

Effects on the *Pocillopora verrucosa* microbiome when in contact with
macroalgae under ocean acidification

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macroalgae under ocean acidification

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LIST OF SYMBOLS AND ABBREVIATIONS

Pulse Amplitude Modulation	PAM
Carbon Dioxide	CO ₂
Principle Coordinate Analysis	PCoA

SUMMARY

Hard coral cover is in decline and this decline has generally coincided with macroalgal proliferation in coral reefs (Gardner et al. 2003, Cheal et al. 2010, De'ath et al. 2012). Coral degradation can be caused by many variables (Hoegh-Guldberg et al. 2007, Anthony et al. 2008, Mumby and Steneck 2008) but this study is focused on potential degradation due to direct competition with allelopathic macroalgae under the effects of future ocean acidification. Allelopathy is the use of chemicals for protection or competitive purposes. It has previously been shown that algae compete with corals through allelopathy, but not if allelopathy causes the microbiome of the coral to enter a diseased state, though there have been several cases of diseased microbiome states observed (Bourne et al. 2009, Mao-Jones et al. 2010, Meyer et al. 2014). As such, it is of interest to determine if the allelopathic competition from algae affects the coral microbiome, leading to a diseased state, and whether these interactions are exaggerated or effected by ocean acidification.

We hypothesize that macroalgal allelopathy effects the microbiome of the reef-building coral *Pocillopora verrucosa* and that these competitive interactions will be affected by the stressor of ocean acidification. We expect the latter because of previous evidence that increased pH causes stress to some species of corals (Anthony et al. 2008). To test this, we used a pre-established scale of algal allelopathy demonstrated in Rasher et al. (2011) and placed corals and algae in contact under ocean acidification conditions for 3 weeks before samples were processed for microbial taxonomy. The initial analyses have demonstrated no significant differences in the abundances of major microbial taxa

compositions for the sampled coral microbiomes when in the presence of the various allelopathic macroalgae, but these are preliminary findings. The data will require finer microbial analysis to determine whether or not there are any significant effects on the coral microbiomes.

CHAPTER 1

INTRODUCTION

Hard coral cover has declined by 80% in the Caribbean with a reduction from 50% to 10% cover in the last three decades (Gardner et al. 2003). The Great Barrier Reef also lost 50% of its live coral cover from 1985 to 2012 (De'ath et al. 2012). Similar declines have occurred globally and these declines have generally coincided with macroalgal proliferation on coral reefs (Cheal et al. 2010). Several studies have determined coral degradation is caused by many variables including ocean acidification, overfishing, pollution, climate change, and disease (Hoegh-Guldberg et al. 2007, Anthony et al. 2008, Mumby and Steneck 2008). As such, the loss of coral cover and topographic complexity has caused a decline in the abundance and diversity of coral-associated fishes (Bruno and Selig 2007, Bridge et al. 2013). The decline in reef fishes due to over fishing, in turn, has led to less algal trimming and greater direct competition between macroalgae and corals.

Previous studies have indicated that macroalgae have varying levels of allelopathy (chemical warfare, often toxicity, used by organisms for protection or competition purposes). Allelopathy can negatively affect corals by causing coral stress and bleaching, although some corals are more susceptible than others to algal chemicals (Rasher et al. 2011, Bonaldo and Hay 2014). Some chemically mediated behavior has been observed in relation to macroalgal flats and the macroalgae related chemicals associated with these behavioral affects are suspected of preventing coral recovery on macroalgae dominated reefs (Rasher et al. 2011, Dixon et al. 2014). In addition to isolating allelopathic

compounds (Rasher et al. 2011), gene expression has been assessed to determine how several species of corals alter expression when in contact with various macroalgae (Shearer et al. 2012). As such, this evidence shows that allelopathic interactions affect coral functioning on a molecular level, possibly causing further coral decline, and increased macroalgal encroachment into coral reef domains (Rasher et al. 2011).

It is not currently known whether the microbes associated with the coral host are influenced by or can mitigate these allelopathic interactions. There have been several studies that looked into the healthy taxonomic composition of coral microbiomes (Mao-Jones et al. 2010, Mouchka et al. 2010, Bourne et al. 2013, Ainsworth et al. 2015), but none that have assessed shifts in the microbiome in relation to the multiple stressors of macroalgal competition and ocean acidification other than Smith et al. (2006), which assessed the presence or absence of microbes on coral health but not under acidification effects. Much like with other organisms, coral microbiomes have been implicated in the maintenance of coral health (Smith et al. 2006, Mao-Jones et al. 2010, Case et al. 2011). It is hypothesized that algae can affect microbial composition by either introducing new microbes to the coral host, causing proliferation of specific microbes of the microbiome into harmful densities, or by causing the chemical killing off of important microbes for coral health.

Though the interactions of macroalgae and corals in relation to recovery and resilience is of great importance to understanding continued reef decline, there is little understanding of how these interactions shift the microbiomes (microbial communities) of corals or how these shifts may harm the coral host. Also, since the oceans currently absorb about half of all carbon emissions, from humans and nature (Feely et al. 2004) it is

not known how these microbial effects may be altered when exposed to ocean acidification caused by increasing CO₂ concentrations (Falkowski et al. 2000, Raven et al. 2005, Ainsworth et al. 2010, Mouchka et al. 2010) but it is known that the increase in CO₂ levels alters the competitive strength of seaweeds on corals (Diaz-Pulido et al. 2011).

This study will utilize deep-sequencing of the bacterial 16S rRNA gene to determine the taxonomic composition of the microbiome of *Pocillopora verrucosa* coral, an important reef building coral in the East Pacific, under competitive pressure from different algal species (*Chlorodesmis fastigiata*, *Amansia rhodantha*, and *Turbinaria ornata*) in high (1000 ppm) and ambient CO₂ treatments. The algae species chosen for this study are related regional variants of the species used in both the Shearer et al. (2014) and Rasher et al. (2011) studies that tested their respective allelopathic tendencies. These algae were selected to represent a gradient of allelopathic potency based on the toxic contact theory of macroalgal competition, this theory proposes that allelopathic lipids are rubbed by contact from the surface of algae onto nearby corals (Rasher and Hay 2010, Rasher et al. 2011). This is the first study on the direct effect of allelopathic algae on the *Pocillopora verrucosa* microbiome. An understanding of how macroalgae and climate change affect coral microbiomes may aid in coral preservation, conservation, and management for the future.

CHAPTER 2

MATERIALS AND METHODS

Field Experiment and Sample Collection:

The experiment was run in Mo'orea, French Polynesia out of the Richard B. Gump Research Station run by the University of California, Berkeley. *Pocillopora verrucosa* corals were collected from the outer reef from 5-10 m by SCUBA, while the various algal species (*Chlorodesmis fastigiata*, *Amansia rhodantha*, and *Turbinaria ornata*) were collected from the fore-reef via snorkeling. The algae species used demonstrate a gradient of allelopathy, as used in Shearer et al. (2014) and Rasher et al. (2011). All corals were cut by a diamond band saw into 3-8 cm nub pieces and were sorted into clonal groups before being glued to a plastic base and allowed to acclimate in the flumes for 3 days in the appropriate CO₂ treatment (ambient or 1000 ppm). 1000 ppm CO₂ was chosen because this is the projected level of CO₂ for the year 2100 (IPCC 2007). pH meters monitored the pH of the flumes and CO₂ was controlled via CO₂ bubbler inputs. The flumes each had the dimensions of 5m x 0.3m x 0.3m. Each coral was wet-weighted pre- and post- experiment. All algae were collected and sorted into small chunks, threaded through 4 mm ropes and were placed in the back of the 4 foot long flumes to acclimate for 3 days.

After that time, each algae clump was attached to the base of a coral sample such that the algae and coral were in direct contact throughout the experiment. There were two controls for the experiment, contact with a plastic aquarium plant and no contact with either algae or a plastic aquarium plant. The contact control had a plastic aquarium plant

threaded through the 4mm rope and attached as the other alga treatments, while the noncontact treatment was a coral without a rope or alga. The experiment was allowed to run for 20 days, with twice daily checks of contact between each algae-coral pair and for replacement of degraded or lost algae as needed. There were a total of 130 coral samples, with 26 corals used for each algal treatment split evenly between the ambient and high CO₂ flumes.

Pulse amplitude modulation fluorometry (PAM) measurements were taken at one day prior to algal contact and days 4, 7, and 23 after algal contact. PAM measurements are generally used to gauge coral health by measuring photosynthetic capability and were used in this experiment to assess allelopathic impact on overall coral health. After the final weighing, all corals were placed in 2 oz. or 4 oz. Whirl-Pak® bags (depending on the coral size) and promptly frozen. The samples were hand-carried back to the Georgia Institute of Technology in a cooler with dry ice. The samples were stored at -80°C until processing.

Sample Extraction and Amplification:

The preserved coral samples were extracted using the PowerSoil® DNA Isolation Kit by MO BIO, as in Baker and Kellogg (2014) (Baker and Kellogg 2014) . Coral samples were chiseled to obtain about 0.25 mg of fragments from each sample for extraction. The chisel and bench surface were sanitized with ethanol between each chiseling event (the chisel being dipped in ethanol between each sample and the bench being wiped down with an ethanol soaked paper towel), which took place in a sterile petri dish. The extractions were run according to the PowerSoil® DNA Isolation Kit instructions. All DNA extracts were tested for DNA content via the Qubit Broad

Spectrum Quantification kit. Any samples that read as “too low”, meaning the DNA concentrations were less than 0.5 ng/ μ L, were re-extracted using a new fragment from the coral sample. Two deionized water controls were pipetted onto a sterile petri dish, pipetted into the PowerSoil extraction tube, and extracted via the same protocol as the coral samples and were treated as the coral samples for sample processing and sequencing. These water controls were intended to give us a control for impurities or contaminations introduced during the processing of the samples. After extraction, a 250 bp DNA fragment spanning the V4 region of the 16S rRNA gene was amplified using the V4-specific primers F515 and R806, as in (Caporaso et al. 2011). The forward and reverse primers were both barcoded and appended with Illumina-specific adapters, as done in (James Kozich 2013). For all PCR amplifications, 3 μ L of extracted DNA was used. Thermal cycling conditions were: initial denaturation at 94°C (3 min), followed by 30 cycles of denaturation at 94°C (45 sec), primer annealing at 55°C (45 sec) and primer extension at 72°C (90 sec), followed by final extension at 72°C for 10 min and a 4°C holding temperature. These Amplicons were then analyzed by agarose gel electrophoresis to verify the length of DNA was 400 bp. The amplicons were cleaned via Diffinity RapidTips, and Illumina adaptor-appended amplicons for each sample were pooled at equimolar concentrations and sequenced on an Illumina Miseq running Miseq Control software v.2.4.0.4, while using the MiSeq reagent kit v2 (500 cycle) with 5% PhiX genomic library control.

16s rRNA Amplicon Analysis:

The sequences were quality controlled by a Phred33 score of Q25. The short fragment sequences (cut off of 100 bp) were removed before merging of the sequences to

achieve longer fragments by the QIIME Trim Galore! app and were then merged using the QIIME FLASH with an overlap of 25 bp. These merged sequences were then run through the software pipeline QIIME v1.8.0 (Caporaso et al. 2010). The reads were concatenated and screened for chimeras using QIIME's identify_chimeric_seqs.py script then non-chimeric sequences were clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using the Greengenes database from the QIIME website. Taxonomy was assigned to OTUs using open reference OTU picking with 97% gene similarity, meaning the microbes are closely related, and QIIME was used to run a core diversity analysis using the script core_diversity_analyses.py for an even sampling depth of 3000. This diversity analysis allowed for visualization of total (alpha) diversity for the samples and principle coordinates analysis (PCoA) unweighted Unifrac beta diversity analysis was used to visualize the compositional differences between samples. The diversity analyses were run with a cut off of 500 reads because this was determined to be the largest number of reads with the fewest number of dropped samples from analysis, which dropped 35 of the 123 successfully sequenced samples from our dataset. The data set is currently under preliminary analysis, but deeper analysis (including the removal of overlapping OTUs between water controls and samples) will aid in better understanding of the subtle shifts in the *Pocillopora verrucosa* microbiome, but have not been completed at this time. An ANOVA will be run after further dataset analysis to test for significance of the variance.

CHAPTER 3

RESULTS

The extractions produced PCR-amplified products of the coral samples with quantifiable DNA (>0.5 ng/ μ L) for 97% (128 out of 132) of the samples but 3% (4 out of 132) of the samples did not amplify enough to be measured by the Qubit Broad Spectrum Quantification kit. All of samples were sequenced (except for two samples suffered primer duplication). In total, 123 samples were successfully sequenced and usable for analysis. The diversity analysis cut off of 500 reads eliminated 35 more samples from our data set, leaving 88 total samples for our preliminary data analysis (16 *Chlorodesmis*, 9 *Dictyota*, 9 *Turbinaria*, 8 plastic controls, 13 empty controls, and 2 water controls). There were over 500 OTUs identified with the *Proteobacteria* making up about 61.5% of all samples (Figure 1). Of the *Proteobacteria* present, *Gammaproteobacteria* made up about 16.9% of the samples (Figure 2), while *Alphaproteobacteria* made up about 28.9% (Figure 2) and *Deltaproteobacteria* made up about 11.0% (Figure 2) of the samples. The PCoA unweighted Unifrac visualizations of the algal treatments and of the CO₂ treatments showed no visual differences in beta diversity among the dominant OTUs (Figure 3 and 4), but further taxonomic analyses could tease out subtle taxonomic shifts in individual OTUs that may co-vary and associate significantly with specific treatments. Alpha rarefaction analysis showed even sampling except for the water controls (Figure 5). Any OTUs present in the extraction blank controls should be removed from the other datasets and the datasets re-evaluated. This allows for identification of only coral associated taxa and finer analysis of shifts in

the abundances of less prevalent taxa, which will be needed to determine a subtle effect of the algal and OA treatments on the *Pocillopora verrucosa* microbiome. An ANOVA also needs to be run to determine if there is significance within the data without the shared OTUs from the water controls.

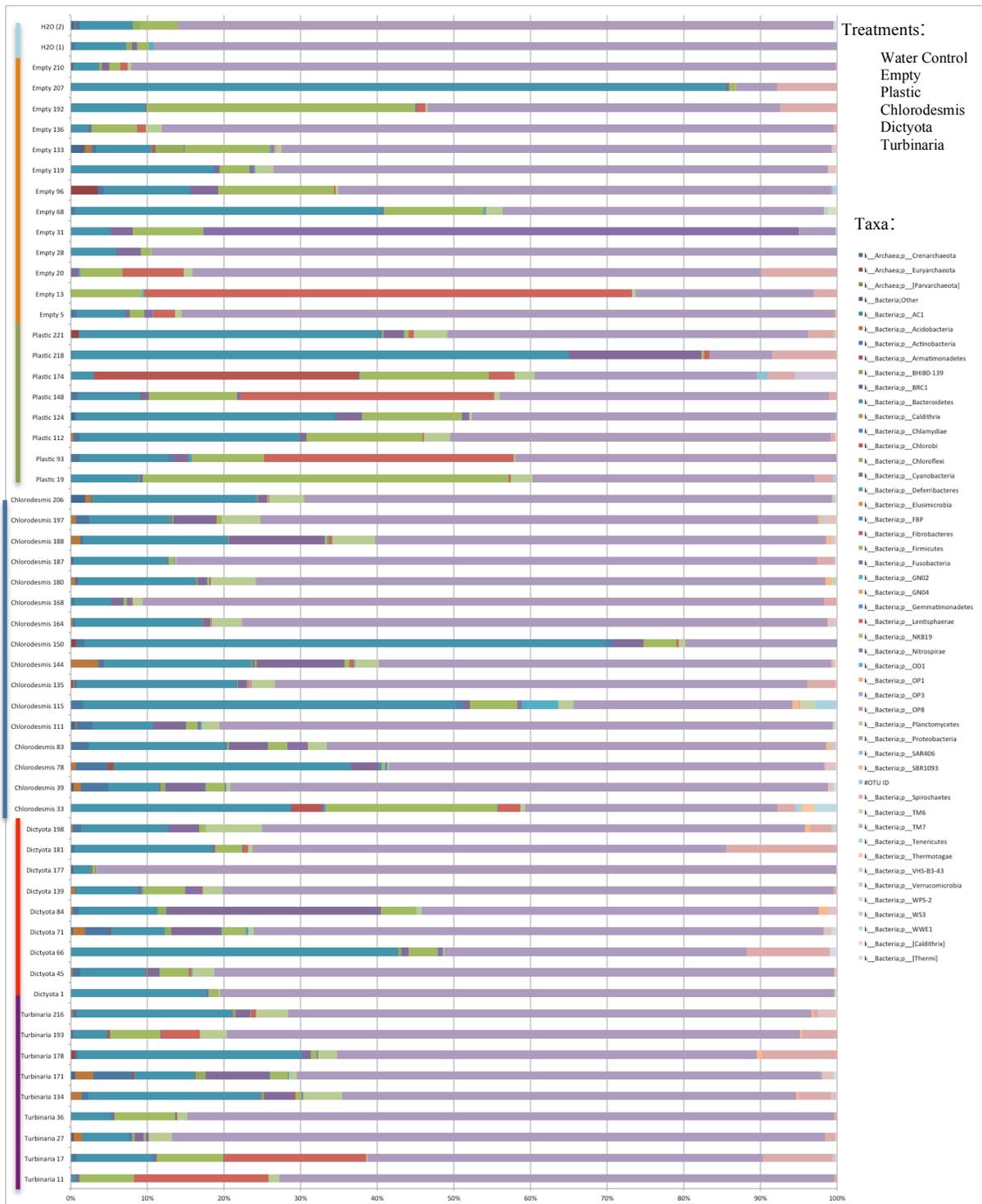


Figure 1. Phylum level taxa summary for all coral samples. Bar charts represent the percent abundance of microbial taxa present in each sample.

Treatments:

- Water Control
- Empty
- Plastic
- Chlorodesmis
- Dictyota
- Turbinaria

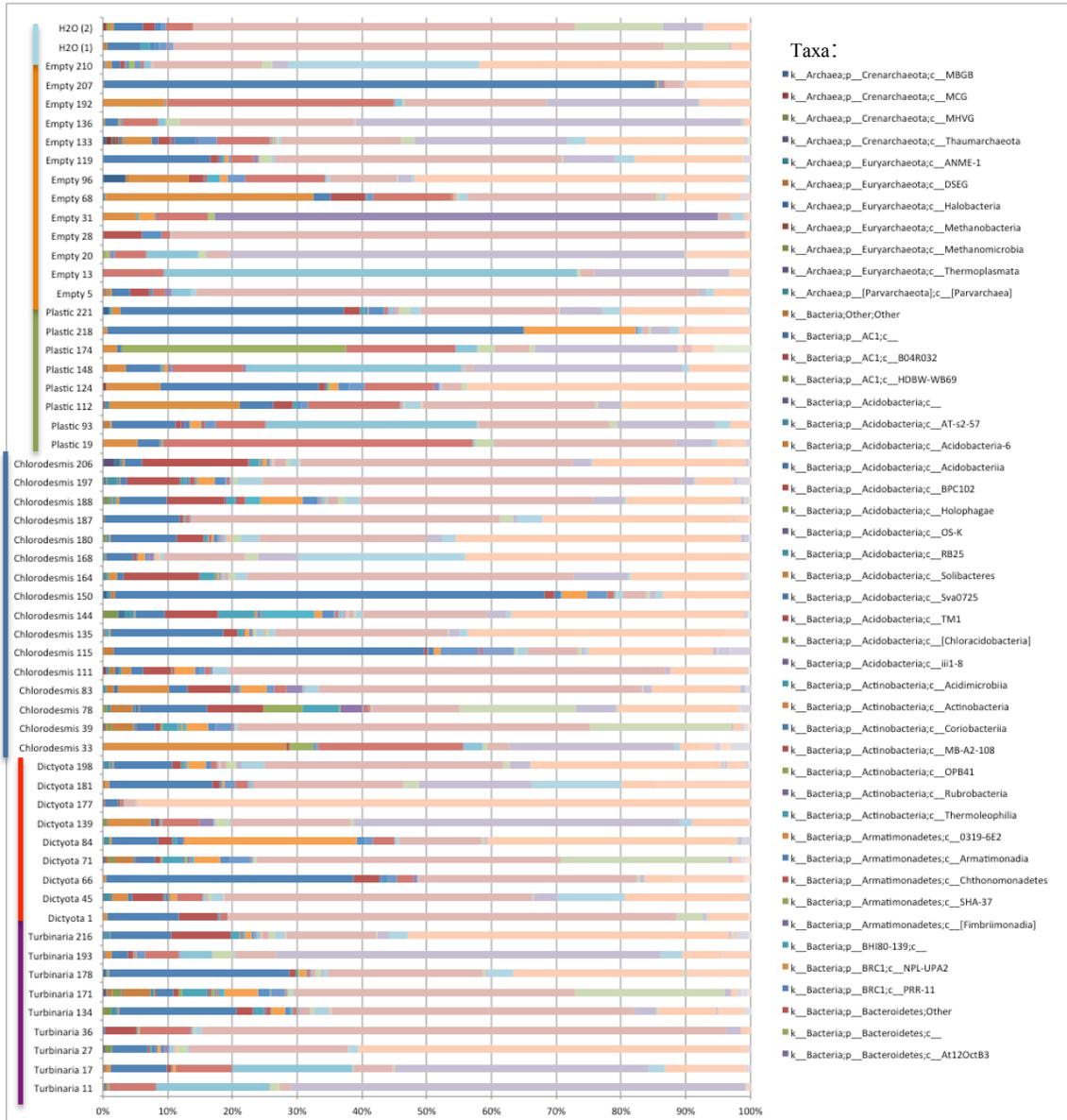


Figure 2. Class level taxonomic summary for all samples. Bar charts represent the percent abundance of microbial taxa present in each sample.

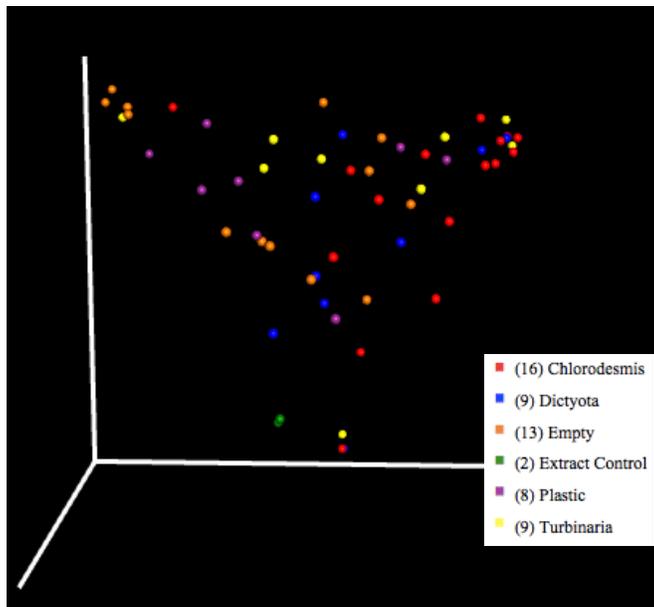


Figure 3. Principle coordinates analysis (PCoA) unweighted Unifrac analyses by Algal Treatment, run with rarefaction of 500.

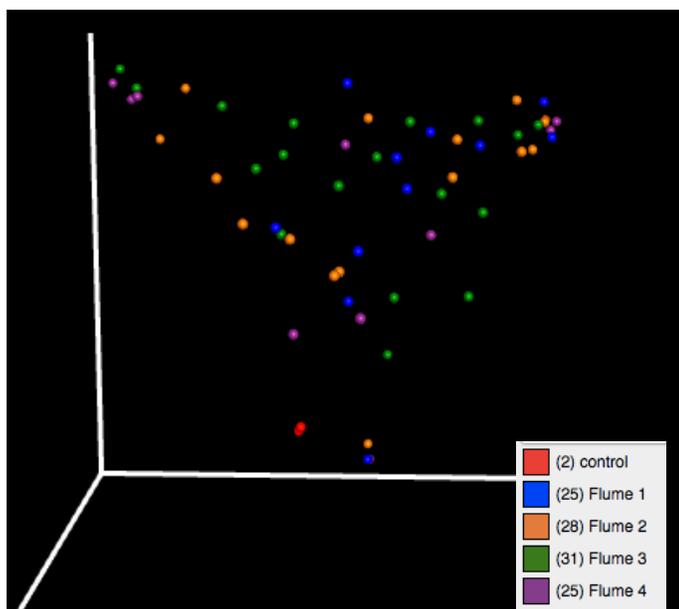


Figure 4. Principle coordinates analysis (PCoA) unweighted Unifrac analyses by flume treatment, run with rarefaction of 500.

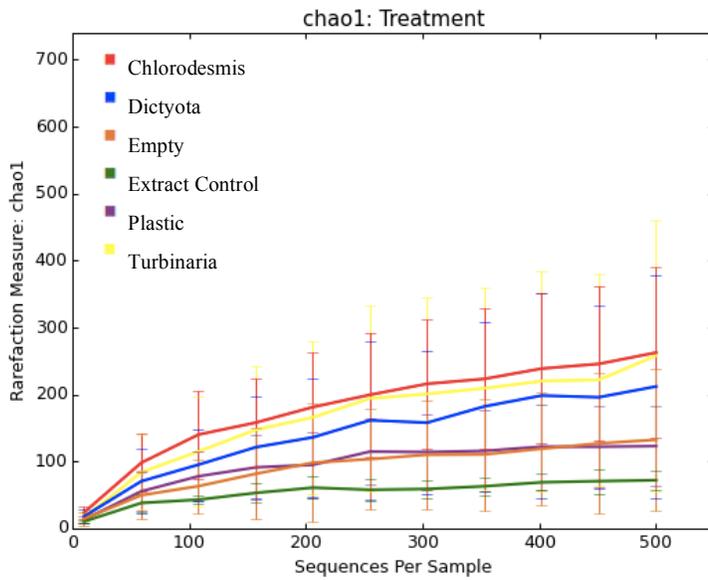


Figure 5. Alpha rarefaction diversity by algal treatment, showing the number of OTUs per sample as a function of sequencing depth. On the y-axis is the number of OTUs per sample, as estimated via the Chao-1 estimator. The error bars indicate the variation in the number of OTUs present in samples of the same algal treatment for the number of sequences in each sample of the treatment.

CHAPTER 4

DISCUSSION

The data do not currently support that allelopathic competition from algae can alter the microbial taxonomic composition of the *Pocillopora verrucosa* microbiome, but further data analysis is still needed to investigate the finer microbial effects. The alpha rarefaction (Figure 5) shows a high level of inclusiveness for the samples and that the water controls have limited microbial diversity. The PCoA unweighted Unifrac plots (beta diversity) show equal differences and a wide spread between samples of the same treatment for the two treatments (CO₂ and algal) (Figures 3 and 4). The taxonomic abundances show large variance between samples of the same algal treatment and flume treatment (Figures 1 and 2), enforcing that there is no shift in dominant taxa from either the presence of algae or ocean acidification conditions. Since these are preliminary analyses, an ANOVA has not been run to determine if there is anything of significance within our data. The taxonomic compositions show (Figures 3 and 4) no notable average shift in composition in the samples. Thus, we did not see any affect of allelopathic algal contact or ocean acidification directly on the composition of the *Pocillopora verrucosa* microbiome. Though, there could be subtle variations of large impact in the microbiome, which have yet to be deciphered from our data. To decipher these impacts it would be required to remove shared OTUs with the water controls from corals sample analyses and to block the corals by individual clones to remove any clone specific taxa from the analyses. Additionally, an ANOVA test of the OTU corrected sample data would be required to determine if there is any significance within the data. After these analytical

steps are taken more solid determinations about the effects of the treatments on the microbiomes of the experimental corals can be made.

Some previous studies have connected changes in the taxonomy of the coral holobiont to disease states (Mao-Jones et al. 2010, Meyer et al. 2014). These coral studies support that microbiomes associated with corals may have just as much importance for overall coral health and indication of coral disease, as the human microbiome does for human health (Peterson et al. 2009, Cho and Blaser 2012). Therefore, it makes sense to pursue the various attributes associated with the microbiome of corals (Ainsworth et al. 2015) and to study what factors alter the community to cause various diseased states. We assess the latter in this study by focusing on how increased CO₂ concentrations (ocean acidification) will affect the intensity of algal-coral competition on a microbial level do to additional coral stressing (Kaniewska et al. 2012). Our preliminary results show that algae do not have a significant effect on the composition of the coral microbiome and as such the competition of algae and corals under ambient versus high CO₂ concentrations does not alter the *Pocillopora verrucosa* microbiome either. This said, further analyses of our results is required to make any solid statements about whether the algal and CO₂ treatments effected the taxa of the microbiomes at a more subtle taxonomic level than preliminary analyses can detect.

One avenue for future research on this effect of algal competition on coral microbiomes would be to do the same experiment with other allelopathic algae, or with different coral hosts, to determine if shifting the taxonomic community of corals is a competitive strategy for algae that occurs in nature. Since, there would need to be a specific mechanism to cause the same shifts in abundances of specific taxa within the

coral microbial community, it would be of import to understand how these shifts are initiated by the algal competitor, either by bacterial transfer or by release of particular competitive chemicals utilized by the specific taxa. Also, if the finer data analysis uncovers subtle shifts in taxa, it would be of interest to determine whether these observed microbial shifts occur over a broad range of corals. If such a general case occurs, then it would be possible to formulate mitigation for these negative interactions on corals and possibly aid in preserving reef function.

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