0.2	
CB4	Control	20.0	-0.2	
CB4	Control	29.9	7.4	-73.3
CB4	Control	37.0	0.6	
CB4	Control	43.0	-2.0	
CB4	Control	49.9	6.7	
CB4	Control	57.9	7.3	-140.4
CB4	Control	62.0	1.3	
CB4	Control	62.2	13.5	-26.6
CB4	Control	62.7	6.2	
CB4	Control	63.0	14.8	-37.3
CB4	Control	63.9	13.0	-48.1

CB4	Control	64.9	7.6	
CB4	Control	65.7	22.4	-35.4

Table S2.5, Experiment 2 plant-by-plant CH4 fluxes

Includes only those fluxes for which the root mean square error (RMSE) of a linear regression was less than 0.5 parts per million CH_4 concentration per hour. Isotope data were only used if the RMSE of the flux rate was less than 0.5 ppm, and the R^2 value of the flux rate was greater than 0.9. Fluxes listed without isotopic composition were either measured on the LGR instrument which did not measure isotopic composition, or they met the RMSE quality control standard but failed the additional R^2 quality control standard used for isotopic data. For the planted boxes which were isotopically labeled it is indicated whether they were labeled during the week 5 event or the week 10 event. The * indicates an isotopic composition measurement was taken after labeling began.

Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
1	Planted, Week 5	9.9	-0.6	
1	Planted, Week 5	14.9	7.0	
1	Planted, Week 5	23.8	5.8	
1	Planted, Week 5	34.8	202.8	25.6*
1	Planted, Week 5	35.7	182.2	26.8*
5	Planted, Week 5	9.9	367.7	-61.2
5	Planted, Week 5	34.1	607.2	-31.6*
5	Planted, Week 5	34.8	515.7	-23.7*
5	Planted, Week 5	35.7	670.5	-25.6*
6	Planted, Week 10	45.9	601.4	
6	Planted, Week 10	49.8	601.3	
6	Planted, Week 10	60.9	693.0	-6.1*
6	Planted, Week 10	62.2	335.2	-2.1*
6	Planted, Week 10	62.7	790.7	25.9*
6	Planted, Week 10	63.0	493.9	13.6*
6	Planted, Week 10	63.9	577.5	14.0*
6	Planted, Week 10	64.8	538.0	34.3*
7	Planted, Week 5	1.8	-0.6	
7	Planted, Week 5	9.9	-27.3	
7	Planted, Week 5	35.0	96.1	-27.6*
8	Planted, Week 10	1.8	-0.2	
8	Planted, Week 10	9.9	3.2	
8	Planted, Week 10	14.9	-64.5	
8	Planted, Week 10	23.9	32.1	
8	Planted, Week 10	29.9	156.5	-61.7
8	Planted, Week 10	43.9	294.1	
8	Planted, Week 10	49.8	435.7	

8	Planted, Week 10	60.9	672.3	-8.8*
8	Planted, Week 10	62.1	356.0	8.0*
8	Planted, Week 10	62.6	347.7	16.6*
Box	Treatment	Time since planting (days)	CH$_4$ Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
8	Planted, Week 10	63.9	373.2	53.8*
8	Planted, Week 10	65.6	358.3	92.5*
11	Simulated plant	1.8	21.2	-81.1
11	Simulated plant	23.9	273.2	
11	Simulated plant	43.9	260.2	
11	Simulated plant	49.8	525.4	
11	Simulated plant	62.6	-0.8	
12	Planted, Week 10	1.9	35.8	-45.9
12	Planted, Week 10	9.9	84.3	-60.8
12	Planted, Week 10	14.9	38.5	
12	Planted, Week 10	23.9	211.2	
12	Planted, Week 10	43.8	611.3	
12	Planted, Week 10	49.9	562.6	
12	Planted, Week 10	54.9	945.7	-62.3
12	Planted, Week 10	60.8	460.6	-32.0*
12	Planted, Week 10	62.1	462.6	-17.7*
12	Planted, Week 10	62.6	504.4	-6.2*
12	Planted, Week 10	63.9	551.6	13.2*
12	Planted, Week 10	65.6	546.4	33.7*
14	Planted, unlabeled	1.8	-3.5	
14	Planted, unlabeled	9.9	-12.1	
14	Planted, unlabeled	14.9	-8.7	
14	Planted, unlabeled	23.9	1.2	
14	Planted, unlabeled	29.9	12.8	-72.7
14	Planted, unlabeled	43.9	44.4	
14	Planted, unlabeled	62.6	-1.1	
15	Planted, unlabeled	10.8	-2.7	
15	Planted, unlabeled	23.9	43.2	
15	Planted, unlabeled	31.9	250.3	-57.8
15	Planted, unlabeled	43.9	418.6	
15	Planted, unlabeled	49.9	558.3	
15	Planted, unlabeled	62.6	-1.7	
16	Simulated plant	10.8	15.3	-73.3
16	Simulated plant	24.0	284.4	
16	Simulated plant	45.8	70.0	
16	Simulated plant	49.9	168.4	

19	Simulated plant	10.9	-1.3	
19	Simulated plant	14.9	-74.3	
19	Simulated plant	24.0	3.4	
Box	Treatment	Time since planting (days)	CH_4 Emissions (mg m^{-2} d^{-1})	δ13 C of emissions (‰)
19	Simulated plant	31.9	6.0	
19	Simulated plant	45.8	10.4	
19	Simulated plant	50.8	1.2	
20	Planted, Week 5	10.9	-1.8	
20	Planted, Week 5	14.9	-16.8	
20	Planted, Week 5	24.7	0.7	
20	Planted, Week 5	35.0	0.8	
21	Planted, Week 10	1.8	-2.3	
21	Planted, Week 10	10.9	-1.8	
21	Planted, Week 10	15.8	3.9	
21	Planted, Week 10	24.7	52.2	
21	Planted, Week 10	31.9	95.6	-63.0
21	Planted, Week 10	45.8	190.0	
21	Planted, Week 10	50.8	243.8	
21	Planted, Week 10	60.9	220.8	-21.2*
21	Planted, Week 10	62.2	277.2	-0.9*
21	Planted, Week 10	62.6	281.7	5.3*
21	Planted, Week 10	63.9	250.5	21.6*
21	Planted, Week 10	64.8	275.6	31.6*
CB1	Control	10.9	36.1	-61.2
CB1	Control	24.8	33.1	
CB1	Control	32.0	69.4	-61.3
CB1	Control	50.9	83.2	
CB1	Control	62.5	0.2	
CB2	Control	10.9	-6.7	
CB2	Control	15.8	-152.5	
CB2	Control	24.8	6.3	
CB2	Control	32.0	432.2	-57.0
CB2	Control	43.9	33.3	
CB2	Control	50.9	31.2	
CB2	Control	62.6	-0.4	
CB3	Control	10.9	6.7	
CB3	Control	24.8	17.6	
CB3	Control	32.0	61.8	-58.2
CB3	Control	43.9	24.1	
CB3	Control	50.9	25.1	

CB3	Control	62.6	-1.5	

Figures S2.4-2.22

All these figures are available as separate files because they are animated GIFs. Each file name includes the figure number and the experiment and box which the data are from. The box numbers correspond to the plant height and CH_4 flux data in Tables S2-S5.

Table S3.1 – List of known biochemical transformations

Alanine	C3H5NO	71.03711384
Arginine	C6H12N4O	156.1011111
Asparagine	C4H6N2O2	114.0429275
Aspartic Acid	C4H5NO3	115.0269431
Cysteine	C3H5NOS	103.0091856
Cystine	C6H10N2O3S2	222.0132859
Glutamic Acid	C5H7NO3	129.0425932
Glutamine	C5H8N2O2	128.0585776
Glycine	C2H3NO	57.02146376
Histidine	C6H7N3O	137.0589119
Isoleucine	C6H11NO	113.0840641
Leucine	C6H11NO	113.0840641
Lysine	C6H12N2O	128.0949631
Methionine	C5H9NOS	131.0404858
Phenylalanine	C9H9NO	147.068414
Proline	C5H7NO	97.05276391
Serine	C3H5NO2	87.03202848
Threonine	C4H7NO2	101.0476785
Tryptophan	C11H10N2O	186.079313
Tyrosine	C9H9NO2	163.0633286

Valine	C5H9NO	99.06841398
acetotacetate (-H2O)	C4H4O2	84.02112943
acetone (-H)	C3H5O	57.03403983
adenylate (-H2O)	C10H12N5O6P	329.0525217
biotinyl (-H)	C10H15N2O3S	243.0803393
biotinyl (-H2O)	C10H14N2O2S	226.0775996
carbamoyl P transfer (-H2PO4)	CH2ON	44.01363872
co-enzyme A (-H)	C21H34N7O16P3S	765.0995656
co-enzyme A (-H2O)	C21H33N7O15P3S	748.0968259
glutathione (-H2O)	C10H15N3O5S	289.0732426
isoprene addition (-H)	C5H7	67.05477526
malonyl group (-H2O)	C3H2O3	86.00039399
palmitoylation (-H2O)	C16H30O	238.2296658
pyridoxal phosphate (-H2O)	C8H8NO5P	229.0140109
urea addition (-H)	CH3N2O	59.02453777
adenine (-H)	C5H4N5	134.0466702
adenosine (-H2O)	C10H11N5O3	249.0861894
Adenosine 5'-diphosphate (-H2O)	C10H13N5O9P2	409.0188541
Adenosine 5'monophosphate (-H2O)	C10H12N5O6P	329.0525217
cytidine 5' diphosphate (-H2O)	C9H13N3O10P2	385.0076207
cytidine 5' monophsophate (-H2O)	C9H12N3O7P	305.0412884
cytosine (-H)	C4H4N3O	110.0354368
Guanosine 5- diphosphate (-H2O)	C10H13N5O10P2	425.0137687
Guanosine 5- monophosphate (-H2O)	C10H12N5O7P	345.0474364
guanine (-H)	C5H4N5O	150.0415848
guanosine (-H2O)	C10H11N5O4	265.081104
deoxythymidine 5' diphosphate (-H2O)	C10H14N2O10P2	384.0123717
thymidine (-H2O)	C10H12N2O4	224.079707

thymine (-H)	C5H5N2O2	125.0351025
thymidine 5' monophosphate (-H2O)	C10H13N2O7P	304.0460394
uridine 5' diphosphate (-H2O)	C9H12N2O11P2	385.9916363
uridine 5' monophosphate (-H2O)	C9H11N2O8P	306.0253039
uracil (-H)	C4H3N2O2	111.0194524
uridine (-H2O)	C9H10N2O5	226.0589716
acetylation (-H)	C2H3O2	59.01330439
acetylation (-H2O)	C2H2O	42.01056471
C2H2	C2H2	26.01565007
Carboxylation	CO2	43.98982928
CHO2	CHO2	44.99765432
condensation/dehydration	H2O	18.01056471
diphosphate	H3O6P2	160.9404898
ethyl addition (-H2O)	C2H4	28.03130015
Formic Acid (-H2O)	CO	27.99491464
glyoxylate (-H2O)	C2O2	55.98982928
carbon/dehydrogenation	C13	1.00335
HYDROGENATION/dehydrogenation	H2	2.015650074
hydroxylation (-H)	O	15.99491464
Inorganic Phosphate	P	30.9737634
ketol group (-H2O)	C2H2O	42.01056471
methanol (-H2O)	CH2	14.01565007
phosphate	HPO3	79.96633236
primary amine	NH2	16.01872408
pyrophosphate	PP	61.9475268
secondary amine	NH	15.01089905
sulfate (-H2O)	SO3	79.95681572
tertiary amine	N	14.00307401

C6H10O5	C6H10O5	162.0528236
C6H10O6	C6H10O6	178.0477382
D-Ribose (-H2O) (ribosylation)	C5H8O4	132.0422589
disaccharide (-H2O)	C12H20O11	340.1005618
glucose-N-Phosphate (-H2O)	C6H11O8P	242.0191559
Glucuronic Acid (-H2O)	C6H8O6	176.0320881
monosaccharide (-H2O)	C6H10O5	162.0528236
trisaccharide (-H2O)	C18H30O15	486.1584707
erythose (-H2O)	C4H8O4	102.031695
transamination (-O)	NH4	2.039459

Figure S3.1 – MSCC

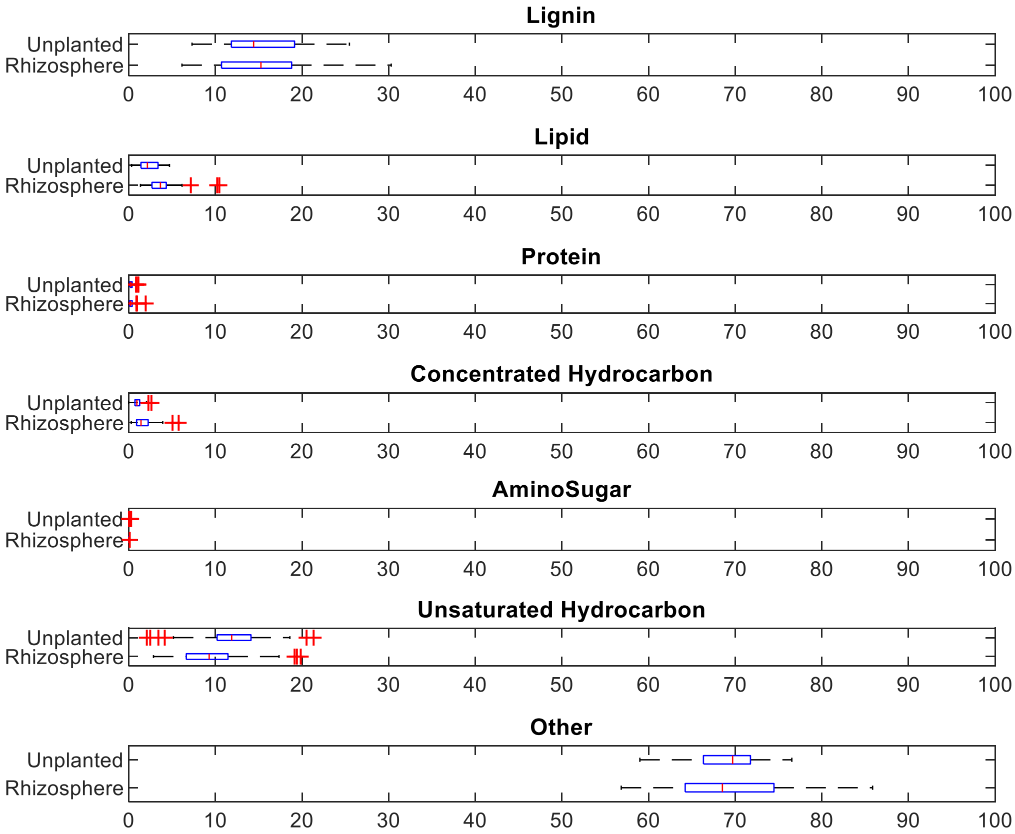

Lignin
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100
Lipid
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100
Protein
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100
Concentrated Hydrocarbon
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100
AminoSugar
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100
Unsaturated Hydrocarbon
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100
Other
Unplanted
Rhizosphere
0
10
20
30
40
50
60
70
80
90
100

Figure S3.2 – Heteroatom classifications

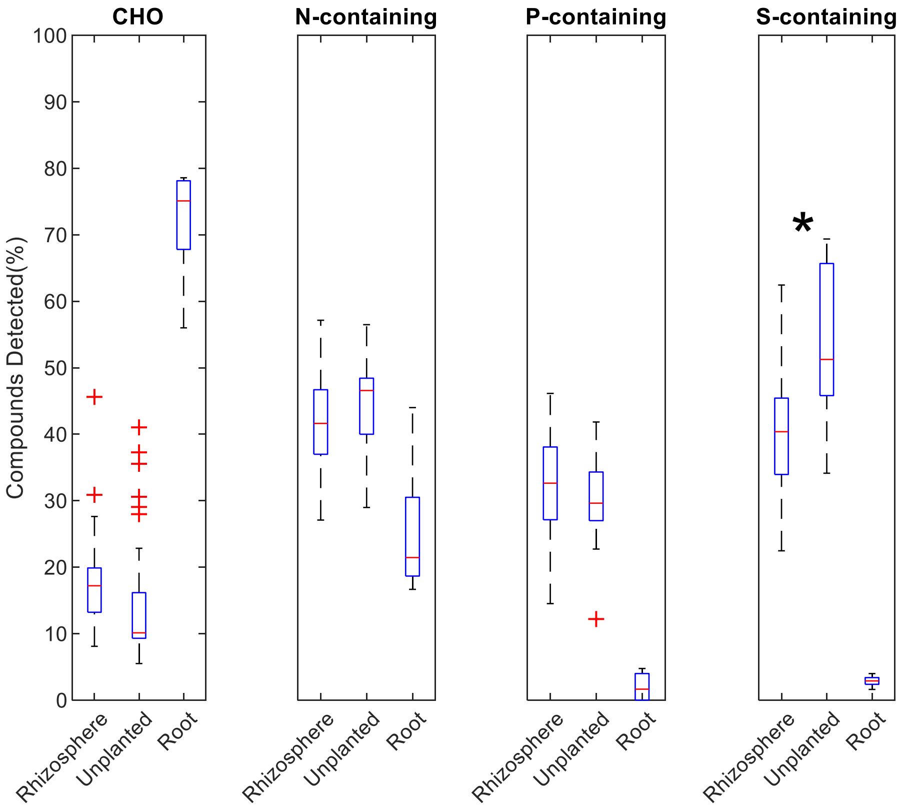


Figure S3.2, number of compounds in each sample that fall into different heteroatom groups for rhizosphere soil, unplanted soil and root surfaces. Among CHO, N-containing, and P-containing compounds the two soil sample types (rhizosphere and unplanted soil) were not significantly different ($p > 0.05$). The unplanted soil had significantly more ($p < 0.05$) S-containing compounds than the rhizosphere, marked with an asterisk. Distributions are of the means of individual samples. Boxes show median with upper and lower quartiles, and tails show all data within 2.7σ of the mean. Outliers outside 2.7σ are marked with "+". Both types of soil data are from FT-ICR-MS analysis of soil extractions, with all three extractions combined to create the complete sample dataset shown here. The root surface data are from LAESI. For rhizosphere n=73, for unplanted soil n=40, and for roots n=6.

Figure S3.3– Atom Ratios

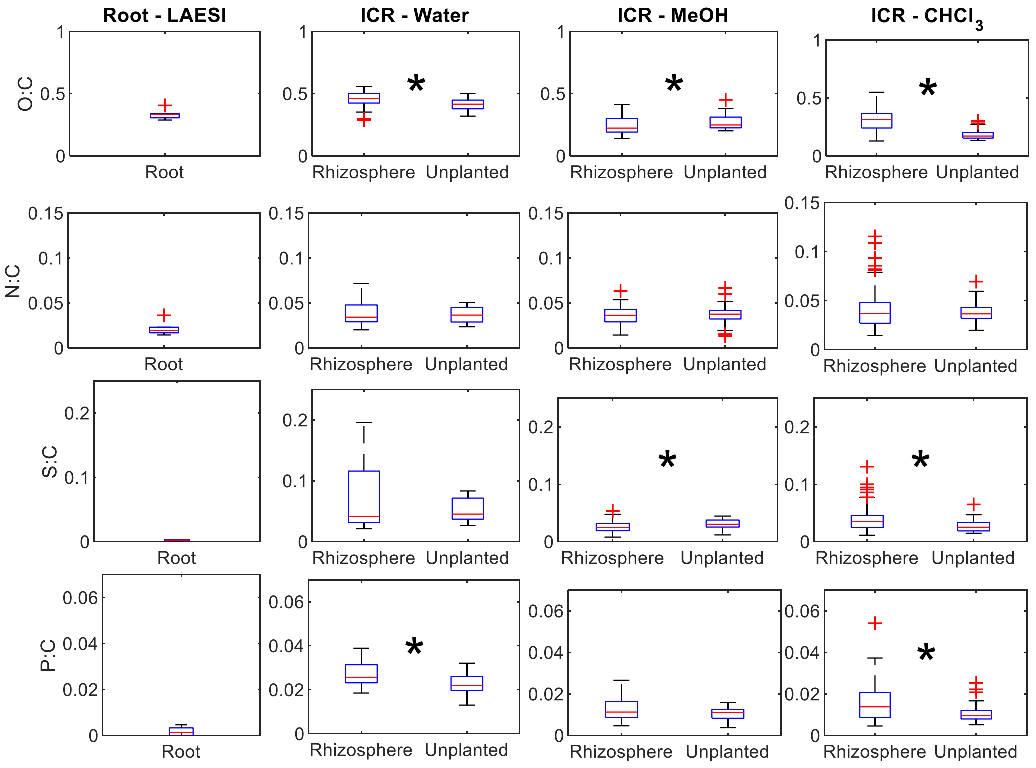


Figure S3.4 – Diversity

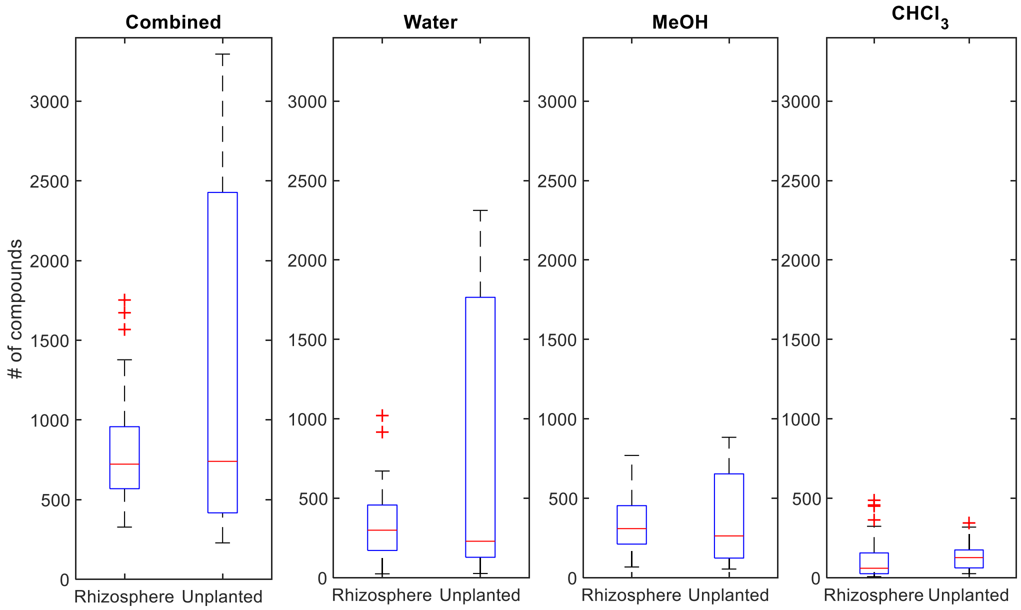


Figure S3.5 – Low Molar Mass PDFs

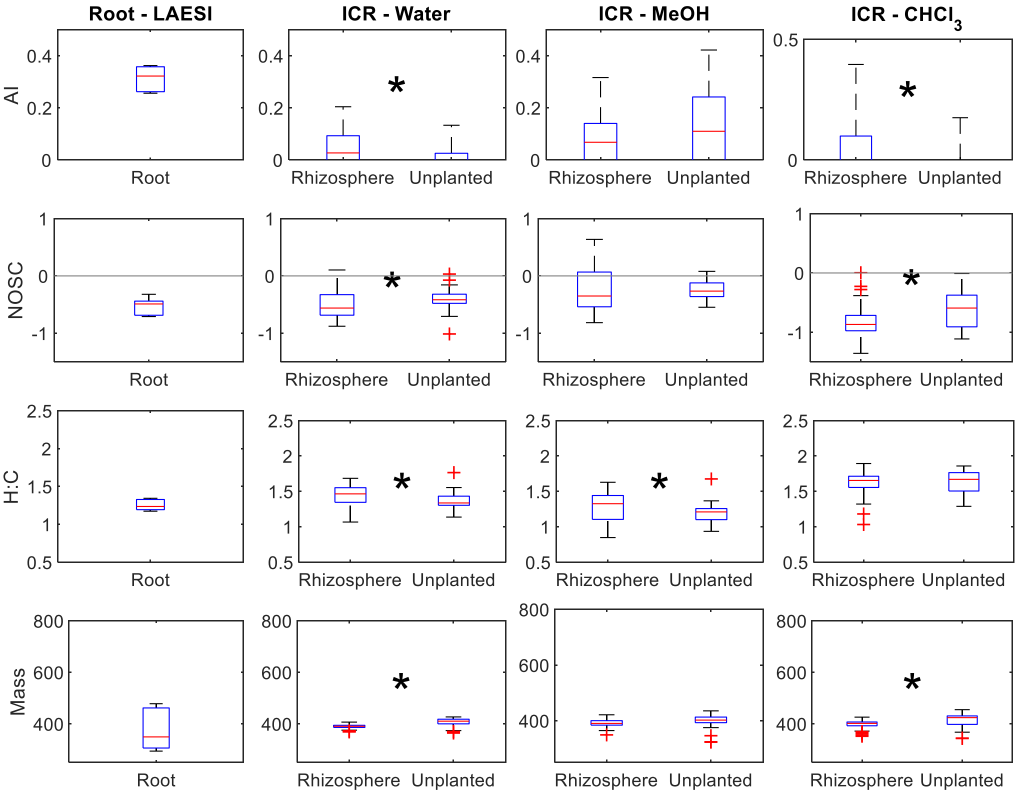


Figure S3.6 – High Molar Mass PDFs

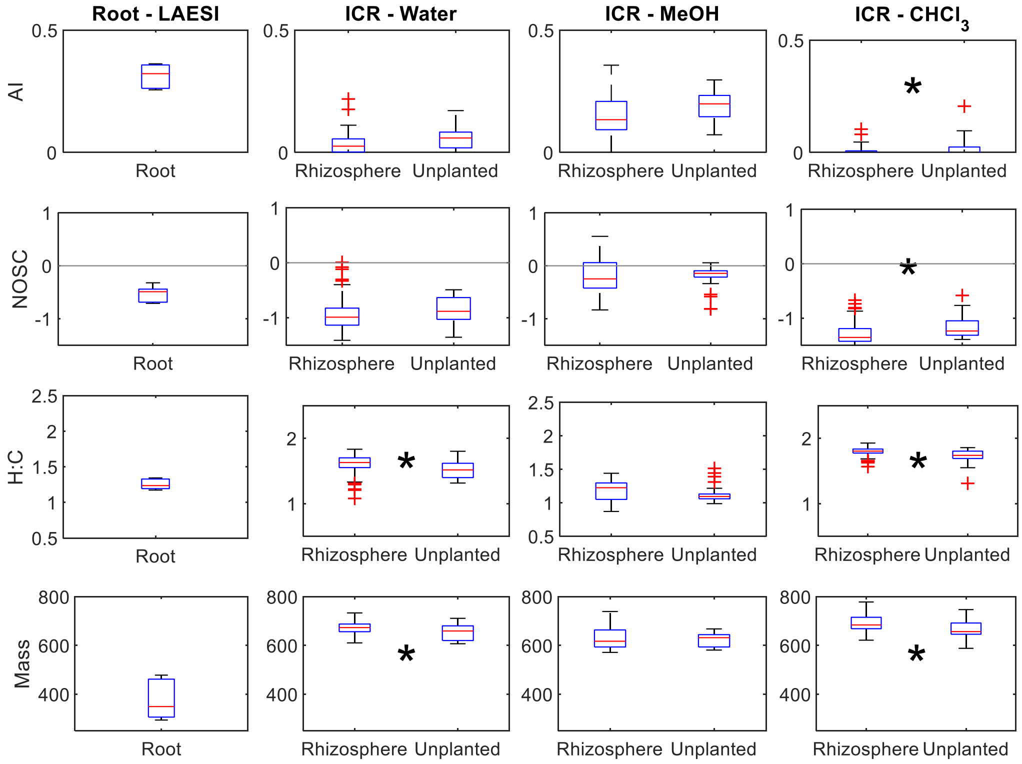


Figure S3.7 – Low Molar Mass compound Classes

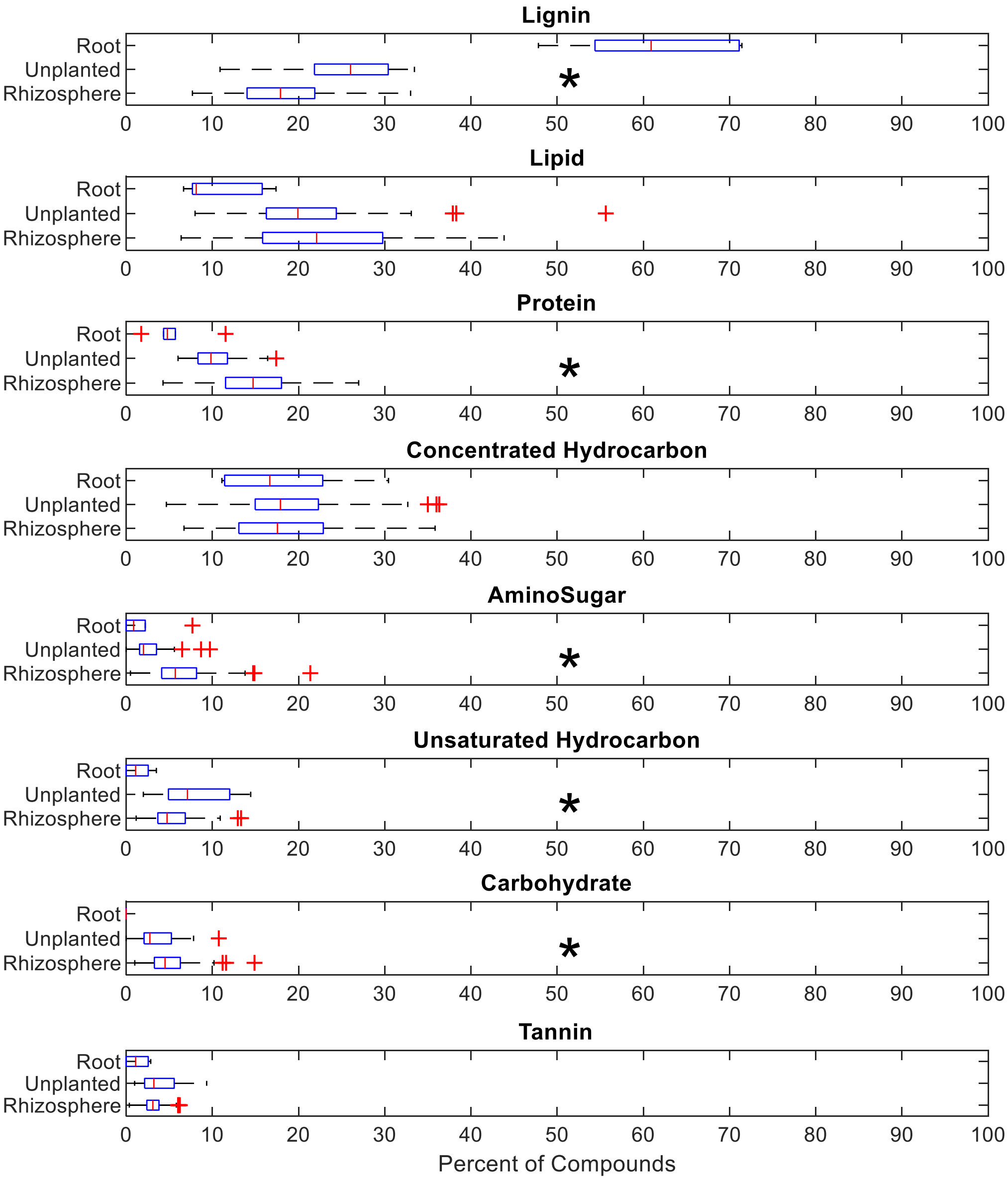


Figure S3.8 – High Molar Mass Compound Classes

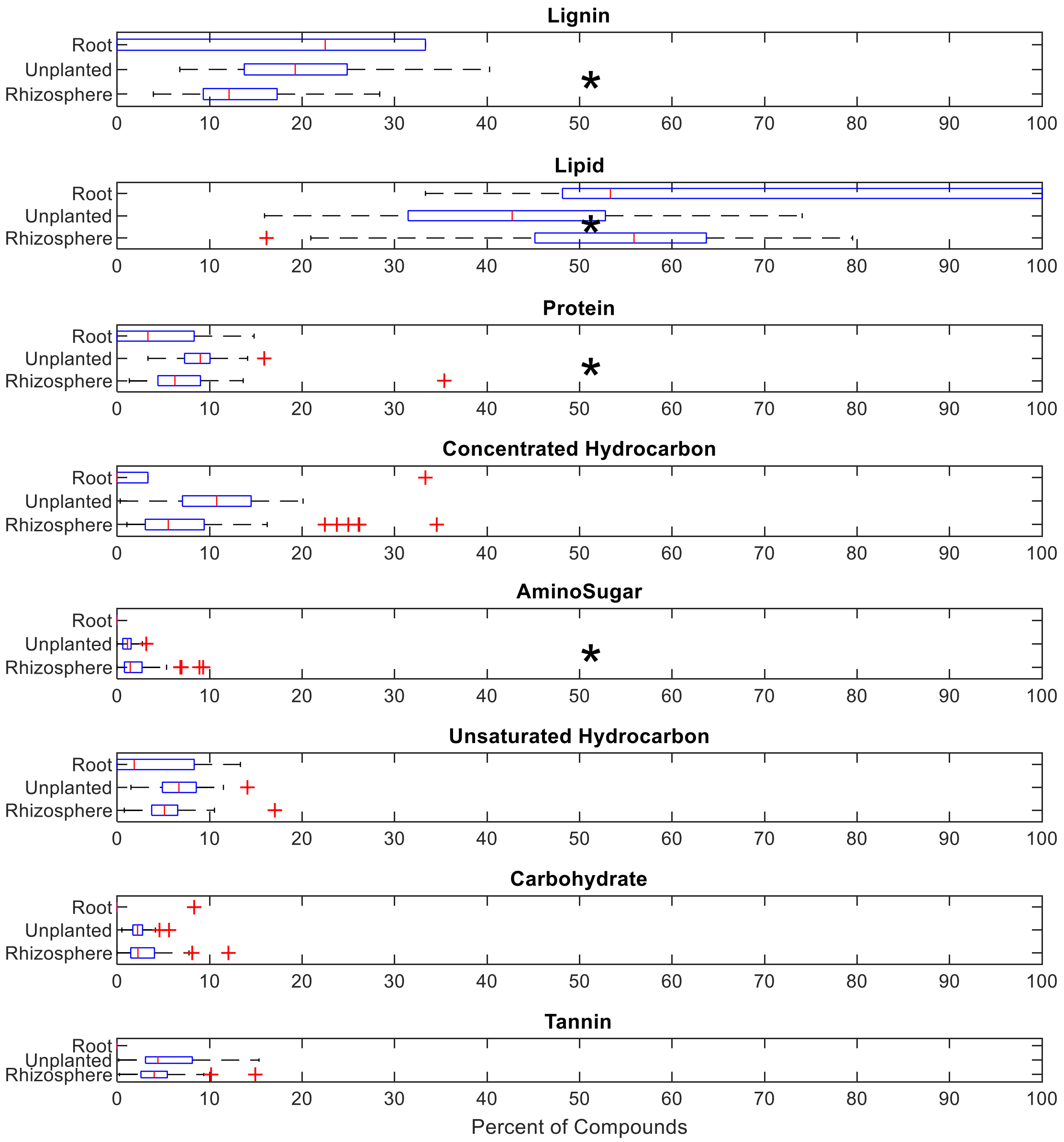
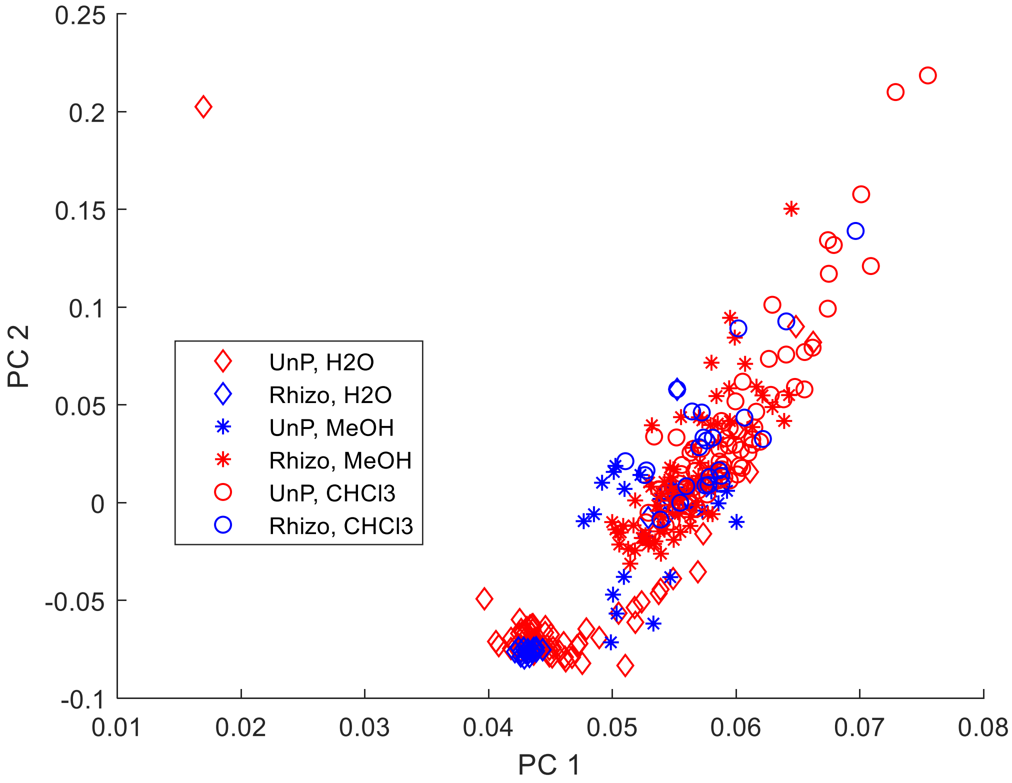


Figure S3.9 – PCA of Microbial Transformations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0.2
0.15
0.1
0.05
0
-0.05
-0.1
PC 2
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
PC 1
UnP, H2O
Rhizo, H2O
UnP, MeOH
Rhizo, MeOH
UnP, CHCl3
Rhizo, CHCl3