Influences of mat layering on methanogenic communities in microbial mats

Lee F. Stanish
University of Colorado, Boulder
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Abstract

Microbial mats are unique habitats that support a large and diverse community that functions based on both well-defined and dynamic physical and chemical gradients. While they occupy relatively small surface areas worldwide, they can be locally abundant in aquatic systems and can produce relatively high amounts of methane and carbon dioxide, which are potent greenhouse gases. In addition, the highest rates of methane production were found in the upper 5mm of the microbial mat, indicating that there is a community of methanogens that can respond to the higher H2 partial pressures present within the mat layer as a result of nighttime nitrogen fixation. This project aimed at understanding the nature of methane production in the cyanobacterial layer of microbial mats. More specifically, the diversity and physiology of methanogenic communities in Sippewissett Salt Marsh microbial mats was examined. The results indicate that the upper and lower mat layers are comprised of somewhat distinctive methanogenic communities that are able to utilize a variety of substrates, including H2/CO2, and that these layers have different physiological responses to changes in substrate availability, which change on a day/night pattern.

Introduction

Microbial mats are among the most ancient microbial fossil remains found on earth and may have played an important role in oxygenating the atmosphere (Allwood et al, 2006). Physically, microbial mats are distinctly laminated, with different microbial communities generally thought to dominate in different layers of the mat. The upper layers are dominated by photosynthetic autotrophs, which consist primarily of cyanobacteria and diatoms. Deeper into the mat, oxygen is quickly depleted and the reducing potential decreases readily. These chemical gradients directly influence community composition by controlling substrate availability. Generally, in the deeper mat layers the highly reduced environment can support methanogenesis.

However, the upper 1mm of microbial mats are extremely dynamic due to the reversal of cyanobacterial metabolism from producing oxygen during the day to respiring CO2 at night. In addition, nitrogen-fixing cyanobacteria actively begin fixing N2 at night, thereby increasing H2 availability. Hoeler et al. (2001) found that methane fluxes are highest in the upper 5mm of hypersaline microbial mats at Guerrero Negro, indicating a previously under-appreciated importance of methanogenic communities in this region of the mat. A similarly unexpected observation was found in sulfate-reducing bacteria, in which SRB's demonstrated a preference for low-oxygen environments and may play a significant role in sulfur cycling at the oxycline (Minz et al., 1999).

The goals of this study were to determine whether methane production was occurring in the upper 1mm of microbial mat and, if so, whether the cyanobacteria influence methanogenic community composition in these systems. Two main appraoches were used to address these questions: first, to examine differences in methane production between the different mat layers on a day/night basis, and second, to characterize the methanogenic communities in the different may layers. Finding significant methane fluxes in the cyanobacterial mat layers could influence nutrient and carbon cycling in these systems, and therefore be an ecologically important methane source and carbon sink in microbial mat ecosystems.

Methods

Microelectrode profiling

5 cm cores were collected during the day on July 7 and at night on July 13, 2008. The cores were immediately brought back to Loeb for microelectrode profiling. The daytime core was illuminated, while the nighttime core was wrapped in foil during the measurements. Oxygen, H_2S, and pH microelectrodes (Unisense) were calibrated according to the manufacturer's instructions. Briefly, the oxygen sensor was calibrated using the anoxic layer of the microbial mat as the zero calibration point, and seawater bubbled with air was used for the upper calibration point. H_2S was calibrated using anoxic pH4 buffer solution for the zero calibration point, while the anaerobic solution spiked with sodium sulfide (final conc = 1uM) served as a second calibration point. The pH electrode was calibrated using pH buffers 4 and 7. Profiles were run using the Sensor Trace Pro software and exported to Excel for analysis.

Cultivation

5 cm cores were collected from the Little Sippewissett Salt Marsh on July 2 and July 7, 2008. The mat layers were carefully excised using a sterile razor blade and transferred to anaerobic enrichment medium containing plain medium (see MBL 2008 manual for recipe) amended with one of four enrichment substrates (Table 1). Plugs of the entire mat collected on July 2 were used to inoculate sample bottle H2/CO2_tot, and were used for subsequent analyses due to loss of the enrichment bottle H2/CO2_U.

Methane production was monitored in all enrichments every two days. Turbid cultures were transferred and monitored as needed. Enrichments growing on TMA and MeOH became turbid after an initial slow growth period, while the H2/CO2 and ACE enrichments grew relatively slowly.

Lifestyle	Substrate
Hydrogenotrophic	H2/CO2
Aceticlastic	30mM Na-acetate
Methylotrophic	62 mM MeOH 40mM TMA

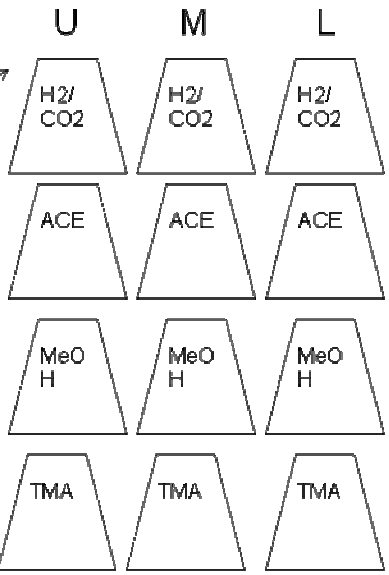

Table 1. Enrichment strategy for methanogens utilizing different metabolic pathways in Little Sippewissett Salt Marsh microbial mat communities. U = upper cyanobacterial layer; M = middle layer between 2-5mm, variable composition; L = lower, unconsolidated sediments below 5mm. TMA = trimethylamine.

Clone library construction

Ten days post-incubation, subsamples of all of the enrichments listed in Table 1 (with the exception of H2/CO2_U, which was replaced by H2/CO2_tot) were collected and DNA was isolated using the MoBio Power Soil DNA extraction kit. PCR was performed using degenerate primers for the mcrA gene (Luton et al., 2002). PCR amplicons from successfully amplified enrichments were cloned into E. coli using the TOPO cloning kit using 50ug/L Kan and X-gal selective LB plates. Successful clones were used to develop clone libraries. The number of sequences obtained from each enrichment and number of sequences analyzed are listed in Table 2.

Phylogenetic analysis

Sequences were imported into Arb and aligned using ClustalW. The alignment was hand-edited and additional mcrA sequences were imported to develop a phylogenetic tree. The tree was rooted using the outgroup member *Methanopyrus kandleri*. The distance matrix criteria were exported from Arb and run using Phylip. OTU's were generated using DOTUR, and SONS and s-Libshuff were used for community comparisons.

Methane production experiment

5 cm cores were collected from Sippewissett Salt Marsh on July 23 and 24, 2008. One core was collected during the day and the other in the early morning before sunrise. Using a sterile razor blade and forceps, the mat was carefully dissected into the upper cyanobacterial layer, the underlying sulfide-rich layer, and the bottom unconsolidated sediment layer. Each layer was transferred to 35ml pre-weighed anaerobic bottles containing the same medium as the enrichment conditions used in the cultivation experiment. A total of 12 enrichment bottles per time period were made. Methane measurements were made using gas chromatography 6 hours and 14 hours post-incubation. The bottles were weighed and the total mass of inoculum was determined. Methane production was normalized per gram of material inoculated.

Enrichment	**# clones sequenced**	**# sequences analyzed**
H2/CO2 entire mat	24	13
H2/CO2 lower	24	18
MeOH upper	16	8
MeOH middle	16	10
MeOH lower	24	20
Acetate upper	16	7
Acetate middle	16	10
TMA upper	16	14
TMA middle	16	13
TMA lower	24	14

Table 2. Number of clones sequenced and used for subsequent phylogenetic analyses

Results

Microelectrode profiling

There were distinct changes in oxygen and H2S concentrations within the mat core during the day compared to night (Fig. 1). During the day, the upper 2mm of the mat are supersaturated with respect to oxygen, while at night oxygen concentrations drop significantly. Much of the mat likely becomes anoxic, which indicates that the nighttime profile may not be accurate. H2S is relatively low throughout the day and is absent in the oxic zone, while at night H2S increases by an order of magnitude and varies throughout the depth of the mat. pH does not vary significantly through the mat during the day or night, and remains slightly basic during the day. The nighttime pH values may be inaccurate due to difficulty in calibrating the sensor.

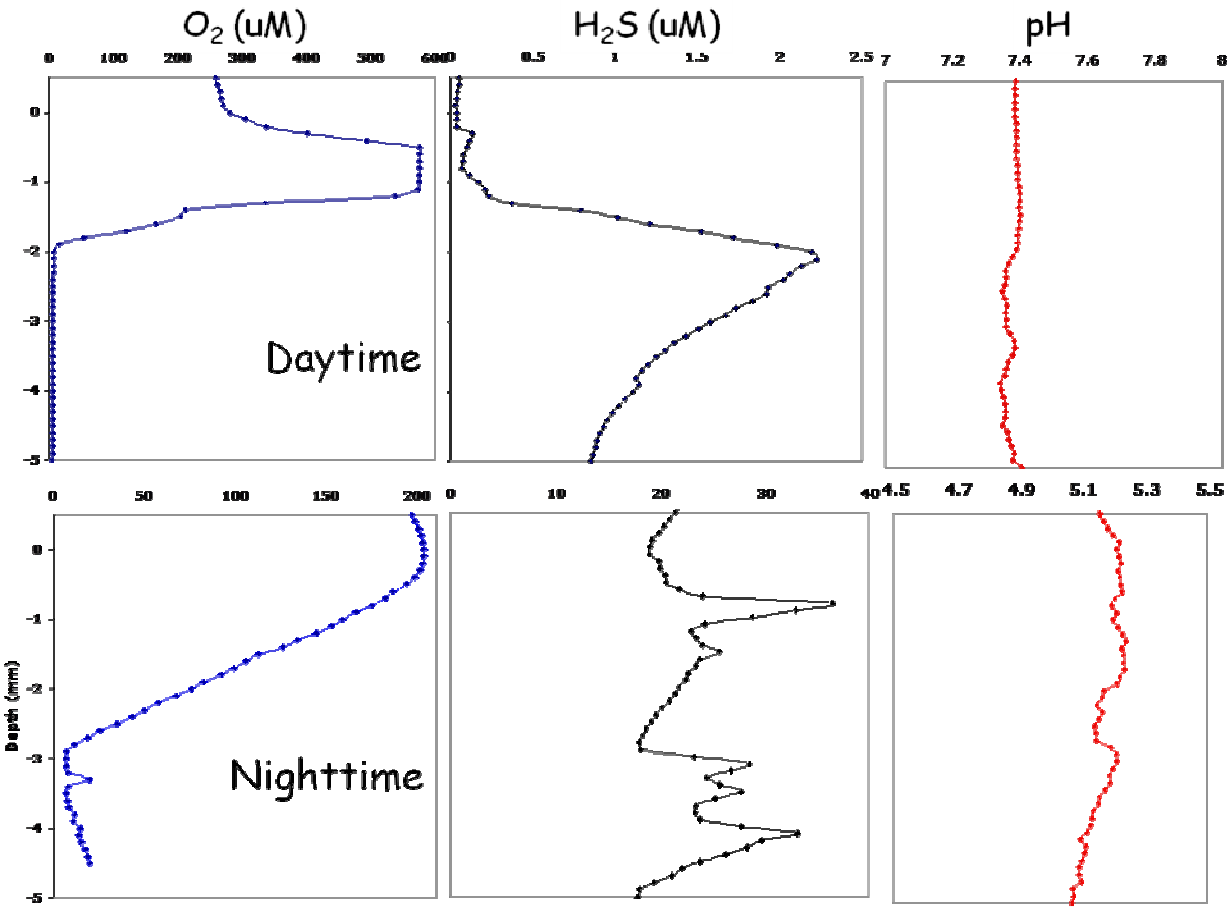

Figure 1. Comparison of microelectrode profiles of O2, H2S, and pH in Sippewissett Salt Marsh microbial mats during the day and night. Note differences in scale between day and nighttime measurements. Note that while the nighttime O2 profile differs from the day, there appears to be an inconsistency in expected concentrations that may result from high H2S concentrations, which can interfere with the O2 electrode. Second, note that the pH of the mat at night is much lower than expected, which may be an artifact of improper calibration. The trend is similar between day and nighttime pH values.

Phylogenetic analyses

A total of 127 sequences from 10 enrichments were used for phylogenetic analysis. A neighbor-joining tree containing representatives from each enrichment and reference sequences was generated (Fig. 7). Sequences obtained from enrichments containing C-1 compounds grouped closely with members of the Family Methanococcoides. Organisms within this group include M. *burtonii* and M. *methylmutens*, which are able to grow on TMA and methanol. Interestingly, a number of sequences from acetate enrichments cluster with this group, even though acetate is not typically used by members of Methanococcoides.

The H2/CO2 enrichments cluster loosely with members of the Family Methanomicrobiales, such as M. *organophilum* and *Methanoculleus thermophilum*. This group is known to use H2 and CO2 as substrates for methanogenesis. Therefore, the enrichments successfully enriched for the target organisms. A number of acetate sequences also cluster in this group, and there are known hydrogenotrophic methanogens in this Family that are also able to utilize acetate.

DOTUR identified 39 OTU's from the pooled clone library, many of which have only one sequence representative. Rarefaction curves for each clone library indicates that most of the enrichment communities are well-represented, with the exception of the TMA enrichment, which does not begin to asymptote (Fig. 3). The estimated richness of each library and the pooled community are presented in Figure 2. The richness estimates reflect the rarefaction curves in suggesting that the TMA enrichments have a much higher richness than the other enrichment communities, and that the pooled community is well-sampled.

Community diversity was then examined by assigning OTU's to either the upper, lower, or mixed sequence pool using output from DOTUR (Fig. 4). Of the 39 OTU's calculated at the 3% divergence level, 24 fall into the lower mat community and 11 fall into the upper mat community, with only 4 OTU's mixed between the upper and lower layers. The majority of the sequences fell into this pooled community, suggesting that the numerically dominant methanogens are present throughout the mat layers. Further, analysis of the community using SONS yields a shared chao value of 5.25, indicating approximately a 5 OTU overlap between the upper and lower mat communities. However, community analysis using s-Libshuff yielded an insignificant difference between the two communities ($P = 0.106$). A more detailed, cultivation-independent analysis is needed.

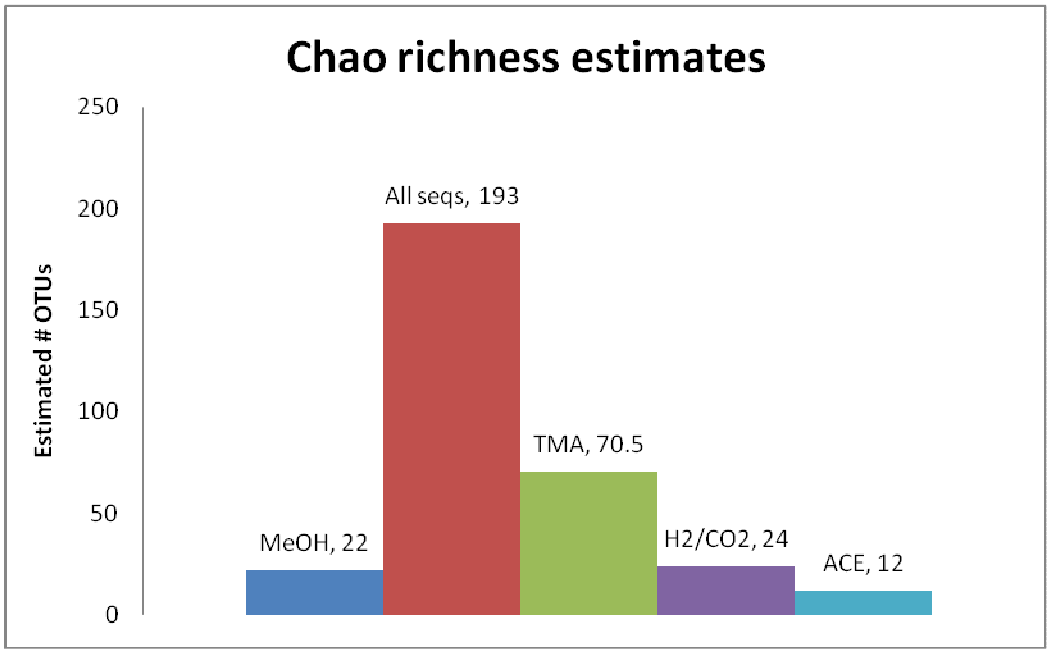

Figure 2. Chao richness estimations for enrichments and for pooled sequences. Sequence similarity cutoff of 3-4% depending on output values.

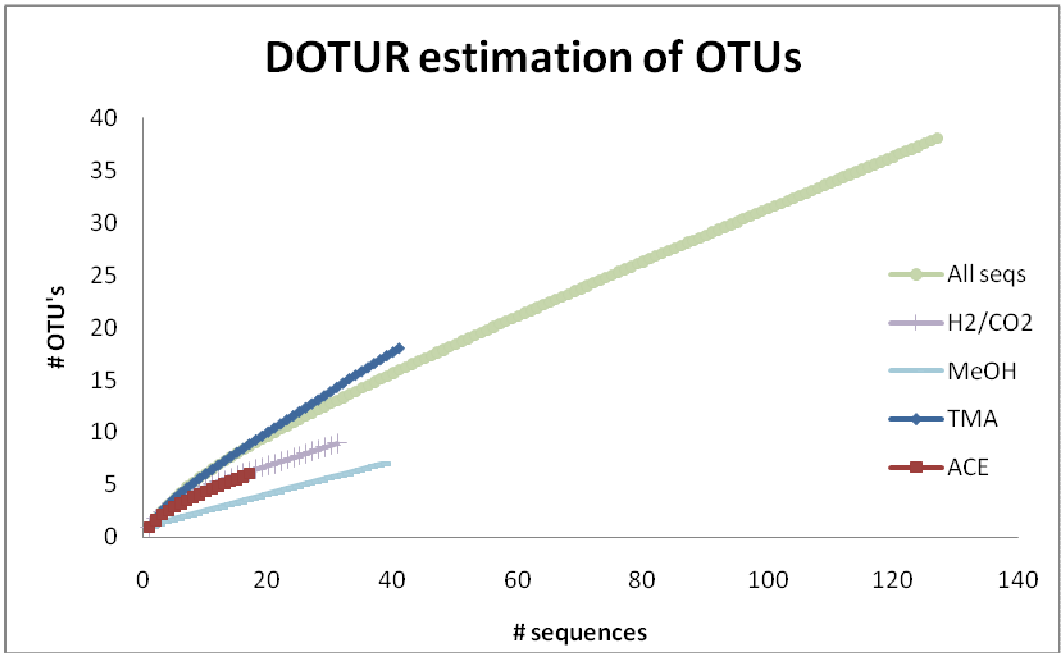

Figure 3. DOTUR estimation of number of OTUs present in each enrichment and in the pooled sequences. Sequence similarity cutoff of 3-4% depending on output values.

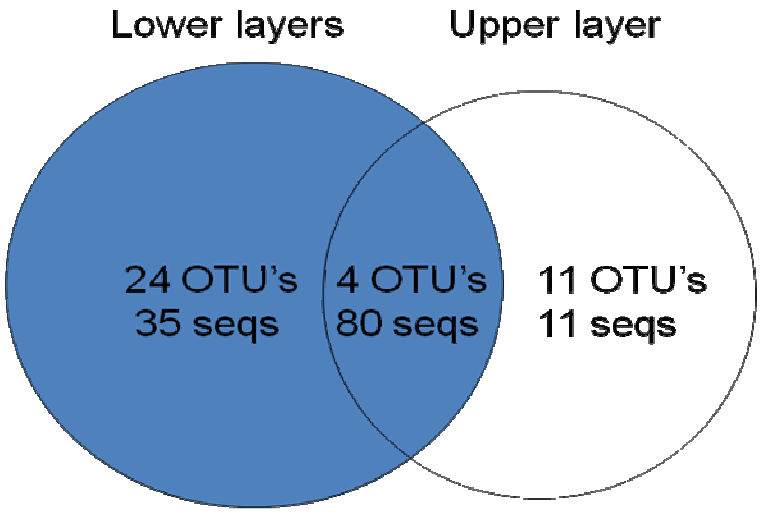

Figure 4. Community comparison of upper and lower mat layers. Sequences from the H2/CO2_tot enrichment were pooled with the upper mat enrichments, however removing these sequences from analysis does not significantly alter the pattern. Lower mat layers included all middle and lower layer sequences pooled together. OTU's defined based on 3-4% sequence similarity.

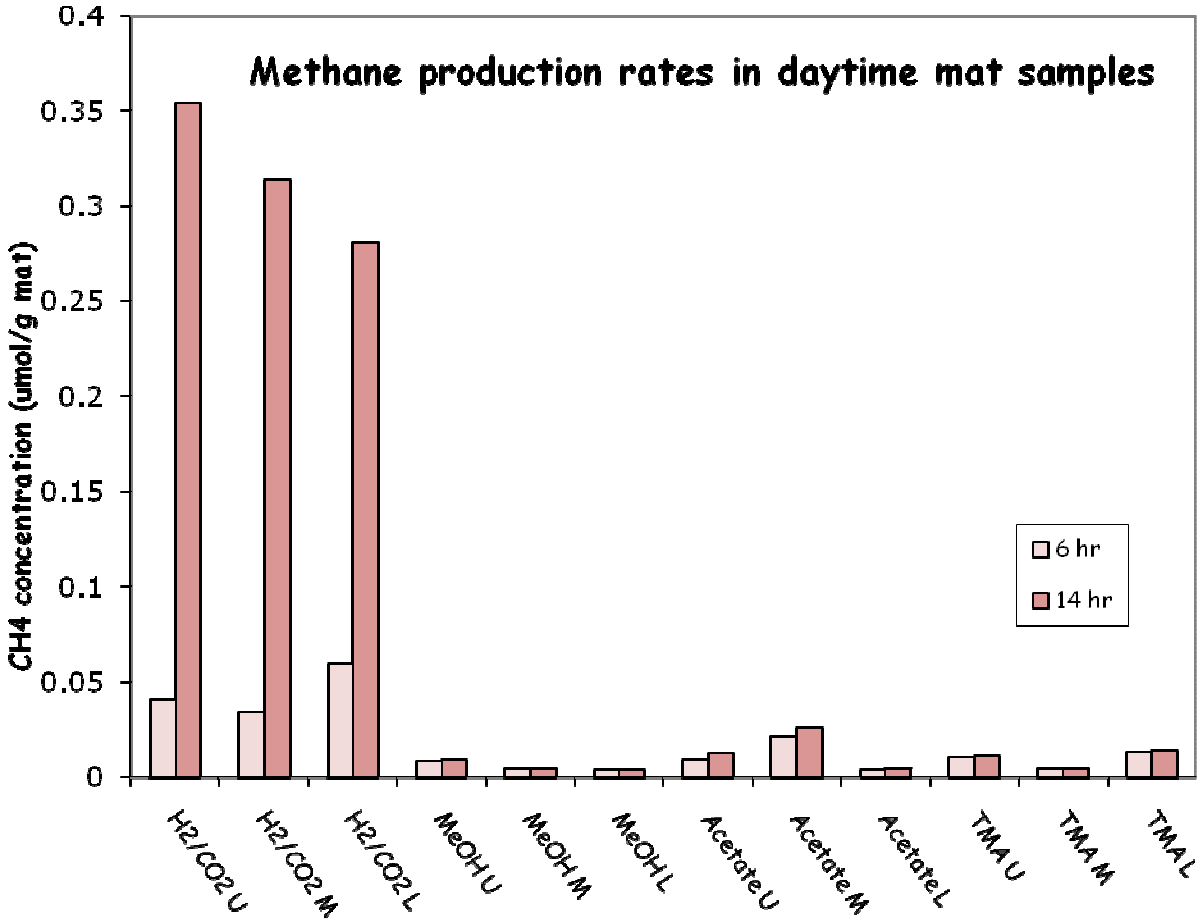

Figure 5. Methane production rates in mat layers collected during the day and incubated with different substrates. Methane concentrations were measured 6 hours and 14 hours post-incubation using gas chromatography. Production rates were normalized to total mass of material incubated.

Methane production experiment

Methane production in the mat microcosms varied between day and night-collected mats (Figs. 5 and 6). During the day, methane production in the MeOH, Ace, and TMA enrichments was relatively low, while in the H2/CO2 enrichments methane production was higher at 6 hours and increased ~8-fold at 14 hours. While replicates would be needed to ensure that these differences were statistically significant, there does not appear to be a significant difference between methane production between the different mat layers.

At night, a different story is evident. In every enrichment, methane production is higher in the upper mat layer than in the lower mat layers. In addition, the huge increase in methane production in the H2/CO2 enrichments between 6 and 14 hours disappears, and methane concentrations in the

enrichments remain stable between the two measurement times.

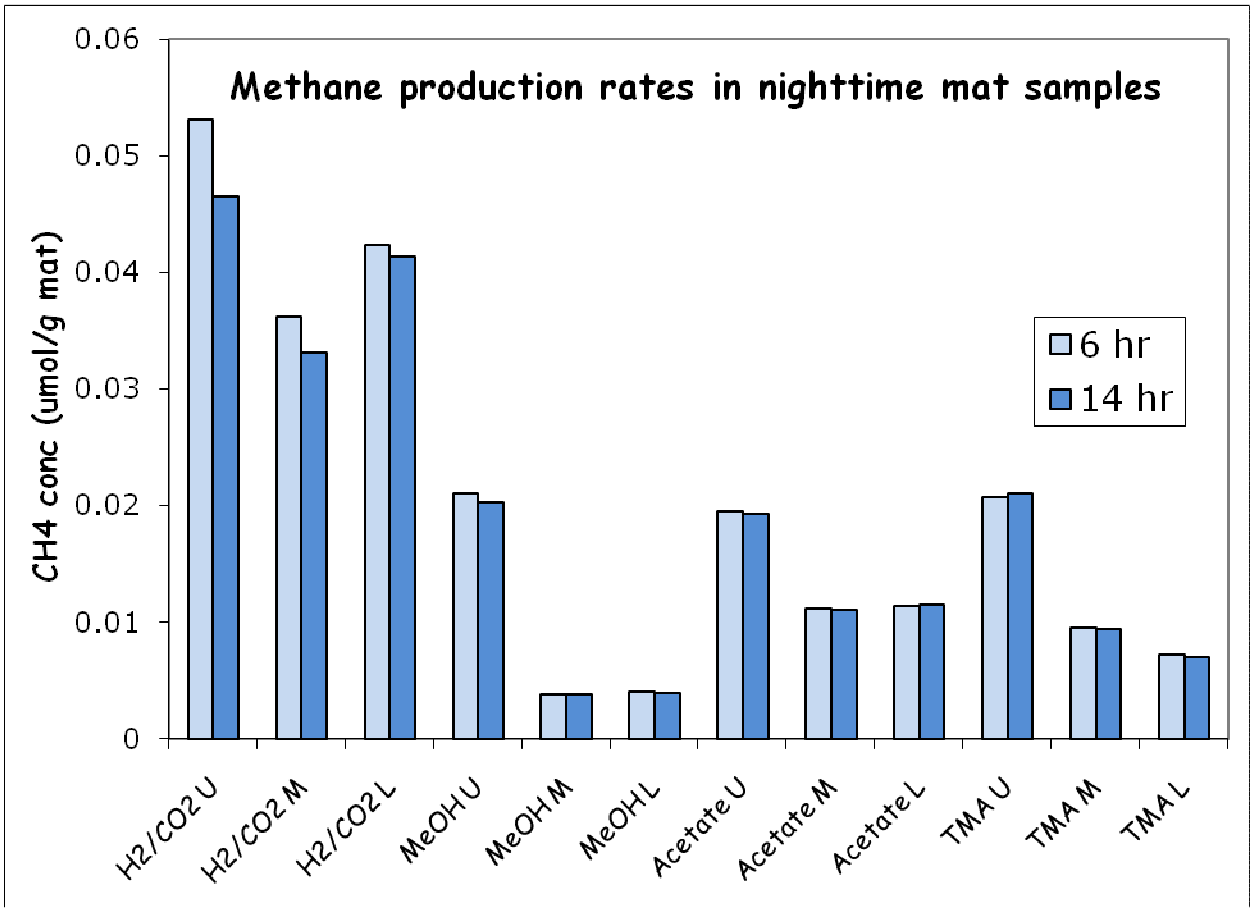

Figure 6. Methane production rates in mat layers collected during the night and incubated with different substrates. Methane concentrations were measured 6 hours and 14 hours post-incubation using gas chromatography. Production rates were normalized to total mass of material incubated.

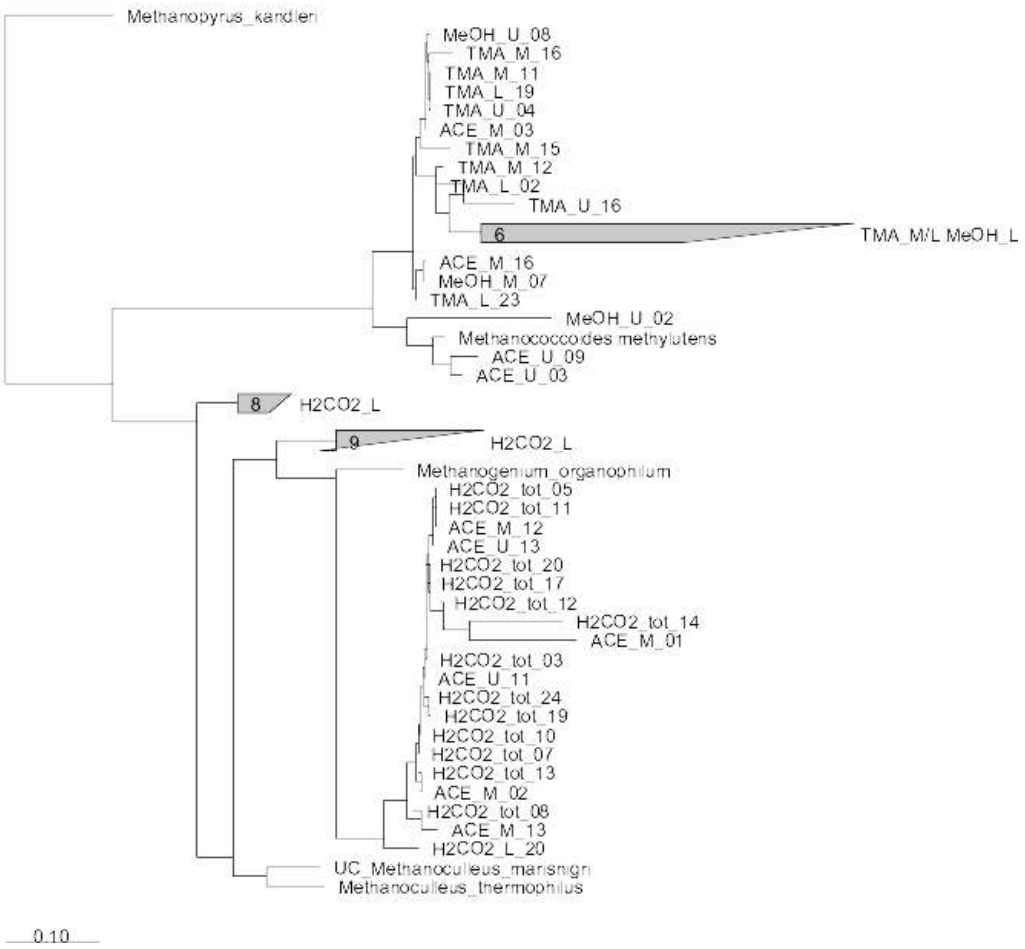

Figure 7. Neighbor-joining tree of representative sequences for all enrichments and mat layers. Described species with the highest Blast similarity to sequences were used as taxonomic references. Most sequences share the highest similarity with uncultured representatives.

Discussion

The goal of this project was to test the findings of Hoeler et al. and others that found that anaeobic activities readily occur in oxic environments when conditions become favorable. The results indicate that microbial mat methanogenic communities in Sippewissett Salt Marsh respond to variation in light and chemical gradients in complex ways. These responses appear to be driven ecologically by the relative stability of the physical and chemical environment, as well as by community composition.

Phylogenetic analysis of cloned representatives from each enrichment and mat layer indicate that both 1) the diversity and species richness with respect to number of operational taxonomic units; and 2) the diversity with respect to low sequence similarities to known and characterized methanogens, is relatively high. This is not surprising considering the highly dynamic system in which these microbes live. Daily and seasonal environmental changes may force organisms to adapt to a dynamic lifestyle and selects for relatively diverse metabolic strategies, such as ability to grow on a variety of compounds. This is evident in the high diversity and richness of methylotrophic methanogens subsisting on TMA.

Community comparison of overlapping OTU's demonstrates that there is minimal overlap between the upper and lower mat layers. This divergence in community structure is largely due to the high number of unique OTU's found in the TMA and H2/CO2 enrichments from the upper and lower layers. There were 3 dominant OTU's that account for 78 of the total 129 sequences. These OTU's aligned most closely with uncultivated representatives within the families Methanosarcinales and Methanobacteriales, respectively. Enrichments using C-1 substrates selected for members of the Methanosarcinales and using H2/CO2 substrate selected for Methanobacteriales, which was expected. Community analysis comparing the upper and lower mat layers was not significant according to slib-shuff ($P = 0.106$), however there are caveats to this analysis, and these data should be repeated before concluding that the upper and lower mat layers support significantly different methanogenic communities.

The short-term methane production experiment demonstrated that 1) microbial mat methanogenic communities have different physiological responses depending on time of day; and 2) the upper cyanobacterial mat layer responds differently than the lower mat layers to changes in access to substrate. Mats collected and assayed during the day did not show distinct variations in methane production related to mat layer. In contrast, there was a marked increase in methane production in the upper mat layers for microbial mats collected during the night under each enrichment condition. This may be due to the 'priming' of the methanogenic communities in the cyanobacterial layer at night. The communities may be adapted to responding quickly to nighttime increases in H2 resulting from N fixation by cyanobacteria. The higher methane production in the upper mat layers in the C-1 enrichments may be the result of residual H2 remaining within the inoculum that allows hydrogenotrophic methanogens to continue methanogenesis, while the lack of increase in methane production at 14 hours would be a result of H2 depletion. The large increase in methane production from 6 to 14 hours in the daytime mat samples using H2/CO2 may be due to growth. However, it is

unclear why the nighttime mats would not also respond in this way. It is possible that the nighttime community had established a basal metabolic rate that was not altered by providing increased substrate. Likewise, the community may regulate methanogenesis on a diurnal pattern. These are two possible explanations that do not exclude other potential mechanisms.

Future Work

An uncompleted goal of this project was to characterize the methanogenic activity within the mats using q-RT-PCR. While RNA was successfully extracted from mat samples collected during the day and at night (see Fig. 8), no further analyses were performed on these samples. An important consideration is that the mcrA enzyme is relatively stable in nature and examination of mcrA expression as an indicator of methanogenic activity may be misleading. However, given the lack of a more suitable gene, it is worth pursuing in order to more fully understand the underlying mechanism responsible for temporal patterns in methanogenesis in microbial mat communities.

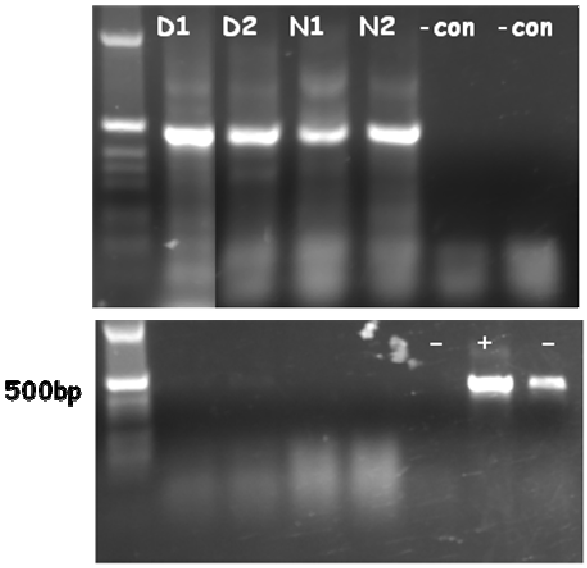

Figure 8. RT-PCR products of mcrA transcripts from Day (D1, D2) and Night (N1, N2) RNA extracts. Top gel: all samples contain amplicons of correct size, procedural control and negative PCR control are blank. Bottom gel: Control PCR confirming lack of DNA contamination in samples. Note unexplained positive band in negative PCR control. Further analysis demonstrated that RNA template does not appear to poison the PCR reaction (not shown), thus positive result in negative control remains

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