ACTIVE METHANE OXIDIZING BACTERIA IN A BOREAL PEAT BOG ECOSYSTEM

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ACTIVE METHANE OXIDIZING BACTERIA IN A BOREAL PEAT BOG ECOSYSTEM

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This work is dedicated to my family

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LIST OF SYMBOLS AND ABBREVIATIONS

S1T3M	S1 Bog, Transect 3, Midway down the transect
MEF	Marcell Experimental Forest
DNA-SIP	Deoxyribonucleic Acid Stable Isotope Probing
ARISA	Automated Ribosomal Intergenic Spacer Analysis
PCR	Polymerase Chain Reaction
pMMO	Particulate methane monooxygenase
sMMO	Soluble methane monooxygenase
pXMO	Sequence divergent particulate methane monooxygenase
QIIME	Quantitative Insights Into Microbial Ecology
MDS	Multidimensional Scaling
ANOVA	Analysis of Variance
SIMPER	Similarity Percentage
dwt	Dry Weight
SSU	Small Subunit

SUMMARY

Boreal peatlands are important ecosystems to the global carbon cycle. Although they cover only 3% of the earth's land surface area, boreal peatlands store roughly one third of the world's soil carbon. Peatlands also comprise a large natural source of methane emitted to the atmosphere. Some methane in peatlands is oxidized before escaping to the atmosphere by aerobic methane oxidizing bacteria. With changing climate conditions, the fate of the stored carbon and emitted methane from these systems is uncertain. One important step toward better understanding the effects of climate change on carbon cycling in peatlands is to ascertain the microorganisms actively involved in carbon cycling. To investigate the active aerobic methane oxidizing bacteria in a boreal peat bog, a combination of microcosm experiments, DNA-stable isotope probing, and next generation sequencing technologies were employed. Studies were conducted on samples from the S1 peat bog in the Marcell Experimental Forest (MEF). Potential rates of methane oxidation were determined to be in the range of 13.85 to 17.26 μmol CH_4 g dwt^{-1} d^{-1}. After incubating with ^{13}C-CH_4, DNA was extracted from these samples, separated into heavy and light fractions with cesium chloride gradient formation by ultracentrifugation and needle fractionation, and fractions were fingerprinted with automated ribosomal intergenic spacer analysis (ARISA) and further interrogated with qPCR. Based on ARISA, distinct banding patterns were observed in heavy fractions in comparison to the light fractions indicating an incorporation of ^{13}C into the DNA of active methane oxidizers. This was further supported by a relative enrichment in the functional gene *pmo*A, which encodes a subunit of the particulate methane monooxygenase, in heavy fractions from samples incubated for fourteen days. Within heavy fractions for samples incubated for 8 and 14 days, the relative abundance of methanotrophs increased to 37% and 25%, respectively, from an *in situ* abundance of

approximately 4%. Phylogenetic analysis revealed that the methanotrophic community was composed of both Alpha and Gammaproteobacterial methanotrophs of the genera *Methylocystis, Methylomonas,* and *Methylovulum*. Both *Methylocystis* and *Methylomonas* have been detected in peatlands before, however, none of the phylotypes in this study were closely related to any known cultivated members of these groups. These data are the first to implicate *Methylovulum* as an active methane oxidizer in peatlands, though this organism has been detected in another cold aquatic ecosystem with consistent methane emissions. The *Methylovulum* sequences from this study, like *Methylocystis* and *Methylomonas*, were not closely related to the only cultivated member of this genus. While *Methylocystis* was dominant in ^{13}C-enriched fractions with a relative abundance of 30% of the microbial community after an eight-day incubation, *Methylomonas* became dominant with a relative abundance of approximately 16% after fourteen days of incubation. The relative abundance of *Methylovulum* was maintained at 2% in ^{13}C-enriched fractions after eight and fourteen days.

CHAPTER 1

INTRODUCTION

Peatlands and Methane

Wetland ecosystems, such as peatlands, make a substantial contribution to the global carbon cycle, releasing 20-40% of the global methane to the atmosphere each year [1]. Peatlands are a particularly abundant in boreal climate zones but with some representation in tropical climate zones. Depending on environmental conditions, peatlands are designated fens and bogs [2-4]. The primary distinguishing characteristic is the source of water input to the ecosystem. Bogs, or ombrotrophic peatlands, receive water inputs solely from precipitation while fens, or minerotrophic peatlands, receive inputs from groundwater, rendering the bogs more nutrient limited than the fens [2, 4]. Predominant vegetation varies with sedges more abundant in fens, while mosses and woody plants make a larger contribution in bogs [4]. Boreal peatlands in particular are characterized by acidic, cold, waterlogged, and anoxic conditions with pH values as low as 3.5 and temperatures which can fall below freezing at the surface [2, 5, 6]. Plant communities in peat bogs are predominated by *Sphagnum* mosses which act to acidify their surroundings and release phenolic compounds which can inhibit microbial metabolism [6].

Due to a combination of the aforementioned physicochemical conditions in peatlands, the microbially mediated breakdown of organic matter proceeds slowly, resulting in a net carbon sink [2, 4, 6, 7]. Overall, peatlands are believed to store approximately one third of the world soil carbon, the equivalent of twenty-five to fifty percent of atmospheric carbon, in an ecosystem which only covers three percent of the earth's land surface area [6, 7].

Methane in Wetlands

Although peatlands act as net carbon sinks, these wetlands comprise a substantial source of methane due to the prevailing anoxic conditions [4]. Methane is an important greenhouse gas with a global warming potential twenty five times that of carbon dioxide over the course of one hundred years but the impact of changing climate conditions on methanotrophic communities remains uncertain [8]. Methane is produced by methanogenic archaea and emitted from wetlands by diffusion, ebullition, and through plants (aerenchyma flux) [4, 9].

Methane emissions from wetlands are highly variable ranging from 80 to 280 Tg CH_4 per year and accounting for 20-40% of the global methane released to the atmosphere [1, 4]. For northern peatlands, estimates average 46 Tg CH_4 per year, or 12.2% of global emissions [10]. Future projections of methane emissions remain uncertain for several reasons. One source of variability is uncertainty in the extent of wetland coverage, particularly wetlands in boreal regions. Another is a lack of understanding about how changing biogeochemical parameters responding to climate change may impact carbon cycling in individual wetlands. For example, temperature is projected to impact the release of greenhouse gases, which could vary substantially in different climate zones [4, 11].

The largest sink of atmospheric methane is chemical oxidation by hydroxyl radicals in the atmosphere which oxidizes about 490 Tg CH_4 per year, or more than 90% of emissions [12]. At the land surface, biological oxidation of methane is carried out by microbes, including both aerobic and anaerobic methane oxidizers, which consume up to 90% of methane produced [13]. While there is some evidence that anaerobic methane oxidation occurs in peatlands large knowledge gaps remain with regard to the importance and mechanism of this process [14, 15]. More likely, a large proportion of methane is oxidized aerobically by methane oxidizing bacteria as it diffuses through aerobic zones in the peat column, typically localized to surface regions or near plant roots [4, 9, 15, 16].

Methane-Oxidizing Bacteria

Aerobic methane oxidizing bacteria, or methanotrophs, are a subgroup of methylotrophic bacteria, which are characterized by their utilization of one-carbon compounds for energy and assimilation [17]. Methanotrophs are phylogenetically located in two phyla: the Proteobacteria and Verrucomicrobia. Within the Proteobacteria, the classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria are known to contain members capable of methylotrophic metabolism. Of these, the Alphaproteobacteria and Gammaproteobacteria as well as some members of the Verrucomicrobia contain members which are capable of oxidizing methane gas [18, 19]. Although members of the Verrucomicrobia have been widely detected in peatlands, none have been definitively linked to methanotrophy and thus more research is needed to ascertain the role of Verrucomicrobia in the carbon cycle of peatlands [20, 21]. Members of the NC10 phylum are also detected in peatlands and capable of anaerobic methane oxidation but their role in the biogeochemical cycles in peatlands remains understudied [22].

Alphaproteobacterial methanotrophs, previously classified as type II methanotrophs, are capable of methane oxidation and carbon assimilation through the serine pathway. By use of methane monooxygenase and methanol dehydrogenase enzymes, methanotrophs insert an oxygen molecule into methane to form methanol, which is subsequently converted to formaldehyde in a 1:1 ratio (1 mole CH_4: 1 mole CH_2O). In methanotrophs possessing the serine pathway, formaldehyde is converted to phosphoglycerate for cellular assimilation and carbon dioxide is fixed in the process [17]. Numerous alphaproteobacterial methanotrophs exhibit facultative heterotrophy, meaning that they are capable of utilizing other C1 compounds or even some multicarbon compounds including acetate and ethanol. For example, within the *Methylocystis* genus, acetate, ethanol, and methanol are utilized; in addition, propanol is utilized by the *Methylocella* genus [18, 21, 23, 24]. Some alphaproteobacterial methanotrophs are believed to be

capable of methane oxidation even at atmospheric concentrations of methane [19, 21, 23, 24].

Gammaproteobacterial methanotrophs, previously classified as type I or type X methanotrophs, assimilate formaldehyde through the ribulose monophosphate (RuMP) pathway. In the RuMP pathway, formaldehyde is converted to glyceraldehyde-3-phosphate, which is then incorporated into cellular material. Unlike the serine pathway, carbon dioxide is not fixed in the RuMP pathway [17]. Although one strain of *Methylomonas* can be adapted to grow on glucose or methanol in the absence of methane, no other gammaproteobacterial methanotrophs to date have been identified as facultative [25].

All methanotrophic bacteria possess a form of methane monooxygenase (MMO). This enzyme exists in two forms: membrane-bound particulate methane monooxygenase (pMMO) and soluble methane monooxygenase (sMMO). Almost all methanotrophs possess pMMO with the exception of a few Alphaproteobacterial methanotrophs that only possess the sMMO form (*Methylocella* and *Methyloferula*) [21]. For methanotrophs that possess both the pMMO and the sMMO expression of each is controlled by copper concentrations [17, 21]. A few Alphaproteobacterial methanotrophs may also express an alternate form of pMMO, pMMO2, which enables these methanotrophs to grow at low methane concentrations [18, 21]. A similar trait is observed within Gammaproteobacterial methanotrophs with the expression of a non-canonical form of the particulate methane monooxygenase, pXMO, which shows some *in situ* expression but requires further investigation as a functional target [26].

Common functional gene targets for methanotrophs are *pmo*A for the particulate methane monooxygenase and *mmo*X for the soluble methane monooxygenase [27]. Environmental samples from peat bogs have been screened in the past for both of these functional targets, and both have been shown to occur *in situ* [15]. Further investigation of the methanotrophic community in peatlands indicated a predominance of

Alphaproteobacterial members, particularly *Methylocystis sp.* for which there are several cultured representatives [15, 16, 20, 28]. More recently the potential contribution of Gammaproteobacterial methanotrophs has been recognized and the first Gammaproteobacterial methanotroph from a peat bog was recently isolated [28, 29].

Methanotrophs in Peatlands

The methanotrophic community in acidic boreal peatlands has been studied in the past with a variety of techniques including cloning, next-generation sequencing, metagenomics, cultivation, microarray analysis, and stable isotope probing (SIP) [5, 13, 15, 16, 20, 28-36]. Across many of these studies the consensus, until more recently, has been that acidic peatlands are dominated by Alphaproteobacterial, or type II, methanotrophs [13, 15, 16, 20, 30, 34-37]. Particularly, *Methylocystis sp.* have been isolated from acidic peatlands and have been identified as dominant methane oxidizers through clone libraries, microarray analysis of the functional gene target *pmo*A, next-generation sequencing and metagenomic analysis of peat, and through SIP experiments [13, 15, 16, 20, 30, 34-37].

In a few SIP studies on peatlands, Gammaproteobacterial, type I, methanotrophs were detected and until recently included primarily *Methylobacter sp.* [16, 38]. In 2011, Kip *et al*. used pyrosequencing of *pmo*A amplicons to show a predominance of *Methylomonas* as well as *Methylocystis* associated with *Sphagnum* mosses [29]. This was further supported with cultivation studies later that year which yielded cultures of *Methylomonas* strains as well as a strain that was related to *Methylosoma* and *Methylovulum*, all of which are Gammaproteobacterial methanotrophs [28]. Even more recent cultivation endeavors from Danilova and Dedysh resulted in a culture of the first described acidotolerant Gammaproteobacterial methanotroph, *Methylomonas paludis,* which was isolated from an acidic boreal peatland; however, within the same study *Methylocystis* was still determined to be the dominant methanotroph, with the Gammaproteobacterial

methanotrophs comprising an insignificant portion of the methanotrophic community [31]. Conversely in another study published in 2014 by Lin *et al*, both Alpha and Gammaproteobacterial methanotrophs (the family *Methylocystaceae* and the genus *Methylomonas* respectively) codominated the methanotrophic sequences in DNA and RNA pyrosequencing reads [5]. Such widely varied accounts of the Gammaproteobacterial contribution to methane oxidation in peatlands suggests that further research is required to gain a better grasp of the extent of the involvement of type I methanotrophs in acidic peatlands.

Significance

As mentioned previously, wetlands are the largest natural source of methane emissions, with peatlands acting as a primary contributor to this methane flux [4]. Although peatlands currently act as net carbon sinks and methane sources, the effects of climate change on these environments are not well understood. Additionally peatlands are not currently included in climate change models in spite of the large carbon store in these ecosystems [2, 4, 11]. In an effort to include peatlands in climate change models and to better understand how these fragile ecosystems may respond to changing climate conditions, it is important to first understand the microbial community composition and how this composition changes with changing environmental conditions [11]. One way to investigate this is to ascertain not only the members of the microbial community present but to also determine which of these members are actively involved in carbon cycling. A mesoscale climate change study called SPRUCE, spruce and peatland responses under environmental change, was implemented to help ascertain the effects of rising temperatures and carbon dioxide concentrations on boreal peatlands. This project is occurring over the course of ten years in the Marcell Experimental Forest (MEF) S1 bog in which the peat and surrounding air is being warmed and the carbon dioxide increased

to monitor the effects of changing climate conditions on numerous aspects of the carbon cycle in boreal peatlands, including methane cycling [39].

Objective and Hypothesis

Within the larger objectives of the SPRUCE project, my objective is to identify the active members of the microbial community involved in methane oxidation at the surface of the S1 bog. The active microbial groups that mediate a particular metabolic process may be effectively characterized through the use of DNA stable isotope probing (DNA-SIP). DNA-SIP has been successfully employed to investigate the methanotrophic community in a wide variety of environments including peatlands [14, 16, 30, 40, 41]. This method can be employed to ascertain the active methanotrophic community involved in methane oxidation by adding ^{13}C-CH_4 to microcosm incubations. Within these incubations methanotrophs participating in methane oxidation oxidize the "heavy" methane and incorporate the ^{13}C into their DNA through fixation of formaldehyde. This enriched DNA can be separated from the ^{12}C or light DNA using cesium chloride gradient formation. The heavy DNA can then be sequenced to determine the microbial community actively involved in oxidizing methane [42]. Alphaproteobacterial, type II, methanotrophs only require two moles of formaldehyde with one mole of CO_2 for use in central metabolism whereas Gammaproteobacterial, type I, methanotrophs require three moles of formaldehyde for use in central metabolism; therefore, the alphaproteobacterial methanotrophs may incorporate the ^{13}C into DNA more quickly than the gammaproteobacterial methanotrophs, indicating a need for multiple time points to assess the active methanotrophic community [17]. Based on aforementioned studies of methanotrophs in peatlands, it was hypothesized that the Alphaproteobacterial methanotrophs (Type II) were actively involved in methane oxidation with only a minor contribution from the Gammaproteobacterial methanotrophs (Type I).

CHAPTER 2

METHODOLOGY

Site Description and Sample Collection

Peat samples were collected at the S1 Bog located in the Marcell Experimental Forest (MEF; N 47°30.476'; W 93°27.162') north of Grand Rapids, MN [43]. This site has been described in detail in other publications [5, 34]. The average pH at the S1 bog is 3.5 - 4 with a salt content below detection limit [5]. Oxygen is depleted to below detection within the top five centimeters of the bog [5].

Samples were collected with a sterilized bread knife from the 0 – 10 cm depth inverval in hollows from S1, transect 3 in July 2012. The collected peat was homogenized and stored at 4°C until use in experiments.

Potential Rates of Methane Oxidation

Ten grams of homogenized peat from the 0 – 10 cm depth interval was added to 150 mL serum bottles in triplicate. Bottles were sealed with blue-butyl rubber stoppers and crimped with aluminum crimp seals. The headspace of each sample was amended with CH_4 (Sigma) to 1% (vol/vol) final concentration. Samples were incubated in the dark at room temperature and methane concentrations were monitored by gas chromatography with a flame ionization detector (GC-FID) on a Shimadzu model GC-2014 equipped with a methanizer over the course of two weeks.

DNA-SIP Incubations

Ten grams of homogenized peat from 0 – 10 cm from S1T3M were added to 150 mL serum bottles in duplicate for each treatment. Bottles were sealed with blue-butyl rubber stoppers and crimped with aluminum crimp seals. Treatments included those for which

the headspace was amended with 1% (vol/vol) 99.9% ^{12}C-CH_4 (Sigma); and others for which the headspace was amended with 1% (vol/vol) 99.9% ^{13}C-CH_4 (Sigma). Headspace concentrations were monitored with GC-FID equipped with a methanizer over two weeks. In parallel, ^{12}C and ^{13}C-CH_4 samples were sacrificed at the initiation of the experiment (T0)[*], after eight days (T1)[†], and after fourteen days (T2). A subsample of 5 grams was removed from each sample and frozen at -80°C until DNA was extracted for further analysis.

Dry weights were calculated by weighing out ~5 grams of peat from the incubation. Samples were then dried in a drying oven at 60°C until a stable mass was obtained.

DNA Extractions

DNA was extracted from frozen peat samples with the MoBio powersoil DNA kit according to the manufacturer's protocol and stored at -20°C for further analysis.

Stable Isotope Probing Procedure: Cesium Chloride Gradient Formation with Ultracentrifugation, Needle Fractionation, and DNA Precipitation

Stable isotope probing was conducted as described by Dunford and Neufeld [42]. In brief, DNA was added to a cesium chloride solution and centrifuged by ultracentrifugation. After 40 hours, samples were removed from the ultracentrifuge and fractionated by needle fractionation into twelve or thirteen fractions and the density of each fraction was determined with a digital refractometer. DNA was precipitated from all fractions with polyethylene glycol and glycogen as a carrier. Precipitated DNA was stored at -20°C for further analysis.

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[*] Samples sacrificed for the T0 sampling were only amended with ^{12}C-CH_4 due to lack of sufficient ^{13}C-CH_4 samples for all three timepoints.
[†] Only one ^{13}C-CH_4 sample was sacrificed at T1 due to lack of sufficient ^{13}C-CH_4 samples.

DNA-Fingerprinting: ARISA and qPCR for *pmo*A

All fractions from heavy and light samples from T1 and T2 were fingerprinted with ARISA. First, ARISA PCR was run on each sample with the S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18 primers. PCR products were run on a 1.5% agarose gel with 1xTBE buffer and successful reaction products were cleaned with the MoBio PCR cleanup kit following the manufacturer's instructions. ARISA PCR products were then run in an Agilent2100 model bioanalyzer and unique bands in heavy fractions were noted from ^{13}C-CH_4 samples to determine the success of the SIP incubation.

All fractions from the ^{13}C-CH_4 sample from the fourteen day incubation were also fingerprinted with qPCR for the functional gene *pmo*A. All fractions were run with A189f/Mb661r primers to target the abundance of *pmo*A genes. Samples were run against a standard curve in a StepOnePlus instrument with 96 wells.

Sequencing and Analysis

One ^{13}C-CH_4 sample from T1 (8 day incubation) and one ^{13}C-CH_4 sample from T2 (14 day incubation) were sequenced with Illumina sequencing technology at the Michigan State Sequencing Facility. Sequences were analyzed in QIIME 1.8.1 (Figure 1). Analyzed sequences were further characterized with Primer6 with SIMPER to identify organisms contributing to the differences between heavy and light fractions. The methanotrophs from all fractions were identified with a maximum-likelihood tree with bootstrap analysis (1,000 replications). Further statistical analyses were conducted in R [44].

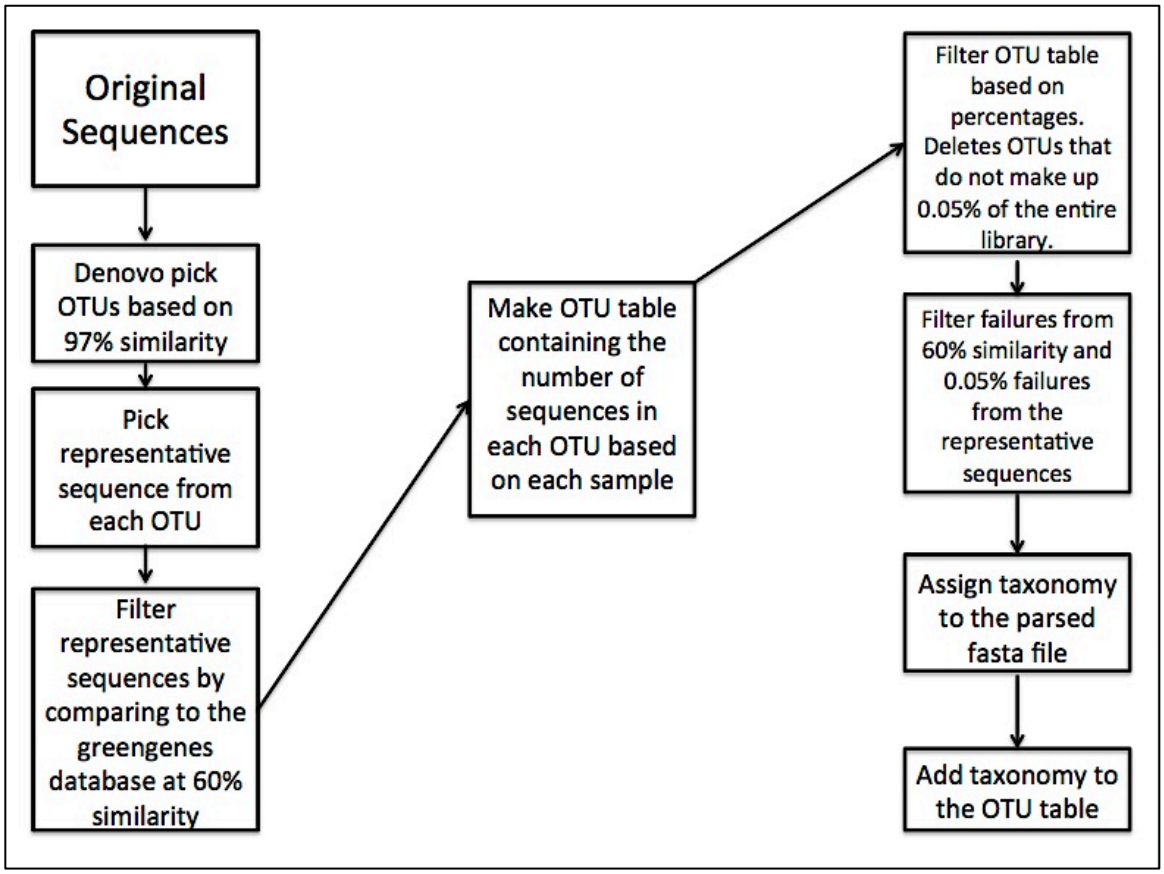

Figure 1: Flowchart for sequence analysis of SSU rRNA gene sequences in QIIME 1.8.1. All samples sequenced were DNA samples.

CHAPTER 3

RESULTS

Potential Rates of Methane Oxidation

The most rapid methane oxidation rates were observed within the first three days of incubation at room temperature. Rates ranged from 13.85 to 17.26 μmol CH_4 g dwt $^{-1}$ d^{-1}. Samples amended with ^{13}C-CH_4 and ^{12}C-CH_4 demonstrated potential oxidation rates of 15.08 ± 2.33 μmol CH_4 g dwt^{-1} d^{-1} and 15.93 ± 1.58 μmol CH_4 g dwt^{-1} d^{-1}, respectively. After two weeks, nearly all of the methane in the headspace had been oxidized (Figure 2). Samples were sacrificed after eight days for time point one, after peak methane oxidation rates were observed, and after fourteen days for time point two, after nearly all of the methane had been consumed.

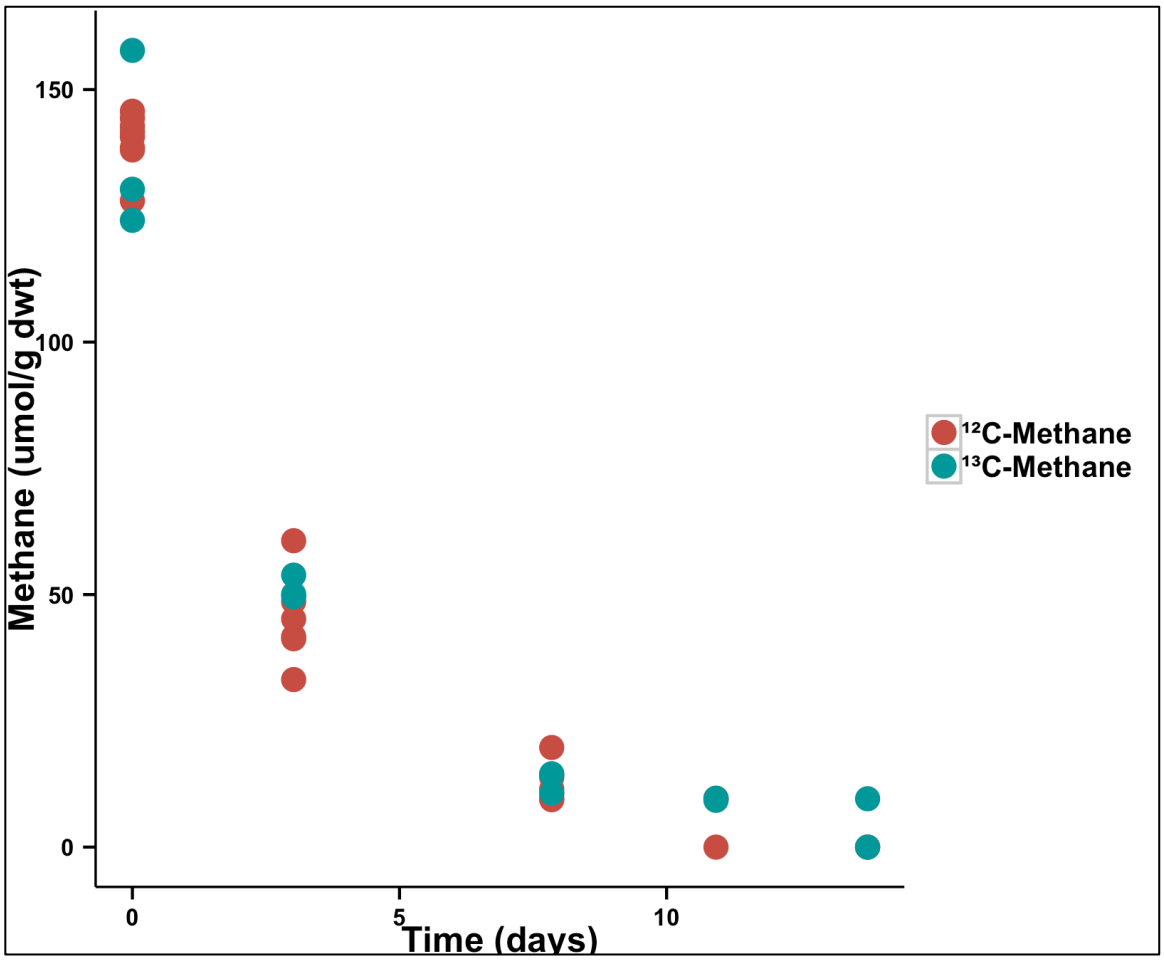

Figure 2: The oxidation of methane with time in the stable isotope probing incubations. Red dots represent ^{12}C-CH_4 treatments whereas blue dots represent ^{13}C-CH_4 amended treatments. The observed methane oxidation rates ranged between 13.85 and 17.26 µmol CH_4 g dwt^{-1} d^{-1}.

DNA-Fingerprinting: ARISA and *pmo*A qPCR

Unique bands were identified in the heavy fractions of ARISA gel images in samples collected after 8 and 14 days of incubation (Figure 3, 4). Fractions were further interrogated using qPCR for the functional gene *pmo*A for the 14-day sample. An enrichment of *pmo*A genes was observed in the heavy fractions in comparison to the light fractions with an abundance of approximately 12% *pmo*A in the ^{13}C-enriched fraction and 0.09% *pmo*A in the lightest, ^{12}C-enriched fraction (Figure 5).

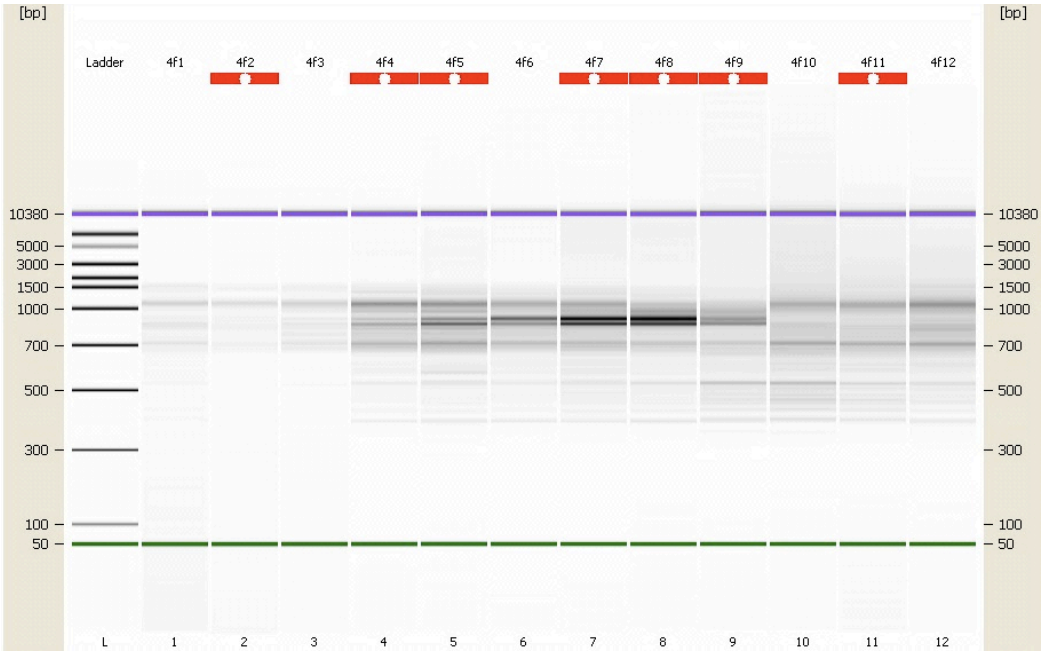

Figure 3: ARISA gel image for the ^{13}C-CH_4 amended treatment after 8 days of incubation. Heavy fractions are labeled 4f1-4f8. Light fractions are labeled 4f9-4f12. Unique bands were observed in fractions 7 and 8 at approximately 980 and 915 base pairs in length.

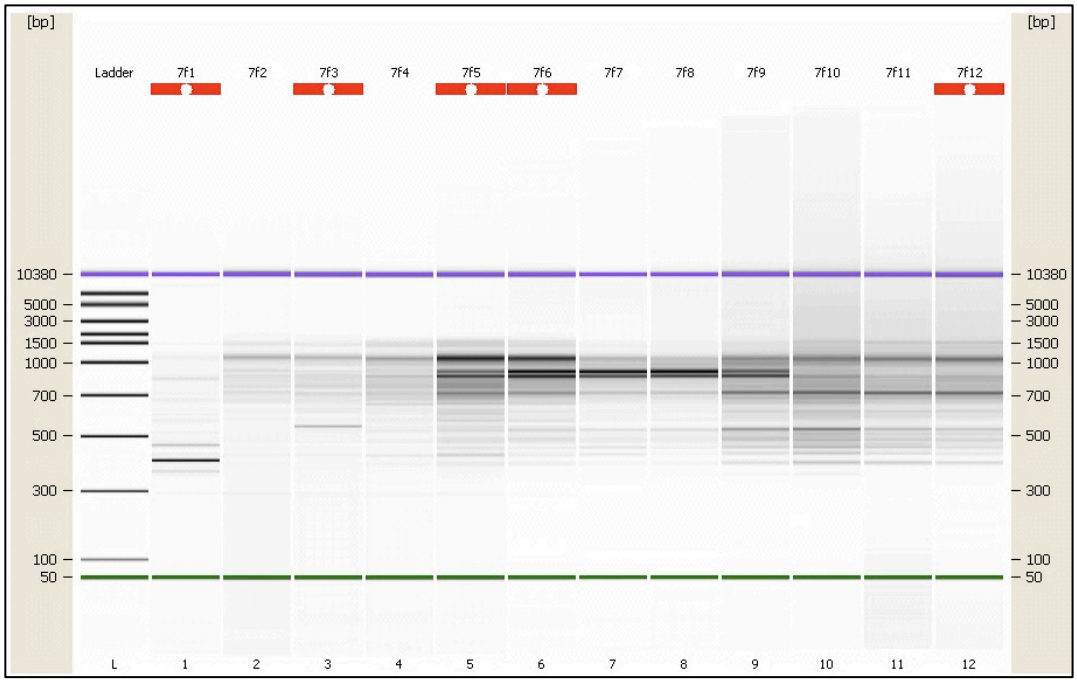

Figure 4: ARISA gel image for the ^{13}C-CH_4 amended treatment after 14 days of incubation. Labels 7f1-7f8 represent heavy fractions. Labels of 7f9-7f12 represent light

fractions. Unique bands were observed in fractions 7 and 8 at approximately 980 and 915 base pairs in length.

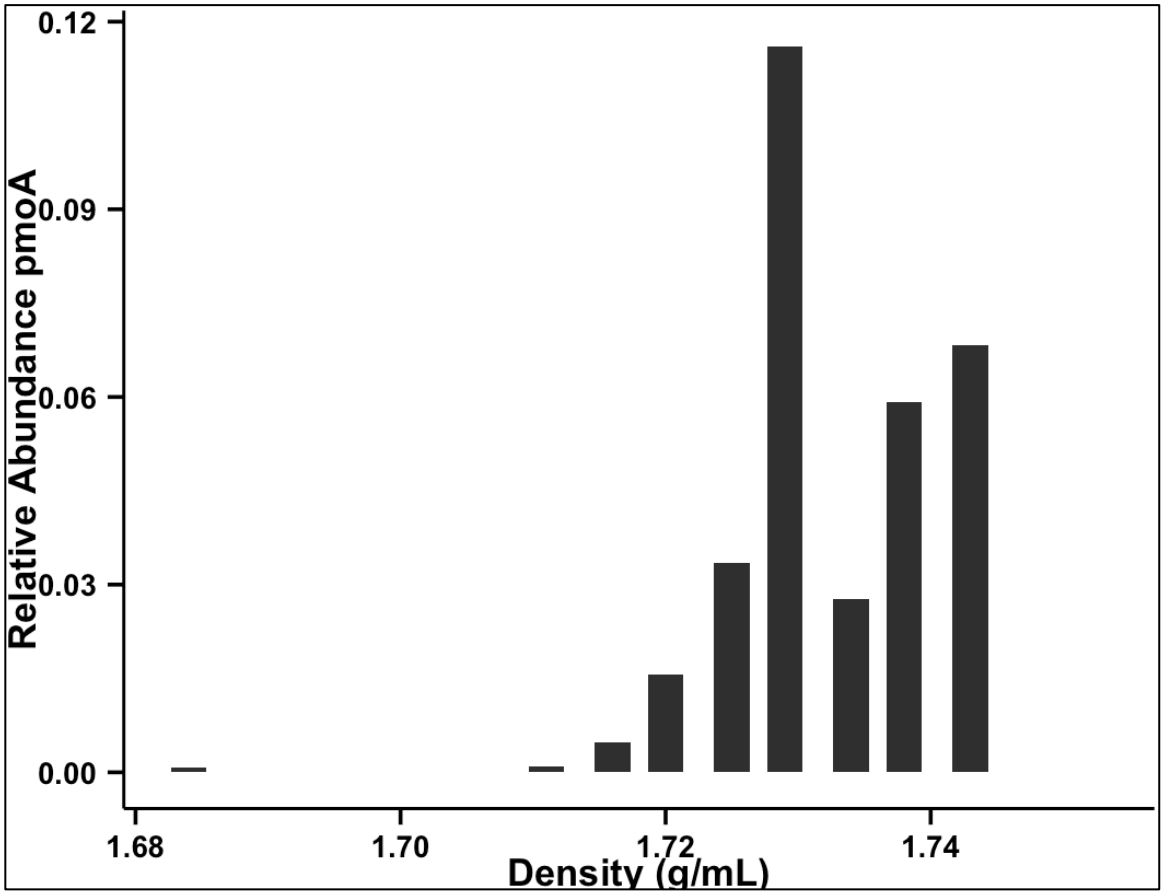

Figure 5: The relative abundance of *pmo*A genes in samples collected after 14 days of incubation. Enrichment was observed in heavy fractions, such as fraction 7 (density=1.729 g mL^{-1}), compared to light fractions such as fraction 10 (density=1.716 g mL^{-1}). Gene abundance of *pmo*A was normalized to rRNA gene abundance as determined by qPCR. Copies of *pmo*A were below detection for fractions one through three.

Sequence Analysis

Shifts in microbial community composition across density fractions were investigated with Bray-Curtis through similarity indices generated from next generation sequencing of SSU rRNA genes. A greater similarity was observed within the time points than among the densities of the samples (Figure 6). Community composition indicated a slight enrichment in Proteobacteria in the heavy fractions after 8 and 14 days of incubation, although this enrichment was not significant (F-value = 0.74, df = 3, p = 0.581) (Figure

7). The Proteobacterial community was composed of the four classes Beta, Delta, Gamma, and Alphaproteobacteria with a predominance of Alpha and Gammaproteobacteria detected (Figure 8). A greater relative abundance of methanotrophs was observed in the ^{13}C-enriched heavy fractions (36%) compared to light fractions (1%) 8 days of incubation (Figure 9). A relative abundance of 10 to 25% of known methanotrophic groups was observed after 14 days in the heavy fractions, while methanotrophs comprised 2% or less of the communities in the light fraction (Figure 9). The differing relative abundance of the methanotrophic community was significantly different across time points and densities (F-value = 7.144, df = 3, p = 0.0439) (Figure 9). In particular, the Alphaproteobacterial community changed significantly with time (p_1) and density (p_2) (F-value = 17.75, df = 3, p-value = 0.00894; Tukey post-hoc $p_1 = 0.0109$, $p_2 = 0.0338$) (Figure 9). The Gammaproteobacterial community did not change significantly with time or density (F-value = 5.099, df =3, p-value = 0.0748) (Figure 9). Based on SIMPER analysis, the organisms contributing to the differences between heavy and light fractions for both time points included Alpha and Gammaproteobacterial methanotrophs. The methanotrophs present in the samples were identified as *Methylocystis, Methylomonas,* and *Methylovulum sp*, none of which were closely related to any currently cultivated organisms (Figure 10). *Methylocystis,* an Alphaproteobacterial methanotroph from the family Methylocystaceae, comprised the majority of the methanotroph population in the heavy fractions after 8 days of incubation while *Methylomonas,* a Gammaproteobacterial methanotroph from the family Methylococcaceae, became the dominant methanotroph after 14 days of incubation (Figure 11). All three genera were enriched in the heavy fractions sampled at 8 and 14 days, although for the Gammaproteobacterial methanotrophs this enrichment was not determined to be significant (Figure 11).

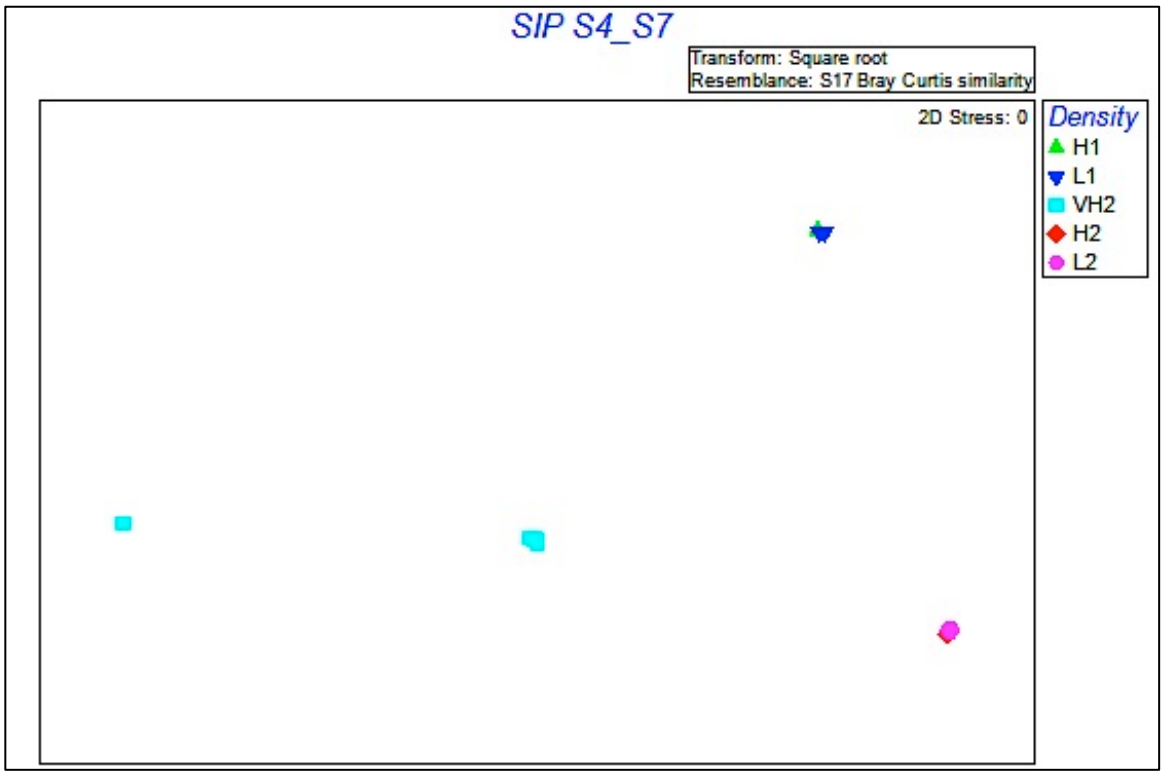

Figure 6: Multidimensional scaling (MDS) plot of the square root transformed Bray Curtis similarity indices for density fractions separated after 8 and 14 days of incubation. Values represent the following treatments and fractions: H1 represent ^{13}C enriched fractions, or heavy density fractions, after 8 days of incubation (green triangles), L1 represent ^{12}C enriched fractions, or light density fractions, after 8 days of incubation (inverse blue triangles), VH2 represent the densest fractions after 14 days of incubation (blue squares), H2 represent ^{13}C enriched fractions after 14 days of incubation (red diamonds), and L2 represent ^{12}C enriched fractions after 14 days of incubation (pink circles).

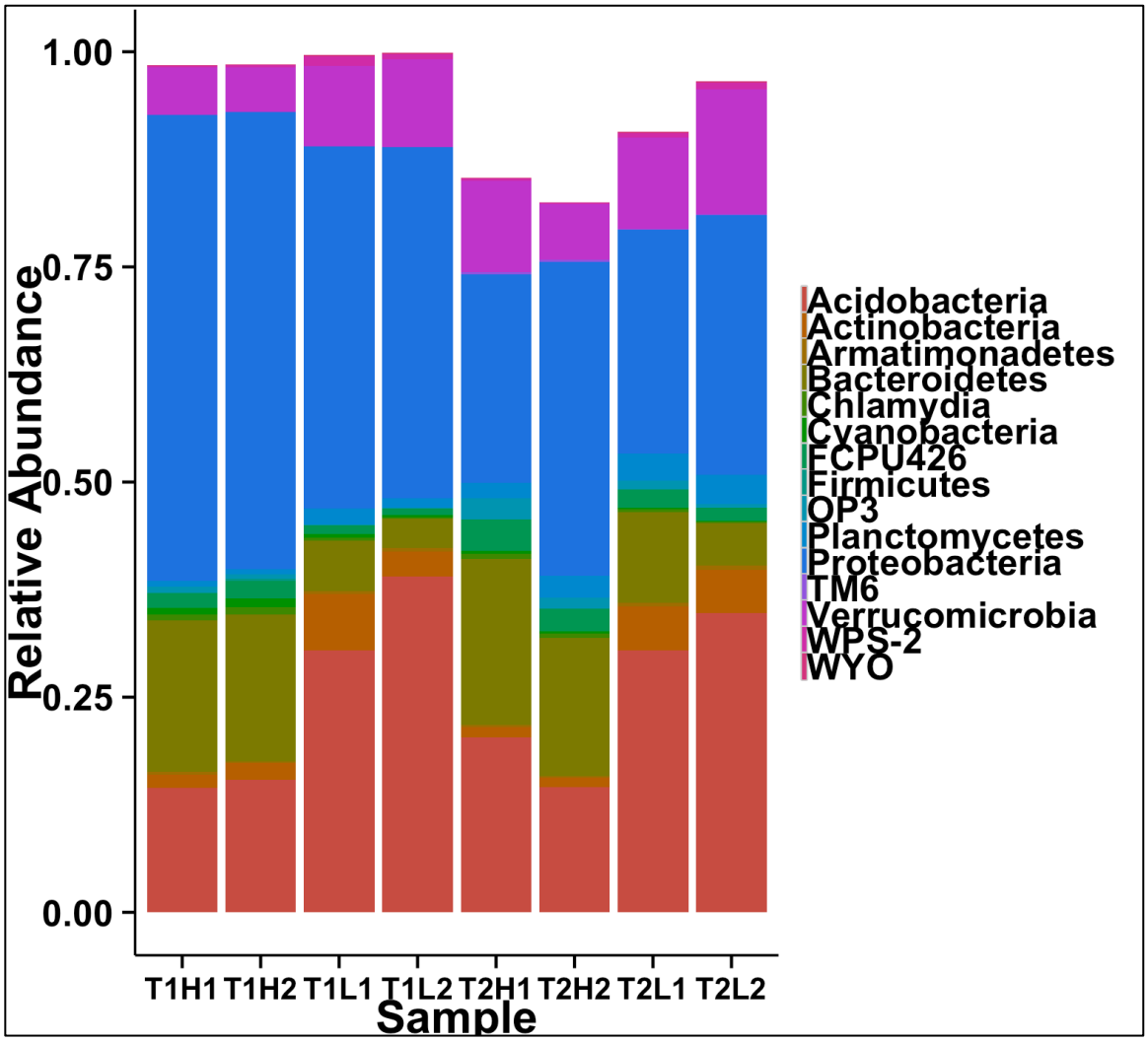

Figure 7: The microbial community composition shifted with the change in density. Shown above is the community composition in the heavy and light fractions, fractions 7 and 8 (H1 and H2, respectively) and fractions 10 and 11 (L1 and L2, respectively), after 8 days (T1) and 14 days of incubation (T2). In particular the Proteobacteria (blue bars) are more abundant in the heavy fractions than the light for both time points. When analyzed with ANOVA in R this shift was not significant (F-value = 0.74, df = 3, p = 0.581).

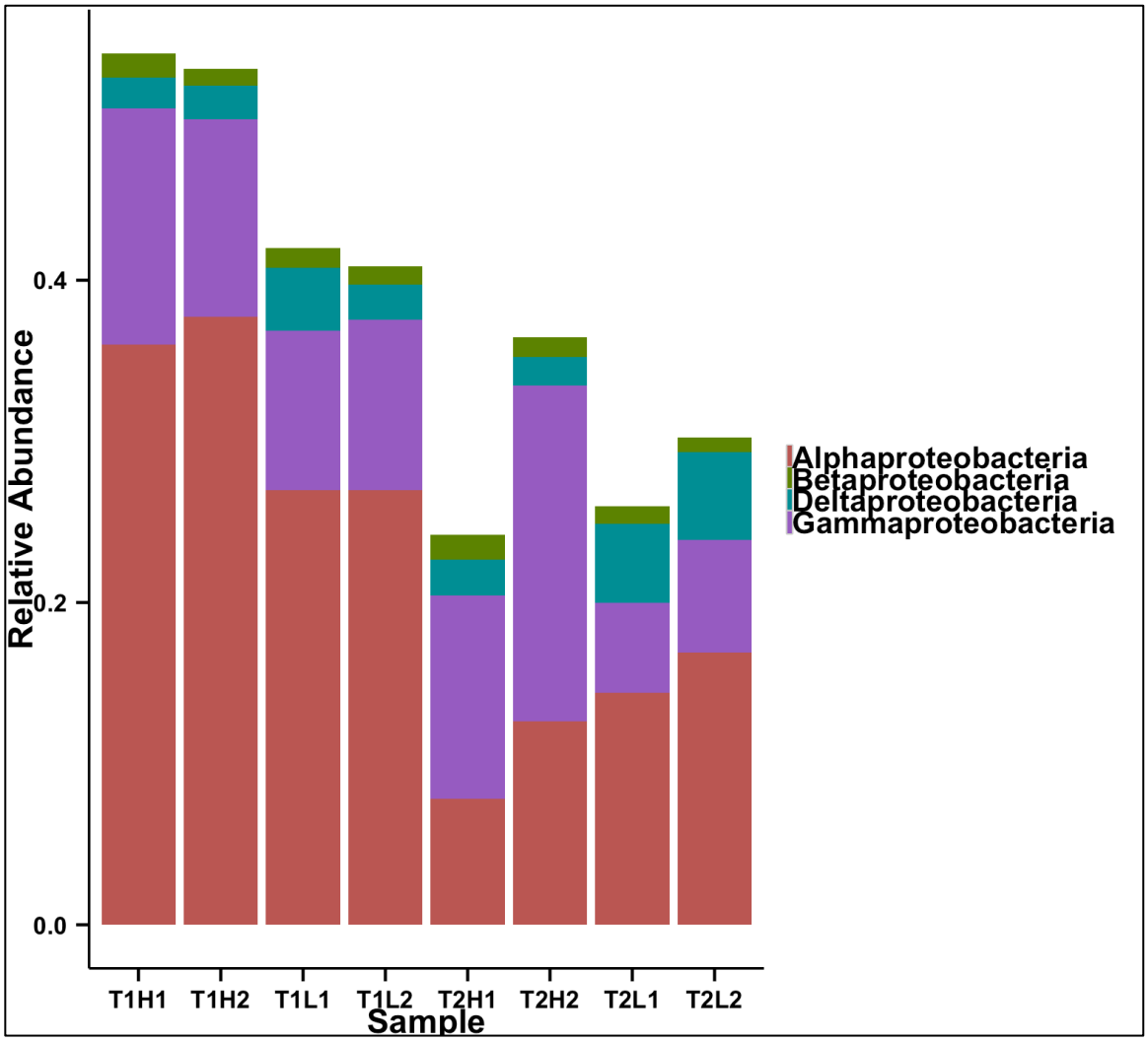

Figure 8: Proteobacterial community composition with phylotypess related to all four classes from both heavy (H) and light (L) fractions after 8 days (T1) and 14 days (T2) of incubation. The classes Alpha (red bars) and Gammaproteobacteria (purple bars) are enriched in heavy fractions relative to light.

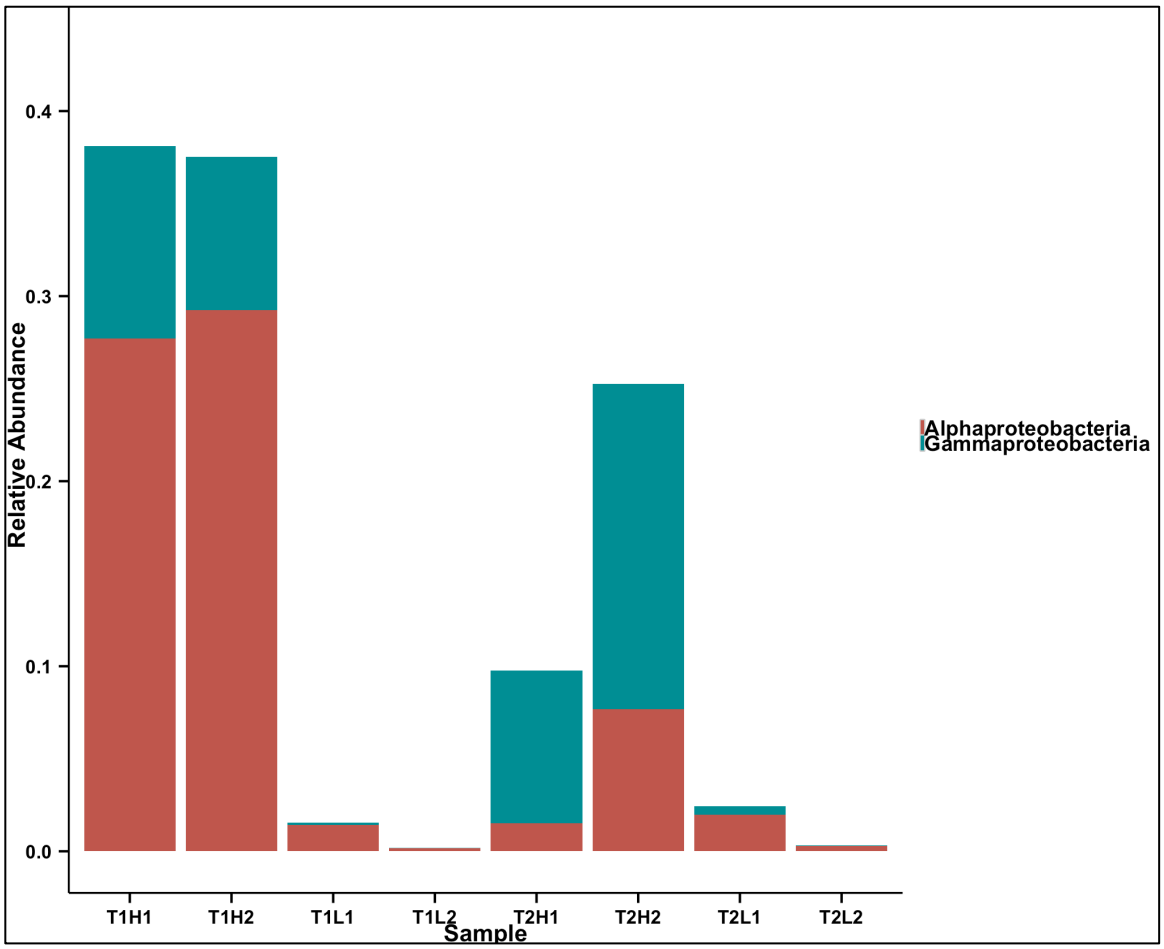

Figure 9: The relative abundance of Alphaproteobacterial and Gammaproteobacterial methanotrophs was greater in heavy fractions (H) compared to light fractions (L) after 8 days (T1) and 14 days (T2) of incubation. ANOVA analysis in R showed a significant difference between the methanotrophic communities in heavy and light fractions (F-value = 7.144, df = 3, p-value = 0.0439) and a significant difference in the Alphaproteobacterial abundance with density (T1 light to T1 heavy; p_1) and time (T1 heavy to T2 heavy; p_2) (F-value = 17.75, df = 3, p-value = 0.00894; Tukey post-hoc $p_1 = 0.0109$, $p_2 = 0.0338$). Regression analysis showed no significant difference with density or time for the Gammaproteobacterial community (F-value = 5.099, df =3, p-value = 0.0748).

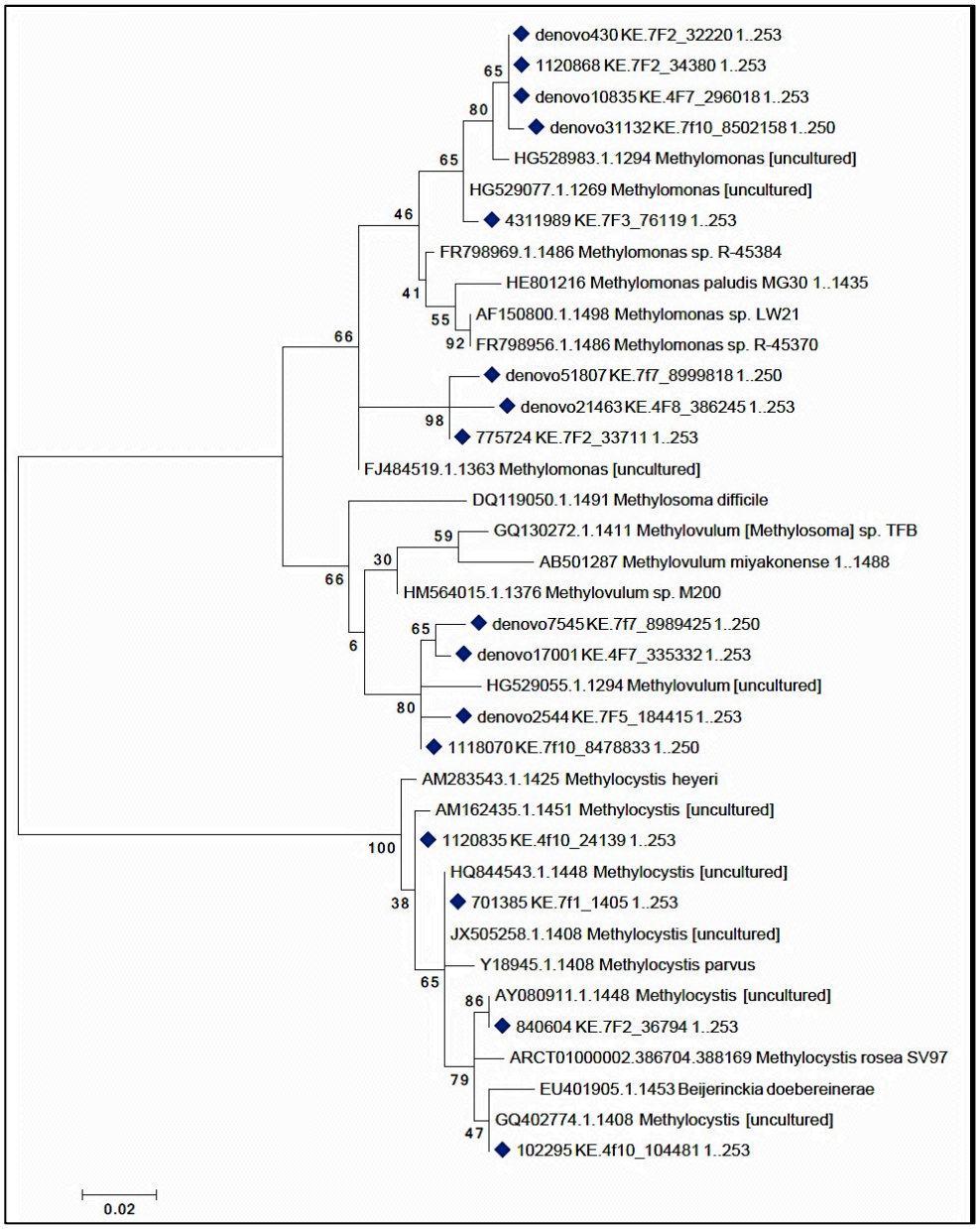

Figure 10: Phylogeny of methanotrophs within SIP fractions (blue diamonds) showing organisms within the Alphaproteobacteria, *Methylocystis sp.*, and the Gammaproteobacteria, *Methylomonas* and *Methylovulum sp.* This phylogenetic tree was prepared with the maximum-likelihood method with bootstrap analysis (1000 replications). None of the phylotypes are closely related to any of the currently cultured members within the respective genus.

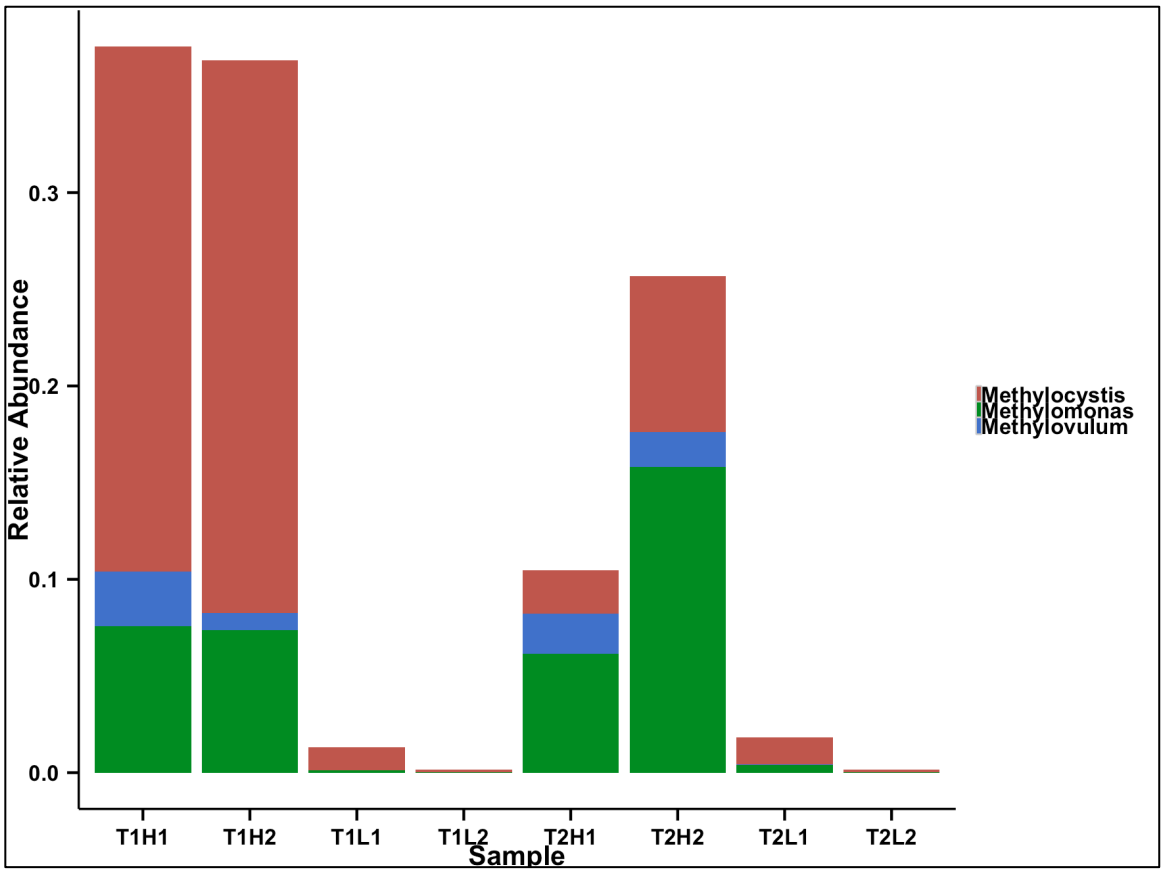

Figure 11: The relative abundance of methanotroph genera in heavy and light fractions shifted with time. The abundance of *Methylocystis* (red bars) changed significantly with time and density (F-value = 17.75, df = 3, p-value = 0.00894). The abundances of *Methylomonas* (green bars) and *Methylovulum* (blue bars) did not change significantly with time or density (F-value = 5.035, df = 3, p-value = 0.0762; F-value = 5.051, df = 3, p-value = 0.07584, respectively).

CHAPTER 4

DISCUSSION

While methanotroph populations have been investigated in peatlands for decades, the active members that mediate methane dynamics remain largely uncertain [13, 15, 20, 28-32]. This work probed the active methanotrophic community in the MEF peat bog at Grand Rapids, MN with stable isotope probing, which circumvents the need for cultivation [42]. Fractions enriched in ^{13}C were directly sequenced with next generation sequencing from samples incubated for 8 and 14 days. The benefit of utilizing next generation sequencing is that the DNA can be sequenced directly instead of relying on clones to capture the full diversity of the microbial community. Additionally, the time course provided the advantage of monitoring the community shift from high methane concentrations (after the 8 day incubation) to lower ones (after the 14 day incubation), which are more representative of potential methanogenesis rates.

The active methanotrophic community was composed of a combination of *Methylocystis* (an Alphaproteobacterial methanotroph), *Methylomonas,* and *Methylovulum* (both Gammaproteobacterial methanotrophs), which were observed to shift with time (Figure 10, 11). The presence of *Methylocystis* was not surprising given the well-documented presence, activity, and cultivated isolates from this methanotroph in acidic peatland ecosystems [13, 15, 20, 29, 30]. The presence and abundance of *Methylomonas* and *Methylovulum* were more surprising. While *Methylomonas* has been detected in amplicon sequences and cultured from peatlands, this genus has not been definitively linked to active methane oxidation in acidic boreal peatlands [28, 29, 31, 32]. *Methylomonas* has been detected in other environments such as a cave system, a soda lake, and landfill cover soil, through the use of SIP [45-47]. Studies in which

Methylomonas was found as an active methane oxidizer share the characteristic of environments that are more neutral to alkaline in pH [45-47]. Peatland methanotroph studies have utilized a variety of methods including microarrays, PLFA-SIP, clone libraries, cultivation methods, and DNA-SIP [13, 15, 16, 20, 30, 31, 33, 38, 45]; however, none of these studies combined DNA-SIP with next generation sequencing of the DNA from heavy and light fractions over a time course. Within the top 10 cm at the S1 bog, potential rates of methanogenesis only reach 0.025 μmol CH_4 g dwt^{-1} d^{-1} [43]. If these potential rates are representative of *in situ* rates of methanogenesis, the methane concentrations in the headspace after 14 days are more representative of the natural environment, lending greater significance to the shifting dominance structure of the methanotrophs (Figure 2, 9). The combination of next generation sequencing and multiple SIP time points enabled detection of the Gammaproteobacterial methanotroph community which can now be shown as key active methane oxidizers in an acidic peatland ecosystem. Further studies should focus on characterizing methanotrophic communities in incubations with lower concentrations of methane that more closely resemble the *in situ* condition.

Perhaps most interesting is the presence of *Methylovulum* in the active methanotrophic community. The first isolate of *Methylovulum miyakonense* was obtained in 2011 and to date no new species within this genus have been characterized [48]. Although originally isolated from forest soil, *M. miyakonense* was also recently isolated from peatland soil; however, none of the strains were shown to grow under acidic conditions, begging the question of the extent of the role *Methylovulum* might be playing in acidic peatland soil [31, 48]. To my knowledge, these are the first data directly linking *Methylovulum* to active methane cycling in peatlands. Although the relative abundance of *Methylovulum* was low in the fractions, there was a distinct enrichment in the heavy fractions compared to the light fractions that was nearly statistically significant (Figure 11).

Several possibilities may explain a seemingly neutrophilic methanotroph participating actively in methane oxidation in an acidic soil environment. One previous suggestion is the existence of neutral microenvironments, such as the endosphere, within the bog system, providing a small niche for *Methylovulum* [31]. The *Methylovulum* sequences present in this experiment were not closely related to *M. miyakonense*, suggesting the existence of yet uncultivated members of this genus that may be acido-tolerant or acidophilic. *Methylovulum* has also been identified as potentially psychrotolerant and capable of oxidizing methane at low concentrations from other environments, suggesting adaptability of this organism to the changing environmental conditions at the surface of boreal peatlands [49].

The methanotrophic community in wetlands plays an important role in the methane cycle by oxidizing methane before it reaches the atmosphere. With changing climate conditions the future role of methanotrophs in wetlands, particularly boreal peatlands, is uncertain. An important step to analyzing the potential impacts of the changing climate on the methane cycle in peatlands is to first identify the microorganisms actively involved in methane cycling. These data take a step toward that goal by identifying the active methane oxidizing bacteria at the S1 bog in the MEF. Active methane oxidizers include representatives from both Alpha and Gammaproteobacteria, including for the first time support that *Methylovulum* and *Methylomonas* are directly involved in methane oxidation at the surface of the peat bog. With these data, the specific bacteria involved in methane oxidation can be targeted for cultivation for future studies on the physiology of these organisms and subsequently the potential effects of climate change on the methane oxidizing community in boreal peat bogs.

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