Estuaries and Coasts
DOI 10.1007/s12237-012-9496-9

Anaerobic Metabolism in Tidal Freshwater Wetlands: II. Effects of Plant Removal on Archaeal Microbial Communities

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Received: 30 July 2011 / Revised: 27 February 2012 / Accepted: 28 February 2012

Abstract The interaction of plant and microbial communities are known to influence the dynamics of methane emission in wetlands. Plant manipulations were conducted in an organic rich (JB-organic) and a mineral rich (JB-mineral) site in a tidal freshwater wetland to determine if plant removal impacted archaeal populations. In concert, a suite of process-based measurements also determined the effects of plant removal on rates of methanogenesis and Fe-reduction. The microbial populations were analyzed with clone libraries of the SSU ribosomal RNA (rRNA) gene from selected plots, and terminal restriction length polymorphism (tRFLP) of the SSU rRNA and the methyl-coenzyme M reductase (mcrA) gene. Overall, methanogenesis dominated anaerobic carbon mineralization at both sites during the most active growing season. A total of 114 SSU rRNA clones from four different plots revealed a diversity of Euryarchaeota including representatives of the Methanomicrobiales, Methanosarcinales and Thermoplasmatales. The clone libraries were dominated by the Thaumarchaeota, accounting for 65 % of clones, although their diversity was low. A total of 112 tRFLP profiles were generated from 56 samples from 25 subplots; the patterns for both SSU rRNA and mcrA showed little variation between sites, either with plant treatment or with the growing season. Overall these results suggest that wetland soil archaeal populations were resilient to changes in the associated surface plant communities. The work also revealed the presence of novel, mesophilic Thaumarchaeota of unknown metabolic function.

Keywords Anaerobic microbial metabolism · Methane · Methanogen · Thaumarchaeota · Euryarchaeota · Tidal freshwater wetland

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Introduction

Tidal freshwater wetlands are inhabited by complex microbial communities that carry out important transformations of carbon and nutrients (Megonigal and Neubauer 2009), and they are home to a relatively high diversity of plants compared to saline tidal wetlands. These ecosystems are strongly influenced by the daily tidal cycles of flooding, but unlike most freshwater wetlands are not subjected to prolonged periods of either full submergence during flooding or desiccation during drought. This makes them good natural laboratories for studying the population and community ecology of microbes involved in dynamic processes under relatively constant environmental conditions (Megonigal and Neubauer 2009).

The submerged soils of tidal freshwater wetlands are generally anaerobic, leading to the accumulation of soil organic matter and simultaneous release of the potent greenhouse gas methane (CH_4). In large part, these ecosystem

Published online: 10 March 2012

processes are regulated by anaerobic decomposition, which is carried out exclusively by microbes. Following a series of initial degradation steps by fermenting bacteria, it is generally assumed that methanogenesis and Fe-reduction dominate anaerobic carbon mineralization in tidal freshwater wetlands (Megonigal et al. 2004). Methanogens belong to the phylum Euryarchaeota in the domain Archaea, while most Fe-reducers are in the domain Bacteria.

These microbial processes tend to be particularly active in the rhizosphere, a complex soil volume influenced by the growth and activity of plant roots. The rhizosphere is generally enriched in labile organic matter due to plant root exudates and decaying root matter. In saturated sediments, bulk rhizosphere soils are anoxic and can be the site of methane production (Lu et al. 2005) or Fe-reduction (Weiss et al. 2005). However, rhizosphere soils can also be enriched in oxygen because plant roots serve as conduits for oxygen, through radial oxygen loss (ROL), into the wetland's otherwise anoxic soil (Neubauer et al. 2008). This results in a complex mosaic where anaerobic and aerobic processes can occur in close spatial associations. The role of ROL in wetland microbial ecology is especially important because it has the potential to establish steep redox gradients that specific groups of microbes can exploit to oxidize methane, Fe(II), or organic matter.

Plant-mediated rhizosphere processes play a critical role in controlling whether iron cycling or methanogenesis dominates freshwater biogeochemistry (Sutton-Grier and Megonigal 2011). In particular, Neubauer et al. (2005) hypothesized that ROL by an active plant community in a tidal freshwater marsh stimulated oxygen-dependent microbial Fe-oxidation, which in turn provided a source of Fe(III) as an electron acceptor for Fe reduction, leading to a net result whereby Fe-reduction continuously suppressed methanogenesis. Only after plants had senesced, and this Fe(III) supply was diminished, did CH_4 production come to dominate. These studies suggest that plant–microbe linkages are important for determining the dominant pathway of microbial respiration in wetland soils and may be a key mediator of CH_4 production in the anaerobic soil environments of tidal freshwater wetlands.

Although there are still ongoing questions about the controls of the microbial processes involved in anaerobic carbon mineralization (e.g., Keller et al. 2012), there is even less known about the microbial communities that drive these processes. A growing body of evidence from other freshwater ecosystems (rice paddies and northern peatlands) suggests that phylogenetically related groups of either hydrogenotrophic (forming CH_4 from hydrogen and CO_2) or aceticlastic (forming CH_4 from acetate) methanogens are generally present in freshwater soils along with members of the order Thermoplasmatales within the class Euryarchaeota (Cadillo-Quiroz et al. 2006, 2008; Lueders et al. 2001). Further, there is evidence that the plant community can influence microbial communities in wetland soils. For example, original cultivation-independent studies done on rice roots showed unique clades of methanogens (Euryarchaota) and a novel group of the phylum Crenarchaeota (now Thaumarchaeota) associated with the roots which were not found in the bulk soil (Grobkopf et al. 1998). The role that archaeal communities play in important anaerobic processes in tidal freshwater wetlands remains largely unknown, despite the unique opportunities these ecosystems provide for investigating the interplay between rhizosphere-associated microbial processes and plant activity.

The goal of this project was to characterize the overall archaeal microbial community including the methanogenic community in two sites in a tidal freshwater wetland located in Maryland, USA. Study plots were established at both sites made up of alternating subplots that contained the natural plant community, or from which the plants had been removed. The size of these 1-m^2 subplots is much larger than the interaction zone of individual plant–microbe associations; thus, this was an ecosystem-scale experiment to test the importance of plant-mediated rhizosphere processes (i.e., ROL) in regulating anaerobic microbial carbon mineralization. The work of Keller et al. (2012) revealed that methanogenesis was the dominant microbial process during most of the growing season at both sites, regardless of the presence or absence of plants. Based on these findings, we hypothesized that (1) given the importance of methanogenesis, in these soils the archaeal community would be dominated by the Euryarchaeota, the archaeal phylum that includes the methanogens; and (2) given the general lack of response of methanogenesis to plant removal, microbial communities in adjacent plots with and without plants would not differ from one another. Despite the potential importance of archaea, especially methanogens, in tidal freshwater ecosystems, this work is unique in characterizing these microbial communities.

Methods

Experimental Plots and Sampling Strategy

Two sites were investigated at the Jug Bay Wetlands Sanctuary (Patuxent River, MD, USA; lat 38.7811, long −76.7131) in 2007 and 2008. The two sites were located approximately 110 m from one another and shared a plant community dominated by *Typha* spp. (cattail) with lesser amounts of *Peltandra virginica* and *Polygonium arifolium*. The 2007 site had an organic-rich soil (58.1±1.7 % organic matter determined by loss on ignition at 550 °C, mean±1 SE, n=6) and is referred to as Jug Bay—Organic (JB-organic), while the 2008 site had a more mineral-rich soil

(29.7±1.2 % organic matter; n=6) and is referred to as Jug Bay—mineral (JB-mineral). At JB-organic a grid of twelve 1.5 m×1.5-m plots was established, where plants were removed from alternating plots by hand clipping during the growing season. At the JB-mineral site twelve 0.5 m× 2.0-m plots were established with plant removal from alternating plots. The details of plot layout, manipulation, and plant removal, as well as sampling protocols are described in Keller et al. (2012). Soil samples for microbial analysis (May to October) were collected from the same set of cores used to measure rates of anaerobic carbon mineralization in the 5–10-cm soil depth. Soils for microbial community analysis were processed in ambient atmosphere using a sterile spatula and forceps to collect and homogenize root-free soil subsamples which were frozen at −80 °C in 15-mL centrifuge tubes until further processing.

DNA Extraction

DNA extraction was done from frozen samples. Soil samples were thawed, and approximately 1 g of soil was removed and treated with a MoBio PowerSoil DNA Isolation kit (MoBio Laboratories, Carlsbad, CA). Samples were incubated for 10 min at 70 °C in the first buffer (C1) before proceeding with the standard kit procedures for extraction. The quantity and quality of DNA was checked by absorbance at 260/280 using a Nanodrop spectrophotometer and by agarose gel electrophoresis. The DNA yield for the samples ranged from 9.5 to 65 ng/μL with a mean value of 36 ng/μL.

tRFLP Analysis

Terminal restriction fragment length polymorphism (tRFLP) is a cultivation independent method that allows rapid discrimination of populations and assessment of diversity within microbial communities (Liu et al. 1997). In this case the analysis was focused on the archaeal community. tRFLP was performed on DNA extracts for the archaeal small subunit ribosomal RNA gene (SSU rRNA) and the methyl-coenzyme M reductase (mcrA) gene, following the general procedures used by Lueders et al. (2001). The conserved McrA enzyme carries out an essential step in methanogenesis and is thought to exist universally in methanogens. The archaeal SSU rRNA was amplified from environmental DNA extracts using Ar20F and FAM-labeled Ar912r primers (Lueders et al. 2001). The DNA was amplified on an Eppendorf thermal cycler using a standard 60–50 °C touchdown program followed by 30 cycles of amplification. The FAM-labeled DNA amplicon was digested with TaqI for 10 h at 65 °C, and the reactions halted with the addition of 0.5 M EDTA (pH 8) to a final concentration of 20 mM. The mcrA gene was amplified from environmental DNA with 5′FAM labeled MCRf and MCRr (Lueders et al. 2001) primers. The thermal cycler conditions were a 3-min initial denaturation at 95 °C, followed by 40 cycles [0:45 at 94 °C, 0:45 at 50 °C, 1:30 at 72 °C], and a 10:00 final extension. The amplicons were digested with Cfr13I enzyme (and Tango buffer, from Fermentas) for 8 h at 37 °C, and the reaction was stopped with a 20-min incubation at 65 °C. Reaction volumes of 15 μL with 0.25 U enzyme were used to digest>1.5 μg DNA of both SSU rRNA and mcrA genes. The FAM-labeled digestions were submitted to the DNA CORE sequencing facility at the University of Illinois, Urbana-Champaign, where they were run on an ABI Prism 3730XL capillary sequencer against ROX1000 standards (for Archaea SSU) or ROX500 standards (for mcrA). The resulting chromatograms showing the fragment patterns were processed with GeneMarker version 1.75 (SoftGenetics).

Clone Libraries Construction and Phylogenetic Analysis

Clone libraries were generated from four different soil samples, two from the 2007 JB-organic site (Oct 2007) and two from the JB-mineral site (July 2008). The libraries were constructed in *Escherichia coli* using a TOPO TA cloning kit (Invitrogen #K4500, Carlsbad, CA) according to the manufacturer's protocols. Cloning was done from the same partial sequences of the Archaeal SSU rRNA gene (amplified with primers Ar20f/Ar912r) as was used for the tRFLP analysis (Lueders et al. 2001). Approximately 50 colonies that were presumed to contain Archaeal SSU rRNA gene inserts were picked from each cloning step. The presence of inserts (approximately 60 % success rate) was confirmed by PCR and gel electrophoresis, and clones containing inserts were sent to Agencourt/Beckman-Coulter Genomics (Beverly, MA) for DNA sequencing.

The successful clone sequences had the cloning vector sequences removed and were aligned and checked for chimeras using Mallard (v. 1.02, Ashelford 2006) and Chimeraslayer (Mothur v. 1.17.3 (Haas et al. 2011)). Flagged chimeras were individually checked using Pintail (on-line version (Ashelford 2006), and sequences identified as chimeric were removed. To establish the phylogenetic relatedness of the SSU rRNA gene sequences, the clones and reference SSU rRNA gene sequences were aligned using the Silva 106 database with the SINA web aligner (Silva 106 release; http://www.arb-silva.de/aligner/ (Pruess et al. 2007)) and then imported into the ARB database (release 5.2, http://www.arb-home.de/ (Ludwig et al. 2004)). Using ARB, the sequence alignments were further refined by hand alignment. Phylogenetic trees were generated using distance matrix methods and corrected with neighbor joining with Jukes–Canter correction. To obtain bootstrap values, 1000 replicate trees were run. For comparison of the SSU rRNA gene sequences to the archaeal SSU tRFLP profiles, artificial digestions were performed on the resulting sequence data in Sequencher version 4.7 (GeneCodes, Ann Arbor, MI) and compared to actual tRFLP fragments.

Bacteria/archaea Q-PCR

Bacteria to archaea ratios were estimated by using a 5′ nuclease quantitative-PCR (Q-PCR) assay adapted from that of Takai and Horikoshi (2000). The probes were labeled with 6-FAM on the 5′ end and Iowa Black FQ quencher on the 3′ end (Integrated DNA Technologies, Coralville, IA). Reactions were carried out in triplicate in 30-μL reactions containing 20 ng gDNA, forward and reverse primer concentration at 800 nM, probe concentration at 200 nM, and 1× universal master mix (Applied Biosystems). The addition of 1 unit of Platinum Taq and 1× ROX (Invitrogen) was used to optimize the signal to noise of the reactions. Standard curves were constructed with equal mixtures of six different linearized plasmids, three archaeal, and three bacterial clones. The plasmids were diluted from 1 to 10^{-6} starting with a 10-ng/μL stock solution along with a negative control. All standard curves had R^2 values of>0.99.

Results

Process Measurements

More details on microbial processes are included in Keller et al. (2012), but the key results are briefly summarized here to provide a context for our microbial community analyses. In the JB-organic soils, rates of potential CO_2 and CH_4 production measured in anaerobic soil slurries at 20 °C were approximately equal over the 2007 and 2008 sampling periods (Fig. 1). This 1:1 ratio of CO_2–CH_4 suggests that methanogenesis was the dominant process (e.g., Roden and Wetzel 1996; Yavitt and Seidman-Zager 2006; Conrad 1999) and that other microbial processes did not play a significant role in anaerobic carbon mineralization at this site regardless of the presence or absence of plants. In the JB-mineral soils, high CO_2–CH_4 ratios observed in April of 2008 suggest that a non-methanogenic process (likely iron reduction) dominated soils before the plant removal treatment was initiated at this site (Fig. 1). By June of 2008, however, the CO_2–CH_4 ratio dropped to~1:1 suggesting that methanogenesis was the dominant process in these soils regardless of plant presence or absence. There was a subsequent rise in CO_2–CH_4 at the end of the growing season indicating that a non-methanogenic process was important in both treatments (Fig. 1).

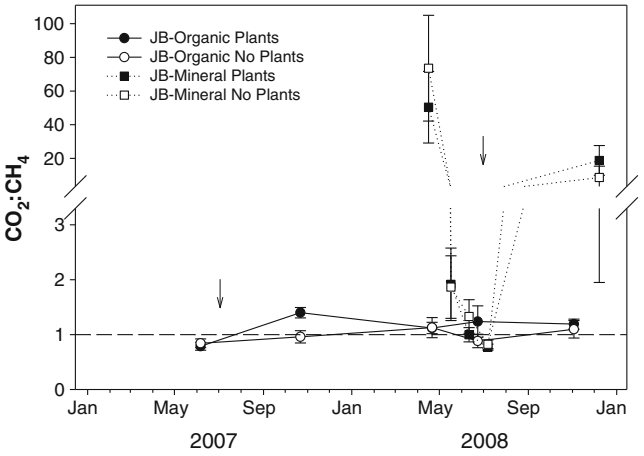
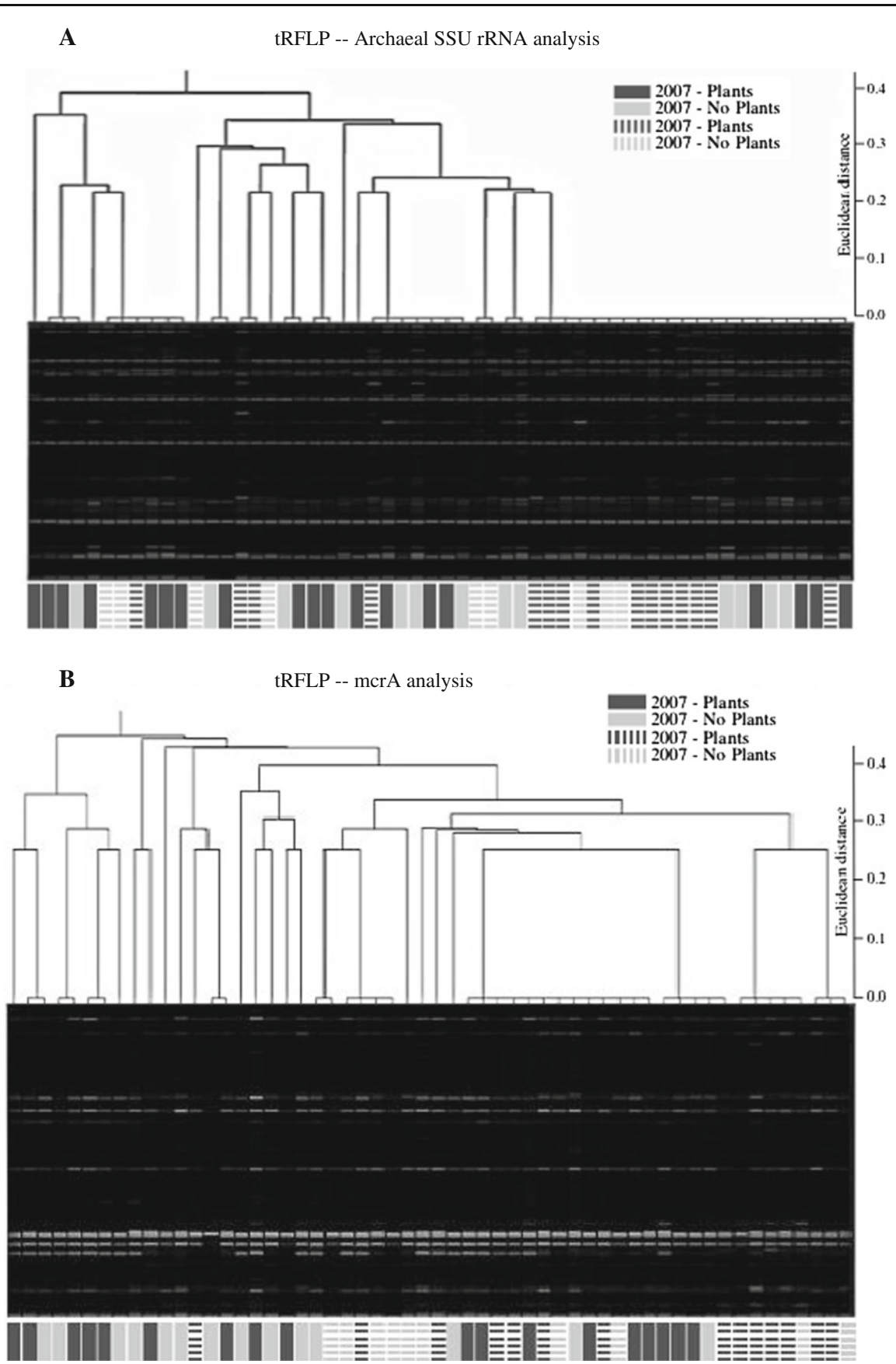


Fig. 1 Ratio of carbon dioxide (CO_2) to methane (CH_4) produced during anaerobic laboratory incubations of root-free soil slurries at 20 °C. Soils were collected from a depth of 5–10 cm below the soil surface. *Arrows* indicate the initiation of plant removal at the JB-organic (2007) and JB-mineral (2008) sites. The *dashed horizontal line* represents a 1:1 ratio of CO_2–CH_4 which is the theoretical value for a methanogenic system (Conrad 1999). Values are means±1 SE (Reprinted with permission from Keller et al. (2012))

tRFLP Analysis

Individual spectra for both the SSU rRNA gene and mcrA gene were reproducible and suggested that while multiple populations could be distinguished, different treatment plots did not show substantial variation from one another, even when compared over subsequent years. Analysis of 57 individual samples from different treatments at different times of year confirmed this trend. The tRFLP analysis of the SSU rRNA gene indicated that overall patterns were similar and that dominant peaks were consistent for all samples, regardless of plot treatment (plants or no plants), soil type (organic or mineral), or time of season (Fig. 2a). Subtle changes in minor peaks appeared to cause much of the differentiation that was observed. The same pattern held for analysis of the mcrA gene (Fig. 2b). While there appeared to be more variation among patterns for the mcrA gene, compared to the SSU rRNA gene, there were no clear seasonal or treatment patterns that could be distinguished. There appeared to be quite good concordance between tRFLP profiles for the archaeal SSU rRNA gene and results from the clone libraries. In Fig. 3, the dominant peaks are identified against putative members of the clone libraries. The most prevalent peaks from the tRFLP profiles corresponded to the Thaumarchaeota, which supports their relative abundance in the clone library. Other peaks could be assigned to different clades of Euryarchaeota that were observed in the clone libraries. There were two prevalent peaks at 197 and 512 bp that did not correspond to any of the clones in the library. It is possible that these peaks represent taxa not present in the clone libraries.

Fig. 2 tRFLP analysis of the Archaeal 16S rRNA gene (**a**) and the mcrA gene (**b**) from 2007 and 2008 plots. Each column represents an individual fragment spectra that has been converted to a barcode for comparative analysis in the dendrogram; samples are shaded for year and treatment (plants or no plants)


A
tRFLP -- Archaeal SSU rRNA analysis
2007 - Plants
2007 - No Plants
2007 - Plants
2007 - No Plants
Euclidean distance
0.4
0.3
0.2
0.1
0.0
B
tRFLP -- mcrA analysis
2007 - Plants
2007 - No Plants
2007 - Plants
2007 - No Plants
Euclidean distance
0.4
0.3
0.2
0.1
0.0

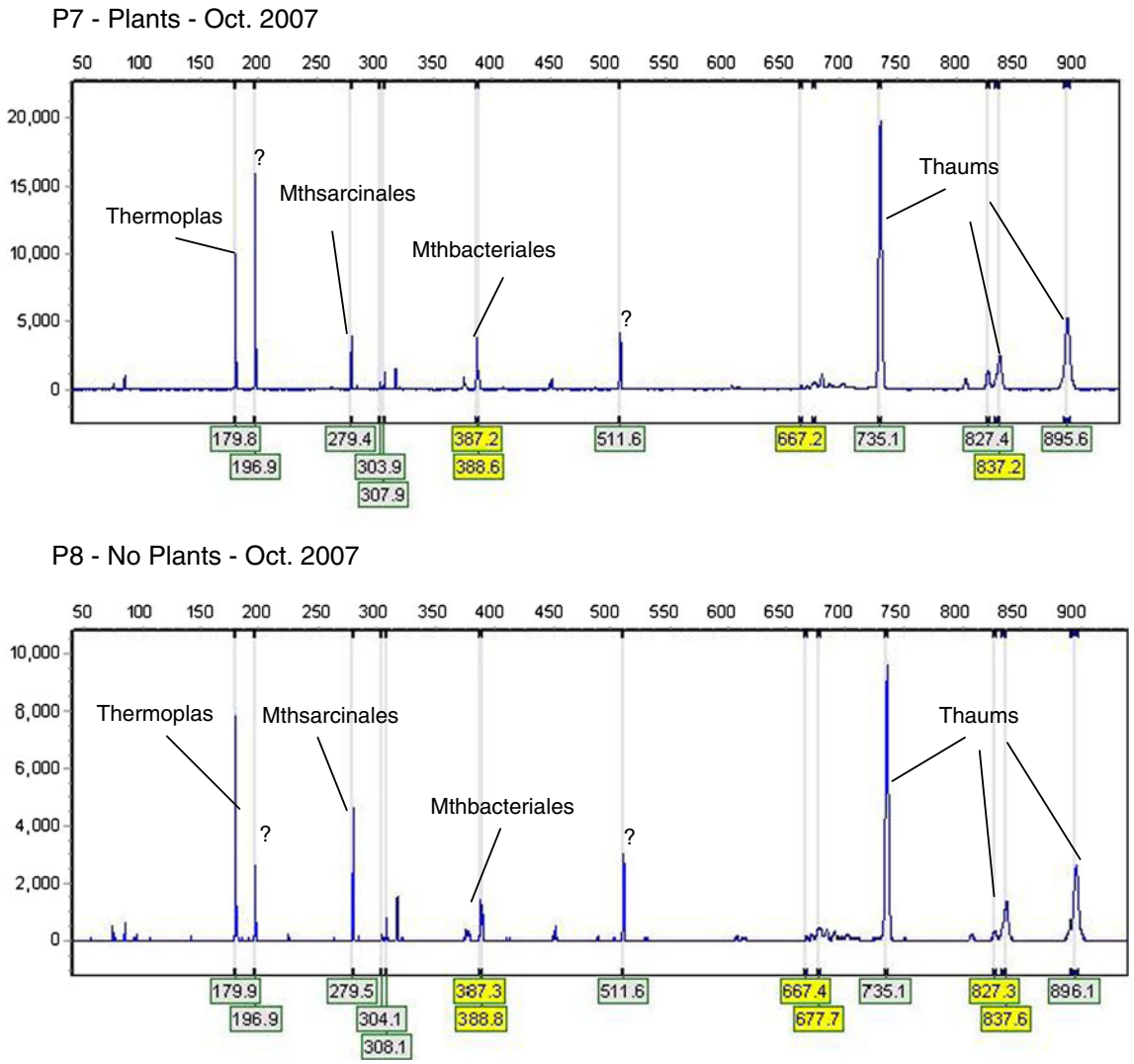


Fig. 3 Examples of individual fragment analysis for two different samples comparing plant and no plant plots in 2007. Overall, tRFLP patterns were reproducible and did not show substantial shifts in detectable populations. Based on in silico comparisons between SSU rRNA clones and tRFLP fragment sizes, dominant fragments corresponding to putative phylogenetic groups are indicated. The *?* indicates fragments that were not identified in the clone libraries, suggesting the presence of an unknown population of Archaea. The represented orders or phyla are abbreviated; see Figs. 4 and 5 for complete names

Clone Library Analysis

A total of 114 nonchimeric clones of the SSU rRNA gene were analyzed from four plots: three with no plants and one with plants from the two different sites (Table 1). Due to the phylogenetically conserved nature of the SSU rRNA, analysis of these clones serves as a proxy to identify and assess the diversity of different microbial populations that are present. From each plot over half the clones belonged to the Thaumarchaeota with the remainder distributed among two different orders of methanogens and the order Thermoplasmatales within the Euryarchaeota. The ratio of archaea to bacteria, based on qPCR, indicated that archaea are abundant, accounting for approximately half the total population at the JB-organic site and about a quarter of the population at the JB-inorganic site (Table 1). Given that methanogenesis, an archaeal process, is dominant at both sites, this result is not surprising; furthermore, the decreased archaeal signal from JB-mineral is consistent with overall rates of methanogenesis being lower at this site (Keller et al. 2012).

Consistent with the tRFLP data, there did not appear to be any obvious differences in distribution of the clones either between the different soil types or between individual plots. A phylogenetic analysis of the clones indicated that the Thaumarchaeota were not closely related to any known species and that despite their abundance in the clone libraries, their overall diversity was relatively low (Fig. 4). A number of the related clones were from a minerotrophic fen, while others were from wetlands and rice paddies, and several came from a petroleum contaminated site in Japan

Table 1 Summary of clone library (rows 4–8) and qPCR (rows 9 and 10) results from the four sites

	Sample site			
	JB-organic		JB-mineral	
	Plants	No plants	No Plants	No plants
Time	Oct-07	Oct-07	Jul-08	Jul-08
Clones[a]	22	25	30	37
Thaumarchaeota	12 (55 %)	14 (56)	21 (70)	28 (75)
M-microbiales	1 (5 %)	4 (16)	2 (7)	2 (5)
M-sarcinales	5 (23 %)	3 (12)	5 (17)	6 (16)
T-plasmatales	4 (18 %)	4 (16)	2 (7)	1 (4)
% Archaea[b]	53.8 (SD=0.8)	48.5 (SD=4.1)	21.9 (SD=0.9)	25.3 (SD=1.5)
% Bacteria[b]	46.2	51.5	78.1	74.7

[a]Represent the total number of clones for each site; these are broken out into respective phylogenetic groupings (with percentage of total) in the rows below

[b]qPCR results for the Archaea–Bacteria ratios, expressed as percent

(see "Discussion" for details). While lacking a tidal influence, fens, wetlands, and rice paddies share in common with Jug Bay anaerobic soils that have active rhizosphere communities.

The clones representing Euryarchaeota were more diverse (Fig. 5). Among the Methanosarcinales, the largest number of clones clustered together with members of the genus *Methanosaeta*, as well as a number SSU rRNA clones from minerotrophic fens in New York state. Among the Methanomicrobiales, several clones were associated with *Methanosphaerula palustris* which also came from a New York fen (Cadillo-Quiroz et al. 2009). Eleven clones belonged to the Thermoplasmatales. Cultured members of this order include lithotrophic and heterotrophic extremophiles; however, these were only distantly related to sequences obtained from the plots.

Discussion

This ecosystem-scale manipulation experiment investigated the impact of plants on the dominant microbial processes involved in mineralization of organic matter and on the microbial communities responsible for these processes. This was done temporally at two tidal freshwater sites in close proximity to one another (approx. 110 m apart), but with different soil types. Most germane to the microbiological analysis was the observation that methanogenesis, as opposed to iron reduction, was the dominant microbial process in the mineralization of subsurface organic matter at both sites during the growing season (Fig. 1). Overall there were no consistent effects of plant removal on the relative importance of methanogenesis as the dominant anaerobic carbon mineralization process at either site over the course of this study (Keller et al. 2012). Given that methanogenesis, a process regulated by the domain Archaea, was the dominant process in these experimental plots, an analysis of archaeal populations was deemed appropriate.

The cultivation-independent approach used here found a diversity of methanogens and other populations of archaea in the marsh soils. However, the most abundant SSU rRNA clones from all samples belonged to the Thaumarchaeota. We were unable to discern any clear pattern of associations of particular archaeal populations to the different plant treatments either temporally or spatially using tRFLP. Furthermore, there were no noticeable differences between the JB-organic (2007) or the JB-mineral samples (2008). The more limited analysis using SSU rRNA clone libraries also did not reveal obvious patterns, thus corroborating the tRFLP findings. The lack of clear patterns between the different sites is consistent with results of biogeochemical process studies that showed that anaerobic carbon mineralization at both sites was dominated by methanogenesis during the majority of the growing season and that there were no significant differences in process rates between plant/no-plant treatments (Fig. 1; Keller et al. 2012). Thus, similar to our initial hypothesis, both the microbial process data and community composition data suggest that the impacts of plant growth and activity, e.g. ROL, on microbial processes, and specifically on the dominant respiratory pathway, were limited over the course of this study. These findings are in opposition to previous work demonstrating that plant productivity and below ground biomass impacted anaerobic microbial metabolism in a potted plant study (Sutton-Grier and Megonigal 2011). Sutton-Grier and Megonigal (2011) did not look specifically at microbial community composition, however, and could not evaluate how observed differences in process rates were linked to microbial populations.

Other studies have had difficulty in correlating microbial population structures with either spatial or temporal variation in either brackish (e.g. saltmarshes) or freshwater tidal systems (e. g. Koretsky et al. 2005; Lasher et al. 2009). A study of ammonia-oxidizing bacteria in tidal freshwater marshes found that population diversity was not affected by the presence or absence of plants; however, population

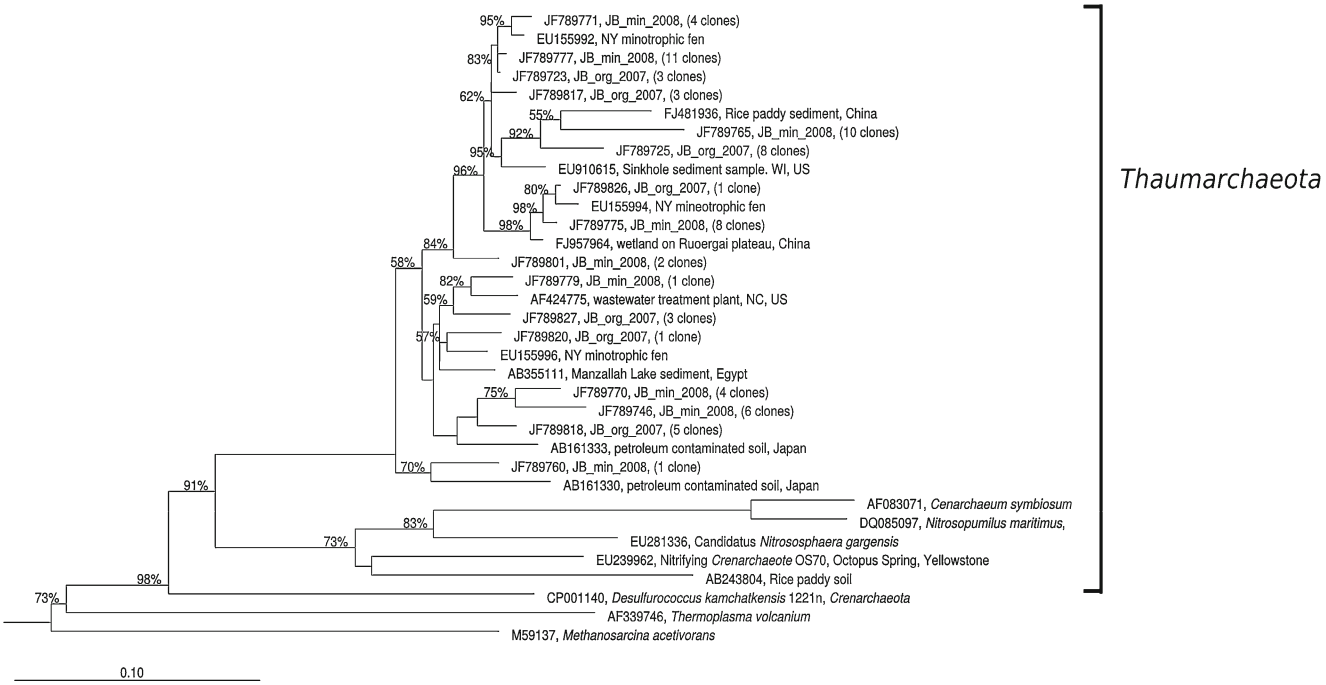


Fig. 4 Phylogenetic tree including representative clones of Thaumarchaeota from the two sites. The clones from this study are labeled JB-mineral or JB-organic. In several instances a tree node was represented by more than one closely related clone; the number of similar clones is denoted in *parenthesis*. Overall, the Crenarchaeota clustered quite closely to one another and were less diverse than the Euryarchaeota. The tree was constructed by the neighbor-joining method; *numbers at branch points* indicate bootstrap values>50 %

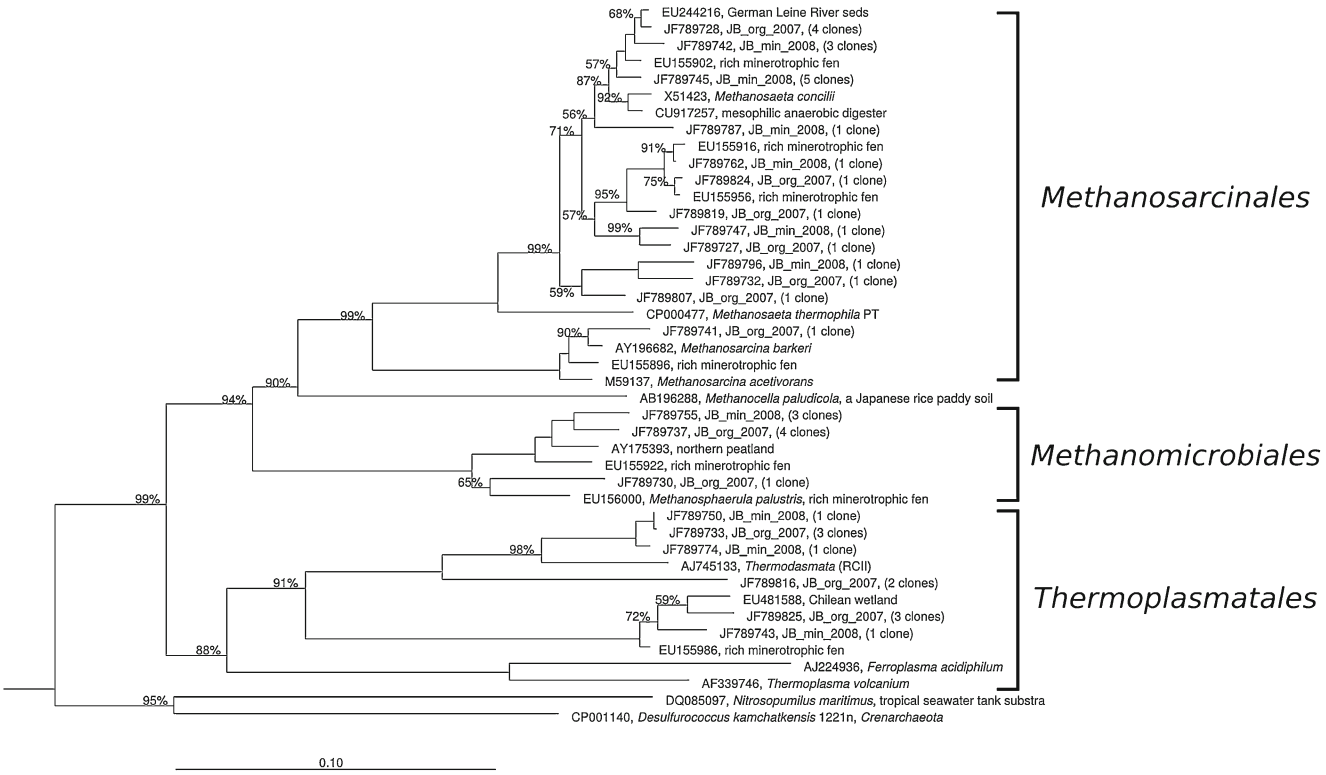


Fig. 5 Phylogenetic tree including representative clones of Euryarchaeota from the two sites. The clones from this study are labeled JB-mineral or JB-organic. In several instances a tree node was represented by more than one closely related clone; the number of similar clones is denoted in *parenthesis*. The clones broadly fell into three groups, related to the Methanomicrobiales, Methanosarcinales, and Thermoplasmatales. The tree was constructed by the neighbor-joining method; *numbers at branch points* indicate bootstrap values>50 %

diversity did show variation based on elevation in the flood plain and with depth (Laanbroek and Speksnijder 2008). Our study investigated microbial populations at similar soil depths, but was not designed to assess effects of elevation changes across the marsh or within samples. A comprehensive study of sulfate-reducing bacteria in saltmarsh sediments found only minor variations in population diversity over an entire growing season (Bahr et al. 2005). The work of Lasher et al. (2009) looked at a large cohort of SSU rRNA clones of bacteria in a Georgia saltmarsh and indicated a seasonal population shifts between summer and winter, but correlations between porewater chemistry and population structure within specific sample sites were difficult to discern. An ecosystem-scale plant manipulation study in a South Carolina saltmarsh that involved plant clipping and addition of nutrients, including nitrogen, showed no distinctive trends in microbial populations corresponding to different treatments. This study looked at phospholipid fatty acid profiles as opposed to phylogenetic or functional genes (Lovell et al. 2001). Similarly, communities of diazotrophic bacteria in this South Carolina saltmarsh seemed quite resilient to perturbation (Bagwell and Lovell 2000).

To our knowledge similar kinds of population analyses for archaea in tidal saltmarshes or tidal freshwater wetlands have not been done. The results presented here indicate that populations of archaea involved in a major biogeochemical process, methanogenesis, do not change substantially over time or in response to plant dynamics, suggesting that over the short term they are quite stable and resilient. It is possible that subtle changes in population structure do take place, but are beyond the ability of our methods to detect in a cost effective manner. A recent study of methanogen population structure in humic bog lakes in Wisconsin found persistent population structures with many shared members; however, analysis of over 2,000 mcrA gene clones did reveal subtle but statistically significant differences in populations between lakes and within vertical profiles for individual lakes (Milferstedt et al. 2010). The rapid development of new, inexpensive high throughput methods for assessing detailed microbial population diversity will likely reveal more such subtle, but important, responses of these communities to change. Nonetheless, our study, along with others discussed earlier in this article, indicates that gross microbial population structure in submerged wetland soils, as well as the overall metabolic response of the community, is resistant to short term perturbation.

The results presented here, while specific for tidal freshwater wetlands, contribute to a growing set of data that indicate that there are specific lineages of archaea associated with freshwater wetland environments. Several recent studies have focused on methanogen populations in bogs and fens. The findings from 16S clone libraries presented here are consistent with the finding that common subgroups within the orders Methanosarincales and Methanomicrobiales predominate in freshwater sediments (Cadillo-Quiroz et al. 2006; Putkinen et al. 2009). The Jug Bay soils had a number of clones that were closely related to the genus *Methanosaeta*. Cultured members of this group are aceticlastic methanogens that utilize acetate in the formation of CH_4. This is consistent with their presence in organic-rich soils where fermentative pathways will result in acetate accumulation. The other methanogenic group represented in the clone libraries is a relative of *M. palustris*, which was isolated from a fen in New York. *M. palustris*, a member of the order Methanomicrobiales, is a hydrogenotrophic methanogen that requires H_2/CO_2 or formate for methanogenesis and will not grow on acetate (Cadillo-Quiroz et al. 2009). We did not find close relatives of the rice cluster I members of the Methanosarcinales (Conrad 2006). Rice cluster I was originally described associated with the roots of rice plants (Grobkopf et al. 1998), and later, in an acidic bog in upstate New York, was shown to be more dominant on the root surfaces or rhizoplane of plants than in bulk soil (Cadillo-Quiroz et al. 2010). Our study did not attempt to separate the root surface from bulk soil. The rhizoplane makes up a small percentage of bulk soil, which may provide an explanation for the lack of rice cluster I clones in this study.

The Jug Bay soils also contained a number of clones related to the Thermoplasmatales. Again, these sequences were related most closely to environmental clones from similar submerged freshwater soils or sediments. This includes members of the rice cluster III that have been found associated with rice paddy soil (Grobkopf et al. 1998; Lueders et al. 2001). An enrichment study using paddy soil identified an organism referred to as *Thermoplasmata* RCIII that was capable of fermenting peptides, and grew in association with methanogens, but could not be isolated as an axenic culture (Kemnitz et al. 2005). This suggests that there may be close, potentially syntrophic associations between different classes of archaea involved in the breakdown and mineralization of organic matter to CH_4 in these soils similar to bacteria–methanogen associations that have been observed previously (Wüst et al. 2009).

The finding of significant numbers of clones of Thaumarchaeota, as well as their abundance, in the tRFLP analysis is interesting. Other investigations of organic rich submerged freshwater sediments (Cadillo-Quiroz et al. 2006; Putkinen et al. 2009) have reported related clones. By SSU rRNA gene phylogeny, these clones are all quite closely related and cluster with group 1.3b members of the Thaumarchaeota, which share the common habitat type of submerged anaerobic soils (Nicol and Schleper 2006). As yet, it is not possible to assign putative metabolic functions to this group of organisms; however, because the clones at Jug Bay are all closely related suggests that they could have a common metabolism. The closest cultured members are

aerobic ammonia oxidizers, and it has been suggested that ammonia oxidation may be a prevalent metabolism for this phylum (Spang et al. 2010; Nicol and Schleper 2006). This current study did not investigate nitrogen dynamics; however, previous work at Jug Bay has shown that there are significant rates of nitrification and denitrification occurring in the marsh during the growing season (Merrill and Cornwell 2000; Greene 2005). A study by Fortunato et al. (2009) looked at populations of ammonia-oxidizing bacteria (AOB) and denitrifying bacteria at Jug Bay. AOB were present at a variety of sites around the Jug Bay sanctuary and showed significant diversity. There appeared to be a seasonal variation in the diversity from summer to winter; however, there was no correlation between diversity of AOB and ammonia concentration (5–20 μM). In general, it appears that ammonia-oxidizing archaea tend to have much higher substrate affinities than AOB, allowing them to grow at lower ammonia concentrations, but at slower growth rates (Martens-Habbena et al. 2009).

While attempting to infer abundances from clone libraries or tRFLP analyses has caveats, it is somewhat surprising that the relative abundance of the Thaumarchaeota is greater than that of the Euryarchaeota. Because methanogenesis is a dominant process in these sediments (presumably carried out solely by the Euryarchaeota), this suggests that whatever these novel Thaumarchaeota are doing may turn out to be an important ecosystem process within the marsh. Because we know so little about these archaea, or what process they may be catalyzing, it is difficult to assess how large scale perturbations, such as those induced by sea-level rise and other effects of climate change, will effect these populations. Likewise, without more study, it is impossible to predict how large-scale population shifts within the Thaumarchaeota might impact overall ecosystem processes in tidal freshwater wetlands.

Acknowledgments We are indebted to Dr. Emily Fleming and Dr. Joyce McBeth for assistance and advice in developing phylogenetic trees. Chris Swarth at the Jug Bay Wetlands Sanctuary allowed unfettered access to our research sites for this project and provided important background references about the Jug Bay ecosystem. We thank Jim Duls, Nicholas Mudd, and Todd Plaia for assistance with field component of this work and laboratory work on this project. The editorial comments of Chris Swarth and Aat Barendregt, as well as two anonymous reviewers, are greatly appreciated. This research was supported by collaborative NSF grant 0516400 to JPM and DE and Smithsonian Post-Doctoral Fellowships to JKK and AESG.

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