

# Impacts of Wetland Plants on Microbial Community and Methane Metabolisms

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## Research Article

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# 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 roots create an environment which favors 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<sub>4</sub>) 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<sub>4</sub> (methanogenesis) and any oxidation of CH<sub>4</sub> (methanotrophy), which may attenuate emissions, are microbially mediated. Therefore, when factors like temperature are cited as influencing wetland CH<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> emissions is partially driven by the carbon in the exudates being processed into CH<sub>4</sub>, the exudates also result in more soil carbon being converted to CH<sub>4</sub> (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<sub>4</sub> 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 (Lenzowski 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<sub>4</sub> 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<sub>4</sub> (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.

Plants have great potential to influence the environment for microbes, including both methanogens and methanotrophs. By doing so, plants impact the amount of CH<sub>4</sub>, a potent greenhouse gas, which is emitted from wetlands. However, plant effects are not uniform and 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<sub>4</sub> 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<sub>4</sub> emission can be due to plant-exudate stimulation of CH<sub>4</sub> 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<sub>4</sub> emissions. In this study, we focused on *Carex aquatilis*, a common wetland sedge shown to increase methane emissions (Schimel 1995; Waldo et al. 2019). 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 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, thus simulating gaseous exchange without the biochemical effects of roots. There were 6 planted boxes, 2 control boxes, and 2 simulated

plant boxes analyzed. Optical oxygen sensors (optodes) measured oxygen concentration around the roots of plants and around the simulated plant roots (Larsen et al. 2011). Methane emissions were monitored throughout the experiment by placing a clear fluxing hood over each box and measuring the rate of CH<sub>4</sub> concentration increase in the headspace. During weeks 5 and 10 of the experiment, 4 plants were exposed to <sup>13</sup>CO<sub>2</sub> by placing a hood on each rhizobox and injecting 99 atom% <sup>13</sup>CO<sub>2</sub> into the headspace over a period of five consecutive days. This <sup>13</sup>CO<sub>2</sub> was photosynthesized and isotopically labeled the plants. Following labeling, root and soil samples were collected under nitrogen. Plants were destructively sampled in both weeks 5 and 10; the unplanted control boxes and simulated plants were only sampled in week 10, at the end of the experiment. Samples collected for chemical analysis were documented in Waldo et al. (2021), and samples collected for DNA analysis and microbe counts are described below.

Soil samples were collected at depths of approximately 5 cm, 20 cm, and 35 cm. All 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 4% paraformaldehyde (PFA). 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 sample was then stored in a 50/50 mix of 70% ethanol and 1X phosphate buffered solution (PBS, Fisher Scientific). 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<sup>3</sup> and 3.2 µm<sup>3</sup> 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  $\mu$ L aliquot was excluded from analysis.

## 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. 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.

The iTags classified microbes to the genus level using the V4 region of 16S rRNA sequences, using primers designed to amplify both bacteria and archaea (FW (515F): GTGCCAGCMGCCGCGGTAA, RV (805R): GGACTACHVGGGTWTCTAAT) (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 available as Online Resource 3.

The metagenomes were sequenced on an Illumina NovaSeq 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 BBDMap 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  $\rho$ , 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 rRNA) and KT443986 (pmoA) (He et al. 2016). The BLAST search only accepted sequences with E-values of  $10^{-20}$  or better for NC10 16s rRNA or 10 for NC10 pmoA.

Specific gene sets found in the metagenomes were used to assess functional differences in microbial populations. To determine whether samples had microbes with aerobic or anaerobic metabolisms present, the number of genes involved in glycolysis (a process which occurs in both anaerobic metabolism) was compared to genes involved in the Krebs cycle (aerobic metabolism). Because glycolysis is also used by aerobes, the glycolysis to Krebs ratio is not equal to the ratio of anaerobes to aerobes. However, there will be a qualitative correlation between the two ratios. 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. To compare methanotrophic metabolisms, methane monooxygenase (MMO) genes were compared. In addition to number of genes, a principle components analysis (PCA) was performed on the MMO gene sets to determine if different types of MMO were used in different samples. PCA was performed in MATLAB (2018b) using the “pca” function and default settings.

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 are presented in Table 1, gene sets for glycolysis are in Table 2, and gene sets used for MMO are in Table 3.

Table 1  
Gene sets used to identify the Krebs Cycle.

Gene	Citation
K00030 isocitrate dehydrogenase (NAD <sup>+</sup> )	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

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

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 (Fig. 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  $\text{CH}_4$  flux (Fig. 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. The most common classes present were Alphaproteobacteria (12.3% of all reads), Acidobacteria (10.0% of all reads), and Deltaproteobacteria (7.8% of all reads). A table showing genus-level results is available as Online Resource 4.

The median percentage of microbes that were methanogens in samples from each box was positively correlated with CH<sub>4</sub> emissions (Fig. 2A,  $p < 0.05$ ,  $\rho = 0.69$ ) as was the percentage of microbes that were methanotrophs, when excluding simulated plants (Fig. 2B,  $p < 0.01$ ,  $\rho = 0.78$ ). Simulated plants were excluded from the correlation test of methanotrophs because in the other three box types (planted boxes from weeks 5 and 10 and control boxes) the oxygen concentrations were low, but in simulated boxes, the oxygen concentrations were higher (Waldo et al. 2019) so the microbes faced a fundamentally different environment. Correlating methanotrophs with CH<sub>4</sub> emissions acts as a proxy for correlating methanotrophs with CH<sub>4</sub> 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<sub>4</sub> 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 (Fig. 2), there was no statistically significant ( $p > 0.05$ ) correlation between the flux of CH<sub>4</sub> 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) than the other box types (Fig. 3A), as was observed in obligate methanotrophs (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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> emissions ( $p < 0.05$ ,  $\rho = .72$ , Fig. 6), indicating more anaerobic 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<sub>4</sub> emissions. Finding more microbes in the rhizosphere of planted boxes harvested in week 10 than in unplanted soil (Fig. 1) 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<sub>4</sub> 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).

Methanogens and methanotrophs are directly involved in CH<sub>4</sub> dynamics. The positive correlation between methanogens and CH<sub>4</sub> emissions (by either number of methanogens or percentage of total microbes, Fig. 2A&C) is straightforward and unsurprising. Other studies have found similar relationships between CH<sub>4</sub> emissions and methanogen abundance (Frey et al. 2011). Because methanogens are the only biologic source of CH<sub>4</sub>, this also makes conceptual sense; the CH<sub>4</sub> has to come from somewhere.

The positive relationship between obligate methanotrophs and CH<sub>4</sub> emissions (Fig. 2B&D) tells us more about the system. Obligate methanotrophs rely on both CH<sub>4</sub> and TEAs to function. Assuming CH<sub>4</sub> emissions are a good proxy for CH<sub>4</sub> availability, the positive correlation indicates that the obligate methanotroph population responded directly to methane availability. The second resource that

methanotrophs need, TEAs, are harder to directly measure, but this study has two lines of evidence that they were a limiting factor in the rhizosphere. First, optical oxygen measurements from the experiment from which these samples were obtained (Waldo et al. 2019) indicated that soil within planted boxes at both time points and within control boxes lacked standing pools of oxygen (Waldo et al. 2019). Second, the ratio of glycolysis to Krebs Cycle genes from the metagenomic data (Fig. 6) indicate the boxes producing the most CH<sub>4</sub> also had a potentially greater prevalence of anaerobes in the microbial community. These data cannot be used definitively because glycolysis is used by both aerobic and anaerobic metabolisms, and anaerobic metabolisms exist that do not use it. However, lacking a more direct measurement of total aerobic versus anaerobic activity it can be used to qualitatively rank samples by relative abundance of anaerobic activity. The increased ratio of glycolysis to Krebs Cycle genes in boxes with high CH<sub>4</sub> emissions implies that when CH<sub>4</sub> emissions are high, the rhizosphere has less oxygen available. The shift from unplanted soil having low CH<sub>4</sub> availability and high TEAs to the mature rhizosphere having high CH<sub>4</sub> and low TEA availability shows a change in what competitive pressures microbes face.

When oxygen demand exceeds supply, competition for oxygen must be more intense. Obligate methanotroph abundance was apparently dependent on the concentration of CH<sub>4</sub>, despite the increasing competition for oxygen in those boxes with high CH<sub>4</sub> emissions and highly anaerobic metabolisms. The samples from simulated plants, however, were taken from sites with standing pools of oxygen (Waldo et al. 2019). The increased supply of oxygen relieved the competition for oxygen and improved the environment for methanotrophs. Because the simulated plants had relatively low CH<sub>4</sub> emissions, either the extra oxygen allowed methanotrophs to thrive at lower CH<sub>4</sub> concentrations or allowed them to oxidize a higher portion of the CH<sub>4</sub> produced. The methane monooxygenase (MMO) analysis showed that the microbes in simulated plant boxes were using more particulate methane oxygenase (PMO) while the rhizosphere microbes in planted boxes were using a soluble MMO to conduct methanotrophy. The reason why PMO would be preferable to MMO in a setting with more oxygen and no root exudates is not immediately clear, but it is further evidence that simulated and real plants had important differences in the environment they created for methanotrophs.

The apparent success of methanotrophs in low-oxygen environments has two potential explanations. First, there was likely some oxygen available. While the optical oxygen sensors showed no detectable oxygen in any of the planted boxes (Waldo et al. 2019) it is well established that wetland plants do transport oxygen through their aerenchyma (Fritz et al. 2011); 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 AOM. While the soil in this experiment likely had very low concentrations of alternative TEAs, as is generally the case in freshwater bogs (Keller and Bridgman 2007; Conrad 2009), there may actually still be high rates of AOM near oxygen sources where an elevated redox state allows for recycling of low concentrations of TEAs (Keller and Bridgman 2007; Segarra et al. 2015). The ANME2d abundance (Fig. 5) supports this idea, showing that at least one genus of AOM-capable

microbes was more abundant in the high-CH<sub>4</sub> environment of the rhizosphere late in the experiment than in the oxygenated environment of the simulated plants.

Facultative methanotrophs, however, displayed a different pattern of abundance than the obligate methanotrophs (Figs. 2–4). Both types of methanotrophs were most common in the simulated-plant boxes where oxygen was most abundant. However, while obligate methanotrophs had a significant positive ( $p < 0.05$ ) relationship with CH<sub>4</sub> emissions whether measured by percentage of genes (Fig. 2B) or by number of microbes (Fig. 2D) in the planted and control boxes, the facultative methanotrophs' correlation was not significant ( $p > 0.05$ ) by either percentage or number of cells (Fig. 3). The ability of obligate methanotrophs to increase in abundance with CH<sub>4</sub> availability while facultative methanotrophs cannot implies that in low-oxygen environments with high rates of metabolic activity, such as the rhizosphere examined in this study (Waldo et al. 2019), obligate methanotrophs were able to out-compete facultative methanotrophs. This outcome is reinforced by the observation that the obligate to facultative ratio was significantly higher in the rhizosphere from the end of the experiment than it was in unplanted control soil (Fig. 4A). Because CH<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> 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<sub>4</sub> availability (Fig. 2B&D), while facultative methanotroph abundance was not (Fig. 6). This finding implies that in low-oxygen, high CH<sub>4</sub> 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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**Availability of data and material:** Metagenomic data from the Joint Genome Institute (JGI) is available through the integrated Microbial Genomes & Microbiomes website at <https://img.jgi.doe.gov> under Study ID Gs0134277 and Project ID Gp0306226. Online Resource 4 contains a summary of the iTag data from JGI.

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

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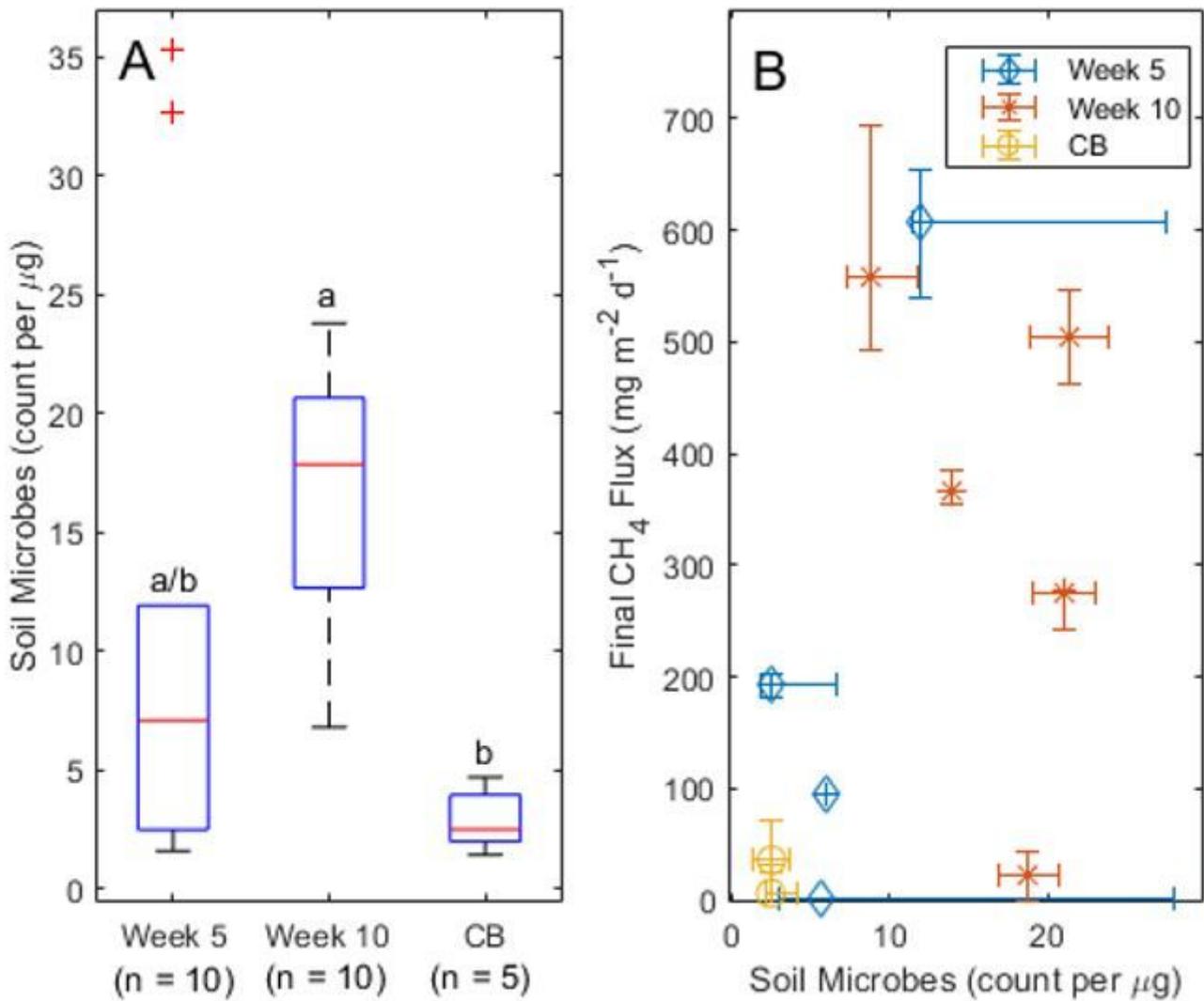
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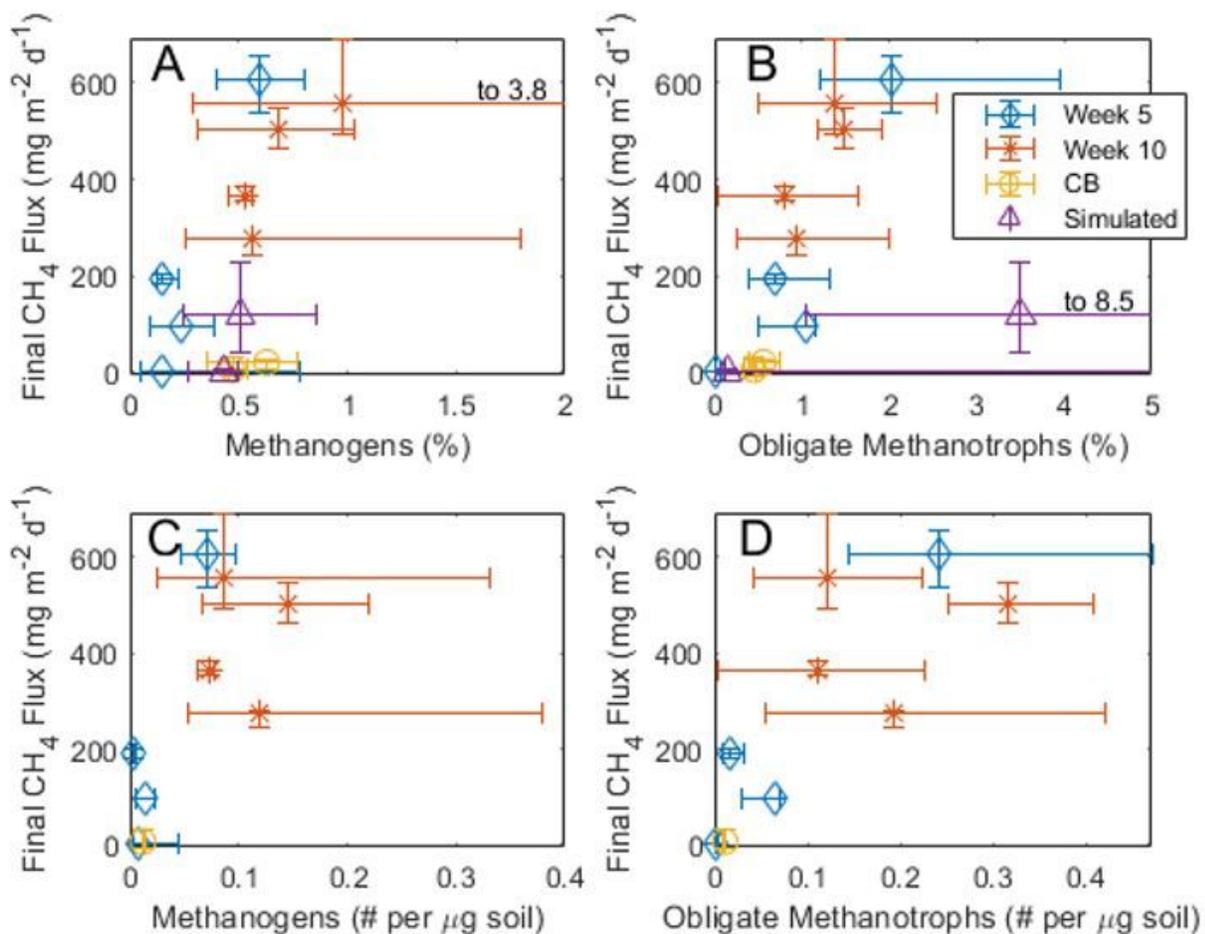
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# Figures



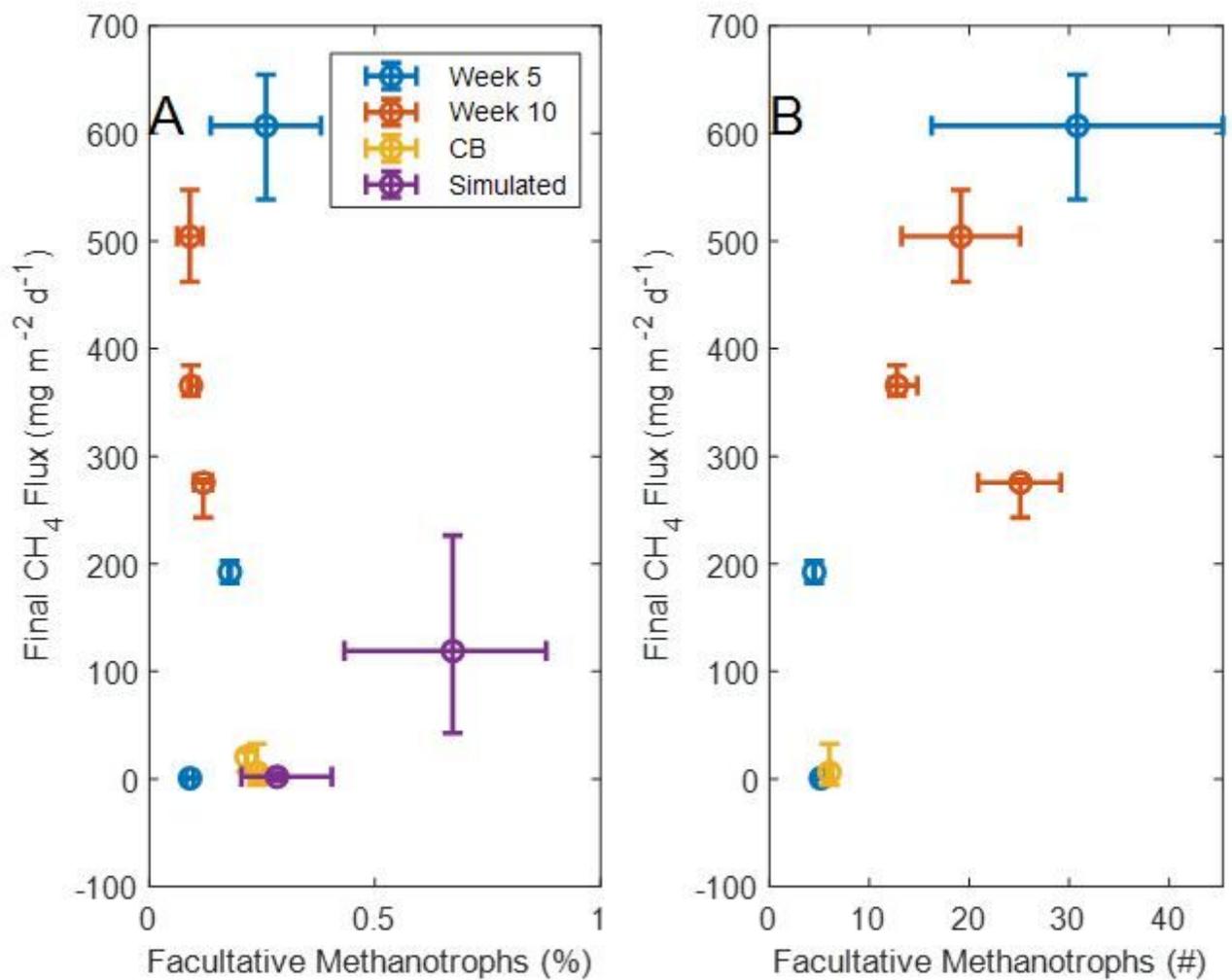
**Figure 1**

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  $2.7\sigma$  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).



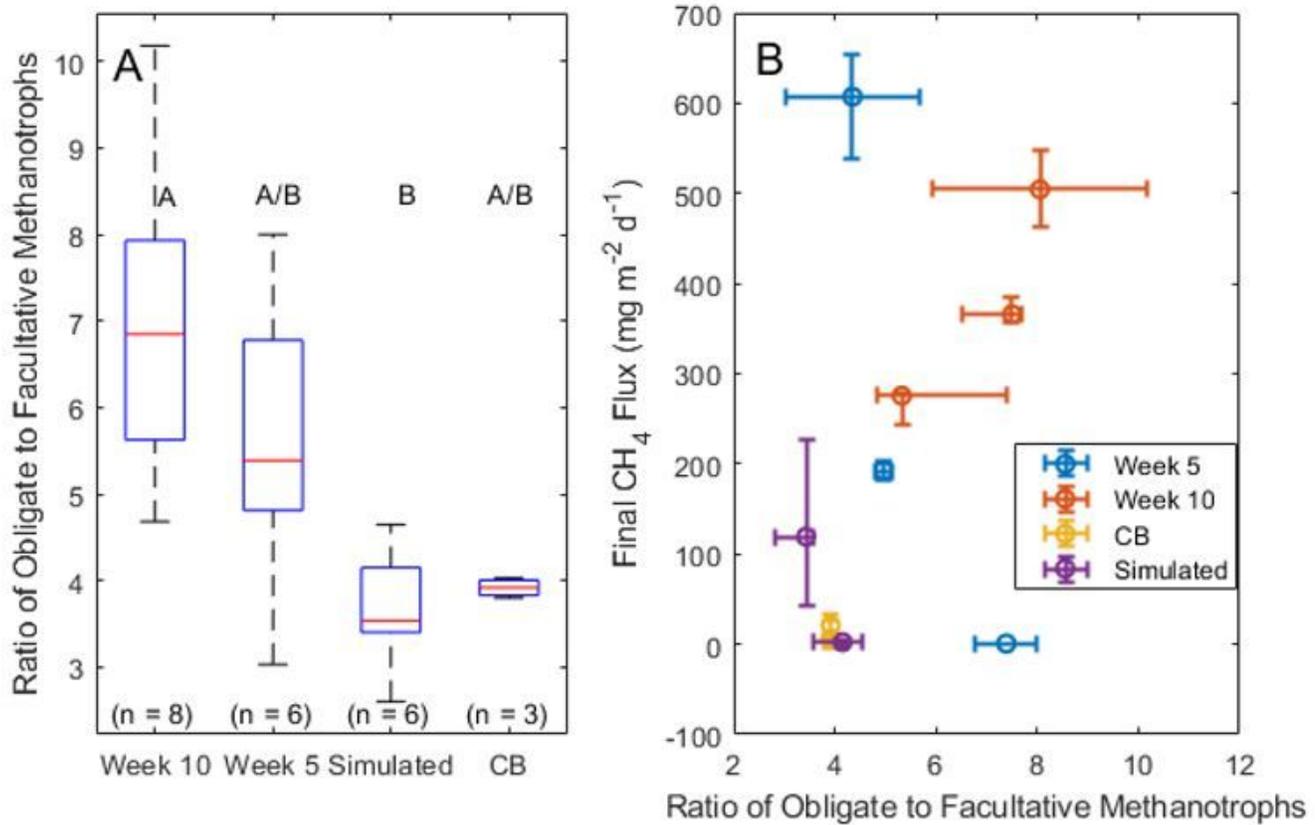
**Figure 2**

CH<sub>4</sub> flux versus (A) methanogen relative abundance, which had a correlation ( $p < 0.05$ ,  $\rho=0.69$ ), (B) methanotroph relative abundance, which had a correlation ( $p < 0.01$ ) when excluding simulated plant boxes, (C) microbe count of methanogens, which had a correlation on the edge of significance ( $p = 0.066$ ,  $\rho=.65$ ), 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).



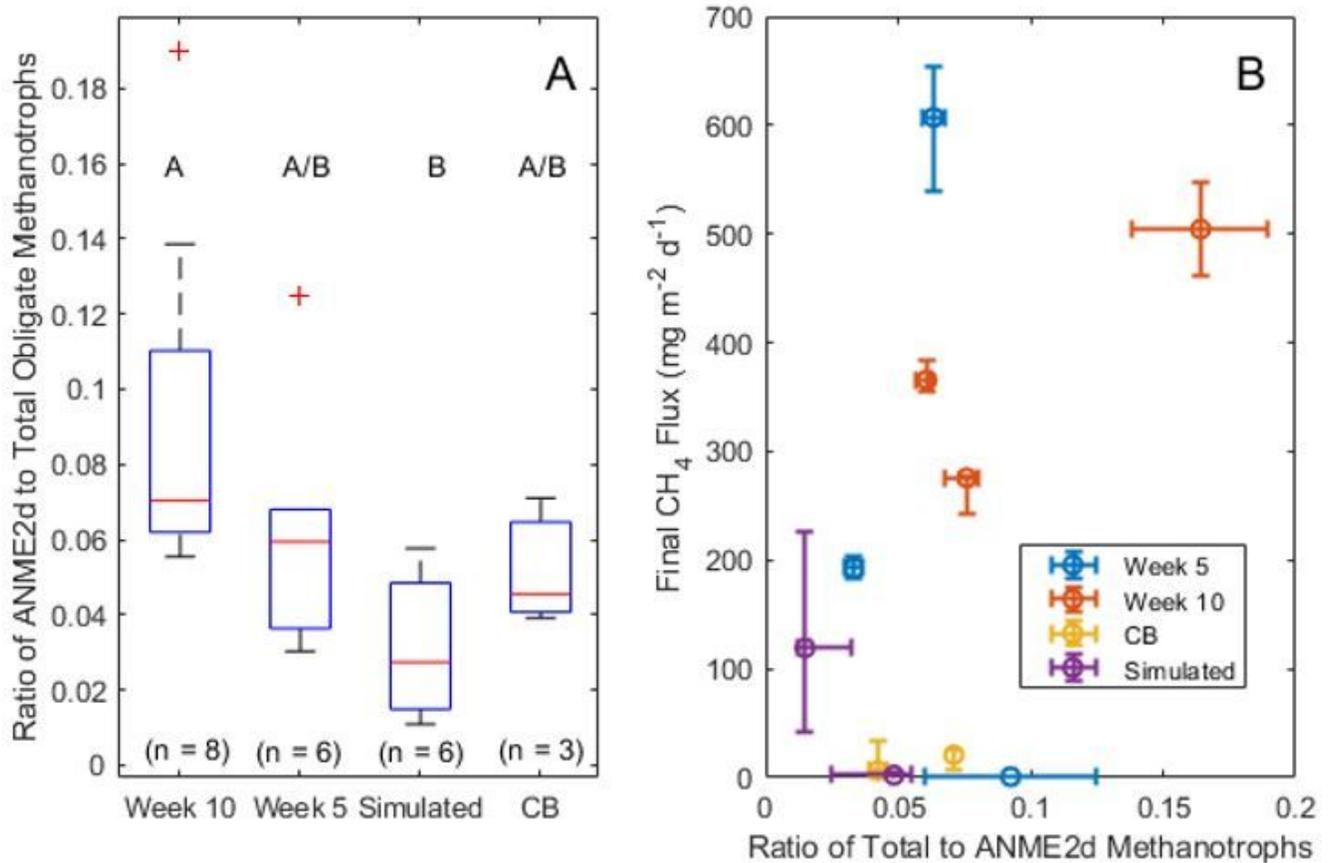
**Figure 3**

CH<sub>4</sub> 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 ( $\rho$ ) 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).



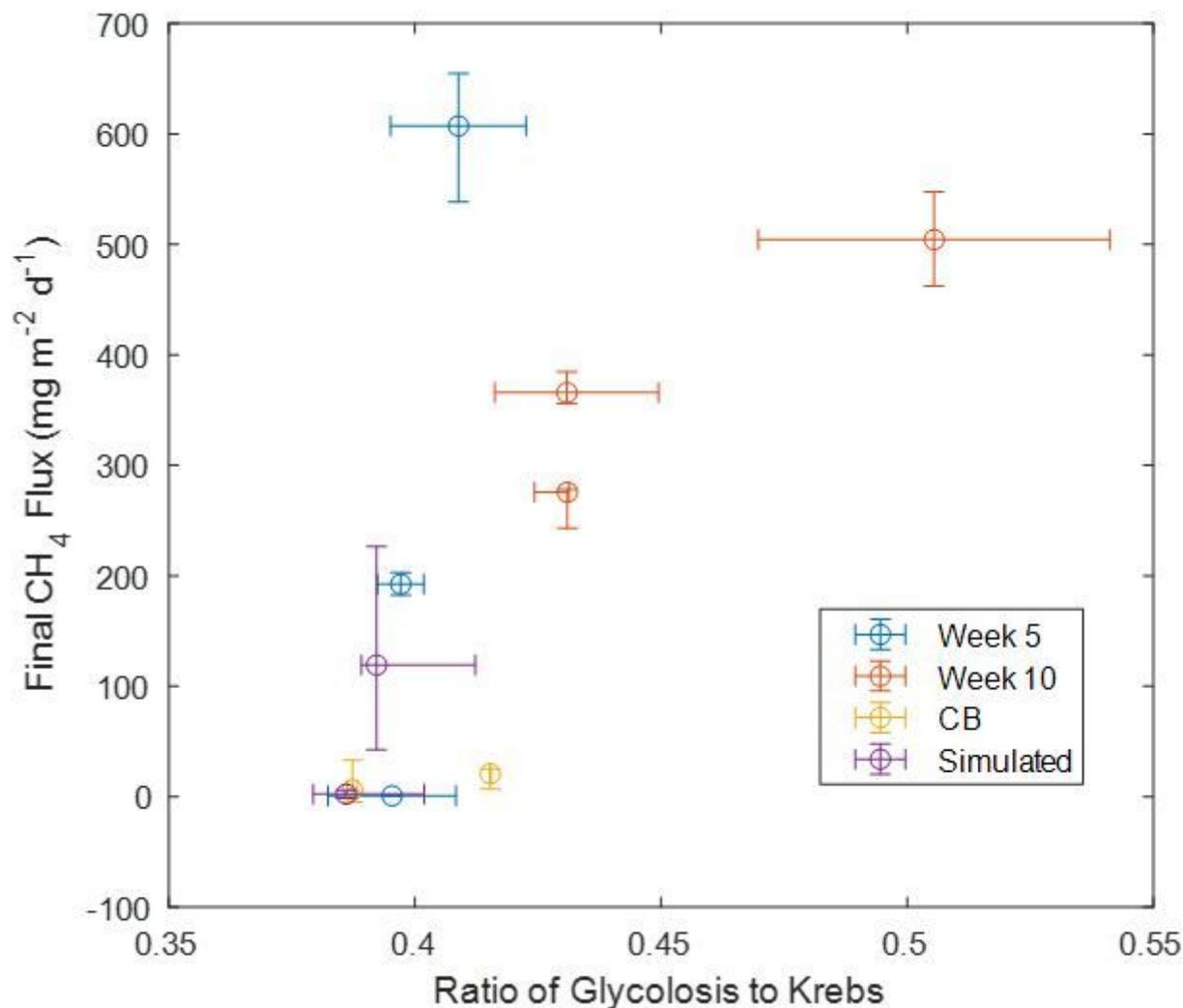
**Figure 4**

(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<sub>4</sub> flux in the final week before harvest. There was no statistically significant correlation ( $p > 0.05$ ), though the Spearman coefficient does indicate a trend ( $\rho=0.63$ ). 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.



**Figure 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<sub>4</sub> flux in the final week before harvest. There was no statistically significant relationship ( $p > 0.05$ ,  $\rho = .16$ ). 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.



**Figure 6**

CH<sub>4</sub> 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 ( $p < 0.05$ ,  $\rho = .72$ ). Each data point is based on 1-7 fluxes (mean 4.2) and 1-3 metagenomes (mean 2.4).

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