Investigating the Effects of Methylphosphonate (or nutrients) on the Microbiome of Macroalgae and the effects of Microplastics on *Sargassum*

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Abstract

Macroalgae are the main basis of the marine food web as well serving as a habitat and nursery for numerous marine species. The microbiome of the macroalgae *S.natans* host bacteria that are able to cleave the C-P bond of Methylphosphonate (MPn), a naturally occurring compound, providing *S. natans* with a much needed phosphorus source. In this study, the microbiome of four other common marine macroalgae-*Padina vikersiae* (petticoat algae), *Caulerpa racemosa* (sea grapes), *Dasya cf. baillouviana* (red algae), and *Cladophora catenata* (green algae) were investigated to see if their microbiome also responded to MPn. The floating macroalgae, *S. natans,* is also impacted by microplastics pollution, however, not much research has been done studying the effects of microplastics on the *Sargassum* microbiome. This study investigated the microbiome of *Sargassum* and microplastics to determine if microplastics can change the microbial community of *Sargassum.* It was first determined which setting on the Waterpik™ worked best to extract the microbiome off the macroalgae. The soft setting worked best for the Petticoat and Grape algae while the pulse setting worked best for the Green and Red algae. Each macroalgae was then incubated with 1420nM of MPn over a 6 day period and it was found that the bacterial abundance increased when introduced to the MPn. Finally, the microbiome of microplastics associated with *Sargassum* was compared to healthy *Sargassum* and microplastics biofilm alone. The bacterial abundance was found to be the highest for the sample consisting of both *Sargassum* and microplastics combined than either the biofilm on plastics or the healthy *Sargassum* microbiome alone. Further bacterial lineage identification was done to see which bacterial lineage dominated the samples. It was found that the copiotroph *Alteromonas* was more associated with the microplastics biofilm while the oligotroph *Roseobacter* was more associated with the *Sargassum* macroalgae.

Introduction

Marine algae are one of the main sources of oxygen production on Earth. A study found that about 50% of all the oxygen humans breathe comes from marine algae (Chapman et al, 2013). These plants produce oxygen as a byproduct of photosynthesis, the process by which carbon dioxide and sunlight are converted into sugars that the organism can use for energy. In the ocean, photosynthetic carbon fixation by floating and benthic algae produce ~ 45 gigatons of organic carbon per year. (Falkowski et al, 1998). It is important to study the microbiome of macroalgae since they are the main basis of the marine food web (Cirri et al, 2019), can provide the macroalgae with essential nutrients (Wilkins et al. 2019) and can produce antimicrobial compounds that protect the algae from disease (Busetti et al. 2017; Longford et al. 2019).

Bermuda is the most northerly coral reef system in the Atlantic and many species of marine (benthic and floating) algae are found along the shallow reefs. (Sterrer 1992). In

Bermuda, *Sargassum natans* (gulfweed), *Padina vikersiae* (petticoat algae), *Caulerpa racemosa* (sea grapes), *Dasya cf. baillouviana* (red algae), and *Cladophora catenata* (green algae), are several types of common marine algae found frequently along the shores of the island, so it is of particular interest to study in Bermuda (Sterrer, 1992). *Sargassum* is a type of macroalgae, or seaweed, that grows in the Sargasso Sea, which is an Atlantic body of water with no land boundaries (Trott et al., 2010; Figure 1). The main species of *Sargassum* in the Sargasso Sea are *S. natans* and *S. fluitans* (Moreira and Alfonso, 2013). *Sargassum* is therefore a vital habitat for ecosystem development for macrobiota, as well as microbial epiphytes (Laffoley et al., 2011). *Padina vikersiae* is found on rocks below the low tide zone, often half-buried in silt. Recent studies have shown that the *Padina* genus are known to be antibacterial and therefore lack bacterial abundance in the microbiome, (Baliano et al, 2016). *Caulerpa racemosa* are found on rocky shores, sea walls and even sandy bottoms, but lie low where wave action is powerful. The *Caulerpa* genus are a host to a rich diversity of bacterial endo- and epibionts (Morrissey et al, 2019). *Dasya cf. bailouviana* is widely distributed in the Caribbean and is known to be euryhaline, able to tolerate a wide range of salinity (10-30 psu) and can tolerate pollution and high concentrations of hydrogen sulfide (Den Hartog, 1964). *Cladorphora catenata* is a green algal species known to be highly, morphologically variable, however no specific studies have been done concerning its microbiome.

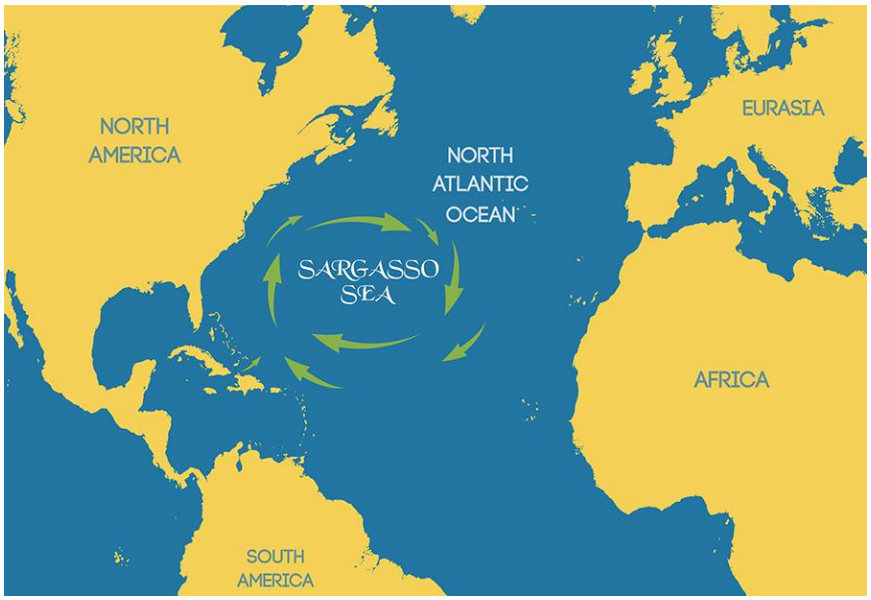

Figure 1. Sargasso Sea location (provided by Wonderopolis.org)

These macroalgae are considered to be an important part of the coastal ecosystem, as they serve as habitat and nurseries for numerous marine species (Laffoley et al., 2011). However, there have been several issues with *Sargassum* blooms occurring during the fall and winter months of the year. Over the past decade, there has been an explosion of *Sargassum* densities that scientists have linked with global climate change and the elevated surface ocean temperature and acidity, (Huffard et al. 2014). There have been questions raised as to whether the latest 2017

invasion of *Sargassum* represents a seasonal abnormality or a shift in ocean currents and climate. These invasions could have significant negative impacts on marine ecosystems, even disrupting entire economic activities of local communities, (Louime et at, 2017)

With the increase in blooms of algae such as Sarassum, it is important to study the environmental and biotic parameters that affect their abundance and distribution. *Sargassum natans* thrive best in areas where there are low concentrations of dissolved phosphorus and nitrogen. They host bacteria such as *Dichthrix*, a nitrogen-fixer, that 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 both the algae and the microbial community and an outgassing of methane to the atmosphere (Karl et al., 2008). In the nutrient deficient waters of the Sargasso Sea, nitrogen and phosphorous limit growth (Cotner et al., 1997; Lomas et al., 2010). However, Sargassum thrives here and it has been suggested that bacteria and *Sargassum* colonies have found a way to coexist in such a way that both organisms are able to thrive (Capone et al., 2008). *Roseobacter* bacteria are found in surface seawater from the Sargasso Sea (Parsons et al., 2012; Sosa et al., 2017). A 2017 study conducted on waters near Hawaii found that *Sulfitobaccter*, a bacteria closely related to *Roseobacter*, is also able to break down the C-P bond in methylphosphonate (Sosa et al., 2017). A study of *S. muticum* in Portugal revealed large abundances of *Rhodobacteraceae* (including *Roseobacter*) and *Loktanella* in September-March (Serebryakova et al., 2018). Although there have been studies looking at *Sargassum sp.* ability to cleave the C-P bond of methylphosphonate, there are no studies looking at the same process done with the other common marine algae.

Microplastics as pollutants have been a common concern in the marine environments for several years. However, not much research has been done studying the dynamics of plastic biodegradation in marine environments, (Morohoshi et al. 2018). Microplastics carry biofilm communities which microbial agents are generally concentrated within, (Parrish et al, 2019). Biofilms are composed of a diverse microorganisms that attach and live on surfaces and are embedded in an extracellular polymeric substance (EPS), (Lage and Graca, 2016). A few studies have shown that plastic biofilms are very diverse and are driven by a complex network of influences such as polymer type, texture, and size of the substratum. One study found that microbial biofilms tend to develop rapidly on plastic, host rapid bacterial colonization and coincide with significant changes in the physicochemical properties of plastic, (Lobelle et al, 2011). Microplastics pollution has been a common problem in Bermuda for the past 50 years, where a study done in 1970 showed that 3,500 pieces of plastics per square kilometer were observed along the island (Damon, 2019). It is found that in the ocean, degrading plastic becomes even more poisonous as it attracts and it can bind with other man-made chemical pollutants. Robbie Smith, a marine ecologist, stated that “there is no quick fix, and that it takes a decade or two for plastic to make its way into the watershed,” (Damon, 2019). With microplastic pollution being a common problem in Bermuda, it would be interesting to see if there is any effect on macroalgae on a microbial level.

In this study, the microbiome of each marine algae was examined. It was hypothesized that the bacterial abundance would increase with additions of methylphosphonate. The changes in the microbiome after incubations with methylphosphonate were also investigated in order to determine if lineages involved in methylphosphonate cleavage became more abundant with increasing methylphosphonate additions. It was hypothesized that the bacterial community would change with methylphosphonate additions. Finally, focusing just on the floating algae, *Sargassum*, it was determined what plastics were associated with *Sargassum* and if plastics found in the oceans could change the microbiome of this floating algae. It was hypothesized that microplastics found in the ocean would significantly change the microbiome of the floating algae, *Sargassum.*

Methods

Method Development

Five algal species were sampled from the dock and offshore. They were immediately placed in seawater from the sample site and back in the laboratory and subdivided into 2g portions. The microbiome was extracted using the Waterpik™ method. Waterpik™ were cleaned with isopropanol (70%), sterile water and sterile seawater prior to use. The Waterpik™ method needed to be adjusted for each of the algae species since the normal setting broke apart several of the algal species. Each algae was tested using each of the three settings-Normal, Soft, and Pulse. The microbial abundance was measured in triplicate for each of the settings and the highest abundance with the smallest standard deviation was chosen to process the frozen samples (Figure 2).

Methylphosphonate Additions

Four algal species (*Sargassum natans* (gulfweed), *Padina vikersiae* (petticoat algae), *Caulerpa racemosa* (sea grapes), *Dasya cf. baillouviana* (red algae), and *Cladophora catenata* (green algae) were sampled from the BIOS dock (32.370213, -64.695513) in August, 2019. Each 2g algal portion was sampled in duplicate for the microbiome at the start of the experiment using a Waterpik™ and 40mLs of sterile seawater into 50mL tubes. Two more 2g algal portions were then incubated in 200mL of sterile seawater for 6 days (Cox, 2019). An additional 2g algal portions were incubated in 200mL of sterile seawater with 1420nM MPn added for 6 days. After 6 days, the microbiome was sampled using a Waterpik™ and 40mLs of sterile seawater into 50mL tubes.

Biofilm of Microplastics

S.natans and *S.fluitans* was sampled from inshore location (Warwick Beach, Bermuda) and offshore location (32° 21.135, 64° 34.842) with and without microplastics associated. The samples were immediately placed in seawater from the sample site and back in the laboratory, they were subdivided into 2g portions. The microbiome and biofilm were extracted in triplicate

using the Waterpik™ method as discussed above using the normal setting and 40mLs of sterile seawater. The samples were divided into the following groups:

- Plastic biofilm
- *S. natans* microbiome with no plastics
- *S. natans* microbiome with plastics
- *S. natans* microbiome with plastics removed
- Plastic biofilm from plastics associated with *S. natans*

DAPI Staining

Samples for cell counts from the macroalgae were taken at the beginning of each trial and from all treatments at the end of each trial, fixed with formalin to a final concentration of 10%, and stored at -80°C. Samples were thawed and 1-2mL were filtered onto Irgalan Black stained 25 mm, 0.2 μm polycarbonate filters (Nucleopore, Whatman) under gentle vacuum (100 mm Hg) and stained with 1 mL of 6, 6-diamidino-2-phenyl dihydrochloride (5 μg mL -1 , DAPI, SIGMA-Aldrich, St. Louis, MO) (Porter and Feig 1980). The filters were mounted onto slides with Resolve immersion oil (high viscosity) (Resolve, Richard-Allan Scientific, Kalamazoo, MI) and stored at -20°C. Slides were then enumerated using an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) under ultraviolet excitation at 100x magnification.

FISH Method

Fluorescent in situ hybridization (FISH) was performed on specific bacterial lineages (Parsons et al. 2015). Bacterial abundance samples 2-3mL were filtered through a 0.2 μm polycarbonate filter under gentle vacuum conditions (~100 mmHg) and stored at -20°C with desiccant. Filters were cut into eighths and washed in 95 % ethanol. Hybridization, wash conditions, and probe sequences using FISH methods are described in Table 1.

Table 1. FISH bacteria probe sequences and wash buffer temperatures for *Alteromonas, Flavobacteriales, Roseobacter*, and *Vibrio*

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
Rhodobacteraceae 536R-Cy3	5'- CAACGCTAACCCCCTCCG-3'	SET 1	52

Vibrio VibSpl-127-Cy3	5'-CCCCACATCAAGGCAATTTC-3'	SET 1	45

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)

Results

Method Development Results

Each algae was different with some more fragile than others. Initial testing using the normal setting on the Waterpik™ resulted in some algal species being destroyed (Grape and Red). For example, the soft setting improved the microbiome abundance by 1.01×10^8 cells/L with only 2.03% loss of weight with the Grape algae (Figure 2). One Way Analysis of Variance (ANOVA) tests were done to test significant differences of the Waterpik settings on the macroalgae: Grape, Green, and Red algae. For the Petticoat algae, a Kruskal-Wallis One way Analysis of Variance on Ranks test was used to test for significant difference for each Waterpik setting. This was done instead of an ANOVA test because it failed the Equal Variance Test($P<0.050$). There were no significant differences between each of the settings on each of the macroalgae. (P.grape=0.193, ANOVA, Single Factor, Figure 2); (P.green=0.801, ANOVA, Single Factor, Figure 2); (P.red=0.666, ANOVA, Single Factor, Figure 2);(P. petticoat=0.537, Kruskal-Wallis, Single Factor, Figure 2)

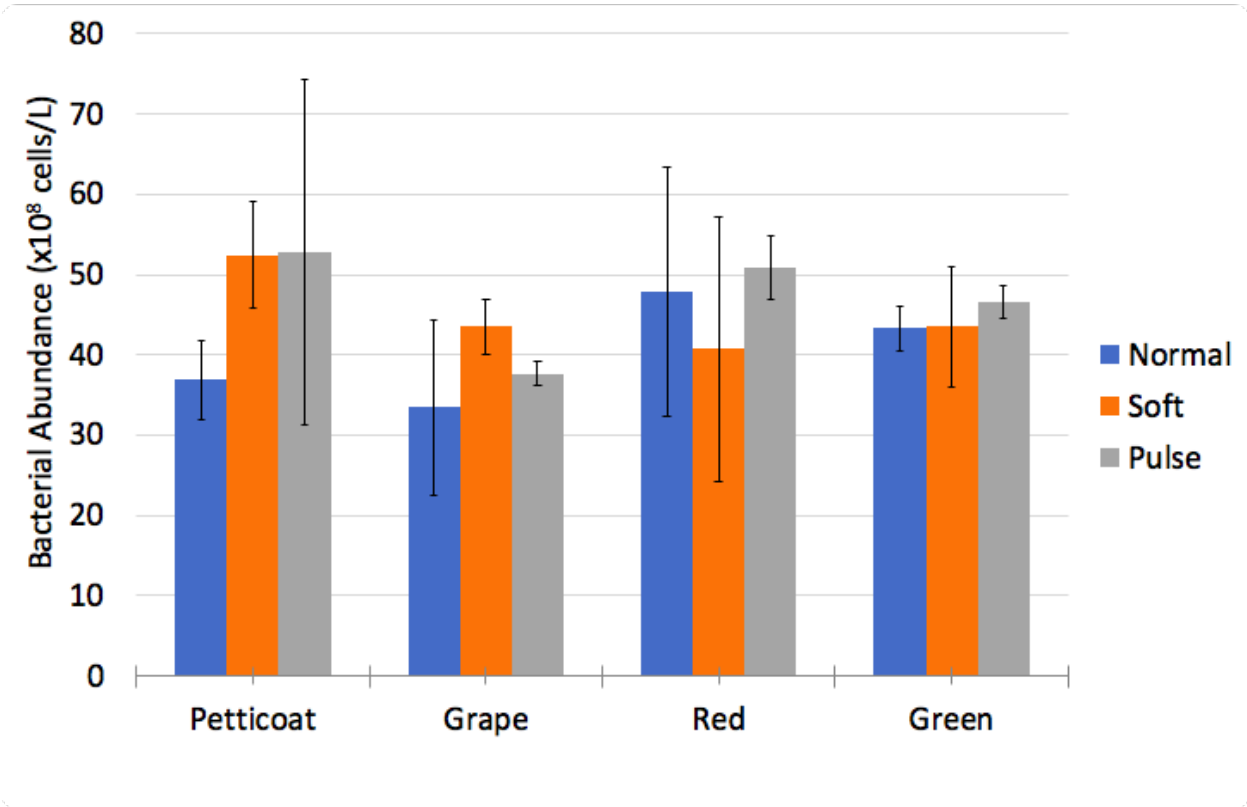

Figure 2. Average bacteria in cells/L (±SD) extracted off the microbiome of each macroalgae compared between each setting-Normal, Soft, and Pulse.

For the benthic algae, the microbiome abundance was similar with Red having the highest abundance at 5.07±0.32 x10^9 cells/L and Green having the lowest at 4.13±0.63 x10^9 cells/L (Figure 3). The floating algae, *S. natans* had the overall lowest microbiome abundance at 3.43±0.59 x10^9 cells/L. A Kruskal-Wallis One Way Analysis of Variance on Ranks Test was attempted to test the significant differences in bacterial abundances of each macroalgae. The test showed that there was a significance (P=0.0281, Single Factor ANOVA, Figure 3). A Dunn's Post Hoc test was performed to show where the significance was. There were significant differences between the following macroalgae: Petticoat vs *S.natans* (P=0.03138, Dunn's Post-Hoc, Table 2); Grape vs Red (P=0.0364, Dunn's Post-Hoc, Table 2); Red vs Green(P=0.03138, Dunn's Post-Hoc, Table 2);and Red vs *S.natans* (P=0.002797, Dunn's Post Hoc, Table 2).

Table 2. Summary of Dunn's Post Hoc Test performed on each of the five macroalgae. P values less than 0.05 were determined significant

	Petticoat	**Grape**	**Red**	**Green**	***S.natans***
Petticoat		0.2093	0.4026	0.1884	0.03138
Grape	0.2093		0.0364	0.9523	0.3698
Red	0.4026	0.0364		0.03138	0.002797
Green	0.1884	0.9523	0.03138		0.4026
S.natans	0.03138	0.3698	0.002797	0.4026	

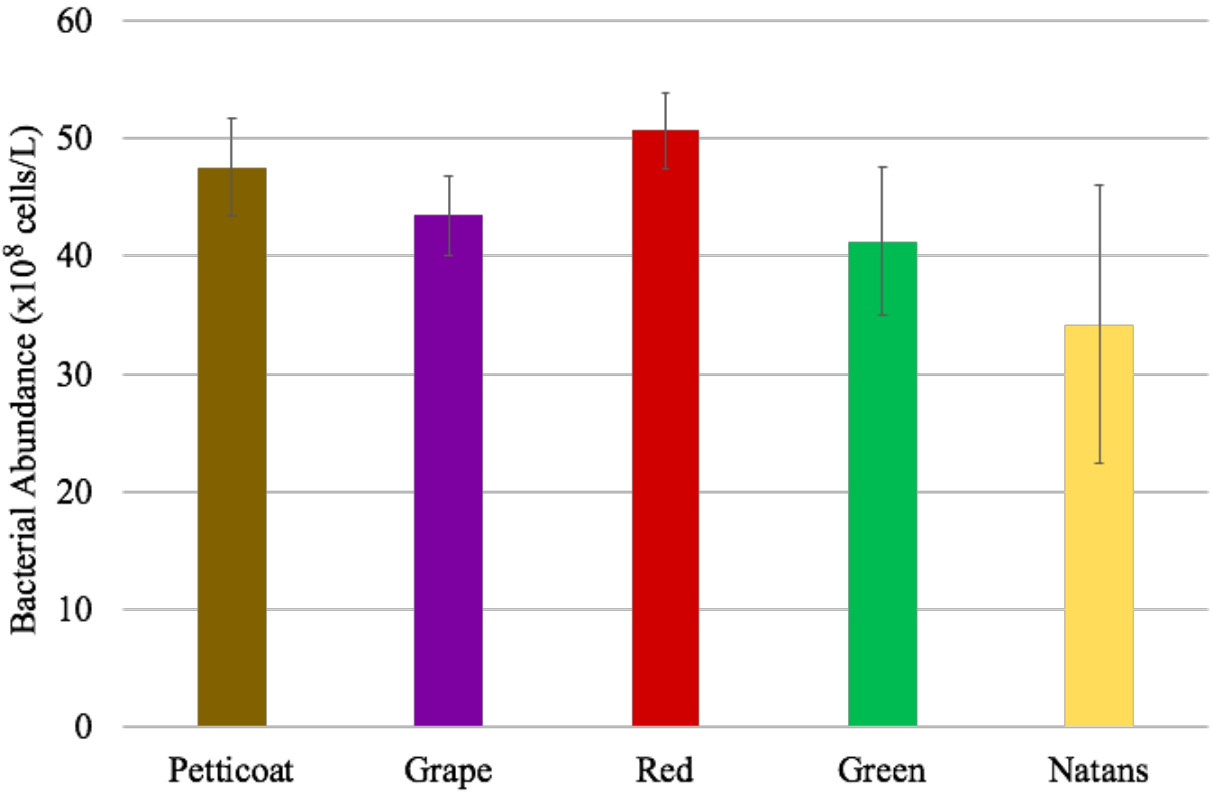

Figure 3. Average bacteria in cells per mL (±SD) of the five common algae in Bermuda (n=4)

Algal Microbiome

Methylphosphonate Additions

One methylphosphonate experiment that tested different algal species was conducted in August, 2019. The five macroalgae were divided into 2g samples at the start of the experiment and the microbiome was extracted using the Waterpik™ method. Two more 2g algal portions were then incubated in 200mL of sterile seawater for 5 days. An additional 2g algal portions were incubated in 200mL of sterile seawater with 1420 nM MPn added for 6 days. The aim of the study was to examine if the bacterial abundances found in the microbiome of each macroalgae increased with the addition of methylphosphonate. Based on the data, it was observed that the bacterial abundance increased with the methylphosphonate additions (Figure 4; Figure S1).

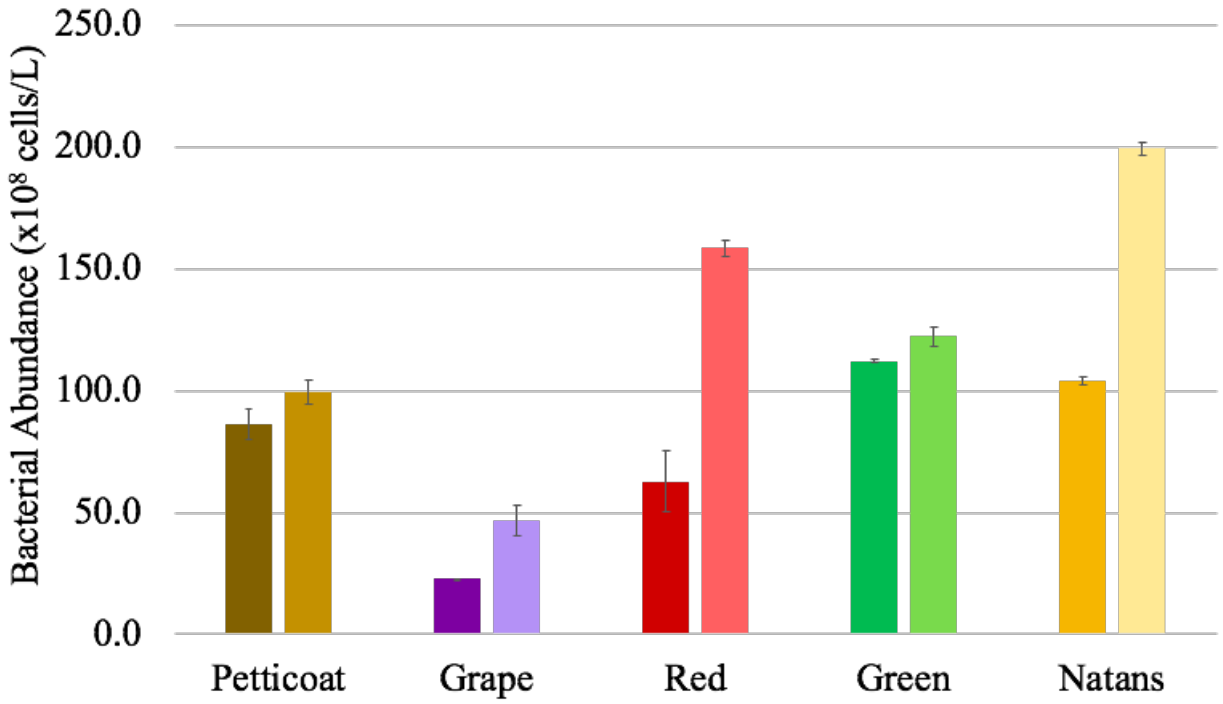

Figure 4. The change in bacteria indicated as means of bacterial cells per mL (±SD) observed at T_f with no MPn additions, and T_f with 1420 nM of MPn. The T_0 values have been subtracted from the T_f values for the duplicate samples. (The darker shade represents the control while the lighter shade represents the MPn additions)

Knowing that there was an increase of bacteria with the additions of methylphosphonate, an ANOVA test was attempted to test if there were any significant differences in bacterial abundances amongst each of the species. However, the test was unable to run because it failed the Equal Variance Test. Because of this, a Kruskal-Wallis One Way ANOVA test was done to test the significant differences between bacterial abundances on each of the macroalgae. It was

found that there were no significant differences between each of the macroalgae (P=0.078, Kruskal-Wallis, Single Factor, Figure 4). There is no significance at the 5% (P=0.05) confidence interval, but there was a significant trend at the 10% (P<0.1). T-tests were used to show significant differences between T_0 and T_f with MPn additions of each macroalgae. Petticoat algae (t=0.144), Green algae (t=0.073), and Grape algae (t=0.032) had no significant difference in bacterial abundance between T_0 and T_f. Red algae (t=0.009) and *S.natans* (t=0.001) had a significant increase in bacterial abundance between T_0 and T_f. Figure 5 shows the actual bacterial difference in bacterial counts between the control and methylphosphonate additions. The red Algae had the highest increase with a bacterial abundance difference of 9.6±1.30 x10^9 cells/L and *S.natans* with a close second of 9.5±0.31 x10^9 cells/L.

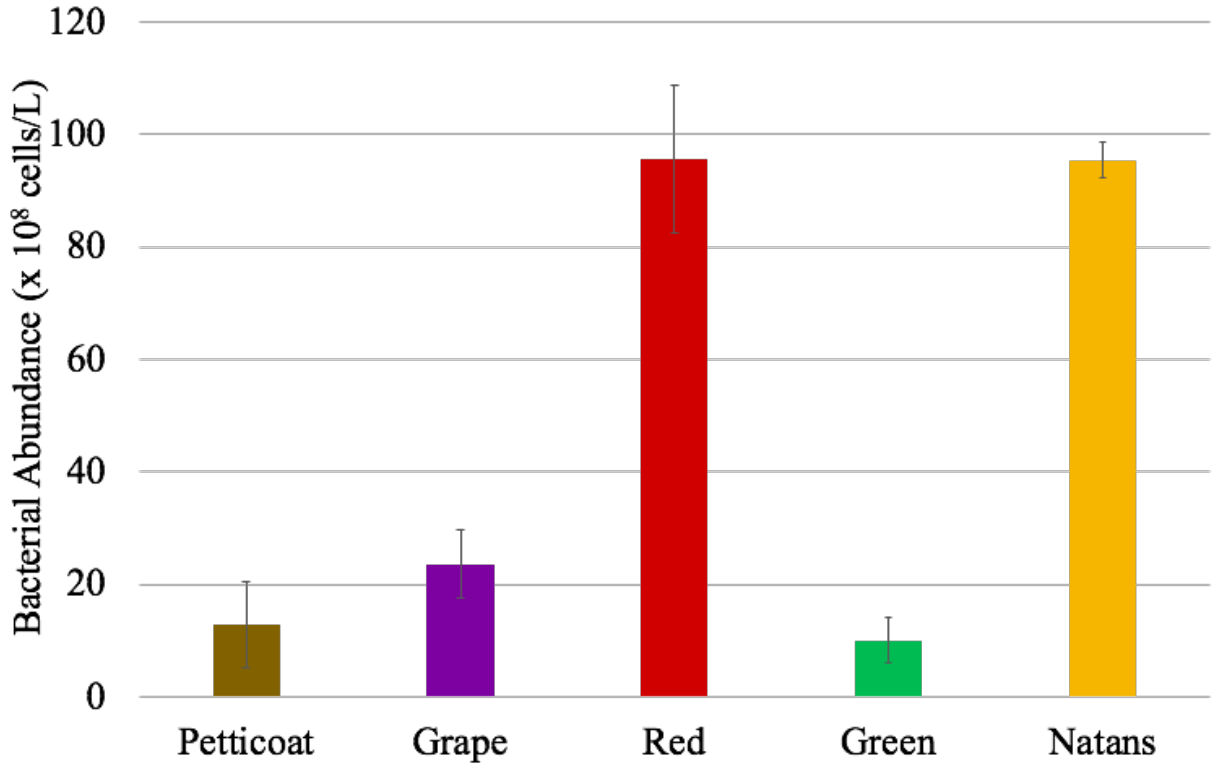

Figure 5. Difference in mean bacterial cells per liter (±SD) from T_f with 1.42mL of MPn and T_f control

Microplastics effect on *Sargassum*

Bacterial abundance on Plastics Biofilm

Sargassum and microplastics samples collected from Warwick Beach, Bermuda were separated into five separate 2g samples:

- **MP (Microplastics)**-Plastic biofilm
- **HS(Healthy *Sargassum*)**-*S. natans* microbiome with no plastics
- **SP(*Sargassum*+Plastics)**-*S. natans* microbiome with plastics

- **SPP(*Sargassum*-Plastics)-***S. natans* microbiome with plastics removed
- **EMP(Extracted Microplastics)**-Plastic biofilm from plastics associated with *S. natans*

The microbiome of each sample was extracted using the Waterpik™ method on the Normal setting.

The microbiome of the *Sargassum* with plastics had the highest abundance of 19.46±1.77 $x10^9$ cells/L (Figure 6). This abundance was 11.43±1.81 $x10^9$ cells/L more than the healthy *Sargassum*. An ANOVA test was used to test significant differences in bacterial abundances between each sample. The average bacteria between each of the samples taken from Warwick Beach were significantly different (P.value= 0.001, ANOVA Single Factor, Figure 6). A pairwise multiple comparison (Tukey Test) was used to compare which samples showed significant differences. Healthy *Sargassum* and Plastics Removed (A) were found to be significantly different from (*Sargassum*-Plastics) and (*Sargassum*+Plastics) (B) and Microplastics (C). Derived variables were created to see if combined variables equaled the combined samples:

1. Healthy *Sargassum* + Micropastics = *Sargassum* + Plastics
2. (*Sargassum* - Plastics) + Plastics Removed = *Sargassum* + Plastics

The combined variable of healthy *Sargassum* + microplastics was lower than the sampled *Sargassum* + plastics and the combined variable of *Sargassum* with plastics removed with the plastics added back in was higher than the sampled *Sargassum*+ plastics.

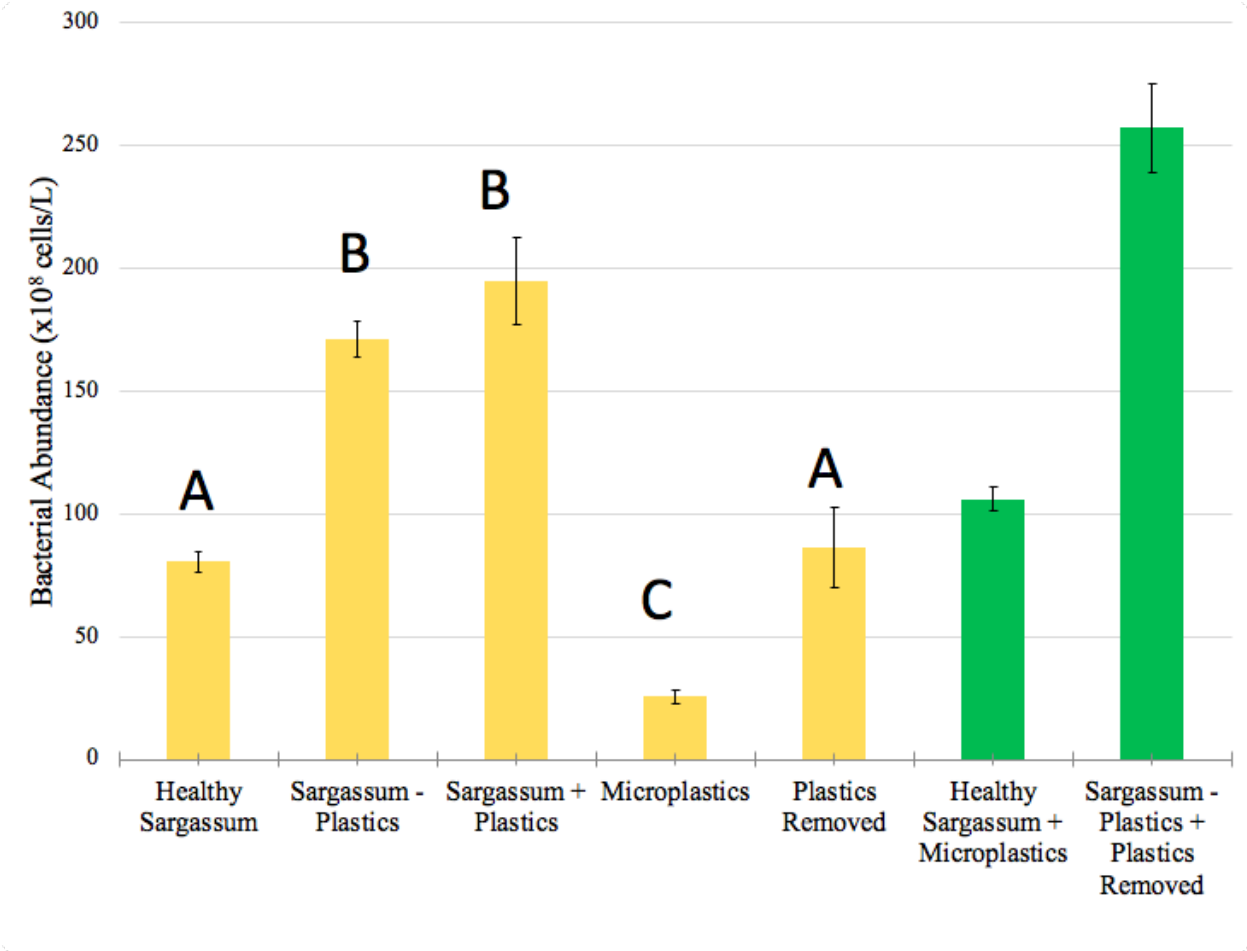

Figure 6: Mean bacteria in cells per mL (±SD) of *Sargassum* associated and not associated with microplastics from Warwick Beach, Bermuda. Green bars represent derived bacterial abundances

The same procedure was done when looking at samples of *Sargassum* associated with microplastics from an offshore location. The samples were separated into 3 separate 2g samples:

- **FP (*Fluitans*+Plastic)**-*S.fluitans* with plastics
- **NP (*Natans* +Plastic)**-*S.natans* with plastics
- **P(Plastic)**-plastic biofilm

The microbiome of the *S.fluitans* with plastics had the highest abundance of 54.58± 8.83 x10^9 cells/L (Figure 7). An ANOVA test was used to test significant differences in bacterial abundances between each sample. The average bacteria between each of the samples taken from the offshore location were significantly different (P.value= 0.004, ANOVA Single Factor, Figure 7). A pairwise multiple comparison (Tukey Test) was used to compare which samples showed significant differences. *Fluitans* +Plastics and *Natans* +Plastics (A) was found to be significantly different from the Plastic Biofilm (B)

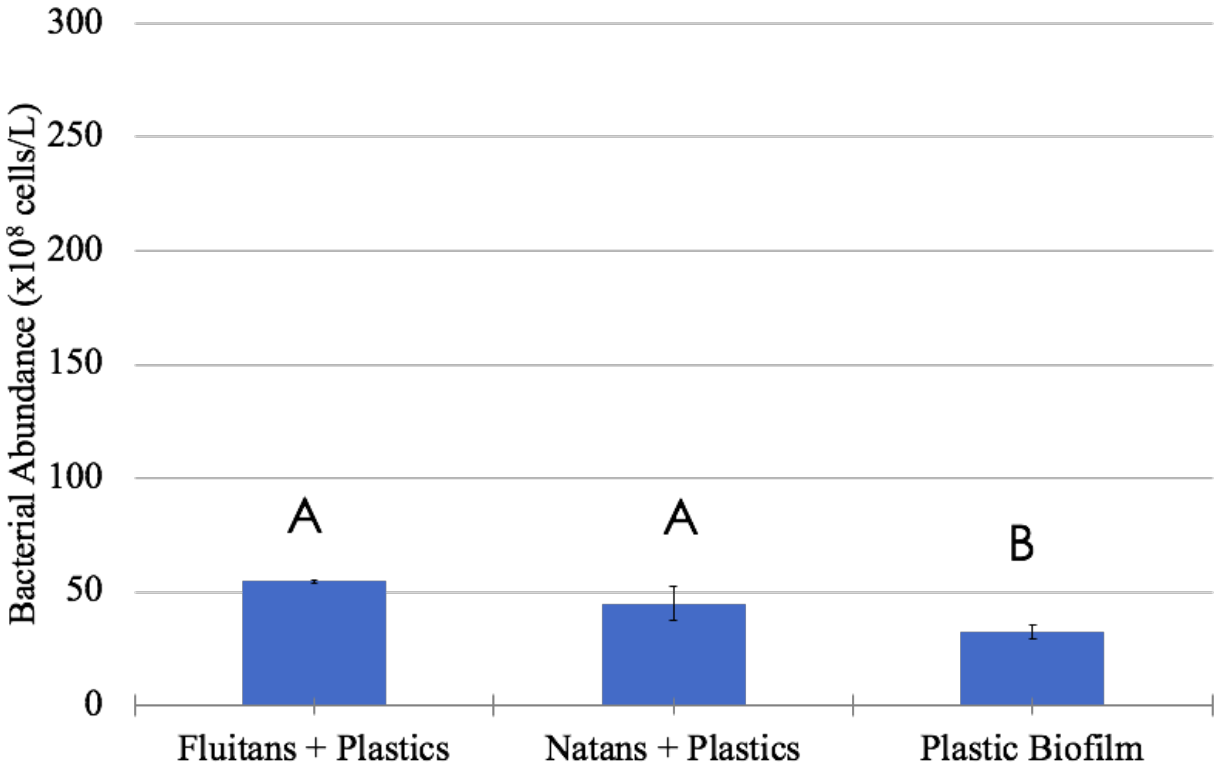

Figure 7: Average bacteria in cells per mL (±SD) of *S.natans and S.fluitans* associated/not associated with microplastics from an offshore location in Bermuda (32° 21.135, 64° 34.842)

Bacterial Lineages on *Sargassum* and plastic

The key bacterial lineages in the microbial community (*Alteromonas, Flavobacteriales, Roseobacter, and Vibrio)* were probed for both inshore and offshore samples (Figure 8). All four lineages were found in the samples. *Alteromonas* was the most abundant bacterial lineage associated with samples that included plastics, specifically *Sargassum* with plastics associated (Figure 7). *Roseobacter* was more abundant in the *Sargassum* samples. Refer to Appendix B (Figure S2) and C (Figure S3) for more figures regarding the bacterial lineages on *Sargassum* and microplastics.

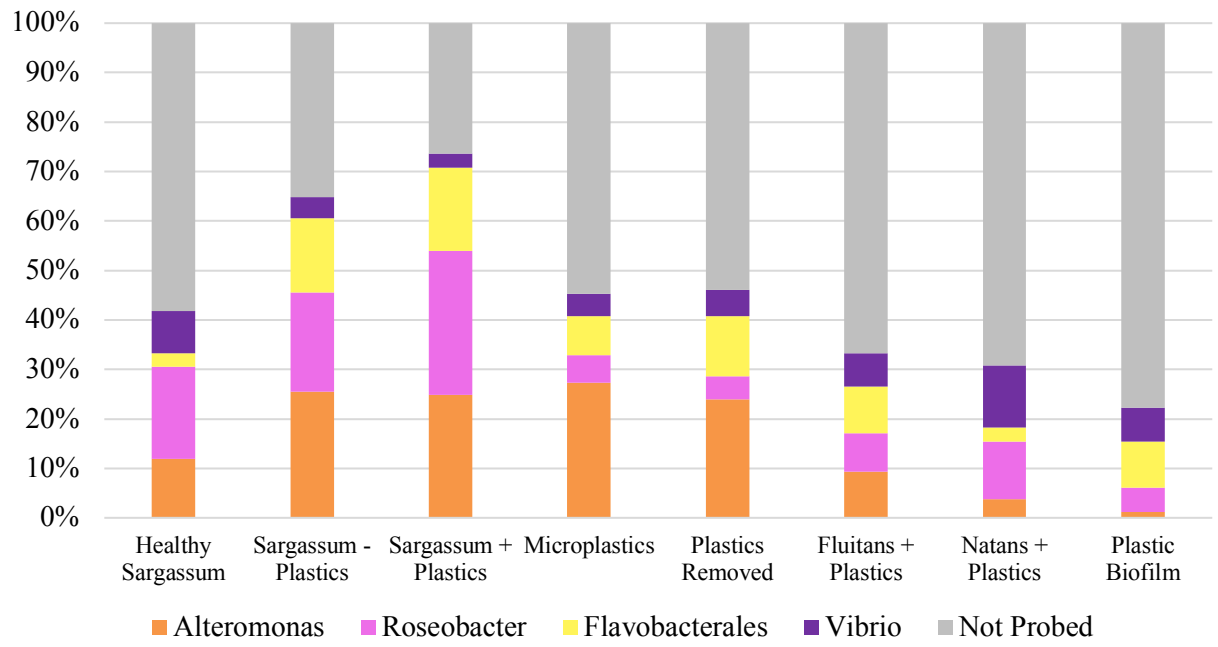

Figure 8. Percentage bacterial abundance to the total bacteria of each bacterial lineage (*Alteromonas, Roseobacter, Flavobacteriales, and Vibrio)* for each sample using FISH protocol

Discussion

Marine algae are important contributors to photosynthesis; providing about 50% of the oxygen consumed in Earth (Chapman et al, 2013). The symbioses between marine algae and their microbiome extends from the organismal level to the ecosystem level (Wilkins et al. 2019). Despite recent research on host-associated microbes, little is known about their interactions with the majority of marine host species (Wilkins et al. 2019). The algal microbiome is a crucial part of the alga holobiont and has a key role in regulating algal populations in nature (Lian et al. 2018). The algal microbiome can promote algal growth by providing essential nutrients (Wilkins et al. 2019). Recent studies have shown that mutualistic algal–bacterial interactions are prevalent in marine systems (Seymour et al. 2017).

<u>Method Development Results</u>

The purpose of performing this method was to see which Waterpik™ setting (Normal, Soft, or Pulse) extracted the microbiome off the macroalgae effectively without destroying the actual macroalgae itself. There were no significant differences between each of the settings (Normal, Soft, and Pulse) on each of the macroalgae. However, it is assumed that significance may have existed if a consistent number of samples were processed. It was difficult to find significant differences between each of the samples because some of them were processed either in duplicate, triplicate, and/or quadruplicate. Based off observations on weight differences before and after blasting, it was decided that the soft setting worked best for the Petticoat and Grape algae while the pulse setting worked best for the Green and Red algae. Based off of

previous experiments, the normal setting deemed effective for *Sargassum natans* (Skinner et al, 2019). Once the most effective setting for extracting the microbiome off each macroalgae was found, it was then used for further experimentation with MPn additions

Methylphosphonate Additions

The algal microbiome host bacteria that can cleave the C-P bond of methylphosphonate, a compound that is abundant in the water column, providing a source of phosphorous to the algae and the algal microbiome and an outgassing of methane to the atmosphere (Karl et al., 2008). The red algae had the highest bacterial increase in the methylphosphonate addition with an increase of bacteria of $9.6\pm1.30 \times 10^9$ while *S. natans* had the second highest increase with $9.5\pm0.31 \times 10^9$ (Figure 5). This result was to be expected with *S.natans* because this is what was found in previous experiments (Cox, 2019). However, this is the first time seeing an increase in bacterial abundance with the Red algae with the additions of methylphosphonate. Based off the data, it was found that there were no significant differences between each of the macroalgae ($P=0.078$, Kruskal-Wallis, Single Factor, Figure 4). However, even though there was no significance at the 5% ($P=0.05$) confidence interval, but there was still a significant trend at the 10% ($P<0.1$). Each of the samples were processed in duplicate. It is assumed that there would be more of a significance had the samples been processed at least in triplicate. T-tests performed on each macroalgae proved that each macroalgae had a significantly higher bacterial abundance when 1420 nM of methylphosphonate was added (Figure 4). Previous experiments looked at the methane production produced by each of the macroalgae and found that red algae also produced the most amount of methane compared to the others (Cox, 2019; Figure 9). Currently, it is unknown whether or not there is a correlation with bacterial abundance and methane production. However, theoretically, it could be possible that the reason for that high production of methane is due to the high abundance of bacteria cleaving the C-P bond on methylphosphonate present in the water. Further bacterial lineage identification needs to be done in order to prove that there are bacteria present able to cleave the bond.

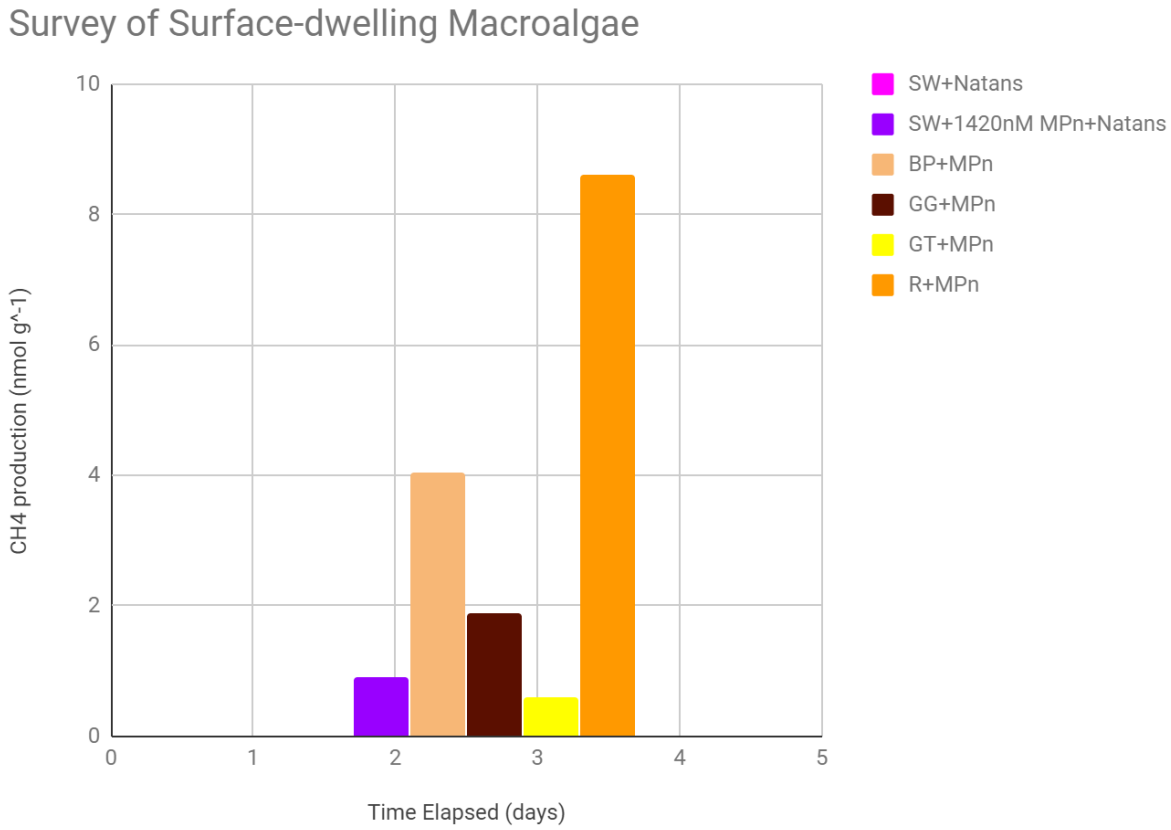

Figure 9. Methane production (nmol g^{-1}) done by each macroalgae with additions of methylphosphonate (Cox, 2019)

Microplastics effect on *Sargassum*

Bacterial abundance on Plastics Biofilm

Microplastics pollution has been a common problem along the coast of Bermuda as well as in offshore locations such as the Sargasso Sea (Damon, 2019). The Sargasso Sea is an Atlantic body of water with no land boundaries (Trott et al., 2010; Figure 1) but instead is surrounded by currents that contain the *Sargassum* as well as any marine microplastics floating on the surface (Hemphill, 2019). These microplastics can carry biofilm communities that include microbes (Parrish et al, 2019). This study looked at the microbiome of microplastics associated with *Sargassum* and compared it to healthy *Sargassum* as well as the microplastics biofilm in order to discover whether the microplastics biofilm impacted the *Sargassum* microbiome. The bacterial abundance was found to be the highest for the sample consisting of both *Sargassum* and microplastics combined than either the biofilm on plastics or the healthy *Sargassum* microbiome alone (Figure 6). This result is to be expected since the microbiome of the sample is the combination of *Sargassum* and plastics, resulting in more bacteria to be extracted. Derived variables, (Healthy *Sargassum* + Microplastics) and ((*Sargassum* -Plastics) +(Plastics removed)), were created to see if these values would potentially equal the same value of (Sargassum +

Plastics). Based off the results, the Healthy *Sargassum* and untouched microplastics combined had less bacteria than the microbiome of *Sargassum* with plastics while when the *Sargassum* with the plastics removed plus the biofilm of the removed plastics combined had higher bacteria that the microbiome of *Sargassum* with plastics (Figure 6). This suggests that a cross-colonization event had occurred where bacteria from the Sargassum moved over to the microplastics and vice versa.

Bacterial Lineages on *Sargassum* and plastic

Further research was done to see which bacterial lineages colonized the microplastics The bacterial lineage, *Alteromonas,* seemed to be the most abundant bacteria present in the microbiome of each sample and *Flavobacteriales* being the second most abundant (Figure 7). *Alteromonas* was found to be more associated with plastics while *Roseobacter* was more associated with *Sargassum*. Studies have shown that proteobacteria including *Alteromonas* are found in the initial stages of marine biofilm formation on microplastics (Lee et al. 2008) while *Roseobacter* has been found associated with phytoplankton and macroalgae (Luo & Moran, 2014). Bacterioplankton trophic strategies are often divided into broad categories defined as either copiotrophs or oligotrophs (Polz et al, 2006; Lauro et al, 2009). Copiotrophs are typically r-strategists that respond rapidly to the input of a limiting nutrient. Studies have shown that *Alteromonas* are well-known copiotrophs that grow rapidly and in high numbers when organic substrates become available, such as freshly produced DOC associated with phytoplankton blooms (Romera-Castillo et al. 2011; Wear et al. 2015). This may be a potential reason for why *Alteromonas* was found to be the most abundant bacteria in the microbiome of each sample. In contrast, oligotrophs are usually characterized as K-strategists that maintain slow, yet continuous growth and are seemingly better adapted to survival in nutrient-limiting environments such as the Sargasso Sea (Klappenbach et al. 2000; Yooseph et al. 2010; Gifford et al, 2013). *Roseobacter* are able to adapt well when nutrients are limited in the marine environment (Lauro et al, 2009; Simon et al, 2017). Other studies of macroalgae have found their microbiome to include members of the *Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes,* and *Cyanobacteria* (Egan et al, 2012). These classes of bacteria include all four of the lineages investigated in this study.

DNA sequencing can be used to determine if any other microbial lineages change in the *Sargassum* microbiome with the addition of plastics. The grey areas indicate a high percentage of different bacterial lineages that were not identified in this study (Figure 8). Thus, there are other possible lineages that might be more associated with the microplastics biofilm than in the *Sargassum* microbiome. Further research needs to be done to identify these lineages.

Bacterial abundance Inshore vs Offshore

This study also looked at the differences in the microbiome between inshore and offshore locations. Based off the data from samples found at Warwick Beach, Bermuda; the *Sargassum* with plastics had a significantly higher bacterial abundance than healthy *Sargassum*. While

microplastics alone had a significantly low bacterial abundance, the microplastics extracted from *Sargassum* seemed to have had a higher abundance, possibly meaning that some of the bacteria found in the *Sargassum* colonized the biofilm of the microplastics (Figure 5). When looking at the data from the offshore location, both types of *Sargassum* had a significantly higher abundance of bacteria (*S.fluitans* with more) than the extracted plastic. However, the extracted plastic still had a decent amount of bacteria within it's biofilm, possibly meaning that some of the bacteria found in the *Sargassum* colonized the biofilm of the microplastics (Figure 6).

Both inshore and offshore samples show that *Sargassum* alone has more bacteria consisted within the microbiome than the microplastics (Figure 6, Figure 7). UV radiation is known to inactivate microorganisms by forming pyrimidine dimers in RNA and DNA, which can interfere with transcription and replication, and therefore, reduce bacterial counts (Goosen and Moolenaar, 2008; Cutler and Zimmerman, 2011). As the *Sargassum* and microplastic samples were washed up on the beach, they were found under piles of sand, protecting the microbiome from direct UV contact. However, the offshore sample was found floating on the surface of the ocean, exposing their microbiomes to UV radiation, therefore decreasing the bacterial abundance on the offshore samples.

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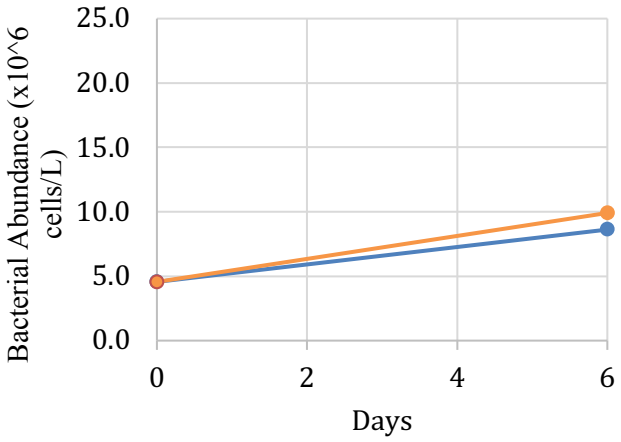
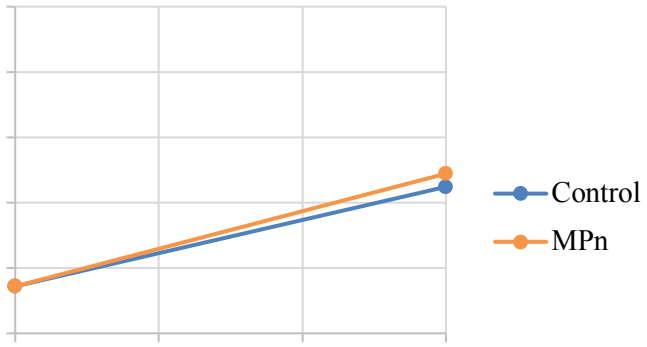
Supplemental

Appendix A.

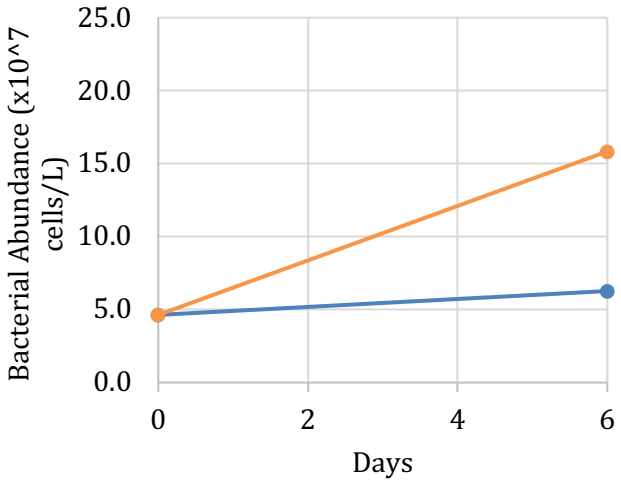
A.) Brown Petticoat Algae



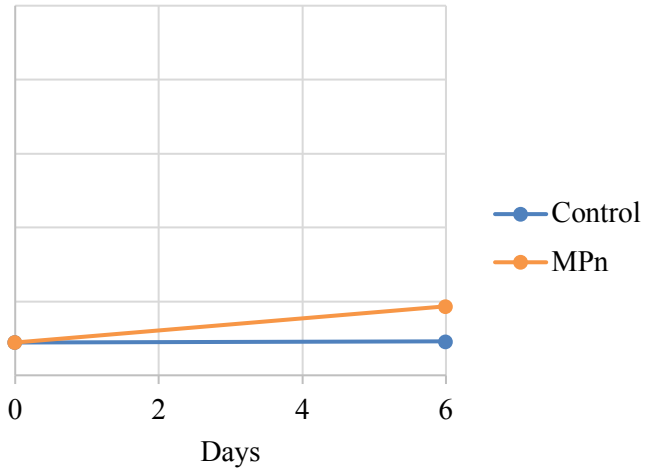
B.) Green Algae



C.) Red Algae



D.) Grape Algae



E.) Sargassum natans

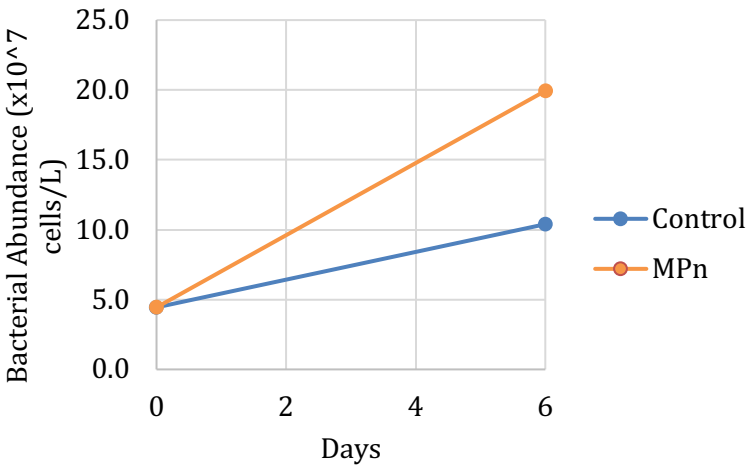


Figure S1. Mean bacteria in cells per mL observed at T_0, T_f with no MPn additions, and T_f with 1 of MPn over a six day incubation period

Appendix B.

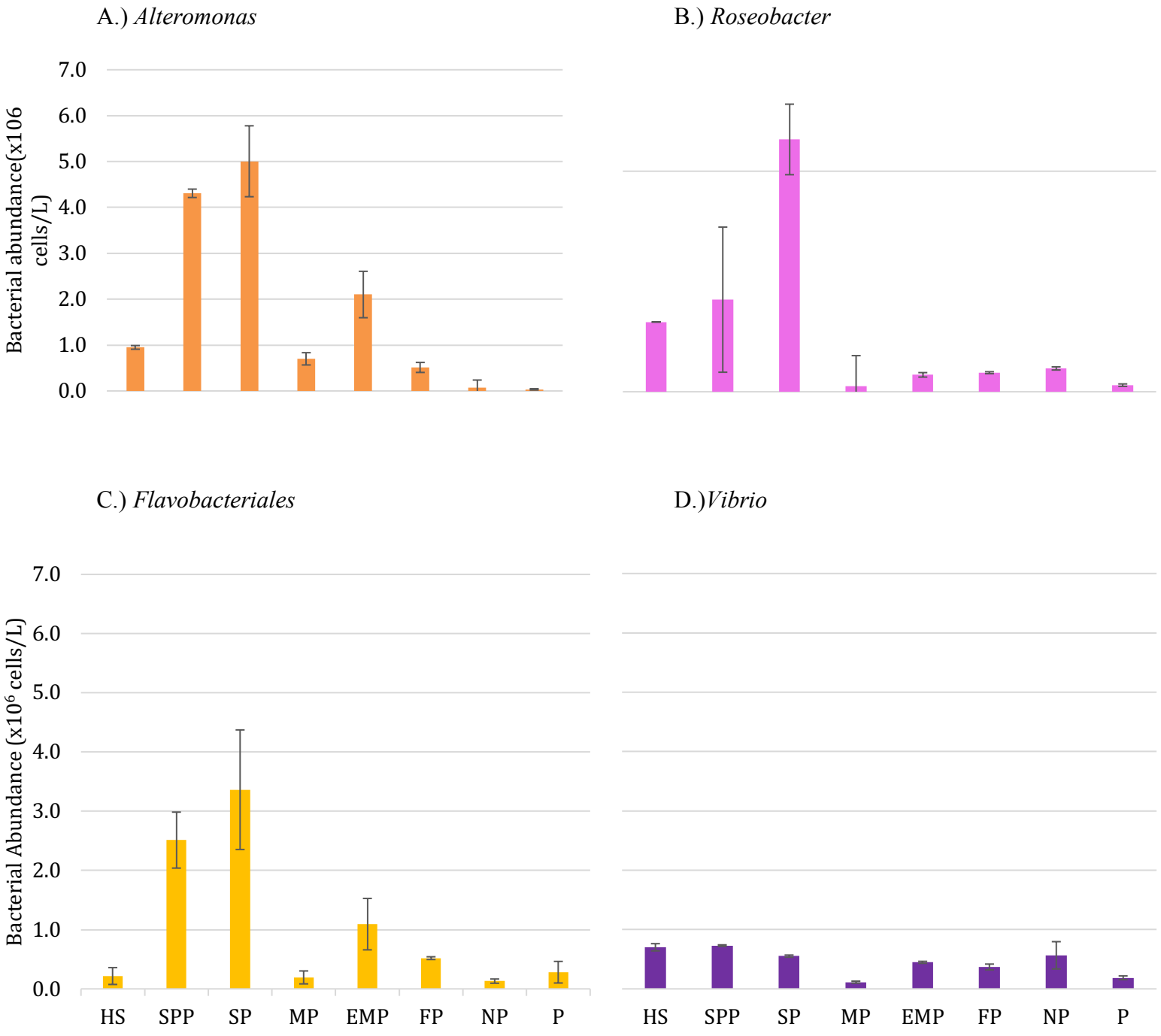


Figure S2. Mean bacterial abundance (±SD) of each bacterial lineage (*Alteromonas, Roseobacter, Flavobacteriales, and Vibrio)* using FISH protocol (see results section for abbreviation meaning)

Appendix C

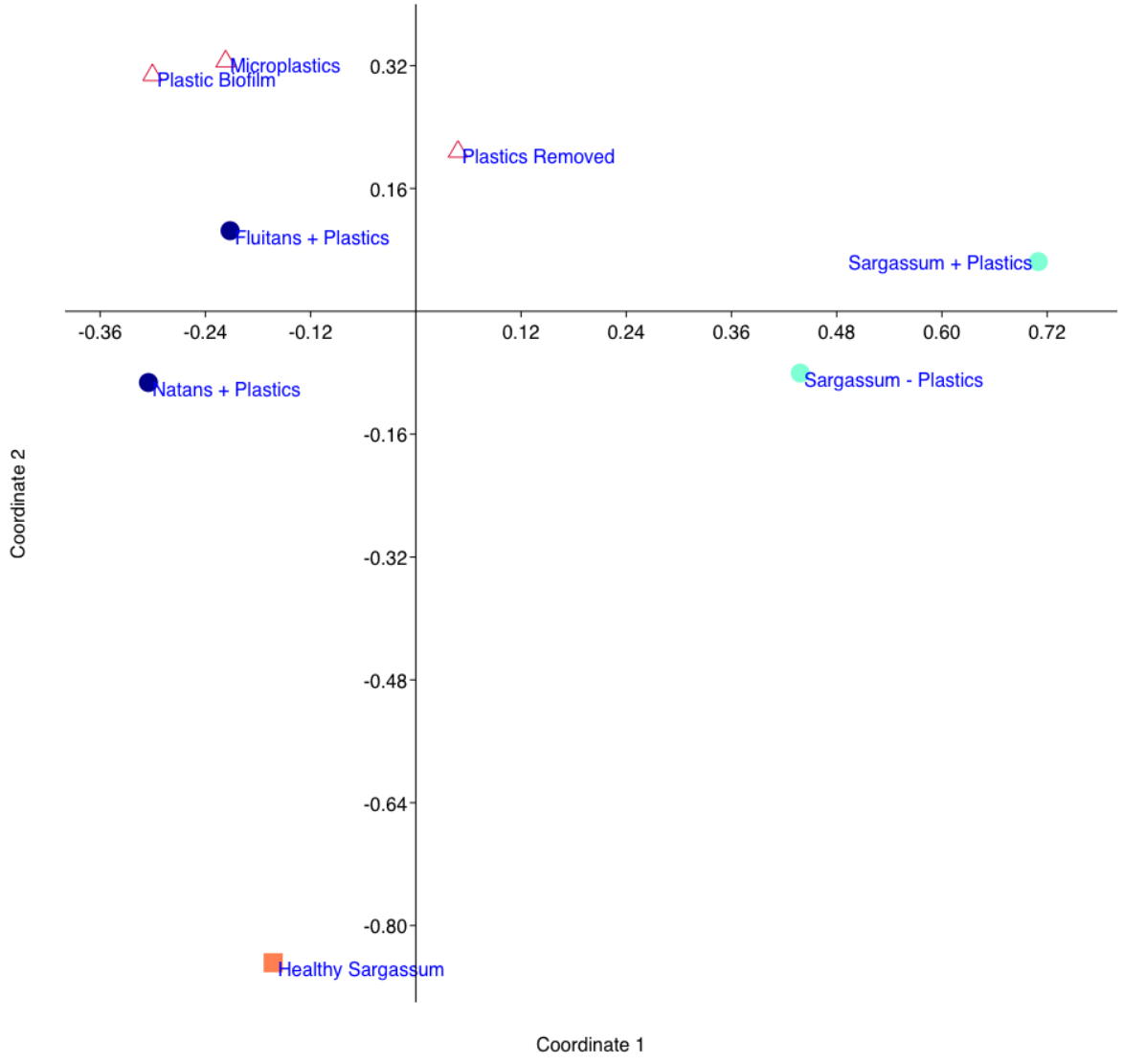


Figure S3. Principal component analysis (PCA) of the bacterial lineage data showing the differences between samples using bray-curtis similarity. Plastics are indicated as red open triangles and cluster in the top left panel. Healthy Sargassum is indicated as an orange filled square and clusters in the bottom right panel far away from the other samples. Inshore Sargassum associated with plastics are aqua filled circles and cluster in the right panels near the axis. Offshore Sargassum associated with plastics are blue filled circles and cluster in the left panels near the axis.