TOO MUCH OF A GOOD THING? THE INFLUENCE OF BENTHIC COMMUNITY & CHRONIC NUTRIENT ENRICHMENT ON REEF MICROBIAL ASSEMBLAGES

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ABSTRACT

Coral reefs are among the most productive ecosystems on the planet and their survival is integral to maintaining the ocean's biodiversity. However, shifts in benthic communities, away from reef building corals toward reefs dominated by fleshy algae are documented worldwide and linked with anthropogenic activities including nutrient pollution and overfishing. It is established that algae produce higher quantities of labile organic matter compared with that of corals resulting in a restructuring of bacterioplankton communities toward less diverse, potentially pathogenic assemblages. Although marine biofilms are lauded for their role in settling invertebrate larvae, including that of corals; the effect of these phase shifts on surface attached microbial communities (e.g. biofilms) has been previously unknown. Submarine groundwater discharge (SGD) is one way that anthropogenic nutrient pollution can enter coastal ecosystems and may potentially deliver microbial populations to these environments. However, until now it was unclear how SGD associated nutrients or the possible delivery of microorganisms might influence reef microbial communities. In this dissertation, I characterize microbial communities associated with SGD and describe the effect of the associated nutrient flux on microbial communities across a coral reef in Maunalua Bay and use tidal influence to inform our findings. At low tide, accompanying the strongest effect of SGD associated nutrients; distinct microbial communities from each sampling location were identified. Samples collected from the SGD spring displayed the highest microbial diversity and contained taxa not found at other sampling locations. Mid-reef samples were enriched with populations of copiotrophic taxa and coincided with a peak in ammonium concentrations, suggesting that SGD associated nutrients stimulate nitrogen metabolism and may shift microbial assemblages on coral reefs. Additionally, to investigate the effect of benthic organism and inorganic nutrients on surface attached microbial

communities, I cultured biofilms with constituent reef primary producers (algae, coral, and sand), factorially crossed with three levels of continuous nutrient enrichment (ambient, low, and high). Samples were collected at 2-week intervals over the course of 6 weeks. Taxonomic comparison of biofilm communities revealed successional trajectories that were divergent from the temporal dynamics of the planktonic community. Both taxonomic and functional profiles were structured by benthic organism and nutrient treatments within biofilm communities. Overall this work demonstrates that both benthic community and nutrient availability influence microbial community structure in both surface attached and planktonic microbial communities.

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CHAPTER 1
GENERAL INTRODUCTION

Overview of the role of microbial communities in coral reef phase shifts

Tropical coral reefs are among the most productive and biologically diverse ecosystems on the planet. However, since the 1950s live coral cover has decreased by 50% (Eddy et al. 2021) and the rate of degradation of coral reefs is likely accelerating (Hughes et al. 2018). While the exact mechanisms for this decline are unclear, evidence suggests that the challenges facing coral reefs are multiple and synergistic (Ellis et al. 2019). Global stressors like warming sea temperatures may exacerbate local stressors such as overfishing and nutrient loading (Zaneveld et al. 2016; Ellis et al. 2019) which are already associated with decreased water quality and thought to promote phase shifts from coral to algal dominated reefs (Fabricius 2005; McCook 1999; Hughes 2003). Understanding how changing reef composition interacts with inputs of nutrients and pollutants is critical for understanding the fate of coral reefs in a changing world.

Productivity in coral reefs is largely dependent on the capture and recycling of nutrients by reef microbial assemblages (Bourne and Webster 2013). Microbial communities in healthy coral-dominated reefs are characterized by diverse microbial assemblages with high proportions of autotrophs (Bruce et al. 2012). Environmental conditions like nutrient availability are associated with shifts in the microbial community. For example, fluctuations in the dominant cyanobacterial families, Prochloraceae and Synechococcaceae are observed in response to nutrient concentration (Frade et al. 2020). High abundances of Prochloraceae are typically associated with oligotrophic environments while Synechococcaceae dominate coastal regions and nutrient rich areas (Weinbauer et al. 2010; Bourne and Webster 2013). In addition to Synechococcaceae, higher proportions of Flavobacteriaceae and Rhodobacteraceae are reported

to positively correlate with increasing nutrient loads (Frade et al. 2020). These groups are often classified as opportunistic pathogens correlated with reefs in poor health (Zaneveld et al. 2016) and, along with other copiotrophic groups such as Vibrionaceae and Enterobacteriaceae, may be abundant on algal-dominated reefs (Haas et al. 2016).

The direct and indirect effects of macroalgae on coral vitality are documented (Birrell et al. 2008) and it is established that organic matter exudates produced by algae are distinct from that of corals both in quantity and composition (Nelson et al. 2013; Quinlan et al. 2018). These differences in organic matter restructure bacterioplankton communities toward less diverse, copiotrophic microbial assemblages (Nelson et al. 2013; Haas et al. 2016), and increased virulence factors (Cárdenas et al. 2018; Haas et al. 2016; Nelson et al. 2013). These types of alterations in the bacterioplankton community are thought to reinforce phase shifts and maintain algal dominance (Haas et al. 2016). Although marine biofilms are recognized as important mediators of coral reef establishment (Webster et al. 2004; Hadfield 2011), little work has been done to understand how marine biofilms may interact with coral- algal phase shifts. Since taxonomic community structure strongly influences biofilm function (Besemer 2015), understanding the role of benthic community in structuring reef biofilm taxonomy and function is an important aspect of understanding phase shifts on reefs.

Submarine groundwater discharge in Hawai‘i

One way that coral reefs may acquire sufficient nutrients to maintain high biodiversity in spite of the typically oligotrophic waters where they are found (Paytan et al. 2006; G. Kim, Kim, and Hwang 2011) is through delivery of terrigenous nutrients. Submarine groundwater discharge (SGD) is a phenomenon wherein fresh meteoric groundwater mixes with saline groundwater that

is entrained in the subterranean estuary before discharging into the surrounding marine waters (Burnett and Dulaiova 2003; Santos et al. 2021). This process results in the expression of nutrient rich waters that are typically cooler, fresher, and often lower in pH than the overlying marine waters. In some areas in Hawai‘i, the contribution of SGD, both in terms of volume and nutrient input, are comparable to that of surface flows (Garrison, Glenn, and McMurtry 2003; Dollar and Atkinson 1992). Studies on coral reefs regarding the biological implications associated with the sustained nutrient load from SGD are limited and varied. In some cases, SGD has been shown to stimulate primary production (reviewed in Santos et al. 2021) and support healthy reefs (Paytan et al. 2006). On the other hand, SGD is also linked with harmful algal blooms (Y.-W. Lee and Kim 2007; Y.-W. Lee et al. 2010), decreased coral vitality (Lubarsky and Silbiger 2018), and associated with higher rates of microbial respiration (Silbiger, Donahue, and Lubarsky 2020). Similarly, higher biomass as well as alterations in benthic macroalgae communities have also been reported in association with SGD (La Valle, Thomas, and Nelson 2019).

Because it provides a link between terrestrial and marine ecosystems, SGD may also serve as a conduit for excess fertilizers or wastewater contaminated groundwater to enter coral reefs thereby facilitating anthropogenic nutrient enrichment. Excessive nutrient loading is correlated with decreases in coral abundance and diversity (Couch et al. 2014; Fabricius 2005), an increase in coral disease (Voss and Richardson 2006; Vega Thurber et al. 2014), and higher abundance of fleshy algae (McCook 1999). Non-point source contamination of groundwater is of special concern in Hawai‘i as much of the groundwater is sourced from shallow, unconfined

aquifers (Whittier and El-Kadi 2014). Onsite sewage disposal systems (OSDS) like septic tanks and cesspools are common throughout Hawai‘i (Whittier and El-Kadi 2014). OSDS leachate along with runoff from areas of excess fertilizer usage or livestock farms may contribute to declining water quality through the contamination of the groundwater aquifers from which SGD is sourced. Studies from around the state link declining water quality with decreases in live coral cover and increases in coral disease (Couch et al. 2014). Nutrient pollution from SGD play key roles in these areas finding elevated stable isotopes that are consistent with nutrient pollution (Richardson, Dulai, and Whittier 2017); and further evidenced by bacterial indicators and dye tracers that prove hydraulic connections with household waste (Wiegner et al. 2021). Specific bacterial populations (i.e. fecal indicator bacteria, FIB) are used to study the relationship between SGD and wastewater pollution, and unique microbial communities are associated with the freshwater-seawater interface (Héry et al. 2014) that may be transported through intertidal sands (Santoro et al. 2006; Cui et al. 2013).

Dynamics of aquatic biofilms

Surface attached microbial communities known as biofilms comprise Bacteria, Archaea, eukaryotic microalgae, and fungi. They are encased in extracellular polymeric substances (EPS) that protect the biofilm community from environmental stressors and facilitate microbial interactions such as cell to cell communication and the sharing of metabolites (Flemming and Wingender 2010). Biofilms are ubiquitous in aquatic environments where they provide ecosystem services including carbon fixation (Wagner et al. 2015) organic matter decomposition (Ardón and Pringle 2007), and nutrient cycling (Battin et al. 2003). Combined with the close

proximity of cells, biofilm EPS allows for the uptake and retention of nutrients from the surrounding environment making biofilms “hot spots” of geochemical cycling (Peter et al. 2011).

Environmental factors such as fluid dynamics and nutrient availability influence microbial community composition (Olapade and Leff 2006; Wagner et al. 2015), cell density (Stoodley et al. 2001), and rate of succession (Stoodley et al. 1998); all of which ultimately influence the ecosystem services provided by the biofilm. Environmental factors such as these can influence biofilm properties even before the community is established. Briefly, submerged surfaces are coated in “conditioning films” of organic- and inorganic- molecules that can alter the chemical properties of the surface and facilitate microbial colonization (Donlan 2002). Physical surface properties combined with water flow over the substrate, setting up boundary layers that, together with conditioning films select for primary colonizers that are motile and capable of attaching themselves to the surface in order to initiate biofilm formation (Flemming and Wingender 2010; Donlan 2002).

Perhaps because pioneering species shift from independent, planktonic lifestyles to surface attached, community lifestyles; biofilms in early successional stages appear to be less specialized in their use of organic compounds (Romaní et al. 2014). In terms of succession, marine biofilms are dominated by Gammaproteobacteria in early stages, compared with later successional stages where Alphaproteobacteria are more abundant, and substantial proportions of Cyanobacteria and Bacteroidetes are commonly reported (J. W. Lee et al. 2008; Webster et al. 2004). This progression follows typical patterns of environmental biofilms where the early stages of biofilm development are thought to be dominated by metabolic generalist that utilize a wide range of dissolved organic carbon (DOC), sourced from surrounding waters. As biofilms mature,

mixed communities form and are often dominated by autotrophs that are capable of supporting heterotrophs within the biofilm community (C. R. Jackson 2003; Romaní et al. 2014).

Interactions within the biofilm community, like the use of allochthonous or autochthonous DOC, can be tightly coupled and are simultaneously dependent on environmental conditions (Ylla et al. 2009; Peter et al. 2011). For example, heterogeneity in flow is correlated with increased microbial diversity (Singer et al. 2010) which supports greater multifunctionality (Peter et al. 2011) and allows for the utilization of a broader range of organic compounds (Singer et al. 2010). In freshwater systems, exogenous labile organic matter is linked with decreased microbial diversity (Ylla et al. 2009) and can decouple interactions between autotrophic and heterotrophic communities (Scott et al. 2008). Furthermore, bioavailability of DOC has been shown to shift microbial communities in mature biofilms (Olapade and Leff 2006) and can influence the uptake and utilization of other nutrients (Suchismita Ghosh and Leff 2013). Organic matter exudates from algae are known to differ in both quality and composition from those of corals (Nelson et al. 2013; Quinlan et al. 2018) and are associated with shifts in the bacterioplankton community (Haas et al. 2016; Nelson et al. 2013). Yet, it is unknown how these differences will affect marine biofilm communities and the ecosystem services they provide.

Objectives of the dissertation

The over-arching goal of this dissertation is to investigate the impacts of changing reef benthic community and inorganic nutrient inputs on coral reef microbial community structure, including both planktonic and biofilm assemblages. This was accomplished both in a controlled lab experiment and in a field experiment which leveraged the natural nutrient gradient supplied

by SGD. **Chapter 1** (this chapter) serves as an introduction and provides brief scientific background for the information that follows.

Chapter 2 sought to characterize microbial communities associated with SGD and describes the effect of the associated nutrient flux on microbial communities over a tidal cycle at Maunalua Bay. Previous work in this location mapped the spatial extent of SGD and its influence on multivariate biogeochemistry of waters in this area, identifying 4 zones of SGD influence (Nelson et al. 2015). For this study, over the course of a tidal cycle, one sample was collected from each of the previously described zones at regular time intervals. Using salinity as a proxy for SGD, geochemical parameters were fit with polynomial regressions to identify parameters that were: associated with SGD, of marine origin, or exhibited non-linear relationships with SGD. 16S rRNA sequences were used to analyze microbial communities. Multivariate methods were used to compare microbial communities between sampling locations, and polynomial regressions were again employed to evaluate microbial populations across sampling sites along a salinity gradient. Geochemistry findings were used to inform microbial community analysis and correlations between non-linear geochemical parameters were coincident with specific microbial populations. Because the geochemical environment is often innately intertwined with the observed microbial communities and because of the temporal dynamics reported in this chapter, the results and discussion section are reported in tandem.

Chapters 3 & 4 investigate the effect of benthic organism and chronic nutrient enrichment in structuring marine biofilm communities. Over the course of 6-weeks, biofilms were cultured on glass slides, suspended in individual aquaria housing one of three benthic primary producers (algae, coral, or sand) and supplied with one of three nutrient treatments (ambient, low, or high). Samples were destructively sampled at 2-week, 4-week, and 6-week

intervals. In reef systems, organic matter exudates from benthic primary producers may function as conditioning films on submerged surfaces, potentially influencing biofilm community structure from early time points.

Chapter 3 uses 16S rRNA sequencing to compare the taxonomic community structure of the resulting marine biofilms over time. Comparisons between planktonic and surface attached microbial communities establish that successional dynamics observed in biofilm communities are independent from the planktonic community. The overall effect of succession, benthic organism, and nutrient amendment on biofilm community structure was evaluated using multivariate methods. Linear mixed models were used in combination with a random forest algorithm to identify and rank microbial populations driving trends in the community structure.

Chapter 4 uses metagenomes from biofilms sampled at 2- and 6-weeks to contrast the effects of time and benthic community on the functional potential of marine biofilms. Metagenomic sequences were uploaded to the open-source web server, MG-RAST for taxonomic and functional annotation. Taxonomic patterns found among the treatments in Chapter 4 (metagenomic data) support the findings in Chapter 3 (16S rRNA dataset): both organism and nutrient additions had significant effects on biofilm community structure at each time point in our study. Samples collected from 2-week biofilms were used to test the effects of benthic organism and nutrient treatment on functional profiles.

Finally, **Chapter 5** concludes this dissertation with a synthesis of the findings, and potential implications. I discuss the successes and short comings of this work and make recommendations to expand on the work and themes presented here.

CHAPTER 2
SUBMARINE GROUNDWATER DISCHARGE INFLUENCES CORAL REEF MICROBIAL POPULATIONS BOTH DIRECTLY AND INDIRECTLY

Abstract

Submarine groundwater discharge (SGD) is an important biogeochemical component of coastal ecosystems and coral reefs worldwide. SGD provides terrestrially derived nutrients to oligotrophic ecosystems and is known to stimulate primary productivity and alter benthic community composition. In this study, we characterize the microbial communities associated with SGD in Maunalua Bay, O‘ahu, Hawai‘i and describe the effect of the associated nutrient flux on microbial communities across a coral reef and in the context of tidal influence. Using salinity as a proxy for SGD, we found linear relationships between salinity and concentrations of geochemical parameters (silicate, SiO_4^{4-}; nitrate, NO_3^-; and phosphate, PO_4^{3-}) typically associated with SGD enriched in groundwater relative to coastal waters and non-linear, mid-reef enrichment of ammonium (NH_4^+) and a specific amino acid-like component of dissolved organic matter, Coble T. These associations were strongest at low tide and coincided with distinct microbial populations. Samples collected from the SGD spring displayed the highest microbial diversity and contained taxa that were not recovered from other sampling locations in the reef. Microbial populations enriched in regions of SGD mixing contained higher abundances of copiotrophic taxa, indicating that SGD-associated nutrients likely stimulate benthic production and may result in shifts of microbial communities at mid-reef locations.

Introduction

Coral reefs are among the most productive ecosystems in the world and their ability to maintain high biomass and rich biodiversity despite the oligotrophic waters where they are commonly found remains an open question in ecology (Alldredge, Carlson, and Carpenter 2013; Nelson et al. 2015). One way that reefs may acquire sufficient nutrients to support this high productivity is through submarine groundwater discharge (SGD). SGD is a process wherein coastal groundwater is discharged directly into the sea through a variety of pathways, including combined land and ocean based hydraulic gradients that drive advective flows (Burnett et al. 2003; Santos et al. 2021). SGD is a common feature of islands in the Hawaiian Archipelago (Knee et al. 2008; Amato et al. 2016) and other volcanic islands (G. Kim, Kim, and Hwang 2011). In some locations (e.g. Kahana Bay, Oʻahu, Hawaiʻi), SGD can supply volumes of water that are comparable with surface water inputs (Garrison, Glenn, and McMurtry 2003; G. Kim, Kim, and Hwang 2011). SGD is widely recognized as an important source of terrestrially derived nutrients in coastal ecosystems and is thought to support coral reefs through the provision of new nutrients in these environments (Paytan et al. 2006). Waters associated with SGD in tropical island settings are generally cooler, nutrient rich, and lower in salinity and pH relative to marine waters (Garrison, Glenn, and McMurtry 2003; L. Li et al. 1999; Michael, Mulligan, and Harvey 2005) and are a common source of organic matter (Nelson 2013, Kim 2017) and dissolved nutrients (Si, N, P, (Dollar and Atkinson 1992)) in coastal ecosystems.

The biological implications of SGD-delivered nutrients are complex and can vary based on local factors including lithology (Rahman et al. 2019), land use (Knee et al. 2010), and reef mixing rates (Silbiger, Donahue, and Lubarsky 2020). While many studies find nutrients associated with SGD can increase primary productivity (Santos et al. 2021), SGD has also been

linked to red tide blooms in areas where dissolved inorganic nitrogen or phosphate has been depleted thereby limiting the growth of diatoms and favoring the growth of dinoflagelllates (Y.-W. Lee and Kim 2007; Y.-W. Lee et al. 2010). Similarly, while slight increases in inorganic nutrients have been shown to promote coral growth, salinity stress and chronic nutrient loading by SGD are associated with mortality (Lubarsky and Silbiger 2018) and shifts in species composition in coral communities (Cárdenas et al. 2018) as well as alterations in community composition and productivity in benthic algal communities (La Valle 2019, 2021). Both SGD (Nelson et al. 2015; J. Kim and Kim 2017) and benthic community organisms can also be sources of organic matter on coral reefs (Quinlan et al. 2018), and alterations in the benthic community or its function are known to shift microbial communities (Nelson et al. 2013). For example, algae are known to produce higher quantities of labile organic matter compared with corals, and nutrient additions exacerbate these differences (Quinlan et al. 2018). These variations in organic matter are implicated in shifting microbial communities toward less diverse, copiotrophic microbial communities that harbor more potentially pathogenic bacteria than those found on coral-dominated reefs (Nelson et al. 2013; Haas et al. 2016). Because nutrients supplied by SGD affect reef biology at all scales, it is therefore important to consider the impact that SGD may have on reef microbial communities. Yet, the extent to which SGD influences the microbial ecology of reef ecosystems remains understudied (Ruiz-González, Rodellas, and Garcia-Orellana 2021).

Microbial communities in tropical island aquifers are distinct from those of surface waters and coastal marine waters (Kirs et al. 2020) and SGD may directly influence microbial communities through advective input of cells. Further, SGD associated nutrients have been shown to alter rates of bacterial respiration and production, and growth efficiency (Johnson and

Wiegner 2014; Carlson and Wiegner 2016). Indirectly, nutrient loads and salinity changes associated with SGD can influence microbial communities through its effect on the broader food web. Lecher and Mackey (2018) found that SGD-triggered phytoplankton blooms promote the growth of certain bacterial groups over others. Further, chronic nutrient enrichment has been shown to stimulate the release of different types of dissolved organic matter from coral reef benthic producers (Quinlan et al. 2018), with cascading impacts on coral reef biofilm microbial communities (Remple et al. 2021).

As a direct link between terrestrial and marine ecosystems, SGD can also deliver terrigenous pollutants to the coastal ocean. In Hawai‘i, much of the ground water is sourced from shallow, unconfined aquifers that are especially susceptible to contamination by non-point source pollution, and several studies have found evidence that suggests SGD is impacted by local land use practices (e.g. agriculture, and onsite sewage disposal systems (OSDS) (Couch et al. 2014; Richardson, Dulai, and Whittier 2017; Whittier and El-Kadi 2014). Together with proven hydraulic connectivity (Abaya, Wiegner, Beets, et al. 2018), these findings have raised public health concerns that SGD may deliver human pathogens, like Enterococcus *spp.* (a species of fecal indicator bacteria, FIB), to marine ecosystems by providing a mechanism for OSDS contaminated groundwater to enter coastal ecosystems. Studies attempting to link FIB with SGD in wastewater impacted areas have found mixed results. While some studies have not found FIB in the expectedly high concentrations needed to definitively link SGD with sewage contaminated waters (Boehm et al. 2014; Boehm, Shellenbarger, and Paytan 2004; Knee et al. 2008), others have successfully used multi-indicator approaches to detect sewage contamination in coastal waters (Abaya, Wiegner, Colbert, et al. 2018; Abaya, Wiegner, Beets, et al. 2018; Wiegner et al. 2021).

In the present study, we analyze biogeochemistry and bacterioplankton community structure across an SGD plume at Black Point in Maunalua Bay, Oʻahu, Hawaiʻi. Using biogeochemical tracers of SGD, we examined the spatial and temporal influence of SGD on microbial community composition. We leveraged the previously described spatial distribution of SGD influence (Nelson et al. 2015) and, together with the temporal dynamics of SGD biogeochemistry (Richardson et al. 2017) at this location, discern nutrient parameters and microbial communities that relate to SGD both directly and indirectly. To characterize temporal variability in microbial ecology, we explored the effect of SGD-associated nutrient fluxes on microbial community structure over the course of a tidal cycle (57 hour experiment). We hypothesize that there is a distinct microbial community associated with SGD and ask whether SGD could be a source of FIB in coastal waters. We further ask how nutrients associated with SGD might indirectly impact reef microbial communities.

Methods

Water sample collection and processing

Black Point is located in Maunalua Bay on the southeastern coast of Oʻahu, Hawaiʻi. SGD fluxes to the nearshore environment at Black Point average 16,000 m^3 d^{-1} (Holleman 2011; Richardson, Dulai, and Whittier 2017). Previous work in this location has mapped out the spatial extent of SGD and its influence on multivariate biogeochemistry of waters in this bay (Nelson et al. 2015). Companion studies examined how the temporal dynamics of SGD influence (1) benthic algal community composition (La Valle, Thomas, and Nelson 2019), (2) coral accretion and erosion (Lubarsky and Silbiger 2018), (3) carbonate chemistry (Richardson et al. 2017), and

(4) coral reef ecosystem metabolism (Silbiger, Donahue, and Lubarsky 2020). SGD enters the coastal ocean at Black Point through point source springs. While Black Point has several springs, most SGD is focused through one dominant discharge location, which we term the "Spring". The Spring is typically submerged but is occasionally exposed during low tide. Waters collected from the Spring are characteristic of groundwater, with lower temperature and salinity and elevated concentrations of NO^{3-}+ NO^{2-}, SiO_4^{4-}, PO_4^{3-}, and Rn, when compared with the surrounding marine waters (Nelson et al. 2015; Richardson, Dulai, and Whittier 2017; Richardson, Dulaiova, and Whittier 2015). Previous work has established four distinct biogeochemical zones that extend outward from the Spring (Nelson et al. 2015). Based on these zones, we established a time series at three locations ~5m ("Transition Zone"), 50m ("Diffuse Zone"), and 120m ("Ambient Reef") from the Spring, in addition to direct sampling of the Spring, along a transect running roughly perpendicular to shore to contrast the temporal dynamics within these zones (Richardson et al. 2017). A piezometer was used to collect samples from the Spring, and discrete bottle samples were taken from all reef sites at approximately 20 cm below the surface at each of the sites every 4 hours over the course of 57 hours for a total of 16 sampling events.

Samples were collected in acid-cleaned 1 L polycarbonate bottles that were triple-rinsed with sample water and immediately transported on ice to the lab for processing. Nutrient, flow cytometry, and salinity samples were subsampled and stored until they could be processed. Flow cytometry samples were immediately fixed to 0.5% paraformaldehyde and stored at -80° C until analysis following Nelson et al. (2015). Salinity samples were refrigerated and analyzed following (Richardson et al. 2017). The remaining water was pumped via platinum-cured silicone tubing using a peristaltic pump through a 0.2 um sterile polyethersulfone filter. The filtrate was subsequently collected for dissolved nutrient analysis in acid-washed 60 mL high

density polyethylene bottles and stored at 4º C and for fluorescent dissolved organic matter (fDOM) in amber glass vials with Teflon lined caps stored at 4º C in the dark away from volatile organics, both of which were analyzed according to Nelson et al. (2015). A total of 520 mL was passed through the filter from each sample, and the cartridge was pumped dry with air and frozen dry until DNA could be extracted for microbial community analysis using the Qiagen DNEasy Powersoil kit according to the manufacturer's directions, replacing the soil with a filter.

Bacterial SSU rDNA (16S) amplicon sequencing and analysis

The V3-V4 hypervariable regions of the 16S rRNA genes were amplified from extracted DNA using primers 341F and 806R with unique paired end oligonucleotide sequence indices assigned to each sample for multiplex sequencing library preparation as described in Kozich (2013). Polymerase Chain Reaction was performed in 25 μL volumes with 1 μL of DNA template. Reaction conditions consisted of an initial denaturing step at 98ºC for 1 minute followed by 30 cycles of denaturation at 98 ºC for 15 seconds, annealing at 55 ºC for 30 seconds, elongation at 72 ºC for 30 seconds, with a final extension at 72 ºC for 1 minute. Equimolar amounts of amplicons from each sample were mixed and purified using the SequalPrep™ Normalization Plate following the manufacturer's protocol and submitted to the Hawai'i Institute of Marine Biology Evolutionary Genetics Core Facility for 600 cycle paired-end sequencing using the Illumina MiSeq V3 chemistry.

Raw sequence data was processed using a custom bioinformatic pipeline detailed previously (Arisdakessian, Cleveland, and Belcaid 2020; Jani et al. 2021). Sequences were denoised into unique amplicon sequence variants (ASVs) at 100% nucleotide identity using the dada2 package in R (Callahan et al. 2016). Reads were truncated at position 260/220

(forward/reverse) and were discarded if they contained one or more bases with quality scores less than 2, or more than 3 expected errors using the *filterAndTrim()* function. The *learnError()* and *dada()* functions were used with default parameters to denoise, and reads were merged using the *mergePairs()* function. Any pairs containing more than one mismatch or an overlap of fewer than 20 bases were discarded. Sequence alignment and annotation were performed in mothur v1.42.3 using the SILVA.nr V132 SSU database. Sequences with start or stop positions outside of the over-all 5th-95th percentile range were discarded. Potential chimera sequences were removed with *chimera.vsearch()*. Taxonomies were assigned using the *classify.seqs()* and *classify.otus()* functions. We identified, quantified, then removed from further analysis all mitochondrial or chloroplast OTUs, as well as sequences without at least a phylum level classification. For subsequent statistical analysis, alpha-, and beta- diversity we randomly subsampled at 1800 sequences per sample using the *sub.sample()* function. Samples containing fewer than 1800 sequences were discarded. We defined microbial operational Taxonomic Units (OTUs) as unique sequences (commonly referred to as amplicon sequence variants or ASVs) using dada2 and refined in R using the lulu package. OTUs were merged if they co-occurred in every sample and one of the two ASVs had a lower abundance than the other in every sample. Finally, we discarded unreplicated OTUs (represented by 2 or less identical sequences across all samples).

Statistical Analysis

We used salinity as a proxy to track the SGD fraction across the reef as the Spring salinity at Black Point is characterized by low salinities with relatively little variability over tidal cycles (mean = 5.11, range = 4.80 – 5.61). To evaluate how biogeochemical and microbial

parameters covaried with SGD across the reef, second degree polynomial regressions were fit to salinity using the *poly* function in R (*stats* package, (R Core Team 2020) to differentiate between water quality and microbial parameters that were both linearly and non-linearly associated with either SGD or marine waters. To evaluate the relative effect strength of linear and polynomial coefficients in each model, standardized regression coefficients (β-weights) were calculated using *lm.beta()* (Behrendt 2014). Response variables that were linearly correlated with salinity (salinity p-value ≤ 0.05) and with a linear β-weight ($\beta_{L)}$ greater than the quadratic β-weight (β_Q) were considered to be conservatively mixing (e.g., associated with SGD if they contained a negative linear coefficient or of marine origin if they contained a positive linear coefficient). Response variables with a significant negative quadratic term and where the absolute value of β_Q was greater than β_L were considered to be nonlinear and non-conservative and enriched in mid-salinities. Two models were created to explore parameter behavior with respect to salinity/SGD: (1) Model 1 describes SGD-associated parameters, and (2) Model 2 describes the parameters associated with mid-salinity ranges. In order to establish where in the reef nonlinear parameters were enriched and the degree to which this pattern was influenced by tidal height, both mid-salinity elements as well as those that contained no significant terms in Model 1 were further interrogated using linear mixed effects models using the *lme4* and *lmeTest* packages in R (Bates et al. 2015; Kuznetsova, Brockhoff, and Christensen 2015) (Model 2): fixed effects included sampling location as a categorical variable (site), tidal height as a continuous variable and their interaction term; sampling event (every ~4 hours) was included as an orthogonal random intercept to account for repeated measures. Prior to statistical analysis, relative abundances of microbial taxa were angular transformed (arcsine of square root). Chemical parameters were log10 transformed when appropriate to best approximate the Gaussian distribution assumed by

each model, though fDOM parameters were normally distributed and thus not transformed. To compare microbial communities between sampling locations, multivariate analyses of site and tidal height were performed for microbial communities using the vegan package in R (Oksanen et al. 2019). Weighted unifrac distance matrices were constructed from ASV relative abundances. Permutational analysis of variance (PERMANOVA) was performed using the *adonis()* function and nonmetric multidimensional scaling with the *metamds()* function from the *vegan* package (Oksanen et al. 2019). Additional pairwise PERMANOVA tests were conducted using the *pairwise.adonis* package (Martinez Arbizu 2017).

Results & Discussion

Surface water and SGD chemistry

Consistent with previous results, the four sampling sites (Spring, Transition, Diffuse, and Reef) exhibited distinct biogeochemistry at low tide when SGD was greatest (Figure 2.1, Table S2.1). At high tide, in the absence of substantial SGD inputs and increased tidal mixing with marine waters, water chemistry was more homogeneous (Figure S2.1, Table S2.1). Ranges and geometric means of biogeochemical parameters collected at each of the four sites during the entire sampling period, including all low and high tide intervals, are displayed in Table 2.1 along with their statistical association to sampling site (e.g. site effect). As reported in Nelson et al. (2015) and Richardson et al. (2017), nutrients were higher in samples collected from the SGD Spring compared with concentrations of nutrients on the reef. In our study, geometric mean concentrations of nutrients at the Spring ($mean_{Spring}$ SiO_4^{4-} = 569 μM, NO^{3-}+ NO^{2-} = 166μM, PO_4^{3-} = 3 μM) were more than twenty times higher than those collected from ambient marine

waters at the Reef site ($mean_{Reef}$ SiO_4 =19.47 μM, NO^{3-}+ NO^{2-} = 2.57 μM, PO_4^{3-} = 0.16 μM). Similarly, humic-like fDOM components and indices were up to two times as high in the Spring when compared to the Reef site ($mean_{Spring}$ Coble A = 0.042, HIX = 5.37, M:C 1.13; $mean_{Reef}$ Coble A = 0.018, HIX = 2.28, M:C = 1.00), consistent with Nelson et al (2015).

Direct and indirect effects of SGD on surface water chemistry

To investigate the potential effects of SGD on surrounding waters, parameters that are (1) negatively linearly correlated with salinity are annotated as "SGD", (2) positively linearly correlated with salinity are annotated as "Marine", and (3) related to salinity via a non-linear quadratic relationship are annotated as "Mid-Salinity".

We examined the effect of tide on SGD-associated nutrient delivery across the four sampling zones at low and high tide (Table 2.2, Table S2.1). Statistically significant relationships between salinity and each of SiO_4^{4-}, NO^{3-}+ NO^{2}, PO_4^{3-}, Coble A, HIX, and M:C at low tide were mirrored by the site effects identified and described above in the overall dataset. These linear correlations suggest that they co-occur with SGD, as expected for many of the listed parameters. At high tide, several of the same parameters (SiO_4^{4-}, NO^{3-}+ NO^{2}, PO_4^{3-}, Coble A, and HIX) showed a linear association with salinity as well but the strength of the regression was reduced, as evidenced by their relative slopes, indicating a decreased effect of SGD. Generally, parameters with large concentration differences, such as those for SiO_4^{4-}, NO^{3-}+ NO^{2}, and PO_4^{3-} between the Spring and Ambient Reef sites, maintained a significant site effect with salinity at high tide, while those with smaller concentration differences did not (Table 2.2).

To determine the indirect effects of SGD on reef biogeochemistry, we also tested for nonlinear relationships between salinity and other parameters as described above by the site

effect denoted “Mid-Salinity”. Two parameters, ammonium (NH_4^+) and Coble T, displayed a significant quadratic relationship with salinity (Table 2.1). These were interpreted as being enriched by interactions between SGD and reef location (Figure S2.2). Similar effects were observed for both nutrient parameters at low tide (Table S2.1). At high tide, there was no difference between concentration of Coble T at any of the sites, but the highest concentrations of NH_4^+ were maintained at mid-reef locations relative to Ambient Reef site.

Microbial communities associated with SGD

Out of a total of 228 bacterial families found in this study, we identified 117 bacterial families that were inversely correlated with salinity and, thus, associated with SGD. For simplicity, the 30 most abundant of these families are displayed (Figure 2.2); a complete list of significantly correlated families is provided in Supplementary Materials (Table S2.2). SGD-associated bacteria were dominated by Alpha- and Gamma-proteobacteria including Sphingomonadaceae, Cellvibrionaceae, and Alteromonadaceae (mean relative abundances in the Spring were 17.38%, 4.74%, and 4.67%, respectively). Most SGD-associated taxa were abundant in Spring samples and rare or absent from samples collected at other locations. This included members of the Deltaproteobacteria and the Patescibacteria, which made up ~2.5% and 3.0% of SGD- associated OTUs, but were below .015% and 0.05% in reef water samples, respectively. A number of rarer taxa were also found only in the Spring, including members of the Families Burkholdariaceae (2.2%), Oligoflexaceae (1.1%), and Ardenticatenaceae (1.6%) which are generally associated with freshwater habitats.

Because a large portion of the total microbial richness was associated primarily with Spring samples, we further sought to identify bacterial families that were persistent outside of the

SGD Spring. These 'persistent' bacterial families were sourced from SGD but found throughout the reef and identified based on their percent change with salinity and their presence in four or more samples. Eight families displayed shallower than average slopes in relationship with salinity (Table S2.2). These families were SGD-associated, persistent on the reef, and included Rhodobacteraceae (mean relative abundance Spring = 6.75%, Transition Zone = 3.77%) and Arcobacteraceae (mean relative abundance Spring = 1.7%, Transition Zone = 0.90%) (Figure 2.2 inset). In freshwater systems, Arcobacteracceae serve as potential FIB (Collado 2008, Lee 2012), and their persistence here could provide further evidence for contamination of groundwater by OSDS leachate (Richardson et al. 2017). Because SGD is often sourced from unconfined coastal aquifers in these and similar volcanic islands, it could provide a mechanism for microbial communities to travel from terrestrial to marine ecosystems. A recent study of Oahu groundwater found good water quality across 37 aquifers and no evidence of sewage-specific biomarkers, though they did note substantial bacterial diversity among groundwater samples and types (Kirs et al. 2020). While (Kirs et al. 2020) found no evidence of FIB in groundwater, soil samples did contain fecal coliforms and Enterococcus. Additionally, because high concentrations of Enterococcus are common in tropical beach sands, it may not be a reliable FIB in tropical locations in general (Fujioka et al. 1998; Cui et al. 2013). The complexity of these results underscore the need for understanding the physical and biological processes that govern the sources and fates of microbial communities in groundwater across diverse environmental settings.

The dominant bacterial families that we found associated with SGD are common in surrounding environments, likely highlighting hydraulic connectivity of this location. Sphingomonadaceae dominated Spring samples and are commonly found in Oahu soils (Kirs et

al. 2020). Cellvibrionaceae and Alteromonadaceae are common in Hawaii beach sands (Cui et al. 2013), and each made up substantial portions of the taxa found in Spring samples. Other groups found primarily in the Spring are commonly associated with subterranean estuaries (STEs). Deltaproteobacteria, such as the Desulfobacteraceae, have been previously reported in coastal aquifers on Oahu (Kirs 2020) and are key members of the STE, noted for their enhanced iron- and sulfur-cycling capabilities(McAllister et al. 2015). Other families of Deltaproteobacteria such as Bdellovibrionaceae, and Oligoflexaceae, are known predators of other bacteria(Waite et al. 2020). Along with Patescibacteria, which are thought to be episymbiotic (Tian, Ning, and He 2020), these microbes likely benefit from the high density of cells found in coastal aquifers (Chen et al. 2019; Hong et al. 2018) and were not persistent in marine waters, where such lifestyles may be difficult to maintain.

Microbial communities associated with the mid-reef environment

To understand how reef biogeochemistry and the indirect effects of SGD influenced microbial populations we identified taxa with significant nonlinear (quadratic) relationships to salinity or sampling site. In all, 19 families were identified (Figure S2.3), and the most prevalent of which are displayed in Figure 2.3. Taxa with a significant non-linear relationship with salinity and sampling site were most abundant in the Transition and Diffuse zones and included Flavobacteriaceae (mean relative abundance Spring = 11.39%, Transition Zone = 33.94%, Diffuse Zone = 31.53%, Ambient Reef = 30.61%) and Rubritaleaceae (mean relative abundance Spring = 0.43%, Transition Zone = 2.47 %, Diffuse Zone 1.23%, Ambient Reef = 0.53 %). Taxa with nonlinear relationships to salinity and sampling site that were also influenced by water level included Vibrionaceae (mean relative abundance Spring = 2.28% Transition Zone = 6.43%

Diffuse Zone = 3.38% Ambient Reef = 2.72%) and unclassified Verrucomicrobiales (mean relative abundance Spring = 0% Transition Zone =0.34% Diffuse Zone = 0.24% Ambient Reef = 0.14%) which were most abundant in the Transition Zone at low tide.

Previous work has demonstrated that SGD increases primary production (Johnson and Wiegner 2014; Adolf et al. 2019) and, at this location, stimulates NEP (Silbiger, Donahue, and Lubarsky 2020). Increased NEP is expected to result in increases in dissolved organic matter (DOM) (Mueller et al. 2016; Baetge et al. 2020). In our study, DOM is captured by the DOC and fDOM measurements, the latter comprising both conservative SGD-associated humic components and a non-linear proteinaceous component (Coble T). While DOC appeared marginally enriched at mid-reef locations, mean concentrations did not differ significantly among sites ($p = 0.90$ and Figure S2.2). Coble T was significantly enriched at mid-reef sites and represents a protein-like form of fDOM that is reportedly elevated in coral reefs (Matthews et al. 1996). Both have been observed to be qualitatively enriched mid-reef at this location in spatial surveys at low tide (Nelson et al. 2015).

Corals have been shown to increase production of Coble T in response to nutrient stimulation (Quinlan et al. 2018), and it is therefore plausible that SGD-driven nutrient enrichment of the reef stimulates coral production of this fDOM component at Black Point. Bacterial remineralization of proteinaceous DOM released by nutrient-enrichment of benthic producers is likely to result in the simultaneous draw down of DOM and the production of NH_4^+. Linear regressions revealed three families with significant linear correlations to NH_4^+: Vibrionaceae, Prolixibacteraceae and Rubritaleaceae (Figure S4). Some mid-reef populations, like Vibrionaceae, are known estuarine taxa and may have a metabolic preference for these mid-salinity ranges. It is notable that populations of copiotrophic Gammaproteobacteria, such as

Vibrionaceae, Coxiellaceae and Psychromonadaceae, or the broad Bacteroidetes family Flavobacteriaceae, appear to be enriched at mid-reef locations, suggesting that there may be more rapid consumption of DOC in the mid-reef sites, and this consumption could mask spatial differences in DOC cycling/production, which may explain why there were no statistically significant spatial trends in DOC across the reef flat. The implication that mid-reef enrichment of NH_4^+ may be driven by microbial remineralization of protein-like fDOM components released by corals under SGD-associated nutrient enrichment deserves further exploration.

Multivariate patterns in microbial community diversity and composition

Overall, sampling location and the interaction of sampling location and tidal height were significant factors influencing microbial community composition ($p < 0.005$) and accounted for 32.5% and 9.2% of the observed variation between microbial communities, respectively (Figure 2.4 a). Pairwise comparisons between sampling locations demonstrate that samples collected from the Spring host the most distinct communities (Table 2.3). At low tide, both the Transition and Diffuse zones were distinct from Ambient Reef site, but at high tide, these differences dampened, and no pairwise differences were observed between the microbial communities collected from each sampling location (Table 2.3). Finally, we tested whether the overall community diversity differed by site. Community dispersion was greatest in Spring samples (Figure S2.4). Microbial communities collected from the Spring also displayed the greatest Shannon diversity, which decreased with distance from the SGD spring (Figure 2.5a). This trend was mirrored by species evenness, which was highest in the Spring compared with all reef locations (Figure 2.5b). Lastly, species richness was significantly higher in samples collected

from the Spring and Transition Zones compared with those collected from the Diffuse Zone and Ambient Reef site (Figure 2.5c).

Hydrological processes that drive SGD likely contribute to the observed diversity and distinct microbial assemblages at the Spring site. Over the tidal cycle, this site undergoes environmental extremes, at times being completely exposed or submerged by the tide. Environmental perturbations, like those described above, and physical processes that influence SGD also subject microbial communities in this microenvironment to bidirectional inoculation of taxa from marine and freshwater endmembers (Ruiz 2020) and confer resilience in existing microbial communities (Cui et al. 2013). Indeed, bacterial populations that were observed exclusively in Spring samples or persistent in mid-reef sites are commonly found in Oahu aquifers (Kirs et al. 2020) and/or associated with STEs (Figure 2.6). However, only a handful of the SGD-associated microbial populations were persistent at mid-reef sites as well, suggesting that physical processes are capable of transporting microbial populations associated with SGD into reef locales. In our study, the salinity from samples collected from the Spring were consistently low which could contribute to the distinct microbial community identified in this location since many freshwater bacteria cannot sustain the metabolic demands of marine environments. Further, other factors (e.g., functional or metabolic) may prevent these organisms from establishing appreciable abundances.

The enhanced microbial richness continued into mid-reef sites where SGD-associated nutrients have been previously mapped (Nelson et al. 2015) and are known to stimulate ecosystem production (Silbiger, Donahue, and Lubarsky 2020). At low tide, microbial populations in the Transition Zone separated from those observed in more marine waters at the Ambient Reef site (Figure 2.4). Copiotrophic bacteria, known to quickly breakdown labile

organic matter, were abundant and coincided with elevated concentrations of NH_4^+ in these locations. This finding suggests that the combination of nutrients associated with SGD and organic matter from reef primary producers are capable of supporting distinct microbial communities. A distinguishable community was observed in samples collected from the Diffuse Zone and Ambient Reef sites. This community was marked by slow growing marine bacteria typically associated with oligotrophic waters, such as SAR11 and SAR116, and further enriched with taxa associated with productive reefs including Endozoicomonadacea and Synechococcales.

Conclusions

SGD is an important phenomenon connecting terrestrial and marine environments. Until now the spatial and temporal effects of the SGD-associated nutrient flux on marine microbial communities had not been described. In our study the highest concentrations of SGD-associated nutrients were observed at low tide, near the SGD spring and decreased with distance away from the Spring. Distinct microbial communities were evident along the SGD gradient and were different from microbial communities observed in both the mid-reef and ambient reef waters. Increased relative proportions of copiotrophic bacteria observed at mid-reef sites coincided with waters that were enriched with ammonium and Coble T, indicating that SGD-associated nutrients indirectly influence microbial populations by stimulating reef benthic producers. At high tide, water chemistry and microbial community structure were largely homogenized due to increased tidal mixing and minimal SGD influence. However, high concentrations of ammonium remained at mid-reef sites relative to ambient marine waters indicating that processes, not captured in this study contribute to the observed trends. In this study, our focus centered on planktonic microbial

communities. However, surveys of the benthic community that include both surface attached microbial communities as well as macro-organisms could greatly benefit future works.

Table 2.1. Water chemistry by sampling site
Geometric mean values of each chemical parameter are displayed. Polynomial regressions were used to identify chemical parameters with significant linear or quadratic relationships to salinity. Parameters with significant linear relationships that were inversely related to salinity were considered to be SGD associated. Significant linear relationships that are positively correlated with salinity are considered to be marine. Parameters with a significant quadratic relationship, were associated with mid-salinity ranges.

Parameter	**Spring**	**Transition**	**Diffuse**	**Reef**	**Parameter Association**	**Significant Site Effect**
Salinity	**5.11**	**25.74**	**29.68**	**33.65**		
NO^{3-}+ NO^{2-} (µmol/L)	165.90	19.89	9.67	2.57	SGD	
Phosphate (µmol/L)	3.22	0.74	0.37	0.16	SGD	
Silicate (µmol/L)	569.40	112.49	55.73	19.47	SGD	
DON SFIA µM	12.80	7.24	4.86	5.27	SGD	
CobleA	0.042	0.033	0.023	0.018	SGD	
HIX	5.37	3.60	3.16	2.28	SGD	
M.C	1.13	1.04	1.01	1.00	SGD	
NH_4 (µmol/L)	0.183	1.054	0.787	0.416	Mid-salinity	Site
CobleT	0.017	0.023	0.014	0.017	Mid-salinity	
Bacterioplankton	34.17	154.55	196.38	194.27	Marine	Site
Eukaryotes	9.34	30.38	28.71	20.06	Marine	
DOC (µM)	36.30	77.60	75.89	67.21	Marine	
Chlorophyll A (µg / L)	0.057	0.592	0.607	0.235	None	Site

Table 2.2. Results of linear regression against salinity by tide
Slopes from linear regressions with salinity are displayed for each chemical parameter (nutrient) by tide. Parameters with significant linear relationships that were inversely related to salinity were considered to be SGD associated. Significant linear relationships that are positively correlated with salinity are considered to be marine. Parameters with a significant quadratic relationship, were associated with mid-salinity ranges. Because Spring samples could not be collected at high tide, significant mid-salinity relationships appear as "SGD".

Nutrient	**Low Tide Slopes**	**Parameter Association (Low Tide)**	**High Tide Slopes**	**Parameter Association (High Tide)**
NO^{3-}+ NO^{2-} (μmol/L)	-303.47	SGD	-20.79	SGD
Phosphate (μmol/L)	-5.84	SGD	-0.62	SGD
Silicate (μmol/L)	-1113.33	SGD	-108.23	SGD
DON.SFIA μM	-16.63	SGD	0.89	
CobleA	-0.05	SGD	-0.01	SGD
HIX	-5.71	SGD	-1.95	SGD
M.C	-0.23	SGD	0.00	
NH_4 (μmol /L)	0.09	Mid-salinity	-0.77	SGD
CobleT	0.00	Mid-salinity	0.01	
Bacterioplankton	306.03	Marine	59.62	
Eukaryotes	19.06	Mid-salinity	18.03	
DOC (μM)	68.53	Marine	23.44	
Chlorophyll A (μg / L)	0.20	Mid-salinity	0.12	

Table 2.3. Pairwise comparisons of microbial communities

Pair	All Data		Low		High	
	R^2	P-value	R^2	P-value	R^2	P-value
Spring vs Transition	0.373	0.0030	0.400	0.008		
Spring vs Diffuse	0.416	0.0020	0.435	0.0030		
Spring vs Reef	0.504	0.0020	0.541	0.0040		
Transition vs Diffuse	0.0758	0.025	0.156	0.011	0.0785	0.76
Transition vs Reef	0.156	0.0020	0.292	0.0030	0.130	0.76
Diffuse vs Reef	0.0454	0.11	0.057	0.22	0.106	0.76

Figure 2.1. Water chemistry at low tide separates samples by collection site
Nutrients that are associated with SGD are inversely related to salinity, and are enriched in the Spring and Transition Zone.

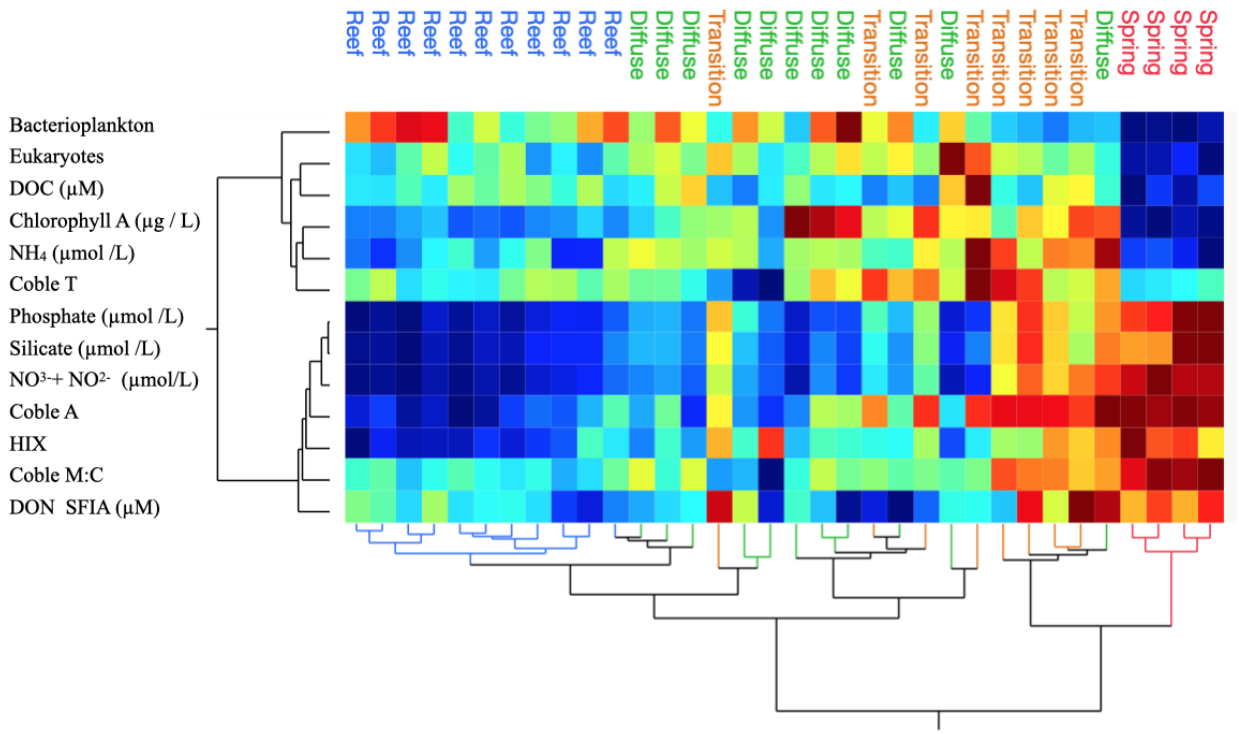


Figure 2.2. 30 Most abundant SGD associated families
117 microbial families are negatively correlated with salinity. These families are dominated by alpha- and gammaproteobacteria, and are highest in Spring samples but decrease linearly with distance from the SGD seep indicating that the subterranean estuary or groundwater may be a source of these microbes. Most of the SGD-associated taxa are found almost exclusively in Spring samples. Only a few taxa are persistent on the reef and found in abundance at other sampling sites. Three representative, persistent taxa are plotted against salinity (inset).

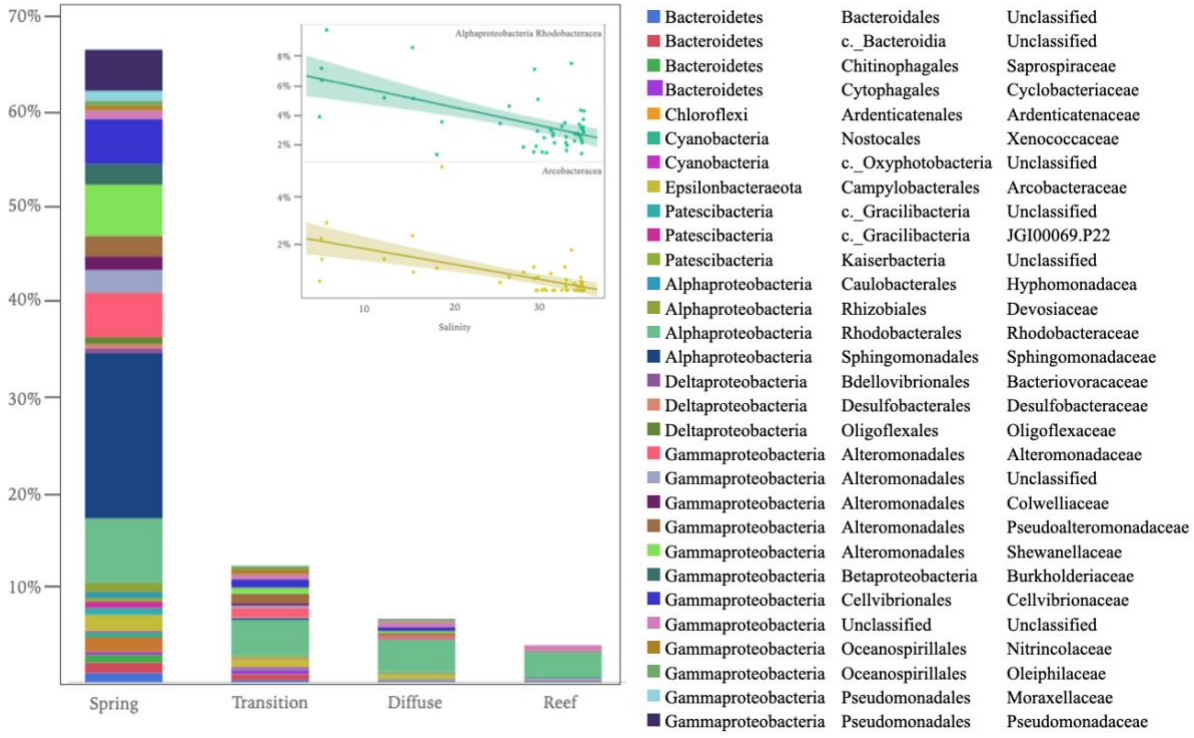


Figure 2.3. Microbial taxa enriched at mid-salinities
19 Families had significant quadratic relationships with salinity. These families were most abundant at mid-reef sites and in samples with moderate salinities.

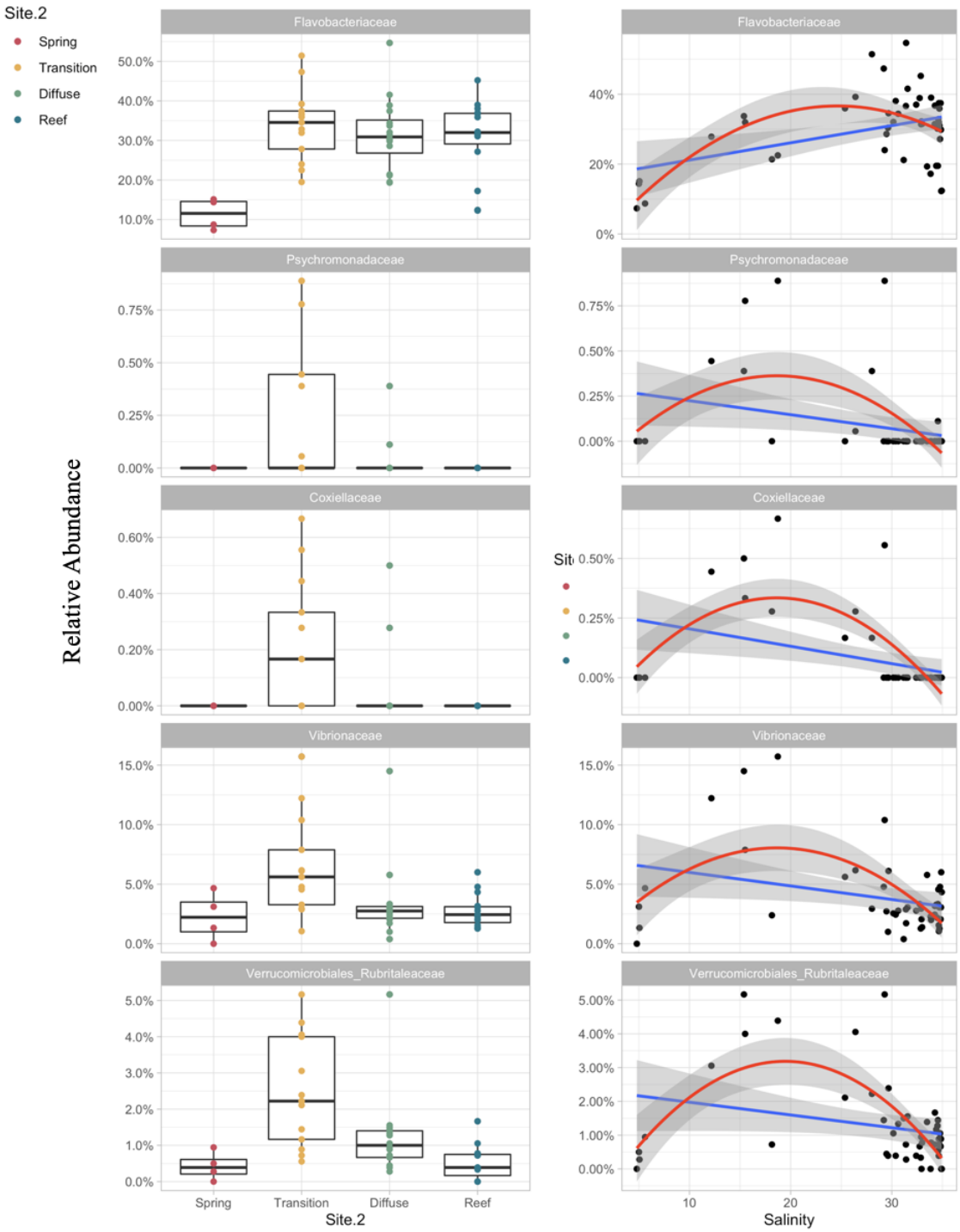
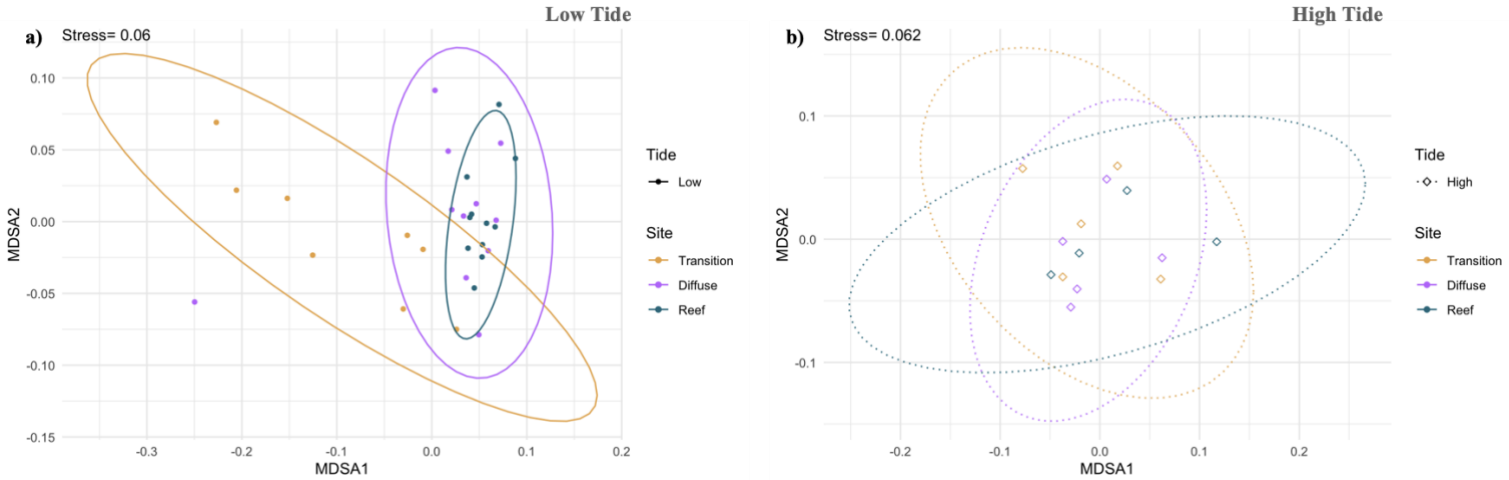


Figure 2.4. Ordinations of microbial communities
Transition Zone, Diffuse Zone, and Ambient Reef microbial communities by tide. Microbial communities of the Transition Zone separate from Diffuse Zone and Ambient Reef waters at low tide, but no difference is observed at high tide.



c) Microbial community variance partitioning

Dataset	Parameter	R^2	P-value
Full	Site:Water level	0.0923	0.005
	Site	0.325	0.001
	Water Level	0.0259	0.074
Low Tide	Site:Water level	0.0929	0.048
	Site	0.417	0.001
	Water Level	0.0195	0.396
High Tide	Site:Water level	0.0816	0.999
	Site	0.133	0.687
	Water Level	0.0694	0.544

Figure 2.5. Microbial diversity by sampling location
Overall microbial diversity, Shannon diversity is highest in the Spring and decreases with distance from SGD seep. Species richness is higher in the Spring and Transition Zone, but evenness is highest in the Spring compared with all other sampling locations.

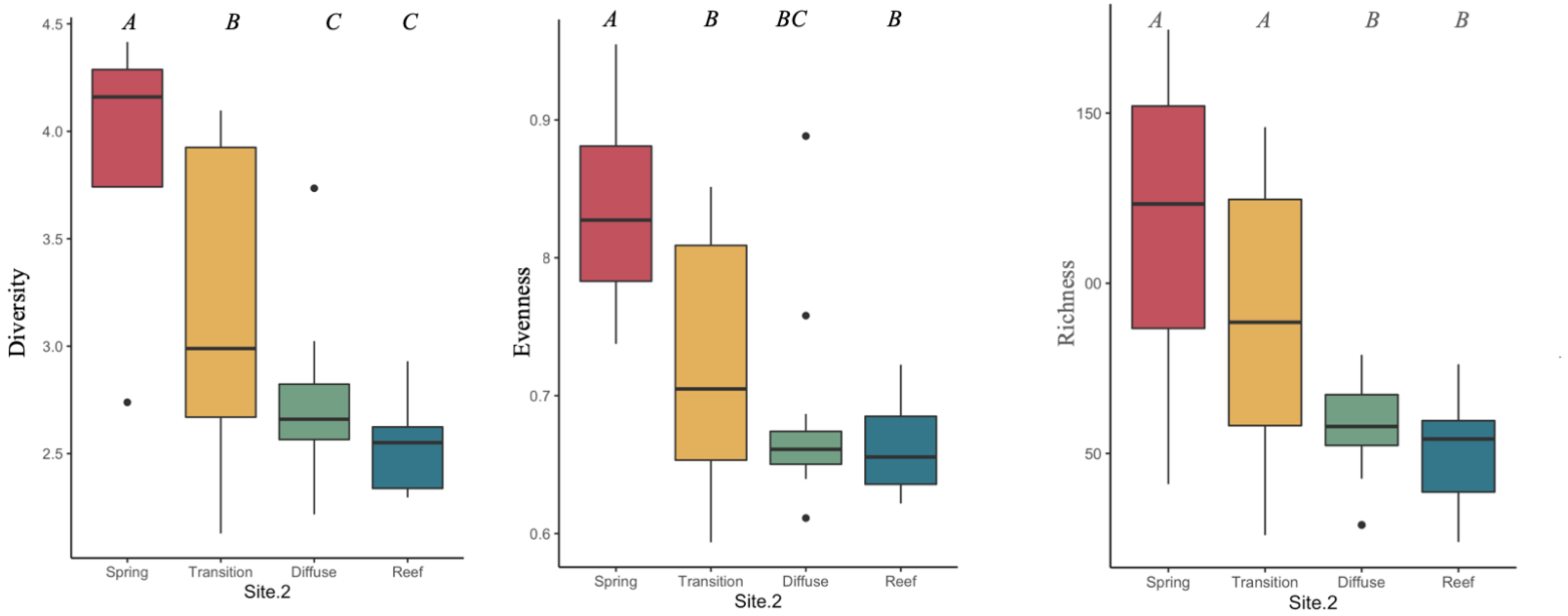
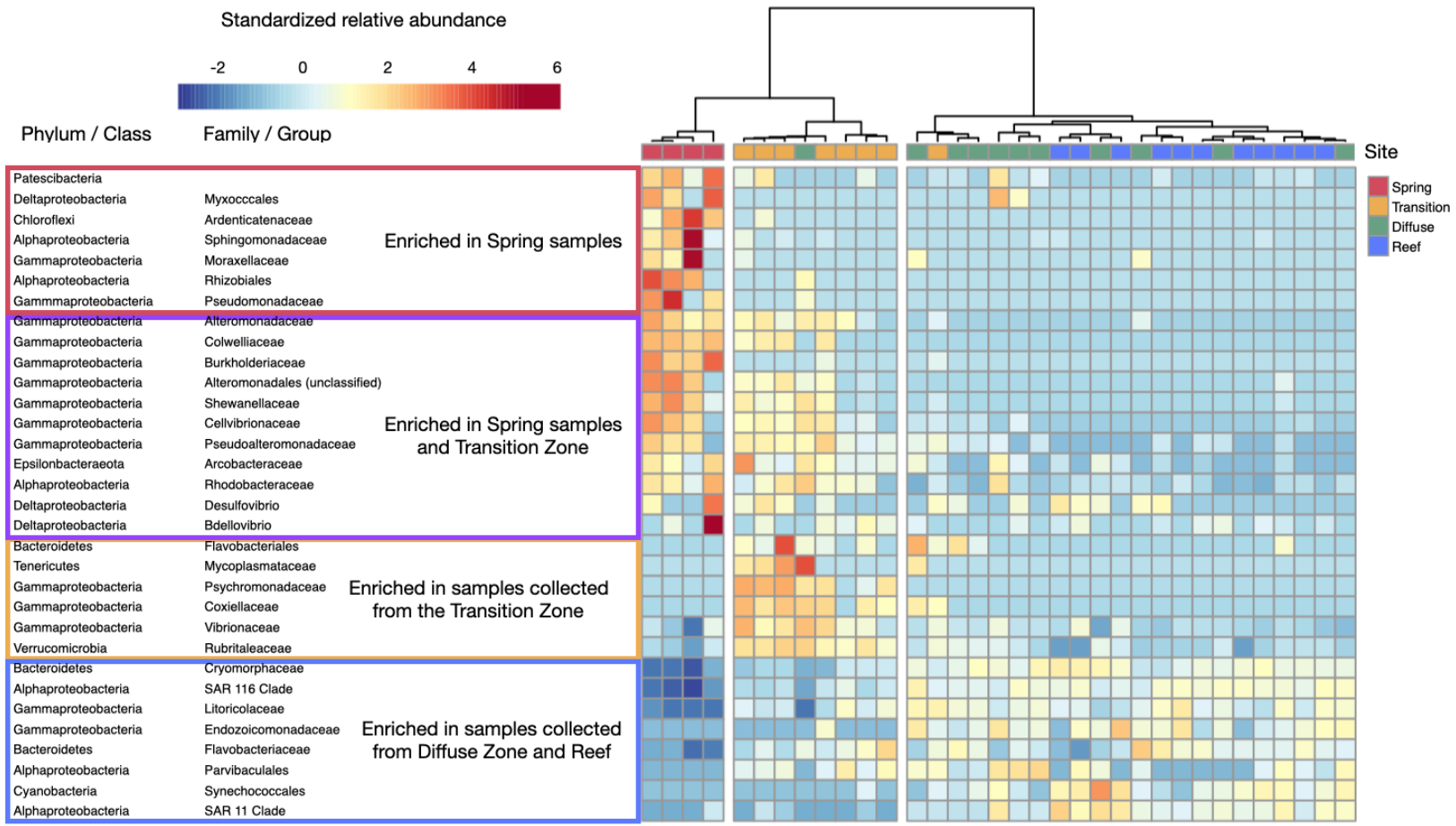


Figure 2.6. Hierarchical clustering of samples according to bacterial community similarity
Relative abundances of each family are z-scored and used to organize each sample type. Sample types are color coded by location (top row); samples collected from the Spring in red, Transition Zone in gold, Diffuse Zone in green, and Reef in blue.



CHAPTER 3
CORAL REEF BIOFILM BACTERIAL DIVERSITY AND SUCCESSIONAL TRAJECTORIES ARE STRUCTURED BY REEF BENTHIC ORGANISMS AND SHIFT UNDER CHRONIC NUTRIENT ENRICHMENT

Abstract

Work on marine biofilms has primarily focused on host-associated habitats for their roles in larval recruitment and disease dynamics; little is known about the factors regulating the composition of reef environmental biofilms. To contrast the roles of succession, benthic communities and nutrients in structuring marine biofilms, we surveyed bacteria communities in biofilms through a six-week succession in aquaria containing macroalgae, coral, or reef sand factorially crossed with three levels of continuous nutrient enrichment. Our findings demonstrate how biofilm successional trajectories diverge from temporal dynamics of the bacterioplankton and how biofilms are structured by the surrounding benthic organisms and nutrient enrichment. We identify a suite of biofilm-associated bacteria linked with the orthogonal influences of corals, algae and nutrients and distinct from the overlying water. Our results provide a comprehensive characterization of marine biofilm successional dynamics and contextualize the impact of widespread changes in reef community composition and nutrient pollution on biofilm community structure.

Introduction

Biofilms are complex communities of surface attached microorganisms that are encased in an extracellular polymeric matrix (Donlan 2002). They are ubiquitous in aquatic environments where they provide ecosystem services including primary production(Wagner et al. 2015), organic matter decomposition (Ardón and Pringle 2007), and nutrient cycling (Battin et al.

2003). The close proximity of cells and the ability of the extracellular matrix to capture and retain nutrients from the surrounding fluid make biofilms “hot spots” of biogeochemical cycling (Dang and Lovell 2016). Extrinsic factors including fluid dynamics and nutrient availability have been shown to influence biofilm community composition, cell density, and rate of succession (Singer et al. 2010; Olapade and Leff 2006; Lawes et al. 2016; Webb et al. 2006). In marine environments biofilms are known to play key roles in the settling and subsequent metamorphosis of invertebrate larvae (Sneed, Ritson-Williams, and Paul 2015; Hadfield 2011), an ecosystem service that is likely governed by the community composition of the biofilm (Sneed, Ritson-Williams, and Paul 2015; Besemer 2015).

Although the importance of marine biofilms is widely accepted, most coral reef microbiology studies have focused on planktonic and symbiotic organisms. Studies focusing on bacterioplankon communities on coral reefs have revealed active and dynamic heterotrophic bacterial assemblages influenced by reef residence time (Nelson et al. 2011; Glasl et al. 2019; Becker et al. 2020), diel ecosystem processes (Kelly et al. 2019), sources and concentrations of organic matter (Nelson et al. 2011; Quinlan et al. 2018; Cárdenas et al. 2018), reef benthic composition (Haas et al. 2016; Kelly et al. 2014; Zaneveld et al. 2016; Morrow et al. 2013), direct coral interactions (Weber et al. 2019; McNally et al. 2017) and nutrient availability (Kelly et al. 2014; Zaneveld et al. 2016). Similarly, populations of potentially pathogenic bacteria and virulence genes increase in the surface mucus layer of corals in response to increased nutrient loads (Shaver et al. 2017; Vega Thurber et al. 2014); and the microbiomes of physiologically sensitive corals may experience an overall decrease in microbial diversity accompanied by an increase in disease-associated microbes with ocean acidification and warming (Grottoli et al. 2018). In combination with other environmental stressors, nutrient pollution in coastal

ecosystems is implicated in shifts away from coral dominated reefs toward those dominated by fleshy algae (Fabricius et al. 2005; Smith et al. 2016). These changes in benthic cover may further impact the physical and chemical environment by altering physiological responses of the benthic community such as coral and algal photosynthesis (Langdon and Atkinson 2005; Ferrier-Pagès et al. 2000), coral and algal organic matter exudation (Quinlan et al. 2018) and ultimately impacting net community production and calcification rates (Silbiger et al. 2018). Organic matter exudates produced by algae have been shown to be compositionally distinct from those of corals (Quinlan et al. 2018; Nelson et al. 2013), potentially driving the restructuring of the planktonic microbial community as reefs shift to algal dominance (Haas et al. 2016; Kelly et al. 2014; Nelson et al. 2013; Mcdole et al. 2012; Dinsdale et al. 2008; Kelly et al. 2012), yet it is unclear how shifts in organic matter may affect biofilm microbial communities.

Because marine biofilms are important in mediating reef processes and maintaining biodiversity (e.g. nutrient cycling, larval settlement, etc.) (Quinlan et al. 2018; Qian et al. 2007; Whalan and Webster 2014), a comprehensive understanding of the taxonomic structure and function of marine biofilms is crucial for adequately assessing the ecosystem services they provide and evaluating their role in maintaining coral reefs. Further, the use of biofilms in the assessment of pollution and ecosystem recovery reveals shifts in microbial community structure and function in response to environmental perturbation (Romani et al. 2016). In this way biofilms can serve as indicators of ecosystem health. Understanding the environmental factors that determine biofilm community assembly and succession are the first steps toward a predictive characterization of biofilm structure and function. As biofilms are sourced from and subject to the surrounding water column (Jones et al. 2007) physical and chemical changes in the overlying water, as well as alterations in the planktonic microbial community, could significantly impact

marine biofilm communities. Succession in marine biofilms has been previously studied (Webster et al. 2004; Sweet, Croquer, and Bythell 2011; Witt, Wild, and Uthickea 2012) finding that surface associated microbial communities differentiate from planktonic communities within hours (Donlan 2002; Webster et al. 2004; Dang and Lovell 2000) and quickly progress from communities of primary settlers to established heterotrophic communities within a matter of days (Datta et al. 2016). The very first stages of biofilm establishment are governed by the population of microorganisms capable of attaching to a surface (Donlan 2002; Datta et al. 2016), which can be affected by physio-chemical properties of the substrate including rugosity, hydrophobicity and the nutrient content of the organic matter coating the surface (i.e. the conditioning film) of the substrate (Donlan 2002).

Presented here are the results of an experiment designed to test the core hypotheses that the organic resources derived from the surrounding benthic organisms and inorganic nutrient availability work interactively and orthogonally to influence biofilm assembly and succession. Over the course of six weeks, we tested the effect of constituent benthic organisms (corals, algae, and sand-associated microphytobenthos) and sustained nutrient loads (continuous micromolar enrichments in nitrate and phosphate at three concentrations) on the successional dynamics of developing marine biofilms on glass slides. We hypothesized that biofilm communities would differ from planktonic communities, that biofilms would differ between benthic organismal treatments, and that nutrient amendments would further differentiate microbial communities

Methods

Sample Collection

Samples of coral, macroalgae, and carbonate sand were collected from the fringing reef around Moku o Loʻe (Coconut Island) adjacent to the Hawaiʻi Institute of Marine Biology (21.435º, -157.787º) on October 12-16, 2015. Collections were in accordance with local regulations; corals were collected under the State of Hawaiʻi Division of Aquatic Resources Special Activity Permit 2015-17 to the Hawaiʻi Institute of Marine Biology. Corals were collected from the fringing reef on the southwest side of the island. Three individual colonies from the two dominant coral species in Kāneʻohe Bay, *Porites compressa* and *Montipora capitata*, were harvested and fragmented to produce 36 coral nubbins, 12 from each of 3 colonies. Each nubbin was buoyant weighed and equally sized nubbins (*P. compressa* = 24.8 ± 5.23g dry weight; *M. capitata* 21.9 ± 5.05g dry weight) were mounted on polystyrene frames using epoxy. Each coral frame held six nubbins (three *P. compressa* , and three *M. capitata*); coral nubbins were allowed to acclimate for 10 days prior to the start of the experiment. Macroalgae (*Gracilaria salicornia*) and sand samples were collected from a low energy, sandy reef flat on the northern side of Coconut Island in less than 1m depth, where *G. salicornia* is abundant and grows unattached to the substrate. Macroalgae samples were cleaned of visible invertebrates and epiphytes then wet weighed and split into 36 equal portions (11.0 ± 0.55g wet weight) and contained in polyethylene mesh boxes. Samples of carbonate sand were collected using a 7.5 cm diameter core, placed in 36 petri dishes, then placed undisturbed in experiment aquaria. More detailed information on sample collection is available from Quinlan (2018) and Silbiger (2018).

Experimental Design

Experimental aquaria were set up in an outdoor mesocosm facility and consisted of 27 six-liter, flow through, acid washed, polycarbonate aquaria which were divided between three, 1300 liter, shaded incubation tanks (9 aquaria per tank) used to maintain constant temperature (Figure 3.1; S3.1). Source water from Kāneʻohe Bay flows into the mesocosm facility first through a sand filter and 20 µm polyethylene cartridge filter before use in our experiment. The filtered seawater was subsequently pumped into 9 nutrient mixing header aquaria (10L) via multi-channel peristaltic pump; header mixing aquaria (3 replicate aquaria of each of three nutrient treatments) were maintained at a 30-minute residence time, cleaned weekly and variously housed 5-15 small (3cm) coral fragments associated with a separate experiment. To create nutrient treatments in header aquaria, a bulk nutrient stock of potassium nitrate and potassium phosphate (3:1 molar N:P) was mixed at the beginning of the experiment and frozen in single use aliquots to maintain continuity throughout the experiment. Every other day, an aliquot of frozen nutrient stock was diluted to the appropriate concentration in seawater and administered to the header aquaria via peristaltic pump to mix. Treatments were maintained at three stable levels, including ambient (averaging 0.1 µM L^{-1} NO_3^- and 0.06 µM L^{-1} PO_4^{3-}) and medium and high enrichments averaging 2.68 and 6.64 µmol L^{-1} NO_3^-, respectively (Quinlan et al. 2018; Silbiger et al. 2018). Nutrient concentrations and N:P stoichiometry (2.46 ± 0.37 SD across all treatments) spanned natural inorganic nutrient conditions measured on reefs across the Hawaiian archipelago (Silbiger, Donahue, and Brainard 2017) and background nutrient conditions during the experiment were consistent with baseline concentrations in Kāneʻohe Bay (Drupp et al. 2011). Each aquarium held one of the three benthic organisms (either four coral frames, four algal mesh boxes, or four petri dishes of sand) and received one of the three nutrient

treatments (ambient, medium, or high nutrient addition) resulting in nine factorially-crossed treatments. Treatment aquaria were maintained at a 5 hour residence time and mixed with a submersible water pump. Each set of nine treatment aquaria was established in one of three independent 1300L flow-through incubator tanks to maintain thermal stability and was monitored over the course of six weeks. This resulted in a total of 27 aquaria comprising three independent replicates of each of the 9 treatments. Each nutrient treatment level in a 1300L tank was fed by an independent mixing header tank to ensure independence. To mitigate tank effects due to weather conditions and variations in light exposure; aquaria sets (blocks of 9 aquaria) were rotated between, and individual aquaria were shuffled randomly within, the larger incubator tanks once per week over the course of the 6-week experiment. All plastics used in this experiment, including aquaria, slide racks, and tubing were acid-washed and soaked for at least 72 hours in flowing seawater to remove plasticizers before starting the experiment.

Biofilm culturing and sampling

Glass slides have been previously shown to provide suitable substrate for marine biofilms and are known to result in more consistent and reproducible communities compared with ceramic tiles (Witt, Wild, and Uthicke 2011). Therefore, biofilms were cultured using glass slides suspended in the upper 10cm of each experimental aquarium (Figure 3.1). Slides were cleaned with alcohol and combusted to remove surface coatings and organic matter prior to the experiment. Slides were evenly spaced in polyoxymethylene slide racks typically used for microscopy staining, with ample space between each slide to allow for water to flow freely around each side of the glass slide. Racks were suspended using nylon line and polypropylene suction cups. Biofilm samples were destructively sampled by removing one glass slide from each

aquarium rack at each sampling point in a manner intended to minimize altering the fluid dynamics surrounding the remaining slides. Sterile polyester tipped swabs (Puritan 25-806 1PD) were used to sample biofilms from each slide; a standardized swabbing technique was used to minimize variability between samples and time points: Each side of a slide was swiped 10 times with a swab, turning the swab one quarter turn every 5 swipes. Slides were discarded after collection, and swab tips were placed in sterile tubes and frozen until DNA could be extracted. For analysis of planktonic bacteria, at each time point 40mL of seawater was collected from each of three replicate aquaria within each organism by nutrient treatment using an acid washed, rubber-free polyethylene syringe, pooled into a single 120mL sample and filtered through a 0.22 μm PES filter, resulting in 9 DNA samples from planktonic organisms at each time point (27 planktonic samples total). Filters were frozen at -80° C until DNA could be extracted. Genomic DNA from biofilm swabs and filters was extracted using the Epicentre MasterPure Complete DNA and RNA Purification Kit (MCD85201) using the protocol outlined by the manufacturer for DNA purification from plasmid or serum samples with the following modifications: samples were rotated throughout the lysis incubation and swabs or filters (depending on sample type) were aseptically removed following the addition of RNase and just before DNA precipitation steps. DNA was re-suspended in 50μL TE buffer. Analysis of dissolved organic matter (DOM) was performed using the 0.22 μm PES filtrate collected synoptically with the samples described here. Findings from the DOM analysis are reported separately(Quinlan et al. 2018).

Bacterial SSU rDNA (16S) amplicon sequencing and analysis

The V3-V4 hypervariable regions of the 16S rRNA genes were amplified using primers 341F and 806R (Klindworth et al. 2013), with unique paired end oligonucleotide sequence

“barcodes" assigned to each sample as described in Kozich (2013). Polymerase Chain Reaction was performed in 25μL volumes with 1 μL of DNA template or no-template control. Reaction conditions consisted of an initial denaturing step at 98 ºC for 1 minute followed by 30 cycles of denaturation at 98 ºC for 15 seconds, annealing at 55 ºC for 30 seconds, elongation at 72 ºC for 30 seconds, with a final extension at 72 ºC for 1 minute. Equimolar amounts of amplicons from each sample were mixed and purified using the SequalPrep™ Normalization Plate following the manufacturer‘s protocol and submitted to the Hawai‘i Institute of Marine Biology Evolutionary Genetics Core Facility for 600 cycle paired-end sequencing using the Illumina MiSeq V3 chemistry.

Raw sequence data was pre-processed into amplicon sequence variants (ASVs) at 100% nucleotide identity using the dada2 package in R version 4.0.3 (Callahan et al. 2016; R Core Team 2020). Reads were truncated at position 260/190 (forward/reverse) and were discarded if they contained one or more bases with quality scores less than 2, or more than 3 expected errors using the *filterAndTrim()* function. The *learnError()* and *dada()* functions were used with default parameters to denoise, and reads were merged using the *mergePairs()* function. Any pairs containing more than one mismatch or an overlap of fewer than 20 bases were discarded. Sequence alignment and annotation were performed in mothur v1.42.3 (Schloss et al. 2009) using the SILVA.nr V132 SSU database (Yilmaz et al. 2013). Sequences with start or stop positions outside of the over-all 5th-95th percentile range were discarded. Potential chimera sequences were removed with *chimera.vsearch()*. Taxonomies were assigned using the *classify.seqs()* and *classify.otus()* functions. We identified, quantified, then removed from further analysis all mitochondrial or chloroplast OTUs, as well as sequences without at least a domain level classification. For subsequent statistical analysis, alpha-, and beta- diversity we randomly

subsampled at 3300 sequences per sample using the *sub.sample()* function. Samples containing fewer than 3300 sequences were discarded. We defined microbial operational Taxonomic Units (OTUs) as unique sequences (commonly referred to as amplicon sequence variants or ASVs) using dada2 and refined in R using the lulu package (Frøslev et al. 2017). OTUs were merged if they co-occurred in every sample and one of the two ASVs had a lower abundance than the other in every sample. Finally, we discarded unreplicated OTUs (represented by 2 or less identical sequences across all samples). We further culled OTUs by discarding those with 10 or fewer sequences, either across all samples or within a given sample. In all, our sample set consisted of 106 samples, including 79 biofilm samples and 27 water samples (Table S2). Post-QC, gamma diversity for the dataset was 18,278 unique OTUs including 14,237 unique OTUs from biofilm samples compared with 4,041 unique OTUs found in water.

Statistical analysis

Multivariate analyses of time and treatment effects on biofilm and planktonic bacterial communities were performed on Bray-Curtis distance matrices constructed from OTU relative abundances using the *vegan* package in R (Oksanen et al. 2019) including permutational analysis of variance (PERMANOVA) with the *adonis()* function and nonmetric multidimensional scaling with the *metamds()* function. Additional pairwise PERMANOVA tests were conducted for biofilm samples within each time point using the *pairwise.adonis* package(Martinez Arbizu 2017). For comparison, all multivariate statistical models were additionally run with weighted Unifrac distance matrices (Lozupone and Knight 2005) and yielded nearly identical results (Table S3). When performing multiple tests, the false discovery rate was controlled by adjusting p-values according to Benjamini and Hochberg (Benjamini and Hochberg 1995). Linear

univariate mixed-effect models were performed using the *lme4* and *lmerTest* packages in R (Bates et al. 2015; Kuznetsova, Brockhoff, and Christensen 2015). Each experimental parameter: time point, benthic organism, and nutrient level, as well as factorial interaction terms, were included as fixed effects in the models, with holding tank and aquarium included as orthogonal random effects to account for environmental differences between experimental holding tanks (Figure 3.1) and repeated measures, respectively. These models were used to determine the response of alpha diversity metrics (richness and evenness) as well as each bacterial population in biofilm samples. Prior to statistical analysis, relative abundances of bacterial taxa were angular transformed (arcsine of square root) to best approximate the gaussian distributional assumptions of the model and the false discovery rate was controlled by adjusting p-values according to Benjamini and Hochberg (1995). Bacterial populations found to have significant fixed effects for time or organism were then screened for associations to specific timepoints or organismal treatments using the *randomForest* function in R (randomForest package, (Liaw and Wiener 2014) to assign discriminant scores (mean decrease in accuracy; MDA). For example, taxa with a significant fixed effect of time (FDR-adjusted $p < 0.05$) were evaluated for their predictive power (MDA score) in a random forest model for the three timepoints. Similarly, to identify taxa that were indicative of biofilms cultured with each benthic organism, we selected OTUs from the linear mixed model that were significantly influenced by benthic organism ($p < 0.05$) or time-organism interactions ($p < 0.05$). Because a positive MDA score indicates a variable performed better than a random permutation of variables when classifying each sample, taxa with positive MDA scores greater than two standard deviations from the mean MDA were interpreted as strongly associated with a treatment category and selected for visualization. Because multivariate nutrient effects manifested most clearly in the biofilms incubated with

coral, mixed-effect models testing for nutrient effects on bacterial populations (those with with significant nutrient or nutrient interaction fixed effects $p < 0.05$) were restricted to coral-associated populations: we pre-screened taxa for association with specific biofilm organismal treatment categories, first using random forest to evaluate all biofilm taxa for strong organismal associations, then selecting taxa with local variable importance scores (MDA) that were higher in coral treatments compared with either sand or algae. Thus, any taxon interpreted as associated with a particular fixed effect category was screened by both linear models and random forest for a robust and conservative assignment.

Results

Multivariate analysis of bacterial community structure variation

Planktonic and biofilm communities differed clearly across all time points and both clustered primarily by time point (Figure 3.2a). Specifically, a strong differentiation was observed between biofilm communities and planktonic communities across all time points (sample type $R^2 = 0.187$, $p = 0.001$), and each time point differed significantly, independent of sample type (time point $R^2 = 0.134$, $p = 0.001$; Figure 3.2c, Model 1 and Table S3.3). Mean bacterial diversity differed significantly between sample types ($p<0.01$), with evenness consistently higher in biofilms (mean = 0.879 ± 0.0479 sd) than in bacterioplankton communities (mean = 0.731 ± 0.0783 sd) at each time point ($p < 0.001$) and richness higher in biofilms by 6 weeks ($p = 0.002$) (mean richness at 6 weeks biofilm = 215.292 ± 82.95 sd, bacterioplankton = 149.596 ± 32.65 sd). Additionally, temporal variation in microbial communities differed between biofilms and plankton (Figure 3.2c, Model 1: sample type × time point $R^2 = 0.067$, $p=0.001$),

indicating that successional patterns observed in the biofilms were orthogonal to changes observed in the planktonic community.

Because of the clear distinction between planktonic and biofilm communities (Figure 3.2a), we additionally evaluated the fixed single and 2-way interactive effects of time, benthic organism and nutrient treatments on biofilm and planktonic communities separately (Figure 3.2b and c, Table S3.3). Since planktonic samples were pooled across replicates and primarily used as a reference for biofilm responses, interaction terms could not be evaluated in the plankton community. Within each sample type (biofilm or bacterioplankton), microbial community structure changed significantly through time (Figure 3.2b: Models 2 and 3, $p_{time} < 0.001$) and more than twice of the explained variance was attributed to time point in the planktonic community than in the biofilm community (Figure 3.2b: Models 2 and 3; R2 = 0.426 and 0.204, respectively). Relative to the strong directional succession of the biofilm community the planktonic community did not exhibit a clear directional trajectory, instead changing stochastically through time (Figure 3.2b). Pairwise PERMANOVA emphasized that successional patterns observed in the biofilm community follow a directional trajectory with greater changes observed between 2 and 6 weeks ($R^2 = 0.240$, $p < 0.001$) than between 2 and 4 or 4 and 6 weeks ($R^2 = 0.152$ and 0.082, respectively, $p < 0.001$) while planktonic communities were roughly equidistant between 2, 4 and 6 weeks ($R^2 = 0.290$ to 0.399, each $p < 0.001$; Figure 3.2b). We hypothesized that the composition of the benthic reef community (henceforth "organism") would influence biofilm communities from early successional stages. We further hypothesized that increasing inorganic nutrients could alter biofilm communities either indirectly, such as by influencing exudates of benthic organisms (Quinlan et al. 2018), or directly by shifting nutrient dependence away from autochthonous (within biofilm, presumably organic) sources toward

environmentally available inorganic sources. Both organism and nutrient manipulations had significant effects on biofilm community structure ($p < 0.001$) across all time points. Moreover, significant 2-way interactions were identified among all three variables ($p < 0.01$); a 3rd degree factorial model was also tested in biofilm communities but no significant three-way interaction ($p = 0.296$) was identified. The variance explained by the temporal effect ($R^2 = 0.204$, Figure 3.2c: Model 3) was greater than that of the organism ($R^2 = 0.123$) or nutrient effect ($R^2 = 0.063$), demonstrating that successional changes over time were the primary overall driver of community differentiation in these biofilms (Figure 3.2b, 2c).

Comparison of biofilm communities between time points

Because biofilm community structure differed most strongly by time point (Figure 3.2), to better clarify how marine biofilms are influenced by benthic organisms and inorganic nutrients we separately analyzed the biofilm community differentiation by organism and nutrient treatments within each time point (Figure 3.3). Both organism and nutrient level were found to significantly impact the resulting biofilm communities at each time point, with organism consistently explaining more variance between biofilm communities than nutrient level (~26% compared with 15%) (Figure 3.3). Organism and nutrient effects were increasingly orthogonal through time and significant interactions between organism and nutrients strengthened over time (2-week $R^2 = 0.140$, $p = 0.003$; 4-week $R^2 = 0.144$, $p = 0.031$; 6-week $R^2 = 0.156$, $p = 0.006$), suggesting that the chronic nutrient amendments integrated their effects across successional processes. To estimate the variability in community composition of a given treatment, multivariate dispersion was measured among time points as well as within each time point grouped by organism or nutrient level (Figure S3.4). The average distance to centroid

(dispersion) increased between 2- and 4-week time points ($p = 0.004$), but dispersion did not change from 4 to 6 weeks ($p = 0.993$). Dispersion was significantly different between organismal treatments only at 6-weeks ($p = 0.048$), where coral treatments displayed a significantly lower average distance to centroid than sand and algae treatments. Biofilms that were subject to a medium nutrient addition displayed significantly lower dispersion at 2-weeks compared with ambient and high-nutrient treatments ($p = 0.0148$), but there were no significant differences among nutrient treatments at later time points.

Univariate linear mixed effect models were used to test hypotheses regarding the effects of time and treatments on alpha diversity estimates (Observed Richness and Shannon Evenness) within biofilm communities (Figure 3.4). Both richness and evenness of biofilm bacterial communities differed significantly among time points, between benthic organisms and their interaction ($p < 0.001$). Evenness additionally responded to nutrients ($p = 0.004$) and this effect differed among organismal treatments ($p = 0.016$). Overall, biofilm bacterial community evenness increased with succession (2-week mean = 0.849 ± 0.0514 sd; 6-week mean = 0.909 ± 0.0268 sd), with significant differences between 2 and 6 weeks within every organismal treatment (pairwise Tukey $p < 0.05$). Richness only significantly increased in biofilms cultured with corals (Coral $p < 0.001$; Figure 3.4a). At each time point, an organismal effect on evenness was observed (2-week $p = 0.012$; 4- and 6-week $p < 0.001$; Figure 3.4a). However, a significant organism effect on richness was only observed at 6 weeks (Figure 3.4a), indicating that organismal impacts on biofilm diversity took time to manifest. Nutrients exhibited effects on evenness at 2- and 6- weeks (2-week $p = 0.03$; 4-week $p=0.4$; 6-week $p = 0.004$) but nutrients only effected richness at 6-weeks (2-weeks = 0.1; 4-weeks $p = 0.3$; 6-weeks $p = 0.04$); a significant organism-nutrient effect on richness was observed at both 2 and 6 weeks ($p < 0.01$)

but this interaction did not manifest in evenness ($p = 0.8$), indicating that nutrients again played a subtle role in diversity effects.

Population Level Analyses

Most bacterial taxa differed at least qualitatively in relative abundance between biofilm and planktonic communities throughout the experiment (Figure 3.2a). Twelve families significantly differed between the two sample types and individually comprised more than 2% of the total reads in either community (Figure S3.5). Surface attached microbial communities were enriched with Rhodobacteraceae, Rhizobiaceae and Hyphomonadaceae of the Alphaproteobacteria, making up ~19%, 5%, and 4% of the biofilm total reads, respectively. Further, Parcubacteria (3%), Nostocales (4%), Microtrichaceae (2%), Saprospiraceae (5.4%) and Pirellulaceae (3.5%) were also significantly enriched in biofilm communities. The free-living community was enriched with SAR11 (8.9%), Vibrionaceae (6.2%) and the Bacteroidetes families Flavobacteriaceae (12.5%) and Cryomorphaceae (16.1%).

To identify bacterial taxa most strongly associated with time and treatment effects in the biofilm community level analyses, we used linear mixed models and a random forest algorithm to identify and rank OTUs that changed significantly. To ensure that our method was not selecting rare taxa, we additionally excluded OTUs with a mean relative abundance < 0.05%. In all, 28 OTUs were selected by our criteria as associating significantly with experimental treatments of successional stage and benthic organism. Of these, the relative abundance of 12 OTUs responded significantly (adj. $p \leq 0.05$) only to time; 3 OTUs responded only to organismal treatments; 7 OTUs responded significantly to organism, time, and the time-organism interaction, and 1 OTU responded to both time and organism treatments, but did not exhibit a

response to their interaction. These treatment associated OTUs indicate that marine biofilms from early time points were more similar to each other than at later time points which diverged according to the benthic organism with which the biofilms were cultured (Figure 3.5). Early time points were marked by members of Parcubacteria, as well as the Saprospiraceae and Rhizobiaceae families, which were nearly absent from later time points (Figure 3.5). Trends could be identified at higher taxonomic levels in later time points, which were enriched with constituents of the Planctomycetes, Actinobacteria, and Acidobacteria phyla (Figure 3.5 and Figure S3.6). Gammaproteobacteria grew more abundant with time in all of the treatments (Figure S3.6a) and were particularly important indicators of biofilms cultured with sand and algae treatments. In these treatments, OTUs classified as Porticoccaceae and Halieaceae of the gammaproteobacterial Cellvibrionales order were enriched in both algae and sand treatments at 4- and 6-weeks (Figure 3.5 and Figure S3.7a). Biofilms cultured with algae were additionally marked by multiple OTUs identified as members of the Rhodobacteracea family (Figure 3.5). Cyanobacteria, especially members of the Nostocales order, were indicative of coral treatments throughout, while members of the Sphingomonadaceae family and the genus Synechococcus were indicative of coral and sand treatments at 4- and 6-weeks (Figure 3.5 and S3.7).

The nutrient effect on biofilm communities was relatively small compared with the effects of succession and benthic organism (Figure 3.2c), and, at the population-level, we found no OTUs consistently responding to nutrient treatments across all organismal treatments at any given time point. However, a significant nutrient-organism interaction was observed in 6-week coral treatments (Figure 3.4). Thus, to further investigate the influence of nutrient amendments within our biofilms, we designed an algorithm to select OTUs associated with coral treatments and used linear models to identify OTUs that significantly responded to nutrient additions. In

general, these coral associated OTUs were statistically associated with either early or late time points, and nutrient amended vs. unamended (ambient) treatments. Of the 23 OTUs that met our criteria, 13 were enriched at late time points (5 from ambient, 8 nutrient amended) and 6 OTUs were indicative of early time points (3 ambient, 3 nutrient amended) (Figure 3.6). Members of the Bdellovibrionales and Phormidesmiaceae are abundant at later time points but tend to decrease with nutrients while OTUs of the Sphingomonadaceae increase with nutrient additions at later time points. Similarly, Puniceispirillales decreased with the addition of nutrients, but were enriched in early time points.

Discussion

This study comprises the first thorough characterization of colonization and differentiation of Bacteria between the plankton and surface attached lifestyles in coral reefs (Figure S3.5). We demonstrate that biofilms exhibit a successional trajectory that diverges from the stochastic temporal dynamics of the bacterioplankton (Figure 3.2), and use this successional trajectory to contextualize the degree to which benthic organismal context and nutrient enrichment influence biofilms (Figure 3.3). Successional dynamics drive much of the variation among biofilms, with benthic context the next dominant driver and nutrient enrichment having the smallest effect (Figure 3.2c). Metrics of diversity, taxonomic richness and evenness change through time and with treatment, demonstrating that taxonomic evenness increases as marine biofilms mature, and that both benthic community member and nutrient availability affect biofilm community richness (Figure 3.4). Our study demonstrates that organic resources from the surrounding benthic community and inorganic nutrient availability independently influence the assembly and succession of marine biofilm communities, and we provide a synthetic overview of

the bacterial clades that differentiate coral and algal biofilms and respond to chronic nutrient enrichment (Figures 3.5 and 3.6).

Biofilm-associated bacteria clades

Microbial communities are strongly influenced by their physical and chemical surroundings. In marine environments, two major lifestyles are commonly observed: free-living bacterioplankton and surface-associated biofilm communities. It is argued that most marine microbes appear to prefer one lifestyle over the other, though preference may depend on environmental conditions (Dang and Lovell 2016). For example, while surface attached communities can be heavily impacted by flow regimes(Stoodley et al. 2001), bacterioplankton may be more influenced by residence time(Nelson et al. 2011) or diel processes (Kelly et al. 2019). Planktonic communities of Bacteria in the ocean are reportedly abundant in Pelagibacterales (SAR11), Puniceispirillales (SAR116), Flavobacteriales, Rhodospirilalles, the Gammaproteobacteria SAR86 clade, Synechococcales, and Actinomarinales (Giovannoni and Rappé 2000; Mohit et al. 2014; Dussud et al. 2018) while surface-attached communities are commonly enriched with Rhodobacterales, Alteromonadales, and specific groups of Cyanobacteria(Mohit et al. 2014; Dussud et al. 2018; Salta et al. 2013). Nutrient input and availability is known to shift both surface attached and planktonic microbial communities. Although bacterioplankton communities in coastal areas can be relatively similar to those found in the open ocean(Rappé, Vergin, and Giovannoni 2000), coastal sites differ markedly in terms of nutrient input and production, and subtle shifts in the bacterioplankton community have been consistently observed in coral reefs relative to the adjacent ocean(Leichter et al. 2013; Yeo et al. 2013; Nelson et al. 2011). Betaproteobacteria appear to be confined to coastal regions(Yeo et al.

2013; Giovannoni and Stingl 2005) and the predominant group of Cyanobacteria may be shifted from Prochlorococcus to Synechococcus in nutrient rich conditions like those found around coral reefs(Nelson et al. 2011; Bourne and Webster 2013; Weinbauer et al. 2010). Our results support previous studies and find high abundances of typical bacterioplankton groups in the water. Because our experiment allowed for filtered seawater to flow through experiment tanks, variability in the bacterioplankton community of the source water likely contributed to the variability observed in our experiment tanks. In comparison biofilm communities were more evenly distributed, at least in part due to their ability to adhere to surfaces and were enriched with taxa that are known to benefit from surface attached lifestyles. Members of the Marine Roseobacter Clade (e.g. Rhodobacteraceae) are known copiotrophs for which surface colonization allows for more rapid response to labile organic matter (Dang and Lovell 2016) and Planctomycetes (e.g. Pirellulaceae) have been found to dominate marine biofilms associated with kelp and are notable for their ability to degrade sulfate polymeric carbon compounds (Bengtsson and Øvreås 2010). As well as taxa that are likely to benefit from the close proximity of cells found in biofilms such as Parcubacteria, which are known for their unusually small genomes and are thought to participate in symbiotic or episymbiotic interactions (He et al. 2021) (Figure S3.5).

Succession in marine biofilm communities

To date, studies investigating surface- or particle-attached microbial communities are typically conducted on found particles (i.e. marine snow or plastic debris) and, in reef ecosystems, focus on epiphytic-biofilms or host-associated microbiomes. Relatively few studies compare the successional stages of marine biofilms, and those that do tend to focus on the short

time periods of primary colonizers and the initial biofilm formation that occurs on time frames of hours to days. This has left a knowledge gap regarding biofilm development and successional dynamics in marine systems and the environmental factors influencing any observed variations. Surface-attached microbial communities are known to provide important ecosystem services (Wagner et al. 2015; Battin et al. 2003) and, in tropical reefs, are considered important factors mediating larval settlement (Hadfield 2011; Webster et al. 2004).

Biofilms in early successional stages appear to be less specialized in the use of organic carbon sources than mature biofilms; indicating that the capacity to use a wide range of organic compounds might be advantageous for pioneering species (Ylla, Canhoto, and Romaní 2014). Further, young biofilms are thought to be colonized by metabolic generalists that source dissolved organic carbon from the surrounding water column (C. R. Jackson 2003; Romaní et al. 2014). While our experiment did not capture the initial pioneering species (hours to days), indicator taxa of our early time points (2 weeks) included known copiotrophic bacteria, capable of utilizing a wide range of carbon compounds, including three OTUs of Bacteroidetes (Figure 3.5). The marine Bacteroidetes are important decomposers and lauded for their ability to breakdown high molecular weight organic matter; two of these OTUs were further identified as Saprospiraceae, a family that is typically only found in surface attached communities in the marine environment, and includes species known to prey on microalgae(McIlroy and Nielsen 2014). Consistent with previous studies that captured these time frames, Alphaproteobacteria were also abundant in early biofilm communities(Webster et al. 2004); and in our study were further identified as Rhizobiales, regarded as important mediators of biofilms formation(Carareto Alves et al. 2014), and Caulobacterales, a well-established group for surface adhesion and monolayer formation(Entcheva-Dimitrov and Spormann 2004; Karatan and Watnick 2009).

Over time, Gammaproteobacteria, Planctomycetes, and Acidobacteria increased in abundance supporting previous findings that indicate these groups differentiate biofilms from the free-living microbial community(DeLong, Franks, and Alldredge 1993; Webster et al. 2004). Important taxa enriched in later time points included the Alpha- and Gammaproteobacteria families Rhodobacteraceae and Cellvibrionales both associated with polysaccharide decomposition. The Rhodobacteaceae are well-established late-successional colonizers of nutrient rich habitats(Buchan, González, and Moran 2005) while the recently established order Cellvibrionales(Spring et al. 2015) includes both established polysaccharide degraders from soil habitats(Gardner 2016) and a number of widespread marine Gammaproteobacterial clades (OM60/NOR5, SAR92) variously documented in diatom blooms associated with both upwelling (Teeling et al. 2012; Wear et al. 2015) and Southern Ocean iron enrichments (Landa et al. 2016). These observations are consistent with well-established concepts of biofilms as communal digestive systems (Zobell 1943) where resources from the surrounding environment are captured and interact with enzymes produced within the biofilm community to efficiently cycle nutrients (Flemming et al. 2016; Battin et al. 2003) and mature biofilms facilitate greater species diversity through habitat diversification and a wider array of resources (Romani et al. 2016).

Influence of Benthic Community on Biofilm Composition and Succession

Resources provisioned from the surrounding benthic community, presumably dissolved organic matter (Quinlan et al. 2018; Haas et al. 2011; Nelson et al. 2013), were the strongest environmental influence on the structure and succession of marine biofilm communities. The bioavailability of DOC has been demonstrated to influence community composition in mature biofilms (Olapade and Leff 2006) and ultimately affects the uptake and utilization of other

nutrients (Suchismita Ghosh and Leff 2013). DOC sourced from algal exudates are known to differ from that of corals in both quantity and composition (Nelson et al. 2013; Haas et al. 2011) and these differences result in a restructuring of the bacterioplankton community from highly diverse taxonomic assemblages to less diverse communities adept at quickly growing on labile carbon compounds (Haas et al. 2016; Nelson et al. 2013). Similarly, while bacterial evenness increased over the course of the experiment, biofilms cultured with sand and algae did not significantly increase in richness (Figure 3.4). Further, OTUs indicative of biofilms cultured with algae were dominated by known copiotrophs (Figure 3.5). In a sister study analysis of dissolved organic matter (DOM) from samples collected concurrently with our biofilm samples revealed that all benthic organisms in our study increased organic matter exudates in response to nutrient addition and corals exude more proteinaceous organic matter than other benthic organisms – specifically, the exudation and accumulation of tryptophan-, tyrosine-, and phenalalanine-like organic matter was enriched in coral treatments (Quinlan et al. 2018). Tyrosine and tryptophan along with other small cyclic compounds have been shown to inhibit biofilm formation (P. de Carvalho and Abraham 2012) by inhibiting cellular communication (Ghosal et al. 2016; Saheli Ghosh, Qureshi, and Purohit 2019) and flagellar motility (Brandenburg et al. 2013). While reduction in biofilm formation and successional dynamics was not observed, it was clear from our study that coral and algal biofilms were distinct throughout the experiment and followed different successional trajectories. Furthermore, proteinaceous organic matter was always enriched in coral treatment relative to the influent seawater but decreased between the two- and four-week time points (Quinlan et al. 2018) and this reduction in organic matter coincides with the shift between early and late microbial communities where coral biofilms differentiate from biofilms cultured with sand and algae. Interestingly, biofilm communities cultured in the

presence of corals were enriched with several taxa known to be important in the breakdown of polycyclic hydrocarbons including the Rhodobacteraceae and Sphingomonadaceae families of Alphaproteobacteria, as well as the Gammaproteobacteria *Marinobacter*, suggesting that polycyclic hydrocarbons comprise a distinct pool of compounds released more by corals than by algae. Together our results provide clear evidence that biofilms also are strongly influenced by the composition of dissolved organic matter in coral reef systems.

Effect of Inorganic Nutrient enrichment on biofilm dynamics

The addition of inorganic nutrients increased the exudation of DOC by each of the benthic organisms in a companion study conducted coincident with this one in these same mesocosms (Quinlan et al. 2018). While nutrient concentration was consistently a significant factor shaping biofilm communities, the nutrient effect on biofilms was relatively small and tended to primarily interact with other experimental parameters rather than serve as a strong primary control over biofilm composition. This emphasizes that organic matter quality, rather than quantity, is likely the dominant structuring force in biofilm composition. In our study, nutrients impacted both measures of diversity (richness and evenness) at 6-weeks. Because of the relatively weak role of nutrients in structuring communities we selected a singular time point and organismal treatment within which we contextualized the impact of nutrient amendments: while evenness significantly increased through time in each of the organismal treatments, a significant increase in richness was observed only in biofilms cultured with corals at 6 weeks, in part due to interactions with nutrient enrichment. During week 6, nutrient additions decreased richness in biofilms cultured with algae but had no significant effect on evenness. For the same time period, nutrient additions had opposing effects on alpha diversity metrics in biofilms

cultured with corals: richness increased with nutrients while evenness decreased. Furthermore, with increased nutrients, populations of bacteria typically associated with oligotrohic coral reefs decreased, including families of Alphaproteobacteria and Cyanobacteria (e.g. Rhodobacteracea and Thalassobius, and Rivularia and Synechoccus) (Nelson et al. 2013; Frade et al. 2020) and shifted toward populations of copiotrophic Flavobacteria.

In freshwater systems bacterial communities have been shown to preferentially utilize inorganic nitrogen sources in the presence of labile organic matter, however when concentrations of labile organic carbon are low, organic nitrogen from recalcitrant carbon sources is preferred as it might serve as both a carbon and nitrogen source for the bacterial community (Suchismita Ghosh and Leff 2013). With evidence that coral exudates are enriched with DOM exhibiting fluorescence characteristics similar to aromatic amino acids (Quinlan et al. 2018) we can hypothesize that at least some portion of the carbon compounds exuded by corals are relatively enriched in nitrogenous compounds. Functional genes directly related to the degradation of aromatic hydrocarbons have recently been linked with various metabolic pathways including those required for nitrogen fixation and sulfate metabolism (Zhang, Hu, and Wang 2019). It is interesting that OTUs from two groups of late-succession marine Bacteria established to metabolize polycyclic compounds, Shingomonadaceae and Rhodobacteraceae (Pinyakong, Habe, and Omori 2003; Ghosal et al. 2016; Dang and Lovell 2016) respond differently to nutrient additions (Figure 3.6); with the former increasing and the latter decreasing), potentially suggesting differences in nutrient requirements or capabilities between the two groups that imply shifts in the aromaticity of polycyclic DOM released by corals under nutrient enrichment. Such a shift would be consistent with our earlier observations of nutrient amendment altering the

composition of coral exudates to increase humic-like components with potentially higher aromaticity (Quinlan et al. 2018).

In coral reefs, anthropogenic stressors including overfishing and nutrient pollution have been implicated in phase shifts from coral dominated reefs toward those dominated by fleshy algae (Fabricius et al. 2005; McCook 1999). Previous research working to identify the underlying mechanisms contributing to these phase shifts have found both direct and indirect effects of algae on coral vitality (Smith et al. 2006; Birrell et al. 2008), and suggested that alterations in microbial community structure could create a feedback loop that maintains the shift from coral to algal dominance (J. B. C. Jackson et al. 2001; Krediet et al. 2013; Haas et al. 2016; Mcdole et al. 2012). While this is likely only one contributing factor, variation in organic matter exudates produced by algae have been shown to be compositionally distinct from those of corals, resulting in a restructuring of the bacterioplankton community from highly diverse taxonomic assemblages to less diverse communities adept at quickly growing on labile carbon compounds (Haas et al. 2016; Nelson et al. 2013). In our study, biofilms cultured with algae were enriched in copiotrophic bacteria throughout the experiment (Figure 3.5), including several types of Rhodobacterales and Flavobacterales, and nutrient additions correlated with decreased richness in mature algal biofilms (Figure 3.4b), suggesting further bias toward a few dominant copiotrophs. Conversely, in biofilms cultured with corals, richness increased throughout the experiment and this trajectory was enhanced by the addition of nutrients. However, in mature biofilms cultured with corals, nutrient additions corresponded with a significant decrease in evenness (Figure 3.4b) and increases in populations of Flavobacteriaceae (Figure 3.6) suggesting that the addition of nutrients can derail some aspects of microbial diversity even in coral-dominated reefs by promoting the growth of copiotrophic organisms. Nutrient enrichment may

be a key factor in initiating these phase shifts, and nutrient enrichment in reef waters increases exudate output from benthic community members and shifts bacterioplankton communities toward less diverse assemblages with increased virulence factors(Haas et al. 2016; Nelson et al. 2013). Nutrient enrichment is also implicated in the progression of coral disease (Bruno et al. 2003) and increased virulence factors of microbes inhabiting the surface mucus layer of corals (Thurber et al. 2009). Taken together, our results indicate that both benthic community structure and nutrients work orthogonally and interactively to influence the composition of coral reef biofilms, suggesting that ongoing microbialization of coral reefs is likely to alter biofilm structure and function.

Conclusion

Biofilms are known to host complex communities of microorganisms that work in concert to perform biogeochemical processes and ecosystem services. In this study we demonstrate that marine biofilms differentiate from the planktonic community and exhibit successional trajectories distinct from their planktonic counterparts. Our results show that differences in organic matter produced by benthic organisms influence marine biofilms from early developmental stages and further differentiate these communities over time. This study further provides evidence that inorganic nutrient additions can shift biofilm communities either through the stimulation of primary producers, thereby reinforcing diverging microbial communities, or by shifting nutrient dependence away from biofilm derived sources, toward environmentally available inorganic sources. Our findings add to the growing evidence that chronic nutrient enrichment of reef ecosystems results in loss of diverse microbial assemblages. Finally, our work illustrates the structure of biofilm communities distinctly associated with coral

and algal dominated reefs, paving the way for future understanding of how ongoing global phase shifts in coral ecosystems may impact key microbial processes crucial for reef resilience in a changing world.

Figure 3.1. Experimental design
Three 1300L flow-through incubation tanks were used to maintain constant temperature for 9 flow-through aquaria each (total 27 experimental aquaria, 6L volume each); a schematic of one tank with 9 aquaria is shown here. Each aquarium held one of three benthic constituent organisms (algae, coral, or sand) and was supplied via peristaltic pump with filtered seawater amended to one of three nutrient treatments (ambient, low, or high) from independent header mixing aquaria (one for each nutrient level in each tank to maintain independence). Biofilms were cultured on glass slides, evenly spaced in slide racks and suspended vertically in each experimental aquarium to allow water to flow around each surface of the slide. Nutrients were measured weekly and were stable throughout the experiment; averages are reported. Additional data in Quinlan et al. (2018) and Silbiger et al. (2018).

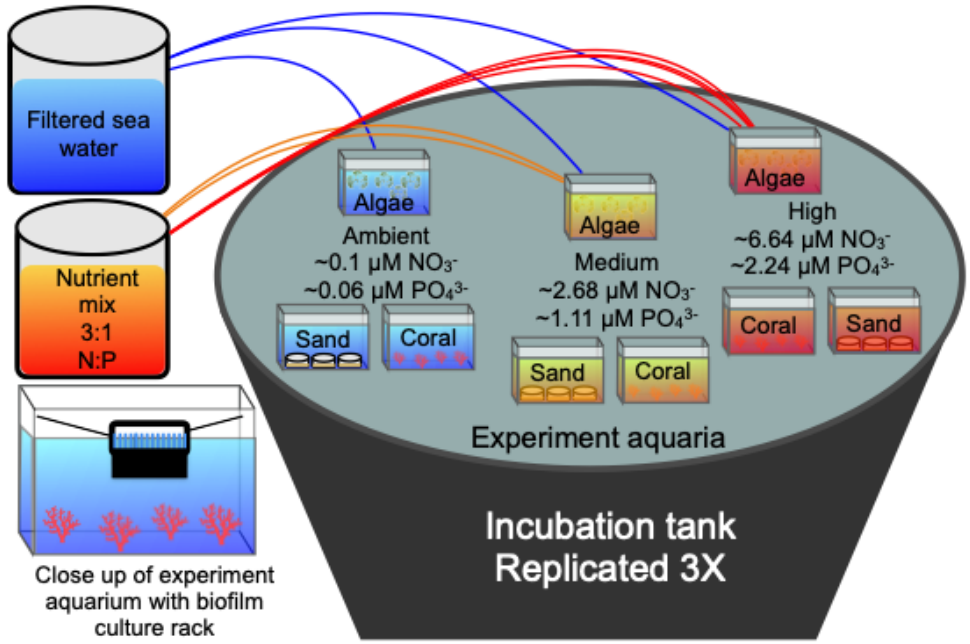


Figure 3.2. Multivariate visualization and statistical differentiation of biofilm and planktonic bacterial communities
Hierarchical clustering (Ward's minimum variance method) organized all OTUs in the study according to distributions of standardized (z-scored) mean relative abundance across treatments. Throughout the 6-week experiment, biofilm communities were distinct from planktonic microbial communities while both clustered by time point (a). Biofilms displayed a successional trajectory not found in the planktonic community (b); data points are color coded by time point (2-weeks = blue, 4-weeks = gold, 6-weeks = red), and shaped by sample type and organism (open = planktonic, closed = biofilm; Triangle = algae, circles = coral, square = sand). Variance partitioning of 3 different PERMANOVA models (c) illustrate the relative influence of sample type, time point, benthic organism and nutrient enrichment on bacterial taxonomic structure of planktonic and biofilm communities. Model 1 combines both planktonic and biofilm communities and emphasizes that sample type is the strongest driver of microbial community composition. Time point has the next largest influence on both planktonic (Model 2) and biofilm (Model 3) bacterial communities. Biofilm microbial communities are more strongly influenced by benthic organism (R2 = 0.123) than nutrient treatment (R2 = 0.063), while these parameters are equally influential in the planktonic community, explaining a much smaller variance (<1%). All tests and model terms shown are significant ($p<0.01$).

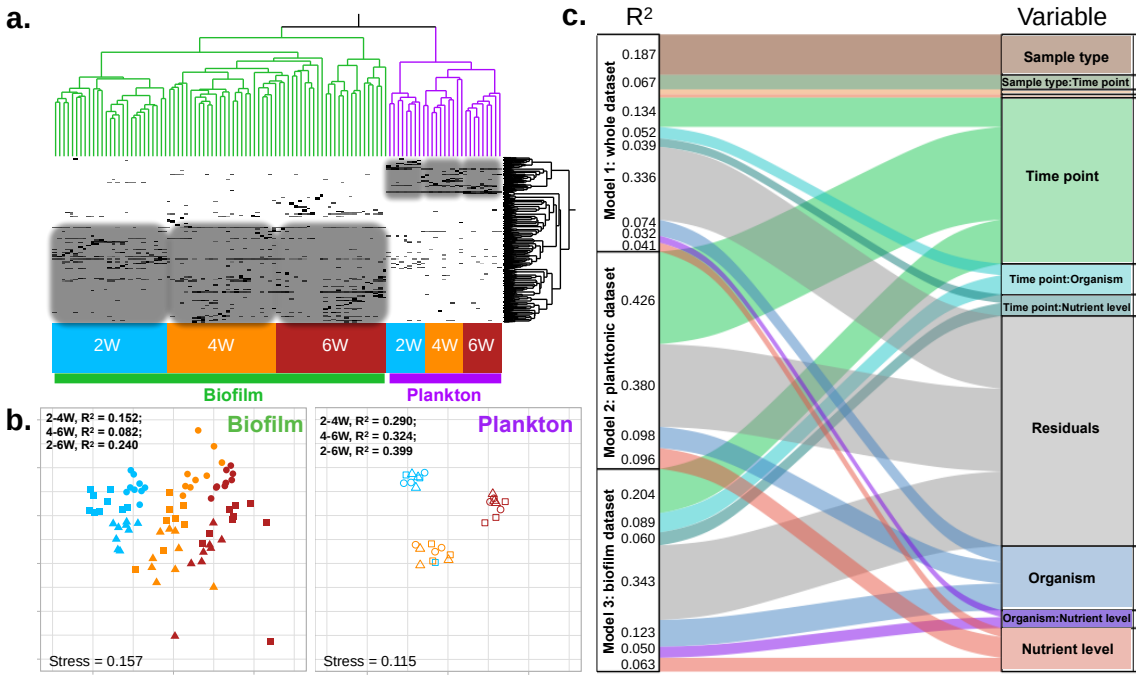


Figure 3.3. Effects of benthic organism and nutrient enrichment on biofilm communities within each time point
At each time point, both nutrient treatment and benthic organism significantly influence biofilm community structure ($p < 0.001$). Organism (a-c) is a better predictor of biofilm community than nutrient level (d-f) across each time point. A significant interaction between organism and nutrient level is also observed at each time point: 2 week ($R2 = 0.140$, $p = 0.003$), 4 week ($R2 = 0.144$, $p=0.031$) and 6 week ($R2 = 0.156$ & $p=0.006$), indicating that nutrients affected community structure differentially according to the benthic organism present.

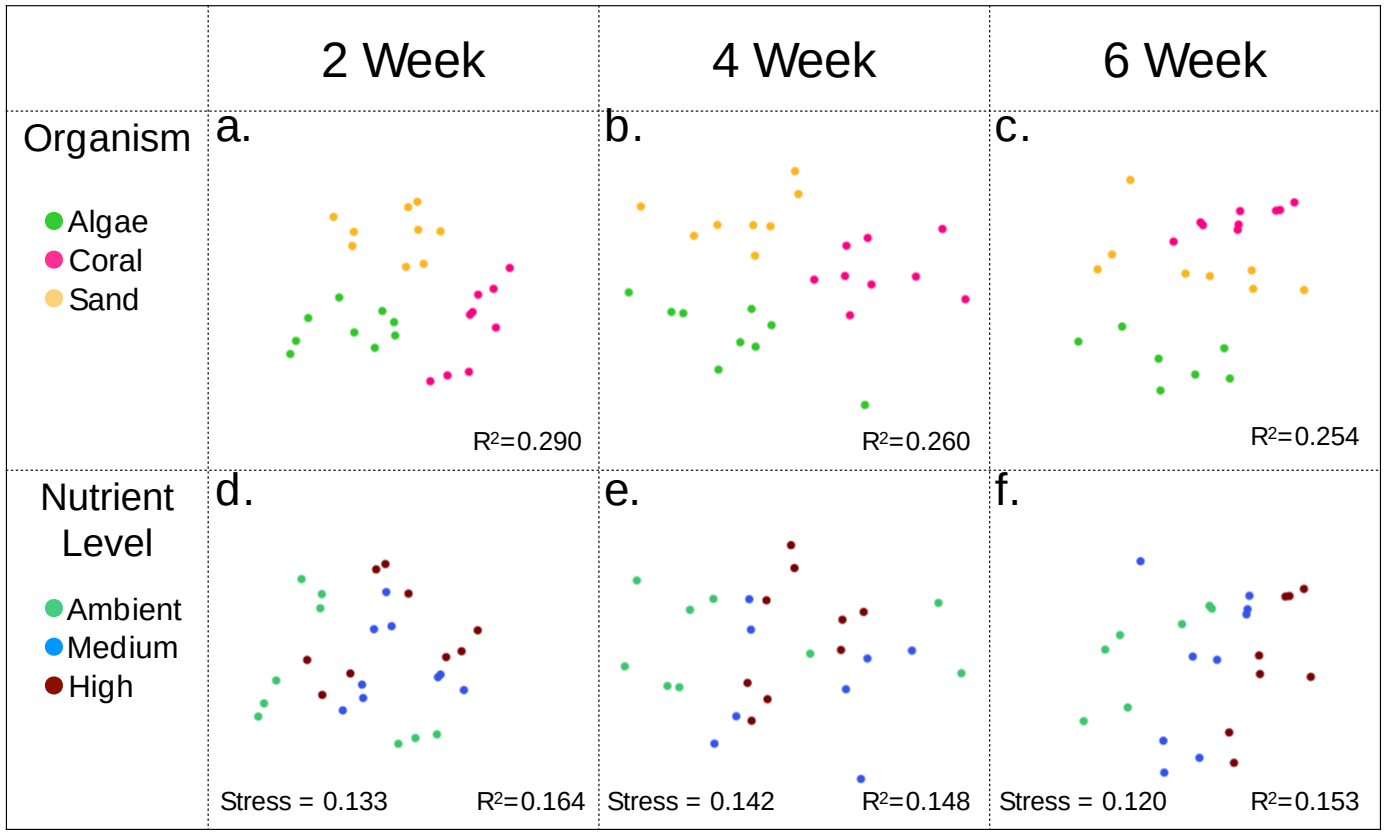


Figure 3.4. Changes in alpha diversity metrics of bacteria richness and evenness in biofilms through time in response to benthic organism and nutrient treatments

a) Richness and evenness significantly increased over 6-weeks (p_{time} <0.001) independent of the benthic organism with which they were cultured (P_{org} <0.001). Black asterisks indicate time points with significant organismal effect (p_{org} ≤0.001). Color coded asterisks indicate an increase in richness or evenness between 2- and 6- weeks (p_{time} ≤0.001; green = algae, pink = coral, yellow = sand)). b) Within each organismal treatment, nutrients differentially impact biofilms at 6-weeks and had opposing effects on richness and evenness in coral treatments.

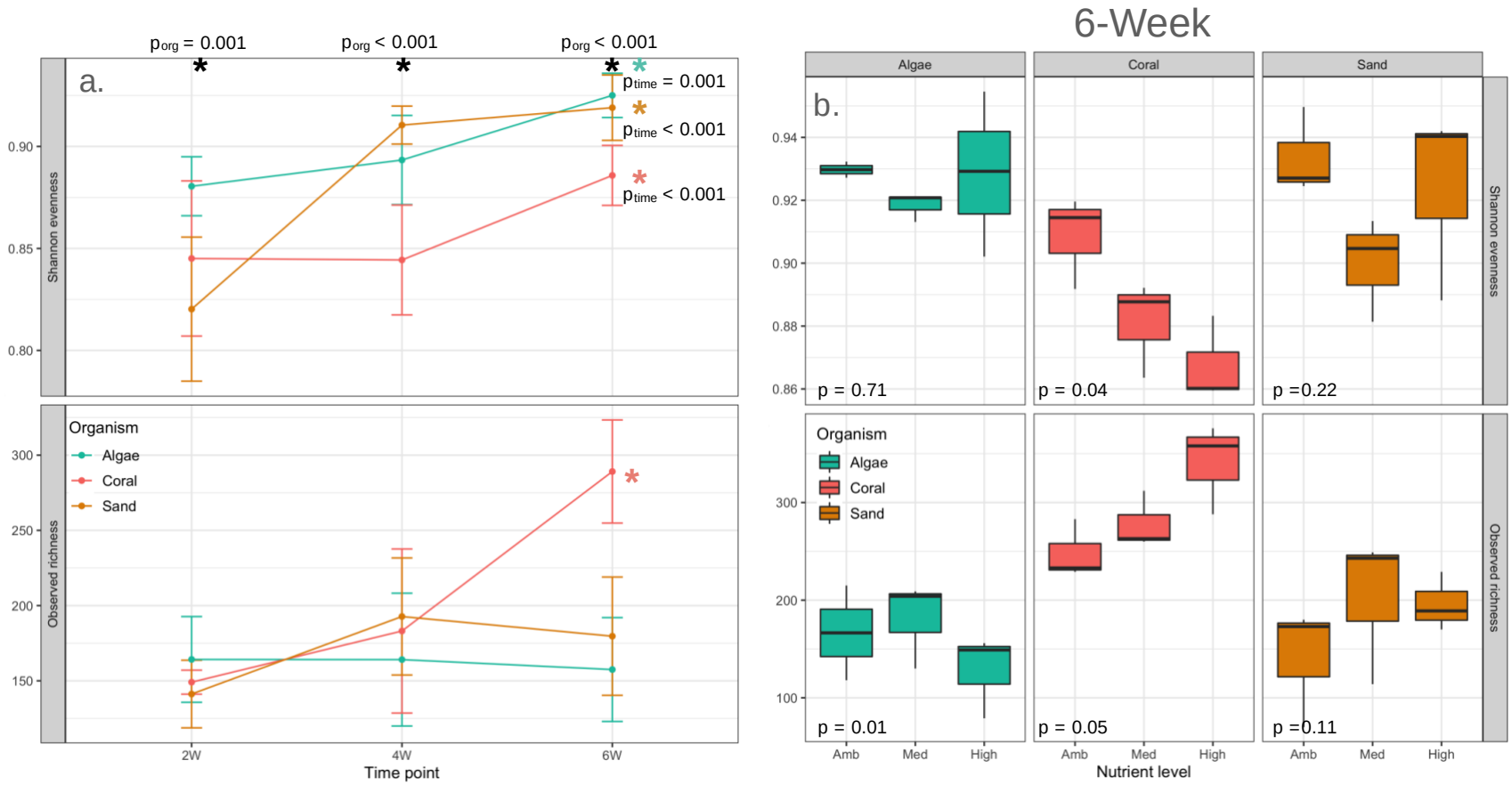


Figure 3.5. Visualization of enrichment of biofilm OTUs associated with treatments and time points
Hierarchical clustering (Ward's minimum variance method) organized statistically selected OTUs according to distributions of standardized (z-scored) mean relative abundance across treatments. OTUs that were both significant by linear mixed model and discriminant by random forest are displayed along the y-axis. Note the stronger similarity between selected OTU enrichment patterns at earlier stages of development compared with later time points. Later time points separate primarily according to benthic organism, and further cluster by time point within these groups.

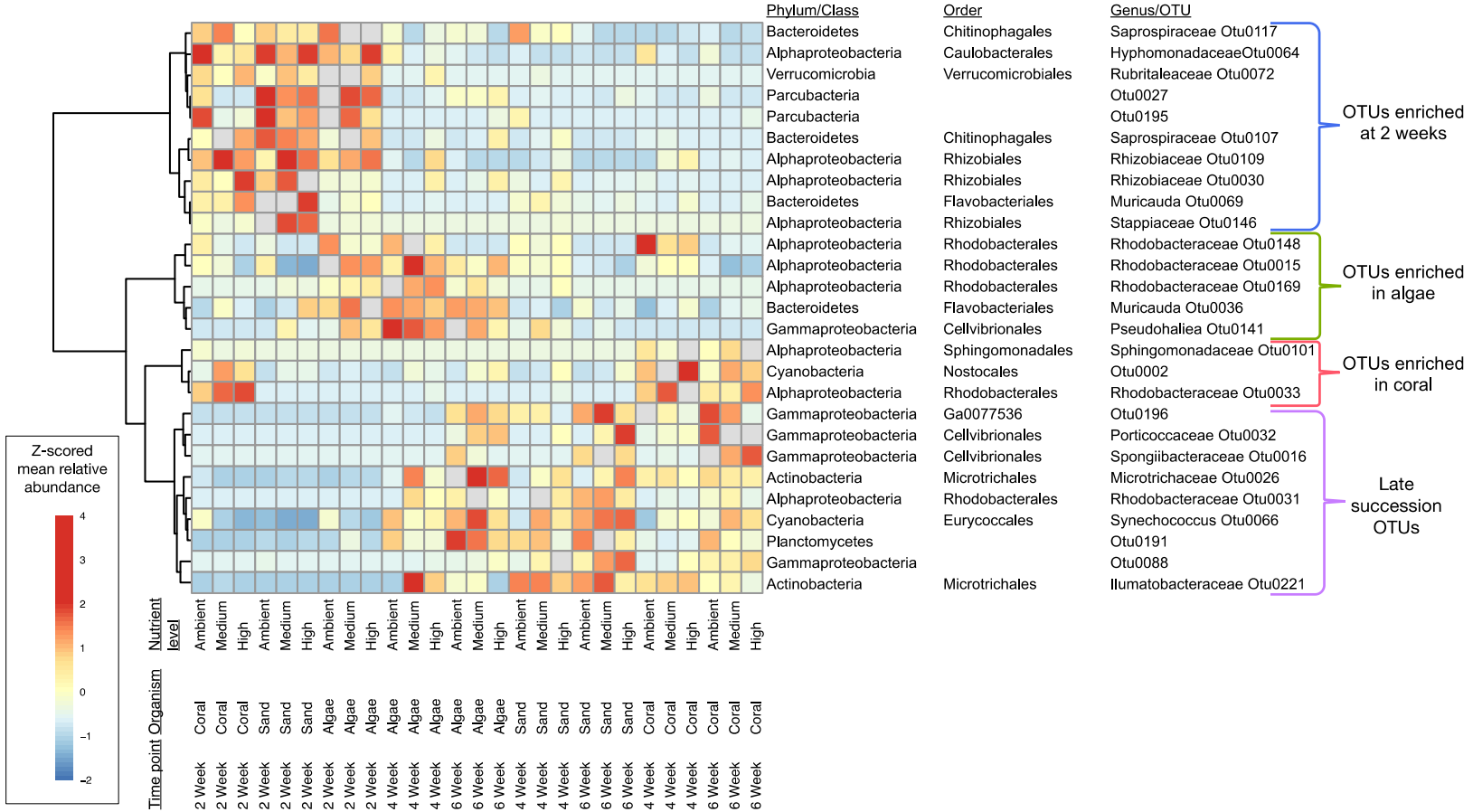
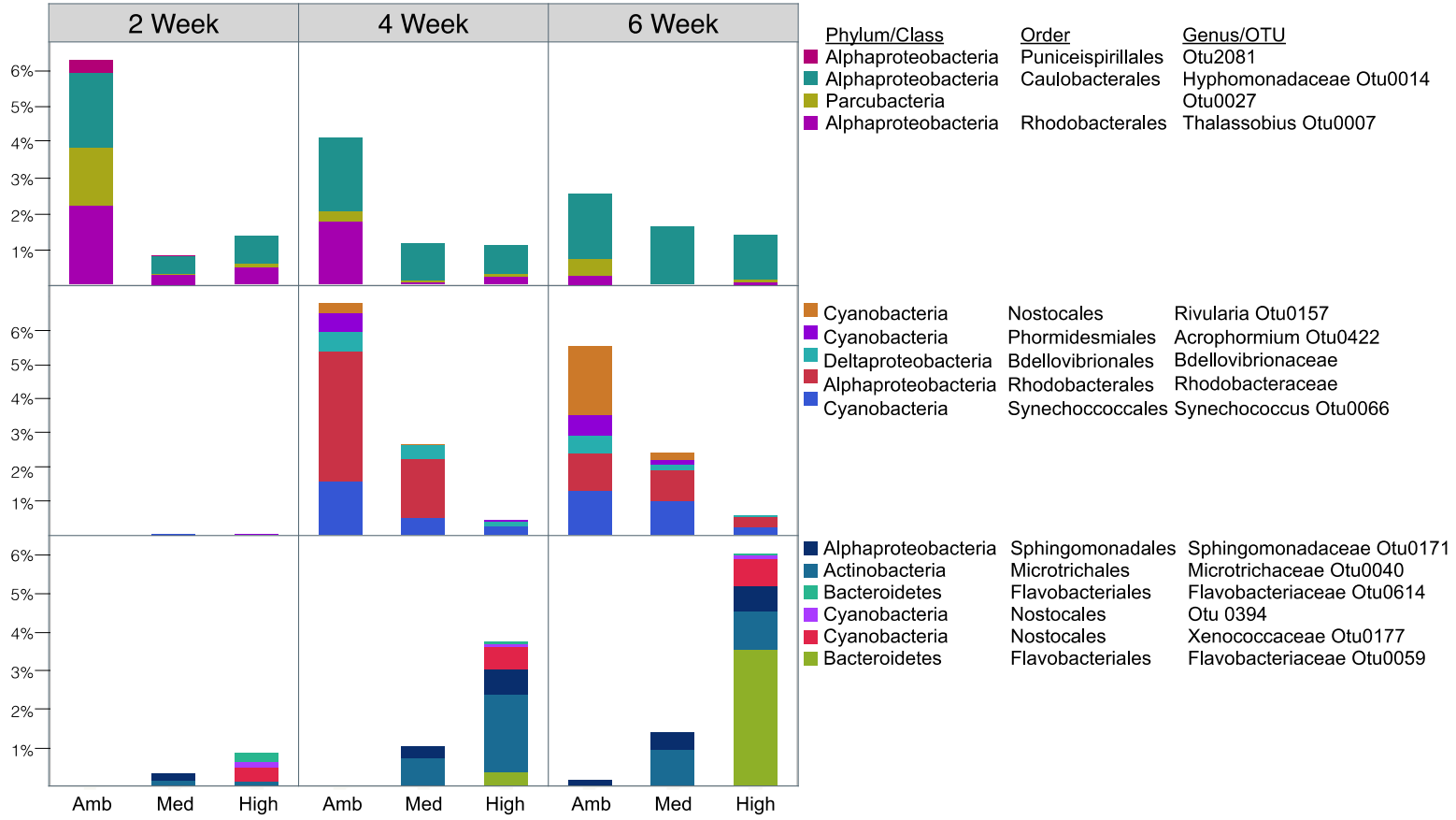


Figure 3.6. Relative abundance patterns of coral-associated biofilm OTUs that responded to nutrients
At top are families with OTUs that associated with earlier time points and decreased with nutrient additions, in the middle are families with OTUs that are associated with later time points that decrease with nutrient additions, and at bottom are families with OTUs associated with later time points that increase with nutrient additions.



CHAPTER 4
BENTHIC COMMUNITIES AND NUTRIENT ENRICHMENT INFLUENCE FUNCTIONAL PROFILES AND GENETIC POTENTIAL OF REEF BIOFILM

Abstract

Marine biofilms are complex microbial communities that provide important ecosystem services and support biodiversity in coral reefs. Compared with planktonic communities, little is known regarding the environmental factors that govern the taxonomic composition and genetic potential of reef biofilms. Biofilms were cultured over the course of 6 weeks in aquaria containing one of three common reef organisms (macroalage, coral, or sand) and factorially crossed with three levels of continuous nutrient enrichment (ambient, medium, and high). After 14 days, biofilms were collected to investigate the effects of benthic community and nutrient additions on the functional profiles of the resulting biofilms. After 6-weeks, a representative subset of biofilms was collected from each treatment to compare the genetic potential of marine biofilms between 2- and 6-weeks. Our results provide evidence that benthic community and nutrient amendments independently influence the functional profiles of marine biofilms while mature biofilms appeared to develop complexity and became more robust over time.

Introduction

Biofilms are important members of aquatic ecosystems where they contribute substantially to global geochemical cycles through primary productivity (Wagner 2015), organic matter decomposition (Ardón and Pringle 2007), and nutrient cycling (Battin et al. 2003). In marine environments, biofilms are largely comprised of diatoms and bacteria (Salta et al. 2013). Along with intrinsic and extrinsic factors such as biofilm age and environmental conditions, the microbial community composition ultimately governs the ecosystem services provided by the

biofilm (Romani, Guasch, and Balaguer 2016). The formation of biofilms is a multi-stage process that is initiated when cells from the surrounding water column adhere to a submerged surface and begin to produce extracellular polymeric substances (EPS). The early stages of biofilm development involve the division of cells and the formation of microcolonies, which may contain one or multiple species depending on nutrient availability (Nielsen et al. 2000). As biofilm development progresses, three-dimensional structures are formed, facilitated by the EPS which serves to keep cells in close proximity to one another, protects the biofilm community and facilitates cell-to-cell interactions (Donlan 2002). Throughout biofilm formation, subpopulations of bacterial species may develop (e.g. motile vs non-motile, metabolically active vs dormant, etc.) and can affect the biofilm structure (Tolker-Nielsen 2015; Leung and Lévesque 2012). Finally, cells from the biofilm reenter the planktonic phase, completing the biofilm cycle.

The stages of biofilm formation are conserved among a wide variety of prokaryotes (Hall-Stoodley, Costerton, and Stoodley 2004). At first glance, biofilm formation may appear to be a highly programmed process with common set of "biofilm genes" expressed in all bacteria within a biofilm. However, a core set of genes has not yet been described, suggesting that biofilm formation is mainly governed by adaptive responses (Tolker-Nielsen 2015). For example, in many bacterial species, initiation of biofilm formation occurs in response to an increase in the levels of the intracellular secondary messenger: cyclic di-GMP (c-di-GMP) (Tolker-Nielsen 2015; O'Toole and Wong 2016). In Vibrio cholera however, small RNA (sRNAs) have also been shown to regulate both biofilm formation as well as chemotaxis and motility (Dang and Lovell 2016). Further, quorum sensing is known to regulate cell density through chemical signals that allow for cell-to-cell communication and it is also known to regulate virulence, antibiotic resistance, and horizontal gene transfer (Paul et al. 2018; Pena et al.

2019). Perhaps because of overlap or redundancy in regulatory pathways, biofilm formation is a dynamic process and its regulation is highly complex (Hall-Stoodley and Stoodley 2002).

Marine biofilms are important members of coral reef ecosystems known to promote the settlement and subsequent metamorphosis of invertebrate larvae (Webster et al. 2004; Hadfield 2011; Whalan and Webster 2014; Sneed, Ritson-Williams, and Paul 2015). However, our current understanding of reef microbial communities is primarily drawn from planktonic and host-associated symbiotic microbial communities. This work demonstrates that reef bacterioplankton communities are influenced by physio-chemical processes including diel cycles (Kelly et al. 2019), reef benthic community (Haas et al. 2016; Zaneveld et al. 2016; Morrow et al. 2013), sources and concentration of organic matter (Quinlan et al. 2018; Cárdenas et al. 2018; Nelson et al. 2013) and nutrient availability (Frade et al. 2020). Nutrient loading is correlated with increases of potentially pathogenic bacterial species and virulence factors in the symbiotic microbial communities from coral surfaces (Shaver et al. 2017; Vega Thurber et al. 2014). In combination with other environmental stressors, nutrient loading in coastal systems is implicated in phase shifts and the reduction of reef building corals in favor of fleshy algae (Diaz-Pulido et al. 2009). Algae are known to exude organic matter that is more biologically labile compared with that of corals (Nelson et al. 2013; Quinlan et al. 2018) and may facilitate the restructuring of the planktonic microbial community to promote algal dominance in reef phase shifts (Haas et al. 2016; Kelly et al. 2014; Mcdole et al. 2012). In freshwater systems, community composition of mature biofilms is influenced by the bioavailability of DOC (Olapade and Leff 2006) and ultimately affects the uptake and utilization of other nutrients (Suchismita Ghosh and Leff 2013). Shifts in carbon source are also known to alter microcolony development(Nielsen et al. 2000) and biofilm 3D structure of young biofilms (Wolfaardt et al. 1994; Klausen et al. 2003).

We have previously demonstrated that organic matter exudates from reef primary producers (algae, coral, and sand) influence marine biofilm community composition. Biofilms cultured over the course of 6-weeks were taxonomically more similar at early time points compared with later time points, and nutrient additions shifted the taxonomic community structure within organismal treatments (Remple et al. 2021). However, it is unclear if organic matter from reef benthic organisms or the addition of inorganic nutrients might influence the functional profiles of marine biofilms. In the present study, we use metagenomics to compare the functional potential of marine biofilms cultured over 6-weeks in the presence of common reef primary producers (coral, algae, and sand) and under sustained nutrient loads (continuous micromolar enrichments of nitrate and phosphate at three concentrations).

Methods

Sample Collection

Samples of coral, macroalgae, and carbonate sand were collected from the fringing reef around Moku o Loʻe (Coconut Island) adjacent to the Hawaiʻi Institute of Marine Biology (21.435º, -157.787º) on October 12-16, 2015. Collections were in accordance with local regulations; corals were collected under the State of Hawaiʻi Division of Aquatic Resources Special Activity Permit 2015-17 to the Hawaiʻi Institute of Marine Biology. Corals were collected from the fringing reef on the southwest side of the island. Three individual colonies from the two dominant coral species in Kāneʻohe Bay, Porites compressa and Montipora capitata, were harvested and fragmented to produce 36 coral nubbins, 12 from each of 3 colonies. Each nubbin was buoyant weighed and equally sized nubbins (P. compressa = 24.8 ± 5.23g dry

weight; M. capitata 21.9 ± 5.05g dry weight) were mounted on polystyrene frames using epoxy. Each coral frame held six nubbins (three P. compressa, and three M. capitata); coral nubbins were allowed to acclimate for 10 days prior to the start of the experiment. Macroalgae (Gracilaria salicornia) and sand samples were collected from a low energy, sandy reef flat on the northern side of Coconut Island in less than 1m depth, where G. salicornia is abundant and grows unattached to the substrate. Macroalgae samples were cleaned of visible invertebrates and epiphytes then wet weighed and split into 36 equal portions (11.0 ± 0.55g wet weight) and contained in polyethylene mesh boxes. Samples of carbonate sand were collected using a 7.5 cm diameter core, placed in 36 petri dishes, then placed undisturbed in experiment aquaria. More detailed information on sample collection is available from Quinlan (2018) and Silbiger (2018).

Experimental Design

Experimental aquaria were set up in an outdoor mesocosm facility and consisted of 27 six-liter, flow through, acid washed, polycarbonate aquaria which were divided between three, 1300 liter, shaded incubation tanks (9 aquaria per tank) used to maintain constant temperature (Figure 4.1; S4.1). Source water from Kāneʻohe Bay flows into the mesocosm facility first through a sand filter and 20 μm polyethylene cartridge filter before use in our experiment. The filtered seawater was subsequently pumped into 9 nutrient mixing header aquaria (10L) via multi-channel peristaltic pump; header mixing aquaria (3 replicate aquaria of each of three nutrient treatments) were maintained at a 30-minute residence time, cleaned weekly and variously housed 5-15 small (3cm) coral fragments associated with a separate experiment. To create nutrient treatments in header aquaria, a bulk nutrient stock of potassium nitrate and potassium phosphate (3:1 molar N:P) was mixed at the beginning of the experiment and frozen

in single use aliquots to maintain continuity throughout the experiment. Every other day, an aliquot of frozen nutrient stock was diluted to the appropriate concentration in seawater and administered to the header aquaria via peristaltic pump to mix. Treatments were maintained at three stable levels, including ambient (averaging 0.1 µM L-1 NO_3^- and 0.06 µM L-1 PO_4^{3-}) and medium and high enrichments averaging 2.68 and 6.64 µmol L-1 NO_3^-, respectively17,32. Nutrient concentrations and N:P stoichiometry (2.46 ± 0.37 SD across all treatments) spanned natural inorganic nutrient conditions measured on reefs across the Hawaiian archipelago91 and background nutrient conditions during the experiment were consistent with baseline concentrations in Kāneʻohe Bay. Each aquarium held one of the three benthic organisms (either four coral frames, four algal mesh boxes, or four petri dishes of sand) and received one of the three nutrient treatments (ambient, medium, or high nutrient addition) resulting in nine factorially-crossed treatments. Treatment aquaria were maintained at a 5 hour residence time and mixed with a submersible water pump. Each set of nine treatment aquaria was established in one of three independent 1300L flow-through incubator tanks to maintain thermal stability and was monitored over the course of six weeks. This resulted in a total of 27 aquaria comprising three independent replicates of each of the 9 treatments. Each nutrient treatment level in a 1300L tank was fed by an independent mixing header tank to ensure independence. To mitigate tank effects due to weather conditions and variations in light exposure; aquaria sets (blocks of 9 aquaria) were rotated between, and individual aquaria were shuffled randomly within, the larger incubator tanks once per week over the course of the 6-week experiment. All plastics used in this experiment, including aquaria, slide racks, and tubing were acid-washed and soaked for at least 72 hours in flowing seawater to remove plasticizers before starting the experiment.

Biofilm culturing and sampling

Glass slides have been previously shown to provide suitable substrate for marine biofilms and are known to result in more consistent and reproducible communities compared with ceramic tiles (Witt 2011). Therefore, biofilms were cultured using glass slides suspended in the upper 10cm of each experimental aquarium (Figure 4.1). Slides were cleaned with alcohol and combusted to remove surface coatings and organic matter prior to the experiment. Slides were evenly spaced in polyoxymethylene slide racks typically used for microscopy staining, with ample space between each slide to allow for water to flow freely around each side of the glass slide. Racks were suspended using nylon line and polypropylene suction cups. Biofilm samples were destructively sampled by removing one glass slide from each aquarium rack at each sampling point in a manner intended to minimize altering the fluid dynamics surrounding the remaining slides. Sterile polyester tipped swabs (Puritan 25-806 1PD) were used to sample biofilms from each slide; a standardized swabbing technique was used to minimize variability between samples and time points: Each side of a slide was swiped 10 times with a swab, turning the swab one quarter turn every 5 swipes. Slides were discarded after collection, and swab tips were placed in sterile tubes and frozen until DNA could be extracted. For analysis of planktonic bacteria, at each time point 40mL of seawater was collected from each of three replicate aquaria within each organism by nutrient treatment using an acid washed, rubber-free polyethylene syringe, pooled into a single 120mL sample and filtered through a 0.22 um PES filter, resulting in 9 DNA samples from planktonic organisms at each time point (27 planktonic samples total). Filters were frozen at -80° C until DNA could be extracted. Genomic DNA from biofilm swabs and filters was extracted using the Epicentre MasterPure Complete DNA and RNA Purification Kit (MCD85201) using the protocol outlined by the manufacturer for DNA purification from

plasmid or serum samples with the following modifications: samples were rotated throughout the lysis incubation and swabs or filters (depending on sample type) were aseptically removed following the addition of RNase and just before DNA precipitation steps. DNA was re-suspended in 50μL TE buffer. Analysis of dissolved organic matter (DOM) was performed using the 0.22 um PES filtrate collected synoptically with the samples described here. Findings from the DOM analysis are reported separately.

Metagenome Sequencing and Analysis

Extracted genomic DNA from a subset of samples was submitted to the Hawai‘i Institute of Marine Biology Evolutionary Genetics Core Facility for library preparation and sequencing using the Nextera XT Library Prep Kit (37 samples). Fragment libraries were sequenced using the Illumina MiSeq with paired-end 600 cycle V3 chemistry kits. DNA concentrations ranged from <0.05-1.84 ng/uL and the mean concentration was 0.728 ng/μL of DNA per sample. The mean number of reads obtained from each sample was 1,154,596; ranging from 241,196 to 2,506,564 reads. Sequences were uploaded to the Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) online server version 4 for taxonomic and functional annotation(Meyer et al. 2008). The RefSeq and SEED databases were selected for taxonomic and functional annotations, respectively(O'Leary et al. 2016; Overbeek et al. 2014). Sequences were annotated using MG-RAST default settings; an average expect value of e^{-5}, percent identity of 60%, and minimum alignment length of 15 nucleotides for taxonomy (refSeq) and 15 amino acids for functional (SEED) profiles.

The RefSeq database follows the classic Linnaean taxonomic structure (kingdom to species). The SEED database serves as a classification system that organizes genes into four

hierarchical levels based on FIGfam protein families. A FIGfam is a set of proteins that are similar in both sequence and function, also known as "isofunctional homologs". For a given sequence, the database returns the "group" (the most general functional category), the "subgroup", the "subsystem", and "function" (the most granular functional category). SEED subsystems are similar to metabolic pathways in their categorical groupings, but can be thought of more generally since the relationships between functional roles is not strictly metabolic. Instead, subsystems further include information such as regulatory relationships and chromosomal clustering relationships (Pusch 2016). For our purposes, we focused primarily on SEED subsystems to draw comparisons between biofilms and contextualized within the broader subgroup when necessary.

Sequences classified as Bacteria dominated biofilm samples accounting for approximately 93 - 97% of all sequences (supplemental Figure S4.1). Because of this dominance and because we have previously thoroughly investigated amplicon-based taxonomic structure of Bacteria in these samples, only bacterial sequences were used in subsequent metagenomic analyses. Samples collected at 2-weeks generally contained more sequences (RefSeq range =74,130 - 829,534, mean = 347,224 ± 176,342 sd; SEED range = 26,034 -275,744 , mean = 117,628 ± 59,694 sd) compared with 6-weeks (RefSeq 71,541 – 870,926, mean = 301,454.2 ± 223,330 sd; SEED 24,717 – 283,772, mean = 100,573 ±72,979 sd). Samples containing fewer than 150,000 RefSeq reads or 50,000 SEED genes were discarded and all organismal (algae, coral, and sand) and nutrient treatments (ambient, medium, and high) were considered in subsequent statistical analyses, removing 3 samples from the analysis. This resulted in duplicate samples at 2 weeks for each combination of organism/nutrient treatment except for sand-ambient nutrient treatment, coral-medium nutrient treatment, and algae-high nutrient treatment; which

were represented by triplicate samples (a total of 21 samples at the 2 week timepoint); comparisons of organismal and nutrient treatments were conducted on samples collected from the 2-week time point. Only a subset of samples were shotgun sequenced from the 6 week timepoint (n=6), tests on organism and nutrient were not done at 6 weeks and comparisons of biofilms between time points were conducted using all organismal treatments but combining nutrient enrichment treatments into nutrient added (N+) or unamended (N0) nutrient treatments. Replicate samples collected at 2 weeks were averaged and compared with the corresponding 6-week sample for an n of 1 for each combination of time point (2 week or 6 week), organism (algae, coral, or sand), and nutrient amendment (N0/N+).

Statistical analysis

Mutivariate analyses of organismal and nutrient effects on biofilm communities at 2-weeks were performed on Bray-Curtis distance matrices constructed separately from relative abundances of RefSeq OTUs and SEED subsystems using the *vegan* package in R (Oksanen et al. 2019) including permutational analysis of variance (PERMANOVA) with the *adonis()* function and nonmetric multidimensional scaling with the *metamds()* function. Models used benthic organism, nutrient level, and their interaction term as fixed effects. Linear univariate mixed-effect models were performed using the *lme4* and *lmerTest* packages in R (Bates et al. 2015; Kuznetsova, Brockhoff, and Christensen 2015). Benthic organism, nutrient level, and their interaction term were included as fixed effects in the models, with holding tank as an orthogonal random effect to account for environmental differences between experimental holding tanks (Figure 4.1). The false discovery rate was controlled by adjusting p-values according to Benjamini and Hochberg (1995). Prior to linear statistical analysis, relative abundances of

bacterial taxa and functional annotations were angular transformed to meet gaussian distributional assumptions.

Because a large number of bacterial taxa were found to have a significant fixed effect for organism, we further ranked the more abundant of these taxa by their importance in classifying organismal treatments using the *randomForest* function in R (randomForest package (Liaw and Wiener 2014). Abundant taxa were identified as those whose mean relative abundance was greater than the overall mean relative abundance, global mean decrease in accuracy scores (MDA) were generated by random forest, and the 30 highest of these were used for visualization.

To compare functional difference between time points, the relative abundance of SEED subsystems were used to estimate fold change differences by calculating the $\log_2$ ratio of 2- to 6-week time points. Subsystems with an average relative abundance greater than 3×10^{-6} were used to calculate proportional fold changes and identify subcategories of interest. For a given subgroup, proportional fold changes were calculated by dividing the number of subsystems displaying a large fold change (i.e. fold change $\geq 2x$) by the total number of subsystems associated with the subgroup in our dataset. All subsystems displaying a large fold change with a proportional fold change ≥ 0.5 were labeled as subgroups of interest. Subgroups with a proportional fold change > 0.3 and where all subsystems displaying a large fold change (at least 2x) were enriched at a single time point were also considered to be subgroups of interest.

Results

Taxonomic community structure and functional profiles of marine biofilms at 2-weeks

Biofilm communities clearly differed by organism in both taxonomic structure (genus) and functional potential (subsystem). Both organism and nutrient treatments significantly influenced the taxonomic and functional traits of biofilms. At the genus level, benthic organism explained ~40% ($p < 0.001$) of the observed variance between samples and nutrient treatment further explained ~26% ($p < 0.001$) of the observed variance. Similarly, benthic organism explained more variance than nutrient treatment between samples annotated by SEED subsystem, accounting for ~24% and 15%, respectively ($p < 0.001$). The interaction of benthic organism and nutrient treatment was not significant in either dataset, indicating that organism and nutrient caused orthogonal shifts in taxonomic structure of the biofilms (Figure 4.2).

Comparison of bacterial populations in 2-week biofilms

To identify organisms that responded significantly to our treatments, we used linear mixed models to identify genera that responded significantly to organism and nutrients. Out of 593 genera, 398 were found to respond to benthic organism and 221 responded to nutrient additions ($p \leq 0.05$). Figure 4.3 shows abundant bacterial taxa that respond significantly to organism or nutrient treatments. Consistent with our previous study, Alphaproteobacteria were abundant in these, 2-week samples; cyanobacteria populations were indicative of biofilms cultured with corals and Flavobacteria were associated with nutrient additions. In the present study, biofilms cultured with corals were enriched with the Cyanobacteria genus, *Acaryochloris* and multiple genera of Rhodobacteraceae. Two genera of Flavobacteriaceae were also enriched in biofilms

cultured with corals but were more abundant in nutrient enrichments. Taxa commonly associated with marine sediments and soils were indicative of biofilms cultured with sand, including multiple genera of Methylophilaceae, Hyphomonadaceae and Bradyrhizobiaceae, all three of which were also found in lower abundance in biofilms cultured with algae. Biofilms cultured with algae were enriched with multiple genera of Deltaproteobacteria, lower abundances of which were found in biofilms cultured with sand.

Genetic potential of 2-week biofilms cultured with coral or algae

Functional profiles of biofilms revealed metabolic shifts influenced by organism and nutrient treatments (Figure 4.4). Out of 1038 subsystems 111 responded significantly to organismal treatments ($p \leq 0.05$). Subsystems involved with sulfur metabolism were more abundant in algal treatments and included genes involved with alkanesulfonate assimilation and release of dimethyl sulfide from DMS. These biofilms were also enriched with genes involved with the Entner Doudoroff (ED) pathway and genes involved with the uptake and utilization of labile sugars, lactose and galactose; and bacterial secretion systems, specifically Type II, Type III, type IV Dot/Icm, and type VI, were also more abundant in biofilms cultured with algae relative to corals.

In coral biofilms, carbohydrate metabolism was marked by a higher proportion of genes involved in pyruvate, alanine, and serine interconversions; maltose and maltodextrin utilization; and the Calvin-Benson cycle. Genes associated with photosynthesis and the production of pigments such as those for bilin and chlorophyll biosynthesis as well as light harvesting complexes were also more abundant in biofilms cultured with corals compared algae. Genetic potential in biofilms cultured with corals were further marked by genes involved with the

metabolism of aromatic compounds. The phenylacetyl CoA catabolic pathway, involved in the degradation of aromatic compounds, and gene families involved with the production of phenylalanine and tyrosine were more abundant in coral biofilms and displayed a significant organism-nutrient level interaction.

From the 1038 subsystems analyzed in our study, 31 subsystems responded significantly to nutrient treatments. Among these were subsystems involved with the metabolism of aromatic amino compounds. The subsystem, phenylalanine and tyrosine branches from chorismate, contains genes involved with the biosynthesis of aromatic amino acids (L-phenylalanine and L-tyrosine) and increased with nutrient additions. Genes involved with the breakdown of aromatic compounds decreased with nutrients including those associated with the cresol degradation and phenol hydroxylase subsystems. Separately, genes involved with iron acquisition were affected by nutrients. Iron acquisition in Vibrio and genes involved with hemin transport decreased with nutrients. However, those involved with the siderophore assembly kit subsystem increased with nutrients.

Changes in functional potential between time points

Fold change analysis was used to study the change in genetic potential over time in marine biofilms. Eight SEED subgroups contained a high proportion of subsystems that differentiated at least 2-fold between time points (Figure 4.5). Within these subsgroups, subsystems containing genes associated with non-ribosomal peptide synthetases (NRPS) and genes involved with quorum sensing increased 2-fold between time points; paerucumarin biosynthesis and biofilm adhesins proteins increased 3-4x. Subsystems classified under the regulation of virulence subgroup included the two-component response regulator of virulence

RseDE and a conserved operon linked to transcriptional regulatory protein tyrR, both of which increased 2-3x between 2- and 6-weeks. Similarly, ESAT-6 protein secretion systems, commonly associated with virulence but also found in non-pathogenic species increased 5-6x at later time points. Genes involved with light harvesting increased over time; genes relating to the phycobilisome and the chlorosome increased 2-fold and 5-fold between time points, respectively. Within the tetrapyrrole subgroup, subsystems involved with light harvesting in phototrophs including bilin biosynthesis and heme biosynthesis increased 2-3x. In heterotrophs, however, tetrapyrroles may be considered catabolic and in our dataset genes associated with chlorophyll degradation increased 3x between 2- and 6-weeks.

Subsystems with large fold changes classified within the dormancy and sporulation subgroup did not display a bias toward either time point. Genes involved with exosporium, biosynthesis of spore pigment, and the spoVS sporulation protein family increased over time, approximately 2x, 4x, and 10x, respectively. However, subsystems containing genes for persister cells and spore germination decreased 2-4x between time points. Interestingly, subsystems involved with phages and prophages, included phage capsid proteins and phage tail fiber proteins that decreased 2-3x between time points.

Discussion

The goal of this study was to characterize and compare the functional potential of marine biofilms cultured with benthic organisms commonly found in reef ecosystems. Our results demonstrate that the dominant organisms in the surrounding benthic community are a primary determinant of both the taxonomic composition and functional potential of marine biofilms. Nutrient additions further impact marine biofilms by shifting their functional profiles possibly in

conjunction with the surrounding benthic community. Mature biofilms developed in complexity as subsystems related to phototrophic communities as well as those associated with sporulation and dormancy increased at later time points. Evidence of phage infection was identified in young biofilms, but decreased by later time points suggesting that biofilms were more robust at 6-weeks than at 2-weeks.

It has been hypothesized that organic matter released as exudates by corals contain higher levels of refractory DOC compared with fleshy algae (Quinlan et al. 2018), as inferred from the observation of reduced growth rates and higher growth efficiencies of bacterioplankton on those substrates (Nelson et al. 2013), while algae tend to exude higher quantities of DOC enriched with labile sugars such as glucose and galactose (Nelson et al. 2013). These differences are known to restructure bacterioplankton communities from highly diverse taxonomic assemblages toward less diverse, copiotrophic communities adept at quickly growing on labile carbon compounds (Nelson et al. 2013; Haas et al. 2016) and are thought to promote the expression of virulence factors (Nelson et al. 2013; Cárdenas et al. 2018). Our recent work demonstrated that exudates from benthic primary producers could influence marine biofilms from early time points resulting in microbial community structures that were more similar at 2-weeks compared with later time points. Biofilms at later time points differentiated according to the benthic primary producers with which they had been cultured and nutrient additions further shifted the resulting biofilm communities (Remple et al. 2021). However, it is unclear how variation in organic matter and nutrients might influence the functional potential of reef biofilm communities.

In freshwater systems, the bioavailability of DOC has been demonstrated to influence community composition in mature biofilms (Olapade and Leff 2006) and ultimately affects the uptake and utilization of other nutrients(Suchismita Ghosh and Leff 2013). Because DOM

produced by autotrophs within the biofilm community support heterotrophic community members, microbial interactions in environmental biofilms are tightly coupled (Espeland and Wetzel 2001; Romaní and Sabater 2000). Nutrient loading or the introduction of labile organic matter can destabilize these interactions as heterotrophic community members may preferentially utilize labile organic matter from exogenous sources (Ylla et al. 2009) and phototrophs may become less reliant on bacterial remineralization of nutrients to meet their metabolic demands (Scott et al. 2008). Therefore, understanding the interaction of organic matter source and nutrient enrichment as influences on benthic marine biofilms is a priority for understanding assembly and successional processes.

Coral biofilms utilize recalcitrant organic matter

Genes involved with photosynthesis and the production of pigments were more abundant in biofilms cultured with corals compared with those cultured with algae. This finding suggests that phototrophic lifestyles were more widespread among dominant Bacteria in biofilms cultured with corals and could indicate stronger coupling between phototrophic- and heterotrophic communities in these biofilms, consistent with decreased bioavailability of exogenous organic matter from corals. Analysis of dissolved organic matter collected concurrently with our biofilm samples are reported in Quinlan et al. (2018) and reveal that corals exuded more recalcitrant organic matter than other benthic organisms in our study, and contained aromatic amino acids – specifically, tryptophan-, tyrosine-, and phenalalanine-like organic matter was enriched in coral treatments. Further, these exudates increased with nutrient additions in coral treatments (Quinlan et al. 2018). Biofilms from these treatments contained a significantly higher proportion of genes involved in the degradation of aromatic compounds including aromatic amine catabolism and

genes from the phenylacetyl CoA catabolic pathway, that further increased with nutrient additions. Genes involved with the biosynthesis of phenylalanine and tyrosine increased with nutrient additions and were significantly enriched in biofilms from coral treatments. Together these findings suggest that biofilms cultured with corals maybe specifically tuned to metabolize organic matter exudates from corals enriched in aromatic compounds and may even contribute to the accumulation of organic matter typically found on coral reefs.

Biofilms from algae utilize labile organic matter and encode virulence factors

Metagenomic comparisons of the central carbohydrate metabolisms in bacterioplankton communities report a higher proportion of genes associated with the Entner-Doudoroff (ED) and pentose phosphate (PP) cycles in algal-dominated reefs (Haas et al. 2016). These pathways are considered to be indicators of copiotrophic taxa as they are less efficient than the Embden-Meyerhof-Parnas pathway which were associated with coral-dominated reefs (Haas et al. 2016). Similarly, our study found a significantly higher proportion of genes involved with the ED pathway in biofilms cultured with algae. Genes involved in glycolysis in archaea were also enriched in algal biofilms, but there was no difference in the abundance of genes involved with the pentose phosphate cycle or glycolysis in bacteria. Biofilms cultured with algae additionally contained a higher proportion of genes involved the uptake and utilization of lactose and galactose, suggesting organisms inhabiting algal biofilms are adept at quickly utilizing labile organic compounds. This is consistent with observations of galactose in exudates of reef macroalgae (Haas and Wild 2010) and enrichment of release relative to corals (Nelson et al. 2013), suggesting that biofilm Bacteria community shifts are responding to the sugar compositional profile of macroalgae.

In marine ecosystems, copiotrophic bacterioplankton have been linked with increased virulence factors (Barott et al. 2012; Cárdenas et al. 2018). Virulence factors impart pathogenicity in microbes and include toxins, exoenzymes, and secretion systems (Brown, Cornforth, and Mideo 2012). In our study, DNA from algal biofilms encoded a significantly higher number of potential virulence factors compared with corals. Most notably, type- III, IV, and VI secretion systems were more abundant in algal biofilms. Both type III (T3SS) and type VI (T6SS) secretion systems are commonly associated with Gram-negative, pathogenic bacteria. The T3SS, also known as the injectisome, promotes the transfer of virulence proteins from bacterial cytoplasm into eukaryotic cells (Galán and Waksman 2018) and is found in a variety of plant and animal pathogens including the coral pathogen, *Vibrio coralliilyticus* (Hoyer et al. 2019). The type VI secretion system (T6SS) transfers toxic effectors to both prokaryotic and eukaryotic cells, and plays an important role in competition and pathogenesis among bacteria (Pena et al. 2019). However, T6SS is also found in non-pathogenic bacteria where it may function in antipathogenesis or, as in the case of Myxococcus xanthus, T6SS could be used in interactions with other bacteria (Jani and Cotter 2010). The Myxococcus genus was more abundant in biofilms cultured with algae in our study and could be a source of the proportionally higher T6SS genes associates with these treatments. The Type IV secretion system (TIVSS) is the most cosmopolitan type of bacterial secretion system and is broadly found across Gram-positive and Gram-negative bacteria as well as archaea (Pena et al. 2019). A particular type of TIVSS called the Dot/Icm system is the major virulence mechanism in Legionella pneumophila and Coxiella burnetiid (Gómez et al. 2013) and homologues of the Dot/Icm system have been described in the fish pathogen Piscirickettsia salmonis. In our study, TIVSS Dot/Icm genes were significantly higher in algal biofilms compared with biofilms cultured with coral. Together, these

findings suggest that algal biofilms carry more potential delivery mechanisms for virulence factors than those on coral dominated reefs, consistent with findings that algal DOM stimulates taxa associated with virulence (Nelson et al. 2013; Cárdenas et al. 2018).

Comparison of functional potential over time

In its simplest form, biofilm development is depicted as a linear process that commences when free-floating cells attach to a submerged surface (attachment), followed by growth into a mature, structurally complex biofilm and culminates in the dispersal of detatched bacterial cells into the surrounding waters (Hall-Stoodley and Stoodley 2002). In mature biofilms, structural complexity is facilitated by extracellular polymeric substances (EPS) which provide stability, mediate adhesion, and protect cells in the biofilm from predation and toxic substances. The EPS further contains compounds such as proteins, extracellular DNA, and particulates (reviewed in (Romani et al. 2016). In our study, samples collected from biofilms at 2-weeks contained a larger proportion of genes associated with phages and prophages including those associated with phage tail fibers and capsid proteins. Although phages can encode cells that degrade EPS, recent studies demonstrate that EPS confers some amount of resistance to infection finding phage infected cells present only in certain areas of the biofilm with lower amounts of matrix (Melo et al. 2020). Interestingly, although relatively young biofilms are expected to contain mostly metabolically active cells, genes associated with persister cells and spore germination were higher in our 2-week samples compared with those collected at 6-weeks. Persister cells are subpopulations of cells that are resistant to environmental stressors through reduced metabolic activity or dormancy. The resistance furnished by persister cells is distinct in that it is not heritable and it is reversible. Because phages require active machinery in a host cell to propagate, their replication

is strongly influenced by the metabolic state of the host cell therefore dormant and persister cells may limit phage replication (Pires, Melo, and Azeredo 2021).

Mature biofilms are expected to contain higher proportions of cells with reduced metabolic capacities (Pires et al. 2017). Within the dormancy and sporulation subgroup, we further found subsystems containing genes involved with exosporium, spore pigment biosynthesis, and the SpoVS protein family. Increases in these genes likely suggest a higher abundance of cells entering dormant or sporulation phases as a natural progression of bacterial life in biofilms. Further, many of the subsystems that increased substantially between time points, appear to function in regulation and maintenance of mature biofilms. For example, biofilm adhesin biosynthesis is a subsystem within the quorum sensing and biofilm formation subgroup that increased 3-fold over time. As the name suggests, this subsystem consists of a collection of genes required for the synthesis and transport of poly-beta-1,6-N-acetyl-D-glucosamine (PGA), a common feature of the biofilm EPS (Lasa 2006) and an adhesin protein involved in cell-to-cell and cell-to-surface adhesion in biofilms (Pena et al. 2019) and is also important in the formation of biofilm architecture and microstructure (Itoh et al. 2008). Additionally, the paerucumarin biosynthesis subsystem increased ~3x between time points. Paerucumarin is known to chelate iron but does not function as a siderophore. Instead, it is known to regulate biofilm formation and has been shown to promote or reduce biofilm formation depending on environmental circumstances (Lin et al. 2016). Finally, genes involved with nonribosomal peptide synthetases (NRPS) doubled between time points and are important in the synthesis of cytostatics and antibiotics (Martínez-Núñez and López 2016) that can promote competition within biofilm communities through inhibition of growth and cellular division and maintain genetic diversity in complex biofilms (Rendueles and Ghigo 2015).

Photosynthesis at later time points

Samples collected at 6-weeks contained more genes associated with light harvesting complexes including those associated with the chlorosome and the phycobilisome suggesting an active phototrophic community and potentially signaling a shift in light availability within the biofilm community. The chlorosome is an extremely efficient light harvesting complex that is particularly adept at functioning in low light conditions (Orf and Blankenship 2013). It is a feature of green sulfur bacteria and some filamentous anoxygenic phototrophs. At later time points, genes involved with the chlorosome increased 5-fold, potentially indicating the development of microenvironments that are characteristic of mature biofilms. In addition to being more abundant in 2-week coral treatments, genes associated with the phycobilisome increased 2x between time points. The phycobilisome is a light harvesting complex that is specific to cyanobacteria, red-algae, cryptophytes, and glaucophytes (Singh 2015). It contains phycobiliproteins that are linked to tetrapyrrole chromophores called bilin and facilitate the phycobilisome in adapting to various light conditions in a process called complementary chromatic adaptation (Singh et al. 2015). Bilins are obtained by modifying hemes through enzymatic activity like those of the heme oxidase (Adir, Bar-Zvi, and Harris 2020). Increases in genes associated with the phycobilisome were accompanied by a 2-3x increase in genes associated with both heme and bilin biosynthesis in biofilms collected at 6-weeks. Although we can not definitively determine if there was organismal treatment effect at the 6-week time point; our previous work demonstrated that relative abundances of cyanobacteria increased at later time points in biofilms cultured with corals (Remple et al. 2021). It is therefore likely that increases in

genes associated with heme-, and bilin biosynthesis, and the phycobilisome are attributed to cyanobacteria populations in biofilms cultured with corals.

Conclusion

Biofilms are dynamic systems with changing taxonomic assemblages and functional profiles that evolve based on environmental conditions. In this study, we show that benthic primary producers and nutrient additions independently influence functional profiles of marine biofilms. Genetic potential of young biofilms in our study appeared to be capable of utilizing the specific organic matter exudates of dominant reef primary producers. Biofilms cultured with coral contained more genes involved with the metabolism of aromatic compounds. Those cultured with algae appeared to contain a higher proportion of genes for the utilization of labile organic matter and involved with bacterial secretion systems. Finally, comparisons of genetic potential over time revealed that young biofilms in our study appeared to ward off phage attacks before developing into complex microbial communities capable hosting both phototrophic and heterotrophic groups.

Figure 4.1. Experimental design & sampling scheme
a) Three 1300L flow-through incubation tanks were used to maintain constant temperature for 9 flow-through aquaria each (total 27 experimental aquaria, 6L volume each); a schematic of one tank with 9 aquaria is shown here. Each aquarium held one of three benthic constituent organisms (algae, coral, or sand) and was supplied via peristaltic pump with filtered seawater amended to one of three nutrient treatments (ambient, low, or high) from independent header mixing aquaria (one for each nutrient level in each tank to maintain independence). Biofilms were cultured on glass slides, evenly spaced in slide racks and suspended vertically in each experimental aquarium to allow water to flow around each surface of the slide. Nutrients were measured weekly and were stable throughout the experiment; averages are reported. Additional data in Quinlan et al. (2018) and Silbiger et al. (2018). b) Replicate samples were obtained from 2-week biofilms and used to investigate the effects of benthic organism and nutrient on functional profiles. To compare functional potential over time, 2-week samples were averaged and compared with representative samples of biofilms collected at 6-weeks.

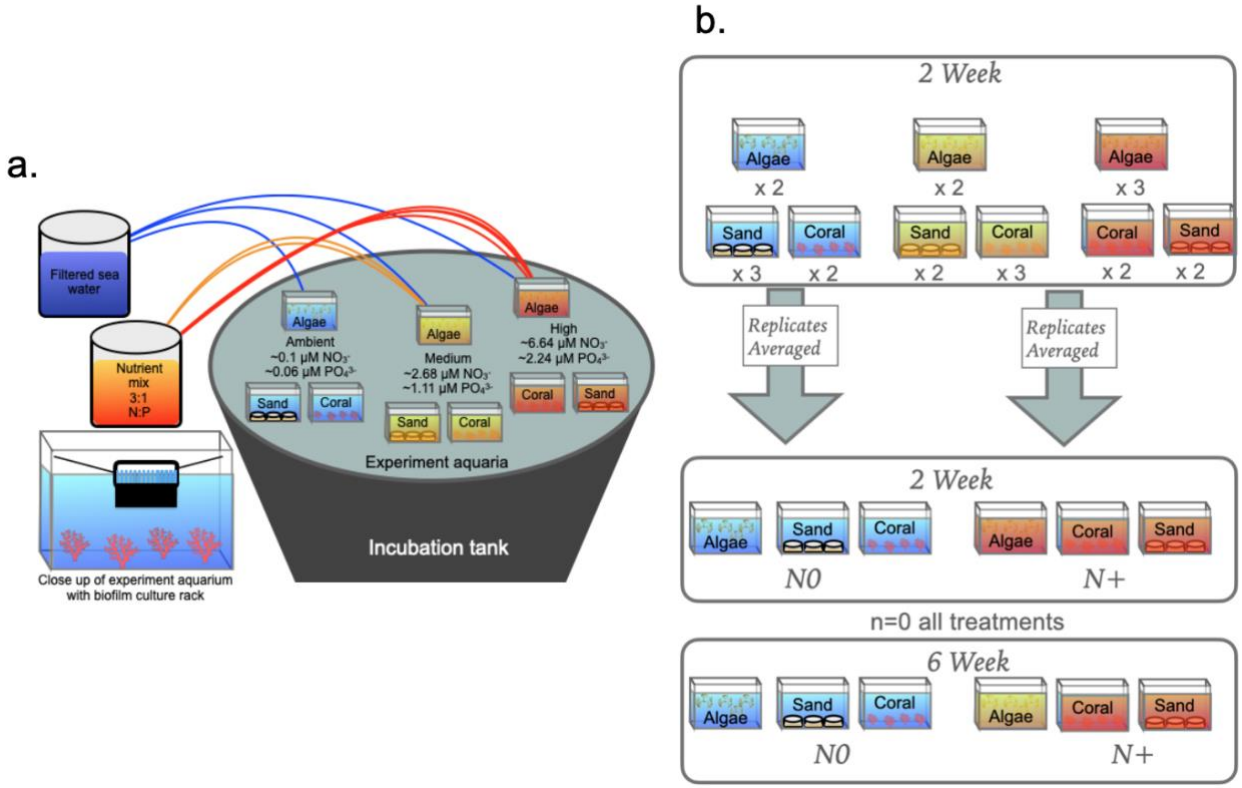
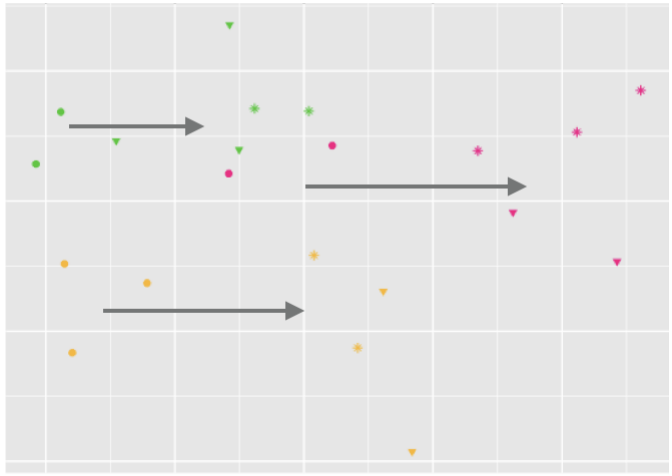
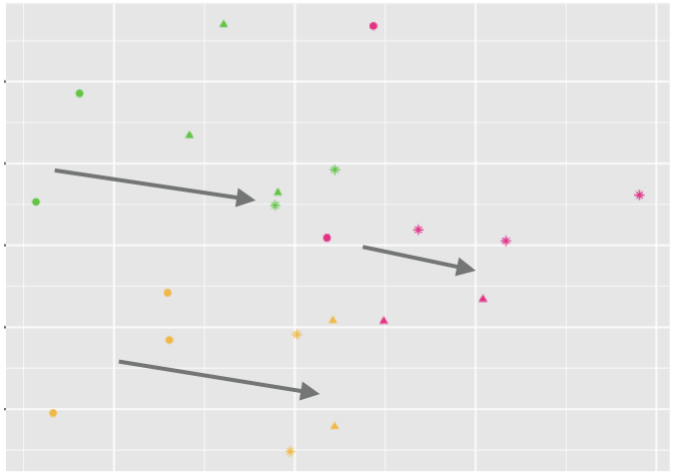


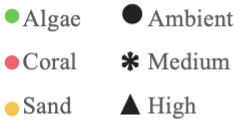
Figure 4.2. Visualization of biofilm OTUs associated with treatments at 2-weeks
Hierarchical clustering (Ward's minimum variance method) organized statistically selected OTUs according to distributions of standardized (z-scored) mean relative abundance across treatments. OTUs that were both significant by linear mixed model and abundant were ranked by random forest and are displayed along the y-axis. Green box denotes taxa that are enriched in biofilms cultured with algae, pink box denotes taxa enriched in corals, and yellow box indicates taxa that are more abundant in biofilms cultured with sand.

a) Taxonomy: RefSeq Genus



b) Functional potential: SEED subsystems





c) Comparision of PERMANOVA results

	Organism		Nutrient		Interaction (O:N)	
	R^2	P-value	R^2	P-value	R^2	P-value
Taxonomy	0.413	<0.001	0.257	<0.001	0.093	0.383
Functional Potential	0.237	<0.001	0.155	<0.001	0.17	0.17

Figure 4.3. Visualization of biofilm OTUs associated with treatments at 2-weeks
Hierarchical clustering (Ward's minimum variance method) organized statistically selected OTUs according to distributions of standardized (z-scored) mean relative abundance across treatments. OTUs that were both significant by linear mixed model and abundant were ranked by random forest and are displayed along the y-axis. Green box denotes taxa that are enriched in biofilms cultured with algae, pink box denotes taxa enriched in corals, and yellow box indicates taxa that are more abundant in biofilms cultured with sand.

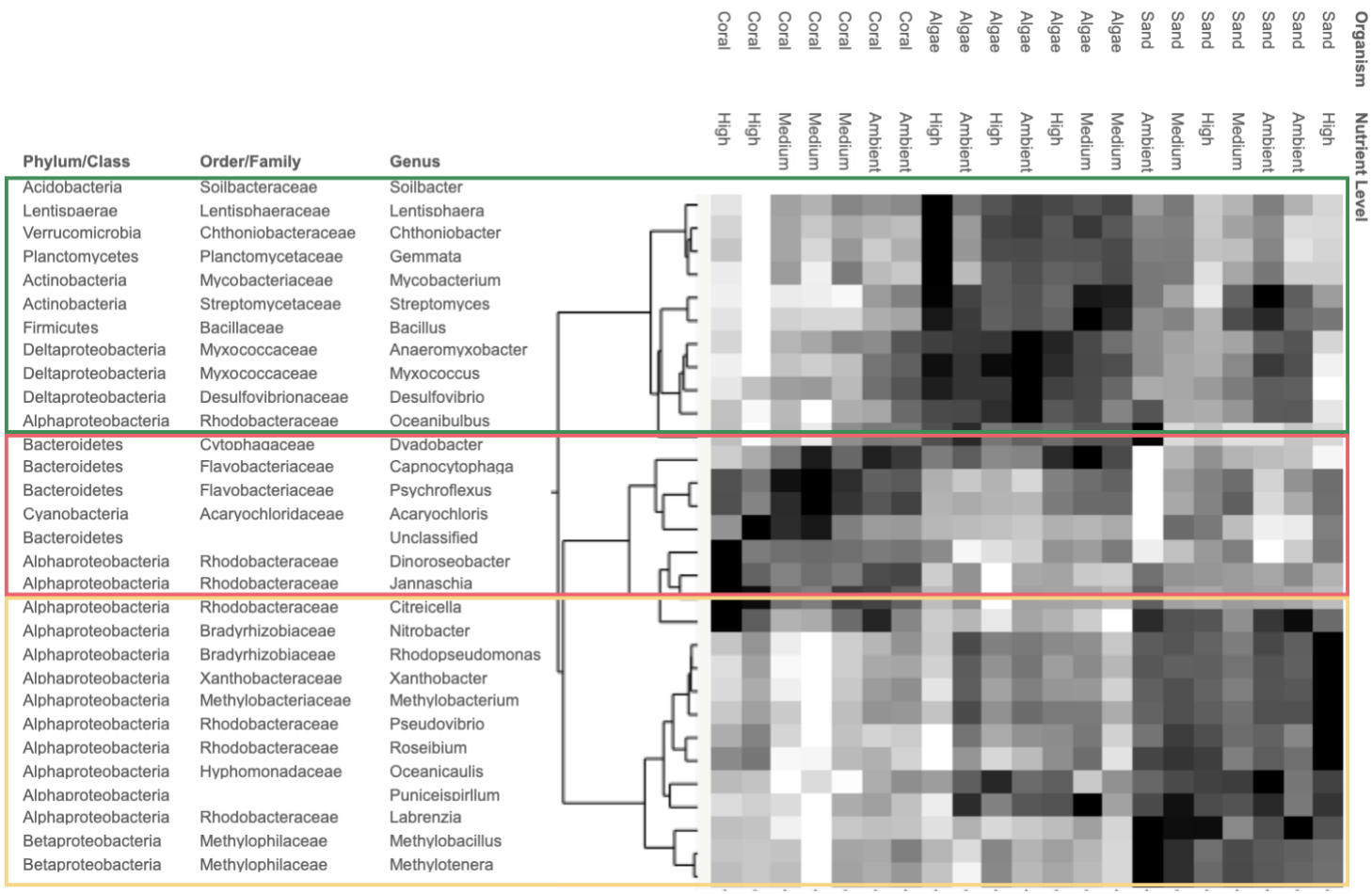


Figure 4.4. Visualization of SEED subsystems at 2-weeks

Heatmap of standardized (z-score) relative abundance by treatment at 2-weeks. Subsystems are organized by SEED subgroup and group. SEED group and subgroups that responded significantly ($p <= 0.05$) to experimental treatments by linear model are marked, blue diamond indicates a group or subgroup that responds significantly nutrient treatment, purple circle indicates a group or subgroup that responds significantly to organism.

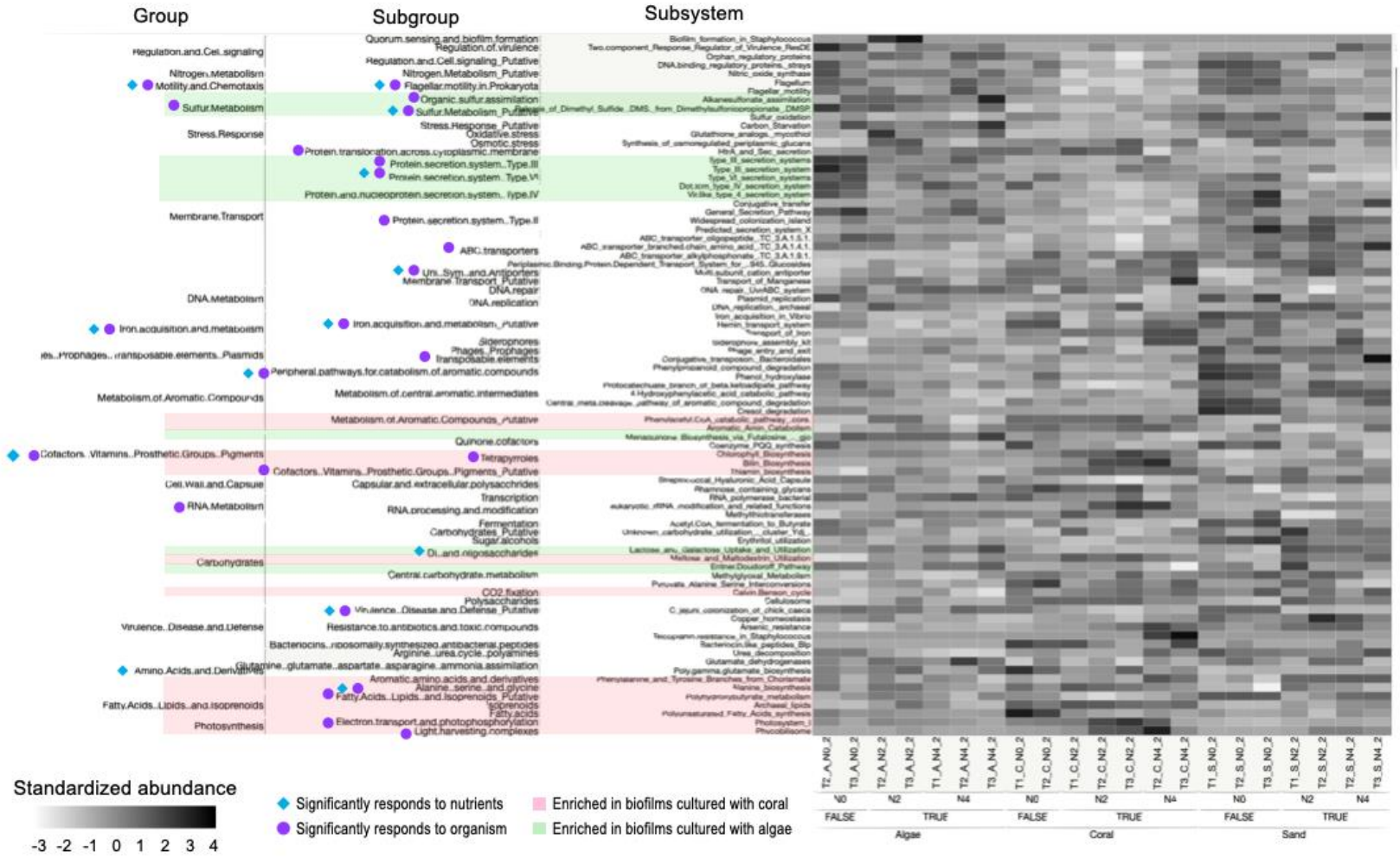


Figure 4.5. Organism and nutrient effect on the functional potential of 2-week and 6-week biofilms
Functional profiles of marine biofilms cultured at 2-weeks and 6-weeks demonstrate that mature (6-week) biofilms differentiate over time, separating by both benthic organism and nutrient treatment.

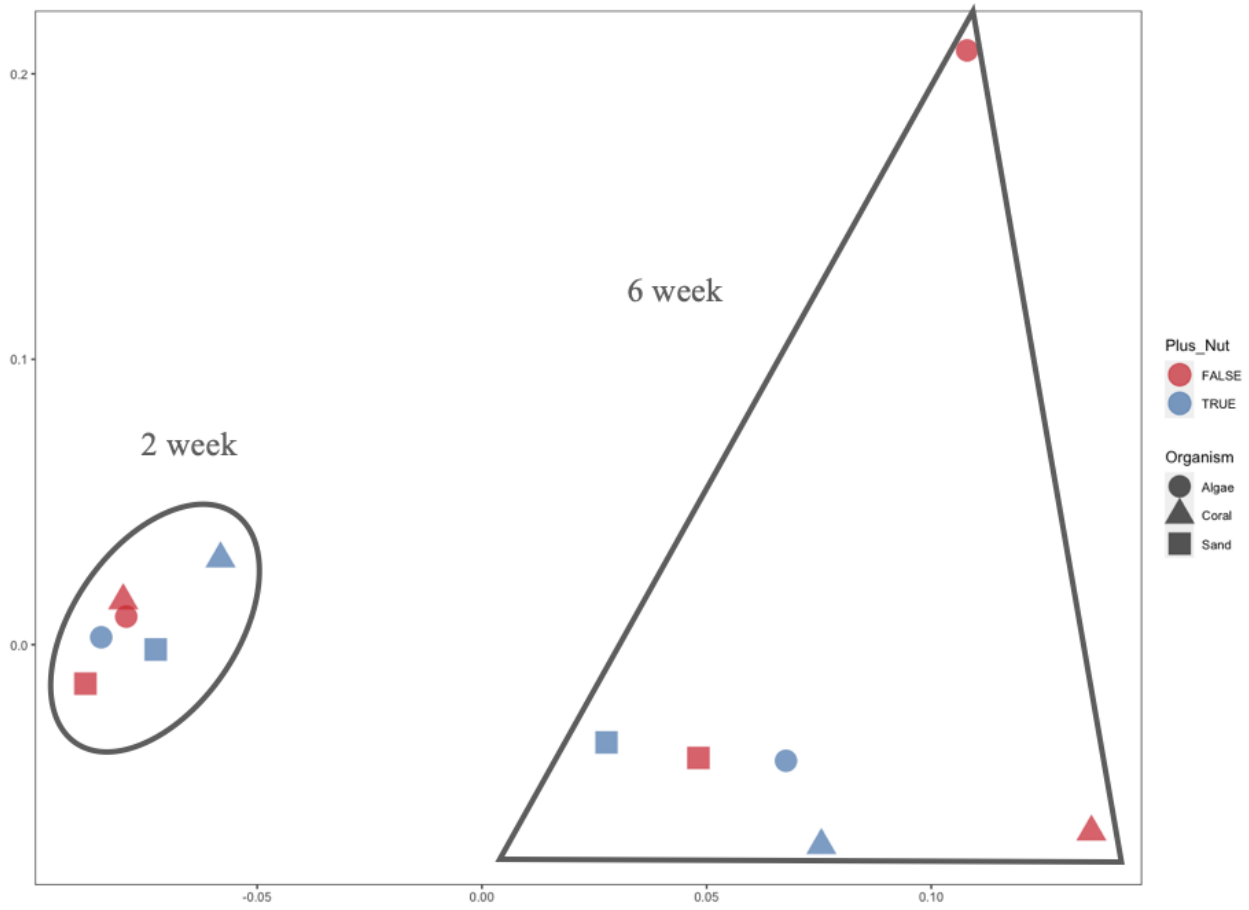
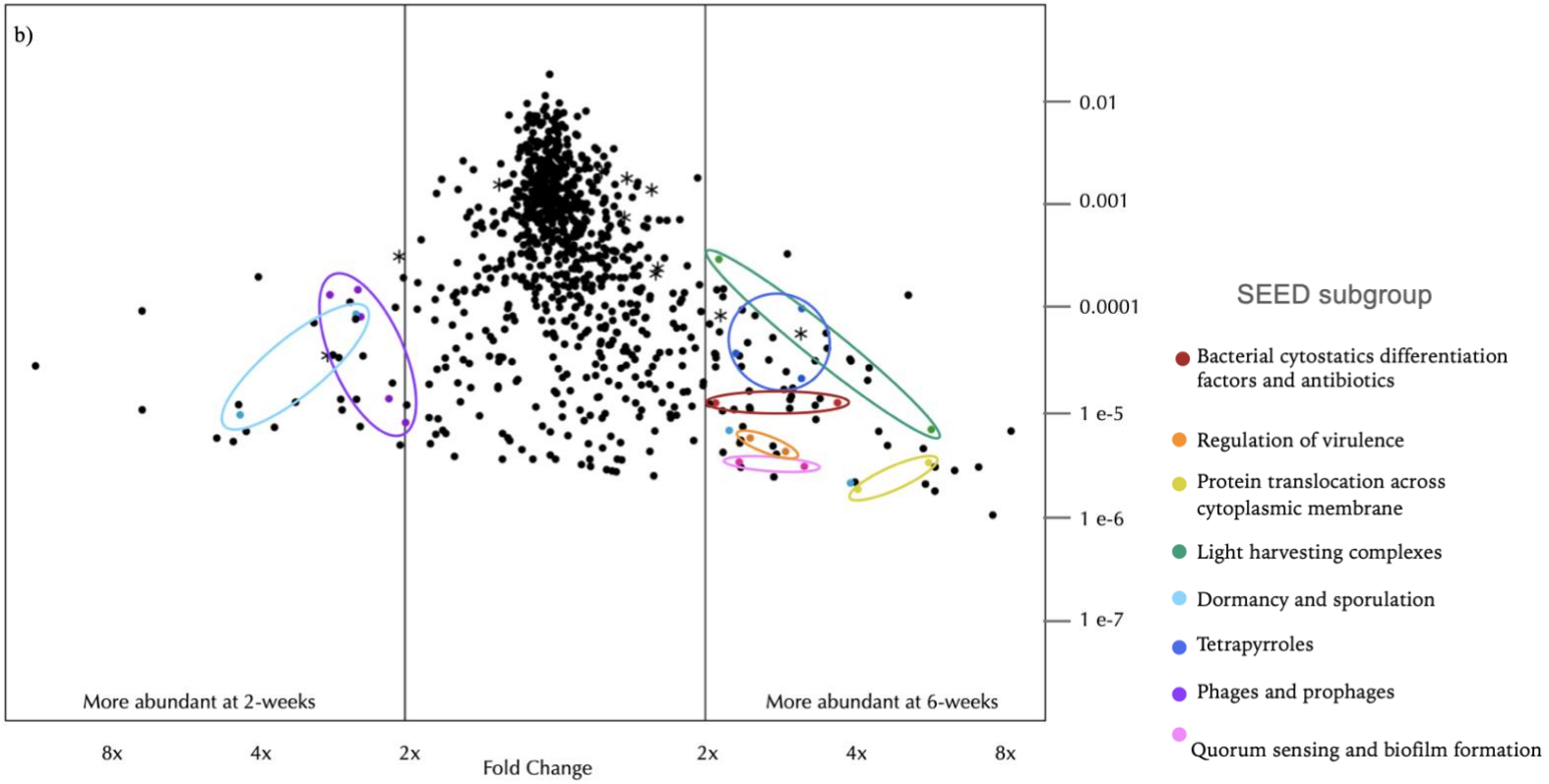


Figure 4.6. Proportional fold change of SEED subsystems between 2-weeks and 6-weeks
Log_2 ratio of SEED subsystems (6-week:2-week) are plotted (x-axis) by the mean relative abundance at 2-weeks (y-axis). Individual SEED subsystems that responded significantly to time are plotted as an asterisk (*). Separately, the log_2 ratio was used to calculate the proportion of subsystems within a subgroup that changed at least 2x between time points. Subsystems are color coded by their subgroup association. Subgroups are color coded if the proportional fold change ≥ 0.5 or proportional fold change ≥ 0.3 and all responding subsystems were associated with a single time point.



CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

Coral reefs are culturally, biologically, and economically important structures, and their survival is paramount to the ocean's biodiversity and the livelihoods of the people that live near them. Over the past 20 years links have been established between anthropogenic stressors, coral-algal phase shifts, and microbial community structure. One such stressor, nutrient loading, is correlated with alterations in planktonic and host-associated microbial communities, but the dynamics governing these changes remain an open and active area of research.

This dissertation furthers our understanding of the dynamics governing reef microbial communities by investigating the role of nutrient loading in shaping reef microbial structure. The work presented here is partitioned in to two main projects; a field study (Chapter 2), characterizes microbial communities associated with submarine groundwater discharge (SGD) and provides evidence that the associated nutrient flux impacts planktonic communities on reefs. In Chapters 3 & 4 I focused on marine biofilms. Biofilms were cultured over the course of 6 weeks with 3 benthic primary producers (coral, algae, and sand) in a nutrient press experiment. Taxonomic and functional profiles were compared to resolve the effects of succession, benthic community, and inorganic nutrients on biofilm community structure. Organic matter exudates were collected alongside these biofilms and were analyzed and reported separately (as outlined in Chapter 3 and reported in Quinlan, Remple, et al. 2018).

The major findings of my dissertation are 1) disparate microbial communities are found along a biogeochemical gradient established by SGD, 2) benthic community organisms work interactively and orthogonally with inorganic nutrients to influence biofilm community structure, and 3) strong successional trajectories in marine biofilms differentiate surface attached microbial

communities from bacterioplankton communities. My findings provide evidence that increased inorganic nutrients shift both planktonic and surface attached microbial communities directly and indirectly, by stimulating benthic primary producers. The simultaneous consideration of both planktonic and surface attached microbial communities could further this line of research considerably.

In chapter 2 I report the findings of a study at Black Point in Maunalua Bay, HI which is among the first characterizations of microbial communities associated with SGD. Compared with other sampling sites, the samples collected from the SGD spring contained the most diverse microbial assemblages however, most of these populations were confined to the SGD spring. Reef locations with the strongest influence of SGD-associated nutrients (i.e. the Transition Zone) hosted microbial communities that were distinct from both the SGD spring and the ambient reef waters, and were enriched with copiotrophic organisms. Similarly, high concentrations of ammonium were observed in mid-reef waters but were much lower in SGD- and ambient reef waters, indicating that ammonium was produced at mid-reef sites. Although we could not discern the source, it is likely that breakdown of organic matter by heterotrophic microbial communities at these locations contribute to the anomalously high concentrations of ammonium observed in these samples. Interestingly, microbial communities at high tide were not different between ambient reef waters and mid-reef locations, but high concentrations of ammonium were observed at mid-reef sites regardless of tide. Because biofilms are anchored in place and tend to be highly metabolically active, future studies like this may benefit from surveying benthic structures and analyzing the surface attached microbial communities alongside samples from the planktonic community.

In Chapters 3 &4, our mesocosm experiment demonstrated biofilm communities diverged from bacterioplankton communities and that both benthic community members as well as inorganic nutrients consistently impact microbial community structure of reef biofilms. In a companion study, analysis of dissolved organic matter (DOM) collected concurrently with our biofilm samples established that 1) all benthic organisms in our study increased organic matter exudates in response to nutrient additions and 2) DOM exudates from corals were enriched with proteinaceous organic matter associated with aromatic amino acids (Quinlan et al. 2018). Functional profiles from young biofilms suggested that the microbial community may be specifically tuned to processing organic matter produced by the benthic community. For example, genes involved with the metabolism of aromatic amino acids were elevated in coral treatments. Taxonomically, these biofilms were more similar than those collected at later time points which further diverged by organismal treatment. Together, these results indicate that differences in biofilm function may become more pronounced over time.

While nutrient additions significantly influenced biofilm community structure at each time point, the effects were not consistent between organismal treatments. In coral treatments, biofilms contained a higher proportion of genes involved with photosynthesis. These genes increased with nutrient additions suggesting that biofilms from corals host a larger autotrophic community than those cultured with other benthic organisms.

Microbial interactions within environmental biofilms are often tightly coupled. In freshwater systems, bacterial communities in biofilms are largely reliant on photosynthate produced within the biofilm community (Espeland and Wetzel 2001; Romaní and Sabater 2000). Organic matter and nutrients are retained and rework within the biofilm matrix prior to being released into the surrounding waters (Battin et al. 2016; Lyon and Ziegler 2009). Nutrient

loading decouples these interactions by decreasing the reliance of phototrophs on bacterial remineralization of nutrients, and heterotrophs become less stressed for photosynthetically derived organic carbon (Scott et al. 2008).

In our study, genes from biofilms cultured with corals, involved with photosynthesis increased with nutrient additions, but genes involved with aromatic amino acid metabolism did not follow these same patterns. The association of aromatic amino acids with coral exudates implies that coral exudates contain nitrogenous compounds and these compounds may be a source of nitrogen in oligotrophic waters. Biofilms cultured with corals contained a higher proportion of genes involved with amino acid metabolism relative to those cultured with other benthic organisms, and suggests that they were capable of utilizing these compounds. However, the disproportionate increase of photosynthesis genes relative to genes involved with aromatic amino acid metabolism could indicate a breakdown of microbial interactions within the biofilm community. By utilizing nitrate from the surrounding water column, autotrophs no longer require nutrient cycling from within the biofilm community to meet their nutrient requirements. On a reef-wide scale, processes like this could result in a loss of the nutrient retention and cycling typically performed by biofilms. Ultimately, these biofilms could become a source of labile organic matter, likely contributing to the types of alterations in the planktonic community that are associated with in phase shifts.

To date, marine biofilms are recognized as important components of coral reef ecosystems. However, their importance is typically contextualized within their ability to directly influence the benthic community through larval settlement or as part of a mutualistic dynamic, as is the case in epiphytic communities and microbial communities found within the coral holobiont. Outside of these duties, marine biofilms are often considered nuisances and studies

are focused on their eradication rather than the dynamics governing their development and community structure. Because of this perspective, foundational research regarding the dynamics governing marine biofilm assembly, development, and function lags compared with work in freshwater systems. While some fundamental concepts can be leveraged from one system to another, freshwater and marine environments are innately different and there is no substitution for foundational work in the present environment.

This body of work establishes baselines of marine biofilm assembly separately from planktonic communities. Further, I have discussed how interactions within biofilm communities might ultimately influence planktonic communities, and shown that analyses of planktonic communities alone cannot fully describe all aspects of microbial ecosystem dynamics.

The effort to research and preserve coral reefs has seen an increasing number of manmade structures are appearing in oceans around the world (i.e. coral nurseries, art installations, artificial reefs, etc.). To aid in these preservation efforts, futures studies should include analyses of biofilms growing on these structures as independent members of the ecosystem. Work to compare autotrophic vs heterotrophic populations, metabolic capacity, and diel patterns in biofilms, as well relating processes in biofilm and planktonic communities could clarify aspects of coral reef function and resilience.

APPENDIX A
SUPPLEMENTARY TABLES AND FIGURES FOR CHAPTER 2

Table S2.1. Comparison of water chemistry by sampling site and tide

Geometric mean values of each chemical parameter are displayed. a) Polynomial regressions were used to identify chemical parameters with significant linear or quadratic relationships to salinity at low tide. b) At high tide, the Spring is submerged and samples are not collected from this location. Therefore, only a linear relationship can be tested.

Parameters with significant linear relationships that were inversely related to salinity were considered to be **SGD** associated. Significant linear relationships that are positively correlated with salinity are considered to be **marine**. Parameters with a significant quadratic relationship, were associated with **mid-salinity** ranges.

a) Low Tide

Parameter	Spring	Transition	Diffuse	Reef	
Salinity	**5.11**	**22.14**	**27.80**	**33.41**	**Association**
NO^{3-}+ NO^{2-} (µmol/L)	165.90	57.24	20.46	3.54	SGD
Phosphate (µmol/L)	3.22	1.36	0.56	0.19	SGD
Silicate (µmol/L)	569.40	249.79	95.72	23.23	SGD
DON SFIA uM	12.80	7.39	4.13	4.96	SGD
CobleA	0.04	0.04	0.02	0.02	SGD
HIX	5.37	3.70	3.10	2.13	SGD
M.C	1.13	1.06	1.03	1.01	SGD
NH_4 (µmol /L)	0.18	1.01	0.77	0.42	Mid-salinity
CobleT	0.02	0.03	0.02	0.02	Mid-salinity
Bacterioplankton	34.17	127.86	191.93	199.21	Marine
Eukaryotes	9.34	25.44	24.38	18.88	Mid-salinity
DOC (µM)	36.30	76.86	71.34	68.15	Marine
Chlorophyll A (µg / L)	0.06	0.53	0.50	0.22	Mid-salinity

b) High Tide

Parameter	Transition	Diffuse	Reef	
Salinity	**32.77**	**34.29**	**34.55**	**Association**
NO^{3-}+ NO^{2-} (µmol/L)	3.67	1.86	0.79	SGD
Phosphate (µmol/L)	0.28	0.16	0.09	SGD
Silicate (µmol/L)	31.39	16.95	10.17	SGD
DON SFIA uM	7.02	6.95	6.56	
CobleA	0.03	0.02	0.02	SGD
HIX	3.44	3.30	2.95	SGD
M.C	1.00	0.96	0.95	
NH_4 (µmol /L)	1.12	0.82	0.41	SGD
CobleT	0.02	0.01	0.01	
Bacterioplankton	209.30	206.55	177.18	
Eukaryotes	40.37	41.13	25.05	
DOC (µM)	78.79	86.94	63.85	
Chlorophyll A (µg /	0.70	0.92	0.30	

Table S2.2. SGD associated microbial families

Kingdom	Phylum	Class	Order	Family
Archaea	Euryarchaeota	Thermoplasmata	Marine_Group II	Marine_Group II fa
Archaea	Nanoarchaeaeota	Woesearchaeia	Woesearchaeia_or	Woesearchaeia_fa
Archaea	Thaumarchaeota	Nitrososphaeria	Nitrosopumilales	Nitrosopumilaceae
Bacteria	Actinobacteria	Actinobacteria	Micrococcales	Microbacteriaceae
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales_unclassified
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinifilaceae
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidia_unclassified	Bacteroidia_unclassified
Bacteria	Bacteroidetes	Bacteroidia	Chitinophagales	Saprospiraceae
Bacteria	Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagales_uncultured
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Bernardetiaceae
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Cyclobacteriaceae
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Cytophagaceae
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Cytophagales_unclassified
Bacteria	Bacteroidetes	Bacteroidia	Cytophagales	Spirosomaceae
Bacteria	Bacteroidetes	Bacteroidia	Flavobacteriales	Crocinitomicaceae
Bacteria	Bacteroidetes	Bacteroidia	Sphingobacteriales	Sphingobacteriales env.OPS_17
Bacteria	Bacteroidetes	Ignavibacteria	Ignavibacteriales	BSN166
Bacteria	Bacteroidetes	Rhodothermia	Rhodothermales	Rhodothermaceae
Bacteria	Calditrichaeota	Calditrichia	Calditrichales	Calditrichaceae
Bacteria	Chlamydiae	Chlamydiae	Chlamydiales	Chlamydiales_unclassified
Bacteria	Chlamydiae	Chlamydiae	Chlamydiales	cvE6
Bacteria	Chloroflexi	Anaerolineae	Ardenticatenales	Ardenticatenaceae
Bacteria	Cloacimonetes	Cloacimonadia	Cloacimonadales	Cloacimonadales
Bacteria	Cyanobacteria	Melainabacteria	Obscuribacterales	Obscuribacterales

Table S2.2. (Continued) SGD associated microbial families

Bacteria	Cyanobacteria	Oxyphotobacteria	Eurycoccales	Eurycoccales
Bacteria	Cyanobacteria	Oxyphotobacteria	Nostocales	Xenococcaceae
Bacteria	Cyanobacteria	Oxyphotobacteria	Oxyphotobacteria	unclassified
Bacteria	Cyanobacteria	Oxyphotobacteria	Phormidesmiales	Phormidesmiaceae
Bacteria	Cyanobacteria	Sericytochromatia	Sericytochromatia	Sericytochromatia
Bacteria	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Arcobacteraceae
Bacteria	Epsilonbacteraeota	Campylobacteria	Campylobacterales	Thiovulaceae
Bacteria	Fibrobacteres	Fibrobacteria	Fibrobacterales	Fibrobacteraceae
Bacteria	Firmicutes	Bacilli	Bacillales	Staphylococcaceae
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae
Bacteria	Firmicutes	Clostridia	Clostridiales	Family
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae
Bacteria	Gemmatimonadetes	BD2.11		
Bacteria	Marinimicrobia	.SAR406		Marinimicrobia
Bacteria	Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae
Bacteria	Omnitrophicaeota	Omnitrophia	Omnitrophales	Omnitrophaceae
Bacteria	Patescibacteria	ABY1	ABY1	unclassified
Bacteria	Patescibacteria	ABY1	Candidatus	Komeilibacteria
Bacteria	Patescibacteria	ABY1	Candidatus	Magasanikbacteria
Bacteria	Patescibacteria	Gracilibacteria	Absconditabacteriales	.SR1.
Bacteria	Patescibacteria	Gracilibacteria	Candidatus	Peregrinibacteria
Bacteria	Patescibacteria	Gracilibacteria	Candidatus	Peribacteria
Bacteria	Patescibacteria	Gracilibacteria	Gracilibacteria	
Bacteria	Patescibacteria	Gracilibacteria		JGI 0000069.P22
Bacteria	Patescibacteria	Parcubacteria	Candidatus	Campbellbacteria
Bacteria	Patescibacteria	Parcubacteria	Candidatus	Kaiserbacteria

Table S2.2. (Continued) SGD associated microbial families

Bacteria	Patescibacteria	Parcubacteria	Candidatus	Liptonbacteria
Bacteria	Patescibacteria	Parcubacteria	Candidatus	Portnoybacteria
Bacteria	Patescibacteria	Parcubacteria	Candidatus	Ryanbacteria
Bacteria	Patescibacteria	Parcubacteria	Parcubacteria	unclassified
Bacteria	Patescibacteria	Saccharimonadia	Saccharimonadales	Saccharimonadales
Bacteria	Planctomycetes	Phycisphaerae	Phycisphaerales	Phycisphaeraceae
Bacteria	Planctomycetes	Phycisphaerae	Tepidisphaerales	Tepidisphaeraceae
Bacteria	Planctomycetes	Planctomycetacia	Pirellulales	Pirellulaceae
Bacteria	Planctomycetes	vadinHA49	vadinHA49	
Bacteria	Proteobacteria	Alphaproteobacteria	Azospirillales	Azospirillaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Hyphomonadaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Kordiimonadales	Kordiimonadaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Micavibrionales	uncultured
Bacteria	Proteobacteria	Alphaproteobacteria	Paracaedibacterales	Paracaedibacteraceae
Bacteria	Proteobacteria	Alphaproteobacteria	Parvibaculales	Parvibaculaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Devosiaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Stappiaceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Xanthobacteraceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillales
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	AB1
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiales
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae
Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bacteriovoracaceae
Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae

Table S2.2. (Continued) SGD associated microbial families

Bacteria	Proteobacteria	Deltaproteobacteria	Bradymonadales	Bradymonadales
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Blfdi19
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Nannocystaceae
Bacteria	Proteobacteria	Deltaproteobacteria	Oligoflexales	0319.6G20
Bacteria	Proteobacteria	Deltaproteobacteria	Oligoflexales	Oligoflexaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadales
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Colwelliaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Methylophilaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Rhodocyclaceae
Bacteria	Proteobacteria	Gammaproteobacteria	CCM19a	CCM19a
Bacteria	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Cellvibrionaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Cellvibrionales	Spongiibacteraceae
Bacteria	Proteobacteria	Gammaproteobacteria	EC3	EC3
Bacteria	Proteobacteria	Gammaproteobacteria	Francisellales	Francisellaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	Incertae
Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria	unclassified
Bacteria	Proteobacteria	Gammaproteobacteria		KI89A clade
Bacteria	Proteobacteria	Gammaproteobacteria	MBAE14	MBAE14
Bacteria	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Marinomonadaceae

Table S2.2. (Continued) SGD associated microbial families

Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Nitrincolaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Oleiphilaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Saccharospirillaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadales
Bacteria	Proteobacteria	unclassified		
Bacteria	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Terrimicrobiaceae
Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	DEV007
Bacteria	WPS.2			

Figure S2.1. Water chemistry at high tide

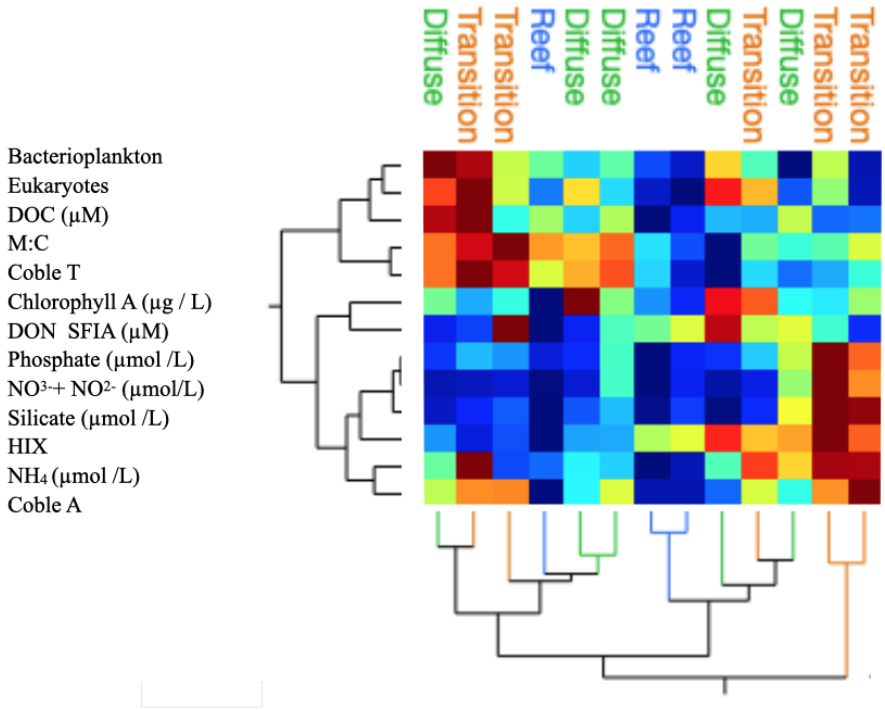


Figure S2.2. Reef chemistry by salinity or site

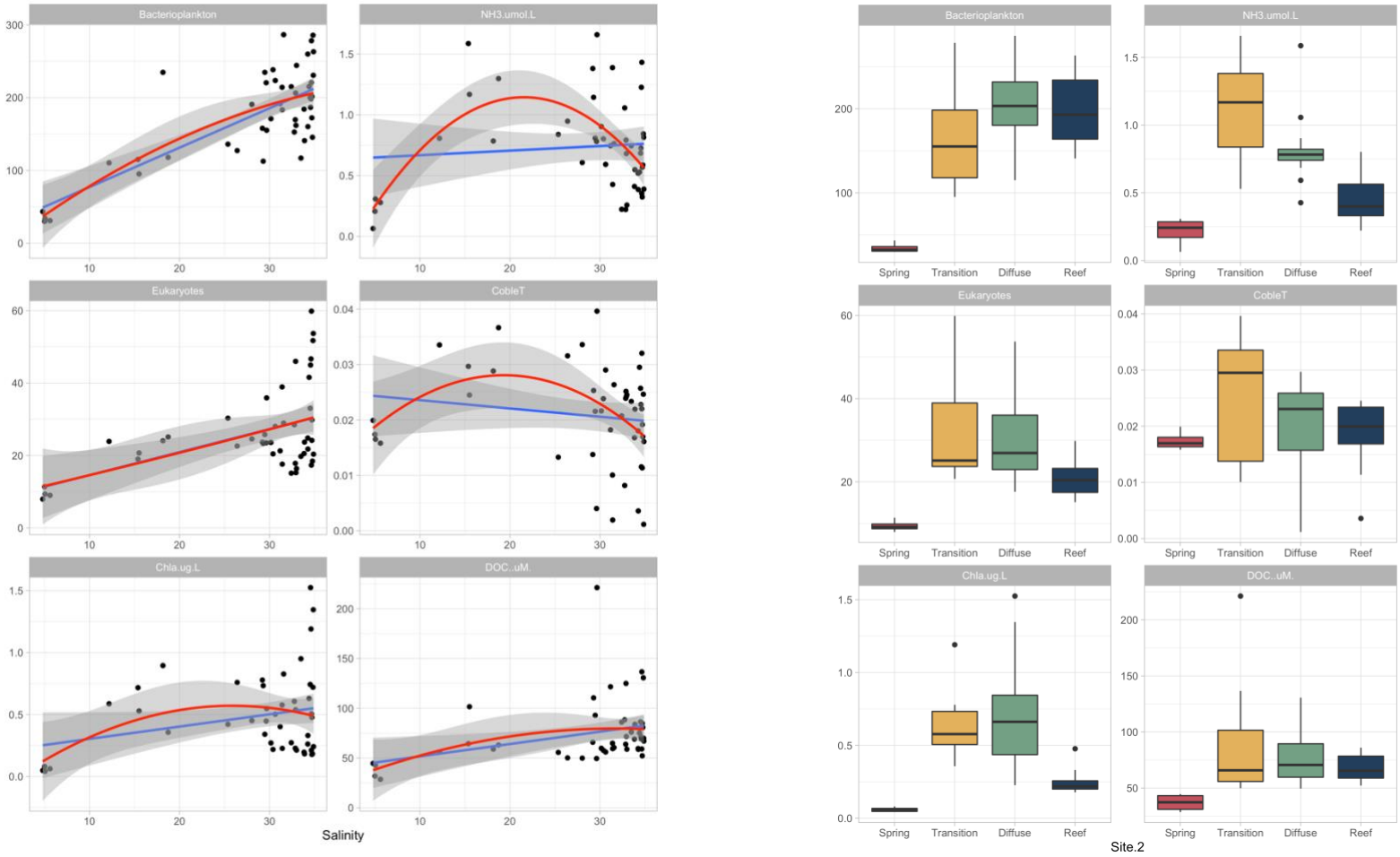
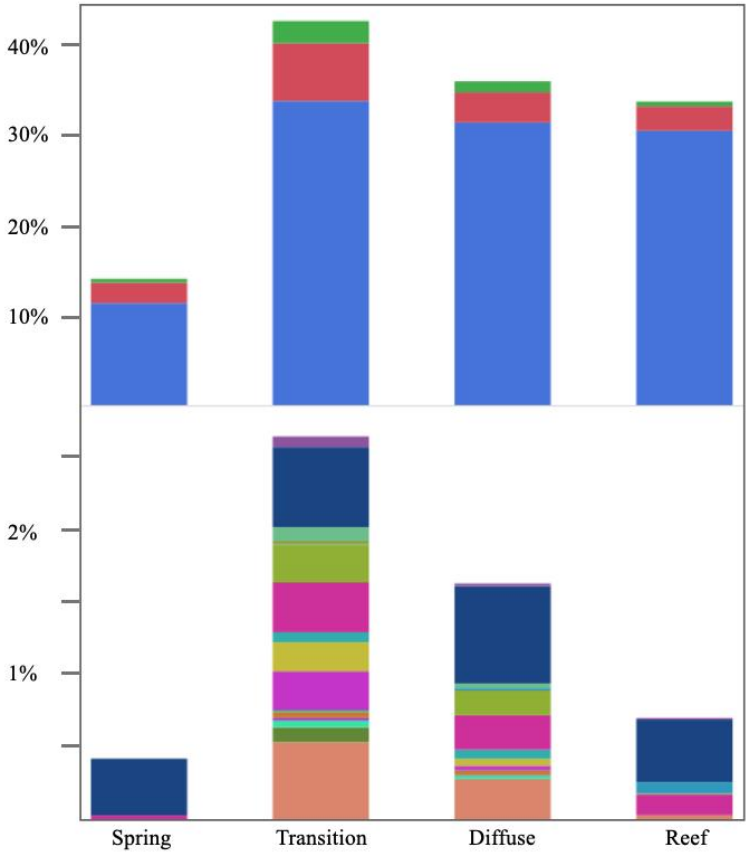
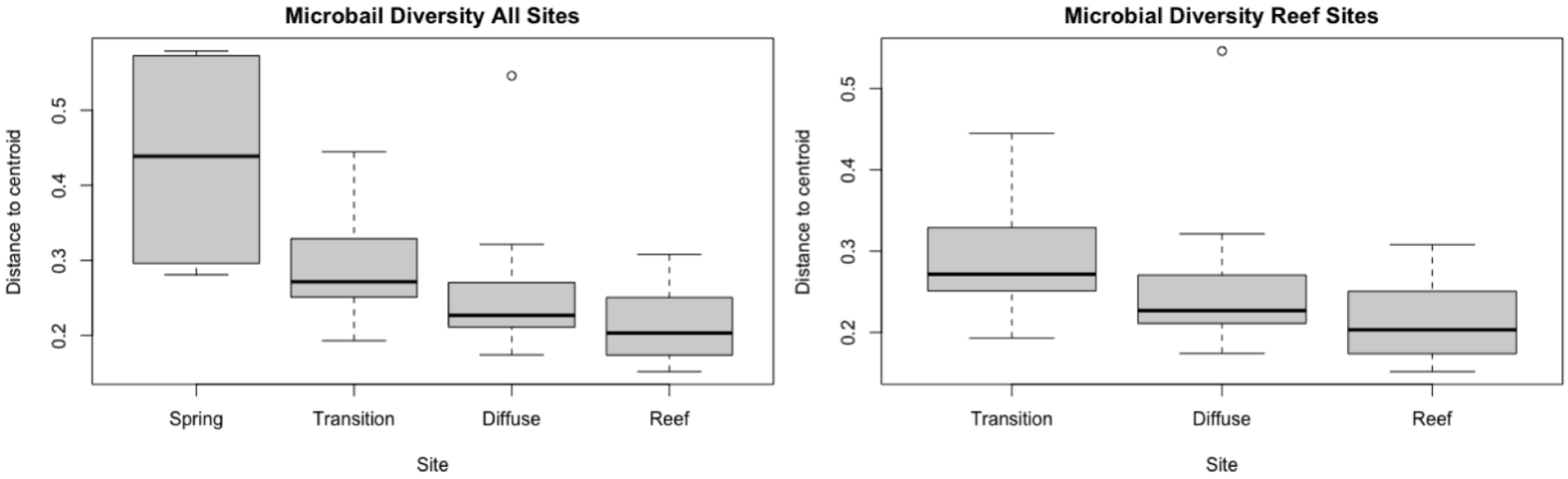


Figure S2.3. Mid-reef microbial families
Taxa that were enriched at mid-reef location relative to the Spring or Ambient Reef waters, had a significant quadratic relationship to salinity or were significantly related to site.



Phylum	Class	Order	Family
Bacteroidetes	Bacteroidia	Flavobavteriales	Flavobacteriaceae
Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Rubritaleaceae
Proteobacteria	Gammaproteobacteria	Cellvibrionales	Halieaceae
Proteobacteria	Gammaproteobacteria	Oceanospirillales	
Bacteroidetes	Bacteroidia	Flavobavteriales	Blattabacteriaceae
Cyanobacteria	Oxyphotobacteria	Nostocales	Geitlerinemaceae
Firmicutes	Clostridia	Clostridiales	
Lentisphaerae	Lentisphaeria	Lentisphaerales	Lentispaeraceae
Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae
Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae
Proteobacteria	Gammaproteobacteria	Alteromonadales	Psychromonadaceae
Proteobacteria	Gammaproteobacteria	Coxiellales	Coxiellaceae
Tenericutes	Mollicutes	Mycoplasmatales	Mycoplasmataceae
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	
Bacteroidetes	Bacteroidia	Bacteroidales	Prolixibacteraceae
Bacteroidetes	Bacteroidia	Bacteroidales	Tannerellaceae
Cyanobacteria	Oxyphotobacteria	Thermosynechococcales	Acaryochloridaceae
Proteobacteria	Gammaproteobacteria	Arenicellales	Areicellaceae

Figure S2.4. Beta dispersion of microbial communities.
Microbial communities collected from the SGD spring show 2x higher dispersion compared with other sites.



APPENDIX B
SUPPLEMENTARY TABLES AND FIGURES FOR CHAPTER 3

Table S3.1. Summary of sequence data by sample type

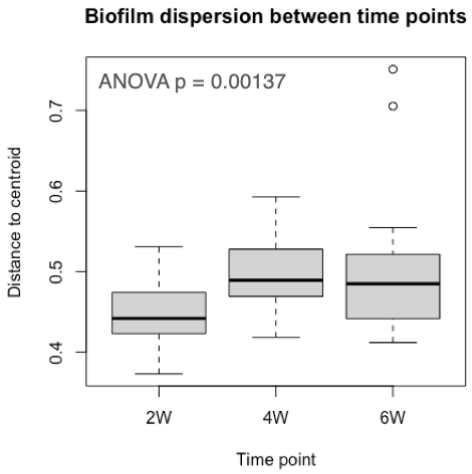
	Total Sequences	Total aligned sequences	Total unique aligned sequences	OTUs passing QC & >10 reads
Total	4,270,844	1,715,955	26,000	18,278
Biofilm N = 79	3,297,825	1,305,303	20,066	14,237
Planktonic N = 27	973,019	410,652	5,934	4,041

Table S3.2. Comparison of Unifrac vs Bray Curtis distance matrices

Predictor	Whole Dataset				Water				Biofilm			
	Unifrac		Bray Curtis		Unifrac		Bray Curtis		Unifrac		Bray Curtis	
Results	R^2	P-Value	R^2	P-Value	R^2	P-Value	R^2	P-Value	R^2	P-Value	R^2	P-Value
	Results of Model 1				Results of Model 2				Results of Model 3			
Sample Type	0.284	0.001***	0.187	0.001***								
Time: Sample Type	0.100	0.001***	0.067	0.001***								
Time	0.126	0.001***	0.134	0.001***	0.651	0.001***	0.426	0.001***	0.242	0.001***	0.204	0.001***
Organism	0.056	0.001***	0.074	0.001***	0.064	0.001***	0.098	0.001***	0.115	0.001***	0.123	0.001***
Nutrient	0.030	0.001***	0.041	0.001***	0.067	0.001***	0.096	0.001***	0.061	0.001***	0.063	0.001***
Time:Org	0.051	0.001***	0.052	0.001***					0.106	0.001***	0.089	0.001***
Time:Nut	0.028	0.006**	0.039	0.001***					0.053	0.019*	0.060	0.07**
Org:Nut	0.024	0.001***	0.032	0.001***					0.047	0.001***	0.050	0.001***

Figure S3.1. Experimental photos and set up
a) Individual aquaria were held in 1300L incubation tanks to maintain temperature. Nutrient treatments and filtered seawater were supplied via peristaltic pump to each experimental aquarium. b) Flow though racks holding glass slides were suspended in each aquarium to culture biofilms.

Figure S3.2. Biofilm dispersion between time points and for each time point between organism or nutrient treatment



Results of Tukey HSD between time points

Time point	Adj. p-value
2 to 4 Week	0.00379
2 to 6 Week	0.00534
4 to 6 Week	0.993

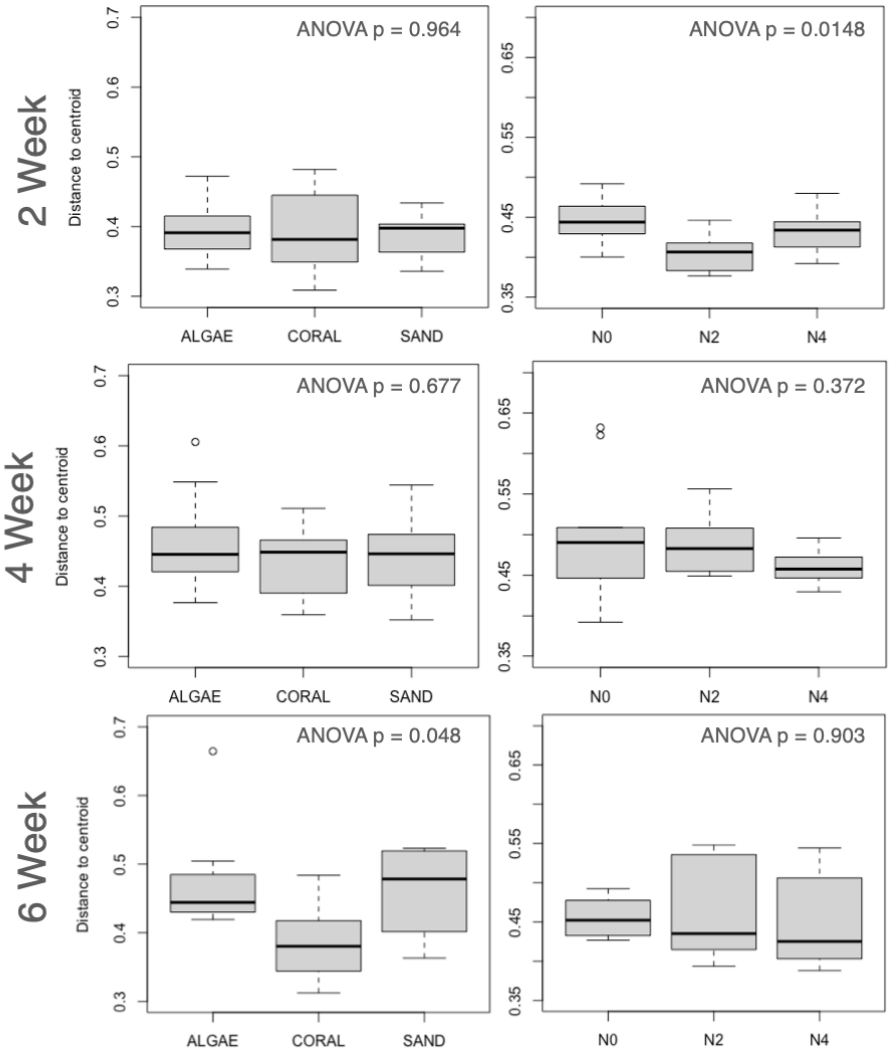


Figure S3.3. Abundant families that differed significantly between biofilm and water samples
Means in each stacked bar are connected by lines to better visualize which taxa increase and which decrease in the context of the overall proportion of the community. Biofilms selected for The Alphaproteobacteria families Rhodobacteraceae, Rhizobiaceae, Hyphomonadaceae as well as the Parcubacteria, Nostocales, Microtrichaceae, Saprospiraceae and Pirellulaceae; Water samples were enriched in SAR11, Vibrionaceae and the Bacteroidetes families Flavobacteriaceae and Cryomorphaceae. Values are means of 79 biofilm and 27 water samples. Shown are the 12 families exceeding a mean of 2% in either sample type and differing significantly across all timepoints (one-way mixed-effect ANOVA with aquaria as a random intercept; FDR-adjusted $p < 0.05$); together they comprise more than half of the total sequence abundance of both sample types. Two additional families not shown, the Alphaproteobacteria Micavibrionaceae and the Gammaproteobacteria Cellvibrionaceae, comprised roughly 2% and 3%, respectively, of the sequences in both biofilm and water but did not differ significantly.

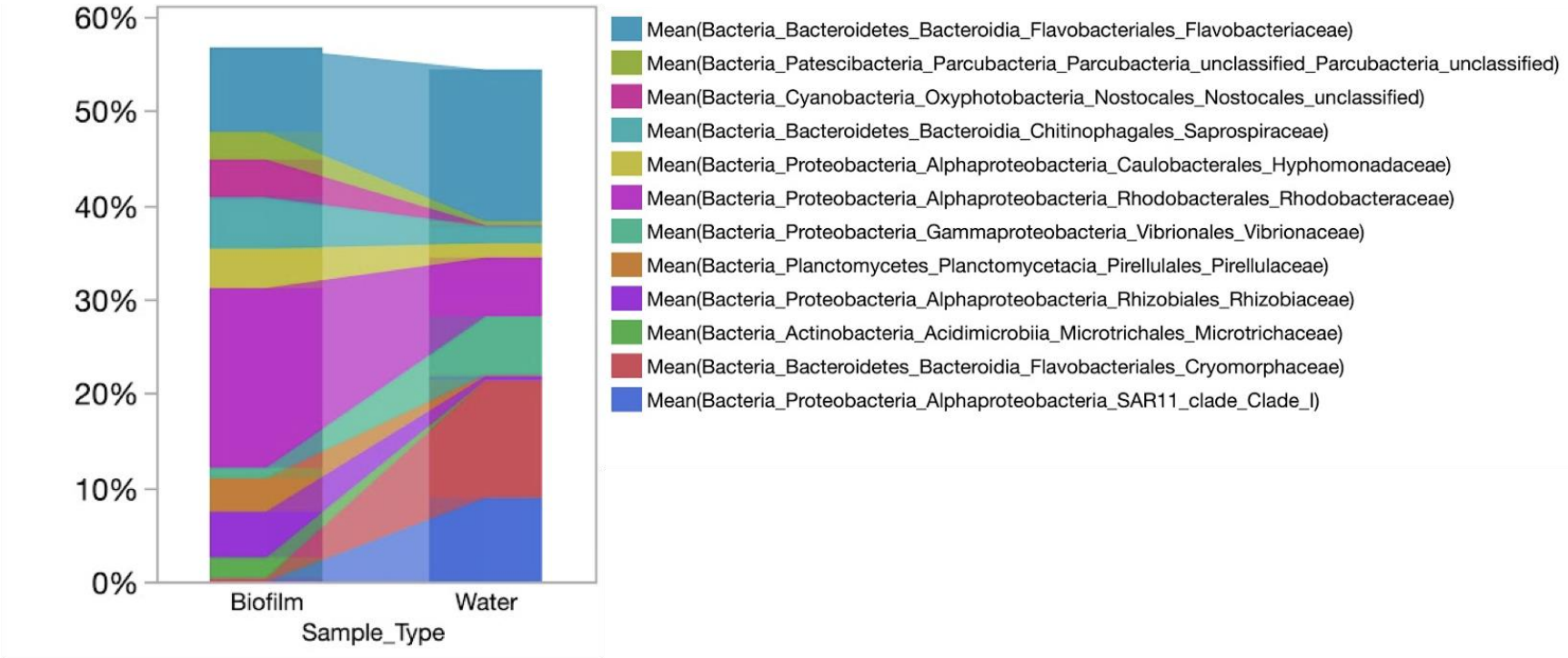


Figure S3.4. Bacterial taxa shifts with time
Marine biofilms were dominated with Alphaproteobacteria at each time point. Over time, Alphaproteobacteria decrease, and an increase in Gammaproteobacteria is evident. Marine biofilms were further enriched with Planctomycetes and Acidobacteria with time.

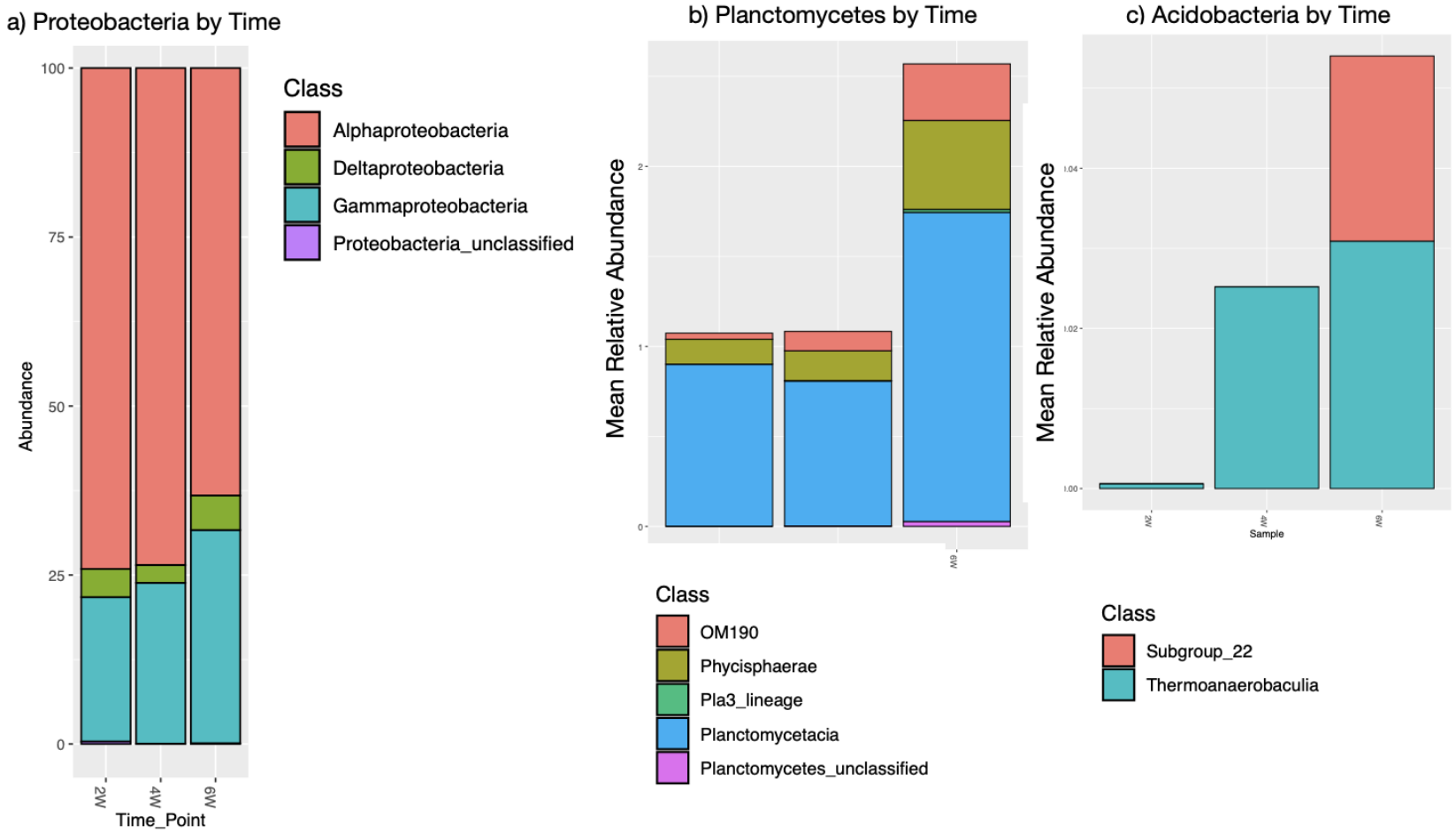
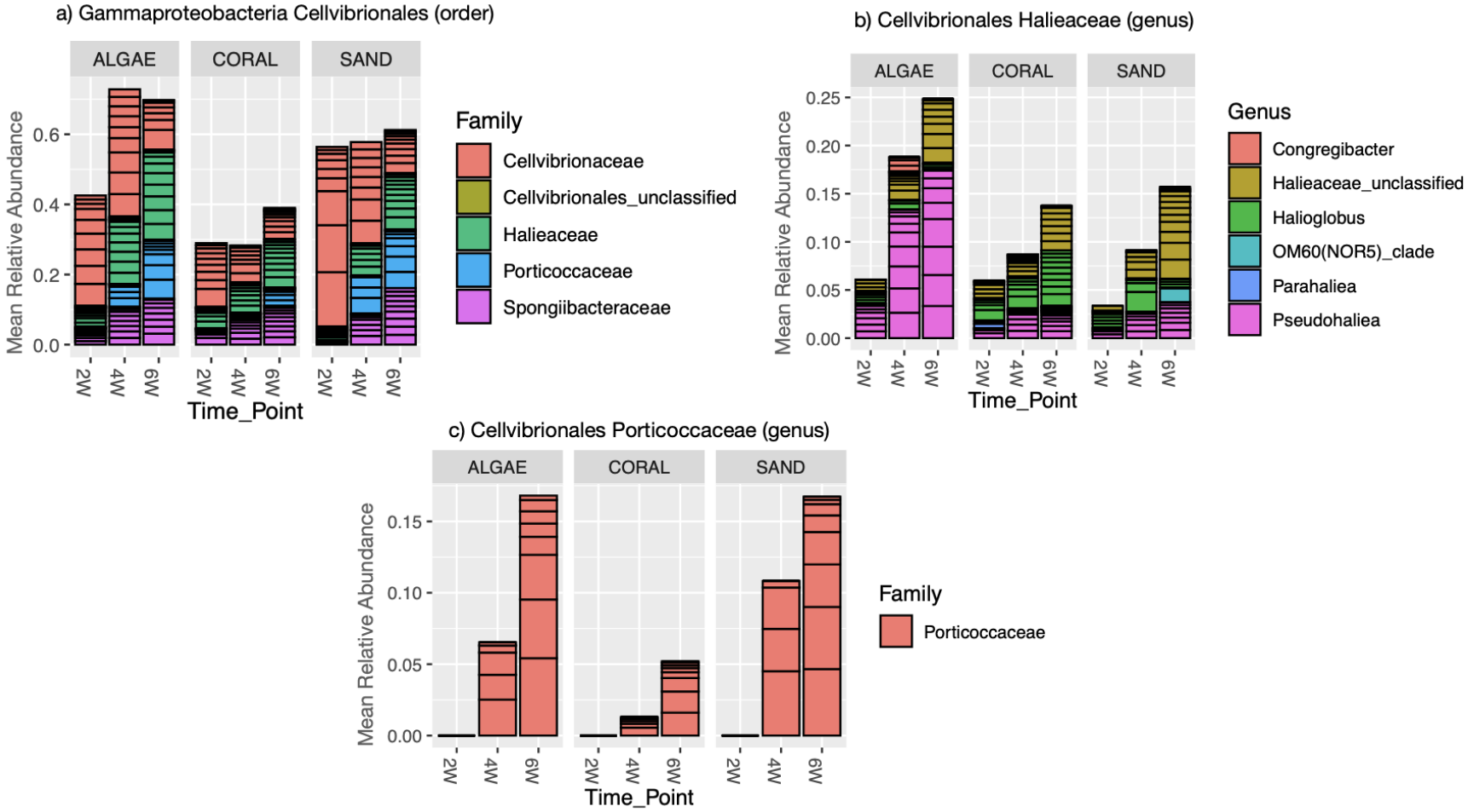


Figure S3.5. Gammaproteobacteria increase in all biofilms as they mature
Within this class, Cellvibrionales were important indicators of biofilms cultured with sand and algae; and ASVs classified as Halieaceae and Porticoccaceae were enriched in algae and sand treatments.



APPENDIX C
SUPPLEMENTARY TABLES AND FIGURES FOR CHAPTER 4

Figure S4.1. Proportion of reads assigned to each domain obtained from metagenomes

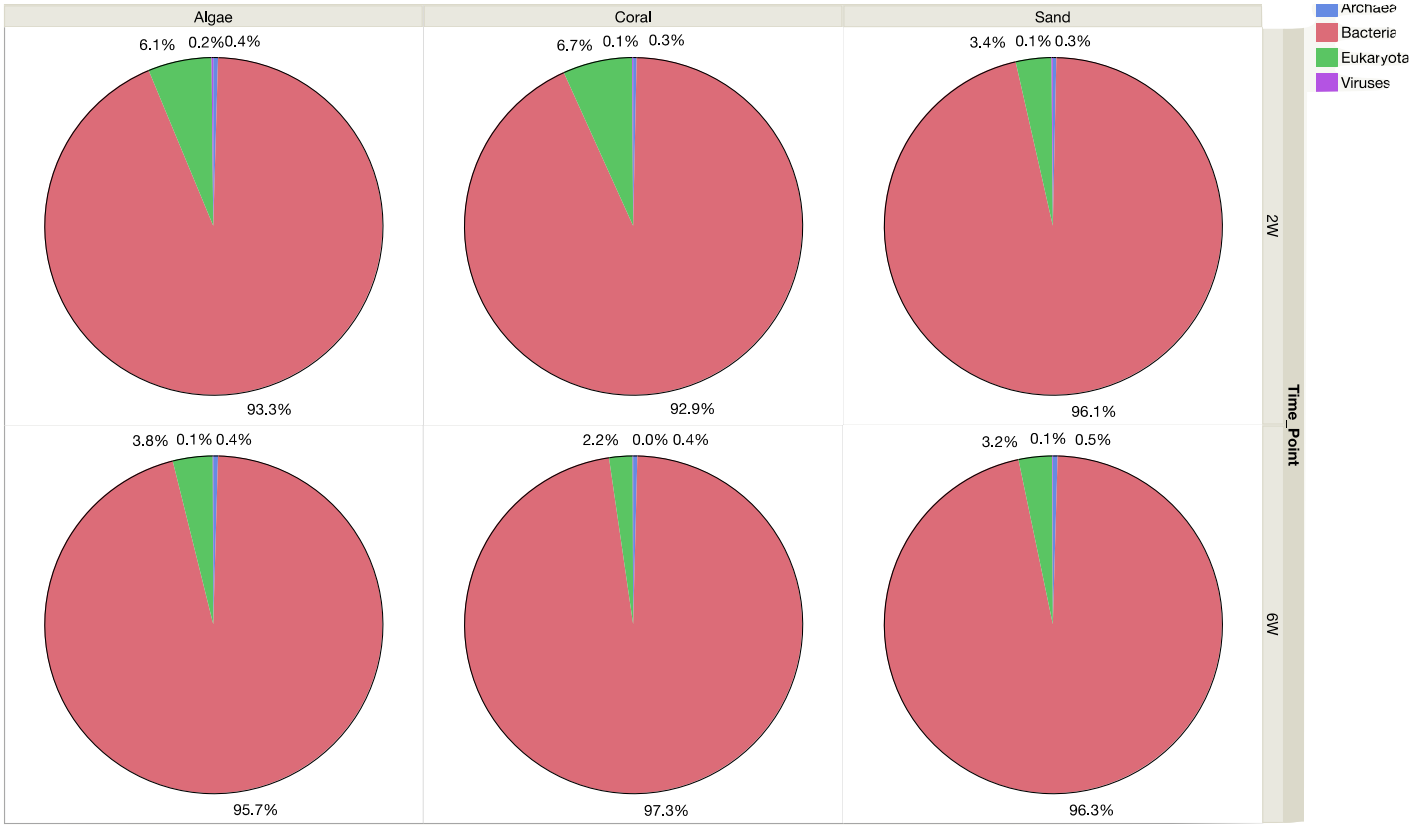


Figure S4.2. Comparison of central carbohydrate metabolisms in biofilms by benthic organisms

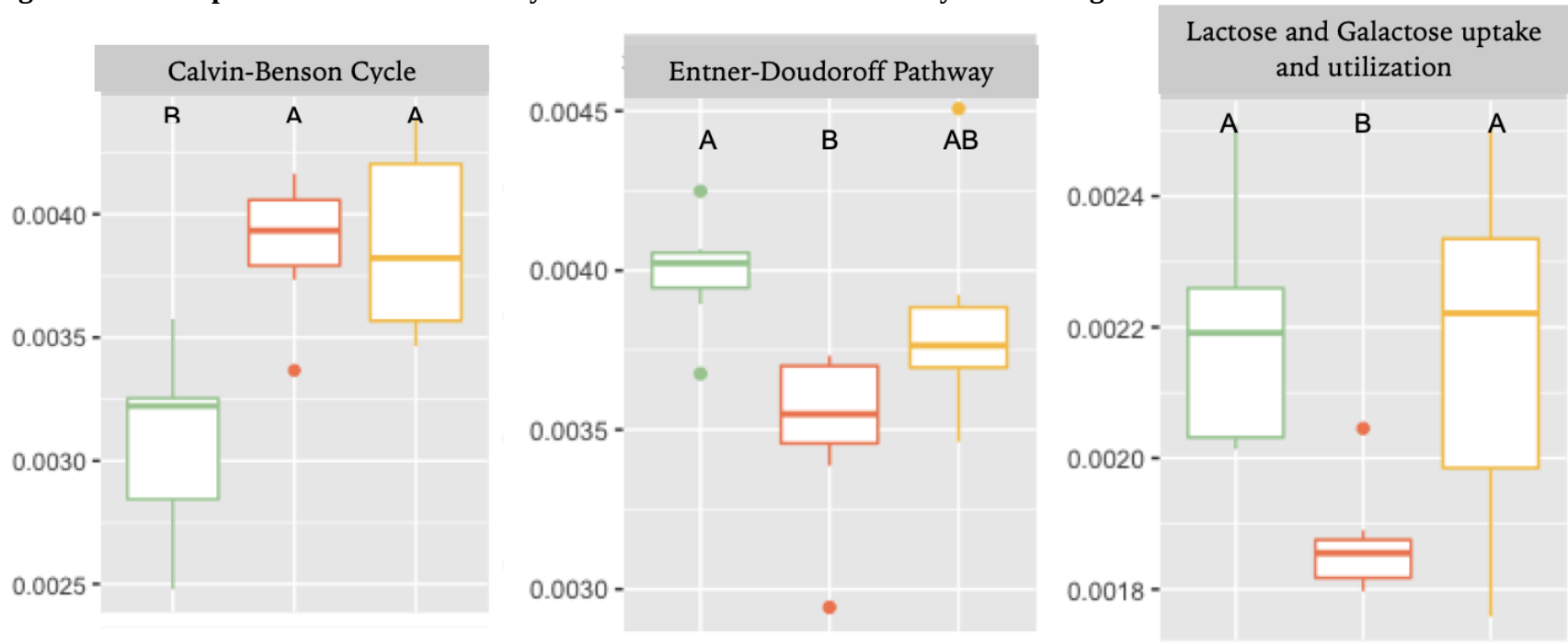


Figure S4.3. Secretion systems elevated in biofilms cultured with algae
Secretion systems were elevated in biofilms cultured with algae compared with those from coral and sand.

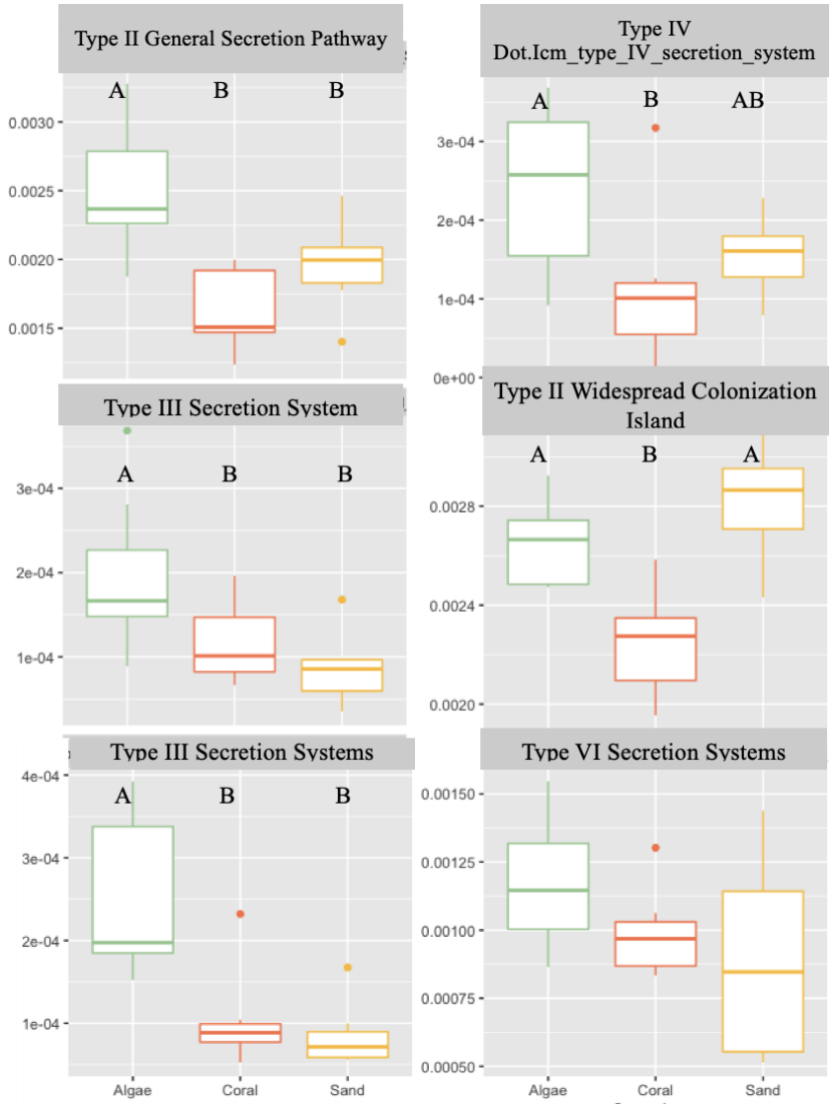
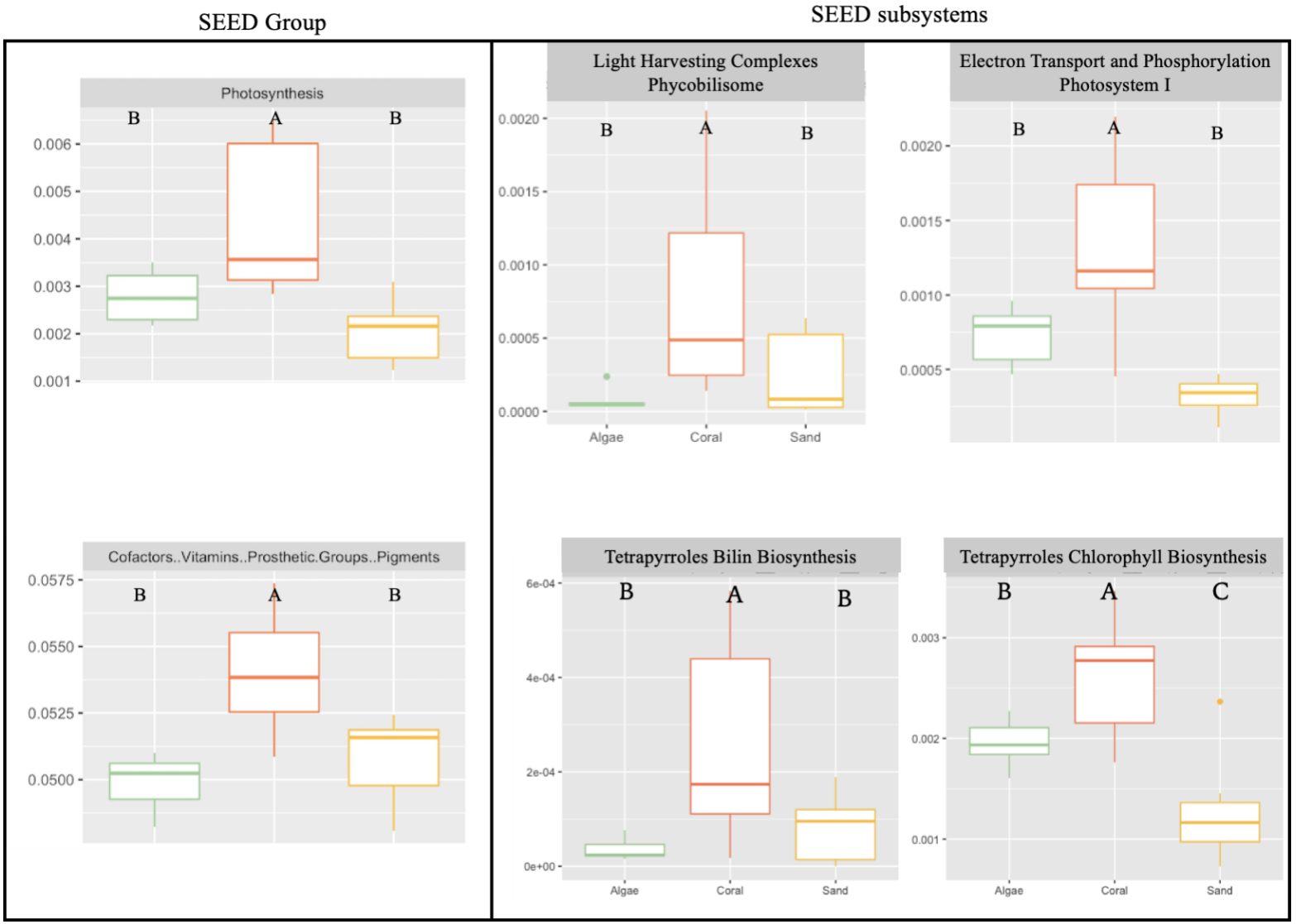


Figure S4.4. SEED groups and subsystems enriched in biofilms cultured with coral



APPENDIX D
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