Methylphosphate Utilization by Trichodesmium

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Abstract

Phosphonates are organophosphorus compounds recalcitrant to degradation. The Carbon - Phosphorus lyase pathway allows certain microbes to make use of such compounds, releasing a hydrocarbon in the process. This process is shown when methane derives from methylphosphonate consumption. Methane liberation from methylphosphonate facilitated by microbial activity has been shown to occur in the oxygen-rich surface ocean around the world. It may provide these bacteria a phosphorus source used to support growth when phosphate is limited. This project tested the hypothesis that the cyanobacteria *Trichodesmium* in wild populations in the Gulf of Mexico and lab cultures use methylphosphonate when phosphate concentrations are low, releasing methane as a byproduct.

Introduction

Nutrients in the surface ocean, particularly nitrogen (N) and phosphorus (P) are needed to perform photosynthesis (Palter et al., 2020), influencing marine carbon (C) cycles and primary productivity rates in the surface ocean. Marine bacteria from the genus *Trichodesmium* are important primary producers in oligotrophic regions, responsible for as much as 15% of total primary production in the Pacific gyres (Dutheil et al., 2018). *Trichodesmium* colonies also support the open-ocean food web by supplying up to 50% of the new nitrogen available (Walworth et al., 2018) through the use of their N-fixation pathways. This mechanism allows a steady nitrogen source for themselves and other organisms around them, making P the limiting factor for local growth (Palter et al., 2020). Since *Trichodesmium* colonies are integral to the marine food web, understanding what promotes growth in these populations is crucial to the entire ecosystem's health (Dutheil et al., 2018).

Specific microbes can use methylphosphonate (MPn), the most prevalent organic phosphonate source in the ocean (Lockwood et al., 2022), as a P source through the C-P lyase pathway (Sosa et al., 2019), a critical process in the oceanic phosphorus redox cycle (Van Mooy et al., 2015). The metabolism of methylphosphonate is also a global mechanism of methane release in the upper ocean (Lockwood et. al., 2022) suggested as the primary cause of the ocean methane paradox (Repeta et al., 2016), making MPn cleavage important to the global methane cycle.

Trichodesmium colonies in the Sargasso Sea have been shown to use MPn when under phosphate stress (Chappell et al., 2006). Through this project, we tested the hypothesis that the cyanobacteria *Trichodesmium* in wild populations in the Gulf of Mexico and lab cultures used methylphosphonate when phosphate concentrations are low, releasing methane as a byproduct. Evidence supporting this hypothesis will be little to no methane production in incubated *Trichodesmium* control conditions relative to the main treatment condition with a methylphosphonate addition, with a greater disparity between groups as phosphate availability decreases.

Methods

Field Experiment Setup

Trichodesmium colonies were collected from the Gulf of Mexico in September 2022 aboard the R/V Atlantis using a 150 µm mesh size net (General Oceanics, Miami, FL). *Trichodesmium* 5 colonies were sorted and rinsed in 0.22 µm filtered seawater collected from the sample site (Figure 1A). Colonies were added to 150 mL glass serum bottles with 125 mL of filtered seawater (FSW) and 25 mL headspace and sealed with a butyl rubber stopper. Five experiments were performed over the duration of the cruise. Each experiment had four treatments: FSW, FSW + *Trichodesmium*, FSW + *Trichodesmium* + MPn, FSW + *Trichodesmium* + MPn + antibiotics (Table 1). For the MPn condition, we added 1 mL MPn (1.92 µM) to each experimental treatment. We also added 1 mL of Ampicillin Trihydrate (0.1 µM), Carbenicillin

(0.1 μM), Chloramphenicol (0.02 μM), Kanamycin (0.05 μM), Neomycin (0.05 μM), Streptomycin sulfate (0.1 μM) and Tetracycline Hydrochloride (0.01 μM) per antibiotic condition bottle. Bottles were incubated in an on-deck flow through an incubator with mesh bags to mimic sea surface conditions. 3 mL of headspace was removed three times and analyzed on GC-FID. 9 ml of headspace was added to replace that volume, and bottles were returned to the incubator.

Latitude	Longitude	Field or Culture	Experiment #	# Time Points	Treatments	Length of experiment (hours): time points
27°18.016'N	87°52.765'W	Field	1	2	FSW, no P addition, MPn, MPn + A	15: 0, 15
27°21.995'N	90°33.965'W	Field	2	3	FSW, no P addition, MPn, MPn +A	24: 0, 12, 24
27°50.862' N	91°24.717' W	Field	3	4	FSW, no P addition, MPn, MPn +A	30: 0, 7, 18, 30
27°50.862' N	91°24.717' W	Field	4	4	FSW, no P addition, MPn, MPn +A	49: 0, 24, 31, 49
27°0.606' N	90°38.330' W	Field	5	3	FSW, no P addition, MPn, MPn +A	40: 0, 16, 40

n/a	n/a	Culture	1	3	Replete YBC II media, P free YBC II media, and MPn + P free YBC II media	332: 0, 120, 332

Table 1.1 Details of experiments performed in the field and with cultures. Description of treatments: FSW consisted of only 0.22 µm filtered seawater with no addition of *Trichodesmium.*

No P addition treatments were 0.22 µm FSW with *Trichodesmium* colonies added. MPn treatments were 0.22 µm filtered seawater with *Trichodesmium* colonies added and 1.92 µM MPn. MPn + A treatments were 0.22 µm filtered seawater with *Trichodesmium* colonies, 1.92 µM MPn, and the antibiotic mix described in text above. Replete YBCII media treatments contained P at 50 µM in KH2PO4 and *Trichodesmium erythraeum* RLI filaments. P-free YBCII media contained no added P and contained *Trichodesmium erythraeum* RLI filaments. MPn + P free YBCII media contained the P-free media with MPn added as P-source at a concentration of 2.5 µM and *Trichodesmium erythraeum* RLI filaments.

Culture Experimental Setup

We performed additional incubation experiments using cultured *Trichodesmium erythraeum* to further test the impact of MPn on the ability of *Trichodesmium* to make use of alternate nutrient sources. Eric Webb kindly provided cultures at the University of Southern California. Cultures of *Trichodesmium* were grown in YBC II media (Chen et al. 1996) in an Innova incubator with 12:12h day/night light cycles and 50 µmol photons $m^{-2}s^{-1}$. For the experimental setup, 800 µL of *Trichodesmium* stock was inoculated into 30 mL polycarbonate bottles containing 20 mL of media and 10 mL of headspace. Bottles were sealed with butyl rubber stoppers (Figure 1B). Culture experiments included triplicate bottles of three conditions: MPn (2.5 µM) + *Trichodesmium* in phosphorus-free YBC II media, *Trichodesmium* in phosphorus-free YBC II media, and replete YBC II media + *Trichodesmium*. Controls without *Trichodesmium* were performed for every treatment. The cultures were incubated for a total of 14 days (332 hours), and methane concentrations in the headspace were sampled at 0 hours, 120 hours (5 days), and 332 hours (approximately 14 days).

Analysis by gas chromatography

All methane production analysis was done using a Shimadzu Gas Chromatography Flame Ionization Detector 14A with an N-octane on res Cil C 1/8" diameter packed 10m column at 40°C. The Gas Chromatograph was run with helium as the carrier gas, and nitrogen was flushed between replicates and samples. Field samples were measured with 3 mL triplicates, and cultures were measured once with 1 mL each. Methane standards were 4.2 ppm, 27 ppm, and 54 ppm. Standard curves were created by graphing the standard peak area against standard ppm, and applying the equation for the linear trendline to the peak areas generated for the samples. A new standard curve was created for every time point.

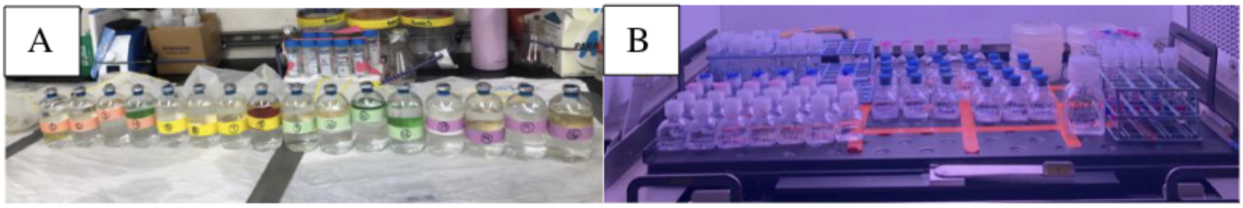

Figure 1. A. Experimental setup for field *Trichodesmium*. B. Experimental setup for lab cultures.

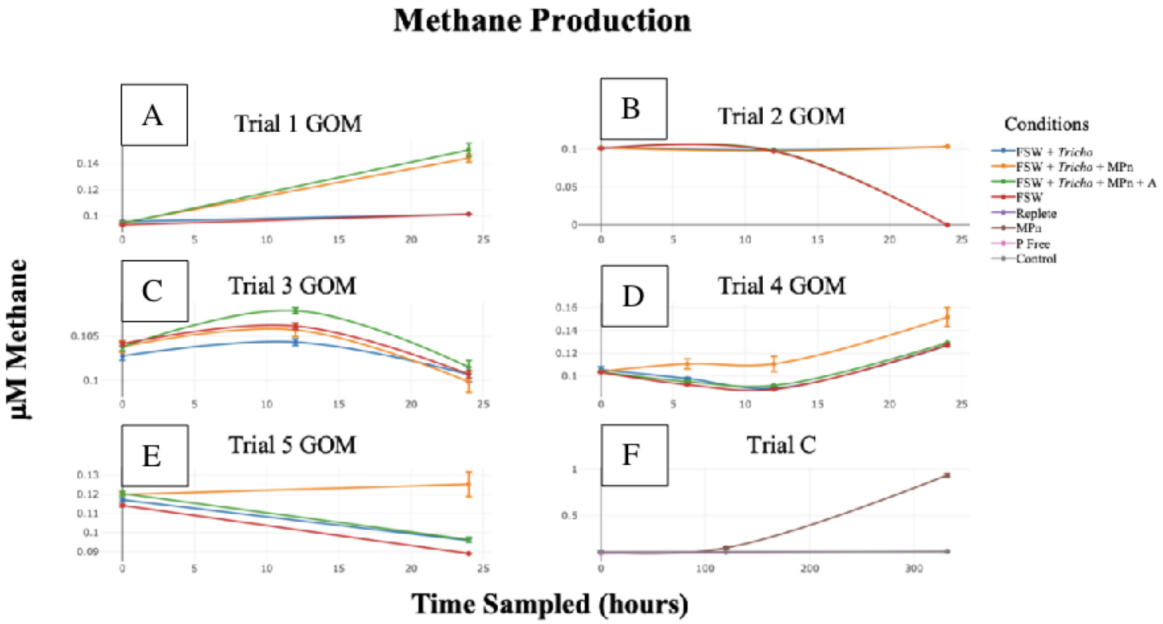

Figure 2. A-F. Methane production measurements in µM for incubation trials over a 24-hour period for field samples and 332-hour period for cultures. Figure 2 A-E. Time series data for wild Trichodesmium in the field.
Figure 2 F. Time series data for culture Trichodesmium in the lab. All incubations use the FSW + Trichodesmium + MPn condition or MPn condition as the experimental condition.

In trial 1 (Figure 2A), Conditions FSW + *Trichodesmium* + MPn and FSW + *Trichodesmium* + MPn + A showed increased methane production over the control treatments. FSW + Trichodesmium + MPn condition resulted in 0.14 μM methane after 24 hours, FSW + *Trichodesmium* + MPn + A condition resulted in 0.15 μM methane after 24 hours and controls FSW and FSW + *Trichodesmium* both resulted in 0.10 μM methane after 24 hours. In Trial 2 (Figure 2B) all conditions resulted in 0.10μM methane after 12 hours, FSW + *Trichodesmium* + MPn and FSW + *Trichodesmium* conditions resulted in 0.10 μM methane after 24 hours, and controls FSW and FSW + *Trichodesmium* + MPn + A were not measured at 24 hours due to instrument error. This absence of data results in a 0 μM methane data point at 24 hours in Figure 2B. In the third trial (Figure 2C), the FSW + *Trichodesmium* + MPn, FSW + *Trichodesmium* + MPn + A, and FSW resulted in 0.11 μM methane after 12 hours, and 0.10 μM methane after 24 hour. FSW + *Trichodesmium* condition resulted in 0.10 μM methane after both 12 hours and 24 hours. In the fourth trial (Figure 2D), FSW + *Trichodesmium* + MPn condition resulted in 0.11 μM methane after 6 and 12 hours and 0.15 μM methane at 24 hours. FSW + *Trichodesmium* + MPn + A condition resulted in 0.09 μM both 6 hours and 12 hours and 0.13 μM methane after 24 hours. Control FSW + *Trichodesmium* condition resulted in 0.10 μM after 6 hours, 0.09 μM after 12 hours and 0.13 μM methane after 24 hours. Control FSW conditions resulted in 0.09 μM after 6 and 12 hours and 0.13 μM methane after 24 hours. In the final field sample trial (Figure 2E) FSW + *Trichodesmium* + MPn condition resulted in 0.13 μM methane after 24 hours, both FSW + *Trichodesmium* + MPn + A and FSW + *Trichodesmium* conditions resulted in 0.10 μM methane after 24 hours and FSW control resulted in 0.09 μM after 24 hours. The lab culture experimental MPn condition (Figure 2F) showed increased methane production over control treatments. MPn media conditions resulted in 0.15 μM methane after 120 hours and 0.93 μM methane after 332 hours. Replete, P-Free, and control bottles resulted in 0.11 μM methane after 120 and 332 hours.

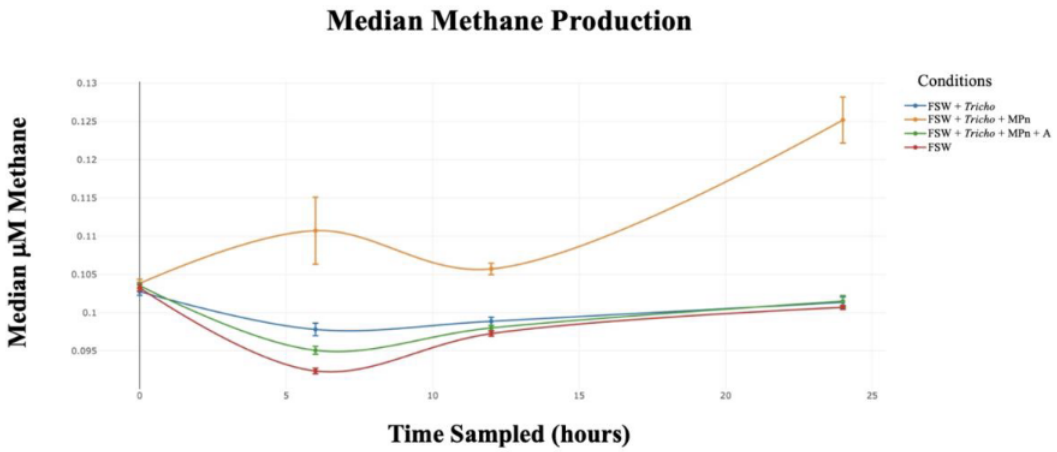

Figure 3. Median methane measurements in µM for wild Trichodesmium in the field with 24-hour incubations. Median of averaged trial measurements across all 5 trials were plotted.

The FSW + *Trichodesmium* + MPn experiment condition shows an increase in methane production over median methane production for the control conditions. Median FSW + *Trichodesmium* + MPn condition resulted in 0.11 µM methane after 6 hours, 0.10 µM methane after 12 hours, and 0.13 µM methane after 24 hours. Median FSW + *Trichodesmium* + MPn + A, FSW + *Trichodesmium,* and FSW control conditions resulted in 0.09 µM-0.10 µM methane after 6 hours, 0.10 µM methane after 12 hours and 0.10 µM methane after 24 hours. Not all trials had measurements taken at 6 hours, so the median of 6 hours, therefore is not representative of every trial.

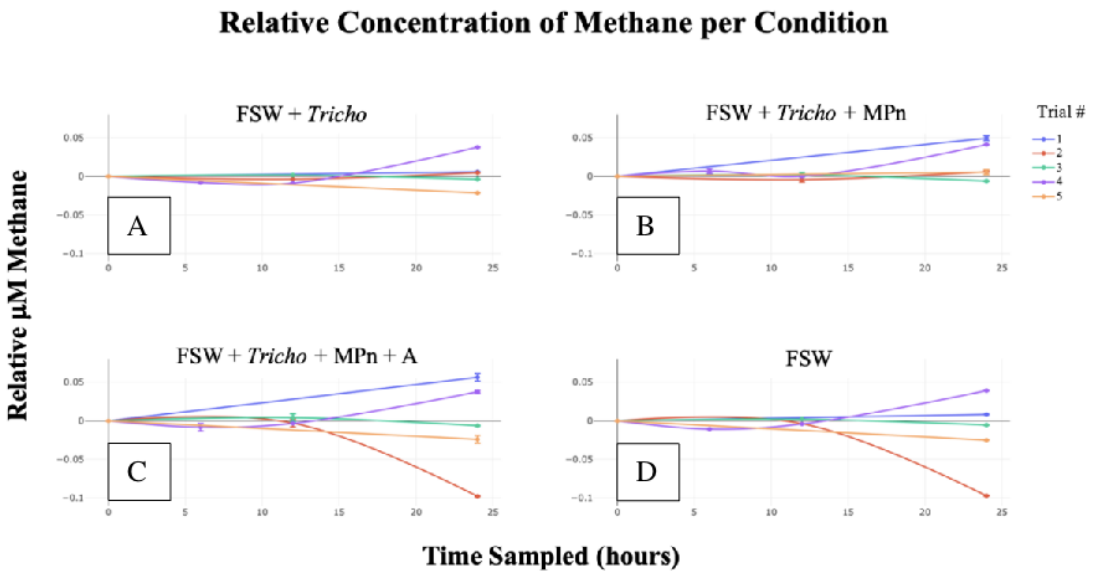

Figure 4. Methane measurements (µM) normalized for wild Trichodesmium in the field with 24-hour incubations. Values are normalized to account for initial methane values in the bottle headspace. The values shown indicate total new methane production per condition and trial.

In the experimental condition FSW + *Trichodesmium* + MPn for wild *Trichodesmium*, Trial # 4 (Figure 4B, Figure 2D) and Trial #1 (Figure 4B, Figure 2A) shows the highest new methane production over 24 hours with a total new production of 0.045 µM methane and 0.050 µM methane respectively. Trials # 2, 3 and 5 (also shown in Figure 2C and Figure 2 E) showed negligible new methane production. Negative values show a decrease of methane in the headspace, likely related to the consumption of ambient methane.

Discussion

The antibiotic and MPn treatments were intended to kill the *Trichodesmium* and any associated epibionts. In Trial # 1 and Trial # 3, the antibiotics likely did not kill the wild *Trichodesmium* because we saw an increase in methane production over 24 hours indicating that the MPn was being used. In Trial # 2, we did not measure any methane production at 24 hours, suggesting that the antibiotic may have effectively killed the wild *Trichodesmium*. In Trial #1 the antibiotic added treatment (Figure 2A and Figure 4C) resulted in 0.15 µM methane after 24 hours which is higher than the MPn only addition experiment condition (Figure 2A) of 0.14 µM methane. This could be due to the antibiotic killing an important epibiont that lived with *Trichodesmium* but not the *Trichodesmium* itself given the importance of the associated microbial community to *Trichodesmium* nutrient acquisition (Lee et al., 2018). Overall, we saw similar levels of production in the MPn + antibiotic treatments and the filtered seawater control (Figure 3), implying that the antibiotics were effective overall at halting activity.

Trial # 2 and Trial # 3 (Figure 2B and Figure 2C) show the least amount of methane production from the MPn only conditions. The experiment conditions in these trials show no appreciable difference to the control conditions. These trials were performed during Hurricane Ian in the Gulf of Mexico; in Trial #1, wild *Trichodesmium* colonies were collected prior to Hurricane Ian in the sample locations, and in Trial # 4 and Trial # 5, wild *Trichodesmium* colonies were collected after Hurricane Ian moved away from our sample locations. For the affected trials, we originally predicted the hurricane stirred up nutrients in the water column; therefore, *Trichodesmium* no longer needed to make use of alternate nutrients. However, nutrient data (not shown here) show no difference in phosphate concentrations throughout the expedition, suggesting that there was no new input of phosphate to the surface waters from the storm event at the locations sampled. The *Trichodesmium* could have been physically agitated from rougher sea surface conditions, potentially impacting their community structure and therefore limiting their ability to acquire nutrients through complex metabolic processes.

Methane is produced by the wild *Trichodesmium* populations non-linearly (Figure 2C and D and Figure 3). In Figure 3, all plots show a slight sinusoidal curve. These fluctuations could be due to *Trichodesmium* being less productive in certain time periods in the day/night cycle or at different growth phases during the incubation. Future experiments looking at diel methane production could further investigate these changes in phosphorus uptake.

Average methane production in field samples ranged from 0.1 μM to 0.15 μM. Methane is produced from methylphosphonate at a 1:1 ratio (Karl et al., 2008). A single mole of phosphorus is made available for uptake for every mole of methane produced. We added MPn at a concentration of 1.92 μM. Median methane produced at 24 hours in the MPn treatment was 0.13 μM indicating that 6.5% of the MPn added was likely converted to bioavailable phosphorus. We expect more MPn would have been consumed if the experiments ran over a longer incubation period. In culture, we added MPn at a concentration of 2.5 μM. The average culture methane produced at 332 hours in the MPn treatment was 0.93 μM indicating that 37% of the MPn added was likely converted to bioavailable phosphorus. The MPn usage rate was approximately twofold higher in the wild *Trichodesmium* than in culture (0.11% $hr^{-1}$and 0.27% hr^{-1}respectively). The wild *Trichodesmium* may be better primed for MPn uptake than culture *Trichodesmium*, which may have never encountered MPn in lab conditions.

Trial # 4 (also shown in Figure 2. A. and Figure 2. D.) produced the highest amount of methane at 0.15 μM methane in 24 hours. Similarly, cultures of *Trichodesmium* also measured 0.15 μM methane in 120 hours. *Trichodesmium* in culture may have been producing methane at a slower rate as they acclimated to the new phosphorus source. An experiment in the Sargasso Sea, with 10 – 20 wild colonies of FSW + *Trichodesmium* + MPN produced a similar 0.20 μM methane after 36 hours and 1.1 μM methane after approximately 72 hours (Karl et al., 2008). *Trichodesmium* trials reached nearly the same concentration as Karl et (2008) (93 μM) but after 332 hours rather than 72 hours. The Sargasso Sea wild *Trichodesmium* may have been better primed to use the MPn than our cultures were. Similarly, if the wild Trichodesmium experiments

in the Gulf of Mexico had run longer than 24 hours, we may have seen methane concentrations increasing past the culture incubation methane levels. A previous experiment with *Trichodesmium* IMS101 produced 0.12 μM methane in the MPn condition after 132 hours (Beversdorf et al., 2010), very similar to our culture *Trichodesmium* trials that reached 0.15 μM methane in the MPn condition in 120 hours. The trend of production over the time course of culture incubations suggests that the *Trichodesmium* may have taken time to adapt to new phosphorus sources and then increased uptake of the MPn once they acclimated.

Based on our *Trichodesmium* culture methane production measurements, we calculated that culture produces approximately 276 μM methane per week. According to the EPA, a single cow produces approximately 260 lbs of methane per year translating to approximately 25,602 μM methane per weekly methane. While *Trichodesmium* methane production is much lower than that of the average cow, their abundance in the surface ocean suggests that they have the potential to contribute a large pool of methane to the atmosphere.

Conclusion

Through this experiment, we could quantify methane production from both wild *Trichodesmium* in the Gulf of Mexico and culture *Trichodesmium*, showing their ability to use the C-P Lyase pathway and produce methane from MPn. We established that this methane production accelerates in both the field incubations (24 hours) and culture trials. (332 hours). Methane production via MPn cleavage in the surface ocean is important to understand regarding the global methane paradox (Repeta et al., 2016) and the global methane cycle. In future experiments, lipid analysis could determine the impacts of different P sources on phospholipid composition. With a deficient phosphorus source, *Trichodesmium* may make fewer Phospholipids, and in P-free conditions, there may be fewer lipids in general due to inhibited growth.

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