Activity of methyl phosphonate consuming organisms and enrichments of methanogens from berry ponds in Little Sippewissett Salt Marsh, MA

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Abstract

Here investigated the production of methane by both aerobic and anaerobic processes in berry ponds of Little Sippewissett marsh and sought to enrich for methanogens present in the sediments of these ponds. Microcosm experiments indicated the occurrence of methane production upon the consumption of methyl phosphonates. We were also able to enrich the a hydrogenotrophyic methanogen from sediments taken from these ponds, confirming that both processes, anaerobic and aerobic production of methane are present in Little Sippewissett marsh.

Introduction

Traditionally, the most studied process of methane production is the one performed by archaea from substrates such as CO_2, H_2, methanol, formate, methylamines and acetate under anaerobic conditions (Thauer et al., 2008). Methane is produced also in salt marshes and marine environments, but it is assumed that the presence of methanogens in these environments is disfavored due to the high contents of sulfur, which favors the growth of sulfur-reducing bacteria, which compete with methanogens for substrates such as CO_2 and H_2 (Orelamb et al., 1982; Thauer et al., 2008). However, some authors relate the production of methane in salt marshes, and, more specifically, in Sippewissett, which raises the question of which microorganisms are contributing to this process (King and Wibe, 1978; Buckley et al., 2008).

Recently the production of methane by aerobic marine environments was proved true, thus presenting an alternative pathway to explain the supersaturation of oceans with methane (Karl et al., 2008). There are evidences from metagenomic data that methyl phosphonates are produced by several marine microorganisms, such as the archeon *Nitrosopumilus maritimus* (Metcalf et al., 2012). Aerobic microorganisms under conditions of phosphate limitation, would consume these methyl phosphonates, thus producing methane. However, to our knowledge, the occurrence of his process has not yet been studied in salt marshes.

Despite of the conditions favoring sulfur-reducing bacteria, traditional anaerobic methanogens can also be found in salt marshes. Some authors found that the use of methanol and methylated amines is a way to avoid competition with sulfur-reducing bacteria by substrates such as CO_2 and H_2 (Oremland et al., 1982). Besides, evidences for the activity of hydrogenotrophic methagens in the mats of in Great Sippewissett Marsh have been related recently (Buckley et al., 2008).

Within this context, this project was designed with two main objectives. The first objective was to evaluate the production of methane in Little Sippewissett Marsh upon the consumption of methyl phosphonates. The second objective was to enrich for methanogens by using the substrate of berry ponds. We chose the berry ponds because these are characteristic formations of Little Sippewissett Marsh an that were not yet studied for the presence of methanogens and for the production of methane by aerobic organisms.

Material and Methods

Sampling sites

Water and sediment samples were taken in Little Sippewissett Marsh, Falmouth, MA, in June 12, 2013, July 8, 2013, and in July 13, 2013, in the first and second berry ponds (Fig. 1). They were taken in the afternoon, at low tide. The

water was taken either with 50 mL falcon tubes, or glass and polystyrene bottles, and stored at 4ºC until processing.

Evaluation of aerobic methane production in berry ponds

This step consisted of two experiments carried out with water and sediments collected from the berry ponds. The aim of these experiments was to evaluate the production of methane by aerobic organisms after the consumption of methylphosponic acid (MPn), which was proven as a pathway to the aerobic production of methane in marine ecosystems.

First experiment

The first experiment was set up in a completely randomized design, with eight treatments and three replicates per treatment. The treatments were water and sediment with the addition of MPn, either with or without BES, or phosphate, and two controls with filtered-sterile water, either with MPn or phosphate.

A hundred and ten milliliters of water were added to 160-mL sterile serum bottles (Figure 2). The water used in the treatments with sediments or in the controls was filtered sterilized with Nalgene filters (0.22 μm). All bottles received filtered-sterilized glucose and sodium nitrate to a final concentration of 1 mM glucose and 16 μM of NO_3^-. Filter-sterilized MPn, PO_4^{-3} (in the form of KH_2PO_4) and BES were added to the final concentrations of 1μM, 1μM and 10 mM, respectively, according to the treatment. Treatments with sediments received 2 mL of the superficial sediment of the berry ponds, which contained particulate matter and berries. All bottles were capped with blue butyl rubber stoppers and crimp-sealed with aluminum closures after the addition of the substrates. They were incubated at 30ºC for a period of two days and measurements were taken daily. A hundred microliters of headspace were taken from each bottle with a glass syringe and injected in a Shimadzu GC-2014 with a FID detector and using N_2 as the carrier gas. The following parameters were set in the machine: R. inlet pressure of 170.1 KPa, R. column flow of 30 mL. min^{-1}, column temperature of 200ºC and injection

temperature of 70ºC. A peak appearing after 0.5 min. was considered as methane, as based on previous calibrations with pure methane, and the area of the peak was recorded and converted to mmol. L^{-1} of methane.

Second experiment

The second experiment was also set up in a completely randomized design, with five treatments and four replicates. The treatments were as follows: addition of MPn (5.5 μM), addition of MPn and phosphate (5.5 μM each), addition of phosphate (5.5 μM), no addition of phosphorus source, autoclaved water and MPn (5.5 μM) and sterile water and phosphate (5.5 μM).

Eighteen milliliters of water were added to 28-mL batch tubes (Figure 2). At this time, the water used in the sterile controls was autoclaved. Each bottle received double filtered-sterilized glucose and sodium nitrate to a final concentration of 0.5 mM glucose and 80 μM of NO_3^-. Double filter-sterilized MPn and PO_4^{-3} were added to the final concentrations of 5.5 μM, according to the treatment. All tubes were capped with Teflon-lined silicone stoppers and crimp-sealed with aluminum closures after the addition of the substrates. They were incubated in a shaker at 27ºC, 180 rpm, for a period of three days and measurements were taken daily. The procedure for gas chromatography was the same used in the first experiment.

Enrichment of methanogens from berry pond sediments

Enrichments for methanogens were set up in seawater media with either sediments or berries. Thirty milliliters of media were added to 160 mL serum bottles sealed with blue stoppers and metal crimps.

The enrichments consisted of seawater media with the addition of sediment (sediment + berries) and H_2+CO_2. The following enrichments were set up with the addition of either sediments or pure berries and with the addition of H_2+CO_2 as based on the results of the first enrichment.

All enrichments were monitored periodically for the production of methane by gas chromatography and by fluorescence microscopy. For the gas chromatography, 100 µL of headspace were taken from each bottle with a glass syringe and injected in a Shimadzu GC-2014 with a FID detector and using N_2 as the carrier gas. The following parameters were set in the machine: R. inlet pressure of 170.1 KPa, r. column flow of 30 mL. min^{-1}, column temperature of 200ºC and injection temperature of 70ºC. A peak appearing after 0.5 min. was considered as methane, as based on previous calibrations with pure methane, and the area of the peak was recorded and converted to mmol. L^{-1} of methane.

DNA was extracted from sediments and from the first enrichment with the PowerSoil Mobio Kit, according to the instructions of the kit, and used for the amplification of the 16S rRNA and *mcr*A genes. 16S was amplified with the primer and conditions presented by Park et al. (2010). *mcr*A genes were amplified with the primers and conditions presented by Luton et al., (2002).

Fluorescence microscopy

Catalyzed Reporter Deposition – fluorescence in situ hybridization (CARD-FISH) was performed on sediments, berries and aliquots of the first enrichment (H_2+CO_2). All samples were fixed in 2% paraformaldehyde for up 12 hours, 10-fold diluted in PBS/EtOH (1:1), palced on ice and sonicated for 20 seconds, at 20% intensity. Berries were mashed before sonication. Then, each sample was filtered with filters on polycarbonate filters with pore diameter of 0.22 µm and stained with DAPI (4',6-diamidino-2-phenylindole) or submitted to hybridization with probes for Archaea (Arch915) for CARD-FISH. For details, see Pernthaler et al. (2004). All slides were visualized in a Zeiss AX-10 Imager M2 fluorescence microscope.

Aliquots of the enrichments were also taken for the preparation of wet mounts and examination of natural fluorescence (420/480 filter) by fluorescence microscopy in a Zeiss AX-10 imager M2 fluorescence microscope.

Results

Evaluation of aerobic methane production in berry ponds

First experiment

On the first day, methane production was observed in all treatments that received MPn, except by the treatments sediment+BES+Mpn and the control with MPn (Figure 3). However, methane was detected in all treatments on the second day. Methane concentrations were especially high (181 μM) in the control with MPn. Treatments receiving phosphate in both water and sediments presented higher methane production if compared to their pairs that received MPn.

Second experiment

The production of methane followed a pattern similar to that of the literature in the case of the second experiment. Methane production was low at the beginning of the incubation, but increased significantly in the treatments that received Mpn in the second and third days (Figure 4). However, the addition of phosphate did not inhibit the production of methane when MPn was added.

Enrichment of methanogens from berry pond sediment

The first millimeters of sediment at the bottom of the berry ponds, containing both particulate matter and berries, were sampled and used for the enrichment of methanogens. We found a significant production of methane in the enrichments incubated with H_2 and CO_2, and we followed this production for over a month (Table 1).

The presence of methanogens was confirmed by the amplification of archaeal 16S rRNA genes and of the *mrcA* gene, which codes one peptide of the methyl coenzyme-M reductase enzymatic complex. These genes were amplified both from the enrichment and sediment, confirming that we were able to enrich for methanogens present in the sediment of the berry ponds.

The examination of cultures at the microscope by using the wavelengths of 420/480 permitted the visualization of small, auto fluorescent coccoid cells, either alone or in pairs (diplococcus) (Figure 6). Methanogens could also be visualized in enrichments to which only berries were added. The presence of these cells was consistent in enrichments prepared on different days, both with sediments or berries from the ponds. The same cells could be observed in the enrichment when examined by CARD-FISH, using the probe Arch915, specific for *Archaea* (Figure 7). However, we were not able to visualize *Archaea* and *Euryarchaeota* in the berries and the sediment because of the strong background in the filters.

Discussion

Salt marshes are a common environment in the East Coast of the United States (Tiner, 1984) and we sought for insights on their potential to contribute to the emissions of methane. We evaluated the production of methane by aerobic organisms upon the consumption of methyl phosphonates in Little Sippewissett Salt Marsh, MA, as well as the presence of 'traditional' methanogens.

In the case of Sippewissett, the presence of ponds containing berries, i.e. consortia between sulfur-reducing and sulfur-oxidizing bacteria, are a particularity of this ecosystem. There is evidence for methane production in microbial mats of Great Sippewissett marsh (Buckley et al., 2008), which raises the question about the occurrence of methane production in the berry ponds, despite the fact that methanogens are usually disfavored in ecosystems rich in sulfur, such as marine environments (Thauer et al., 2008). Besides, it was recently shown that the consumption of methyl phosphonates by aerobic microorganisms is a significant source of methane in marine environments (Karl et al., 2008) and it is possible that aerobes present in these ponds might be producing methane.

Even though not conclusive, our results indicate the potential for methane production upon the consumption of methyl phosphonates in the berry ponds. It is worthy noting that methane emissions after incubation of samples with methyl

phosphonates were higher than those found by Karl et al. (2008). Besides, sequences of the gene *phnJ*, involved in the pathway of methyl phosphonate consumption, can be found in the mat (Suter, 2012), showing that organisms involved in anaerobic methanogenesis are present in Sippewissett, and suggesting that they might be also present in the berry ponds.

However, we emphasize that new experiments must be carried out in order to provide sound conclusions about the evaluated process. An issue we faced was the high variability of GC readings. We applied an analysis of variance (data not shown) in order to detect whether there was a significant difference among the treatments, but the high variability (coefficients of variation were higher than 100%) contributed to the lack of statistical differentiation among treatments. Also, some of the controls and treatments receiving phosphate presented production of methane, which has no clear explanation. It is possible that small cells passed the filters, so we made sure to autoclave the water used in the controls of the second experiment. Even though, we still had problems with the measurements due to issues with the GC.

Our results also show that methanogenic archaea are present in the sediments of the berry ponds and indicate that these microorganisms may also be in the berries. Previous reports show that *Euryarchaeota* 16S rRNA genes were found in berries by the application of culture-independent techniques (Wilbanks et al., 2010). Sequences closely associated to methanogens can also be found in a microbial mat metagenome from Little Sippewissett built by previous students of the MBL Microbial Diversity course. However, more examination is necessary in order to confirm the presence of methanogens in the berries. We cannot rule out the carryover of sediment remains when incubating the media with berries. So more work must be done in other to confirm whether the methanogenic archaea is part of the berry consortium.

Based on the morphology, i. e. cocci in single cells or arranged in pairs, physiological traits, e.g. hydrogenotrophic metabolism, and on the description of the

environment from where it was enriched, the enriched archaea possibly belong to the genus *Methanococcus*. More tests as well as sequencing of 16S rRNA and *mcr*A genes must be done in order to confirm the taxonomic position of this archaea. The presence of a hydrogenotrophic archaea in Little Sippewissett supports the observation that methane production takes place in this ecosystem as previously reported by Buckley et al. (2008). However, these authors detected methanogenic activity in the mats, while we worked with the berry ponds. Similarly to what occurs in the mats, N_2 fixation by Cyanobacteria and fermentation could be the source of H_2 to these archaea (Buckley et al., 2008).

Conclusions and future directions

Our work shows evidences of aerobic methane production in the berry ponds of Little Sippewissett Marsh, MA, and shows that hydrogenotrophic methanogens are present in the sediments of the ponds. These topics should be further investigated in order to reveal details of the metabolism of methane in this environment. Due to technical issues, especially regarding the variability in GC measurements, new experiments must be run in order to confirm the existence of aerobic methane production in the ponds. Several questions remain to be answered, such as (1) if the methanogens are part of the berry consortium or are only in the sediments; (2) the isolation and characterization of these hydrotrophic methanogens; (3) what is the contribution of both aerobic and anaerobic production of methane, as well as of metanotrophic microorganisms, to methane emissions in the marsh; and (4) the correlation between changes in the physicochemical variables of the ponds and the activity of methanogens.

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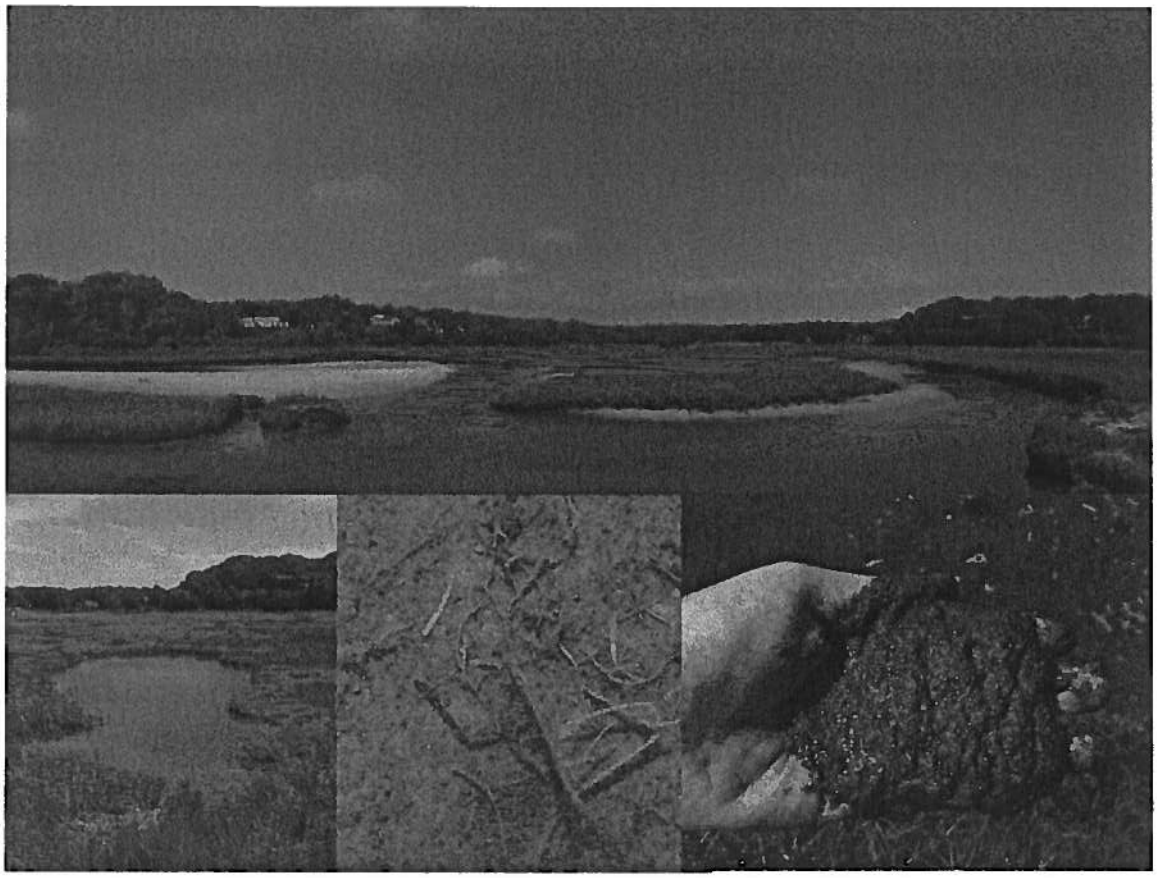

Figure 1. Top: panoramic view of the Little Sippewissett marsh, Woods Hole, MA (top); Bottom, from left to right: berry pond 1, berries on the surface of the sediment and sediment taken from the pond.

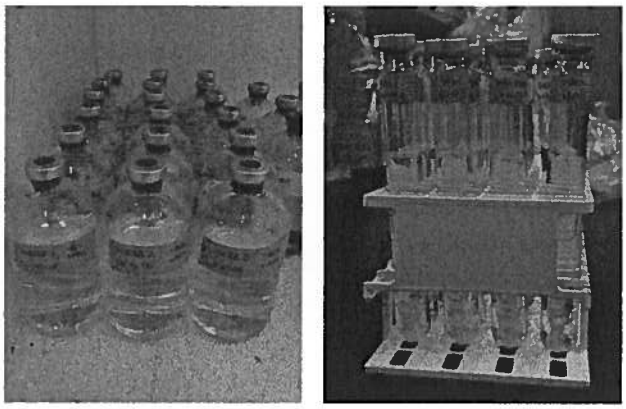

Figure 2. First (left) and second (right) experiments to evalute the production of methane upon the consumption of methylphosphonates..

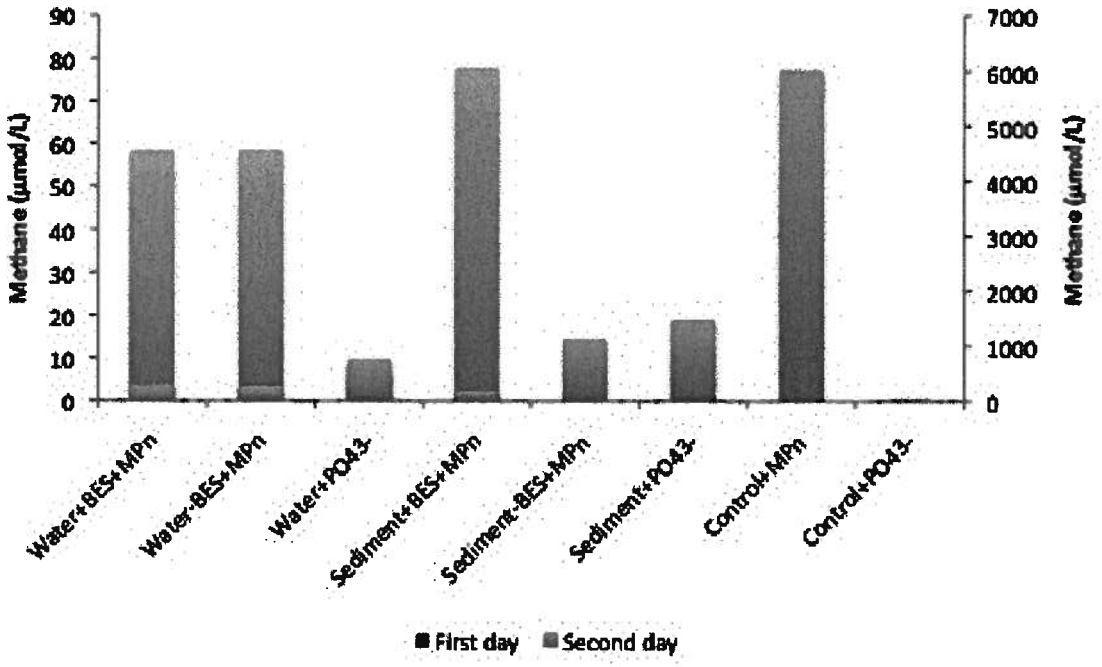

Figure 3. Methane production by the consumption of methyl phosphonates by aerobic microorganisms. Data from the first experiment. Production for the first data is presented in the left y axis, while production for the second day is presented in the right y axis.

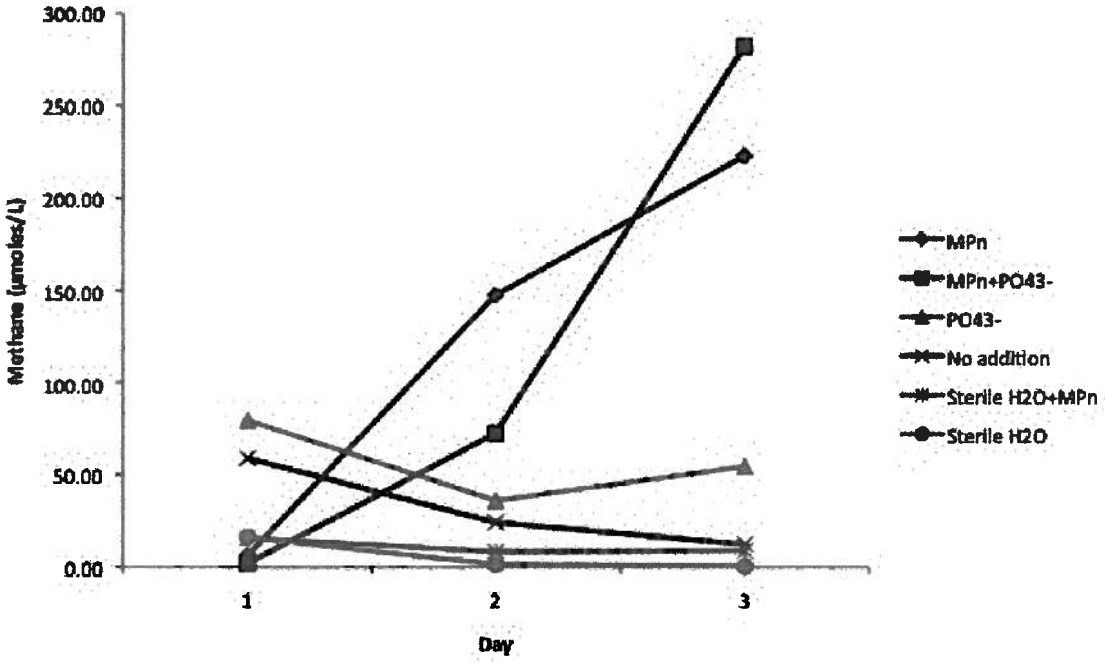

Figure 4. Methane production by the consumption of methyl phosphonates by aerobic microorganisms. Data from the second experiment.

First enrichment
06/12/2013

"Berries"
07/03/2013

"Pure berries"
07/03/2013

Figure 5. Different bottles used for the enrichments of methanogens with sediments and berries.