Impacts of The Wetland Sedge *Carex aquatilis* on Microbial Community and Methane Metabolisms

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Code availability: Statistical analysis performed using MATLAB r2018b, scripts available upon request.

Keywords: methane, wetlands, bog, microbial priming, root exudates, climate change.

Abstract

Aims Microbial activity in the soil of wetlands is responsible for the emission of more methane to the atmosphere than all other natural sources combined. This microbial activity is heavily impacted by plant roots, which influence the microbial community by exuding organic compounds and by leaking oxygen into an otherwise anoxic environment. This study compared the microbial communities of planted and unplanted wetland soil from an Alaskan bog to elucidate how plant growth influences populations and metabolisms of methanogens and methanotrophs.

Methods A common boreal wetland sedge, *Carex aquatilis*, was grown in the laboratory and DNA samples were sequenced from the rhizosphere, unplanted bulk soil, and a simulated rhizosphere with oxygen input but no organic carbon.

Results The abundance of both methanogens and methanotrophs were positively correlated with methane emissions. Among the methanotrophs, both aerobic and anaerobic methane oxidizing microbes were more common in the rhizosphere of mature plants than in unplanted soil, while facultative methanotrophs capable of utilizing either methane or other molecules became relatively less common.

Conclusions These trends indicate that the roots in this experiment created an environment which favored highly specialized microbial metabolisms over generalist approaches. One aspect of this specialized microbiome is the presence of both aerobic and anaerobic metabolisms, which indicates that oxygen is present but is a limiting resource controlling competition.

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. 2013). 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 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 that respond to a host of environmental variables. 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 types of plants growing in the wetland (Robroek et al. 2015). The majority of microbial species present in wetland soil samples, as in most environments, are uncultured (Ivanova et al. 2016).

Plants impact the wetland microbial community through two primary modes. First, plants exude carbon compounds from their roots which may be more biodegradable than the other 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). While this increase in CH_4 emissions is partially

driven by the carbon in the exudates being processed into CH_4, the exudates also result in more soil carbon being converted to CH_4 (Waldo et al. 2019). This phenomenon is known as the microbial priming effect (Fontaine et al. 2007; Kuzyakov 2010; Ruirui et al. 2014; Ye et al. 2015). The plant growth cycle is seasonal, so changes in root exudation over the plants' life cycle impacts CH_4 emissions even when factors such as temperature are kept constant (Neue et al. 1997).

The second effect that wetland plants have on the microbial environment is leakage of oxygen into the soil from 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 quickly enough that it does not accumulate in the soil (Waldo et al. 2019; Turner et al. 2020), it can influence microbial communities by facilitating the 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. As with root exudation, oxygen transport changes over time as plants grow throughout the season, and different species of plants allow for varying amounts of oxygen transport (Schimel 1995). The balance between the dynamic effects of root exudation and oxygen transport will control what types of microbial CH_4 metabolisms are favored.

In addition to the traditional model of aerobic obligate methanotrophs, the rhizosphere also supports two other methanotrophic metabolisms. Once considered insignificant in wetlands (Conrad 2009), recent work has shown that anaerobic oxidation of CH_4 (AOM) is

common in freshwater wetlands (Segarra et al. 2015). Though it may be common, AOM is performed by a limited number of microbes, primarily the ANME2d anaerobic archaea (Haroon et al. 2013) and bacteria of the NC10 phylum (He et al. 2016). To avoid the use of oxygen, AOM relies on alternative terminal electron acceptors (TEAs). In freshwater bogs, rain is the primary source of water and nutrients; groundwater is not available to transport TEAs into the wetland. The continued availability of non-oxygen TEAs without transport into bogs can be explained by recycling and regeneration of the TEAs within the wetland (Keller and Bridgham 2007). This recycling requires an ultimate electron sink that is used to regenerate the TEAs used by anaerobic methanotrophs. Plants can supply that electron sink by leaking oxygen from their roots which is used to generate a variety of TEAs in the relatively oxidized rhizosphere (Keiluweit et al. 2016).

The second non-traditional methanotrophic metabolism within the rhizosphere is facultative methanotrophy. 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* that can also use 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). Because the rhizosphere is a dynamic soil zone where the balance of microbial activity, root exudation, and oxygen availability may change over time, the ability to use different carbon sources for energy could be a competitive advantage.

The plant effects described above can either increase (Shannon and White 1994; Joabsson et al. 1999; Popp et al. 2000; Whalen 2005) or decrease (Schipper and Reddy 1996; Fritz et al. 2011; Lenzewski et al. 2018) CH_4 emissions. Decreases driven by plants are due to increased methanotrophy (Schipper and Reddy 1996; Fritz et al. 2011; Lenzewski et al. 2018), while increases in CH_4 emission can be due to plant-exudate stimulation of CH_4 production (Chanton et al. 2008; Waldo et al. 2019; Turner et al. 2020) and/or increased transport through aerenchyma (Shannon and White 1994; Joabsson et al. 1999). Determining metabolisms fostered by the presence of roots can be used to build a mechanistic understanding of why some plant species increase while other decrease CH_4 emissions. In this study, we focused on *Carex aquatilis*, a common wetland sedge shown to increase methane emissions (Schimel 1995; Waldo et al. 2019). Our hypothesis was that the plants would increase populations of both methanotrophs and methanogens, but that the community composition would shift as well as simply grow. We compared the microbial communities of planted and unplanted wetland soil to elucidate how *Carex* growth influenced populations of methanogens and methanotrophs, with special focus on the different forms of methanotrophy.

Materials and Methods

Experimental Setup

This investigation used samples collected during a previous study, Waldo et al. (2019), which described the experimental setup in detail. Briefly, *Carex aquatilis*, a common boreal wetland sedge, were grown for 10 weeks in rhizoboxes (48cm tall, 20cm wide, 5cm thick) filled with 4.5 L per box of peat collected from a thermokarst bog in central Alaska. There was one

147 plant per box, grown from a nursery seedling. There were also two unplanted box types:
148 control boxes with peat alone, and simulated plants that utilized silicone tubes to transport
149 gases, thus simulating gaseous exchange without the biochemical effects of roots. There were 9
150 planted boxes, 2 control boxes, and 2 simulated plant boxes analyzed. More replicates were
151 used for planted boxes than unplanted box types (control and simulated) because of the
152 additional element of randomness introduced by having different individual plants in each box.
153 Optical oxygen sensors (optodes) measured oxygen concentration around the roots of plants
154 and around the simulated plant roots (Larsen et al. 2011). Methane emissions were monitored
155 throughout the experiment by placing a clear fluxing hood over each box and measuring the
156 rate of CH_4 concentration increase in the headspace. During weeks 5 and 10 of the experiment,
157 4 plants of the 9 were exposed to $^{13}CO_2$ by placing a hood on each rhizobox and injecting 99
158 atom% $^{13}CO_2$ into the headspace over a period of five consecutive days. This $^{13}CO_2$ was
159 photosynthesized and isotopically labeled the plants. The isotope treatment was used to create
160 the carbon source model of Waldo et al. (2019), and is not considered a key factor in the aims
161 of this study. Following labeling, root and soil samples were collected under nitrogen. Plants
162 were destructively sampled in both weeks 5 and 10; the unplanted control boxes and simulated
163 plants were only sampled in week 10, at the end of the experiment. The 9th planted box, which
164 was never isotopically labeled, was also harvested in week 10. Samples collected for chemical
165 analysis were documented in Waldo et al. (2021), and samples collected for DNA analysis and
166 microbe counts are described below.

167 Soil samples were collected at depths of approximately 5 cm, 20 cm, and 35 cm. All
168 samples were collected inside a gasbag filled with high-purity nitrogen. 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 DNA sequencing. In planted boxes, roots and associated rhizosphere soil were collected. In control and simulated plant boxes, soil was collected.

Fluorescence Microscopy

Fluorescence microscopy was used to enumerate the microbes in samples from the rhizosphere and unplanted soil, but not in samples from boxes with simulated plants due to finite access to instrumentation. For planted boxes, root sections were cut from each sampling location. Root sections were sonicated in centrifuge tubes filled with 4% paraformaldehyde (PFA) for 60 seconds, after which the root sections were removed and placed in a 50/50 mix of 70% ethanol and 1X phosphate buffered solution (PBS, Fisher Scientific). Soil dislodged from root samples was classified as rhizosphere soil (White et al. 2015), and was recovered by centrifugation (20 minutes at 15,000 g). The rhizosphere sample was then also stored in ethanol/PBS mixture. For unplanted boxes, the protocol was the same, except the sample was not sonicated or centrifuged during PFA incubation. All samples were then stored at -20 C before being shipped on dry ice to the Environmental Molecular Sciences Laboratory (EMSL) where they were stored at -80 C until analysis.

For microbe counting, the samples were thawed and either the entire rhizosphere pellet (for plant samples) was used, or an aliquot of bulk soil (for control box samples) was taken that had similar volume to that of a typical rhizosphere pellet. To the soil sample, 0.3 to 0.4 g of sterile garnet beads were added with enough water to bring the total volume up to 1.5 mL. This

mixture was then vortexed for 45 seconds. In a fresh tube, 98 µL of the mixture was combined 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. These measurements were used to calculate the dry mass of soil per volume of water. Mass-normalized cell density was calculated by dividing the total cell count by the mass of solids in the droplet which was imaged. Any sample which had less than 0.5 mg of soil in the 98 µL aliquot was excluded from analysis. In total, 10 samples from each harvest of planted boxes as well as 5 samples from control boxes were successfully quantified. See ESM 5 for a list of specific samples analyzed.

DNA Sequencing

For DNA sequencing, approximately 1 mL of soil was collected from each sample site for all three treatment types. DNA was extracted from the peat using a MoBio PowerSoil kit, with modifications made to optimize the kit for extractions from peat soils (See Online Resource 1). A DNA quality check was conducted according to the Department of Energy Joint Genome Institute (JGI) “iTag Sample Amplification QC SOP” v. 1.3 (Online Resource 2). Briefly, an aliquot of the DNA was amplified using PCR; the PCR product was visualized on an agarose gel compared to size standards. The primers used for both the quality check and for the actual iTag

analysis used the V4 region of 16S rRNA sequences, using primers designed to amplify both bacteria and archaea (FW (515F): GTGCCAGCMGCCGCGGTAA, RV (805R): GGACTACHVGGGTWTCTAAT) (Rivers 2016). DNA was stored at -20 C until transport to JGI for analysis. The DNA samples were shipped to JGI on dry ice. Once there, the samples were processed to produce one of two sequencing products: iTags or metagenomes. Not all samples collected were sequenced, due to sample quality control and/or sequencing resources available. A total of 26 samples were sequenced for metagenomes, and 70 for iTag. For a list of samples sequenced for iTag, see the headers on ESM 4. For a list of metagenomes, see ESM 5, which also contains an explanation of sample naming conventions.

The iTags classified microbes to the genus level 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 available as Online Resource 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).

Statistical Analysis

All tests to determine whether multiple groups of data were or were not from the same distribution were done first using a mixed-effects model (“fitlme” in MATLAB R2018b) in which

the box was a random variable, and the box type was the test variable. The mixed-effects model used only returns whether a difference between groups exists, not which groups are different. When a significant difference existed in the data, the Kruskal-Wallis test was used to determine between which groups the difference existed, performed using the "kruskalwallis" function in MATLAB (R2018b). All tests for relationships or trends within a dataset were done using a Spearman Rank Correlation Coefficient with the "corr" function in MATLAB (R2018b). The Spearman Rank Correlation returns both a p-value, indicating statistical significance, and ρ, indicating direction and strength of monotonic correlation.

Sequence Data Analysis

The iTag data was analyzed for the frequency of methanogens and methanotrophs. For methanogens, the classes Methanobacteria and Methanomicrobia were included. 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 also counted as facultative methanotrophs. Similarly, the iTag data did not identify any taxa that are documented to perform AOM, so the metagenomic data were used to isolate the candidate genus *Candidatus Methanoperedens*, which contains ANME2d anaerobic methanotrophs (Haroon et al. 2013). Bacteria of the NC10 phylum also perform AOM but were not identified in the metagenomic phylogeny through IMG. Instead, NC10 presence was determined through a BLAST search for sequences from the GenBank database of the National Center of Biotechnology Information (NCBI) under accession numbers KU891931 (16S

255 rRNA) and KT443986 (pmoA) (He et al. 2016). The BLAST search only accepted sequences with
256 E-values of 10^{-20} or better for NC10 16s rRNA or 10 for NC10 pmoA.

257 Specific gene sets found in the metagenomes were used to assess functional differences
258 in microbial populations. To determine whether samples had microbes capable of conducting
259 respiration or fermentation, the number of genes involved in glycolysis (a process which occurs
260 in both metabolisms) was compared to genes involved in the Krebs cycle (used in respiration,
261 but not fermentation). Because glycolysis is used in both metabolisms, the ratio is not a linear
262 measure of the relationship between the two metabolisms. However, there will be a qualitative
263 correlation between the two ratios. For the Krebs cycle, only those genes involved in the first
264 oxidation were used because that limited the number of genes involved and focused the
265 results. To compare methanotrophic metabolisms, methane monooxygenase (MMO) genes
266 were compared. In addition to number of genes, a principle components analysis (PCA) was
267 performed on the MMO gene sets to determine if different types of MMO were used in
268 different samples. PCA was performed in MATLAB (2018b) using the “pca” function and default
269 settings.

270 The gene sets were identified through the KEGG Orthology (Kanehisa and Goto 2000;
271 Kanehisa et al. 2016). The gene sets used for the Krebs Cycle are presented in Table 1, gene sets
272 for glycolysis are in Table 2, and gene sets used for MMO are in Table 3.

273 Table 1. Gene sets used to identify the Krebs Cycle.

Gene	Citation
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

274

275 Table 2. Gene sets used to identify glycolysis.

Gene	Citation
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
K15635 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Johnsen and Schönheit 2007

276

277 Table 3. Gene sets used to identify MMO.

Gene	Citation
K10944 methane/ammonia monooxygenase subunit A	Holmes et al. 1995; Stolyar et al. 1999; Norton et al. 2002
K10945 methane/ammonia monooxygenase subunit B	Stolyar et al. 1999; Norton et al. 2002
K10946 methane/ammonia monooxygenase subunit C	Stolyar et al. 1999; Norton et al. 2002

K16157 methane monooxygenase component A alpha	Murrell et al. 2000
K16158 methane monooxygenase component A beta chain	Murrell et al. 2000
K16159 methane monooxygenase component A gamma chain	Murrell et al. 2000
K16160 methane monooxygenase regulatory protein B	Murrell et al. 2000
K16161 methane monooxygenase component C	Murrell et al. 2000
K16162 methane monooxygenase component D	Murrell et al. 2000

Results

Fluorescence Microscopy

Ten weeks after the start of the experiment, rhizosphere soil samples had a significantly ($p < 0.05$) higher concentration of microbes than did the unplanted control box samples (Figure 1A). The rhizosphere soil collected during week 5 of the experiment did not have a significantly different number of microbes from rhizosphere soil collected in week 10 or from the control box soil. 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, there was not a statistically significant correlation between microbe count and CH_4 flux (Figure 1B, $p > 0.05$).

iTag

The 16S rRNA iTag analysis produced a median of 537,000 reads per sample, with an interquartile range of 244,000 to 630,000. From these data, 838 genera of microbes were identified in the samples. A breakdown of microbial community composition to the Class level

292 is shown in Table 4. An additional table showing genus-level results is available as Online
293 Resource 4.

294 Table 4. Most Common Classes Identified by iTag.

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

295

296 The median percentage of microbes that were methanogens in samples from each box
297 was positively correlated with CH_4 emissions (Fig. 2A, $p < 0.05$, $\rho=0.69$) as was the percentage of
298 microbes that were methanotrophs, when excluding simulated plants (Fig 2B, $p < 0.01$, $\rho=0.78$).
299 Simulated plants were excluded from the correlation test of methanotrophs because in the
300 other three box types (planted boxes from weeks 5 and 10 and control boxes) the oxygen
301 concentrations were low, but in simulated boxes, the oxygen concentrations were higher
302 (Waldo et al. 2019) so the microbes faced a fundamentally different environment. Correlating

methanotrophs with CH_4 emissions acts as a proxy for correlating methanotrophs with CH_4 availability in the rhizosphere.

When microbe count data was used with the percentages to find the total number of each type of microbe, there was a positive correlation between CH_4 flux and methanotroph count ($p < 0.01$, $\rho=0.87$, Fig. 2D), but the correlation with methanogen count was on the edge of significance ($p = 0.07$, $\rho=.65$, Fig. 2C). The number of methanogens and methanotrophs were also significantly correlated with each other ($p< 0.05$, $\rho=.31$). Microbe count data was not available for all samples that were sequenced, so the number of replicates was smaller in the count analysis, and no microbe counts were conducted on samples from simulated plant boxes.

Metagenomes

The metagenomic data were used to identify functional genes and taxa which could not be identified in the iTag data. Facultative methanotrophs comprised less than 1% of all samples (Fig. 3A). In contrast to the obligate methanotrophs (Figure 2), there was no statistically significant ($p > 0.05$) correlation between the flux of CH_4 in the final week before harvest and either the percentage of facultative methanotrophs (Fig. 3A, with simulated boxes $\rho=-0.16$ or excluding simulated boxes $\rho=0.10$) or the number of facultative methanotrophs (Fig. 3B, $\rho=0.62$). However, the percentage of microbes that were facultative methanotrophs in simulated plant boxes was greater ($p < 0.05$ by mixed-effects model and Kruskal-Wallis run on the same dataset presented in Fig. 3A) than the other box types, as was observed in obligate methanotrophs (Same data as presented in Fig. 2B, $p < 0.01$).

The ratio of obligate to facultative methanotrophs was significantly larger ($p < 0.05$) in rhizosphere samples from week 10 than in simulated boxes, while the other two treatment types (control boxes and rhizosphere samples from week 5) had intermediate ratios that were not significantly different ($p > 0.05$) from the ratios in any other treatment (Fig. 4A). There was no significant correlation ($p > 0.05$) between the ratio of obligate to facultative methanotrophs and the flux of CH_4 in the final week before harvest (Fig. 4B).

The ratio of ANME2d archaea, which are capable of AOM, to total obligate methanotrophs was significantly larger ($p < 0.05$) in rhizosphere soil from week 10 than in the simulated plant boxes, with rhizosphere soil from week 5 and control boxes having an intermediate ratio (Fig. 5A) — as was seen with the ratio of facultative to obligate methanotrophs. There was no significant relationship ($p > 0.05$, $\rho=.16$) between the ratio of ANME2d to total methanotrophs and the flux of CH_4 in the final week before harvest (Fig. 5B). The BLAST searches did not return any matches for the NC10 pmoA genes and the NC10 16s sequences returned did not display any statistically significant relationships with other relevant data (data not shown). The lack of pmoA gene detections, even at low match quality, indicates that the 16s sequences may not be derived from NC10 bacteria. For this reason, the NC10 BLAST results were omitted from further analyses and all discussion of AOM are related to the ANME2d results.

The ratio of genes involved in glycolysis to those involved in the Krebs Cycle was positively correlated with CH_4 emissions ($p < 0.05$, $\rho=.72$, Fig. 6), indicating more fermentation activity in boxes with greater methane emissions.

There were no statistically significant ($p > 0.05$) differences in the percentage of MMO genes between box types. However, the PCA revealed that a single principle component could explain 99% of the variation in MMO genes among the samples. This component was defined by higher frequencies of genes coding for all three subunits of a particulate methane monooxygenase (PMO) (Holmes et al. 1995; Stolyar et al. 1999; Norton et al. 2002) and lower frequencies of the other six MMO-coding genes, which include a regulatory protein and several components of a soluble MMO (Murrell et al. 2000). The PMO-correlated component had significantly ($p < 0.05$) higher scores in simulated plant boxes than in either harvest of real plants. The control boxes were not significantly different ($p > 0.05$) from any other group.

Discussion

Both total microbial population and community composition play a role in explaining the impact of plant roots on CH_4 emissions. Finding more microbes in the rhizosphere of planted boxes harvested in week 10 than in unplanted soil was expected. The first study based on this same experiment found that more root exudates were being added to the soil during week 10 than during week 5 (Waldo et al. 2019). 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 later in the experiment 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, as is well established (e.g., Diaz-Raviña et al. 1988).

366 Methanogens and methanotrophs are directly involved in CH_4 dynamics. The positive
367 correlation between methanogens and CH_4 emissions (by either number of methanogens or
368 percentage of total microbes) is straightforward and unsurprising. Other studies have found
369 similar relationships between CH_4 emissions and methanogen abundance (Frey et al. 2011).
370 Because methanogens are the only biologic source of CH_4, this also makes conceptual sense;
371 the CH_4 has to come from somewhere.

372 The positive relationship between obligate methanotrophs and CH_4 emissions tells us
373 more about the system. Obligate methanotrophs rely on both CH_4 and TEAs to function.
374 Assuming CH_4 emissions are a good proxy for CH_4 availability, the positive correlation indicates
375 that the obligate methanotroph population responded directly to methane availability. The
376 second resource that methanotrophs need, TEAs, are harder to directly measure, but this study
377 has two lines of evidence that they were a limiting factor in the rhizosphere. First, optical
378 oxygen measurements from the experiment from which these sample were obtained (Waldo et
379 al. 2019) indicated that soil within planted boxes at both time points and within control boxes
380 lacked standing pools of oxygen (Waldo et al. 2019). Second, the ratio of glycolysis to Krebs
381 Cycle genes from the metagenomic data indicate the boxes producing the most CH_4 also had
382 more microbes capable of fermentation relative to respiration. While anaerobic respiration
383 processes exist, in nutrient-poor bogs such as the environment simulated here the alternative
384 TEAs are ultimately replenished from oxygen *(see Introduction for analysis supporting this*
385 *conclusion)*. Therefore, lacking a more direct measurement of total aerobic versus anaerobic
386 activity, the glycolysis to Krebs ratio can be used to qualitatively rank samples by whether
387 metabolic activity is ultimately dependent or independent on an environmental oxygen source.

388 The increased ratio of glycolysis to Krebs Cycle genes in boxes with high CH_4 emissions implies
389 that when CH_4 emissions are high, the rhizosphere has less oxygen available. The shift from
390 unplanted soil having low CH_4 availability and high TEAs to the mature rhizosphere having high
391 CH_4 and low TEA availability shows a change in what competitive pressures microbes face.

392 When oxygen demand exceeds supply, competition for oxygen must be more intense.
393 Obligate methanotroph abundance was apparently dependent on the concentration of CH_4,
394 despite the increasing competition for oxygen in those boxes with high CH_4 emissions and
395 highly anaerobic metabolisms. The samples from simulated plants, however, were taken from
396 sites with standing pools of oxygen (Waldo et al. 2019). The increased supply of oxygen relieved
397 the competition for oxygen and improved the environment for methanotrophs. Because the
398 simulated plants had relatively low CH_4 emissions, either the extra oxygen allowed
399 methanotrophs to thrive at lower CH_4 concentrations or allowed them to oxidize a higher
400 portion of the CH_4 produced. The methane monooxygenase (MMO) analysis showed that the
401 microbes in simulated plant boxes were using more particulate methane oxygenase (PMO)
402 while the rhizosphere microbes in planted boxes were using a soluble MMO to conduct
403 methanotrophy. The reason why PMO would be preferable to MMO in a setting with more
404 oxygen and no root exudates is not immediately clear, but it is further evidence that simulated
405 and real plants had important differences in the environment they created for methanotrophs.

406 The apparent success of methanotrophs in low-oxygen environments has two potential
407 explanations. First, there was likely some oxygen available. While the optical oxygen sensors
408 showed no detectible oxygen in any of the planted boxes (Waldo et al. 2019) it is well
409 established that wetland plants do transport oxygen through their aerenchyma (Fritz et al.

410 2011); oxygen was simply used so rapidly in all cases that it did not accumulate enough for the
411 optodes to detect it. Second, the obligate methanotrophs may have been performing AOM.
412 While the soil in this experiment likely had very low concentrations of alternative TEAs, as is
413 generally the case in freshwater bogs (Keller and Bridgham 2007; Conrad 2009), there may
414 actually still be high rates of AOM near oxygen sources where an elevated redox state allows
415 for recycling of low concentrations of TEAs (Keller and Bridgham 2007; Segarra et al. 2015). The
416 ANME2d abundance supports this idea, showing that at least one genus of AOM-capable
417 microbes was more abundant in the high-CH_4 environment of the rhizosphere late in the
418 experiment than in the oxygenated environment of the simulated plants.

419 Facultative methanotrophs, however, displayed a different pattern of abundance than
420 the obligate methanotrophs. Both types of methanotrophs were most common in the
421 simulated-plant boxes where oxygen was most abundant. However, while obligate
422 methanotrophs had a significant positive ($p < 0.05$) relationship with CH_4 emissions whether
423 measured by percentage of genes or by number of microbes in the planted and control boxes,
424 the facultative methanotrophs' correlation was not significant ($p > 0.05$) by either percentage
425 or number of cells . The ability of obligate methanotrophs to increase in abundance with CH_4
426 availability while facultative methanotrophs cannot implies that in low-oxygen environments
427 with high rates of metabolic activity, such as the rhizosphere examined in this study (Waldo et
428 al. 2019), obligate methanotrophs were able to out-compete facultative methanotrophs. This
429 outcome is reinforced by the observation that the obligate to facultative ratio was significantly
430 higher in the rhizosphere from the end of the experiment than it was in unplanted control soil.
431 Because CH_4 is generated in anoxic environments, obligate methanotrophs could gain a strong

advantage over their facultative competitors if they are able to conduct methanotrophy when oxygen concentrations are limiting. It has been hypothesized that obligate methanotrophs 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 AOM that the facultative methanotrophs could not.

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. 2A&C) and were most common in the rhizosphere, genetic indicators of oxygen limitation were highest in the boxes with the highest CH_4 emissions (Fig. 6), and microbial populations were largest in number when the most root exudates were available (Fig. 1, Waldo et al. 2019).

Obligate and facultative methanotrophs responded unevenly to the experimental conditions, demonstrating differing metabolic strategies. Both types of methanotrophy were most abundant around the simulated plants where oxygen was abundant; however, in the rhizosphere and control box soil where oxygen was limited, obligate methanotroph abundance was correlated with CH_4 availability (Fig. 2B&D), while facultative methanotroph abundance

was not (Fig. 6). This finding 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 AOM.

The net effect of these various impacts is that the *Carex* plants studied here greatly increased methanogen abundance, and therefore likely methanogenesis, but also increased methanotroph abundance, and likely methanotrophy. The rhizosphere became a region of intense competition for oxygen, implying that in the rhizosphere of a plant species with a higher rate of oxygen transport through aerenchyma the methanotroph abundance, and likely related rate of methanotrophy, could increase correspondingly.

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Fig. 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 (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. Each datapoint is one box, error bars are upper and lower quartiles determined from multiple samples measured from each box. Each data point is based on 1-3 microbe counts (mean 1.8) and 1-7 fluxes (mean 4.2).

Fig. 2 CH_4 flux versus (A) methanogen relative abundance, which had a correlation ($p < 0.05$, ρ=0.69), (B) methanotroph relative abundance, which had a correlation ($p<.01$) when excluding simulated plant boxes, (C) microbe count of methanogens, which had a correlation on the edge of significance ($p = 0.066$, ρ=.65), and (D) microbe count of methanotrophs, which had a correlation ($p<0.01$). Each datapoint is one box median, error bars are upper and lower quartiles determined from multiple samples measured from each box. Each flux value is calculated from 1-7 fluxes (mean 4.2). Each percentage value is calculated from 3-7 samples (mean 4.8) and each count is calculated from a combination of that sample's percentage and 1-3 total microbe counts (mean 1.8).

Fig. 3CH_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 relationship. However, the Spearman coefficient (ρ) for number of facultative methanotrophs was 0.62, indicating a trend, so the lack of significance was likely due to the low number of replicates. Each data point is based on 1-7 fluxes (mean 4.2), 1-3 metagenomes (mean 2.4), and for (B) 1-3 total microbe counts (mean 1.8).

Fig. 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 CH_4 flux in the final week before harvest. There was no statistically significant correlation ($p > 0.05$), though the Spearman coefficient does indicate a trend (ρ=0.63). Each data point is based on 1-7 fluxes (mean 4.2), 1-3 metagenomes (mean 2.4) for facultative methanotrophs, and 3-7 iTag samples (mean 4.8) for obligate methanotrophs.

Fig. 5 (A) Boxplots comparing the ratio of ANME2d archaea to total obligate 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 ANME2d to total methanotrophs and the CH_4 flux in the final week before harvest. There was no statistically significant relationship ($p > 0.05$, ρ=.16). Each data point is based on 1-7 fluxes (mean 4.2), 1-3 metagenomes (mean 2.4) for ANME2d archaea, and 3-7 iTag samples (mean 4.8) for obligate methanotrophs.

Fig. 6 CH_4 emissions in the week prior to harvest versus the ratio of genes involved in glycolysis to genes involved in the Krebs cycle were positively, though not necessarily linearly, correlated

730 ($p < 0.05$, ρ=.72). Each data point is based on 1-7 fluxes (mean 4.2) and 1-3 metagenomes
731 (mean 2.4).

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