Investigations into Methane Production via Methylphosphonate Demethylation and Acetoclastic Methanogenesis in Oxic Freshwater

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Abstract

Methane production has been conventionally viewed to only occur in anoxic environments, however, there is evidence of methanogenesis in oxic water columns of freshwater lakes. Several studies have indicated that methylphosphonate (MPn) demethylation and acetoclastic methanogenesis could potentially be the most significant mechanisms for methane production in oxic water columns of freshwater lakes relevant to atmospheric emission. This study investigates these two pathways and aims to determine which one is dominant. **I hypothesize that MPn demethylation will be the dominant pathway since this pathway gets enhanced by the water's natural conditions. More specifically, the MPn demethylation pathway is induced by phosphate starvation and phosphate is often the limiting nutrient in these environments.** To test this hypothesis, I incubated different experimental treatments of pond water containing additions of MPn, acetate (Ac), phosphate, and/or nitrate. The dominant pathway was determined by comparing the stoichiometric ratios between MPn or Ac added and methane production. The stoichiometric ratio revealed that each mole of Ac produced more methane than each mole of MPn. Both pathways produced significantly more methane with an increase in temperature, which revealed that temperature is an important driving factor. Ultimately, the results revealed that as temperature changes, the main driving factors for methane production in oxic water columns through the MPn demethylation and acetoclastic methanogenesis pathways may change as well. This study allows us to better understand the magnitude of methane production from oxic water columns and, in turn, reconstruct the methane cycle to incorporate this process and better predict future atmospheric methane trends.

Introduction

The discovery of methane production in oxic water columns in freshwater lakes contradicts the traditional knowledge that methane production strictly occurs in anoxic environments (Günthel et al. 2020; Bogard et al. 2014). This newly discovered phenomenon generally occurs in large lakes where methane emissions from anoxic sediments into bottom waters is unlikely to affect surface concentrations (Bogard et al. 2014; DelSontro et al. 2017; Morana et al. 2020). Because of this, the near-surface waters must locally produce methane to be able to sustain methane supersaturation. Most past studies investigated the specific microbial methane production pathways (Grossart et al. 2011; Carini et al. 2014; Khatun et al. 2019), the effects of nutrient additions and environmental change (DelSontro et al. 2017; Hartmann et al. 2020), and the presence or absence of this phenomenon in various locations (Morana et al. 2020). As a result of these prior studies, MPn and acetate (Ac) have been a substrate of interest. Recently, marine bacteria that use methylphosphonate (MPn) as a sole source of phosphate have been found in the water column (Carini et al. 2014; Karl et al. 2008). MPn is known to be produced in the water column by archaea, bacteria, and invertebrates that harbor genes encoding the MPn synthase enzyme (Carini et al. 2014). Although MPn in the water column is at low concentration, several studies proposed that aerobic decomposition of MPn could be a main pathway for methane production in phosphate-stressed marine habitats (Carini et al. 2014; Khatun et al. 2019; Karl et al. 2008; Repeta et al. 2016). This pathway would likely be driven by microorganisms, specifically cyanobacteria, diatoms, and green algae, that possess the C-P lyase genes which utilize organophosphates when phosphate starved and produce methane as a by-product of the demethylation of phosphonic acids (Khatun et al. 2019). Another possible

dominant pathway is acetoclastic methanogenesis, which is driven by cyanobacteria, algae, and attached archaea (Bogard et al. 2014; DelSontro et al. 2017). These microorganisms enable direct transfer of Ac from autotrophs to methanogenic archaea and their activity has been confirmed at the molecular level in diverse oxic environments (Bogard et al. 2014; Grossart et al. 2011). Bogard et al. (2014) conducted a study comparing hydrogenotrophic and acetoclastic methane production by methanogenic archaea in the oxic water column of a temperate lake using floating mesocosms open to the atmosphere. They used apparent isotopic fractionation factors to qualitatively distinguish whether methane is produced from Ac or CO_2 reduction and found that acetoclastic methanogenesis was likely dominant (Figure 1 from Bogard et al. 2014). The likely source of Ac in the water column would be through photolysis and dissolved organic carbon decomposition pathways that produce acetate as an end product (Zhuang et al. 2019).

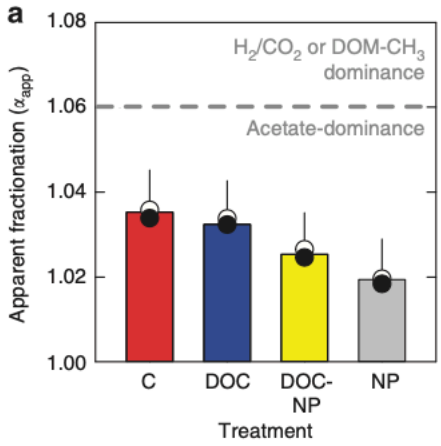


Figure 1 | Methane production through acetate vs CO_2 reduction Apparent fractionation factors were used to find whether methane was produced from acetate or CO_2 reduction. Four different experimental treatments were studied: control (C), dissolved organic carbon (DOC), dissolved organic carbon and nutrients (DOC-NP), and nitrogen and phosphorous (NP). This figure suggests that the acetoclastic pathway strongly dominated in all treatments and increased in importance with the addition of nutrients. The white circles represent the maximum associated isotopic fractionation factor, and the black circles represent the minimum. The apparent fractionation was estimated from a highly conservative scenario analysis indicated by the error bars. Apparent fractionation values were estimated on the seventh day of the experiment. This figure was from a study conducted by Bogard, Matthew J, et al. "Oxic Water Column Methanogenesis as a Major Component of Aquatic CH_4 Fluxes."

Several studies have indicated that MPn demethylation and acetoclastic methanogenesis could be the leading mechanisms behind methane production in oxic water columns of freshwater lakes, but there are no current studies that directly compare the two pathways. Both pathways produce more methane during the summer stratification period, but the MPn demethylation pathway is induced by phosphate starvation, so excess nitrogen inputs can enhance the phosphate limitation of lakes which further promotes methane production (Günthel et al. 2020; DelSontro et al. 2017; Carini et al. 2014; Morana et al. 2020). On the other hand, Zhuang, Guang-Chao, et al. (2019) studied acetate as a microbial and carbon energy source and found that the uptake of acetate by SAR11, a marine bacteria known to produce methane, is not

controlled by nitrogen or phosphate concentrations. It has also been shown that microorganisms have access to nitrogen from below the thermocline but not enough phosphate, since there is less phosphate around the thermocline during the stratification period (Khatun et al. 2019). It seems, then, that the MPn demethylation pathway should be the higher contributor because the MPn demethylation pathway gets enhanced by the lake's natural conditions during the stratification period.

I conducted a study to examine the hypothesis that MPn demethylation is the dominant pathway by comparing the stoichiometric ratios between added MPn or Ac and methane production. Oxic water methane production was investigated using water sampled from a pond located on the property of the University of Rochester's C.E.K. Mees Observatory in Ontario Country, NY. This environment was chosen for sampling due to ease of access at the late winter/early springtime of sampling. The pond had a high nitrogen to phosphate ratio (N:P) at the time of sampling, which suggests that phosphorous could be the limiting nutrient. Although there are generally very low concentrations of both MPn (Morana et al. 2020) and Ac (Tang et al 2014) in freshwater environments, knowing the stoichiometric ratios between both these substrates and methane will allow us to gauge the significance of these pathways on methane production. I used different experimental treatments of pond water containing additions and combinations of either MPn, Ac, phosphate (P), nitrate (N) or none. These were used to evaluate how adding different concentrations of the substrates and nutrients affect methane production. This study explores the ability of these pathways to produce methane in the spring, so both the light and dark incubations were repeated at three different temperatures to mimic the natural variability in temperate Northern Hemisphere springtime (6°C, 12°C, 18°C). These incubations allow us to assess how varying water temperatures affects methane production. To control for the effects of phosphate, which typically inhibits the MPn decomposition pathway, its concentration was measured using a low range phosphate checker before and after the incubation. It has been determined that nitrogen enhances the MPn pathway, so nitrate was also measured before and after the incubation using a nitrate ion-selective electrode to see how methane concentration changes with time and with other nutrient additions. Both phosphate and nitrate were added in varying amounts to the samples in the 12°C experiment, in order to observe how the changing nutrient concentrations affect methane production. Finally, methane production was measured using a Gas Chromatography with a Flame Ionization Detector (GC-FID) to be able to calculate the stoichiometric ratio between MPn or Ac and methane.

Although studies on oxic water column methane production during high phytoplankton growth are ongoing (Hartmann et al. 2020), the precise biochemical pathways remain undetermined (Günthel et al. 2020). With freshwaters contributing about 122 Tg/yr of methane emissions to the atmosphere -- or about 20% of global totals -- it's important to understand how these unknown biochemical pathways affect methane production (Günthel et al. 2020). With current information on these biochemical pathways, lakes' contribution to methane production is expected to increase with rising temperatures in certain regions and higher nutrient concentrations caused by increased eutrophication (Günthel et al. 2020). Determining the stoichiometric ratios of the possible dominant substrates to methane allows us to better understand the magnitude of the methane production from oxic water columns and, in turn, reconstruct the regional and global methane dynamics to incorporate this process and better predict future atmospheric methane trends.

Methods

Field Measurements

Sampling site characteristics and sampling

The water samples were collected from a pond located on the property of the University of Rochester's C.E.K. Mees Observatory (see Images 1 and 2). The pond is shallow and located in south Ontario County near Rochester, New York. The pond is about 45 m in length, 48 m in width, and has a combined surface area of 1,420 m^2. This information was calculated using Google Maps.

Samples for the incubation experiments were collected on the morning of April 7th around 10:30 AM from the southwest end of the pond (42.702500, -77.404661) from the surface water. The air temperature was 48°F with 89% relative humidity, and 0.01 inches of precipitation fell that day (weather data from weather.gov). Surface water was collected in two 30 L carboys and taken back to the laboratory.

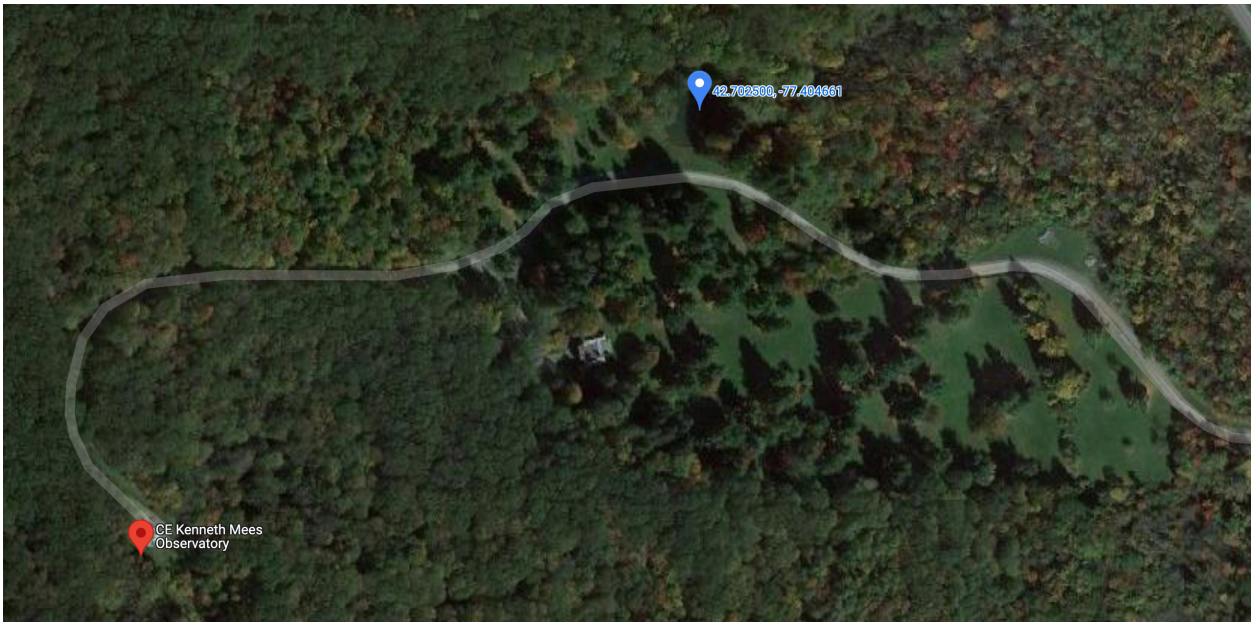


Image 1 | Sampling pond Google Earth image of the pond that was sampled (pinned in blue).

Image 2 | Photograph of the pond during sampling

Environmental parameters

Initial phosphate and nitrate concentration data for environmental parameters were obtained the same day as sampling after the samples were taken back to the laboratory. Nutrient concentrations were measured using the Hanna low range phosphate checker to measure phosphate and an Oakton Cole-Palmer nitrate ion-selective electrode to measure nitrate. Water samples for nutrient analyses were taken directly from the carboys, 10 mL for the phosphate and 50 mL for the nitrate measurement. Initial methane concentrations were measured using the nitrogen headspace displacement method and measured with an Agilent 5860 Gas Chromatograph with a Flame Ionization Detector (GC-FID) (Leonte et al. 2017). The average concentration from five vials of pond water was used to find the initial concentration of methane.

Each 500 mL vial were filled to the top with unamended pond water and capped with a rubber stopper. A 109 mL headspace was added using a 1.5-inch syringe to take out 109 mL of water and another 1.5-inch syringe was inserted at the same time to insert 109 mL of methane-free nitrogen. 1.56 mL of 8 mol/L NaOH was then added to the solution to inhibit biotic methane production. The vials were placed in the 12°C light incubator to equilibrate for 24 hours. Afterwards, the concentrations were measured using the GC-FID.

Experimental Steps

Methane concentration measurements

Methane concentration was measured before and after the incubation. Water for methane concentration analysis was collected in 250 mL vials and filled to the top with care taken to expel any bubbles. Stock solutions for MPn, Ac, nitrate, and phosphate were then added to their respective vials using a syringe as prescribed for each experiment, and the vials were capped with rubber stoppers to prevent contamination with the ambient air. The vials were incubated for five days with half the vials incubated in the light and others in the dark. On the fifth day of the incubation period, 60 mL of methane-free nitrogen was introduced, and an empty syringe was used to pull out the displaced water to create a gaseous headspace. All biological activity in the vials was then halted through the addition of 1 mL of 8 mol/L NaOH and the vials were placed back in their respective incubators for another twenty-four hours to allow the headspace and the aqueous solution to equilibrate.

Methane was measured using an Agilent 5860 GC-FID. 10 mL of headspace was taken from each vial, displaced by 10 mL of sparged water, and 3 mL of the gas was injected into the sample loop in duplicates. The sample gas was then transferred from the sample loop into a capillary column (length = 15 m, i.d. = 0.32 mm, Agilent) using helium as a carrier gas (flow rate = 2 mL). The oven temperature of the Agilent 5860 GC-FID was set at 40°C, the temperature was held there for 2.75 mins, then ramped up to 70°C for 1.5 mins during each run. Methane concentrations were then calculated by fitting the results to a calibration curve generated using a nitrogen blank and two standards of methane concentrations of ten and a hundred parts per million (ppm) in triplicates. Using solubility data from Wiesenburg and Guinasso (1979), temperature and salinity of the water, and the volumes of the gaseous headspace and water, the measured methane concentration in the headspace was converted to the true dissolved methane concentration in the samples.

Phosphate measurements

Phosphate concentrations were measured before and after the incubation using a Hanna HI713 Phosphate Low Range Checker with a detection range of 0.00 to 2.50 ppm, resolution of 0.01 ppm, and reading accuracy of ±0.04 ppm ±4% at 25°C. Water samples used to measure phosphate were taken out during the addition of a headspace to measure methane. An empty syringe was inserted in the vial, while a syringe filled with methane-free nitrogen was inserted to replace the displaced water. To measure for phosphate, 10 mL of the sample was added to the phosphate checker's cuvette. The cuvette was then placed in the phosphate checker to zero the meter. Once the cuvette was zeroed in the phosphate checker, it was removed and the contents of one packet of the H1713-0 Phosphate Low Range reagent from the kit was poured in the cuvette. The cuvette was shaken for about two minutes or until the reagent was completely dissolved. After dissolving the reagent, the cuvette was placed back into the phosphate checker to find the concentration of phosphate in ppm.

Nitrate measurements

Nitrate concentrations were measured before and after the incubation using an Oakton Cole-Palmer nitrate ion-selective electrode with a concentration range of $7x10^{-6}$ to 1.0 M (0.5 to 62,000 ppm) and accuracy of ±2%. The low-level nitrate measurement procedure was used. To prepare the electrode, the rubber cap covering the electrode tip was removed and filled with the filling solution (0.1M $(NH_4)_2SO_4$) to just below the fill hole. The electrode was then gently shaken to remove any air bubbles that may be trapped in the nitrate membrane. Before the calibration, seven standards of NO_3^{-1} (1 µM, 2 µM, 4 µM, 6 µM, 9.9 µM, 29 µM, and 48 µM) and the low-level Ionic Strength Adjuster (ISA) ($4x10^{-3}$ M $(NH_4)_2SO_4$) were prepared. The low-level ISA was prepared by adding 20 mL of the standard ISA (0.4M $(NH_4)_2SO_4$) to 100 mL of deionized (DI) water. To begin the calibration, 1 mL of the low-level ISA was added to 100 mL of DI water in a beaker. The beaker was then placed on a magnetic stirrer to ensure the solution was being stirred at a constant rate for each standard. The electrode tip was placed in the solution being careful to not touch the bottom of the beaker and avoid bubbles on the tip. Then, 0.1 mL of the first standard was added to the solution. After the reading stabilized, the mV was recorded. Between each standard, the electrode was rinsed with DI water and blotted dry. These calibration steps were repeated for all seven standards, up to a final concentration of 48 µM (Table S1). To measure nitrate in the pond water, 60 mL of pond water was placed in a 150 mL beaker and 0.6 mL of the low-level ISA was added into the beaker. The beaker was then placed on the magnetic stirrer and the electrode tip was lowered into the solution. The mV reading was recorded once the reading stabilized. This process was repeated post-incubation for the samples with nitrate additions.

Incubations

To investigate the importance of MPn and Ac pathways on oxic water methanogenesis, six incubation experiments were performed on the same pond water collected from a pond located on the property of the University of Rochester's C.E.K. Mees Observatory. Within the six incubation experiments, seven main experimental treatments ($+1\mu M$ MPn, $+5\mu M$ MPn, $+10\mu M$ MPn, $+0.1\mu M$ Ac, $+2.5\mu M$ Ac, $+5\mu M$ Ac, control) and seven auxiliary treatments ($+1\mu M$ P, $+4\mu M$ P, $+8\mu M$ P, $+9\mu M$ N, $+14\mu M$ N, $+19\mu M$ N, initial CH_4, NaOH treatment) were used to evaluate how adding different concentrations of nutrients affect methane production. In addition to the main experimental treatments, there was one control treatment for each temperature and light setting, five vials to measure initial methane concentration, and one NaOH experiment. The control served as a comparison group to ensure that the methane concentration in the vials with substrate and nutrient additions deviate from the values from the unamended water. To obtain the initial methane concentration, five vials were sterilized with 8 mol/L NaOH at the beginning of the incubation and measured for methane. The vials for the NaOH treatment experiment contained six vials (vial #32 - 37) containing the highest substrate and nutrient addition concentrations (10 µM MPn, 5 µM Ac, 5 µM MPn + 8 µM P, 5 µM MPn + 19 µM N, 2.5 µM Ac + 8 µM P, and 2.5 µM + 19 µM N). The vials in the NaOH treatment experiment were sterilized halfway through the incubation to ensure that the 8 mol/L NaOH treatment was successful at halting biological activity. Three incubators were set up, with one set at 6°C, another set at 12°C, and the last one set at 18°C. These temperatures were chosen to mimic the varying temperatures of this region's spring season, and the incubators were set at three different temperatures to observe methane production with varying conditions.

The bottom of each incubator was lined with cardboard so the relatively colder walls of the incubator would not disproportionately cool the vials. For each temperature setting, the incubators were given a grow light with an automatic timer, set to cycle on and off every twelve hours on the full spectrum light setting, designed to mimic natural sunlight. To prepare for the incubations, stock solutions of each concentration and the sterilizing solution (8 mol/L NaOH) were made so that only one mL of each solution would need to be added to the 250 mL vials to reach the desired concentration (see Tables S2 and S3).

Seventy-two 250 mL vials and five 500 mL vials were filled to the top with unfiltered pond water from the carboys. Each 250 mL vial of pond water was then amended with their respective concentration of MPn, Ac, Na_3PO_4 (P), $NaNO_3$ (N), or none by using a 1.5-inch syringe to inject 1 mL of the stock solution into the vial. The vials were then capped with a rubber stopper, being careful to expel any air bubbles, and incubated in different experimental treatments as shown in Figure 2. For the first experiment, pond water was amended with different concentrations of MPn, Ac, or neither. Then, the vials were incubated with a grow light that cycled on and off every twelve hours at 6°C for five days. For the second experiment, the process for the first experiment was repeated, but the vials were incubated in the dark instead of light by placing the vials under a cardboard box in the incubator to prevent light from the grow lamp from coming in contact with the vials. Phosphate has been known to inhibit methane production while nitrate has been known to enhance methane production, so these nutrient additions were made in the third and fourth experiment to observe how MPn and Ac pathways respond to nutrients. For the third experiment, pond water was amended with different concentrations and combinations of MPn, Ac, P, N, or none and incubated with light at 12°C (see Figure 2). The third experiment was repeated but incubated in the dark for the fourth experiment. For the final set of incubations, the pond water for the fifth experiment was amended with different concentrations of MPn, Ac, or neither and incubated at 18°C. The same process was repeated in the dark for the sixth experiment. To measure the initial concentration of methane, the five 500 mL vials (vial #27 – 31) were filled with unamended pond water, capped with a rubber stopper, and a 109 mL headspace of methane-free nitrogen was immediately added using one syringe and another to take out the displaced water following the technique by Magen et al. (2014). After the headspace was added, the vials were immediately sterilized with 1.56 mL of 8 mol/L NaOH to stop biological activity and methane production. The five vials used to measure the initial methane concentration were then placed in the 12°C incubator under the grow light to equilibrate. To confirm the effectiveness of using the 8 mol/L NaOH to halt biological activity, the remaining six 250 mL vials (vial # 32 – 37) were amended with their respective concentration of substrate and/or nutrient as indicated in Figure 2. These vials were then placed in the 12°C incubator under the grow light. After forty-eight hours of incubating, a 60 mL headspace of methane-free nitrogen was added to the vials. They were then preserved with 1 mL of 8 mol/L NaOH and placed back in the incubator to equilibrate. On day four of the incubation, 10 mL of headspace was taken out of the initial methane concentration vials using a syringe and the concentrations of methane were measured using a GC-FID. On day five of the incubation, a 60 mL headspace of methane-free nitrogen was added to the rest of the vials and the vials were preserved with 1 mL of 8 mol/L NaOH. The vials, now with a headspace, were placed back in the incubator for twenty-four hours to equilibrate the headspace and the aqueous phase. The 60 mL of incubated pond water taken out of the vials from adding the headspace were used to measure phosphate and nitrate for the vials with nutrient additions. Phosphate and nitrate were measured using the low range phosphate checker and nitrate ion-selective electrode, respectively. Over the next seven

days, 10 mL of the headspace was taken out of the rest of the vials, displaced by 10 mL of sparged water, and used to measure the concentration of methane.

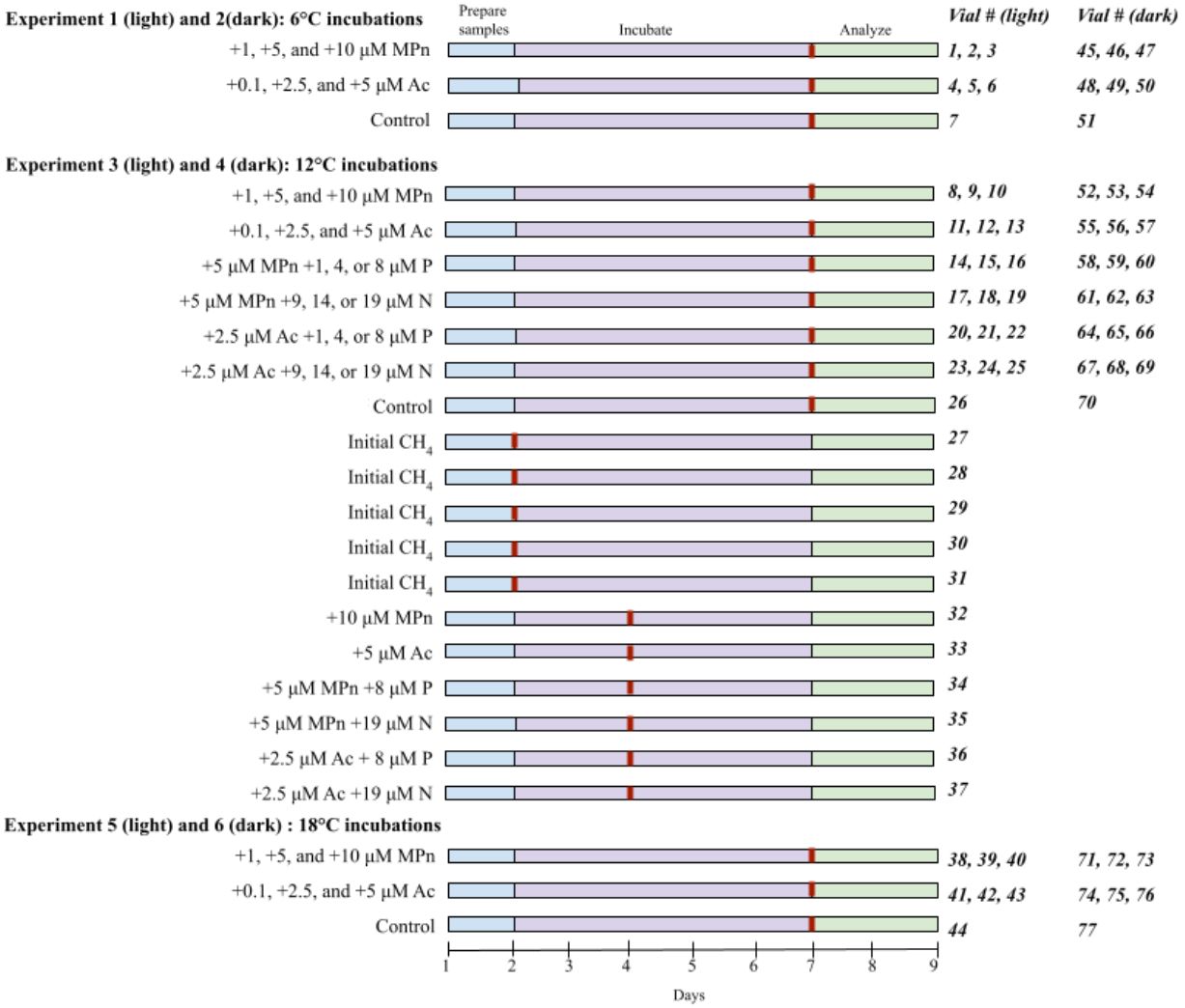


Figure 2 | Timeline of the six experiments. Day 1 "Prepare samples" specifically indicates the process of preparing the vials for incubation from filling the vials with pond water to adding the correct concentration of substrates and nutrients. Day 2 – 7 "Incubate" indicates the five-day period of time the vials were incubated. The red line indicates the day that the vials were sterilized with 8 mol/L NaOH**.** Day 7 -9 "Analyze" were the days that the samples were measured and analyzed for methane concentration.

Results and Discussion

Environmental setting

The pond on C.E.K. Mees Observatory had an initial nitrate concentration of about 35.8 µM and an initial phosphate concentration of < 0.42 µM. The average of the five measured initial methane concentration was 15.19 nM (see Table 1). These control vials served as a comparison group to determine how much the vials with chemical additions deviated from the starting values.

Initial Methane Concentration	
Vial #	**[CH_4] (nM)**
27	13.54
28	14.42
29	12.27
30	20.78
31	14.95
Average	15.19 ± 3.28

Table 1 | Initial methane concentration This table shows the results from measuring the initial methane concentration. To obtain the initial concentration, five vials of unamended water were sterilized with 8 mol/L NaOH at the beginning of the incubation and the initial methane concentration was measured using the GC-FID.

Methane production experiments

Total methane production

Methane concentrations from the vials containing MPn additions ranged from 11.05 nM to 368.9 nM and 12.32 nM to 402.8 nM for Ac additions. The total methane production for each sample was determined using Eq(1):

$$[CH_4]_{Prod} = [CH_4]_{Meas} - [CH_4]_{init}\ , \qquad \text{(Eq. 1)}$$

where $[CH_4]_{Prod}$ is the total methane production from the vial, $[CH_4]_{Meas}$ is the dissolved methane concentration from the vial, and $[CH_4]_{init}$ is the initial original dissolved methane concentration measured (see results in Table 2). In most experiments, the methane production from the control treatments were higher than the methane production from vials containing substrate additions. However, when MPn was paired with nutrients and incubated under light, all of the nitrate additions and the highest and lowest addition of phosphate produced more methane than the control. The additions of nitrate created a larger N:P ratio which could lead to phosphate starvation. This trend is similarly seen in previous studies and indicates that MPn demethylation is enhanced during the day. However, the reasons for why the highest addition of phosphate produced more methane are unclear. Damm et al. (2010) found that in the central Arctic Ocean, methane production occurred in phosphate-replete waters whereas no methane accumulation was observed in phosphate-starved waters. Therefore, it is possible that, under certain conditions, low N:P ratio enhances the ability of bacteria to compete for phosphate, therefore producing methane. While MPn is inhibited by higher phosphate additions, there may be other pathways of production taking place. The Ac addition produced more methane than the control with all additions of phosphate under the light treatment, the lowest addition of phosphate in the dark treatment, and the lowest and highest additions of nitrate in the dark treatment. Out of all the treatments, Ac paired with the lowest addition of phosphate and highest addition of nitrate in the

dark produced the most methane. This may indicate that the acetoclastic pathway gets enhanced by phosphate starvation in the dark. Overall, the additions of both nutrients to Ac and nitrate to MPn increased methane production under the light experiments.

The stoichiometric ratio of each substrate (CH_4:MPn, CH_4:Ac) and each substrate and nutrient pairing (CH_4:MPn:P, CH_4:MPn:N, CH_4:Ac:P, CH_4:Ac:N) were calculated to determine the molar ratio of methane produced for each experiment (see Table 3). This was done by normalizing the methane produced per one mole of added substrate (MPn or Ac) or nutrient (phosphate or nitrate), then averaging the three vials with the same substate and/or nutrient additions. Contrasting previous studies, additions of phosphate to MPn increased methane production and additions of nitrate to MPn decreased methane production under the light incubation. This reveals that methane production through MPn gets enhanced by additions of phosphate and inhibited by additions of nitrate. Since these findings contrast previous studies (Günthel et al. 2020; DelSontro et al. 2017; Carini et al. 2014; Morana et al. 2020), the trend of MPn demethylation being enhanced by phosphate starvation may depend on other factors involved such as temperature or different MPn and nutrient concentrations. Comparing the incubations at different temperatures, the highest temperature produced more methane in the light than the dark, so it's possible that the nutrient additions would only enhance methane production for the MPn and Ac pathway for higher temperatures. Although the lowest temperature incubation (6°C) also produced more methane in the light than the dark, it's likely that this temperature is too low for biological activity to occur to produce methane since the range of methane produced in the 12°C incubator is about four times higher than the 6°C experiment. For Ac, it was observed that additions of both phosphate and nitrate to Ac decreased methane production, which indicate that nutrients inhibit the acetoclastic methanogenesis pathway. Directly comparing MPn and Ac, it was observed that each mole of Ac produced more methane than each mole of MPn in all treatments, except when Ac was paired with nitrate in the light incubation. It was also observed that methane production from Ac consistently increases in the dark, but not in the light, and MPn methane production consistently increased in both light and dark treatments.

6°C					
Light			**Dark**		
Vial #	**Addition (µM)**	**[CH_4] (nM)**	**Vial #**	**Addition (µM)**	**[CH_4] (nM)**
1	1 MPn	19.47	45	1 MPn	14.97
2	5 MPn	11.05	46	5 MPn	19.93
3	10 MPn	29.2	47	10 MPn	11.26
4	0.1 Ac	27.78	48	0.1 Ac	15.57
5	2.5 Ac	12.32	49	2.5 Ac	13.44
6	5 Ac	29.6	50	5 Ac	20.27
7	control	29.15	51	control	20.88

12°C							
Light				**Dark**			
Vial #	**Addition (µM)**	**[CH_4] (nM)**	**N or P (µM)**	**Vial #**	**Addition (µM)**	**[CH_4] (nM)**	**N or P (µM)**

8	1 MPn	32.58	N/A	52	1 MPn	183.1	N/A
9	5 MPn	48	N/A	53	5 MPn	154.6	N/A
10	10 MPn	45.21	N/A	54	10 MPn	161.4	N/A
11	0.1 Ac	16.63	N/A	55	0.1 Ac	163.5	N/A
12	2.5 Ac	29.59	N/A	56	2.5 Ac	54.48	N/A
13	5 Ac	73.23	N/A	57	5 Ac	44.57	N/A
26	control	68.96	N/A	70	control	191.2	N/A
14	5 MPn + 1 P	94.26	<0.42 P	58	5 MPn + 1 P	101.2	<0.42 P
15	5 MPn + 4 P	66.49	1.79 P	59	5 MPn + 4 P	119.2	1.05 P
16	5 MPn + 8 P	82.5	6.53 P	60	5 MPn + 8 P	106.5	5.79 P
20	2.5 Ac + 1 P	104.3	0.42 P	64	2.5 Ac + 1 P	231.9	<0.42 P
21	2.5 Ac + 4 P	69.65	2.21 P	65	2.5 Ac + 4 P	98.63	2.32 P
22	2.5 Ac + 8 P	78.33	7.05 P	66	2.5 Ac + 8 P	143.8	6.74 P
17	5 MPn + 9 N	97	35.73 N	61	5 MPn + 9 N	157.4	47.89 N
18	5 MPn + 14 N	80.23	38.60 N	62	5 MPn + 14 N	75.31	52.33 N
19	5 MPn + 19 N	106.5	43.81 N	63	5 MPn + 19 N	110.3	54.94 N
23	2.5 Ac + 9 N	62.06	32.45 N	67	2.5 Ac + 9 N	196.4	48.14 N
24	2.5 Ac + 14 N	59.55	36.39 N	68	2.5 Ac + 14 N	183.3	51.66 N
25	2.5 Ac + 19 N	41.22	52.59 N	69	2.5 Ac + 19 N	237.4	54.43 N

18°C					
Light			**Dark**		
Vial #	**Addition (µM)**	**[CH_4] (nM)**	**Vial #**	**Addition (µM)**	**[CH_4] (nM)**
38	1 MPn	368.9	71	1 MPn	206.4
39	5 MPn	294.7	72	5 MPn	247.9
40	10 MPn	286	73	10 MPn	259.8
41	0.1 Ac	281.6	74	0.1 Ac	202.3
42	2.5 Ac	283.3	75	2.5 Ac	309.4
43	5 Ac	402.8	76	5 Ac	323.8
44	control	434.3	77	control	359.2

Table 2 | Methane concentration results This table shows the results of the dissolved methane, phosphate, and nitrate concentration for each vial in each experiment after the initial methane concentration (15.19 nM) was subtracted from them. The concentration of methane was measured using a GC-FID, phosphate was measured using a low-range phosphate checker, and nitrate was measured using a nitrate ion-selective electrode.

6°C					
Light			**Dark**		
CH_4	**MPn**	**Ac**	**CH_4**	**MPn**	**Ac**
0.0082	1		0.00669	1	

0.0962		1	0.055		1

12°C									
Light					Dark				
CH_4	**MPn**	**Ac**	**P**	**N**	**CH_4**	**MPn**	**Ac**	**P**	**N**
0.0155	1				0.0767	1			
0.0403	2.291		1		0.0481	2.291		1	
0.00737	0.391			1	0.00955	0.391			1
0.0642		1			0.555		1		
0.0438		1.145	1		0.0915		1.145	1	
0.00443		0.195		1	0.0158		0.195		1

18°C					
Light			Dark		
CH_4	**MPn**	**Ac**	**CH_4**	**MPn**	**Ac**
0.152	1		0.0939	1	
1.00329		1	0.737		1

Table 3 | Stoichiometric ratios This table shows the stochiometric ratio of methane to substrate to nutrient for a 5-day period. The ratios may change if a longer incubation period were implemented. The stoichiometric ratios were normalized to either the nutrient, if there are phosphate or nitrate additions in the experiment, or the substrate if there were no nutrient additions.

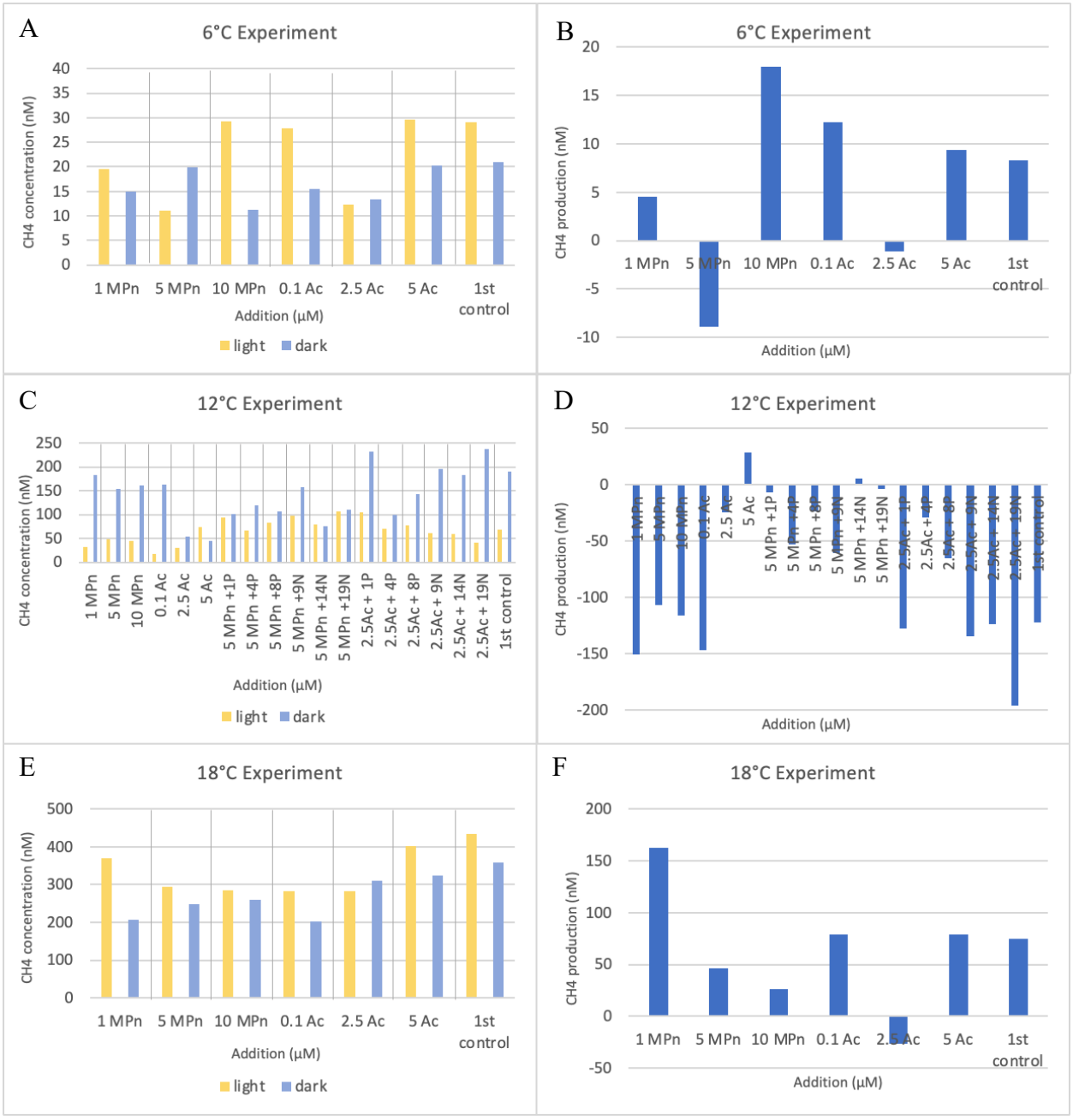
Light influences on methane production

To determine the influence that light has on methane production, the difference was calculated using Eq(2):

$$[CH_4]_{diff} = [CH_4]_{Prod(l)} - [CH_4]_{Prod(d)}\ , \qquad \text{(Eq. 2)}$$

where $[CH_4]_{diff}$ is the difference in light and dark methane production, $[CH_4]_{Prod(l)}$ is the value calculated from Eq(1) for the light vials, and $[CH_4]_{Prod(d)}$ is the value calculated from Eq(1) for the dark vials (see Graph 1 for the results). Through this calculation, it was observed that MPn produced about 124.44 nM more methane in each dark treatment compared to the light in the 12°C experiment and about 78.5 nM more methane in each light treatment compared to the dark in the 18°C experiment. It is known that the rate of photosynthesis increases with higher temperatures, so the increase in methane production in higher temperatures and under light may be due to an increase in the rate of photosynthesis. In the Ac treatments, Ac produced approximately 10.77 nM and 79.15 nM more methane, respectively, in each light treatment in the 6°C and 18°C experiments except for the 2.5 Ac additions. The increase in Ac methane production under the light could also be due to an increase in the rate of photosynthesis. However, the methane production from the 2.5 Ac additions in all three temperature experiments did not follow this pattern as the concentration of methane was higher in the dark experiment than under the light experiment (vial #5, 12, 42). This may be due to natural variability since only the lowest Ac addition in the 12°C experiment showed a significant difference between light and dark methane production (146.87 nM more methane production in the dark vial). When

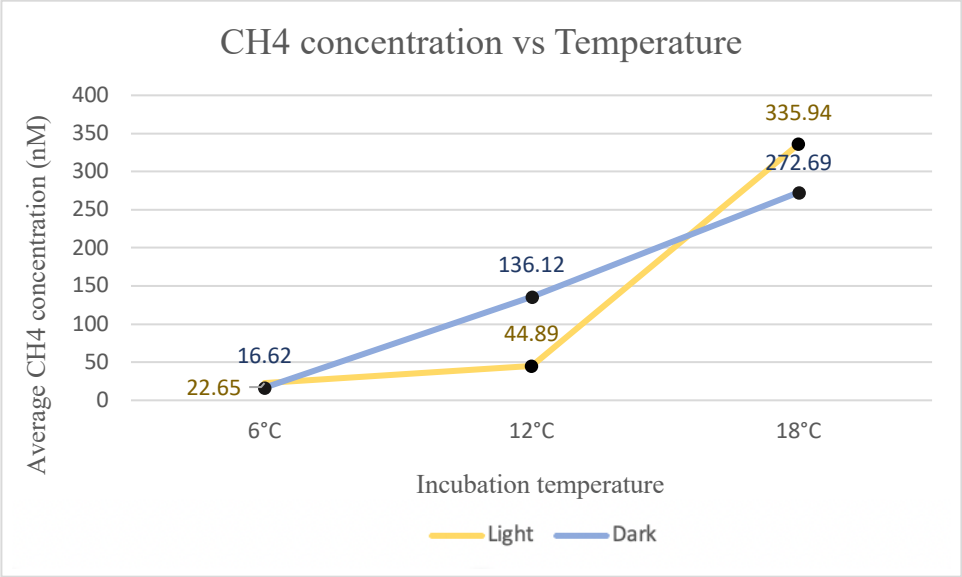
paired with nutrients, MPn paired with phosphate produced an average of about 27.88 nM more methane in the dark and MPn produced about 60.4 nM more methane in dark with the lowest nitrate addition. However, there was not a significant difference between the light and dark methane production in higher nitrate additions. Since phosphate has been known to inhibit MPn demethylation, it is likely that MPn paired with phosphate lead to a lower rate of photosynthesis. The lower rate of photosynthesis would cause the light vial to decrease in methane production compared to the dark vial. Regarding the higher additions of nitrate to MPn not having a significant difference between light and dark production, this may be caused by other methanogenesis pathways not examined in this study such as direct production from cyanobacteria or initiation by other substrates such as dimethylsulfoniopropionate (DMSP) (Bižić, M., et al. 2020; Damm, E., et al. 2010). On the other hand, both Ac paired with phosphate and nitrate produced about 74.02 nM and 151.42 nM more methane, respectively, in dark with the nitrate additions producing more in the dark than the phosphate additions (see Graph 1). Lower phosphate and higher nitrate concentrations seem to promote methane production in the acetoclastic pathway in the dark, which indicates that this pathway may be induced by phosphate starvation. Another possibility for methane being produced more in the dark than the light with nutrient additions in both MPn and Ac pathways is that photosynthesis may be consuming nutrients that the methanogenic bacteria require in order to generate methane. Therefore, at reduced nutrient loads, less methane production can occur.



Graph 1 | Substrate and nutrient addition experiment results Graphs A, C, and E show the methane concentration for each addition with the corresponding light and dark vials next to each other. The values used for these graphs were taken from Table 2. Graphs B, D, and F show the difference between the methane production from the light vial and the methane production from the dark vials (Eq (2)). The positive numbers indicate how much more methane was produced in the light vials than the dark and the negative numbers indicate how much more methane was produced from the dark vials than the light.

Temperature influences on methane production

MPn has been known to produce more methane in the summer months when temperatures are highest (Günthel et al. 2020), so it was expected that as temperature increased, there was generally more methane production (see Graph 2). There was also a larger increase with methane production in the light treatment compared to the dark as temperature increased (see Graph 2). Light combined with higher temperature increases the rate of photosynthesis, which likely led to higher methane production. It was observed that methane production was approximately 6.47 nM, 8.55 nM, and 17.95 nM lower for the middle concentration addition of MPn (5 µM) for all temperatures (6°C, 12°C, and 18°C, respectively) compared to the lower and higher concentrations of MPn. This was likely due to natural variability since the difference is not significant. On the other hand, methane production was approximately 20.85 nM and 64.9 nM lower for the middle concentration addition of Ac (2.5 µM) for the lower temperatures (6°C and 12°C, respectively) compared to the lower and higher concentrations of Ac. Overall, the results show that temperature increases caused the largest increase in methane production, so the largest factor controlling methane production in this study was temperature changes. Also, since the substrate/nutrient and light trends change drastically in each different temperature experiment, it is likely that different methane production pathways, not necessarily MPn demethylation or acetoclastic methanogenesis, were being observed at each different temperature.



Graph 2 | Methane concentration vs temperature This graph shows the methane concentration increase with temperature. The methane concentration from the vials without nutrient additions for each experiment were averaged together and plotted in a line graph.

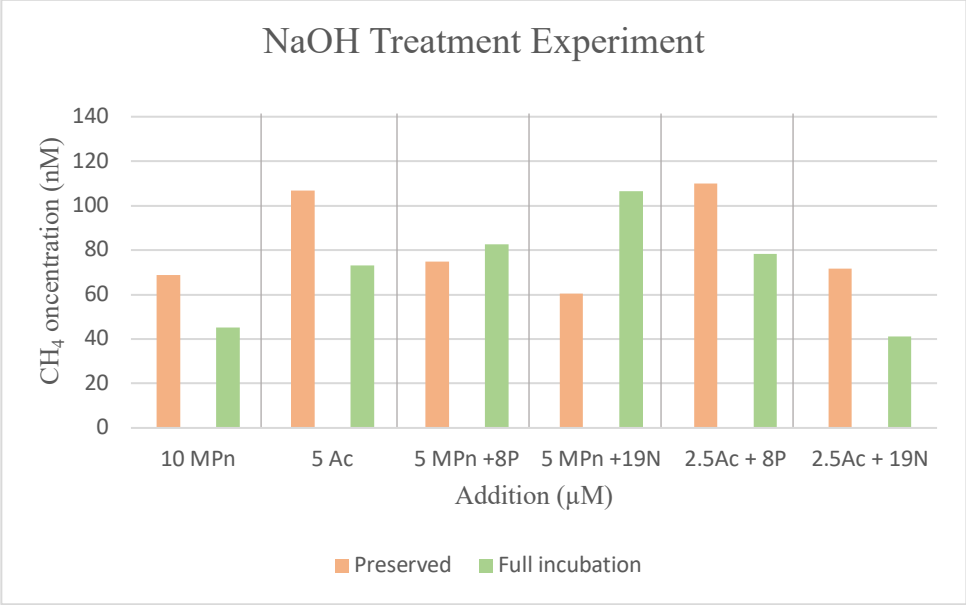
NaOH treatment experiment

The vials for the NaOH treatment experiment consisted of vials containing the highest substrate or nutrient concentrations (10 µM MPn, 5 µM Ac, 5 µM MPn + 8 µM P, 5 µM MPn + 19 µM N, 2.5 µM Ac + 8 µM P, and 2.5 µM + 19 µM N). These six vials were sterilized on day two of the incubation period to determine if 8 mol/L NaOH was an effective treatment to halt biological activity. The results are shown in Table 4 and the comparison between the NaOH treatment vials and the full incubation (5 days) vials are shown in Graph 3. The majority of the

sterilized treatment vials produced more methane than the fully incubated vials except for the vials containing MPn and nutrient pairing additions. This potentially displays the natural variability of methane production, the influence of methane oxidation, or displays that using 8 mol/L NaOH was not completely sterilizing.

NaOH treatment experiment		
Vial #	**Addition (µM)**	**Produced [CH_4] (nM)**
32	10 MPn	68.89
33	5 Ac	106.7
34	5 MPn + 8 P	74.87
35	5 MPn + 19 N	109.9
36	2.5 Ac + 8 P	60.55
37	2.5 Ac + 19 N	71.66

Table 4 | NaOH treatment experiment results This table shows the results of the original dissolved methane concentration for each of the six vials after the initial methane concentration (15.19 nM) was subtracted from the measured concentration. The concentration of methane was measured using a GC-FID.



Graph 3 | NaOH treatment vs full incubation This graph shows the results of each vial that was sterilized with 8 mol/L NaOH on day two of the incubation compared to the fully incubated vial (five days) containing the same substrate and/or nutrient addition.

Conclusion

The goal of this study was to determine whether MPn demethylation or acetoclastic methanogenesis would produce more methane and determine whether either of the two pathways would produce enough methane to make a significant impact on atmospheric methane emissions.

I hypothesized that the MPn demethylation pathway would produce more methane than the acetoclastic methanogenesis pathway since the MPn demethylation pathway was previously observed to be enhanced by the lake's natural conditions. Based on my hypothesis, I expected methane production from MPn to increase with lower phosphate and higher nitrate additions, however, this was not the case in this study. The stoichiometric ratio indicated that Ac supplied more methane per mole than MPn and that MPn was not enhanced by phosphate limitation in this experiment. Since the nutrient additions were only added to one of the three temperatures, this may indicate that MPn demethylation being enhanced by phosphate starvation may depend on other factors such as temperature or other substrate and nutrient concentrations. Surprisingly, most light experiments revealed that more methane production was being produced in the dark than the under light, especially with nutrient additions to Ac. Methane production was shown to be most influenced by increases in temperature, and the increase in temperature led to a significant increase in methane production under the light. This trend could indicate that temperature is a large driving factor, and the main substrate or methane production pathway that drives methane production may depend on the temperature of the water. This study did not investigate the microbial community structure present in the water samples, choosing instead to explore the ecosystem responses. Nonetheless, changes in the microbiome, such as seasonal or chemical changes, will likely influence methane production. Regarding whether either the methanogenesis pathways studied would make a significant impact on the methane cycle, the control treatment generally produced more methane than the treatments with additions, so the additional methane production, if any, from MPn and Ac are likely not significant in the temperate Northern Hemisphere springtime.

This study provides an initial, first-order understanding of methane production mechanisms in oxic waters. Further studies are required to determine whether the drivers of methane production shifts with temperature increases and if nutrients at higher temperatures have a different affect from the nutrient additions at lower temperatures. Identifying the type of microbial organisms in the water sample and the cell count would also be beneficial in determining which methane production pathways are most likely to occur. Furthermore, considering the vials containing the middle additions of MPn and Ac (5 MPn and 2.5 Ac) produced less methane than the lowest and highest substrate additions, future studies are required to determine whether this trend is controlled by factors such as light, temperature, nutrients, if this trend continues at higher temperatures, or if it's due to natural variability in methane production and oxidation. Future studies are also required to determine whether the stoichiometric ratio of methane produced by the addition of MPn and Ac changes significantly for incubations over five days.

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Appendix

Standard #	Concentration (µM)	Volume added to 100 mL of DI water (mL)
1	1	0.1
2	2	0.1
3	4	0.2
4	6	0.2
5	9.9	0.4
6	29	2.0
7	48	2.0

Table S1 | Calibration standards for low-level nitrate measurements This table shows the nitrate standards used for the low-level nitrate measurement calibration and the amount of each standard added to 100 mL of deionized water.

Stock solution #	Chemical	Grams added to 500mL	V of stock vial (mL)	C of stock (nmol/mL)	V of stock added to pond water (mL)	Final C of pond water (nmol/mL)	V of pond water vial (mL)
1	MPn	0.01895	500	305.448	1	1	296.87
2	MPn	0.0949	500	1529.66	1	5	296.87
3	MPn	0.18985	500	3060.12	1	10	296.87

4	Ac	0.00204	500	29.9824	1	0.1	296.87
5	Ac	0.051005	500	749.633	1	2.5	296.87
6	Ac	0.102015	500	1499.34	1	5	296.87
7	P	0.021071	500	296.858	1	1	296.87
8	P	0.084	500	1183.43	1	4	296.87
9	P	0.168	500	2366.86	1	8	296.87
11	N	0.115	500	2706.2	1	9	296.87
12	N	0.18	500	4235.79	1	14	296.87
13	N	0.244	500	5741.85	1	19	296.87

Table S2 | Stock solution calculations and data This table shows the values used to calculate the stock solution for each substrate and nutrient addition where V = volume and C = concentration.

NaOH solution #	Chemical	Grams added to 100mL	V of stock vial (mL)	C of stock (µmol/mL)	V of stock added to pond water (mL)	Final C of pond water (µmol/mL)	V of pond water vial (mL)
14	NaOH	31.99	100	8002.139	1	32	256.87
15	NaOH	31.99	100	8002.139	1.56	32	391

Table S3 | 8 mol/L NaOH solution calculation and data This table shows the values used to calculate the stock solution used to sterilize the vials where V = volume and C = concentration. NaOH solution #13 was used to preserve vials #1-26 and 32-77 and NaOH solution #14 was used to preserve the initial methane concentration vials #27-31.