Methylphosphonate and Sargassum Impact on the Sargasso Sea Microbiome

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ABSTRACT

Pelagic *Sargassum* are an ecologically important macroalgae that contains both macrobiota and microbial epiphytes. *Sargassum* thrives in the nutrient depleted conditions in the Sargasso Sea where dissolved phosphorus and nitrogen occur in very low concentrations. Certain bacterial lineages are known to be able to cleave the C-P bond of Methylphosphonate (MPn), a compound abundant in the water, producing bioavailable phosphorus for Sargassum growth. This study investigated the response of the Sargassum microbiome to different concentrations of MPn and the impact of *Sargassum natans* dissolved organic matter (DOM) and MPn on the microbiome of *S. natans* and the surrounding seawater of the Sargasso Sea. The abundance of *Alteromonas, Flavobacteriales, Rhodobacteraceae, SAR 11, SAR 324,* and *Vibrio* were analyzed to identify what specific lineages found in the Sargasso Sea would be affected by additions of MPn, *Sargassum* DOM and both MPn and *Sargassum* DOM. The Sargassum microbiome numbers increased with increasing MPn concentrations with no real changes in the lineages investigated. The ocean microbiome grew five times more in the DOM+MPn treatment, three times more in the MPn addition, and twice more in the DOM addition when compared to the control. Copiotrophic lineages, *Alteromonas* and *Flavobacteriales* were elevated in the DOM treatments while *Rhodobacteraceae* and *Vibrio* were elevated in the MPn additions.

INTRODUCTION

Sargassum Seaweed is a type of pelagic algae native to the Sargasso Sea, it has two species, S. natans and S. fluitans which are holopelagic species characterized by numerous blades and gas-filled pneumatocysts. These characteristics allow them to exist in rafts at the air-sea interface creating a floating habitat for numerous organisms (Lapointe et al. 2014). Sargassum supports a diverse community of seabirds and pelagic fish, commonly residing along migratory paths of humpback whales and sea turtles (Huffard *et al*., 2014). In recent years, the biomass of Sargassum has been increasing at a dramatic rate in the Sargasso Sea and unprecedented quantities of decomposing Sargassum have been washing up upon Carribean coastlines (Johnson *et al*. 2013). The economic impact of larger Sargassum blooms has become evident on the tourism sectors of many Carribean and Central American countries. For instance, in a recent statement by the Mexican President, the country expects to spend $2.7 million on beach cleanup annually, and a drop of tourism by 30% in some affected areas. (Fleming, 2019)

Previous publications have aimed to link Sargassum biomass growth with nutrients discharged from major river deltas or ocean upwelling. A massive increase in the 90's was traced back to nutrients from the Mississippi River (Smetacek, Zingone *et al*., 2013). More recently, researchers have hypothesized that nutrient discharge from the Amazon River (exacerbated by deforestation and fertilization), and upwelling along the coast of West Africa are tied to the creation of the Atlantic Sargassum belt, a stretch of Sargassum that extends from West Africa to the Gulf of Mexico (Wang *et al*., 2019). It is possible that compounds like Methylphosphonate (MPn) are then carried via ocean currents from these sources to areas of higher latitude where Sargassum grows. Typically, Sargassum would not be able to use raw MPn, but certain lineages of bacteria able to cleave the C-P bond in MPn into useful Phosphate and Methane have been found on the Sargassum microbiome (Susilowati et al. 2014). This suggests that the Sargassum microbiome has adapted to the typically Phosphorus-deficient environment of the Sargasso Sea, giving some lineages of bacteria the advantage of being in a mutualistic relationship with their host organism (Ulrich et al. 2018). Several bacterial lineages of interest to this experiment were *Alteromonas, Flavobacteriales, Rhodobacter, Vibrio, SAR 11, and SAR 324.* A recent study found that *Vibrio* and members from the *Pseudoalteromonas* were found in high concentrations when extracted from *Sargassum* (Susilowati et al. 2014). SAR 11 is a common Ocean microbe that has been studied for evidence of a capability to break down Methylphosphonate (Carini et al. 2014).

This study aims to study the effect of increasing concentrations of Methylphosphonate on the Sargassum microbiome and the effect of additions of Sargassum DOM, Methylphosphonate, and both Sargassum DOM and Methylphosphonate have on the microbial environment of ocean seawater. Sargassum was incubated in flow through aquaria with sterile seawater and concentrations of Methylphosphonate from 0nM to 1420nM.The response of the microbiome to the various Methylphosphonate conditions was measured using microscopy. Ocean bacterioplankton were cultured in the presence or absence of Sargassum DOM and Methylphosphonate, over 7 days. Samples were taken every 12 hours for three days and the bacterioplankton abundance and community structure measured. This project aims to determine if the Sargassum microbiome responds to Methylphosphonate, if the microbiome is responsible for cleaving the C-P bond of Methylphosphonate and determine if Sargassum DOM can be utilized by ocean bacteria. A potential outcome of this research is to help balance the global carbon budget. The world's waters produce an estimated 2% of the total amount of methane released into our atmosphere (Reeburgh 2007), and Methylphosphonate-cleaving bacteria living on the Sargassum microbiome could play a significant role in the ocean Carbon cycle.

METHODS

Experimental Design

Sargassum MPn addition experiments

To test how the microbiome was changed due to the addition of Methylphosphonate, a series of trials were conducted to test how differing amounts of Methylphosphonate would affect the bacterium abundance on Sargassum. To do this samples of Sargassum weighing approximately 2g were added to serum bottles filled with 100mL of water. Different amounts of Methylphosphonate were added to each sample set and the bottles were over pressured by adding 20mL of air. The bottles were then left to incubate for a period of 3-7 days.

Sargassum DOM stable isotope probing experiment

Samples of Sargassum were incubated with 13C labeled bicarbonate as the source of carbon dioxide. The 13C label would be converted to 13C labeled DOM by the Sargassum via photosynthesis. This 13C labeled DOM would then be taken up by the bacteria making their DNA heavier, allowing it to be separated by centrifugation prior to sequencing.

This experiment used ocean surface water collected from the BATS site at 32.147948, -64.492048 on Thu 10 Oct 2019 03:03:03. Carboys were filled with 8.4L 0.2µM filtered sea water and 3.6L whole seawater and incubated at 26 °C (SST at time of retrieval was 26.34 °C). Experimental conditions were created through the addition of 20µM Isotope-labeled S. Natans DOM and/or the addition of 70nM MPn. The summer trials conducted at BIOS illuminated a direct relationship between higher levels of [MPn] and an increase in the number of cells in the microbiome of Sargassum Seaweed. The work also suggested the microbiome population became affected dramatically at a concentration around 100 nM MPn. The addition of 70nM MPn was decided upon so that the +MPn conditions would be around the 100nM critical point observed in previous experiments after added to the basal levels of MPn observed in the Sargasso Sea.

Treatment	Volume	Filtrate	Whole Water	Addition
A, B	11-12L	Surface (8.4L)	Surface (3.6L)	None / Control
C, D	11-12L	Surface (8.4L)	Surface (3.6L)	Control + MPn(70nM)
E, F	11-12L	Surface (8.4L)	Surface (3.6L)	+ S. Nat DOM (20µM)
G, H	11-12L	Surface (8.4L)	Surface (3.6L)	+ S. Nat DOM (20µM)+ MPn (70nM)
TOTAL	**44-48L**	**33.6L**	**14.4L**	

Table 1: This table shows the four experimental conditions created for the experiment. The conditions were done in duplicate. Conditions with +MPn were spiked with 70 nM MPn and conditions with +DOM

were spiked with 20 µM C13 labeled DOM. Note that these concentrations were added in excess upon that which is found naturally in the Sargasso Sea.

Experimental Timetable

Timepoint	Day	Date	Time	DAPI	Probe	TOC	Nuts	DOC	DCNS	DNA	PAR TOC
T0	0	10/10/19	20:00	X	X	X	X	X	X	X	
T1[2]	0.5	10/11/19	9:00	X							
T2	1	10/11/19	20:00	X	X						
T3	1.5	10/12/19	9:00	X							
T4	2	10/12/19	20:00	X	X	X	X	X	X		X
T5	3	10/13/19	15:00	X							
T6	4	10/14/19	15:00	X	X	X	X	X	X	X	X
T7	5	10/15/19	15:00	X	X						
T8	7	10/17/19	15:00	X	X	X	X	X	X	X	X

Table 2: This table shows the times when the microbiome was sampled for various information. DAPI slides were taken at every time point to create a smooth growth curve.

Sample Collection and Analysis

Samples taken for TOC, DOC, DCNS, and Nutrients were shipped to UCSB for further analysis. Total organic carbon (TOC) and dissolved organic carbon (DOC) will be determined via high temperature combustion on a modified Shimadzu TOC-V (Shimadzu Scientific Instruments, Columbia, MD). Dissolved amino acids (DCNS) will be analyzed through high-performance liquid chromatography (Dionex, Thermo Scientific). Nutrients will be analyzed by a continuous flow nutrient analyzer. DNA will be extracted using phenol chloroform and ethanol precipitation and send to UCSB for Illumina sequencing analysis.

Bacterioplankton abundance (DAPI)

10mL Samples from the Carboys were taken and filtered through a polycarbonate 0.2µm 25mm filter previously stained with Irgalan black (SIGMA-Aldrich, St. Louis, MO USA) under light vacuum (5-7 mm Hg). The filters were then stained in darkness with 4', 6-di-amidino-2-phenylindole dihydrochloride (5 ug ml^{-1} DAPI, SIGMA-Aldrich, St. Louis, MO USA) and placed on a microscope slide using Resolve immersion oil and stored at -20C until further use (Porter and Feig, 1980). Total bacterioplankton abundance was determined using an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) under ultraviolet excitation at 100x magnification. At least 400 cells (10 fields) were counted for bacterioplankton abundance and normalized per mL of seawater (Steinberg et al., 2001).

Fluorescence in situ hybridization (FISH

The abundance of *Alteromonas, Roseobacter, Flavobacteriales* and *Vibrio* was quantified using the FISH method in (Parsons et al., 2012) adapted from (Morris et al. 2002; Morris et al. 2004). Following filtration through 0.2um polycarbonate filters, samples were submerged in 95% ethanol to dehydrate prior to probing for bacteria including *Alteromonas, Roseobacter,*

Flavobacteriales and *Vibrio* and using probe sequences specific to each bacteria listed in table 1. Filters were washed twice at 10 minutes per wash using SET 1 buffer solution (20 $mmol^{-1}$ Tris-HCL, 70 $mmol^{-1}$ NaCl, 5 $mmol^{-1}$ EDTA, pH 7.4) heated to 52°C. Filters were then placed on slides to which 20 ul of 6-diamidino-2-phenylindole dihydrochloride (DAPI, SIGMA-Aldrich, St. Louis, MO USA) in citifluor solution was added. Filters were analysed under Cy3 (550 nm) and UV wavelength sets adapted from the methods of Carlson (2009).

Bacteria	**Probe sequence**	**Wash buffer**	**Wash Temperature [°C]**
Alteromonas AC-137R-Cy3	5'- TGTTATCCCCCTCGCAAA-3'	SET 1	52
Flavobacteriales	5'-GGACCCTTTAAACCCAAT-3'	SET 3	46
Rhodobacteracea e 536R-Cy3	5'- CAACGCTAACCCCCTCCG-3'	SET 1	52
SAR 11 441R-Cy3	5'-TACAGTCATTTTCTTCCCCGAC-3'	SET 2	46
SAR 324	5'-GCCCCTGTCAACTCCCAT-3'	Archaea	46
Vibrio VibSpl-127-Cy3	5'-CCCCACATCAAGGCAATTTC-3'	SET 1	45

Table 3: FISH bacteria probe sequences, and wash buffer temperatures for *Alteromonas, Flavobacteriales, Roseobacter* SAR 11, SAR 324, and Vibrio

Catalyzed Reporter Deposition Fluorescence in situ hybridization (CARD-FISH)

Methodology for CARD-FISH conducted to probe for SAR11and SAR324 was based on (Teira et al., 2004) and modified in (Parsons et al., 2015). Polycarbonate filters containing samples were embedded in agarose prior to permeabilization with 0.5M lysozyme. Washes were conducted in SET 2 buffer solution (20 $mmol^{-1}$ Tris-HCL 70 $mmol^{-1}$ NaOH, 5 $mmol^{-1}$ EDTA, pH 7.4) at 52C for 10 minutes followed by a wash in PBS for 10 minutes. DAPI citifluor was added as described in the FISH protocol and imaged under Cy3 (550 nm) and UV light, using methods adapted from Carlson (2009).

Image Analysis

Image analysis using an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) was used to process FISH and CARD-FISH slides excited with Cy3 (550 nm) and UV wavelengths as previously described. The image capturing was performed using a Toshiba (Irvine, CA, USA) CCD video camera with a Pro-series capture kit version 4.5 (Media Cybernetics, Bethesda, MD, USA) and processed with Image Pro software (version 4.5; Media Cybernetics) as previously described (Carlson et al., 2009; Parsons et al., 2015).

Statistical Analysis

Averages and Standard Deviations were calculated using Microsoft Office and Google Sheets. Student T-test using one tail were used to test hypothesis and calculated using a macro in Google Sheets.

RESULTS

Sargassum MPn addition experiments

Three MPn addition experiments were conducted in July and August, 2019. Sargassum was incubated in sterile seawater for three to seven days and sampled for bacterial abundance at the end of the trials. The bacterial abundance includes bacteria and archaea found in the incubation water and on the Sargassum microbiome (Figure 1). Trial 2 began on July 17th and ran until July 23rd, Trial 3 began on August 16th and ran until August 27th, and Trial 4 began on August 26th and ran until September 2nd. The length of the trial was 6 days. The aim of this trial was to reproduce the findings of the previous trials that suggested the microbiome of Sargassum was impacted by Methylphosphonate. Trial 4 was different from previous trials in that it focused on a smaller range of Methylphosphonate concentrations (below 100 nM). Based on all of the data compiled from Trials 2, 3, and 4, we were able to see that the bacterial community changed dramatically once the concentration of MPn reached above 100nM. This concentration of 100nM became the target concentration for the +MPn condition in TWIP. Previous findings that the Sargasso Sea's innate concentration of MPn is between 50-70nM (Martinez et al, 2013; Carini et al, 2014) led us to spike our +MPn conditions with 70nM MPn creating a condition between 120-140nM MPn. This was done to attempt to create a significant difference between the microbial growth in the MPn Control and +MPn conditions. Naturally occurring levels of DOC are 70µM in the Sargasso Sea (Carlson et al, 2004; Steinberg et al. 2001). The +DOM conditions were spiked with 20µM Stable Isotope labeled Sargassum DOM to create a significant difference from the DOM Control conditions, and to allow an excess of isotope-labeled material to be analyzed post-experiment.

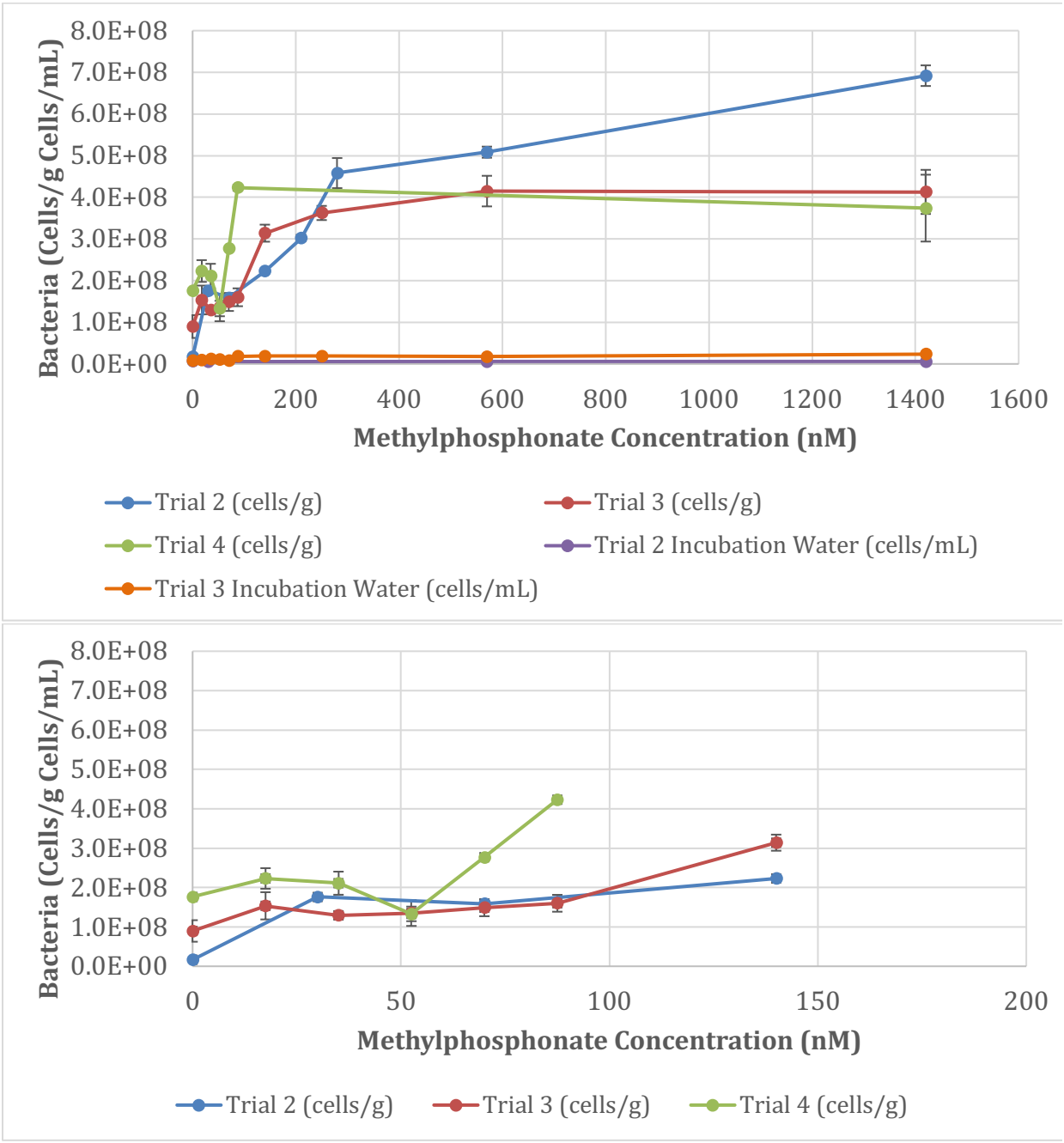

Figure 1. (Top) Graph of gross number of cells per gram of Sargassum counted at the end of incubation periods for the summer trials. Incubation water for Trials 2 and 3 measured in cells/mL of water. (Bottom) Graph shows same data as first graph, on a reduced x axis scale. This highlights the tendency of the microbiome to begin to react substantially to Methylphosphonate additions once the concentration reaches 70-100nM. This trend is seen most clearly from Trial 4's data which tested across a small MPn concentration gradient, and for a shorter amount of time than the other trials.

Trials 2,3, and 4 showed significant increases between the MPn control condition and 87.5nM MPn and between the MPn control and 1420nM MPn. Trials 2 and 3 show significant

increases between 87.5 nM MPn and 1420 nM MPn. However, Trial 4's microbial abundance does not increase significantly from 87.5 nM MPn to 1420 nM MPn. This suggests that during the course of Trial 4, the microbiome holistically stopped responding to Methylphosphonate when levels exceeded 87.5 nM.

Treatment Comparison	Trial 2*	Trial 3	Trial 4
0nM vs 1420nM	<0.00001	<0.00001	<0.00001
0nM vs 87.5nM	<0.00001	<0.00001	<0.00001
87.5nM vs 1420nM	<0.00001	<0.00001	0.034

Table 4. Shows the results of T Test when testing the null hypothesis that the number of bacteria counted at the compared concentrations could belong to the same population against the alternative hypothesis that there was a higher population of bacteria present at the higher concentration. This data shows that during Trial 4, the bacterial count data is not statistically different at the 0.01 confidence level when comparing concentrations 87.5 and 1420 nM. *Trial 2 was tested against 70nM instead of 87.5nM.

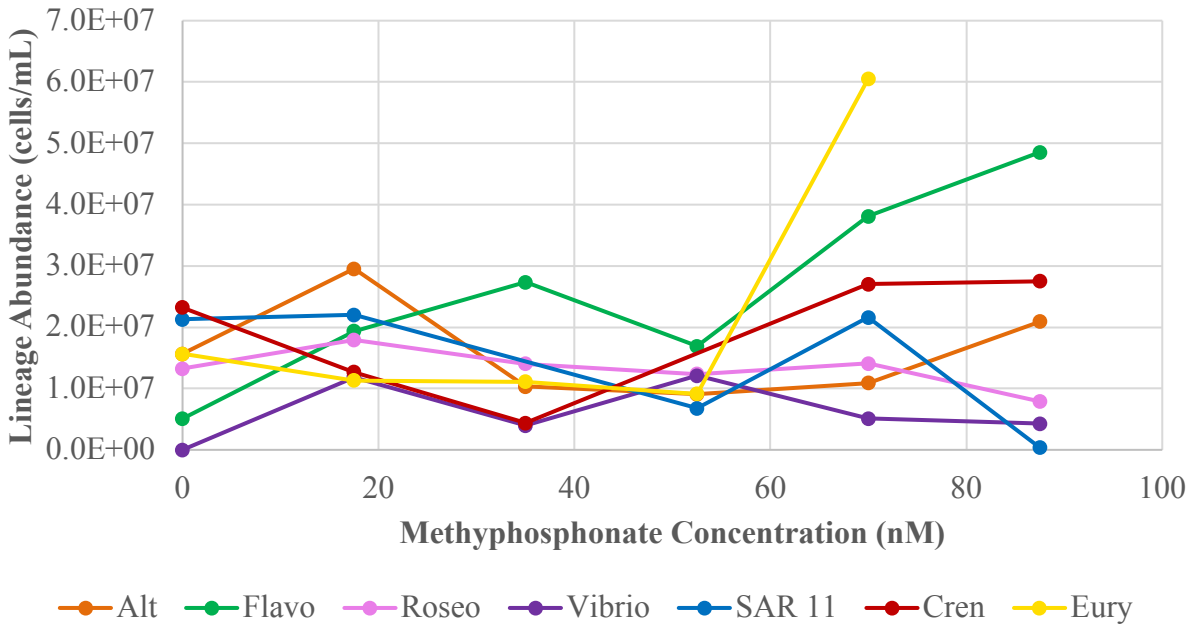

Figure 3. FISH/CARD FISH data from Trial 4 represented as # cells/gram of Sargassum.

From the FISH and CARD FISH data for Trial 4, the abundance of key members of the Sargassum microbial community, *Alteromonas, Flavobacteriales, Vibrio, Rhodobacteraceae, SAR 11, Thaumarchaeota(Cren),* and *Euryarchaeota* were assessed at each concentration of

Methylphosphonate. From this data, we can see small changes in the abundance of bacterial lineages at small incremental changes in [MPn]. Lineages *Euryarchaeota* and *Flavobacteriales* showed the most increase at the higher concentrations. Probe data for [MPn] = 1420 nM not shown.

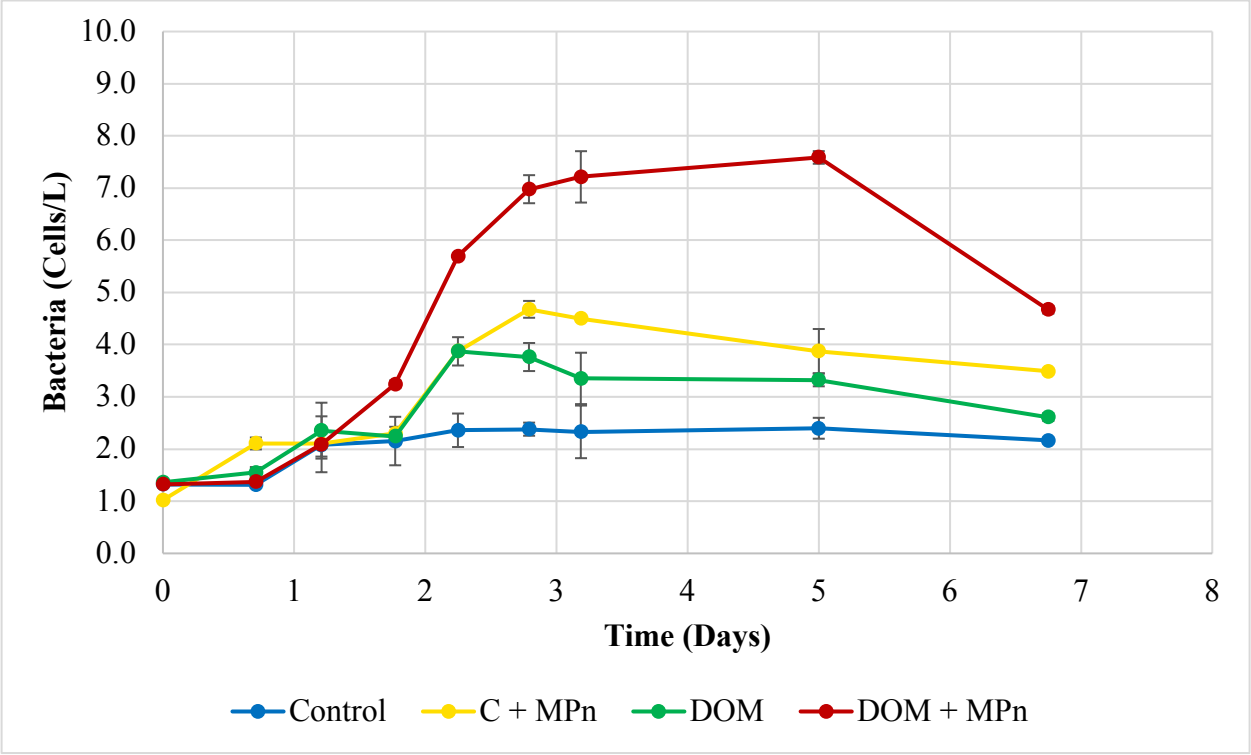

Figure 4. Graph shows DAPI cell counts in for the averages of each of the four conditions at nine time points throughout the course of the experiment.

The +DOM+MPn double addition showed the greatest bacterial growth increasing from 1.3 x 10^8 cells/L on Day 0 to 7.5 x 10^8 cells/L on Day 5. On Day 3, when each condition had reached the bacterial stationary phase, the double addition had grown 1.5 times the amount of the +MPn condition and 1.9 times the amount of the +DOM condition. When compared to the control, all treatments were significantly different (MPn: $p = 0.010$, $n = 18$; DOM: $p = 0.003$, $n = 18$; MPn + DOM: $p = 0.001$, $n=18$). The DOM addition was significantly different from the MPn + DOM addition ($p = 0.010$, $n = 18$) while the MPn was not significantly different from the MPn + DOM addition ($p = 0,048$, $n = 18$).

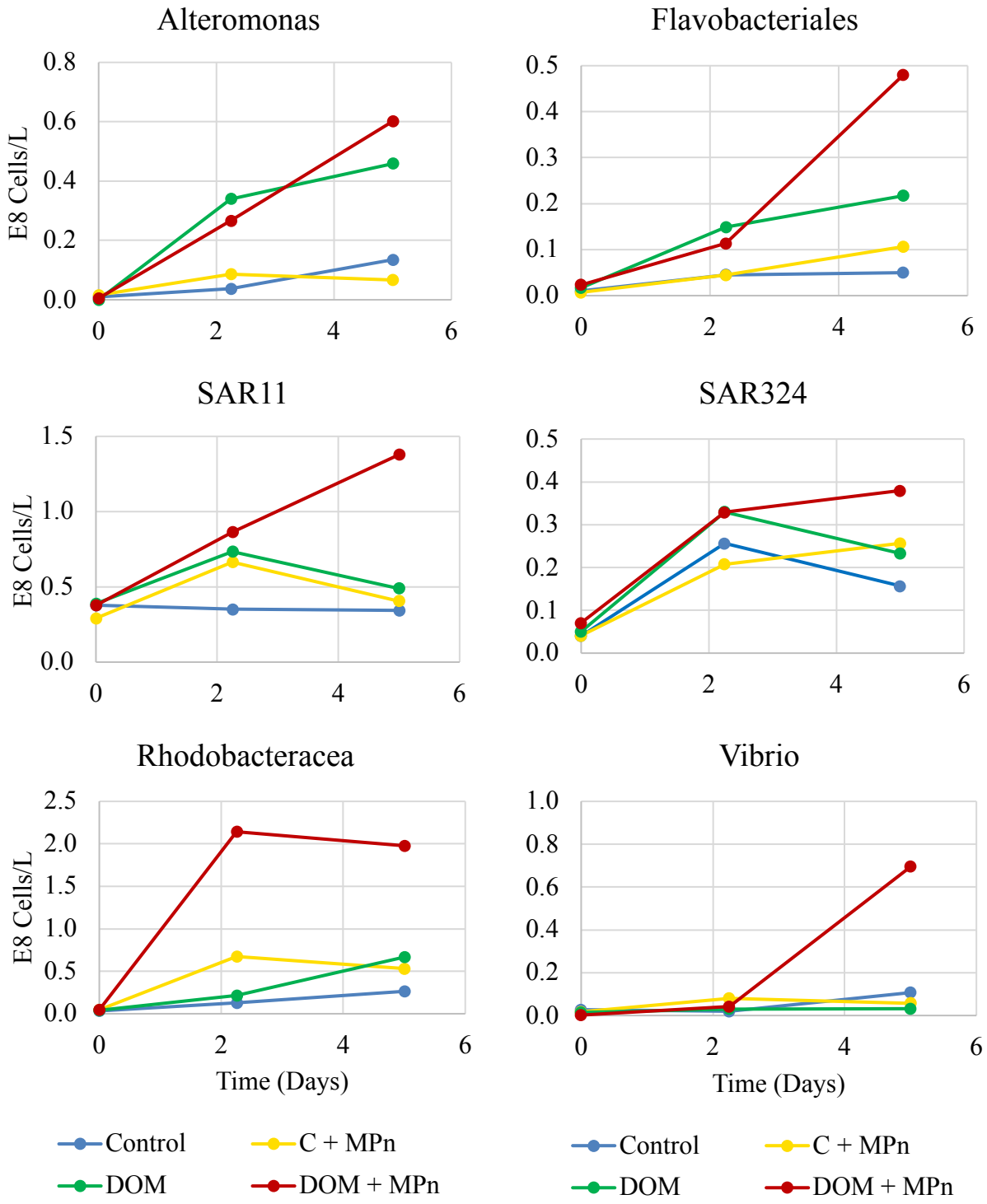

Figure 5. FISH/CARD FISH data represented as a number of cells per liter of incubation water. All lineages that were probed for grew in +DOM+MPn condition. SAR 324 grew in al conditions. SAR 11 grew a smaller similarly in the +DOM and +MPn conditions, while Flavobacteriales and Alteromonas grew to a higher abundance in the +DOM condition than in the +MPn condition. Substantial Rhodobacteracea and Vibrio growth was only seen in +DOM+MPn condition.

FISH and CARD FISH data for TWIP illuminates which species of bacteria responded to the different conditions. Opportunistic and copiotrophic lineages *Flavobacteriales* and *Alteromonas* grew in conditions positive for Sargassum DOM. SAR 324 grew in every condition, and oligotrophic lineages capable of cleaving Methylphosphonate's C-P bond like *SAR 11* and copiotrophic lineages *Vibrio*, and *Rhodobacteraceae* grew well in the double addition.

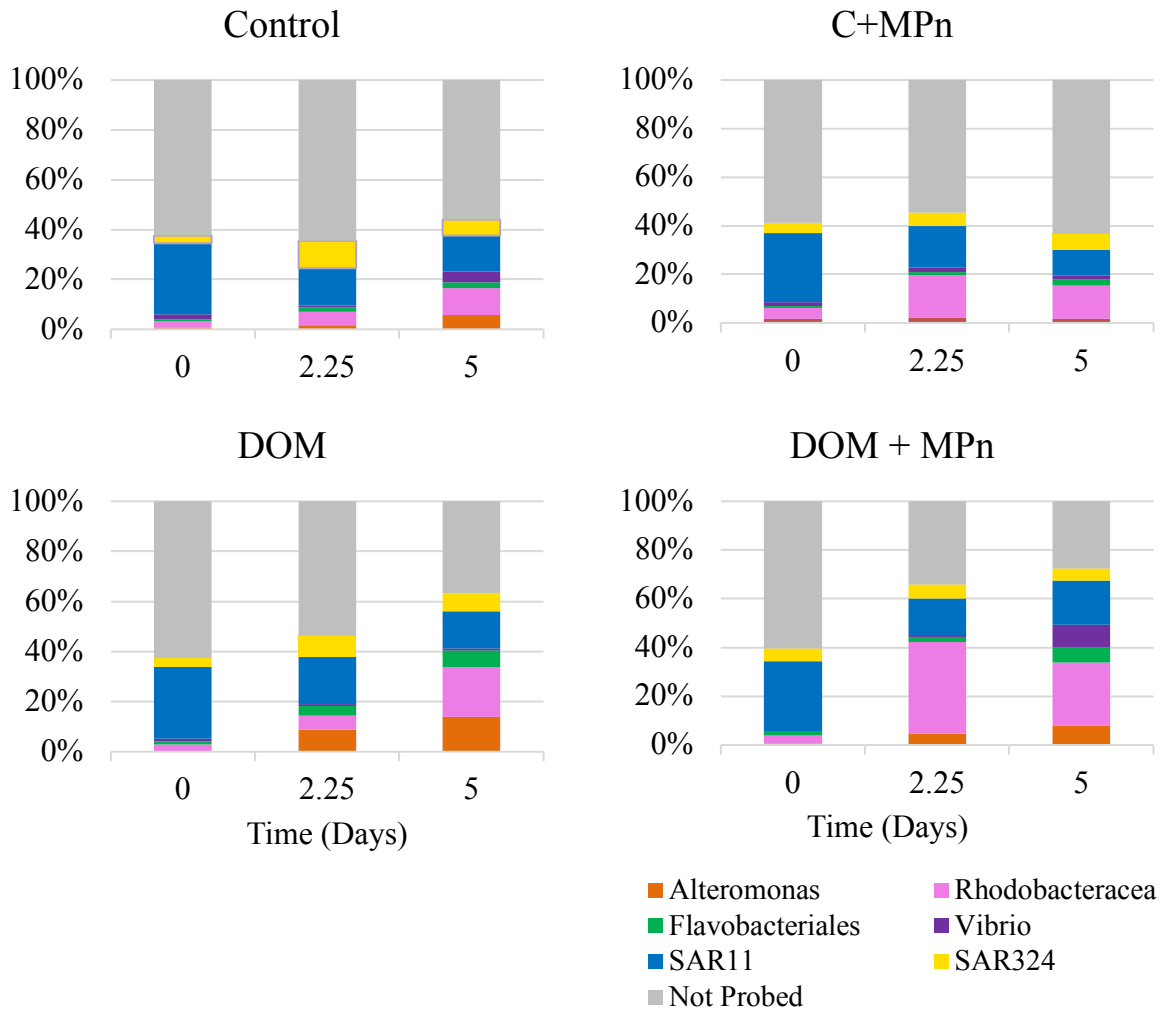

Figure 6. Stacked bar charts for the % abundance of each bacterial lineage to the total bacterioplankton population of the microbiome of each condition (Control, MPn, DOM and MPn + DOM) at T=0, T=2.25, and T=5 days.

DISCUSSION

The waters within the Sargasso Sea are characterized as being nutrient poor (Steinberg et al., 2001). However, this gyre is home to a large variety of both flora and fauna found within the Sargasso Sea (Laffoley et al., 2011). The brown floating algae, Sargassum natans thrives in the Sargasso Sea even though nitrogen and phosphorous limit growth (Cotner et al., 1997; Lomas et al. 2010). It is hypothesized that members of the Sargassum microbiome can cleave the C-P bond of Methylphosphonate, a compound that is abundant in the water column, producing methane and phosphonate. This provides a source of phosphorous to the microbial community and an outgassing of methane to the atmosphere (Karl et al. 2008).

The response of the Sargassum microbiome to Methylphosphonate

There have not been many studies that have investigated the Sargassum microbiome. The bacteria Dichthrix, a nitrogen-fixer, can be found within the Sargassum microbiome (Capone et al., 2008). Alteromonas was found within the Sargassum microbiome but did not increase with increasing concentrations of Methylphosphonate (Figure 3). Since Alteromonas are well-known copiotrophs that grow rapidly when labile organic substrates become available (Romera-Castillo et al., 2011), they may have responded to dissolved organic matter produced by the Sargassum. Roseobacter bacteria are found in surface seawater from the Sargasso Sea (Parsons et al., 2012; Sosa et al., 2017). These bacteria have the ability to cleave the C-P bond in Methylphosphonate and release P nutrients into the water column. A 2017 study conducted on waters near Hawaii found that Sulfitobaccter, a bacteria closely related to Roseobacter, is able to break down the C-P bond in Methylphosphonate (Sosa et al., 2017). A study of S. Muticum in Portugal revealed substantial temporal shifts in the microbiome with large abundance of Rhodobacteraceae (including Roseobacter) in September-March (Serebryakova et al., 2018). Rhodobacteraceae was present in the Sargassum microbiome but did not increase in abundance with increasing Methylphosphonate additions (Figure 3). In contrast, the copiotroph, Flavobacteriales did increase in abundance with increasing concentrations of Methylphosphonate (Figure 3). The Archaea, Thaumarcheota and Euryarcheota did respond to increasing Methylphosphonate suggesting these lineages are important to the Sargassum microbiome and may be involved in cleaving the C-P bond of Methylphosphonate. This finding confirms previous suggestions that microbes of the clave Thaumarchaeota could cleave MPn (Metcalf et al. 2012). SAR11, a free-living aerobic heterotrophic Alphaproteobacteria that account for 15-40% of all bacteria cells throughout the water column (Eiler et al., 2009; Giebel et al., 2011; Morris et al., 2002; Rappe et al., 2002; Schattenhofer et al., 2009) accounted for 24% of the total bacteria within the Sargassum microbiome at the start of the trial but decreased in abundance with increasing Methylphosphonate additions (Figure 3).

The response of ocean bacterioplankton to Sargassum DOM and Methylphosphonate

The lineages that grew well in the DOM conditions, *Alteromonas* and *Flavobacteriales* are well-known copiotrophs that grow rapidly when labile organic substrates become available (Romera-Castillo et al., 2011). However, overall the +DOM condition grew less than expected relative to the +MPn condition. This means either Sargassum was less labile than was expected, or bacteria were even more responsive to small changes in [MPn] than anticipated. The bacteria that grew in +MPn conditions such *Rhodobacteraceae*, *Vibrio*, *SAR 11* and *SAR 324* all have been previously suggested to be capable of cleaving MPn (Metcalf et al. 2012; Ulrich et al. 2018; Carini et al 2014).

While Sargassum DOM and Methylphosphonate both individually have the capability to enhance microbial growth in ocean waters and change the makeup of the microbial community, an environment with both Sargassum DOM and Methylphosphonate will grow more oligotrophic bacteria able to cleave the C-P bond resulting in a higher microbial population. Every lineage that was probed for in this experiment increased in abundance over the length of the trial for the DOM+MPn condition.

The response of the Sargassum microbiome to Methylphosphonate additions suggested that the addition of 100nM+ of Methylphosphonate was enough to change the makeup of the microbiome. This was reconfirmed in the experiment that tested the response of ocean bacterioplankton to Methylphosphonate and Sargassum DOM. However, this experiment also showed that Sargassum DOM at a basal MPn concentration is just as important to bacterial growth as an elevated level of MPn. Moreover, it was shown that having both an elevated level of MPn and Sargassum DOM greatly increased the growth of the bacterial community.

Future Work

In the future, this experiment could be upscaled dramatically to account for numerous other nutrient conditions and concentrations which could impact the ocean microbial community.

Previous work has suggested that at higher MPn concentrations, above 1000 nM, it is possible the differences in the makeup of the microbiome may become more apparent from activation of the phn operon in oligotrophs that are ubiquitous to the Atlantic Ocean such as SAR 11 (Ulrich et al. 2018). Ulrich notes that the through phn operon activation proteins responsible for Methylphosphonate catabolism and intracellular Methylphosphonate transfer will be coded for by the microbial genome.

The Sargasso Sea, in addition to being Phosphorous poor, is also deprived of many sufficient Nitrogen sources. A possible area of study may be the effect of a supply of nitrogen on the microbiome of Sargassum or ocean surface water. Data collected from the summer trials suggests that supplying ocean microbes with a sufficient nitrogen source may exacerbate the growth of the microbiome (Cox, 2019). Seeing as nitrogen sources are common in ammonium-containing fertilizers similar to those containing MPn, a repeat of this experiment with stable isotope labeled ammonium could prove useful. Additionally, some of the lineages that were

probed for, including SAR 11 and Thaumarchaeota, are ammonia-oxidizing bacteria (Metcalf et al. 2012; Treusch et al, 2005).

It may be worthwhile to include a logarithmic gradient of MPn concentrations e.g. +70, +700, +1400, +7000 nM MPn conditions. This gradient could possibly create conditions under which some bacteria thrive and others die out. Another nutrient worth including in this experiment would be ammonium, seeing as previous work by Cox has shown that its presence or absence could change the makeup of the Sargassum microbiome. Using 13C labeled MPn and 15N labeled NH_4 may also prove beneficial to track where these nutrients are being taken up, and would expound upon the novelty of this project as a Stable Isotope Probing experiment.

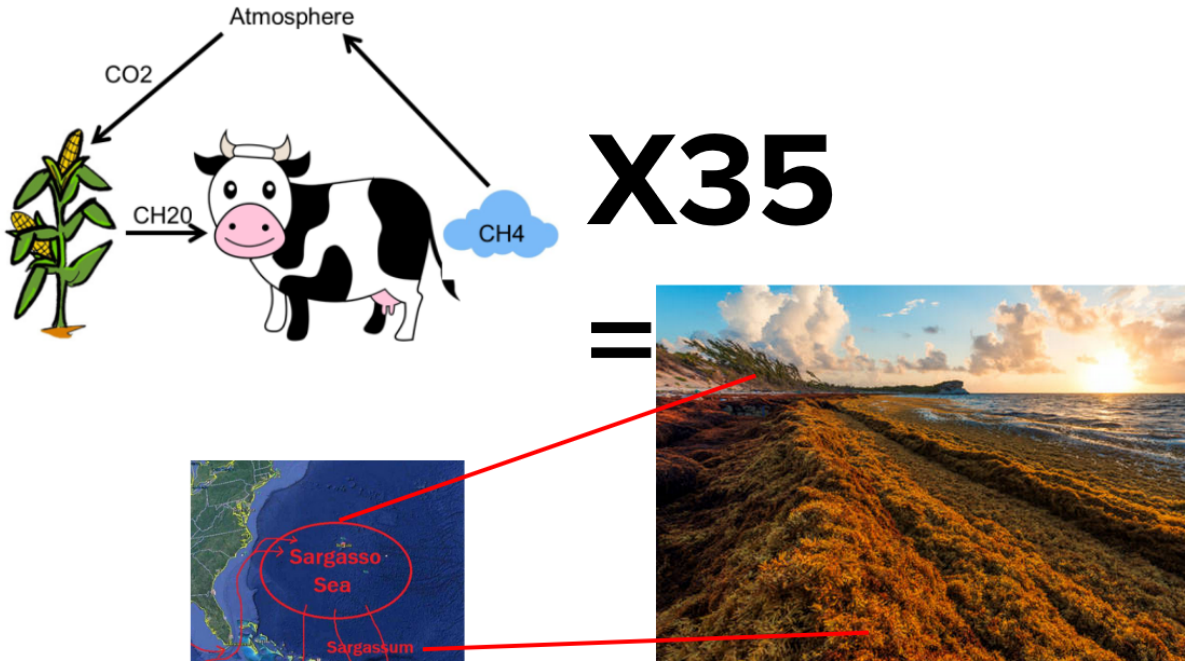

Figure 7. Based on work performed at BIOS during Summer 2019, we extrapolated the amount of methane produced on Sargassum during the length of the trial to the total amount (10 million tonnes) of Sargassum living in the Sargasso Sea (Milledge et al. 2016; Cox 2019). In total, 3.5 metric tons of methane are produced from all of the bacteria living on all of the Sargassum in the Sargasso Sea per year. This is roughly equivalent to the amount of methane produced by 35 cows.

Other Work

- Assess the growth rates of Sargassum under various nutrient conditions after ideal conditions are found for select microbes in the Sargassum microbiome. Other conditions can be created to mimic higher concentrations of MPn seen at the outlet of major river deltas or areas of ocean upwelling.

- Culture microbes relevant to Sargassum microbiome in lab and expose them to various concentrations of MPn to measure growth rates.
- Create reporter constructs for operons (Pho, pdh) responsible for Methylphosphonate cleavage and transport in ocean microbes, followed by culture and exposure to varied concentrations of MPn (low = basal-100nM; med = 1000-2500nM; high = 6000-10000nM)
    - Knock-down of genes responsible for in vitro P_i usage could ease creation of sufficient culture medium as described in (Ulrich et al. 2018)
- Find bacteria capable of organic MPn synthesis. One can imagine a microbial interaction whereby MPn is synthesized at a rate faster than it can be broken down. If this type of relationship could be maintained, it could prove to be an efficient way to deprive Sargassum of its currently hypothesized Phosphorus source through the C-P bond lysis in Methylphosphonate by bacteria on its microbiome.

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