Effects of Macroalgae on Coral-Microbial Associates on Caribbean Coral Reefs

by

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Abstract

Scleractinian corals harbor eukaryotic and prokaryotic organisms that form dynamic mutualistic, parasitic and commensal associations with the coral host and exhibit substantial genetic and ecological diversity. The microorganisms that inhabit the surface mucosal layer, tissues, and calcium carbonate skeleton of corals may provide the first line of defense against microbial infection, and evidence suggests that they play an intrinsic role in host fitness and disease susceptibility. Protection may be provided through the production of potent antibiotics or by filling an otherwise available niche open to infection by opportunistic pathogens. As corals are increasingly affected by catastrophic epizootics and bleaching events, it becomes increasingly important to understand the composition of coral-microbial assemblages and what causes the shift from a healthy to a diseased state. Often we correlate disease stressors such as high water temperature, sedimentation, nutrient loading, and overfishing, all of which may inhibit a corals innate ability to mediate an optimal microbial assemblage. However, as corals succumb to an early death there is an increase in colonizable substratum, which has provided an ideal environment for the proliferation of macroalgae, sponges, and other competitive dominants under present-day conditions. Coral-associated microbiota may be severely affected by the physical encroachment and allelochemicals exuded by encroaching organisms. This dissertation examines what is generally known about coral-microbial associations, briefly touching on coral disease and coral-algal interactions, followed by a laboratory assay that examines the allelochemical activity of common macroalgal extracts on coral reef microorganisms (Chapter 2). Additionally, I describe the species-specificity and geographic continuity of microbial assemblages associated with two common Caribbean coral species, *Montastraea faveolata* and *Porites astreoides,* in Belize, Florida, and St. Thomas (Chapter 3). Finally, I specifically examine the effect of encroaching macroalage and their allelochemicals on coral microbiota (Chapters 4 & 5). Better understanding of healthy coral-microbial associations and the ability of competing benthic organisms to physically or chemically shift these assemblages is critical to predicting reef resilience and the future of coral reef health.

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DGGE	Denaturing Gradient Gel Electrophoresis
FKNMS	Florida Keys National Marine Sanctuary
MF	*Montastraea faveolata*
MRPP	Multi-Response Permutation Procedure
nMDS	Non-metric Multidimensional Scaling
NOAA	National Oceanic and Atmospheric Association
PA	*Porites astreoides*
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
SCUBA	Self Contained Underwater Breathing Apparatus
SML	Surface Mucus Layer
TAE	Tris Acetic Acid EDTA buffer
OTU	Operational Taxonomic Unit

Chapter 1

Review: Coral-Microbial Symbioses and Macroalgal Mediators

Introduction

Coral reefs are biodiversity hotspots of great ecological and economic significance. Scleractinian corals, hydrocorals, and crustose coralline red algae construct the primary framework of these living monoliths that build structures of such magnitude they can be recognized from space. Coral reefs cover 280,000 km^2 in tropical and subtropical waters and their topographic complexity and unique primary production support millions of different organisms (Knowlton and Jackson 2001, Spalding et al 2001). However, increasing anthropogenic and environmental stressors are causing substantial declines in the biodiversity and abundance of hermatypic corals worldwide, causing such severe changes in community composition that it has become possible to witness the death of a reef in one human life-time (Wilkinson 2004). Recent research has revealed that the dynamic and highly diverse consortia of coral-associated microorganisms may be implicated in both the decline and recovery of global coral populations.

The coral holobiont includes the coral tissues, endosymbiotic zooxanthellae, protists, bacteria, archaea, viruses, and endolithic algae and fungi. The holobiont encompasses all microbiota that inhabit host surface mucus, tissues and calcium carbonate skeleton. Best known and previously considered to be the most ecologically relevant are dinoflagellates in the genus *Symbiodinium* (zooxanthellae). These obligate photosynthetic symbionts live within coral gastrodermal tissues at high densities ($>10^6$ cm^{-2}) and provide > 90% of a coral's carbon and nutritional requirements (Muscatine and Porter 1977). Scleractinian corals owe much of their success as reef-builders to this efficient microbial symbiosis. A number of clades of *Symbiodinium* can live within one host species or even within a single coral colony (Knowlton and Rohwer 2003). However, we will focus on non-eukaryotic coral associates that also have the potential to play a critical role in coral biology, although studies of their extensive diversity and influence on coral ecology have only just brushed the surface.

In general, marine prokaryotes are highly diverse but morphologically simple (rods, spheres, filaments) and ~1-2 μm in size. Bacteria are broadly categorized based on their fundamental carbon (e.g. autotroph, heterotroph) and energy sources (e.g. phototroph, chemotroph). Their ribosomal ribonucleic acid (rRNA) molecules are highly conserved, abundant and found in all bacterial cells. By comparing one rRNA copy to another it is possible to distinguish regions of base sequence that never change and regions that are only found in members of the same kingdom, division, genus or species. These intricate levels of variability allow microbiologists to design probes with varying specificity that are then applied to the study of microbial diversity and ecology (Giovannoni and Rappé 2000). Techniques such as fluorescent *in situ* hybridization (FISH) combined with spectral imaging have revealed the structural environment and enumerated the abundance of microorganisms around coral lesions and tissues (Ainsworth et al. 2007). The operational taxonomic units (OTU's) of extremely diverse microbial assemblages can now be quantified using fingerprinting techniques that are arguably the most time- and cost-effective method to observe clear differences between microbial communities. Presently, ingerprinting methods include denaturing gradient gel electrophoresis (DGGE, Rohwer et al. 2002), which relies upon sequence and denaturing characteristics; terminal restriction fragment length polymorphism (T-RFLP, Liu et al. 1997) of conserved genes, which also relies on heterogeneity in restriction digest sites among OTU's; and automated ribosomal intergenic spacer analysis (ARISA, Fisher and Triplett 1999), which relies on 16S-23S intergenic spacer (ITS) region heterogeneity. The analysis of the16S (small subunit) rRNA and the 16S–23S ITS regions have been effective in addressing questions about the phylogeny, evolution, and population diversity of prokaryotes (Boyer et al. 2001). These techniques rely heavily on the ability to directly compare properties associated with each taxon, thus it is necessary to be able to identify each taxon and precisely compare taxon between gels. Therefore, fingerprinting methods have gained further recognition with the advent of affordable and efficient sequencing over the past decade and the ability to accurately compare OTU's within and between gels.

Molecular techniques are critical because the vast majority of marine bacteria (80%) in seawater are represented by only a small number of as-yet-unculturable bacterioplankton clades (Giovannoni and Rappé 2000). However, it is reasonable to hypothesize that a far greater percentage of bacteria can be cultured from nutrient-rich benthic surfaces such as coral mucus,

algal thalli, and sponge epithelial, than from the surrounding oligotrophic ocean waters (B. Jonas pers. comm., Jensen et al. 1996). Solely molecular based techniques provide information on the types of microbiota present, but can introduce bias in favor of certain bacteria over others based on ease of DNA extraction, the selection of probes/primers, and PCR amplification (Forney et al. 2004). Additionally, imprecise estimates of OTU sizes across multiple samples can arise due to run characteristics of polyacrylamide gels, mobility of size standards, and peaks that appear as doublets or are spread out. Thus, to reliably elucidate structure and function of microorganisms in complex communities, manipulative experiments need to utilize both cultured isolates derived from culture media that mimics *in situ* conditions, and molecular-based techniques described above.

Diverse populations of bacteria and archaea possibly co-evolved with host corals (Mitchell and Chet 1975, Ducklow and Mitchell 1979, Shashar et al 1994, Rohwer et al. 2001, Kellogg 2004, Wegley et al. 2004, and Rosenberg 2007). Microbial populations within coral mucosal and tissue layers are 100-1000 fold higher than marine pelagic populations (Rosenberg et al. 2007). Coral mucus itself is a nutrient rich medium that serves as a protective boundary layer between the coral tissues and external environment (e.g. water column, irradiance, encroaching organisms), and is loosely defined as a polysaccharide-protein-lipid complex secreted by ectodermal mucocytes onto the coral surface (Brown and Bythell 2005, Figure 1.1). Mucus aids in heterotrophic feeding, calcification, locomotion, reproduction, sediment shedding, gas exchange, as well as pathogen protection (Brown and Bythell 2005, Ritchie 2006). All corals secrete mucus although it varies in quantity and composition depending on species and abiotic parameters (e.g. water quality and temperature). It is unknown whether this variability is due to variations in the compliment of zooxanthellae (Rowan et al. 1997) or variations in the metabolism of the host coral. Much of the fixed carbon that makes up the mucus layer originates from endosymbiotic *Symbiodinium* sp. and serves as a rich food source for bacteria (Ritchie and Smith 2004). Initial culture-based studies provided evidence that Caribbean coral species may harbor unique species-specific, mucus-associated microbial populations (Ritchie and Smith 1997). By providing a stable growth medium for bacteria (helpful or harmful), mucus may play a role in a coral's innate immune function, enhancing susceptibility or providing protection from pathogens (Ducklow and Mitchell 1979, Banin et al. 2001, Lipp et al. 2004).

There is debate over whether coral associated microbes are a symbiosis or simply an

opportunistic association with water column microbial populations (Rohwer et al. 2002). Beneficial roles microbes may play in the physiological function of the coral holobiont include nitrogen fixation, carbon fixation, nutrient accumulation, antibiotic production, pathogen protection and prevention of fouling and colonization (Shashar et al. 1994, Lesser et al. 2004, Rohwer et al. 2002). Alternatively, microbes have been increasingly implicated in coral disease and reef degradation. Marine mortalities due to disease outbreaks have increased in frequency and severity and there has been a reported increase in the number of novel disease occurrences (Harvell et al. 1999). The number of identified and reported coral diseases have exponentially increased since the first incidence of disease in 1965 (Antonius 1973, Sutherland 2004). Thus the study of coral health and disease has been the driving force for the recent emphasis on the field of coral microbiology (Rosenberg 2007). The lack of baseline and epidemiological information has hindered our understanding of the relative importance of specific bacteria and environmental factors in the spread of disease epizootics. In addition to examining the recent literature on the beneficial and pathogenic roles microorganisms may play in coral health and disease, this review further examines the potential for interacting marine organisms to directly or indirectly influence coral-microbial assemblages.

Microbial Mutualisms

Microorganisms have been implicated in the health and disease of higher organisms for hundreds of years. The recent advent of culture-independent molecular techniques has demonstrated that in many cases the number of symbiotic microorganisms and their collective genetic information far exceeds that of their host (Zilber-Rosenberg and Rosenberg 2008). A 'symbiosis' was broadly defined in the mid 19th century by Anton de Bary as 'the living together of two different species.' The Human body is an ideal example, supporting thousands of microbial symbionts that play such important roles they were coined the 'forgotten organ' (Relman and Falkow 2001), and a second human genome project was suggested termed the 'Human Microbiome Project' (http://nihroadmap.nih.gov/hmp/). Although microorganisms are often associated with pathogens and disease, they can serve particularly beneficial roles in the marine environment. Symbiotic microorganisms protect their hosts from settlement and attachment of biofoulers and/or pathogenic invaders (Lopanik et al. 2006), in some cases providing nutritional products (Shashar et al. 1994), in addition to protection from predators

(Nyholm et al. 2004).

Reshef and colleagues (2006) originally suggested 'The Coral Probiotic Hypothesis' to describe the dynamic relationship between host coral and associated microorganisms. The probiotic theory suggested that the coral host selects for the most advantageous holobiont (the host and its symbiotic microbiota) in the context of prevailing environmental conditions. This theory was developed to explain how the coral, *Oculina patagonica,* developed resistance to infection by the pathogen, *Vibrio shiloi,* even though corals do not produce antibodies (Reshef et al. 2006). Zilber-Rosenberg and Rosenberg (2008) developed the Hologenome Theory, as a generalization of the Probiotic Hypothesis, and proposed that the holobiont, with its hologenome (sum of genetic material in the host and microbiota), are considered a unit of selection in evolution. The theory is based on several generalizations, 1) all animals and plants establish symbiotic relationships with microorganisms, 2) symbiotic microbiota are transferred between generations, 3) the association affects the fitness of the holobiont within the environment, and 4) variation in the hologenome can be caused by changes in either the host or microbiota genomes. Under environmental stress, the symbiotic microbial community can change rapidly. The theory suggests that variation in the diverse microbial symbionts can support or hinder rapid adaptation of the holobiont. However, the hologenome theory has been criticized heavily because it emphasizes the bleaching response of *O. patagonica,* a non-native temperate coral, to *V. shiloi* and possibly 'over simplifies' the complex relationship between host and symbionts(s) (Leggat et al. 2007). But, these theories may begin to explain the evolutionary success of corals and moderate predictions of their demise as human impacts continue to alter coral reef ecosystems (Rosenberg et al. 2007).

Beneficial coral-associated microorganisms can provide nutritional by-products and protection from disease (Sorokin 1973, Ducklow and Mitchell 1979, Shashar et al 1994, Koh 1997, Lesser et al. 2004). Tropical corals live in warm oceans containing very low concentrations of organic material. Bacteria are able to assimilate nutrients at very low concentrations and the high microbial biomass (bacterial and dinoflagellate) can serve as additional direct and indirect heterotrophic food sources for corals (Schiller and Herndl 1989). Coral mucus effectively traps particulate matter and bacteria from the water column and transports the material along the coral surface via ciliary currents (Coles and Strathman 1973). The amount of organic carbon assimilated each day is ~10-20% of the carbon content within the

polyp body. Bacteria may also provide other limited nutrients such as organic phosphorus and essential vitamins. For example, synthesis of vitamin B_{12} requires 9 separate enzymatic steps that have only been characterized in prokaryotes. *Halomonas* bacteria (presumed residents of corals), supply essential B_{12} to several species of macroalgae (Croft et al. 2005). Coral surface bacteria also may attract microbe-feeding zooplankters, which in turn serve indirectly as another coral food source (Schiller and Herndl 1989).

Photosynthetic products, such as fixed-carbon, are supplied by the coral zooxanthellae, but not the nitrogen required for synthesizing amino acids, purines, and pyrimidines (Reshef et al. 2006). Nitrogen fixation is a process exclusively carried out by prokaryotes, and evidence suggests that some coral-associated microbes provide the coral with fixed-nitrogen. These bacteria were originally isolated from the coral skeleton and were suggested to benefit from fixed-carbon excreted by coral tissues while supplying fixed-nitrogen to the coral (Shashar et al. 1994). Recently, unicellular, non-heterocystic, cyanobacteria were also isolated from within the epithelial cells of the framework-building coral *Montastraea cavernosa* (Lesser et al. 2004). These cyanobacteria appeared to be surrounded by host membranes and to coexist alongside the zooxanthellae where they express the nitrogen-fixing enzyme nitrogenase (Lesser et al. 2004). Within the coral tissues cyanobacteria can take advantage of glycerol as an energy source, the primary carbon product translocated by zooxanthellae (Lesser et al. 2004). The coral environment is optimal for the separation of photosynthesis and nitrogen fixation because coral tissues become extremely hypoxic at night when zooxanthellae are not photosynthesizing but the coral tissues continue to uptake oxygen. Along with providing a limited nutrient essential to the synthesis of cellular building blocks, phycoerythrin can become uncoupled from the photosynthetic apparatus of cyanobacteria and serve as a storage pool of fixed-nitrogen. When uncoupled phycoerythrin is in contact with glycerol it results in strong fluorescence and the subsequent elimination of quenching associated with energy transfer from phycoerythrin reaction centers (Lesser et al. 2004). High nitrogen fixation found in coral skeletons (Shashar et al., 1994), and coral tissues (Lesser et al., 2004) coupled with the conversion of nitrates/nitrites to ammonium by fungi may drive nutrient cycling in the coral holobiont (Wegley et al., 2007).

Coral-associated bacteria may provide a final benefit by preventing opportunistic infections through the occupation of an otherwise available niche and by producing antibacterial agents (Koh 1997). In the absence of microbial colonization, many organisms are unable to fight

infections from pathogenic bacteria and viruses (Shanmugam et al. 2005). It was demonstrated that 30% of bacteria isolated from corals have antibiotic properties (Castillo et al. 2001). Antibiotics and antifungals produced by bacteria on coral surfaces could have a symbiotic function (e.g. *Pseudoalteromonas* sp.)(Ritchie 2006). Epibiotic bacteria may only produce active antibiotics when they grow in mixed consortia; consequently such functions have been difficult to elucidate (Munn 2004). Ritchie (2006) demonstrated that *Acropora palmata* corals may be populated by three functional groups of bacteria: 1) Residents, which were resistant to the innate antimicrobial activity of *A. palmata* mucus and could be putative symbionts, 2) Visitors, transient bacteria trapped from the water column in coral mucus and susceptible to mucus antimicrobial activity, and 3) Water column bacteria, which were cultured directly from the adjacent water column and have the ability to come in contact with the coral (Figure 1.2).

Coral mucus indiscriminately traps particles and microbes that pass by in the water column (Wild et al. 2004). Therefore, visitors and water column bacteria may be commensal microorganisms that do no good or harm to the holobiont, but under changing environmental conditions and increasing stress, bacteria may become opportunistic pathogens. Interestingly, many of the resident bacteria isolated from *A. palmata* samples also were known to produce antimicrobial activity (Figure 1.2). Furthermore, the antibiotic property of bacteria within the coral mucus was not present during a bleaching event caused by heightened seawater temperatures. It was demonstrated that the coral-associated bacterial assemblage shifted from one dominated by commensal and/or mutualistic *Pseudomonas* sp. to one dominated by *Vibrio* sp., the genera associated with several coral diseases (Ritchie 2006, Table 1.1). These results support the hypothesis that environmental changes have the ability to rapidly alter the bacterial species composition of the holobiont and may lead to increased virulence of bacterial pathogens and or decreased host defenses, ultimately leading to mortality.

Microbial Pathogens

Emerging diseases and increasing virulence of known diseases are largely responsible for the 30% decline in worldwide coral cover over the past 30 years (Hughes et al. 2003). Despite improved efforts to identify the primary pathogens responsible for infection, most studies remain dependent on the macroscopic disease appearance for diagnosis (Ainsworth et al. 2007). Coral diseases continue to exponentially increase in frequency and severity and there has been a

reported increase in the number of novel disease occurrences (Harvell et al. 1999, Sutherland et al. 2004). For example, White Pox Disease (WPD) exclusively targets *Acropora palmata,* and was first documented in 1996 within the Florida Keys. Since its appearance, populations of *A. palmata* have experienced devastating losses of living cover averaging 88% (Sutherland and Ritchie 2004). In May 2006, the United States listed both *A. palmata* (Elkhorn coral) and *A. cervicornis* (Staghorn coral) as threatened under the Endangered Species Act due to their widespread decline throughout the Caribbean. The increase in disease occurrence is due in part to a better awareness of coral health but also has been correlated with increasing environmental stressors (e.g. elevated seawater temperatures, nutrient enrichment, sedimentation) (Harvell et al. 2002, Sutherland et al. 2004). There are approximately 30 dynamic coral diseases and syndromes described worldwide and only 6 causative agents have met Koch's postulates, and been isolated and characterized (Table 1.1, Sutherland et al 2004, Weil et al. 2006). Furthermore, very little is known about the manner in which these diseases are contracted or spread from one colony to another. This review briefly highlight aspects of several diseases of global significance and experimental results from the past 5-10 years, but in no way attempts to examine every disease and syndrome described to date. It also is important to note that the visual description and causative agents of many of these diseases are still quite transitive and sometimes change on an almost daily basis. Known diseases and syndromes are summarized in Table 1.1, please refer to the reviews listed after Table 1.1 for further information.

Coral bleaching is one of the most globally prevalent threats to coral reefs and generally coincides with an increase in seawater temperature. As global temperatures have steadily risen over the past century due to global warming, so have the incidence and severity of bleaching events (Berkelmans & van Oppen 2006). Bleaching refers to the disruption of the symbiosis between the coral host and its endosymbiotic zooxanthellae and results in loss of the algal symbionts and/or the algal pigments. Bleaching is most notably induced by high temperatures and irradiance, which causes the zooxanthellae to over-photosynthesize producing reactive oxygen species (ROS) that are toxic to the coral tissues, thus the coral may expel or eradicate the zooxanthellae for protection from ROS degradation (reviewed in Jokiel 2004 and Veron 2008). Other causes implicated in coral bleaching are low salinity (Goreau 1964), sedimentation (Peters 1984), exposure to cyanide (Cervino et al. 2003), decreased seawater temperatures (Muscatine et al. 1991), and bacterial infection (Kushmaro et al. 1996, 1997; Ben-Haim and Rosenberg 2002).

In some instances, the bacteria themselves are the putative cause of coral bleaching. The bacterium, *Vibrio shiloi* was shown to cause bleaching in the Mediterranean coral *Oculina patagonica* (Kushmaro et al. 1996, 1997). *V. shiloi* was chemotactic to mucus produced by *O. patagonica* and bound to the β-galactoside containing receptor in the coral mucus. When temperatures were above 25°C, *V. shiloi* would penetrate the epidermal layer of coral tissues and replicate to densities $>10^8$ cells per cm^3 (Kushmaro et al. 1998). At high densities *V. shiloi* produced a proline-rich toxin that inhibited photosynthesis of the zooxanthellae and resulted in bleaching (Rosenberg and Falkovitz 2004). A second bacterium, *Vibrio coralliilyticus,* caused bleaching and tissue lysis in the branching reef coral, *Pocillopora damicornis* (Ben Haim and Rosenberg 2002). Bleaching occurred at temperatures from 24-25°C, and as seawater temperature increased to 27-29°C, coral tissue lysis and colony mortality occurred. There has been some difficulty replicating these observations over the past 5 years, which may indicate that the relationship has adapted or evolved (Rosenberg et al. 2007). It also is unknown whether the mechanisms causing coral death are a result of increased virulence and abundance of *Vibrio* sp. or host susceptibility due to thermal stress. Intriguingly, additional evidence presented by Ritchie et al. (1994), demonstrated that *Vibrio* spp. represented 30% of the bacterial isolates enumerated from bleached *Montastraea annularis* corals, but was never isolated from healthy colonies. Bourne et al. (2008) used culture-independent techniques to examine the microbial composition of *Acropora millepora* colonies over 2.5 years, including an extensive bleaching event in 2002. Prior to visual bleaching signs the microbial community shifted, revealing a correlation between increasing temperatures and the appearance of *Vibrio*-affiliated sequences. After the bleaching event, the microbial profile shifted back to a fingerprint similar to those obtained prior to bleaching. These results suggest that shifts in microbial community composition can act as indicators of stress prior to visual signs on the reef, but the mechanistic cause of the change in microbial composition remains unknown (Bourne et al. 2008, Rosenberg et al. 2008). Temperature anomalies of warm water and other associated environmental factors correlated with coral bleaching may be synergistically linked to shifts in coral-microbial assemblages and disease occurence (e.g. growth anomalies), thus it is important to include bleaching as one of the environmental parameters collected when studying disease causation (McClanahan et al. 2009).

Yellow blotch/band disease (YBD) also involves the disruption of the symbiosis between

the coral and zooxanthellae. Yellow lesions of paling coral tissue are a result of decreasing chlorophyll and a reduction/lysis of zooxanthellae cells. As with other coral diseases, a clear link has been established between the rate of YBD progression and high sea surface temperature (Cervino et al. 2005). Disease lesions are also associated with the presence of a *Vibrio* spp. core group (Cervino et al. 2008). Four *Vibrio* spp. (*V. rotiferianus, V. harveyi, V. alginolyticus* and *V. proteolyticus*), were isolated from diseased corals and inoculated onto healthy corals. The four *Vibrio* spp., individually or as a consortium, appeared to target the zooxanthellae and cause disease signs similar to YBD in the healthy corals tested (Cervino et al. 2008).

Black band disease (BBD) is considered one of the most visually distinct and destructive diseases impacting multiple framework-building corals worldwide and was first reported in 1973 (Antonius 1973). The primary pathogen(s) is believed to be a consortium of interacting microorganisms that may always be present on or near coral tissues. The distinct black band is hypothesized to form a stratified biofilm along the coral surface and is dominated by cyanobacteria, sulfur-reducing, and sulfur-oxidizing bacteria. Coral tissue is deteriorated as a result of high sulfide levels (0.8 mM) that form an anoxic environment along the coral surface (Ainsworth et al. 2007). One of the putative BBD-associated cyanobacterium, *Phormidium corallyticum*, was identified in 58% of apparently healthy coral samples indicating that disease pathogens may always be present and only become pathogenic when in the presence of other members of the microbial consortia, or when pollution and/or seawater temperature reach an optimal environment increasing their virulence (Klaus et al. 2007). Recently, Sekar et al. (2008) used length heterogeneity PCR (LH-PCR) profiling techniques to assess the entire microbial community associated with BBD samples collected from 9 coral species in 3 Caribbean regions based on a hypervariable region within the 16S rRNA gene (Mills et al. 2006). This is one of the only studies to characterize the entire microbial community associated with BBD and the results support the hypothesis that BBD is composed of variable members of distinct physiological and toxin-associated bacterial groups. These studies again suggest that microbial variation and virulence appear to be correlated with environmental stress (e.g. increased nutrients, sewage run-off).

White-band, white-plague, white-pox diseases and white-syndrome have been identified worldwide and have rapid and devastating effects on prominent framework-building and branching coral species. Bruno and colleagues (2007) documented a highly significant

relationship between the frequency of warm temperature anomalies and of white syndrome outbreaks using 6 yrs of coral disease and coral cover data across 48 reefs in Australia's Great Barrier Reef. White syndrome was described as either an additional emergent disease, or a group of diseases, among Pacific reef-building corals. Disease outbreaks followed warm years, but only on high (>50%) cover reefs, which suggests that host density also contributes to the potential for outbreaks (Bruno et al. 2007). White diseases/syndromes are generally described as a lesion of white or yellow, recently exposed coral skeleton that is conspicuously separated from apparently healthy coral tissue. Cytological and microbial studies of white lesions were conducted at the Marine Biological Laboratory in Eilat, Israel in the Gulf of Aqaba. Within the white lesions δ-Proteobacteria, *Cytoflaga-flavobacterium* groups, and communities of *Vibrio* spp. were identified (Ainsworth et al. 2007). Several *Acropora* spp. of coral were infected with white syndrome-like symptoms that moved quickly across affected corals. However, microscopic analysis of the lesion-tissue interface was surprisingly devoid of any significant bacterial population. Differences in the presence-absence of microbial populations appear to distinguish between diseases (e.g. plague, pox and band) and syndromes (Ainsworth et al. 2007).

White Plague Disease type II (WPD-II) has been reported to affect more than 40 different coral species (Weil et al. 2006). The putative bacterial pathogen of WPD II, *Aurantimonas corallicida*, was isolated from the coral, *Dichocoenia stokesi,* (Richardson et al. 1998; Denner et al. 2003) and is one of the few examples for which Koch's postulates was fulfilled (Table 1.1). A recent study documented a shift in microbial community structure in response to disease, with an accumulation of ribotypes similar to pathogens or bacteria previously isolated from diseased, injured or stressed marine invertebrates (Sunagawa et al. 2009). However, *Aurantimonas corallicida*, was not identified, suggesting that *M. faveolata* colonies sampled in this study may have been affected by a disease of yet unknown etiology that may have resulted in (or be a result of) an increase in opportunistic pathogens (Sunagawa et al. 2009). Because these diseases have far-reaching ecological impacts and they appear to have few macroscopic differences, it is particularly important to apply molecular techniques toward identifying the causative agents.

Many of the previously described diseases and syndromes may be caused or exacerbated by a number of proposed factors including elevated seawater temperatures (ET) (Rosenberg and Ben-Haim 2002), UV-exposure (Coles and Seapy 1998), increasing coral density (Bruno et al. 2007), water quality conditions such as elevated nutrients and fecal contamination (Bruno et al.

2003, Voss and Richardson 2006), introduction of novel microbes (Shinn et al. 2000), vectors or reservoirs for disease causing pathogens (Nugues et al. 2004, Sussman et al. 2003), longtime residence of fish/invertebrate feces on live coral surfaces (Weil 2004) (Table 1.1). Of particular interest is the accumulating evidence that marine organisms may be implicated in coral susceptibility to disease (Sussman et al. 2003, Nugues et al. 2004). Marine organisms have the potential to act as vectors or reservoirs, serving both to transmit and spread disease on local and regional scales. Some examples of possible disease vectors are the polychaete fireworm, *Hermodice carunculata,* which was shown to harbor the bleaching pathogen, *Vibrio shiloi,* in its gut (Sussman et al. 2003). The corallivorous nudibranch *Phestilla* spp. has also been implicated as a potential disease vector in Australia. Various microbes colonized coral fragments after *Phestilla* grazing, including *Paramecium* ciliates and *Beggiatoa* spp. bacteria (a member of the black band disease consortium), subsequently leading to epidermal tissue loss (Dalton & Godwin 2006). Corallivorous fishes such as the Caribbean butterflyfish, *Chaetodon capistratus,* acted as a vector for black-band disease in the coral *Montastraea faveolata* via direct oral and/or indirect fecal transmission (Aeby & Santavy 2006, Figure 1.3). Rotjan and Lewis (2008) further review the impact of corallivory on tropical reefs. The green alga, *Halimeda opuntia*, harbored the causative agent of WPD II, *Aurantimonas corallicida,* and contact with *Montastraea faveolata* colonies correlated with visual signs of disease and the presence of *A. corallicida* (Nugues et al. 2004). Other potential disease vectors include the corallivorous snails, *Coralliophylla abbreviata and C. carribbaea*, parrotfishes, and damselfishes that directly interact with diseased and non-diseased colonies. All these variables, acting alone or in combination, can rapidly change spatially and temporally, making it difficult for researchers to identify a single cause(s) driving reef degradation. The final section in this review focuses on competition-induced stress with encroaching macroalgae and the potential avenues by which these organisms may serve as vectors and/or drivers of coral disease.

Macroalgal-Microbial Interactions

Environmental stressors have been directly correlated with coral disease prevalence and changes in coral-microbial assemblages (Ritchie 2006, Bruno et al. 2007, Table 1.1). Therefore, competition-induced stress, in addition to direct physical or chemical mediation of coral-associated microbes, may further confound the physiology and resilience of the coral holobiont

(Ritchie and Smith 1995a, b, Geffen and Rosenberg 2005). Marine invertebrates such as corals have innate protective mechanisms such as physiochemical barriers (mucus), cellular defenses and humoral defenses (reviewed in Sutherland et al. 2004, Mydlarz et al. 2006, Miller et al. 2007). Additionally, microorganisms form symbioses that further protect the coral host, but when this association is disrupted, corals may be exposed to infection from opportunistic bacteria, fungi, and viruses (Rohwer et al. 2002, Shanmugam et al. 2005). The Probiotic Hypothesis (Reshef et al. 2006) and Hologenome Theory (Rosenberg et al. 2007) suggest that the hologenome (sum of the genetic information of the host and its microbiota) may adapt rapidly and with great versatility to changing environmental conditions. In this manner, beneficial adaptations could occur more freely than when dependent solely on genetic mutations and host selective processes. For example, individuals of the freshwater cnidarian, *Hydra*, actively select and shape the microbial communities within their epithelial layers, and these bacteria play an important role in the *Hydra*'s innate immune response (Fraune and Bosch 2007). Therefore any organism and/or environmental stressor with the ability to rapidly alter microbial populations potentially may shift the symbiotic continuum from mutualistic to parasitic relationships. I will review evidence documenting the destructive effect of proliferating macroalgae on present-day reefs and discuss new evidence describing how macroalgae also interact and compete with coral-associated microorganisms.

Macroalgae are one of the major competitors with corals on tropical reefs and long-term ecological studies have continued to reveal declines in live coral cover and increase in algal abundance (Birkeland 1997). This potentially irreversible phase-shift from a coral-dominated reef to an algal-dominated reef is facilitated by high levels of dissolved nutrient from terrestrial run-off and lack of control by herbivores as a result of overfishing and herbivore die-offs (e.g. *Diadema*, Carpenter & Edmunds 2006, Littler et al. 2006, reviewed by Fong & Paul 2011). A greater algal biomass results in increased physical contact and competition between corals and algae, which is known to negatively affect corals at all life history stages.

Macroalgae inflict competitive damage on stony corals via several mechanisms that may simultaneously or synergistically have an effect on coral-associated microoganisms. McCook et al. (2001) reviewed and categorized the available literature on coral-algal competition and listed six specific processes of competition. The six potential mechanisms of competitive inhibition between corals and algae proposed by McCook et al. (2001) are briefly outlined with any

additional processes recently defined. Algae may engage in exploitation competition with corals by interfering with the settlement of larvae through space preemption or biofilm inhibition (Birrell et al. 2005, Mumby et al. 2006, Box and Mumby 2007, Vermeij et al. 2009). Interference competition may reduce coral growth or increase mortality, including shading, chemically mediated competition (allelopathy), preference of settling larvae for algal surfaces (deNys et al. 1991, Miller and Hay 1996, Littler and Littler 1997, Nugues and Szmant, 2006, Vermeij et al., 2009), abrasion, whiplash, basal encroachment (Coyer et al. 1993, Lirman 2001, Box and Mumby 2007), and increased localized sedimentation as a result of reduced water flow (Nugues and Roberts 2003). Therefore phase shifts from coral-dominated reefs to landscapes of higher algal biomass occur both through decreased recruitment of corals by reducing larval settlement, and through impacts on the post-settlement survival of juvenile and adult corals (Hughes and Tanner, 2000, Kuffner et al. 2006). Physical and chemical interactions among macroalgae and corals have the potential to disrupt coral-associated microbial symbionts, potentially exposing the coral to infectious disease (Nugues et al. 2004). Thus, I will examine physical and chemical coral-algal interactions at all life-history stages and their potential to mediate microbial assemblages.

Chemical metabolites produced by macroalgae, microalgae, and cyanobacteria may kill or damage coral larvae prior to settlement, or deter coral larvae from exploring a habitat and settling (reviewed in Birrell et al. 2008a). No metamorphosis of coral larvae from *Stylophora pistillata* or *Acropora palifera* occurred in the presence of the foliose brown alga, *Lobophora variegata*. During this experiment, planulae of both species quickly stopped swimming, sank to the bottom of the laboratory assay containers, and never metamorphosed (Baird & Morse 2004). Morse et al. (1996) conducted a similar experiment and also found that acroporid larvae did not settle in the presence of *L. variegata*. Kuffner et al. (2006) demonstrated that the presence of 3 species of *Lyngbya* cyanobacteria, and the brown algae *Dictyota pulchella* and *Lobophora variegata*, all negatively impacted larval settlement in the brooding coral *Porites astreoides,* and in some cases caused increased mortality of new recruits. Birrell et al. (2008b) experimentally demonstrated that waterborne chemicals from three different algal taxa were responsible for enhancing or inhibiting larval settlement of the broadcast spawning coral, *Acropora millepora.* These experiments demonstrate a correlation between the presence of macroalgae and/or macroalgal exudates and the inhibition of coral settlement. Vermeij and colleagues (2009) are the

first to demonstrate a potential mechanism driving the affect of macroalgae on coral recruitment success. Larvae from the common Hawaiian coral, *Montipora capitata*, were used to examine the independent and combined effects of algae and microbes on coral recruitment success. It was found, as in previous studies, that the presence of macroalgae reduced survivorship and settlement success of planulae. However, with the addition of a broad-spectrum antibiotic, ampicillin, the negative effects were reversed and coral settlement increased. This evidence suggests that macroalgae can indirectly cause planular mortality by influencing the microbial biofilm on settlement surfaces; either by enhancing microbial concentrations or by weakening the coral's resistance to microbial infections (Smith et al. 2006, Vermeij et al. 2009). Macroalgae may both prevent metamorphosis of settled planulae and inhibit coral settlement prior to reaching the reef substratum.

Similar to corals, macroalgae are rich in organic material and present an ideal substratum for growth of microorganisms. Microorganisms often protect macroalgae by producing metabolites that prevent biofouling (Boyd et al. 1999, Armstrong et al. 2001). However, just as with corals, macroalgae are open to microbial attack by disease causing pathogens. Despite the presence of algal diseases (e.g. Red Spot, White Rot), and nutrient-rich thalli that have the potential to host pathogens and biofoulers, reports of widespread algal mortality are uncommon, suggesting that either outbreaks of algal disease have gone unnoticed or algae have acquired mechanisms, and/or microbial symbionts, to aid in resisting infection (Lane and Kubanek 2008).

Several strategies have been identified that aid macroalgae in defending themselves from bacterial colonization and disease. One approach is to disrupt growth and/or attachment of parasites with physical defenses such as mucilaginous coverings, outer cell layer shedding, and erosion of the distal ends of blades (Mann 1973, Nylund and Pavia 2005). Macroalgae can also produce oxidative bursts, the release of reactive oxygen species, when threatened by microbial colonization (Weinberger 2007). Another mechanism of resistance is the use of secondary metabolites as chemical defenses (Boyd et al. 1999, Engel et al. 2006, Puglisi et al. 2007). These compounds are found in sponges, macroalgae, bryozoans and a host of other marine invertebrates, and can serve both primary functions (e.g. components of the cell wall, precursors to holdfast adhesion, and reproduction) and secondary functions (allelopathy, antimicrobial agents, antifouling and/or UV screening agents) (reviewed in Paul and Ritson-Williams 2008). Allelopathy, or the production of inhibitory compounds, has received recent attention as an

important function of natural products from sponges and macroalgae on reef-building corals. Many macroalgae are known to produce natural products that protect them from herbivores (Paul and Hay 1986, Nagle and Paul 1999, Paul et al. 2001, 2007), and these same compounds can play a critical role in competitive interactions with corals and their microbial associates.

Researchers have only recently examined the antimicrobial and/or stimulatory activity of secondary metabolites from marine algae against ecologically relevant marine microorganisms. *Delisea pulchra* is one of the few macroalgae that has been extensively studied, and is known to produce halogenated furanones, which resemble acylated-homoserine lactones (AHL). AHLs are used in bacterial communication signaling for swarming and attachment. Halogenated furanones are competitive inhibitors of the transcription regulator, LuxR, which regulates bacterial swarming and subsequent attachment (Maximilien et al. 1998, Manfield 1999). Thus *Delisea pulchra* is one example of how macroalgae can prevent tissue damage by harboring secondary metabolites that inhibit the growth, survival, virulence, and reproduction of fouling organisms. The red alga, *Asparagopsis armata,* also produces bromoform and dibromocetic acid, which were both active against marine *Vibrio* spp. (Paul et al. 2006). Furthermore, *A. armata* utilizes a delivery system that ushers the release of antibiotic metabolites to the algal surface. When halogenated metabolites were experimentally removed from *A. armata,* the thallus became fouled with significantly higher densities of epiphytic bacteria (Paul et al. 2006). Because these compounds were more active against bacteria isolated from algae lacking brominated metabolites than algae producing normal amounts of these compounds, it is possible that these compounds would also be active against coral-associated bacteria that came in contact with the algal thallus.

Cetrulo and Hay (2000) conducted a survey of activated defenses in 42 seaweed species and determined that damaging the algae before extraction caused a detectable chemical change in 70% of the study algae. Thus coral-algal abrasion may indirectly induce concentrated chemical defenses similar to herbivory. Extracts from an Atlantic green alga and Pacific green and red algae showed activity against all microorganisms tested, but the natural products that are responsible for the antimicrobial activity are largely unknown (Puglisi et al. 2004). In an extensive follow-up study, lipophilic and hydrophilic extracts from 54 species of marine algae and 2 species of seagrasses collected from the Indo-Pacific coral reefs were tested in growth inhibition assays against ecologically relevant microorganisms, including saprophytic fungi,

saprophytic stramenopiles, and a pathogenic bacterium (Puglisi et al. 2007). Overall, 95% of the macroalgae tested were active against one or more microorganisms, however broad-spectrum activity demonstrated < 50% of algae tested. In particular, extracts from the green alga *Bryopsis pennata* and the red alga *Portieria hornemannii* inhibited all assay microorganisms. These results provide persuasive evidence that antimicrobial chemical defense is widespread among marine plants and algae, but appears to be species specific, varying by algal species and microbial susceptibility (Puglisi et al. 2007). Therefore, encroaching macroalgae have the ability to have species-specific effects on the diversity and abundance of coral-associated microorganisms, potentially influencing disease susceptibility (Nugues et al. 2004, Smith et al. 2006, Puglisi et al. 2007).

Macroalgae not only inhibit microorganisms via allelochemical antibiotics, but also stimulate microbial growth, which may have an opposite but equally detrimental effect on coral tissues and coral-associated microbes. In a small experimental study, Smith et al. (2006) showed that the release of high levels of dissolved organic carbon (DOC) by macroalgae caused explosive bacterial growth on adjacent coral surfaces, and led to a zone of hypoxia that induced coral tissue death. In a water quality study, high levels of DOC caused coral mortality and increased microbial growth rates by an order of magnitude within the coral mucus layer (Kline et al. 2006). These results contend that coral-associated microbes are carbon limited and that anthropogenic nutrient enrichment or encroaching macroalgae may directly increase the amount of labile DOC and enable microbes to break down more complex and previously unavailable carbon sources via co-metabolism, leading to uncontrolled microbial growth. It is believed that elevated microbial growth rates cause coral mortality by oxygen depletion, accumulation of poisons (e.g. hydrogen sulfide, secondary metabolites), and/or microbial predation on weakened coral polyps (Segel and Ducklow 1982, Smith et al. 2006). However, macroalgae may need to be in direct contact with coral tissues to result in high enough DOC levels to influence microbial proliferation (Vu et al. 2009) or the transference of pathogens (Nugues et al. 2004). Based on the available evidence, the effect of coral-algal contact on microbial assemblages would be exacerbated by low flow and high temperature conditions (Smith et al. 2006, Bruno et al. 2007).

Outcomes of coral-algal interactions are quite complex and rely on the species-specific competitive potential of both the coral and the alga (Titlyanov et al. 2007). Since coral populations have precipitously declined on many reefs over the past three decades while algal

abundance has increased, competitive interactions between corals and macroalgae are frequent at all life history stages and in need of further research. It is likely that phase-shifts to higher algal biomass not only result in strong negative interactions with adult corals but a decrease in coral recruitment by reducing larval settlement and post-settlement survival, thus macroalgae can alter the overall structure of reef communities (Hughes and Tanner, 2000; Kuffner et al., 2006). Furthermore, as the literature suggests, the competitive stress that macroalgae and other encroaching organisms (e.g. sponges, zoanthids) promote likely diminishes energy reserves that are critical to normal immune function, fecundity, and resilience of healthy corals, potentially contributing to chronic bleaching events and disease infection (Ritson-Williams et al. 2009)

Conclusion

Over the past decade there has been substantial progress made toward defining the abundance and diversity of microorganisms on coral reefs. Both culture-dependent and -independent methods have been utilized to elucidate the composition and function of coral-associated bacteria and Archaea. These studies have shown that corals favor specific populations of microbial associates that play an important role in nutrition and disease-resistance. We have discussed how small changes in environmental conditions can lead to dramatic changes in microbial community composition and structure. The microbial population of the coral holobiont can be further altered when exposed to environmental stress. Alterations in the natural microbiota may influence adaptability and/or disease and bleaching susceptibility. The field of coral microbiology is still largely in the descriptive phase, and only with further experimental manipulations will we begin to comprehend the dynamic ecological functions and interactions among microorganisms, their coral host, and the surrounding reef environment. The causative agent responsible for shifts from healthy to diseased corals has only been established in a few model systems and the biology of coral viruses and Archaea has only briefly been explored. The impact of other competing marine organisms, such as macroalgae, requires further manipulative field assays to determine the true ecological impacts on the entire coral holobiont. However, it is apparent that the coral holobiont represents an intricate web of associations that interact and change spatially and temporally (Figure 1.4). It is necessary to identify the role of each organism in the association and the environmental parameters involved in order to make precise management predictions about the status of today's coral reef ecosystems.

Table 1.1: All reported coral diseases and syndromes and the organisms associated with the infection. Koch's postulates fulfilled for those in bold print. Listed abiotic stressors associated with heightened susceptibility or virulence. Table adapted from Sutherland et al. (2004) and Weil et al. (2006).

Disease (D)/ Syndrome (S)	Putative Pathogen	Type of Microbe	Abiotic Stressors	Reference
1. Aspergillosis (D)	*Aspergillosis sidowii*	Fungus	Elevated Temp (ET), Sediment, Pollution	Smith et al. 1996
2. Atramentous necrosis (D) (Initial)	*Vibrio coralliilyticus*	γ-proteobacteria	ET, Sediment	Jones 2004, Anthony et al. 2008
Atramnetous necrosis (D) (Band)		Biofilm/ mixed consortia	" "	
3. Black Band (D)	*Phormidium coralyticum*,	Cyanobacteria	ET, Eutrophication,	Antonius 1973, Cooney et al. 2002, Voss &
	Trichodesmium sp.	Cyanobacteria	Sediment, Pollution,	Richardson 2006, Viehman et al. 2006, Richardson
	Desulfovibrio,	δ-proteobacteria	Fecal contaminants	2007, Myers et al. 2007, Sekar et al. 2008
	Beggiatoa sp.	γ-proteobacteria		
4. Bleaching	*Vibrio sp.*	γ-proteobacteria	ET, Irradience, flow	Brown 1997, Bourne et al. 2008
5. Bleaching, Induced (D)	***Vibrio shiloi***	γ-proteobacteria	ET	Kushmaro et al. 1996, Rosenberg & Falkovitz 2004
6. **Bleaching, Induced (D)**	***Vibrio coralliilyticus***	**γ-proteobacteria**	ET, Pollution	Ben-Haim & Rosenberg 2002, Rosenberg etal. 2008
7. Brown Band (S)	?	Fungi		Yarden et al. 2007
8. Ciliates (S)	*Halofolliculina*	Protozoan		Cro´quer et al. 2006
9. Dark Spot (S)	*Entocladia endozoica*	Chlorophyte microalga		Goreau et al. 1998, Gochfeld et al. 2006
10. Dark Band (S)	?			
11. Fungal Protozoan (S)	*Trichoderma,Clodosporium*	Fungi	ET	Cerrano et al. 2000
	Penicillum, Humicola	Fungi		
	Ciliates	Protozoan		
12. Necrosis, Patchy (S)	?			
13. Necrosis, Rapid Tissue (D)	*Vibrio harveyi, Vibrio sp.*	γ-proteobacteria	ET, Sediment	Luna et al. 2007
Shut Down Reaction (Aquaria)				Antonius 1977
14. Neoplasia (S)	?			
15. Pink-line (S)	*Phormidium valderianum*	Cyanobacteria		Ravindran et al. 2001
16. Rapid Wasting (D)	?			
17. Red Band Consortium (D)	*Phormidium, Oscillatoria*	Cyanobacteria consortium		Sussman et al. 2006
18. Ring Syndrome (S)	?			
19. Skeletal Tissue Anomalies	*Petrarc madreporae*	Crustacean	UV Radiation	Squires 1965, see Sutherland et al. 2004,
	Podocotyloides stenometra	Trematode		Domart-Coulon et al. 2006,
	Aspergillosis sydowii	Fungus		McClanahan et al. 2009
	Entocladia endozoica	Chlorophyte microalga		
20. Skeletal Eroding Band	*Halofolliculina corallasia**	Protozoan* and Fungi		Antonius & Lipscomb 2000, Yarden et al. 2007
21. Tumors (D)	*Entocladia endozoica*	Chlorophyte microalga		Breitbart et al. 2005
22. Yellow Band (D)	*Vibrio rotiferianus, V. harveyi,*	γ-proteobacteria	ET	Korrubel & Riegl 1998, Bruckner & Bruckner 2006
	V. alginolyticus, V. proteolyticus			Cervino et al. 2008 (*Vibrio* core-group)
23. Yellow Blotch (S)	*Vibrio alginolyticus*	γ-proteobacteria		Santavy and Peters 1997
White Diseases				Review Ainsworth et al. 2007
24. White Band Type I (D)		Gram (-) bacterium		Gladfelter 1982
25. White Band Type II (D)	***Vibrio harveyi/***	**γ-proteobacteria**		Ritchie and Smith 1998
	V. charchariae			
26. White Plague Type I (D)		Gram (-) bacterium	ET and	Dustan 1977
27. White Plague Type II (D)	***Aurantimonas coralllicida***	**α proteobacteria**	Fecal contaminants	Richardson et al. 1998a, b, Denner et al. 2002
	Alteromonad, Vibrionaceae	γ-proteobacteria		Sunagawa et al. 2009
28. White Plague Type III (D)				Richardson et al. 2001
29. White Plague (Red Sea)	*Thalassomonas loyana*	γ-proteobacteria		Ainsworth et al. 2007
30. White Pox (D)	***Serratia marcescens***	**γ-proteobacteria**		Holden 1996

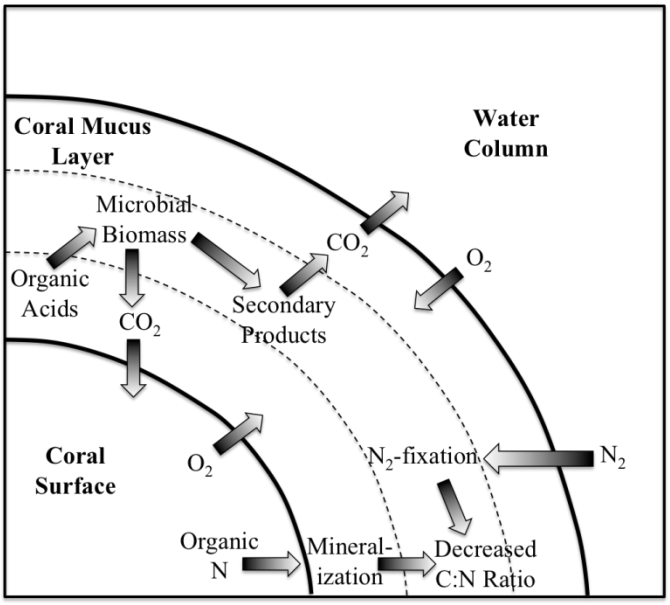


Figure 1.1: Surface mucopolysaccharide layer (mucus) forms a boundary layer between coral tissues and the surrounding reef water, functioning in exchange of gases, nutrients, and secondary metabolites. Mucus may become aerobically stratified in low flow habitats at night when coral tissues are consuming oxygen and zooxanthellae and/or cyanobacteria are not respiring. Adapted from Ritchie and Smith (2004)

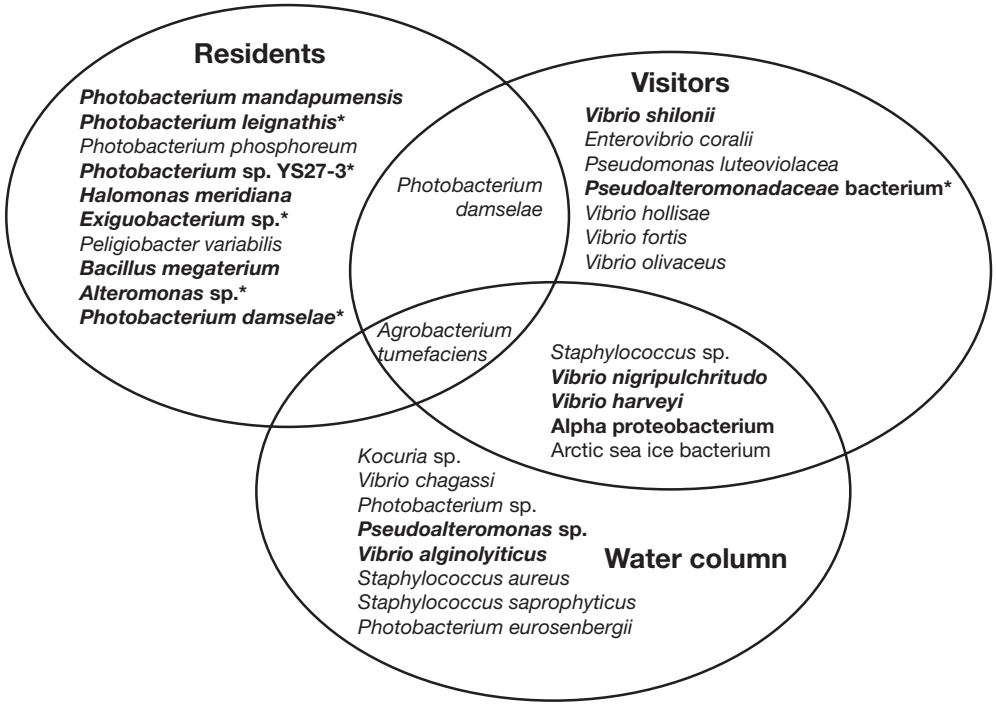


Figure 1.2: Residents represent bacteria selected-for on mucus treated medium as putative coral symbionts. Visitors selected on control media with no coral mucus. Water column bacteria selected from the water column (not coral surface) on control media with no mucus. Overlap represents bacteria common to different treatments and sources. Bold represents antimicrobial producing bacteria. *Bacteria producing antibiotic active against *Serratia marcescens* strain PDL110. Adapted from Ritchie (2006).

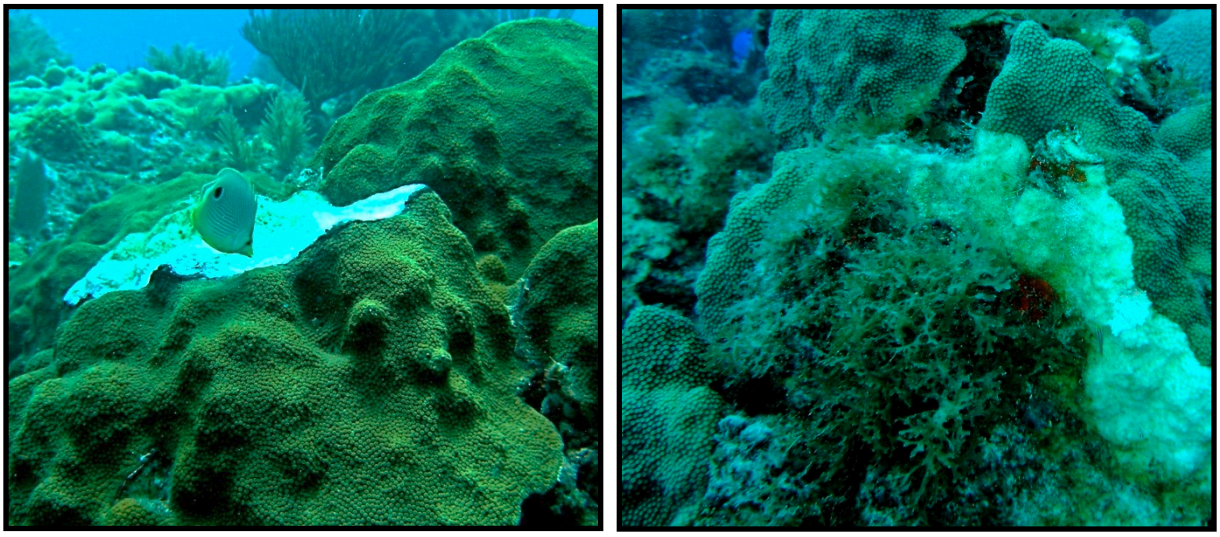


Figure 1.3: Left, Caribbean butterflyfish, *Chaetodon capistratus*, ingesting and releasing mouthfuls of BBD infecting a large *Montastraea faveolata* colony. Right, brown algae in the genus, *Dictyota* sp., as a possible vector for white disease on *Monstastraea faveolata Photos: K. Morrow. FLK, Aug. 2008*.

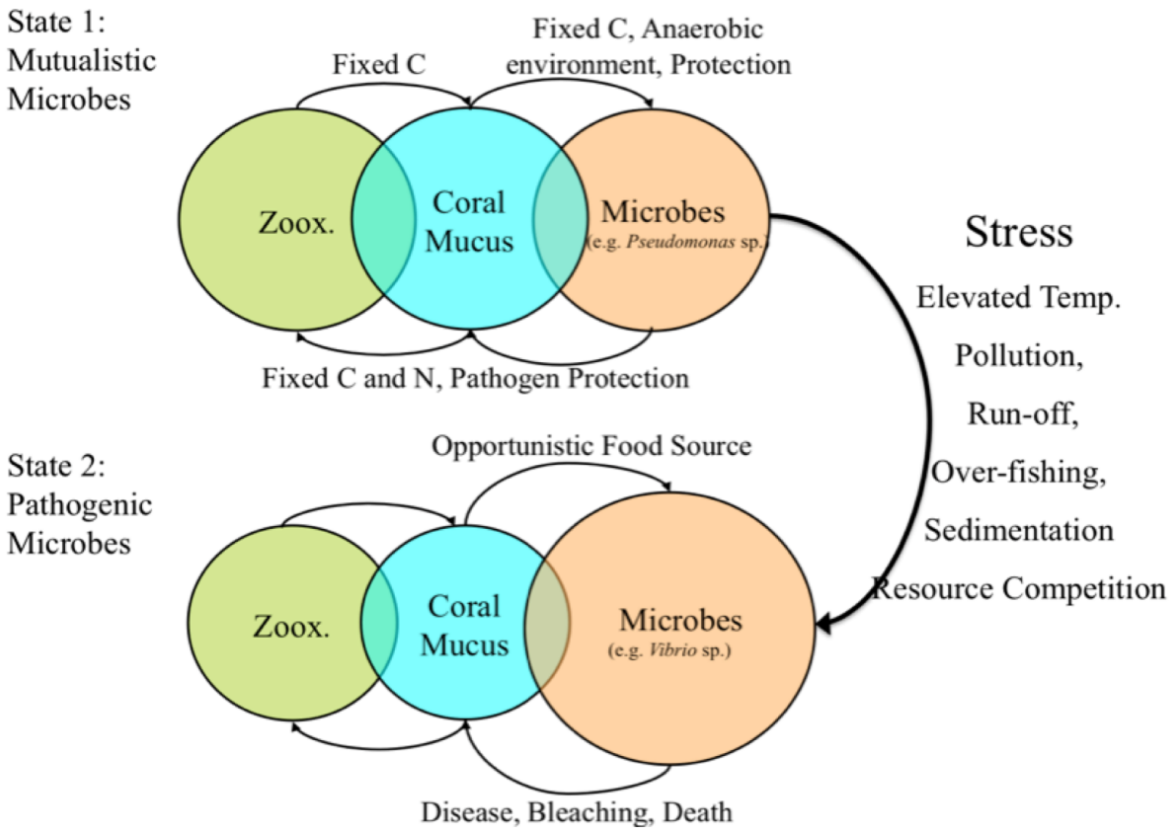


Figure 1.4: Conceptual model representing the flow of resources among components of the coral holobiont. *State 1* – Healthy coral holobiont represented by a symbiotic equilibrium among zooxanthellae, coral tissues, and microoganisms. Environmental and anthropogenic stress induces a shift to *State 2* – Reduced coral and zooxanthellae physiology and an elevated occurrence and severity of coral disease results in a change in overall reef community structure.

Chapter 2

Allelochemicals produced by Caribbean macroalgae and cyanobacteria have species-specific effects on reef coral microorganisms

Introduction

Macroalgae are major competitors with corals and other benthic organisms on tropical reefs, especially where rates of herbivory are low and nutrient enrichment is high (Fong and Paul 2011; Rasher and Hay 2010). Competition can occur through several direct and indirect physical and chemical (allelopathic) mechanisms (reviewed in Chadwick and Morrow 2011). Therefore, benthic community structure on coral reefs can be strongly influenced by allelopathy (chemically mediated competition). A well-known function of macroalgal compounds is to provide chemical protection from abundant and diverse herbivores (Paul and Hay 1986; Nagle and Paul 1999; Paul et al. 2001). Previous studies suggest that as herbivorous fishes and invertebrates selectively remove palatable species of macroalgae, they are replaced by unpalatable, chemically defended seaweeds (Tsuda and Kami 1973). The results of this selective herbivory are commonly seen on many coral reefs that have undergone phase-shifts to increasing dominance by chemically defended seaweeds, including species of *Halimeda, Dictyota, Lobophora* and cyanobacteria of the genus *Lyngbya* (Rogers et al. 1997; McClanahan et al. 1999, 2000; Fong and Paul 2010). Members of these 3 chemically-defended genera are now the most abundant macroalgae on many reefs in Florida and the Caribbean (Rogers et al. 1997; McClanahan et al. 1999, 2000; Kuffner et al. 2006), reaching 7-17% cover at some sites (K.M. Morrow pers. obs.).

Seaweeds further utilize physical and chemical regulatory strategies to prevent bacterial and fungal colonization (Mann 1973; Nylund et al. 2005; Engel et al. 2006; Lane et al. 2009). The microbial diversity and abundance within the marine environment varies in response to changing environmental conditions (e.g., temperature, pH, nutrients, pollutants) (Wommack et al. 1999), and bacterial symbionts may allow host organisms to respond more efficiently to these changes (Reshef et al. 2006; Rosenberg et al. 2007). Specifically, corals are believed to harbor an abundant and diverse, species-specific assemblage of microorganisms (Rohwer et al. 2002).

It has been suggested that the coral host regulates and selects for the most advantageous microbial symbionts in the context of prevailing environmental conditions (Reshef et al. 2006; Zilber-Rosenberg and Rosenberg 2008). Thus, variation in microbial symbionts can support or hinder adaptation of the holobiont (coral host and associated microorganisms). In the absence of microorganisms, many eukaryotic organisms may be less able to fight infections from pathogenic bacteria and viruses (Shanmugam et al. 2005). Microorganisms likely prevent infection by producing antibacterial and antifungal compounds in addition to occupying niches that would otherwise be available to opportunistic pathogens (Koh 1997; Castillo et al. 2001; Ritchie 2006). Corals also may rely on microbial symbionts for nutritional requirements and efficient adaptation to environmental change (Shashar et al. 1973; Ducklow and Mitchell 1979; Lesser et al. 2004; Croft et al. 2005). Thus it is critical to understand the extent to which chemically-defended competitors, such as macroalgae and cyanobacteria, can affect coral-associated microbial assemblages.

Many allelochemicals can inhibit microbial growth, but these compounds rarely have been tested against ecologically-relevant microorganisms. For example, the red alga *Delisea pulchra* produces halogenated furanones that resemble acylated-homoserine lactones (AHL) and are used in bacterial signaling (Kjelleberg et al. 1997). These halogenated furanones function by inhibiting bacterial quorum sensing, through disruption of a transcription regulator, LuxR, which regulates bacterial colonization and biofilm formation (Maximilien et al. 1998; Manfield 1999). Thus, *D. pulchra* can prevent tissue damage by harboring compounds that inhibit the survival, virulence, and reproduction of fouling organisms. Another well-studied red alga, *Asparagopsis armata,* produces bromoform and dibromacetic acid, both of which are active against marine *Vibrio* bacteria that often are pathogenic (Paul et al. 2006). When halogenated metabolites were experimentally removed from *A. armata,* the thallus became fouled with significantly higher densities of epiphytic bacteria (Paul et al. 2006). Finally, another red macroalga, *Callophycus serratus,* was shown to produce bromophycolides and callophycoic acids that inhibit the growth of *Lindra thalassiae*, a marine fungal pathogen (Lane et al. 2009). Therefore, macroalgae such as *D. pulchra, A. armata,* and *C. serratus* employ species-specific chemical metabolites and harbor microbial assemblages that are resistant to compounds produced by the alga. Marine plants and animals frequently harbor species-specific microbial assemblages that are distinct from the assemblages found in the surrounding environment and are important to host defenses

(Baker and Orr 1986; Wahl and Hay 1995; Rohwer et al. 2002). Several other studies have surveyed both tropical (Ballantine et al. 1987; Engel et al. 2006) and temperate macroalgae (Cetrulo and Hay 2000) and illustrate the ubiquitous nature of marine chemical defenses.

Macroalgal compounds not only inhibit but also can stimulate microbial growth, which may have an equally rapid and detrimental effect on organisms living in close proximity to the algal thalli. Little is known about the effects of stimulatory chemicals or primary metabolites on specific microorganisms, but several studies have examined the general effects on *in situ* microbial populations. In an aquarium study, Smith et al. (2006) showed that the release of high levels of dissolved organic carbon (DOC) by macroalgae caused explosive bacterial growth on adjacent coral surfaces, and led to a zone of hypoxia that induced coral tissue death. High levels of DOC in the water column also lead to coral mortality, and increased microbial growth rates by an order of magnitude within the coral mucus layer (Kline et al. 2006). Elevated microbial growth rates appear to cause coral mortality through depletion of oxygen, accumulation of poisons (e.g., hydrogen sulfide, secondary metabolites), and/or microbial predation on weakened coral polyps (Segel and Ducklow 1982; Smith et al. 2006). However, high concentrations of DOC and metabolites may cause elevated and detrimental microbial growth only under certain conditions, including direct coral-algal contact, low water flow in interstitial microenvironments, and/or high temperature stress (Smith et al. 2006; Bruno et al. 2007; Vu et al. 2009).

Here we report the results of bacterial growth assays using crude extracts from six common Caribbean macroalgae and two benthic cyanobacteria for inhibitory and stimulatory activity on coral- and algal- associated bacteria. We screened 16 crude extracts, eight lipophilic (non-polar) and eight hydrophilic (polar), from each algal and cyanobacterial species against 54 bacterial cultures that were isolated from Caribbean reef corals and macroalgae, to identify the specific response of each bacterial culture to these algal extracts. We hypothesized that the macroalgae and cyanobacteria produce both inhibitory and stimulatory compounds that may alternatively affect both beneficial and detrimental coral reef microorganisms. As macroalgae become more prolific on present-day coral reefs, this information is needed to understand the mechanisms of competition among reef organisms on both macro- and microscopic scales. Particular species of encroaching macroalgae may have more or less severe effects on corals and their associated microorganisms, and it is important to determine how these interactions alter the ability of reef-building corals to respond and adapt to changing environmental conditions.

Methods

Sample collection and preservation

Macroalgae and cyanobacteria were collected from coral reefs adjacent to the Mote Marine Laboratory, Summerland Key, Florida (the brown alga *Dictyota menstrualis* and the green alga *Halimeda tuna*), the University of the Virgin Islands MacLean Marine Science Center, St. Thomas (the red alga *Acanthophora spicifera,* the brown alga *Lobophora variegata,* and the cyanobacterium *Lyngbya majuscula*), and the Smithsonian's Carrie Bow Cay Field Station in Belize (the brown algae *Dictyota sp.* and *Dictyota pulchella,* and the cyanobacterium *Lyngbya polychroa*). Collections were made by hand on SCUBA at depths of 8-15 m in April 2003 (*D. pulchella*), July 2005 (*D. menstrualis*), and the remainder between May-August 2008 (*A. spicifera, Dictyota sp., H. tuna, L. majuscula, L. polychroa, L. variegata).* All samples were placed in plastic zip-lock bags at depth and brought to the surface, then placed in seawater-filled coolers and transported back to the laboratory (< 3 hrs). Clean plants, free of substantial epiphyte growth or other macroscopic material, were frozen at -20 ºC. Samples of the green alga *H. tuna* were flash-frozen in liquid nitrogen to prevent degradation of the diterpenoid compounds (Paul and Van Alstyne 1992). All frozen algal samples were transported on ice to the Smithsonian Marine Station in Fort Pierce, FL for further chemical extraction and analysis. *A. spicifera* was selected because it does not contain known chemical defenses and was not expected to inhibit marine bacteria in these assays. All other species of macroalgae and cyanobacteria were selected because they are suspected to produce potent allelochemicals.

Microbial samples were collected with 5 ml plastic-tipped syringes on SCUBA from coral mucus and algal surfaces on the above reefs in the Florida Keys, St. Thomas, and Belize at 8-15 m during May-August 2008. Two common species of Caribbean reef-building corals (*Montastraea faveolata* and *Porites astreoides*) and 2 common macroalgae (*D. menstrualis* and *Halimeda opuntia*) were sampled for associated microbes. Samples were collected from a 5 x 5 cm^2 surface area on all corals and macroalgae after gentle agitation, which encourages sloughing of the viscous mucus and reduces aspiration of seawater into the syringe (Ritchie 2006). Samples were collected from 3 locations along a gradient of coral-algal interaction: A) algal surfaces interacting with corals, B) coral mucus touching the algal thalli, and C) coral mucus 5 cm from the algal thalli. They also were collected from two control areas: X) coral mucus not in

contact with algae or any other sessile organisms, and Y) algae not in contact with corals or any other sessile organisms (Figure 2.1). Extracts from the brown alga *Dictyota* sp. and the green alga *H. tuna* were incorporated into non-toxic Phytagel and placed on separate corals from those sampled for the coral-algal gradient during a 3-day concurrent field experiment in Belize in October 2008. Coral mucus samples were collected from underneath experimental gels and also 5 cm away from the gels. Syringe samples were brought to the surface, placed in seawater- filled coolers, and transported back to the laboratory where they were immediately processed (< 1 hr). At the field station, a subsample (100 µl) of each microbial sample was spread-plated onto artificial seawater agar (FSWA; 1 L sterile seawater, 0.43 g beef extract, 0.64 g NaCl, 0.43 g peptone, and 15 g agar). After 24-48 hrs of growth at room temperature, single colonies were picked and triple-streaked for isolation. Mixed and isolated cultured bacteria were transported back to Auburn University for further analysis. From ~250 isolated bacterial cultures, 54 unidentified strains were maintained consistently in culture. Thus, these 54 cultures were chosen for bacterial assays to test the effects of algal extracts.

16S rRNA Gene Sequencing

Genomic DNA was extracted from the above 54 bacterial isolates using the MOBIO UltraClean® Microbial DNA Isolation Kit. PCR was conducted using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG) and 1492R (5′-GGYTACCTTGTTACG ACTT) (Medlin et al. 1988). The thermalcycling conditions were as follows: initial denaturation (5 min at 95 ˚C); 30 cycles of denaturation (30 s at 95 ˚C), annealing (1 min at 55 ˚C), and elongation (1 min at 72˚C); then a final extension step (7 min at 72 ˚C). The resulting amplicons were evaluated for yield and size by electrophoresis through a 1% (w/v) agarose gel and staining with ethidium bromide. PCR fragments of the correct size were sequenced in both directions by the Lucigen Corporation (Middleton, WI) using dideoxy sequencing chemistry via capillary electrophoresis. A consensus 16S rRNA sequence was determined for each cultured isolate using CromasPro v.1.42 (Technelysium Pty, Tewantin, Australia), and compared to the GenBank nr/nt database using BLASTn to identify the nearest neighbor for each respective bacterial isolate (http://www.ncbi.nlm.nih.gov/BLAST/).

Compound Extraction

All biochemical extractions were conducted at the Smithsonian Marine Station in Fort Pierce, FL during Sept.-Dec. 2008. Crude extracts were obtained from *A. spicifera, D. menstrualis, D. pulchella, Dictyota* sp., *L. variegata*, *H. tuna, L. majuscula* and *L. polychroa*. Frozen bulk samples of macroalgae and cyanobacteria were first wet-weighed and then lyophilized over several days. Then freeze-dried samples were weighed and covered with a 1:1 ethyl acetate:methanol (lipophilic or non-polar extract) solvent solution over 3 consecutive 24-hr periods, followed by three 24-hr extractions in 1:1 ethanol:de-ionized water (hydrophilic or polar extract). Extracts were filtered to remove large fragments of organic material and the solvents removed via rotary evaporation at 35˚C. Extracts then were dried overnight in a Thermo-Savant speed-vac concentrator and frozen (-20 ºC) for later use in the bacterial assays.

Extracts were tested at concentrations approximating those naturally found in macroalgal and cyanobacterial tissues based on algal wet weight (g), similar to methods used by Puglisi et al. (2007) (Table 2.1). Extract concentrations were also determined as g dried extract per g of sample tissue. Extracts were re-suspended to these estimated natural concentrations in artificial seawater (FSW) media with 2.5% ethanol added. Dissolved extracts were pre-filtered using a 0.8 µm (185 mm) circle filter (Whatman), followed by secondary filtration through a 0.22 µm filter (Millepore), attached to a vacuum pump. Using aseptic technique, filtered extracts were serially pipetted into a 96-well plate, including a blank for background absorbance of each extract, and FSW with and without ethanol for use as growth controls for each bacterial culture. Cultures were inoculated directly into extract-containing media and controls as described below.

Bacterial Bioassays

Macroalgal and cyanobacterial hydrophilic and lipophilic crude extracts were tested for activity against coral reef bacteria using a microdilution plate assay, a liquid culture method based on spectrophotometric readings of cellular growth developed by Gruppo et al. (2006). First, bacteria were re-streaked onto fresh FSWA 48 hrs prior to experimentation. From fresh cultures, a single colony was picked and placed in 2 ml of sterile liquid FSW in a 10 ml glass test tube. Test tubes were sealed and incubated at 33 ˚C while shaking at 215 rpm overnight. After 24 hrs of growth, a 150 µl sample from each tube was sub-cultured into each 96 well plate containing algal extract media. Each 96-well plate tested 6 extracts and 2 controls (FSW media

with and without ethanol) against 12 bacterial cultures. Five plates were used to test all 54 bacteria (12 cultures per plate x 5 plates). All extracts were plated in triplicate (n = 3) except for *Dictyota* sp., *H. tuna,* and *L. polychroa*, which were plated in duplicate (n = 2), due to limited extract availability. A single plate containing FSW media + extract was plated to control for background absorbance due to the dark coloration of some extracts. Culture wells were mixed by carefully pipetting their contents, then sealed with a breathable sealing film (Aeraseal^{TM}), and incubated at 33 $^{\circ}$C while shaking at 215 rpm for 48 hrs. Plates were transported to Harbor Branch Oceanographic Institute, and each well's absorbance at OD_{570} was determined using a BMG-labtech NOVOstar microplate reader. Prior to each reading, wells were shaken again to resuspend any settled material. A total of 20 OD_{570} readings were taken for each well and averaged for a final optical density for each well. The sterile cover had to be removed for accurate readings, thus time series data were not taken to reduce the risk of contamination. However, because the initial bacterial inoculum was below the limit of detection, an initial spectrophotometric reading was unnecessary.

Data Analysis

Initially, the background absorbance of each extract was subtracted from the optical density of respective culture wells, to control for extract absorbance. To compare growth rates among bacterial cultures, the percent change in growth was calculated by subtracting the optical density (OD) for each treatment from the OD in the corresponding control well (FSW media + solvent), dividing by the control well OD, and multiplying by 100. Mean percent change ± SE of all duplicate and triplicate plate readings are reported for all bacterial culture treatments.

To compare levels of antibacterial or stimulatory activity among macroalgal or cyanobacterial extracts, the mean percent change of each cultured bacterium type was examined as a function of extract type (termed 'Extract'), source of bacterial culture ('Source'), species of coral, alga, or algal extract the bacteria were cultured from ('Species'), and bacterial taxon ('Bacteria', Table 2.3) using a 4-way analyses of variance (ANOVA) in the statistical program 'R' (R Development Core Team 2009). Tukey pair-wise comparisons were performed to evaluate growth and extract effects.

Results

All extracts from six species of Caribbean macroalgae (6 hydrophilic extracts plus 6 lipophilic extracts) and two species of cyanobacteria (2 hydrophilic extracts plus 2 lipophilic extracts, n = 16 extracts total) exhibited inhibitory and/or stimulatory activity against one or more of the 54 assay bacteria (Table 2.1). Overall, three of the sixteen (19% overall) macroalgal and cyanobacterial extracts exhibited broad-spectrum antibacterial activity, and extracts from 10 macroalgae and two cyanobacteria (75% overall) exhibited broad-spectrum stimulatory activity, while the remaining one *H. tuna* extract exhibited little inhibitory or stimulatory activity. We arbitrarily defined broad-spectrum activity as causing 25% more or less growth in comparison to the control in > 50% of the bacteria assayed. Specifically, two hydrophilic extracts from brown macroalgae demonstrated broad-spectrum antibacterial activity (*D. menstrualis, L. variegata*), and one lipophilic extract from a macroalga inhibited > 50% of bacteria assayed (*Dictyota* sp. from Belize, Table 2.1). Conversely, of the 16 total extracts surveyed, six hydro- and six lipophilic extracts stimulated the growth of > 50% of bacteria assayed. Two macroalgae (*D. pulchella, A. spicifera*), and both cyanobacteria (*L. majuscula, L. polychroa*), had both hydro- and lipophilic extracts that stimulated microbial growth (e.g. >50% growth, Table 2.1). The lipophilic extract from the green alga *Halimeda tuna* had the most limited effect on assay bacteria, with 56% of bacteria neither inhibited nor stimulated.

The 54 bacterial isolates were categorized based on the environmental culture source (i.e., coral mucus, algal surface, interaction zone, etc.), and included members of the alphaProteobacteria, gammaProteobacteria, firmicutes, and actinobacteria phyla (Table 2.2a). Both of the isolates most similar to the putative pathogen *V. shiloi* were cultured from algal thalli (Table 2.2a). On average, 21% ± 2.4 (mean ± SE) of bacteria from all environmental sources were inhibited by algal extracts, 11% ± 1.8 (mean ± SE) were not affected, and 68% ± 3.2 (mean ± SE) were stimulated. Bacteria cultured from coral mucus exposed to *Halimeda tuna* and *Dictyota* sp. lipophilic extracts exhibited the most growth (82 %) and least inhibition (14 %) in comparison to other culture sources (Table 2.2b).

The 4-way ANOVA revealed significant variation in bacterial growth with all 4 main factors: Extract ($F_{15, 384} = 30.147$, $P < 0.001$), Source ($F_{6, 384} = 27.672$, $P < 0.001$), Species ($F_{7, 384} = 13.584$, $P < 0.001$), Bacteria ($F_{11,384} = 10.599$, $P < 0.001$), and also revealed several interaction effects (Table 2.3). Tukey pair-wise comparisons of effects of the 16 extracts on bacterial

growth indicated significant differences in the effects of *L. variegata* hydrophilic versus lipophilic extracts (lower CI = -1042.280985, upper CI = -285.474940, $P < 0.001$, Figure 2.2). All Tukey pair-wise comparisons among and between algal extracts are detailed in Table 2.4. The brown alga *L. variegata* (hydrophilic extract) most inhibited bacterial growth rates (mean -105% ± 8.8 SE), and the cyanobacteria *L. majuscula* (hydrophilic) stimulated the highest mean growth rates among all bacteria assayed (mean +759% ± 98.0 SE, Figure 2.2). On average, bacteria in the genus *Pseudoalteromona*s and other members of the order Alteromonadales demonstrated the most growth across all algal extracts surveyed (>700%). Bacterial growth also varied with culture origin: the most rapid growth occurred in bacteria isolated from corals interacting with crude extracts of *H. tuna* lipophilic compounds (~ +800% growth), and the slowest growth in bacteria cultured from the surfaces of living *H. opuntia* green algae and *D. menstrualis* brown algae.

Hydrophilic extracts from the brown alga *D. menstrualis* preferentially inhibited *Vibrio* spp. (Figure 2.3). This extract inhibited 30 bacteria (56%), of which 22 were *Vibrio* spp. The five *Vibrio* spp. isolates that demonstrated growth in response to *D. menstrualis* extracts had the following nearest neighbors based on 16S rRNA comparison: a) *V. shiloi/ V. mediterranei*, a putative coral pathogen (97.5% 16S rRNA gene identity), b) *V. harveyi/ V. rotiferianus* (99.4% 16S rRNA gene identity), c) *V. harveyi/ V. campbellii* (99.7% 16S rRNA gene identity), d) *V. harveyi/ V. communis* (99.4% 16S rRNA gene identity), and e) *V. harveyi/ V. rotiferianus* (99.7% 16S rRNA gene identity, Figure 3). Additional analysis of cultures with nearest neighbors most similar to *V. shiloi* (n = 2) and *V. harveyi* (n = 10), two putative coral pathogens, indicated that twelve extracts (75%) stimulated *V. shiloi* and only two inhibited the growth of this bacterium (lipophilic *H. tuna* and *Dictyota* sp.). Ten extracts (63%) stimulated *V. harveyi* growth, three (19%) inhibited *V. harveyi* growth, and three had no effect on the putative pathogen. Lipophilic extracts from the Belize brown alga, *Dictyota* sp., were the only ones that inhibited both putative *Vibrio* pathogens.

Discussion

Coral populations have declined precipitously on many tropical reefs over the past three decades while algal abundance has increased, exacerbating competitive interactions between corals and macroalgae at all life history stages. Phase-shifts to higher algal biomass on reefs

result not only in stronger negative interactions with adult corals, but also a decrease in coral recruitment by reducing larval settlement and post-settlement survival (reviewed in Birrell et al. 2008; Ritson-Williams et al. 2009), indicating that macroalgae alter the overall structure of reef communities (Hughes and Tanner 2000; Kuffner et al. 2006). The results of the present study reveal another potential mechanism by which macroalgae may impact coral reef community structure. We show here that algal and cyanobacterial crude extracts both positively and negatively impact coral reef-associated bacteria and likely affect natural assemblages of coral-associated bacteria. Several macroalgae exhibited broad-spectrum activity, while others had species-specific effects on particular taxa or groups of bacteria (e.g., *Vibrio* spp.).

Two of the macroalgae produced hydrophilic compounds that exhibited broad-spectrum antibacterial activity (*D. menstrualis, L. variegata*). However, previous studies have suggested that inhibitory compounds are primarily lipophilic (Ballantine et al. 1987; Steinberg et al. 2001), but we found that 25% of the examined hydrophilic extracts inhibited the majority of bacteria assayed, and all hydrophilic extracts inhibited one or more bacteria (Table 2.1). Hydrophilic (polar) compounds are more readily solubilized in the water column than are lipophilic compounds. Thus, these macroalgal-derived metabolites have a greater potential than lipophilic compounds to inhibit microbial symbionts on organisms in contact with or downstream from algal thalli. These inhibitory effects are determined in part by the location of compound production, in that surface-emitted compounds are further mediated by water flow. Low-flow and interstitial microenvironments thus could experience a build-up of compounds leading to rapid changes in microbial assemblages on interacting organisms.

Antibacterial activity is exhibited by metabolites produced by many marine plants from temperate and tropical locations around the world (reviewed in Goecke et al. 2010). An antibacterial disc diffusion assay of 102 Puerto Rican macroalgae demonstrated at least some antibacterial activity in 64% of lipophilic extracts examined. The distribution of activity was relatively even (63-71%) among the major algal divisions, but was consistently higher in several orders (83% in Dictyotales, 76% in Caulerpales; Ballantine et al. 1987). In another extensive study of 54 species of marine algae and 2 species of seagrasses collected from Indo-Pacific coral reefs, 95% of the extracts demonstrated antimicrobial activity; however, broad-spectrum activity was demonstrated in <50% of samples. In particular, extracts from the green alga *Bryopsis pennata* and the red alga *Portieria hornemannii* inhibited all assay microorganisms, which

included one pathogenic bacterium (*Pseudoaltermonas bacteriolytica*), 2 saprophytic stramenopiles (e.g., oomycete water molds) and 2 fungi (Engel et al. 2006; Puglisi et al. 2007). Thus, antimicrobial chemical defense is widespread among marine plants and algae, and may be species-specific to both algal and microbial taxa.

Most previous studies on the microbial impacts of algal compounds have focused on their antibacterial activity; however, the majority of algal extracts surveyed in this study stimulated bacterial growth, which also has the potential to cause significant and detrimental effects on reef corals and thus should not be overlooked. Cole (1982) hypothesized that cyanobacteria within the phytoplankton stimulate bacterial growth via three mechanisms for transfer of organic material from algae to bacteria: 1) microbes may parasitize algal cells, 2) microbes may obtain nutrition from the decomposition of dead cells, and 3) dissolved organic carbon (DOC) released from algae during cell growth (lysis, excretion, autolysis) may also be available to bacteria. That study also intuitively predicted that productive aquatic habitats likely support pathogenic and enteric microorganisms that can grow by using algal organic matter (Cole 1982). Recent research has confirmed these early ideas, and demonstrated that high levels of DOC can cause coral mortality and increase microbial growth rates by an order of magnitude within the coral mucus layer (Kline et al. 2006). Smith et al. (2006) showed that macroalgal-induced microbial growth on coral surfaces could lead to a zone of hypoxia that induces coral tissue death. Thus, many coral-associated microbes likely are carbon-limited, and if encroaching macroalgae increase the amount of labile DOC present, microbes may break down complex and previously unavailable carbon sources via co-metabolism, leading to uncontrolled microbial growth. Two macroalgae and two cyanobacteria in the present study produced both hydro- and lipophilic compounds that stimulated the growth of the majority of bacteria assayed. Microbial growth can cause coral mortality through oxygen depletion, accumulation of poisons, and/or microbial predation on weakened coral polyps (Cole 1982; Segel and Ducklow 1982; Smith et al. 2006). However, macroalgae may need to be in direct contact with coral tissues to result in high enough DOC and other metabolite levels to influence microbial proliferation and/or coral bleaching and subsequent tissue death (Vu et al. 2009; Rasher and Hay 2010). Antibacterial and/or stimulatory effects of coral-algal contact on associated microbes likely would be exacerbated in low flow and high temperature microclimates (Smith et al. 2006; Bruno et al. 2007). This study may have selected for bacteria that can take advantage of allelochemicals and primary metabolites, such as

DOC, when we applied crude extracts from *Halimeda tuna* and *Dictyota* sp. directly to coral surfaces in Belize. The bacteria cultured from under these extracts exhibited the highest growth and least inhibition in comparison to all other cultures sources, including surfaces of algal thalli (Morrow et al. *pers. obs.*).

This is the first study to determine the direct effects of compounds produced by macroalgae and cyanobacteria on a diversity of bacteria cultured directly from reef corals and macroalgae. Only about 0.1-1.0% of marine microbial taxa are estimated to be cultured on a laboratory medium such as seawater agar, but we hypothesize that this percentage is much higher for coral-associated bacteria due to the relatively nutrient-rich environment they inhabit (Amman et al. 1995). Future studies are needed that also incorporate culture-independent analyses to examine the effects of macroalgal compounds on a wider diversity of coral-associated microbes. Here we documented exponential growth in bacterial taxa cultured from coral mucus that was exposed to *H. tuna* extracts. In contrast, bacteria cultured from the surfaces of *Halimeda* and *Dictyota* macroalgae exhibited the smallest amount of growth in comparison to bacteria cultured from un-manipulated coral mucus, indicating that algal-associated bacteria may be less stimulated by these extracts than are coral-associated bacteria. The highest overall growth rates were among bacterial taxa within the order Alteromondales (Phylum gamma-Proteobacteria), some of which are thought to be resident bacteria of corals (Ritchie 2006). Within the Alteromondales, members of the genus *Pseudoalteromona* had the highest growth in comparison to controls, and are potential coral visitors and disease-causing pathogens (Ritchie 2006). Some members of the Pseudoalteromonadaceae, a small family within the gamma-Proteobacteria, produce algicidal compounds (Egan et al. 2001; Lovejoy et al.1998; Ivanova and Mikhailov 2001; Mayali and Azam 2004), and may be associated with Yellow Blotch Disease (Cervino et al. 2004) and white plague-like diseases in corals (Sunagawa et al. 2009). Finally, some of the slowest growers are potential coral residents, including *Exiguobacteria* and *Roseobacter* spp. (Ritchie 2006). Coral reef-associated bacteria were strongly affected by macroalgal and cyanobacterial extracts during the short time-course of this experiment (48 hrs). Thus, reef seaweeds, although sometimes ephemeral, have the potential to rapidly alter coral-associated microbial assemblages and to potentially induce or exacerbate coral disease.

Approximately half the bacterial types that were cultured from corals and macroalgae were members of the genus *Vibrio* (Phylum gamma-Proteobacteria). Members of this genus

often cause human diseases (e.g., *Vibrio cholerae*, *Vibrio parahaemlyticus* and *Vibrio vulnificus*), and can be virulent marine pathogens. In stony corals, *Vibrio* spp. are associated with yellow blotch/band disease (*V. alginolyticus, V. rotiferianus, V. harveyi, and V. proteolyticus*; Cervino et al. 2004, 2008), white band Type II (*V. harveyi, V. charchariae*; Ritchie and Smith 1998), tissue necrosis, rapid tissue loss, and shut down reaction (*V. coralliilyticus, V. harveyi*; Antonius 1977; Jones et al. 2004; Luna et al. 2007; Anthony et al. 2008). *Vibrio* species also are affiliated with diseased *Porphyra* and *Laminaria* macroalgae (Wang et al. 2008). *Vibrio coralyticus* causes tissue lysis of the reef-building coral *Pocillopora damicornis*, spreading so rapidly in some cases that all coral tissue is destroyed in < 2 weeks (Ben Haim and Rosenberg 2002). One of the bacteria isolated in the current study was most closely related to *V. shiloi/ V. mediterranei,* a bacterium associated with *O. patagonica* bleaching in the Mediterranean Sea. A recent study identified several genes related to virulent functions in *V. shiloi* that were strongly induced by exposure to crushed coral tissue (e.g., Zot toxin, superoxide dismutase; Banin et al. 2003; Reshef et al. 2008). Ten other bacterial isolates examined in this study were strains of *V. harveyi,* which is implicated in yellow blotch/band disease, white band Type II, and rapid tissue disease. The majority of the macroalgal and cyanobacterial extracts stimulated both of these potential pathogens. Notably, hydrophilic compounds produced by *D. menstrualis* preferentially inhibited *Vibrio* spp. relative to other bacteria assayed. *D. menstrualis* extracts inhibited 83% of the 28 *Vibrio* spp. tested, but not the putative coral pathogen, *V. shiloi*. Individuals of *D. menstrualis* exhibit relatively less fouling than do other macroalgae, and their surface extracts include the terpenoid compounds pachydictyol A and dictyol E that deter settlement by the epiphytic bryozoan *Bugula neritina* (Schmitt et al. 1995). However, although *Dictyota* sp. and *D. menstrualis* exhibited natural antibacterial activity in this study, *D. pulchella* produced both hydro- and lipophilic compounds that stimulated bacterial growth, suggesting metabolite differences among species even within the same genus. Furthermore, *V. shiloi* was one of the only bacterial isolates that experienced exponential growth in the presence of *D. menstrualis* (hydrophilic) extracts which otherwise inhibited almost all other *Vibrio* spp. assayed. These results indicate specific effects of macroalgal compounds on particular taxonomic groups of bacteria. Coral microorganisms may be stimulated or inhibited depending on the type of macroalgae and associated metabolites they contact (reviewed in Goecke et al. 2010), but further

study is needed to understand the dynamics of these relationships, using a combination of laboratory and *in situ* studies involving both culture-dependent and -independent methods.

Future research is needed to elucidate the mechanisms by which allelochemicals influence coral-microbial associations, aside from microbial growth inhibition and stimulation. Allelochemicals may regulate microbial composition and abundance by suppressing surface colonization (Chet and Mitchell 1976; Amsler et al. 2001) or attachment and colonization of microbial cells (Steinberg et al. 2001). Future studies also are needed to determine whether these compounds are released into the water column, and at what concentrations, as well as whether the compounds are sequestered near the surface and/or deep within the tissues of algal thalli where they would have more limited effects on interacting organisms. The present results provide evidence that common Caribbean macroalgae produce broad-spectrum as well as species-specific compounds with diverse impacts on a large variety of coral reef bacteria, including three putative coral pathogens. Because the diverse microbial assemblages associated with corals are thought to play an integral part in their innate immune responses (Rosenberg et al. 2007), any shifts in the natural microbial assemblages on corals will contribute to the dynamics of coral health and disease. We have shown here that blanket statements cannot be applied to describe the outcomes or mechanisms of competition among corals, macroalgae, and cyanobacteria on reefs, particularly in terms of the dynamic interactions among their associated microorganisms and allelochemicals.

Table 2.1: The percent of bacterial isolates (n = 54) that were either inhibited, stimulated, or did not change ('0') their growth rates compared to those of control bacteria, in response to hydro- and lipophilic extracts from six species of Caribbean macroalgae and two species of cyanobacteria. Growth was classified as inhibited/stimulated if >25% different from control growth. Bolded percents indicate the main effects of each type of extract. The natural concentration of algal extract (mg) per algal wet weight (g) is listed below each species name.

Hydrophilic (Polar)	L. *variegata*	*H. tuna*	*Dictyota* sp.	*L. polychroa*	*D. pulchella*	*D. menstrualis*	*A. spicifera*	L. *majuscula*
Concentration (mg/g)	29.9	17.8	16.5	27.7	34.5	27.5	31.2	12.9
% Inhibited	**93**	14	6	29	13	**52**	4	4
0	6	4	6	10	15	7	0	4
% Stimulated	2	**82**	**88**	**61**	**72**	41	**96**	**93**
Lipophilic (Non-polar)								
Concentration (mg/g)	16.5	7.5	78.7	28.7	27.5	55.9	14.9	13.8
% Inhibited	2	35	**73**	24	19	7	6	17
0	4	**55**	6	24	19	33	7	4
% Stimulated	**94**	10	20	**51**	**63**	**59**	**87**	**80**

Table 2.2: a) Types of examined bacteria and their environmental sources: 1) Coral Controls, coral mucus not in contact with macroalgae or other sessile organisms (corresponding to X in Fig. 2.1), 2) Near Algae, coral mucus collected 5cm from interacting macroalgae (corresponding to C in Fig. 2.1), 3) Touching Algae, coral mucus collected from under macroalgae (see B in Fig. 2.1), 4) Algal Surfaces, bacteria cultured from the surface of algal thalli (see Y in Fig. 2.1), 5) Near Extract, coral mucus collected 5cm from experimentally applied crude extracts (coral mucus 5 cm away from D in Fig. 2.1), and 5) Under Extract, coral mucus collected from under experimentally applied crude algal extracts (See D in Fig. 2.1). The shaded bacterial species are putative marine pathogens. The replicate number of coral colonies sampled and the corresponding number of bacterial cultures are listed in the first two rows. b) Mean percentage of bacteria from each environmental source that was inhibited, stimulated or not affected by the 16 algal and cyanobacterial extracts.

Culture source	Coral control	Near algae	Touching algae	Algal surface	Near extract	Under extract
n = # of coral colonies sampled	**8**	**5**	**6**	**8**	**4**	**4**
Total # of cultured isolates	**17**	**5**	**9**	**11**	**5**	**6**
Gamma Proteobacteria	1					
Order Alteromondales						
Alteromonas sp., A. macleodii	4				1	
Pseudoalteromonas sp., *P. prydzensis*	1					5
Shewanella sp.	1					
Order Vibrionales						
Photobacterium eurosenbergii				1		
Listonella pelagia			1			
V. sinaloensis	1					
V. harveyi	**7**		**1**	**3**	**1**	
V. brasiliensis, V. campbellii, V. charchariae, V. communis, V. fischeri, V. harveyi or *V. rotiferianus,*	1	2	5	5		
V. shiloi or ***V. mediterranei***			**1**	**1**		
V. parahaemolyticus or ***V. campbellii***		**1**				
Total *Vibrio* spp.	**9**	**3**	**7**	**9**	**1**	**0**
Alpha Proteobacteria				1		
Order Sphingomonadales; *Erythrobacter*		1			1	
Order Rhodobacterales; *Roseobacter*			**1**			
Firmicutes						
Exiguobacterium sp. or *E. profundum*					2	1
Salinicoccus roseus		1				
Actinobacter: *Micrococcus flavus*	1					
% Inhibited	26	22	19	27	21	14
% Not affected	13	10	13	8	16	4
% Stimulated	60	69	68	65	63	82

The bolded bacterial species are putative marine pathogens

Table 2.3: 4-way ANOVA of % change in bacterial growth with four factors: 1) Extract, the type of macroalgal or cyanobacterial extract, 2) Source, the location on the reef from where bacteria were originally cultured, 3) Species, the species of coral or macroalga from where bacteria were originally cultured, 4) Bacteria, the bacterial taxonomic grouping.

4-way ANOVA	*df*	SS	MS	*F* value	Pr(>*F*)	Significance
Extract	15	63,252,552	4,216,837	30.1465	<0.001	***
Source	6	23,223,888	3,870,648	27.6716	<0.001	***
Species	7	13,300,811	1,900,116	13.5841	<0.001	***
Bacteria	11	16,309,686	1,482,699	10.5999	<0.001	***
Extract*Source	90	26,728,568	296,984	2.1232	0.001	***
Extract*Species	99	20,587,917	207,959	1.4867	0.004	**
Source*Species	5	4,351,113	870,222	62.212	<0.001	***
Extract*Bacteria	159	48,916,193	307,649	2.1994	<0.001	
Source*Bacteria	3	5,634,701	1,878,234	13.4276	<0.001	***
Species*Bacteria	3	490,769	163,590	1.1695	0.321	
Extract*Source*Species	69	75,152,538	1,089,167	7.7865	<0.001	***
Extract*Source*Bacteria	45	7,182,557	159,612	1.1411	0.255	
Extract*Species*Bacteria	45	2,255,647	50,125	0.3584	0.999	
Residuals	384	53,713,181	139,878			

Table 2.4: Tukey post-hoc pair-wise comparisons between the growth responses of 60 bacterial isolates to polar and non-polar macroalgal and cyanobacterial extracts (* = P < 0.05, ** = P < 0.001 and *** = P < 0.0001).

Table 4 Tukey post hoc pair-wise comparisons between the growth responses of 60 bacterial isolates to polar and non-polar macroalgal and cyanobacterial extracts

Extract name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. L. avariegata P																
2. L. variezata NP	***															
3. H. tuna P																
4. H. tuna NP		***														
5. Dictyota sp. *P*																
6. Dictyota sp. *NP*		***														
7. L. polychroa P		*														
8. L. polychroa NP																
9. D. pulchella P	**															
10. D. pulchella NP	*															
11. D. menstrualis P	***		*	***	*	***		***								
12. D. menstrualis NP	*															
13. A. spicifera P	***			***		***										
14. A. spicifera NP	***		***	***	*	***										
15. L. majuscula P	***		***	***	***	***	***	***	***	***		***				
16. L. majuscula NP	***			***		***		**								

* = $P < 0.05$, ** = $P < 0.001$, and *** = $P < 0.0001$

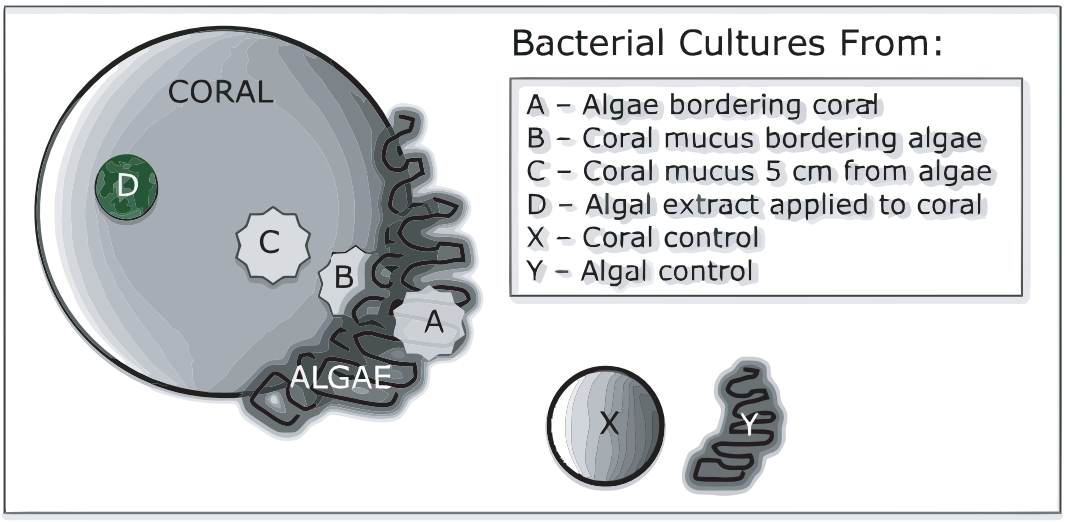


Figure 2.1: Microbial samples were collected from 6 areas on stony corals and macroalgae in Belize, Florida, and the USVI during May-Aug 2008. Samples were collected from A) macroalgal thalli (*Dictyota menstrualis* or *Halimeda opuntia*) in contact with corals (*Montastrea faveolata* or *Porites astreoides*), B) coral mucus in contact with macroalgae, C) coral mucus 5 cm away from coral-macroalgal interaction zones, D) coral mucus interacting with crude algal extracts, X) coral controls isolated from contact with macroalgae and other sessile organisms, and Y) macroalgal controls isolated from contact with corals and other sessile organisms. Each sampled coral or alga was at least 3 m from other sampled individuals on the reefs. See text for collecting details.

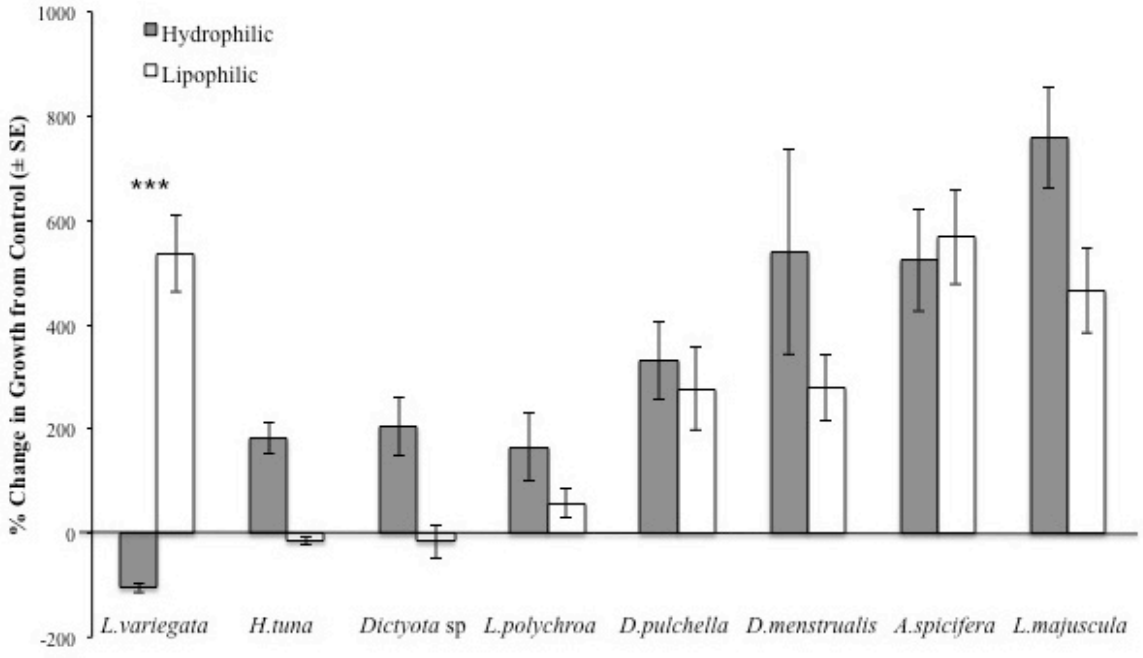


Figure 2.2: Mean ± SE of the percent change in growth of 54 bacterial isolates from Caribbean stony corals and macroalgae, in response to crude hydro- and lipophilic extracts from macroalgae and cyanobacteria. Tukey pair-wise comparisons between hydro- and lipophilic extracts only revealed a significant difference for extracts from *L. variegata* (*** = $P < 0.001$). Cross comparisons for Tukey post-hoc tests between and among algae and cyanobacteria are listed in Table 4.

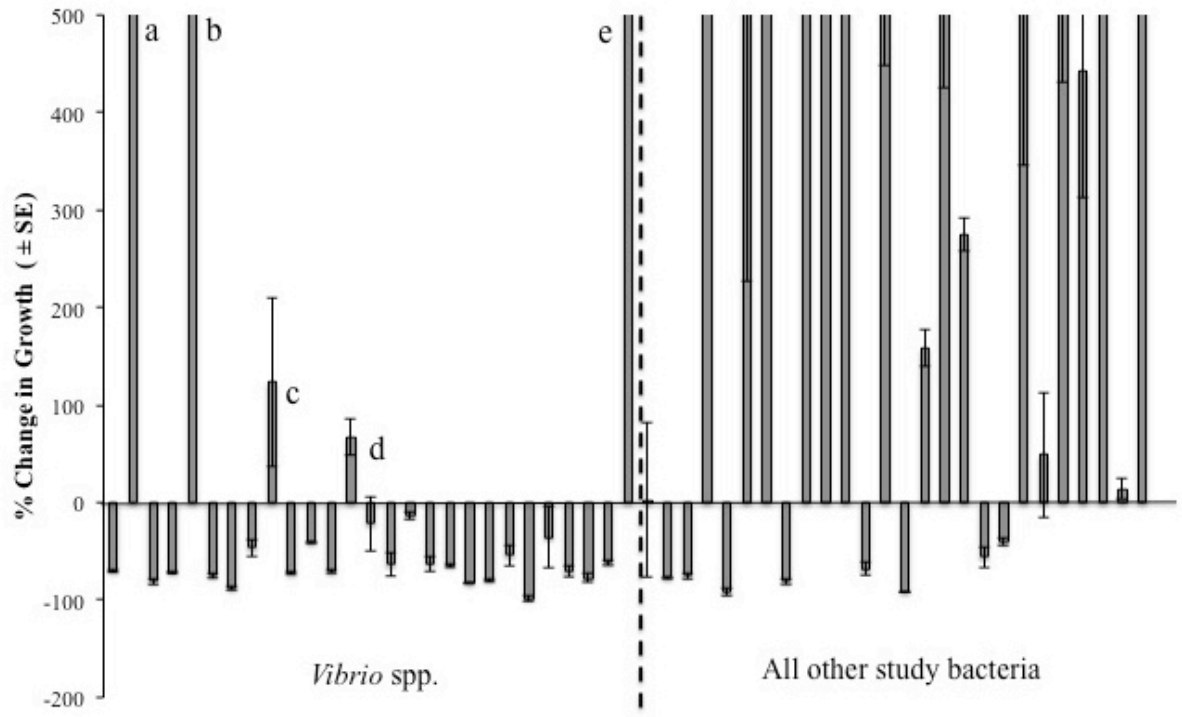


Figure 2.3: Effects of *Dictyota menstrualis* (hydrophilic) extract on the growth of bacterial cultures in comparison to controls (no extract). Growth is illustrated only up to 500%, to reveal the details of inhibitory effects. This extract inhibited 83% of 28 *Vibrio* spp. bacteria tested. The five *Vibrio* strains stimulated by *D. menstrualis* extracts were most closely related to: a) *V. shiloi/mediterranei*, b) *V. harveyi/rotiferianus*, c) *V. harveyi/ campbellii,* d) *V. harveyi/ communis,* and e) *V.harveyi/rotiferianus.*

Chapter 3

Bacterial associates of two Caribbean coral species reveal species-specific distribution and geographic variability

Introduction

Most eukaryotes are now believed to associate with a diverse assemblage of microbial symbionts that aid in their development and health (Xu 2003). These microbes may be ecologically important, in that they appear to contribute to the ability of reef-building corals to adapt and evolve to changing environmental conditions (Reshef et al. 2006; Rosenberg et al. 2007b). Advances in molecular microbiological approaches over the past decade have revealed the ubiquity and diversity of host-microbial associations (Ruby et al. 2004). The bacterial assemblages found within the surface mucus layer (SML) of healthy corals are an important part of a larger holobiont that is composed of the coral (skeleton, tissues, and SML), along with a wide variety of other associated microbial taxa, including eukaryotes, Archaea, viruses, and fungi (Rohwer et al. 2002). Coral microorganisms exhibit substantial genetic and ecological diversity, and are believed to contribute to the overall health of the coral host (Ducklow & Mitchell 1979; Rosenberg et al. 2007a; Medina 2011).

Over the past 30 years, there has been a ~30% loss of coral cover worldwide, as corals become increasingly affected by catastrophic epizootics and bleaching events, often exacerbated by stress associated with global climate change (Harvell 2002; Harvell et al. 2007), water pollution (Szmant 2002), and overfishing (Jackson 2001). There are approximately 30 documented coral diseases and syndromes, with only 5 etiologic agents identified by Koch's postulates (Sutherland et al. 2004, see Chapter 1 Review). The dearth of information on coral-specific pathogens is confounded by the complexity of prokaryotic/eukaryotic associations, which typically involve complex consortia of microorganisms (e.g. Black Band Disease, Yellow Band Disease), making it impossible to identify a single pathogen. In addition, the dynamics and diversity of coral-associated microbiota are influenced by the constantly-changing coral surface microenvironment. Shifts in coral microbial assemblages have been linked to bleaching (Pantos et al. 2003; Bourne et al. 2007), thermal stress (Littman et al. 2010), irradiance (Muller & van

Woesik 2009), disease (reviewed in Bourne et al. 2009), and changes in dissolved organic nutrients (Kline et al. 2006; Smith et al. 2006). Shifts in both microbial diversity and metabolism also have been related to their proximity to human populations (Dinsdale & Rohwer 2008), demonstrating that geographic location may indirectly influence coral reef health through microbial mediation.

Microorganisms are found throughout the coral holobiont (e.g. skeleton, tissues, and SML) and appear to be regulated in part by the coral host (Breitbart et al. 2005; Kline et al. 2006). Recent evidence suggests that the diversity of coral bacterial communities may also be tightly coupled with the taxa of *Symbiodinium* sp. that also inhabit the coral host (Littman et al. 2010; Medina 2011). Coral microbes are thought to benefit the host by providing nutritional by-products, protein and nitrogenous compounds (Lesser 2004; Wegley et al. 2007), and also likely by synthesizing essential vitamins (Croft et al. 2005). Microbial symbionts may also protect corals from disease by preventing opportunistic infections through the occupation of otherwise available niches, and by producing antibacterial agents (Koh 1997; Ritchie 2006; Nithyanand & Pandian 2009; Shnit-Orland & Kushmaro 2009). The dynamic relationship that exists among members of the coral holobiont may allow for greater adaptation to changing environmental conditions than possible via mutation and selection of the coral host alone (Reshef et al. 2006; Zilber-Rosenberg & Rosenberg 2008). Just as humans are thought to have co-evolved with their gut microorganisms (Xu 2003), corals probably coevolved with their microbial symbionts, which likely fill a critical and beneficial role in coral colony immune function.

Although evidence suggests that congeneric coral species associate with similar microorganisms (Rohwer et al. 2002; Medina 2011), the metabolic functions and specificity of coral-microbial associations have turned out to be less predictable than initially hypothesized (Hansson et al. 2009; Shinichi Sunagawa et al. 2009; Daniels et al. 2011). Coral microbiota may vary among reef locations that differ in water quality (Klaus et al. 2005) and water depth (Klaus et al. 2007). The operational taxonomic units (OTUs) of bacteria in the surface mucus layer of *M. faveolata* assemblages vary on both spatial (reef to reef) and temporal (month to month) scales, further suggesting that microbial assemblages are sensitive to surrounding environmental conditions (Guppy & Bythell 2007). Coral microbiota also are believed to be thermally sensitive, losing their protective antibacterial properties at sustained temperatures above 28-30°C (Ritchie 2006). Bordering macroalgae might serve as a bacterial vector, as *Halimeda*

macroalgae were observed to transfer white plague type II disease to exposed *M. faveolata* colonies (Nugues et al. 2004). Thus, in addition to the host coral species, environmental factors such as water quality, location, depth, temperature, and other sessile organisms in close proximity may have significant effects on microbial diversity. Therefore, shifts in coral microbiota could serve as bio-indicators of environmental change and disease. Although we have made significant progress over the past decade, we still lack information about the host-specificity and stability of healthy coral-microbial assemblages, including how and why they change across large spatial scales and gradients of anthropogenic impact. Investigating the microbial assemblages associated with coral species over large geographic scales provides insight into which species maintain the most robust microbial associations, leading to hypotheses about overall reef resilience and contributions of microorganisms to coral health.

The present study investigated the bacterial ribotype (16S rRNA gene) diversity associated with the SML of healthy *Montastraea faveolata* and *Porites astreoides* corals at three sites across the Caribbean in: the Florida Keys, St. Thomas, and Belize. These three sites span the Caribbean Sea and are exposed to decreasing anthropogenic impacts and increasing distance from the mainland, respectively. Only coral colonies that appeared entirely healthy, uniform in color, and free from interaction with adjacent corals, invertebrates, and macroalgae were selected for the sampling reported here. This is the first study to control for biological interactions in the sampling regime, and to examine healthy coral microbiota over a large spatial scale. Structural bacterial ribotype diversity was visualized by denaturing gradient gel electrophoresis (DGGE), in which band presence/absence provided a molecular fingerprint of approximately 25 coral colonies of each species. The bacterial assemblages associated with the SML of a representative subset of these colonies (n = 12) were analyzed using 454 barcoded-pyrosequencing of 16S rRNA gene amplicons.

Materials and Methods

Sample Collection

Microbial samples were collected from the surface mucus layer (SML) of apparently healthy *Montastraea faveolata* (MF) and *Porites astreoides* (PA) coral colonies on reefs adjacent to 4 sites: (1) Mote Marine Laboratory, Summerland Key, Florida (In-shore, Wonderland Reef in May 2009; MF n = 10; PA n = 12), (2) Summerland Key, Florida, (Offshore, Looe Key Reef in

August 2009; MF n = 3; PA n = 3), (3) MacLean Marine Science Center of the University of the Virgin Islands, St. Thomas, U.S. Virgin Islands (Flat Cay Reef in July 2009; MF n = 4; PA n = 4), and (4) Carrie Bow Cay Field Station of the Smithsonian Institution, Belize (Southwater Cay Reef in August 2009; MF n = 7; PA n = 7). The four sampling sites vary in distance from the mainland: Florida in-shore (5 km), Florida offshore (12 km), St. Thomas (2.5 km), and Belize (20 km). Collections were made on SCUBA at 5-15 m depth using sterile 5 ml syringes. A 5 x 5 cm area of mucus on the coral surface was gently agitated using the plastic-tip of the syringe, which encourages sloughing of the viscous mucus and reduces aspiration of seawater (Ritchie 2006). Sterile nitrile gloves were worn during collection to reduce human bacterial contamination, and syringes were capped after collection to prevent further seawater contamination. Mucus was collected from the sun-facing surface of *M. faveolata* and *P. asteroides* colonies that were > 1 m apart and not obviously interacting with any other coral, invertebrates, macroalgae or benthic cyanobacteria. Syringes were placed in seawater-filled coolers and transported back to the laboratory (< 3 hrs) where they were immediately processed for transport and subsequent culture-independent analyses. Syringes were placed tip down in test-tube racks for ~15 minutes to allow the mucus to settle to the bottom, then 2 ml of concentrated mucus was transferred to cryovials and centrifuged at 10,000 x *g* for 10 min. The seawater supernatant was poured off and the remaining mucus pellet frozen at -20°C. Mucus pellets from *M. faveolata* and *P. astreoides* were transported to Auburn University and thawed at 4°C prior to DNA extraction using the MOBIO Ultraclean® Microbial DNA Isolation kit (Carlsbad, CA), according to the manufacturer's instructions, with an additional (10 min) heating step at 64°C to increase DNA yield. Extracted DNA was stored at -80°C until PCR amplification.

PCR amplification and DGGE protocol

Universal bacterial primers 27F-GC (5' -CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG CAG AGT TTG ATC MTG GCT CAG-3') and 518R (5' -ATT ACC GCG GCT GCT GG-3'), were used to amplify the 16S rRNA gene using as template the genomic DNA extracted from coral mucus. The forward primer was modified to incorporate a 40-bp GC clamp for resolution on a denaturing gradient gel electrophoresis (DGGE) system (Muyzer et al. 1993; Ferris et al. 1996). These primers amplify a 491-bp section of the 16S

rRNA gene of members of the domain *Bacteria*, including the highly variable V1-V3 regions (Ashelford et al. 2005; Huse et al. 2008). All PCR was performed on a thermal cycler (model: Mastercycler® ep gradient, Eppendorf, Hauppauge, NY) as follows: 12.5 µl EconoTaq® PLUS GREEN 2X Master Mix (Lucigen, Middleton, WI), 0.5 µl of each 20 µM primer, and adjusted to a final volume of 25 µl with nuclease-free water. Strip tubes, master mix and nuclease free water were UV-irradiated for 20 minutes prior to the addition of primers under sterile conditions in a laminar flow hood to reduce contamination (Miller et al. 2002). DNA template was added during an initial 'hotstart' of 3 min at 94°C, followed by a 'touchdown' PCR protocol, in which the annealing temperature was decreased from 65°C by 1°C every cycle until reaching a final temperature of 54°C, at which temperature 35 additional cycles were performed as follows; 94°C for 45 sec, 54°C for 45 sec, and 72°C for 1.5 min; and 1 final cycle at 94°C for 45 sec, 54°C for 45 sec, and 72°C for 7 min followed by cooling to 4°C. PCR products were analyzed by agarose gel electrophoresis (1% w/v agarose) stained with ethidium bromide and visualized using a UV transilluminator.

Samples were separated using a conventional vertical gel electrophoresis apparatus (Hoefer model SE600, Hoefer Inc.,San Francisco, CA) warmed with a tank heater (Lauda model M6a; Brinkmann Instruments, NY) modified for use as a DGGE system. PCR products were loaded onto an 8% acrylamide gel and run with 0.5 x TAE buffer (Tris base, acetic acid, EDTA) and a 35-60% linear denaturing gradient of formamide and urea. Gels were electrophoresed at 60°C, first for 15 min at 50 V, and subsequently for 10 hrs at 100 V (or 1000 V·hrs) in the DGGE system (Sigler et al. 2004). After electrophoresis, the gels were stained for 30 min with SYBR-Gold nucleic acid stain at a 1:10,000 dilution ratio (Invitrogen, Carlsbad, CA) in TAE buffer, rinsed, and imaged using an AlphaImager HP gel documentation system (ProteinSimple, Santa Clara, CA). Images were saved as 8-bit TIFF files, alignment, normalization, band class identification, and statistical analysis using Bionumerics V. 5.0 (Applied Maths, Austin, TX).

Uniquely dominant and distinct bands were dabbed with a sterile pipette tip and placed directly into PCR strip tubes containing UV-sterilized nuclease free water. Bands were re-amplified with the previously described touchdown protocol using the 27F/518R primer set without the GC clamp. PCR products were analyzed with agarose gel electrophoresis (1% w/v agarose) stained with ethidium bromide and visualized using a UV transilluminator. An ammonium acetate-ethanol precipitation was performed and the resulting product amplified

using the BigDye® sequencing reaction: 1.0 µl of BigDye®, 1.5 µl of 5x Buffer, 0.5 µl of 10 µM 27F, 4 µl of nuclease free water, and 3 µl of template DNA. The following thermalcycler conditions were used: 95°C for 30 s, 50°C for 30 s, and 60°C for 4 min, at which temperature 30 additional cycles were performed. PCR products were purified using the BigDye® XTerminator Purification Kit (Applied Biosciences) and shipped to the Smithsonian Institution Laboratories of Analytical Biology (Suitland, MD) for sequencing. The Smithsonian Institution performed high-throughput (96-well) Sanger sequencing on an ABI sequencer. Sequences were trimmed using CLC Genomics Workbench (CLC Bio, Cambridge, MA), compared to the GenBank nr/nt database by BLASTn and those sequences with > 96% identity and E-values < 1 x 10^{-20} were accepted for downstream analysis.

DGGE analysis

DGGE images were imported into Bionumerics V 5.0 (Applied Maths) and subjected to a series of steps to allow multiple gel images to be reliably compared at one time: 1) each sample lane was identified, 2) a background subtraction was applied, 3) each lane was normalized to the reference standards run on each gel, and 4) each band was identified and quantified. Sample comparison and band matching was initially conducted in Bionumerics, in which band classes were constructed based on optimal position tolerance and optimization settings. A bifurcating hierarchical dendrogram and similarity matrix representing sample clusters was constructed for each coral species using the WARD algorithm and DICE coefficients derived from the band alignment. A binary matrix based on band presence/absence was exported from Bionumerics, converted to a distance matrix and analyzed using the R statistical package (Ihaka and Gentleman 1996). Kruskal's nonmetric multidimensional scaling (nMDS) analysis and permutational multivariate analysis of variance (PERMANOVA) were used to assess the multivariate relationships among and between binary DGGE profiles. The nMDS analysis was used to arrange multivariate data in a two-dimensional plane based on similarity coefficients between different samples. The Euclidean distances (we used Jaccard) between points in an nMDS plot are inversely proportional to the similarity of the samples. The number of dimensions (*k*) were determined by first running a Scree plot, which shows stress (i.e. an inverse measure of fit to the data) as a function of dimensionality (McCune and Grace 2002). Kruskal's stress formula was used as an informal method of determining the appropriate number of dimensions

(McCune and Grace 2002). These data were analyzed using the metaMDS and Adonis utilities within the Vegan package R. MetaMDS is unique in that it calls on isoMDS to perform nMDS, but also searches for the most stable solution by performing several random starts (we used 20, Ihaka and Gentleman 1996). The relationship among samples is represented in a plot of the first two dimensions of the nMDS results.

PCR and Pyrosequencing preparation

A 456 bp region of the 16S rRNA gene that includes the highly variable regions V3 and V4 was selected for barcoded-pyrosequencing using phosphorothioate primers (Ashelford et al. 2005). Phosphorothioate primers have a single phosphorothioate bond at the 3' termini that improves amplification of DNA sequences by DNA polymerases with proofreading activity (Skerra 1992). Samples were amplified using the bacterial forward primer 347F, which included the primer B adaptor for pyrosequencing and the unique 10 bp MID-barcode on the 5' end (5'-CGTATCGCCTCCCTCGCGCCATCAG-MID-GGAGGCAGCAGTRRGGAAT-3'), in addition to the bacterial reverse primer 803R (5' –CTACCRGGGTATCTAATCC-3'). Barcode sequences can be found in supplemental material (Table S1). All PCR reactions were performed using an Eppendorf Master cycler ep gradient thermal cycler as follows: 25 µl *Pfu* (proofreading) DNA polymerase Master Mix, 2 µl of each 12.5 µM primer, 3-4 µl template depending on DNA concentration, and adjusted to a final volume of 50 µl with nuclease-free water. Amplifications were conducted with the following conditions: initial 'hotstart' of 2 min at 95°C, followed by a 'touchdown' PCR protocol in which the annealing temperature was decreased from 62°C by 1°C every cycle until reaching a touchdown temperature of 51°C, at which temperature 30 additional cycles were performed as follows; 95°C for 40 sec, 51°C for 40 sec, and 72°C for 1 min and 15 s; and 1 final cycle at 95°C for 40 s, 51°C for 40 s, and 72°C for 10 min followed by cooling to 4°C. PCR products were purified using an ammonium acetate/ethanol precipitation after which, the concentration of each sample was quantified using a Nanodrop 1000 (ThermoScientific, Wilmington, DE). The 12 samples with unique barcodes were diluted to equimolar concentrations (16 ng/µl), pooled, and sequenced using the Roche 454 FLX sequencer with Titanium chemistry at Engencore (Columbia, SC).

Pyrosequencing Analysis

Sequences were first trimmed using the CLC Genomics Workbench (CLC Bio) with the following parameters, and any sequences not matching these criteria were excluded from downstream analysis: minimum quality score of 0.01 (99.9% quality), minimum sequence length of 200 bp, no ambiguous bases in the sequence or mismatches in the primer sequence. Samples were imported and analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al. 2010). Sequences were then grouped into operational taxonomic units (OTUs) with > 97% identity threshold. Using the Ribosomal Database Project (RDP) pipeline within QIIME, sequences were aligned with 'PyNast' and grouped using a complete linkage clustering method. The sequences were clustered by 'uclust,' which creates "seeds" of sequences that generate clusters based on percent identity. Finally, representative sequences from each OTU were selected and taxonomic identity was assigned to each sequence using the RDP taxonomic classifier at 90% confidence (Wang et al. 2007).

Sequences were analyzed using a number of descriptive and statistical methods within the QIIME pipeline. After bacterial libraries were rarefied so that sequencing effort did not affect diversity comparisons, the following alpha diversity metrics were determined: total observed species (OTUs), predicted species (Chao1), and Shannon-Wiener Diversity (H'). A beta diversity distance matrix was computed from the previously constructed OTU table based on the RDP taxonomic classifier. Weighted UniFrac distances were used to construct 3D principal coordinate analysis (PCoA) plots and UPGMA clusters (Unweighted Pair Group Method with Arithmetic mean, also known as average linkage). Jackknifed beta diversity metrics using 110 sequences per sample were used to directly measure the robustness of individual UPGMA clusters and clusters in PCoA plots.

For comparison, QIIME sequences were analyzed for diversity by two additional methods: the Blast2Go database (http://www.blast2go.org/) and the SILVA comprehensive ribosomal RNA database (http://www.arb-silva.de/). These databases were searched using the Cornell Computation Biology Services Unit BioHPC Web Computing Resource with P-BLAST. Sequences with a >100 bp alignment, >96% identity, and an E-value < 1×10^{-20} were accepted for downstream analysis. QIIME bacterial sequence libraries were further analyzed using BLASTn against two different databases of 16S rDNA sequences composed of: 1) diseased coral-associated bacteria, and 2) healthy coral-associated bacteria (Mouchka et al. 2010). The number of significant hits that met the previously defined parameters were tallied.

Results

DGGE Analysis

DGGE analysis of bacterial assemblages associated with the SML of *M. faveolata* (n = 26) and *P. astreoides* (n = 26) coral colonies from all 4 coral reef sites (Belize, St. Thomas, and the Florida Keys in-shore and offshore) revealed that those associated with *M. faveolata* clustered tightly and separately from *P. astreoides,* based on non-metric Multi-Dimensional Scaling (nMDS; Figure 3.1, Cluster A). In contrast, when all samples were analyzed together, SML samples from *P. astreoides* clustered more consistently by site than as a whole. Samples from the Florida Keys in-shore site at Wonderland Reef were distinct from all other coral samples (Figure 3.1, Cluster B). The remaining *P. astreoides* samples from Belize and St. Thomas clustered together and independently from Florida samples (Figure 3.1, Cluster C). The three *P. astreoides* samples from the Florida Keys off-shore site at Looe Reef clustered more closely to the *M. faveolata* samples than did other *P. astreoides* samples. Permutational MANOVA confirmed the results of the nMDS analysis, in that bacterial assemblages varied significantly both among coral species and sampling sites ($P < 0.01$, $F = 4.45$ and $F = 2.35$, respectively; Table 3.1). The significant variation among sites likely was driven by the patterns in the microbial assemblages on *P. astreoides*, rather than those on *M. faveolata*.

The majority of samples from *M. faveolata* in Belize and St. Thomas clustered together based on hierarchical cluster analyses in Bionumerics 5.0 (Figure 3.2, Clusters 2 & 3), similar to the *P. astreoides* groupings (Figure 3.1 & 3.2). However, the microbial assemblages associated with *M. faveolata* exhibited considerable inter- and intra-site variability, ranging from ~10% average similarity among samples from Belize and Florida (Figure 3.2, Cluster 1) to ~66% average similarity among samples from St. Thomas (Figure 3.2, Cluster 3), suggesting greater between-coral variability of the microbial assemblages in Belize and Florida than in St. Thomas. Samples from *P. astreoides* had more inter-site variability and less intra-site variability than did the *M. faveolata* samples, ranging from an average of ~49% similarity among Florida in-shore samples (Cluster 1) to an average of ~87% similarity among Florida offshore samples (Figure 3.2, Cluster 3). There was a distinct demarcation between *P. astreoides* SML samples from Florida in-shore reefs versus those at all other sites, with an average of - 48% similarity between hierarchical clusters (Figure 3.2).

454-Pyrosequencing Analysis

Sample data provided by DGGE allowed us to select a representative subset (n = 12) for barcoded pyrosequencing that illustrated overall community patterns seen among the 52 initial samples. We excised and sequenced 43 clear, unique, and/or dominant bands from DGGE gels and 26 yielded sequences that were of sufficiently high quality for taxon identification, with the remainder excluded mainly due to contamination from adjacent bands. The DGGE band sequences were affiliated with bacterial taxa in three phyla, including bacterial taxa in the phylum *Cyanobacteria*, within the genera *Synechococcus* (n = 7) and *Prochlorococcus* (n = 2), the γ–*Proteobacteria*, within the genera *Edwardsiella* (n = 1), *Oceanspirillales* (n = 13), and *Escherichia coli* (n = 1), as well as an uncultured α-*Proteobacteria* (n = 1) and one representative within the phylum *Actinobacteria*.

We were unable to obtain significant amplification from one *M. faveolata* sample from Belize; thus it was removed from further analyses. From our remaining 11 samples, QIIME reported a total of 8,547 sequence reads, and each library contained between 397 and 1375 reads (~777 reads/sample), with an average read length ranging from 212 to 436 base pairs (bp) per sample, after primer and barcode removal (Table 2). The reads per sample using the SILVA and BLAST2GO databases yielded a similar number of hits and are also reported (Table 2). Samples from *P. astreoides* surface mucus had an average of 851 (QIIME) and 794 (SILVA) reads, while those from *M. faveolata* had an average of 735 (QIIME) and 611 (SILVA) reads per sample. All 16S rDNA sequences from the barcoded-pyrosequencing analysis were submitted to the NCBI sequence read archive under the accession numbers XXXXX to XXXXX.

Diversity of bacteria

Alpha diversity metrics were computed on the denoised data using the QIIME pipeline. Bacterial diversity as determined by the Shannon–Weiner index (H') was highest for *M. faveolata* and ranged from 4.2-6.1, while H' was much lower and almost non-overlapping for *P. astreoides*, at only 0.8-4.3. The highest overall bacterial diversity was at St. Thomas for both corals, where $H' = 6.1$ for *M. faveolata* and 4.3 for *P. astreoides* surface mucus (Figure 3.3a). The largest range, of 50-145 operational taxonomic units (OTUs), was for microbes in *M. faveolata* surface mucus, with a predicted (chao1) range of 76-402 (Figure 3.3b). A much lower

range of 11-85 OTUs occurred in *P. astreoides* surface mucus, with a predicted (chao1) range of 26-225 (Figure 3.3b).

Composition of bacteria

Coral-associated bacterial assemblages were dominated by sequences affiliated with the phylum *Proteobacteria*, followed by *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria*, and *Firmicutes* (Figure 3.4). In general, five bacterial families were common among both *M. faveolata* and *P. astreoides* samples: 1) Haellaceae, a member of the Oceanospirillales, and 2) Enterobacteriaceae both within the γ-subdivision of *Proteobacteria*, 3) Rhodospirillaceae within the α-subdivision of *Proteobacteria*, 4) Comamonadaceae within the Burkholderiales and members of the β-subdivision of *Proteobacteria*, and finally 5) Flavobacteriaceae members of the *Cytophaga-Flavobacteria-Bacteroides* (Table 3, Figure 3.5). The most abundant genus from *M. faveolata* samples was *Edwardsiella* (4% of total sequence reads), a member of the Enterobacteriales within the γ-*Proteobacteria*. Other common genera within *M. faveolata* mucus were *Curvibacter* (3%), *Mesorhizobium* (3%), *Acinetobacter* (2%), *Bacteroidetes* (1.5%), *Flavobacterium* (1.3%), and *Vibrio* spp. (1.2%), including *V. vulnificus, V. harveyi, V. campbellii,* and *V. alginolyticus.* The dominant genus from *P. astreoides* samples was *Endozoicomonas* (90%), within the order Oceanospirillales and a member of the γ–*Proteobacteria*. The next most abundant genera were *Edwardsiella* (3%), *Plesiomonas* (1%), and *Cetobacterium* (0.5%, Figure 3.5).

The abundance of sequences similar (at a > 96% identity threshold) to coral disease-associated and black band disease-associated bacteria was generally higher in the *M. faveolata* libraries than in those from *P.astreoides*; however, the abundance of sequences that affiliated with a database of healthy-associated bacteria was also higher in *M. faveolata* libraries (Table 2). Very few sequence similarities were observed in any of the examined microbial databases for the microbes associated with *P. astreoides,* likely because Caribbean *Porites* sp. were not assessed in any of the studies that composed the meta-analysis from which these databases were constructed (Mouchka et al. 2010). However, the analysis did include four studies that examined bacterial assemblages associated with members of the Caribbean *Montastraea* species complex (Table S2). Thus, for *M. faveolata* a greater number of sequences affiliated with diseased coral-associated bacteria (41.5 %) than with healthy coral-associated bacteria (2.5%) within the

databases examined. The high abundance of *M. faveolata* sequences affiliated with the coral disease database was particularly evident in St. Thomas samples, where an average of 41.5% of sequences were affiliated with the disease database, compared to much lower percentages in Belize (26.4%) and Florida (20.5%, Table 2). Despite the overall lower similarity for Florida sample sequences with the disease database, a higher number of black band disease database-specific sequences occurred in the Florida Keys samples (7.7%) compared to the other site averages (Table 2).

Phylogenetic distance between coral-associated bacterial communities

Principal coordinate analysis (PCoA) of the weighted UniFrac distance showed that *M. faveolata* and *P. astreoides* each harbor characteristic bacterial communities. All of the *P. astreoides* samples clustered to the right of the graph along the primary axis (66% of the variability) and away from the *M. faveolata* samples on the left of the graph. The *P. astreoides* samples also clustered in the second dimension (15% of the variability), but *M. faveolata* samples did not have a similar clustering in the second dimension (Figure 3.6). The bacterial communities associated with both corals showed some clustering by site, indicating that they vary somewhat among geographic locations (Figure 3.6).

Discussion

Our results provide the first analysis, using culture-independent methods on a large number of coral samples (n = 53), of structural patterns in the bacterial assemblages of healthy coral species among disparate regions of the Caribbean. This study also is unique in revealing the microbial assemblages of coral colonies that are apparently healthy and free of interaction with macroalgae, corals, or other macroinvertebrates, using DGGE. Although PCR-DGGE analysis has many known biases and drawbacks, it is an efficient and economical method to assess large-scale patterns among multiple microbial samples. Examination of DGGE-derived patterns allowed us to select a smaller number of representative samples for barcoded-pyrosequencing analyses. Thus, from a comparison of these two culture-independent methods, we believe that the bacterial assemblages documented in our pyrosequencing results are likely to be characteristic of those associated with the corals examined in all three regions of the Caribbean. Our results strengthen the body of knowledge that indicates that corals generally

harbor species-specific assemblages of microbial organisms (Rohwer et al. 2002; Kvennefors et al. 2010). The data presented here illustrate two significantly different clusters of bacterial ribotypes associated with either *M. faveolata* or *P. astreoides* colonies. However, contrary to earlier hypotheses (Rohwer et al. 2002), this study also indicates that coral-associated bacteria may maintain species-specificity and still vary somewhat across geographic and/or environmental gradients, as has recently been demonstrated for other coral species (Littman et al. 2010; Sunagawa et al. 2009).

The host species we examined are two of the most common reef-building (Hexacorallia:Scleractinia) corals in the Caribbean Sea, but belong to distinct phylogenetic lineages separated by 240-288 million years of evolutionary divergence (Romano & Palumbi 1996; Medina et al. 2006). Recently, Sunagawa and colleagues showed that bacterial assemblages were more similar within phylogenetic clades of coral hosts, suggesting that *M. faveolata* (Short/Robust clade) and *P. astreoides* (Long/Complex clade) should have distinct bacterial associations (Sunagawa et al. 2010). These two coral species also vary in reproductive life history strategy (Brooder vs. Spawner), which may alter their mode of acquiring bacterial symbionts (vertical vs. horizontal transmission). Furthermore, *M. faveolata* is affected by a range of bacterial diseases, including but not limited to White Plague, Dark Spot, Black Band, Yellow Blotch/Band, and Red Band, whilst *P. astreoides* has been associated with only two diseases: White Plague and Yellow Blotch/Band (Garzón-Ferreira et al. 2001). In fact, massive reef building corals are often susceptible to the greatest number of diseases, especially species within the genus *Montastraea*: *M. annularis* (9 diseases), *M. faveolata* (6 diseases), and *M. franksi* (5 diseases; reviewed in (Lafferty et al. 2004). On the contrary, Indo-Pacific corals in the genus *Porites* are known to detect invasion by endolithic fungi and respond by walling off the site of fungal penetration with layers of calcium carbonate (Le Champion-Alsumard et al. 1995, Ravindran et al. 2001). Furthermore, the external cell layers of another member of this genus, *Porites compressa,* were completely devoid of adhering microbes, although the mucus layer still maintained a diverse assemblage of microorganisms (Johnston & F Rohwer 2007). Thus, *Porites* spp. may inherently maintain a discriminating relationship between coral host tissues and associated microbes.

We found the surface mucus layer of *P. astreoides* corals to be dominated (> 90%) by members of the Oceanospirillales within the γ–*Proteobacteria*; the majority with highest

similarity to the genus *Endozoicomonas* within the family Hahellaceae. This result corroborates the previous research of Rohwer and colleagues (2002) that also documented Oceanospirillales to be a major constituent of *P. astreoides* microbial communities. Recent studies have also found Oceanospirillales to be a dominant associate of *P. astreoides* larvae, suggesting that these bacteria are vertically transferred from the parent colony (K. Sharp, pers. comm.). Oceanospirillales are a dominant member of the heterotrophic marine microbial environment, with pigments and a distinct coccoid resting stage (Jensen et al. 2010). These bacteria are aerobic heterotrophs that can utilize constituents of coral tissues and mucus such as organic acids, amino acids, ammonium, and Dimethylsulfoniopropionate (DMSP; Garrity et al. 2005; Raina et al. 2009). DMSP is an organic sulfur compound and plays an important role in the global sulfur cycle by contributing to cloud formation through the production of dimethyl sulfide (DMS; Charlson et al. 1987; Malin et al. 1996). High concentrations of DMSP and DMS have been found within animals that harbor symbiotic algae, such as scleractinian corals and giant clams, providing a potential link between DMSP-degrading Oceanospirillales and corals (Broadbent et al. 2002; Broadbent and Jones 2004; Van Alstyne et al. 2006). Preliminary evidence also indicates that *Porites* spp. are capable of hosting DMSP-degrading organisms because the *dmdA* gene, which is the most highly represented gene for DMSP degradation in the Global Ocean Survey (GOS) database (Howard et al. 2006, 2008), was present in both *P. astreoides* (Wegley et al. 2007) and pH-stressed *P. compressa* microbial metagenomes (Vega-Thurber et al. 2008; Thurber et al. 2009; Jean-Baptiste Raina et al. 2010). Whether these organisms are providing a benefit to their coral hosts through the degradation of DMSP and other organic compounds requires further research. Significant support now exists for the presence of a symbiotic relationship between *P. astreoides* and members of Oceanospirillales, particularly given the known association between members of Oceanospirillales and invertebrates and parasitic insects (Elston 1986), as well as with the *Osedax* polychaete, a worm that feeds upon whalebones (Goffredi et al. 2005).

Within the order Oceanospirillales, family Hahellaceae, there is currently only two described species of the genus *Endozoicomonas* identified from the *P. astreoides* SML in this study. The cultured members of this genus include *E. elysicola*, isolated from the sea slug (*Elysia ornate*) off the coast of Izu-Miyake Island, Japan (Kurahashi & Yokota, 2007), and *E. montiporae,* isolated from the encrusting pore coral (*Montipora aequituberculata*) off the coast

of southern Taiwan (Yang et al. 2010). Recently, Jeong and colleagues sequenced the genome of another member of this family, *Hahella chejuensis*, and discovered genes responsible for the biosynthesis of a pigment (prodigiosin), which has lytic activity against a red-tide dinoflagellate, *Cochlodinium polykrikoides* (Jeong 2005). Prodigiosin has been known for centuries as a cytotoxic compound showing a broad range of activity, but this is the first evidence of algicidal activity (Fürstner 2003). Thus, it is important to determine whether *Endozoicomonas* spp. can actively invade coral tissues and whether these bacteria have the potential to antagonize or potentiate coral symbionts (i.e. *Symbiodinium* sp.). Bacterial pigments within the SML may provide a benefit to the coral, functioning as protective agents against solar radiation or protozoan grazing (Matz et al. 2004). The cultured Hahellacaea can also reduce nitrate to N_2 (i.e. denitrification), an important process in the nitrogen cycle. Further research is needed to define the distribution and potential symbiotic association of the *Endozoicomonas*-like bacteria that comprise the majority of *P. astreoides* surface mucus-associated bacterial taxa. We hypothesize that the more stable bacterial community associated with *P. astreoides*, with high relative abundance of Oceanospirillales taxa, may contribute to the lower frequency of coral disease observed for *P. astreoides* compared to *M. faveolata* corals (Garzón-Ferreira et al. 2001; Lafferty et al. 2004).

Bacterial assemblages in the surface mucus layer of *M. faveolata* corals were significantly more diverse than those of *P. astreoides*. A relatively high abundance of bacteria associated with *M. faveolata* were similar to known nitrogen-fixing taxa: *Cyanobacteria* (5%), Rhizobiales (11%), and Burkholderiales (14%). Shashar and colleagues were the first to detect coral-associated bacteria with the required *nifH* gene to fix nitrogen in corals (Shashar et al. 1994). Five percent of the coral SML samples from both St. Thomas and the Florida Keys contained sequences that displayed high similarity to *Synechococcus* sp. and other uncultured cyanobacterial sequences. Another study of the bacteria associated with the conspecific coral, *M. cavernosa,* also found coccoid cyanobacteria related to *Synechococcus* sp. and *Prochlorococcus* sp. within the coral tissues (Lesser 2004). These symbionts are not believed to be pathogens as are other cyanobacterial species related to *Oscillatoria* sp. or *Phormidium* sp., which are among the consortia of bacteria associated with black band disease (Cooney et al. 2002), reviewed in (Bourne et al. 2009). Nitrogenase activity has been found in *M. cavernosa* colonies (Lesser 2004) and subsequent studies found that endosymbiotic *Symbiodinium* sp. were actively using

the products of nitrogen fixation (Lesser et al. 2007). In the present study, > 5% of the samples from all three locations had sequences similar to Burkholderiales within the β-subdivision of Phylum Proteobacteria and Rhizobiales within the α-subdivision of Proteobacteria. *M. faveolata* samples from Belize were dominated by these two orders with approximately 25% of sequences aligning with the Rhizobiales and 44% with the Burkholderiales. Both of these groups are known to fix nitrogen in terrestrial and mangrove systems, but have yet to be examined in corals (Gomes et al. 2010). *Rhizobia* sp. have long been known to form nitrogen-fixing symbioses with leguminous plants and *Burkholderia* sp. also have recently been shown to nodulate legumes, becoming the first β-*Proteobacteria* to form nitrogen-fixing symbioses (Chen et al. 2003). Other studies using *M. faveolata* and *Montipora* sp. as model organisms have expanded the concept of nitrogen-fixing symbionts within the coral holobiont beyond Cyanobacteria to a wide-range of bacterial taxa within the α- and γ-*Proteobacteria*, *Firmicutes*, *Spirochetes*, and Archaea, including three classes of Euryarchaeota (Olson et al. 2009; Kimes et al. 2010). Clearly there is still much to learn about the diversity of nitrogen-fixing prokaryotes and their role in coral microbial communities, but our data suggests that both alpha- and beta-rhizobia may provide fixed-nitrogen to *M. faveolata,* in addition to the known genera within the Cyanobacteria.

Although high biological diversity is typically associated with a healthy biome, the relatively high bacterial diversity associated with *M. faveolata* in comparison to *P. astreoides* may not be entirely beneficial. We detected a large number of sequences with > 96% identity to sequences within a database of disease-associated bacteria within the *M. faveolata* SML (Mouchka et al. 2010), particularly in samples from St. Thomas. However, the highest abundance of sequences that affiliated with Enterobacteriales was found in coral species samples from the Florida Keys. Prevalence of Enterobacteriales is often associated with fecal contamination (Wu et al. 2010), and indicates the presence of other enteric bacteria, such as *Serratia marcescens,* the etiological agent of White Pox disease on *Acropora* coral species in the Caribbean (Patterson et al. 2002). Furthermore, we also found the highest abundance of sequences related to two other disease-affiliated orders, Rhodobacterales and Rickettsiales, in *M. faveolata* Florida Keys samples. A recent meta-analysis of coral-associated bacterial assemblages showed that Rhodobacter are globally associated with several coral diseases (e.g. black band, white plague, white band disease) and two destructive conditions (e.g. atramentous necrosis, cyanobacterial patches; Mouchka et al. 2010). Members of the Rickettsiales have yet

to be linked to coral disease, but are associated with a number of other marine diseases afflicting invertebrates, particularly abalone (Lafferty & Kuris 1993; Moore et al. 2000). These results support our field observations that the *M. faveolata* colonies selected for sampling were generally healthy and unaffected by disease, but the St. Thomas and Florida Keys corals may be experiencing greater levels of environmental stress and/or anthropogenic-derived microbial influx than are those at our site in Belize, causing a shift in their microbial assemblages to a less stable, potentially pathogenic state. Corals in St. Thomas and Florida may be more susceptible to disease than those in Belize, due to exposure to environmental stressors and higher abundances of opportunistic and potentially pathogenic bacteria in the overlying water column.

Coral-associated bacterial diversity in both *M. faveolata* and *P. astreoides* surface mucus samples was greatest in St. Thomas, contrary to our original hypothesis that the Florida Keys samples would have the highest abundance and diversity of bacteria because Florida reefs are exposed to a large number of snorkelers (2.86 million/yr) and divers (0.8 million/yr; NOAA 2000). The Florida coast is second only to California in terms of absolute and percent of population change, increasing by 7.1 million people, a 75% increase, from 1980 to 2003 (Crossett et al. 2004). Higher human population densities inherently lead to increased nutrient enrichment and sedimentation on adjacent reefs. High nutrient loads are believed to fertilize r-selected opportunistic and potentially pathogenic bacterial taxa (e.g. *Vibrio* spp.), allowing them to become dominant on otherwise healthy corals (Bruno et al. 2003, Kline et al. 2006). It is possible that the pattern documented is an example of an intermediate disturbance model, whereas diversity peaks at an intermediate level (intensity or frequency) of small-scale disturbances (Connell 1978; Sousa 1979). In the U.S. Virgin Islands high levels of runoff and the resulting sedimentation can be attributed to recent increases in housing development and road construction, particularly in St. John and St. Thomas where steep mountain slopes allow rapid runoff (Brooks et al. 2007). Heavy rainfall can also overload existing sewage systems, which results in intermediate and severe pollution of coastal waters. Additional studies have directly linked shoreline development in St. Thomas to increased sedimentation during periods of heavy rainfall (Nemeth and Nowlis 2001). These point-source disturbances during rainfall events may have a lasting effect on the coral-associated microbial assemblages in St. Thomas, increasing their overall diversity in comparison to Belize and the Florida Keys.

The greatest variability among SML bacterial samples for both coral species occurred on Wonderland Reef, Florida (in-shore), whereas the least between-coral variability was in Belize and St. Thomas. Although we did not measure environmental parameters such as nutrients or sedimentation, the high sedimentation rates and anthropogenic impacts due to increasing population density along in-shore reefs within the Florida Keys (Crosset et al. 2004), likely causes higher bacterial loads and physiological stress than on the reefs of St. Thomas and Belize. It is also interesting that the pyrosequencing results suggest less inter-site variability among *P. astreoides* samples than *M. faveolata* samples, when the DGGE results suggest greater inter-site variability among *M. faveolata samples.* The scale of taxonomic resolution, which is lower for fingerprinting approaches, likely drives this discrepancy. We believe that overall, *P. astreoides* experiences less variability and is more robust in its microbial affiliations than *M. faveolata.* We base this conclusion on the low level of taxonomic bacterial diversity, the high-level of inter-site affiliation among pyrosequencing samples, and the high level of intra-site affiliation among DGGE samples. Species-specificity was evident among *M. faveolata* colonies from all three sites, however higher bacterial diversity coupled with high levels of inter- and intra-site variability suggests that these corals are more susceptible to environmental perturbation and less able to mediate a specific microbial assemblage, particularly in comparison to *P. astreoides.* We suggest that *P. astreioides* may have a mechanism for fostering a specific bacterial community within its tissues or mucus based on earlier studies (Johnston & Rohwer 2007), in addition to transferring bacterial symbionts from the parent colony to the planula larva (K. Sharp, pers. comm.). Furthermore, *P. astreoides* consistently associates with clade A *Symbiodinium* spp., whereas *M. faveolata* is more variable (Table 2). It is known that *Symbiodinium* spp. release carbon exudates (Ikeda & Miyachi 1995) and arabinose (Ducklow and Mitchell 1979b) into coral mucus. Thus, because coral mucus composition is thought to play an important role in shaping coral microbial assemblages (Ritchie & Smith 2004), variation in photosynthetic activity and products by different *Symbiodinium* clades may contribute to the variability observed within *M. faveolata* microbial assemblages.

We demonstrate here that two prevalent Caribbean corals, *M. faveolata* and *P. astreoides,* harbor unique and specific assemblages of bacteria in their SML. However, we also document small shifts in the microbial assemblages, which relate to the Caribbean sampling site. Changes in environmental parameters (e.g. temperature, excess nutrients, sedimentation) correlate with

incidents of coral disease, and identification of the microorganisms that compose healthy coral assemblages will allow us to better determine coral stressors and disease causation. Guppy and Bythell (2007) suggest four factors that may influence bacterial assemblages within the SML of corals: 1) production of antimicrobial chemicals, 2) supply of bacteria from the water column, 3) environmental conditions, and 4) mucus composition and production rate (Brown & Bythel 2005). With the inclusion of more recent literature, we also suggest: 5) host coral *Symbiodinium* assemblages (Littman et al. 2010), 6) prior exposure to bleaching and disease (Muller et al. 2008), and 7) potential viral and bacteriophage involvement (Vega Thurber et al. 2008; Efrony et al. 2007). Corals exposed to high levels of anthropogenic and environmental stress, such as those adjacent to St. Thomas and the Florida Keys, likely experience physiological and biochemical changes that alter their microbial assemblages long before visible disease symptoms occur. The shift documented here in *M. faveolata* assemblages from high levels of potentially beneficial nitrogen-fixers (e.g. *Rhizobiales, Burkholderiales*) in Belize to disease-associated taxa (e.g. *Rhodobacteriales* and *Enterobacteriales*) in the Florida Keys, demonstrates how studies of healthy coral microbial communities can be used to indicate reefs that require immediate remedial action to reduce coral stress (e.g. improving sanitation, reducing sedimentation). Thus, this type of study provides important indicator tools for early identification of coral stress. Illumination of the dynamics of coral-microbial-environmental interactions, particularly for key coral species that are dominant members of the reef community across geographic and environmental gradients, thus can contribute important information to support global reef recovery efforts.

Table 3.1: Permutational multivariate analysis of variance (PERMANOVA) results for differences among DGGE samples as a function of coral species (*M. faveolata* and *P. astreoides*) and Site (Belize, St. Thomas, Florida in-shore, and Florida off-shore).

	df	SS	MS	F	R2	Pr(>F)
Species	1.00	1.54	1.54	4.45	0.08	0.01**
Residuals	50.00	17.24	0.35		0.92	
Total	51.00	18.78			1.00	
	df	SS	MS	F	R2	Pr(>F)
Site	3.00	2.41	0.80	2.35	0.13	0.01**
Residuals	48.00	16.37	0.34		0.87	
Total	51.00	18.78			1.00	

Table 3.2: Relevant attributes and sequencing hits for 11 pyrosequencing samples. The *Symbiodinium* sp. clade associated with each sampled coral colony determined via T-RFLP. Number of reads per database (1) Ribosomal Database Project (RDP) within QIIME, (2) BLAST2GO database, and (3) SILVA comprehensive ribosomal RNA database. Relative abundance (%) of sequences that affiliate at >96% identity to any sequence within a database of potential (1) Disease pathogens, (2) Black Band Disease (BBD) pathogens, and (3) Healthy associated bacteria for each coral mucus sample.

Libraries were analysed by BLASTn or P-Blast. Disease, BBD, and Healthy values are the percentage of the sequences in each library that were similar to the listed coral diseases. The most abundant of each are in bold. The coral disease-associated and healthy-associated bacterial databases were obtained from Mouchka and colleagues (2010).

Species	**Site**	***Symbiodinium***	**RDP**	**SILVA**	**BLAST2GO**	**Disease**	**BBD**	**Healthy**
M. faveolata	BLZ	A	736	696	685	26.4	2.4	6.0
M. faveolata	STT	D	1014	819	821	**67.9**	4.9	4.1
M. faveolata	STT	D	456	403	390	15.1	3.8	0.9
M. faveolata	FLK	B	949	704	650	33.2	**12.6**	2.9
M. faveolata	FLK	B	397	200	217	8.2	2.7	**10.6**
		AVG	**735.0**	**611.0**	**605.5**	**41.5**	**4.4**	**2.5**
P.astreoides	BLZ	A	1375	1348	1344	3.4	0.0	1.6
P.astreoides	BLZ	A	809	770	788	0.5	0.1	2.2
P.astreoides	STT	-	531	490	495	1.5	0.5	0.0
P.astreoides	STT	A	688	569	576	**6.8**	**3.4**	0.1
P.astreoides	FLK	A	490	417	402	1.9	0.4	0.3
P.astreoides	FLK	A	1102	1056	1043	4.1	0.5	**5.6**
		AVG	**850.8**	**794.3**	**800.8**	**3.1**	**1.0**	**1.0**

Table 3.3: Classification of the 10 most abundant bacterial groups associated with the coral *Montastraea faveolata* and *Porites astreoides*, listed from most to least abundant. Classification based on SILVA database analysis. Groups are bolded when found in the top 10 most abundant groups of both coral species.

P. astreoides	***M. faveolata***
Gamma; Oceanospirillales; Hahellaceae (88%)	**Flavobacteria (17%)**
Gamma; Enterobacteriaceae (6%)	**Alpha; Rhodospirillaceae (17%)**
Bacteroidetes; Flavobacteriaceae (1%)	**Beta; Comamonadaceae (15%)**
Alpha; Rhodospirillaceae (1%)	Alpha; Rhodobacteraceae (7%)
Fusobacteria; Fusobacteriaceae (1%)	**Gamma; Hahellaceae (7%)**
Beta; Comamonadaceae (0.5%)	Alpha; Phyllobacteriaceae(6%)
Gamma; Alteromonadaceae (0.4%)	Cyanobacteria; Synechococcus (5%)
Gamma; Vibrionaceae (0.4%)	**Gamma; Enterobacteriaceae (4%)**
Firmicutes; Bacillaceae (0.3%)	Gamma; Moraxellaceae (4%)
Alpha; Phyllobacteriaceae (0.2%)	Alpha; Methylobacteriaceae (4%)

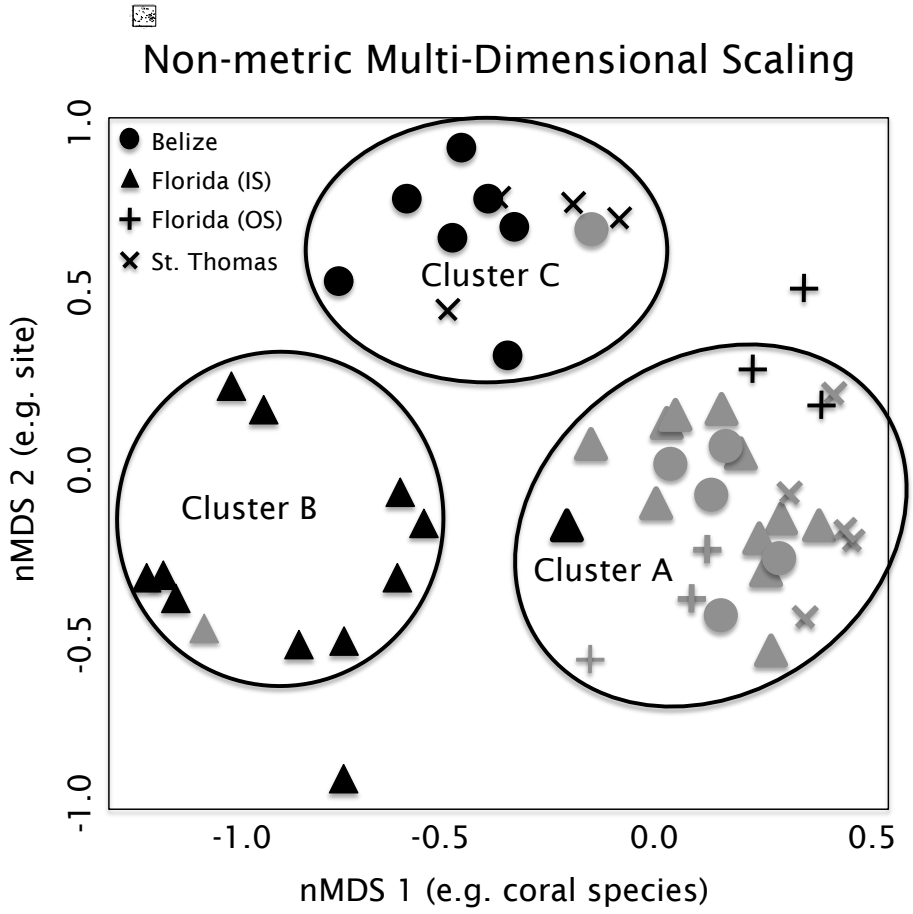


Figure 3.1: Non-metric multidimensional scaling of the first two dimensions based on Jaccard distances. Distance matrix from DGGE gels images (band presence/absence) of *M. faveolata* (solid shapes, n = 26) and *P. astreoides* (open shapes, n = 27) SML microbial assemblages from all three Caribbean field sites: Belize, Florida in-shore (IS), Florida off-shore (OS) and St. Thomas, USVI. Cluster A represents the majority of *M. faveolata* samples. Cluster B represents the majority of *P. astreoides* samples from the Florida in shore (IS) site and Cluster C represents most *P. astreoides* samples from Belize and St. Thomas.

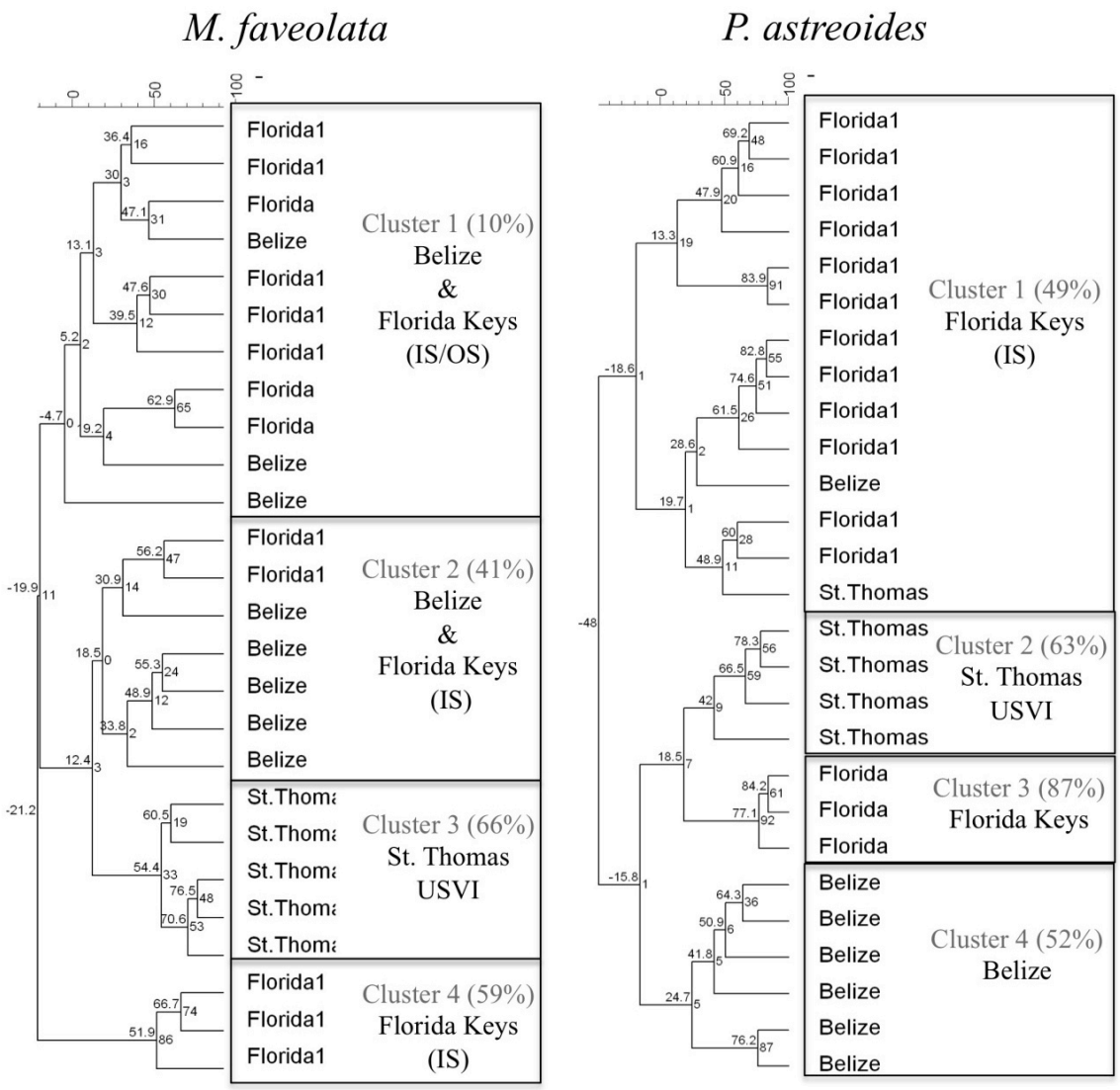


Figure 3.2: Hierarchical cluster analyses using WARD algorithms and DICE coefficients based on band presence/absence from DGGE fingerprints. Bacterial assemblages from *M. faveolata* (n = 26) and *P. astreoides* (n = 27) were analyzed separately. Clusters 1-4 represent most similar bacterial samples within each species with the average percent group similarity within parentheses. Exterior tree numbers are percent similarity and interior tree numbers are bootstrap support based on 1000 iterations.

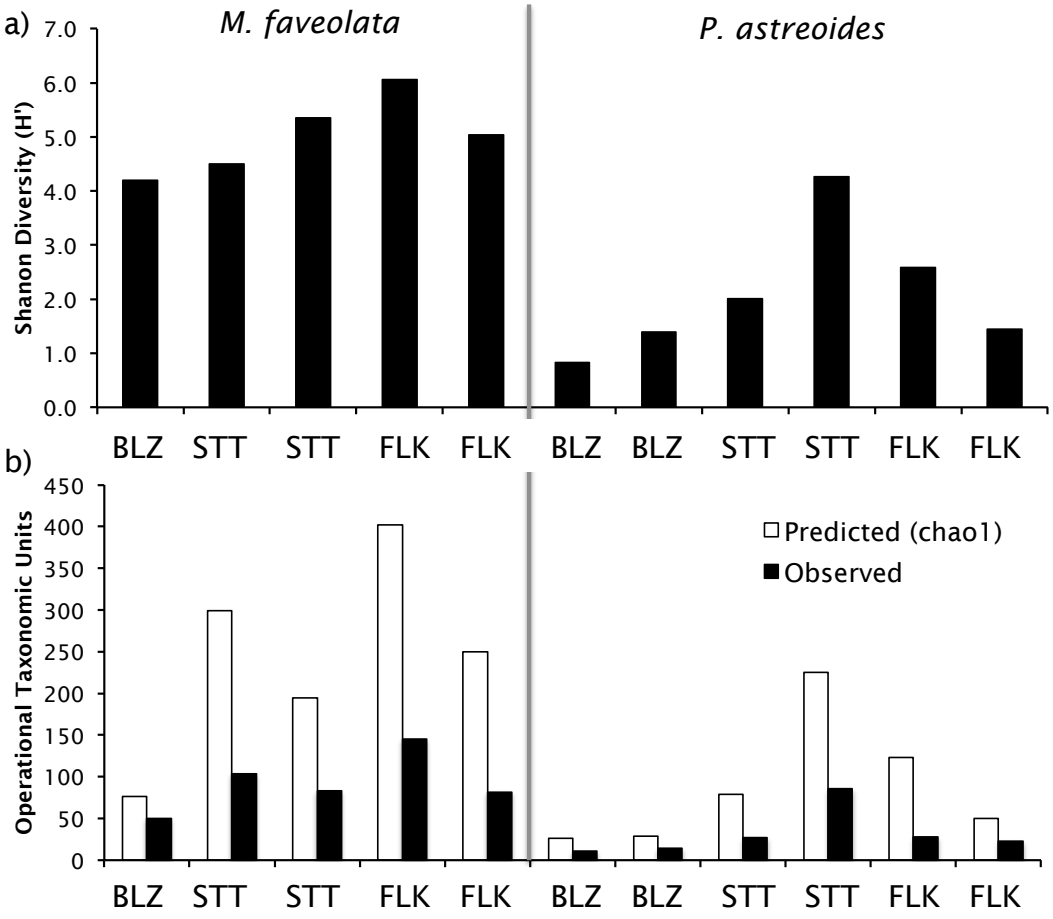


Figure 3.3: Alpha diversity of bacteria associated with the SML of *M. faveolata* and *P. astreoides* corals from Belize (BLZ), St. Thomas U.S. Virgin Islands (STT), and the Florida Keys (FLK): (a) Shannon-Weiner diversity of bacteria from coral mucus samples. (b) Number of OTUs predicted (Chao1) and observed . OTU's were grouped at >97% similarity based on RDP classified results.

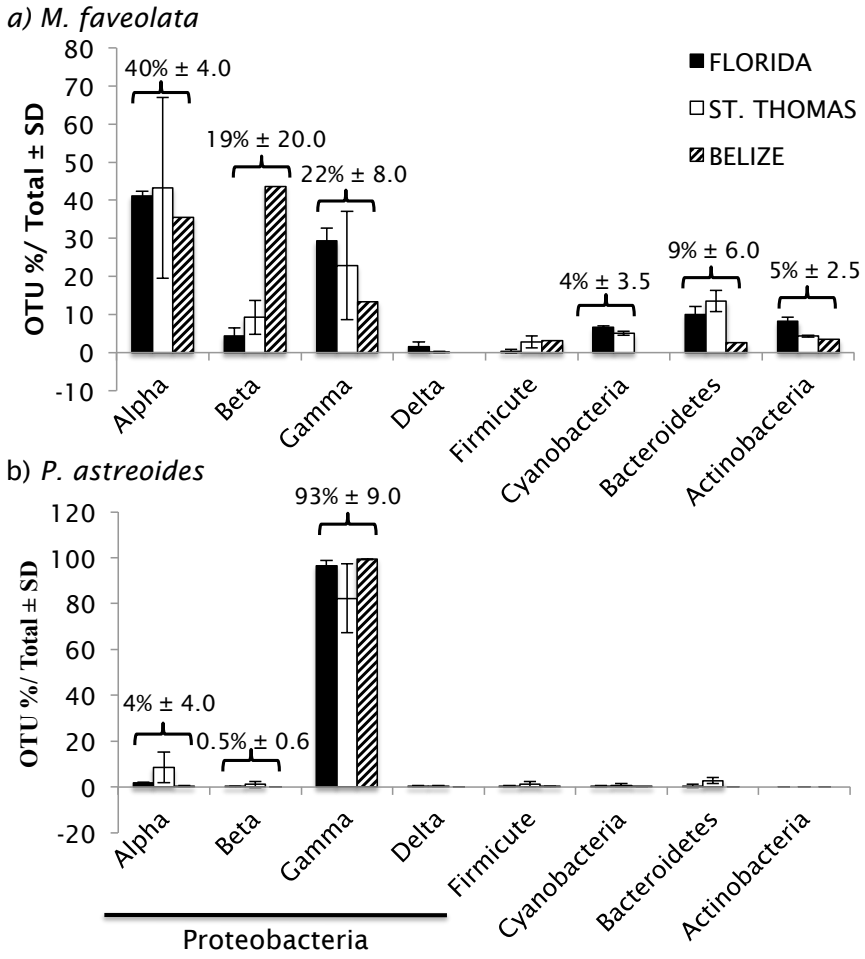


Figure 3.4: Relative abundance of the most common phyla associated with the SML of *M. faveolata* and *P. astreoides* corals in the Florida Keys, St. Thomas U.S. Virgin Islands and Belize, collected May-September 2009. Average percentage of total OTU's ± SD given for the most dominant Phyla.

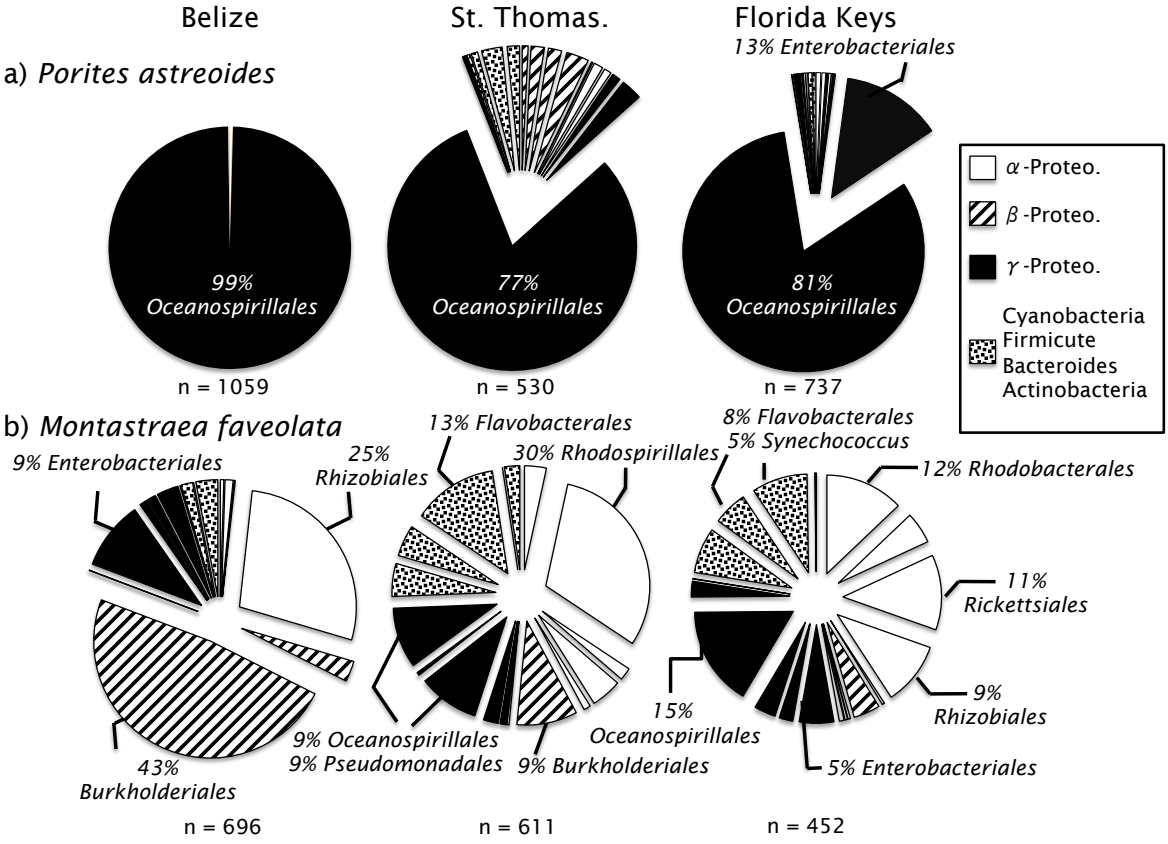


Figure 3.5: Percentage of OTU's related to the nearest Class level affiliation for *Porites astreoides* and *Montastraea faveolata* coral surface mucus samples from Belize, St. Thomas, and the Florida Keys based on SILVA database hits. Pie charts are shaded by Phyla affiliation (Alpha- (α), Beta- (β), gamma- (γ) Proteobacteria, and Cyanobacteria, Bacteroidetes, Actinobacteria, and Firmicutes).

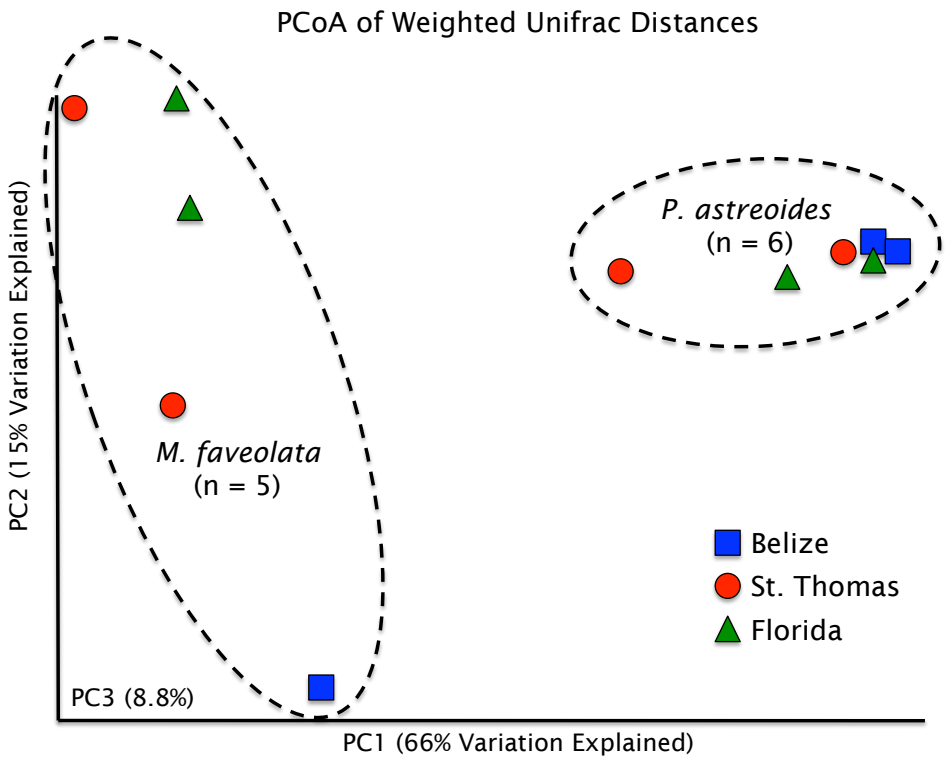


Figure 3.6: Principal coordinate analysis (PCoA) of weighted UniFrac distances based on Ribosomal Database Project (RDP) classified results.

Chapter 4

Coral-algal competition mediates coral-associated microorganisms *in situ*

Introduction

For at least the last half-century coral reefs have been undergoing global degradation due to increasing natural and anthropogenic impacts. The intensity and frequency of stressors, including global climate change, have rapidly increased over recent years (Hughes & Connell 1999; Hoegh-Guldberg et al. 2007; Pandolfi et al. 2011). Frequent disturbances such as hurricanes/cyclones, predation outbreaks, diseases and mass bleaching events reduce the percent cover of living corals, and without recovery, the available space is colonized by sponges, soft corals, and macroalgae that are often competitively dominant in less than ideal conditions (Norström et al. 2009, Chadwick and Morrow 2011). This transition is termed a phase-shift from a coral-dominated landscape to one that is dominated by other benthic organisms. Phase-shifts are also often associated with low herbivory (from disease and/or overfishing) and nutrient enrichment (from run-off and coastal eutrophication, see Relative Dominance Model in Littler et al. 2006; 2009). The resilience of particular coral species to recover from and/or resist disturbance and subsequent phase-shifts may serve as an indicator of variation in future recovery rates among coral reefs.

Macroalgae are frequently the competitive dominants that drive phase-shifts, thus "coral-macroalgal phase-shifts" is widely used to describe unusually low levels of coral cover and a persistent state of high macroalgal cover. Several studies and reviews have described the negative effects of macroalgae and phase-shifts on coral reefs (Done 1992; Hughes & Connell 1999; Hughes et al. 2003; Hughes et al. 2007; McCook et al. 2001; McManus & Polsenberg 2004; Birrell et al. 2008; Bruno et al. 2009). Potential competitive mechanisms of macroalgae include: shading, abrasion, whiplash, and basal encroachment (Coyer et al. 1993; Lirman 2001; Box & Mumby 2007), increased localized sedimentation (Nugues & Roberts 2003), interfering with the settlement of coral larvae through space preemption and/or biofilm production (Birrell

et al. 2005; Birrell et al. 2008; Box & Mumby 2007; Vermeij et al. 2009), and allelopathy or chemically-mediated competition (deNys et al. 1991, Morrow et al. 2011). Macroalgae can cause physical stress (Quan-Young & Espinoza-Avalos 2006), decreased photosynthetic ability (Titlyanov et al. 2007), and reduced fecundity (Foster et al. 2008) in corals, and harbor potential coral pathogens (Nugues et al. 2004). The ability of macroalgae to competitively damage stony corals also depends on the species of macroalgae (Maypa & Raymundo 2004; Birrell et al. 2008), and other compounding factors such as irradiance, sedimentation, dissolved nutrients, and level of herbivory on the reef.

Over the past several decades, the incidence of coral disease has increased, with a striking correlation with increasing macroalgal cover (Goreau et al. 1998; Harvell et al. 1999; Harvell 2004; Weil & Smith 2006). The enhanced need for disease research coupled with the application of molecular techniques made the study of coral-associated microbes more efficient and less cost prohibitive. From these studies has come the recognition of the significant role microorganisms play in the physiology of both healthy and diseased corals. The term holobiont was coined to describe the dynamic relationship between coral host and microbial symbionts, including the *Symbiodinium,* Bacteria, Archaea, viruses, and protozoa (Rohwer et al. 2002). Corals are believed to associate with diverse assemblages of microorganisms that are distinct from the water column and play a dominant role in host health and metabolism (Ritchie & Smith 1997; Rohwer et al. 2001; 2002; Frias-Lopez et al. 2002; Wegley et al. 2004; Bourne et al. 2009; Rosenberg et al. 2007b; Sunagawa et al. 2010; Morrow Chapter 3). Many corals likely associate with species-specific microbial assemblages (Rohwer et al. 2002; Sunagawa et al. 2010), however there is evidence to suggest that these assemblages change across geographic gradients (Littman et al. 2009; Sunagawa et al. 2009), after bleaching events (Bourne et al. 2007), when housed in aquaria (Kooperman et al. 2007), and/or when exposed to stressors (Thurber et al. 2009), potentially enhancing opportunistic infections. Despite the many important roles we know coral microbes play in host physiology and health through the production of antibiotics (Ritchie 2006) and biogeochemical cycling (Lesser 2004; 2007; Wegley et al. 2007; Raina et al. 2009; Kimes et al. 2010), it remains unclear what factors mediate the types of microbiota associated with corals. The coral holobiont may adapt to changing environmental conditions by shifting its resident microbial assemblages, a theory termed the coral probiotic hypothesis and subsequently the hologenome theory (Reshef et al. 2006; Rosenberg et al. 2007a). However, the

microorganisms that coexist in the various compartments of the coral holobiont, including the surface mucus layer (SML), tissues, and skeleton, are thought to be somewhat transitional and influenced by a combination of resident and transient microbial associates, as well as environmental conditions and benthic interactions (Sweet et al. 2010).

Macroalgae are hypothesized to mediate coral reef microorganisms through the release of dissolved organic carbon (DOC; Smith et al. 2006), allelochemicals (Morrow et al. 2011), and the provision of a diverse reservoir of potentially pathogenic microorganisms (Nugues et al. 2004; Barott et al. 2011). Increased organic carbon loading has been shown to kill corals (Kuntz et al. 2005; Kline et al. 2006), and the addition of antibiotics can prevent or delay this mortality, thus carbon-induced elevation of microbial growth rates and consequent oxygen depletion are likely to blame (Smith et al. 2006). Macroalgae may need to be in direct contact with coral tissues to result in high enough DOC levels to influence microbial-driven hypoxia (Vu et al. 2009; Barott et al. 2009) and/or the transference of pathogens (Nugues et al. 2004). It is also believed that excess labile carbon excreted by benthic macroalgae stimulates microbial activity in the water column (Haas et al. 2010). Thus, on a larger scale, reefs dominated by macroalgae are known to have lower levels of oxygen in the overlying water column due to the higher abundances of heterotrophic bacteria and potential pathogens (Haas et al. 2010; Dinsdale & Rohwer 2008).We also know that common Caribbean macroalgae produce potent allelopathic chemicals that suppress corals (Rashar & Hay 2010), and act as broad-spectrum antibacterial and stimulatory agents with activity against coral reef microorganisms (Morrow et al. 2011). Through a combination of these mechanisms, macroalgae may influence overall reef resilience by suppressing coral health and altering the resident microbial community at small to large scales, potentially leading to increased coral disease, mortality, and additional algal proliferation.

The goal of this study was to determine whether contact with macroalgae correlated with shifts in coral-associated microbial assemblages at both the coral-algal interface and on a larger colony-wide scale. We were also interested in whether these interaction patterns varied among geographic regions in the Greater Caribbean Sea. Microbial shifts were documented by analyzing denaturing gradient gel electrophoresis (DGGE) profiles from samples collected along natural coral-algal interaction gradients *in situ*. Coral colonies free of encroaching macroalgae or other benthic invertebrates were used as outgroup controls for each geographic location. Surface mucus layer (SML) microbial samples were collected in Belize, the Florida Keys, and St.

Thomas U.S.V.I. along interaction gradients between two ubiquitous Caribbean corals (*Montastraea faveolata* and *Porites astreoides*), and adjacent foliose brown macroalgae (*Dictyota* menstrualis), and calcareous green macroalgae (*Halimeda opuntia*). These macroalgae were chosen because they are known to compete with corals and to reduce coral growth rates, increase coral tissue mortality, and produce potent allelochemicals that are active against coral reef microorganisms (Ballantine et al. 1987; Lirman 2001; Beach et al. 2003; Rashar & Hay 2010; Morrow et al. 2011). We show that the presence of macroalgae correlates with shifts in *M. faveolata* microbial assemblages more often than *P. astreoides*. We also found that coral microbial assemablages of both species were less stable in Florida than at sites further south in the Caribbean at Belize and St. Thomas.

Methods

Coral-Algal Diversity Surveys

Surveys of coral diversity and benthic interactions were conducted at all three sampling sites: South Water Cay Reef adjacent to the Smithsonian's Carrie Bow Cay Field Station in Belize (October 2008 and August 2009); Flat Cay Reef adjacent to the University of the Virgin Islands MacLean Marine Science Center, St. Thomas, U.S. Virgin Islands (July 2008 and 2009), and the Florida Keys reef tract from the Dry Tortugas National Park to Carysfort Reef in the lower keys (September 2009). Surveys in Florida were conducted aboard the NOAA R/V Nancy Foster, as part of the Coral Health and Diversity Cruises, run by the Florida Keys National Marine Sanctuary (FKNMS) under direction of the National Oceanic and Atmopheric Association (NOAA). Therefore, the Florida surveys were more extensive, and differed somewhat in methodology, than those conducted in Belize and St. Thomas. Only a portion of the NOAA-FKNMS dataset is reported here and the remainder will be reported in later publications.

NOAA-FKNMS surveys were conducted using a radial arc method developed for coral disease studies (Santavy et al. 2001; Santavy et al. 2005). On SCUBA, divers installed a stainless steel rod at previously defined study sites (see Santavy et al. 2001) and fastened a 12 m line to the rod by a carabineer that rotated freely. One diver pulled the line taut and slowly moved the line in an arc around the fixed central point. Two additional divers surveyed the circular band transect between the 8 and 10 meter mark, which encompassed an area of 113 m^2. Previous

studies determined that only the 8 to 10 m segment is necessary to obtain a reliable estimate of coral disease (Mueller et al. 1998; Santavy et al. 1999; Santavy et al. 2001). One diver recorded the number of colonies of each coral species and whether they displayed signs of bleaching or disease, while the second diver measured the length, width, and height of the first 10 colonies of each species encountered within the arc. Corals were included in the survey if ≥ half of a coral colony was within the 8-10 m belt. The second diver also determined whether the first 10 encountered colonies of each species were interacting with *Dictyota* sp., *Halimeda* sp., *Lobophora* sp., sponges, tunicates, predation (e.g. corallivorous snails), and/or other benthic organisms.

The radial arc method required a large number of divers, and predrilled installation sites for the central rod, thus we adopted a simpler but similar method for surveys conducted in Belize and St. Thomas. A 25 m linear band transect was established parallel to the reef crest with a haphazard starting location. One diver swam each band transect in both directions recording the number of colonies of each coral species within 1-m on either side of the transect tape, which encompassed a total area of 50 m^2. The first diver also recorded whether a colony displayed signs of bleaching and/or disease, in addition to recording benthic interactions, as above. A second diver swam the same band transect and placed a 0.25 m^2 divided quadrant at 10 locations along each band transect, approximately every other meter and alternating sides of the transect. The most abundant benthic component in addition to the number of coral recruits within each of 25 squares was identified to the genus level and recorded. Average percent cover per m^2 of each benthic component, the percentage of total corals, and the percentage of benthic interactions with corals were quantified for all three sites.

Microbial Sample Collection

Microbial samples were collected from the surface mucus layer (SML) of apparently healthy *Montastraea faveolata* (MF) and *Porites astreoides* (PA) coral colonies interacting with macroalgae at 3 sites: South Water Cay Reef adjacent to the Smithsonian's Carrie Bow Cay Field Station in Belize (August 2009); Flat Cay Reef adjacent to the University of the Virgin Islands MacLean Marine Science Center, St. Thomas, U.S. Virgin Islands (July 2009), and from; Looe Key Reef in the Florida Keys, which was one of the sites included in the benthic survey as part of the the NOAA Florida Keys National Marine Sanctuary sponsored coral census cruise

(September 2009). The three coral reef sampling sites varied in distance from the mainland: Belize (20 km), St. Thomas (2.5 km from the island), and the Florida Keys (12 km). Collections were made on SCUBA at 5-15 m depths using sterile 5 ml syringes that were capped before and after sample collection. A 5 x 5 cm area was gently agitated on the surface of each coral using the plastic-tip of the syringe, which encourages sloughing of the viscous coral surface mucus and reduces aspiration of seawater (Ritchie 2006). Each syringe was capped before and after collection and sterile nitrile gloves were worn during collection to reduce human or seawater bacterial contamination.

Samples were collected from the SML of coral control colonies (A) of *M. faveolata* and *P. asteroides* that were > 1 m apart and not obviously interacting with other corals, invertebrates, macroalgae or benthic cyanobacteria (n = 3 in Belize and Florida, n = 5 in St. Thomas; Figure 4.1). Samples were also collected from the interaction zone between coral tissues and *Dictyota menstrualis (*brown fleshy algae) and *Halimeda opuntia (*green calcareous algae) as follows: B) coral SML 5-cm from the interaction zone (n = 3), C) coral SML in direct contact with macroalgae (n = 3), and D) surface of macroalgae in direct contact with the coral colony (n = 3, Figure 4.1). Finally, a 5th microbial control sample was taken from the surfaces of *D. menstrualis* and *H. opuntia* thalli that were not contact with other corals, invertebrates or macroalgae (n = 3 in Belize and Florida, n = 5 in St. Thomas; Figure 4.1).

After collection, syringes were placed in seawater-filled coolers and transported back to the laboratory (< 3 hrs) where they were immediately processed for transport and subsequent culture-independent analyses. Syringes were placed tip down in test-tube racks for ~15 minutes to allow the mucus to settle to the bottom, then 2 ml of the concentrated mucus was transferred to cryovials and centrifuged at 10,000 x *g* for 10 min. The seawater supernatant was poured off and the remaining mucus pellet frozen at -20°C. Microbial samples were transported to Auburn University on ice and thawed at 4°C prior to DNA extraction using the MOBIO Ultraclean® Microbial DNA Isolation kit (Carlsbad, CA), according to the manufacturers instructions, with an additional (10 min) heating step to 64°C to increase DNA yield. Extracted DNA was stored at -80°C until PCR amplification.

PCR Amplification and DGGE Analysis

Universal bacterial primers 27F-GC (5' -CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG CAG AGT TTG ATC MTG GCT CAG-3') and 518R (5' -ATT ACC GCG GCT GCT GG-3'), were used to amplify the 16S rRNA gene from bacterial isolated genomic DNA from coral mucus. The forward primer was modified to incorporate a 40-bp GC *clamp* for resolution on a denaturing gradient gel electrophoresis (DGGE) system (Muyzer et al. 1993; Ferris et al. 1996). These primers amplify a 491-bp section of the 16S rRNA gene of members of the domain *Bacteria*, including the highly variable V1-V3 regions (Ashelford et al. 2005; Huse et al. 2008). All PCR was performed on a thermalcycler (model: Master cycler epgradient, Eppendorf, Hauppauge, NY) as follows: 12.5 µl EconoTaq PLUS GREEN 2X Master Mix (Lucigen, Middleton, WI), 0.5 ul of each 20 uM primer, and adjusted to a final volume of 25 µl with nuclease-free water. Strip tubes, master mix and nuclease free water were UV-irradiated for 20 minutes prior to the addition of primers under sterile conditions in a laminar flow hood to reduce contamination (Millar et al. 2002). DNA template was added during an initial 'hotstart' of 3 min at 94°C, followed by a 'touchdown' PCR protocol, in which the annealing temperature was decreased from 65°C by 1°C every cycle until reaching a touchdown temperature of 54°C, at which temperature 35 additional cycles were performed as follows; 94°C for 45 sec, 54°C for 45 sec, and 72°C for 1.5 min; and 1 final cycle at 94°C for 45 sec, 54°C for 45 sec, and 72°C for 7 min followed by cooling to 4°C. PCR products were analysed by agarose gel electrophoresis (1% w/v agarose) stained with ethidium bromide and visualised using a UV transilluminator.

Samples were separated using a conventional vertical gel electrophoresis apparatus (model Hoefer SE600, Hoefer Inc.,San Francisco, CA) warmed with a tank heater (Lauda model M6a; Brinkmann Instruments, NY) modified for use as a DGGE system. PCR products were loaded onto an 8% acrylamide gel and run with 0.5 x TAE buffer (Tris base, acetic acid, EDTA) and a 35-60% linear denaturing gradient of formamide and urea. Gels were first electrophoresed at 60°C for 15 min at 50 V, and subsequently for 10 hrs at 100 V (or 1000 V·hrs) in the DGGE system. After electrophoresis, the gels were stained for 30 min with SYBR-Gold nucleic acid stain at a 1:10,000 dilution ratio (Invitrogen, Carlsbad, CA) in TAE buffer, rinsed, and imaged using an AlphaImager HP gel documentation system (ProteinSimple, Santa Clara, CA). Images

were saved as 8-bit TIFF files followed by alignment, normalization, band class identification, and statistical analysis using Bionumerics V. 5.0 (Applied Maths, Austin, TX).

Band Excision and Sequencing

Uniquely dominant and distinct bands were dabbed with a sterile pipette tip and placed directly into PCR strip tubes containing UV-sterilized nuclease free water. Bands were re-amplified with the previously described touchdown protocol using the 27F/518R primer set without the -GC clamp. PCR products were analyzed with agarose gel electrophoresis (1% w/v agarose), stained with ethidium bromide and visualized using a UV transilluminator. An ammonium acetate-ethanol precipitation was performed and the resulting product amplified using the BigDye® sequencing reaction; 1.0 µl of BigDye®, 1.5 µl of 5x Buffer, 0.5 µl of 10 µM 27F, 4 µl of nuclease free water, 3 µl of Template. The following thermocycler conditions were used; 95°C for 30 s, 50°C for 30 s, and 60°C for 4 min, at which temperature 30 additional cycles were performed. PCR products were purified using the BigDye® XTerminator Purification Kit (Applied Biosciences) and shipped to the Smithsonian Institution Laboratories of Analytical Biology (Suitland, MD) for sequencing. The Smithsonian Institution performed high-throughput (96-well) sequencing on an ABI sequencer. Sequences were trimmed using CLC Genomics Workbench (CLC Bio) and blasted against the NCBI Blastn database and those sequences with > 96% identity and E-values < 1×10^{-20} were accepted for accepted downstream analysis.

DGGE Image Analysis

DGGE images were imported into Bionumerics V 5.0 (Applied Maths) and subjected to a series of steps to allow multiple gel images to be reliably compared, these were: identify sample lanes, apply a background subtraction, normalize to the reference standards run on each gel, and identify and quantify bands. Sample comparison and band matching was initially conducted in Bionumerics, in which band classes were constructed based on optimal position tolerance and optimization settings. A bifurcating hierarchical dendrogram and similarity matrix representing sample clusters was initially constructed for each coral-algal interaction at each site using the WARD algorithm and DICE coefficients derived from the band alignment. A binary matrix

based on band presence/absence was exported from Bionumerics, converted to a distance matrix and analyzed using the R statistical package (Ihaka and Gentleman 1996).

Kruskal's nonmetric multidimensional scaling (nMDS) analysis and permutational multivariate analysis of variance (PERMANOVA) were used to assess the multivariate relationships among and between binary DGGE profiles for each coral-algal species pair at each site. A highly significant PERMANOVA results may suggest that coral and macroalgal microbial samples are distinct and that the macroalga did not affect the coral colony microbial assemblage. Thus, biological significance may be different from statistical significance. nMDS arranges multivariate data in a two-dimensional plane based on similarity coefficients between different samples. The Euclidean distances (we used Jaccard) between points in an nMDS plot are inversely proportional to the similarity of the samples. The number of dimensions (k) was determined by first running a scree plot to determine stress (i.e. an inverse measure of fit to the data) as a function of dimensionality. Kruskal's stress formula was used as an informal method of determining the appropiate number of dimensions (McCune and Grace 2002). These data were analyzed using the metaMDS and Adonis utilities within the Vegan package in R. metaMDS is unique in that it calls on isoMDS to perform nMDS, but also searches for the most stable solution by performing several random starts (we used 20; Ihaka and Gentleman 1996). The relationship among samples is represented in a plot of the first two nMDS dimensions.

A final analysis of the data was done using Multiple Response Permutation Procedure (MRPP) and related Meandist functions within the Vegan package in R. MRPP determines whether the 5 sampling locations (A-E; Figure 4.1) form distinct groups based on their DGGE profiles. In an MRPP analysis samples are *a priori* assigned to 2 or more groups (in our case 5 groups – coral control, algal control, coral near, interaction zone, algae near; Figure 4.1) and is used to calculate a distribution of the average intra-group distance by randomly permutating the data n times (in our case n=5000), which determines a p-value for the observed intra-group distance in the data. Additionally, an A parameter is calculated that relates the observed intra-group average distance to the mean of the calculated distribution. When the value of A is close to 1 it is indicative of very tight groupings. We performed MRPP separately on each of the 4 coral-algal (*M. faveolata* and *P. astreoides* paired with *D. menstrualis* and *H. opuntia*) interactions at all three sites from which we collected samples. Additionally, the function, Meandist, calculates a matrix of mean within-cluster dissimilarities (diagonal) and between

cluster dissimilarities (off diagonal elements). Meandist was used to construct histograms of result matrices based on the within-group and between group dissimilarities. Horizontal lines within the histogram are drawn at the level of mean between-cluster dissimilarity and vertical lines connect within-cluster dissimilarities to this line (Van Sickle 1997).

Results

Coral-Algal Survey Results

We surveyed an approximate area of 310 m^2 in Belize, 350 m^2 in St. Thomas, and 2147 m^2 in the Florida Keys (Table within Figure 4.2a). The highest number of coral species were recorded in Florida (n = 29), possibly due to the vast area censused with NOAA-FKNMS (Table within Figure 4.2a). On average, the most common coral genus recorded at all three sites was *Montastraea* sp. (26% ± 7.6 SE), followed by *Agaricia* sp. (21.46% ± 13 SE). In Belize, 3.2 corals per m^2 were recorded and the most common coral genus was *Agaricia* sp. (33%), followed closely by *Siderastrea* sp. (27%) and *Porites* sp. (21%). In St. Thomas, 3.3 corals per m^2 were recorded and the most common coral genus was *Montastraea* sp. (39%) followed by *Porites* sp. (30%) and *Siderastrea* sp. (15%). In Florida, 0.89 corals per m^2 were recorded and the most common coral genus was *Montastraea* sp. (27%), followed by *Agaricia* sp. (24%) and *Porites* sp. (22%; Figure 4.2a). The greatest percentage of corals interacting with benthic organisms was recorded on Florida reefs (120% of corals surveyed) followed by Belize (58%) and St. Thomas (28%; Figure 4.2b). Corals were often interacting with more than one benthic component, thus the interaction percent could exceed 100%. A particularly high number of coral-algal interactions occurred on Florida reefs in association with macroalgae in the genera *Dictyota* sp. (70% of corals) and *Halimeda* sp. (40% of corals; Figure 4.2b).

Analysis of the NOAA-FKNMS survey showed that *Siderastrea* sp. corals interacted with the greatest percentage and number of benthic components, followed by *M. franksii* and *Diploria* sp. (Figure 4.3a). Again, on average *Dictyota* (70% ± 4.8) and *Halimeda* (33% ± 3.8) macroalgae most frequently interacted with the most coral species in the survey (Figure 4.3a, b). Corals that are generally found in deeper water (e.g. *M. franksii, C. natans*) tended to interact with *Lobophora* brown algae more frequently than with other macroalgae in the study. Of the most common coral species recorded, those that most commonly interacted with the surveyed benthic components were *Siderastrea* sp. (231%), *C. natans* (190%) and *M. franksii* (178%). The

corals that interacted with brown macroalgae in the genus *Dictyota* sp. >80% of the time were: *Siderastrea* sp., *Diploria* sp. and *M. meandrites* (Figure 4.3a). The corals that interacted with green macroalgae in the genus *Halimeda* sp. > 50% of the time were: *Siderastrea* sp., *Madracis* sp. and *Diploria* sp. The study corals *M. faveolata* and *P. astreoides* interacted with *Dictyota* sp. > 70% of the time and with *Halimeda* sp. > 25% of the time. However, *M. faveolata* interacted with both macroalgal species more frequently than did *P. astreoides* (Figure 4.3a).

In terms of variation among area surveyed within the Florida Keys (Dry Tortugas National Park, Key West region, and Upper, Middle, and Lower Keys), there are surprisingly few differences in the type or frequency of interaction between corals and benthic components surveyed (macroalgae, cyanobacteria, sponges, tunicates or predatory snails; Figure 4.3b). Disease was observed in < 2% of corals at all sites surveyed (Belize, St. Thomas, and Florida; Figure 4.2b and Figure 4.3b).

DGGE Results

In terms of the bacterial assemblages associated with the SML of *M. faveolata* and *P. astreoides* coral colonies that formed natural interactions with *D. menstrualis* and *H. opuntia* macroalgae, the PCR-DGGE profiles revealed a diverse bacterial assemblage in all samples. Hierarchical cluster analyses of DGGE band classes showed that these coral control samples grouped closely together at all three sites (Belize, St. Thomas, and Florida; Figure 4.4), providing additional evidence of host-microbial species-specificity. The nMDS and Meandist dendrogram results further confirmed the similarity among coral control samples for both coral species at each site (Figure 4.5 and 4.6).

PERMANOVA results for all coral-algal interactions at St. Thomas revealed highly significant differences, suggesting that coral- and macroalgal-associated bacteria are distinct and that neither of the study macroalgae significantly shifted the coral-associated microbiota at this site (Table 4.1). nMDS plots and Meandist dendrograms also indicate little change in the coral colony microbial assemblage as a result of encroaching macroalgae at St. Thomas. The only significant shift was found at the interaction zone between *M. faveolata* corals and *D. menstrualis* brown macroalgae, reflected in the nMDS plots and Meandist dendrograms (Figure 4.5 and 4.6).

Results for *M. faveolata* coral-algal interactions at Belize and Florida revealed less variation than at St. Thomas. These results suggest that macroalgae shifted the coral-microbial assemblage to one that is more similar to macroalgae and less similar to the coral control composition (PERMANOVA; Table 4.1). Hierarchical cluster analyses (Figure 4.4) and dendrograms constructed using the function Meandist (Figure 4.6) illustrate changes to the coral colony microbiota that are more similar to macroalgal microbial profiles. nMDS plots further illustrate these shifts away from coral control assemblages (Figure 4.5a and 4.5c). The most evident shifts in microbial assemblages, within the colony and interaction zone SML, are seen in Florida for both *M. faveolata*-algal interaction pairs.

Results for *P. astreoides* suggest that macroalgae have less effect on the microbiota associated with the entire coral colony SML than with *M. faveolata*, but macroalgae do cause clear shifts in the SML microbiota within the coral-algal interaction zone (Figure 4.4). Hierarchical cluster analyses (Figure 4.4), nMDS plots (Figure 4.5), and Meandist dendrograms (Figure 4.6) all provide evidence that the coral microbiota at the interaction zone are more similar to macroalgal-associated than to coral-associated microorganisms. However, in one instance, the entire coral colony microbial assemblage associated with *P. astreoides* colonies interacting with *H. opuntia* in Florida was shifted to reflect *H. opuntia* microbiota. All statistical results clearly reflect this shift in colony bacteria (Figures 4.4b, 4.5c, and 4.6). It is notable that overall, *H. opuntia* caused a greater number (3:6) of coral colony microbial shifts than did *D. menstrualis* (2:6), except for at St. Thomas where no alga appeared to alter coral-associated microorganisms. In terms of overall variation among sites, coral-algal interactions at Florida (3:4) caused more apparent changes in overall coral colony microbial assemblages than at either Belize (2:4) or St. Thomas (0:4; Figure 4.6).

Sequencing Results

Variation in the dominant types of bacteria in the assemblages associated with 3 distinct regions along natural coral-algal interaction gradients, in comparison to coral and algal control samples, was revealed using PCR-DGGE of the 16S rDNA gene. Of the 136 bands excised and sequenced from DGGE gels, only 30 sequences were identifiable, presumably due to contamination from adjacent bands during band excision. However, these 30 clear sequences closely matched those of common bacteria from our previous pyrosequencing study that

examined the microbial assemblages of both *M. faveolata* and *P. astreoides* (see Chapter 3). Sequences from *M. faveolata* colonies were most related to Cyanobacteria in the genera *Synechococcus* (n = 3) and *Prochlorococcus* (n = 1), alphaProteobacteria (n =1), betaProteobacteria (n = 1), and *Firmicutes* in the genus *Bacillus* (n = 4). The only sequence successfully identified from *P. astreoides* corals was related to *Oceanospirillales* sp., which we had previously found to compose >90% of the SML within healthy *P. astreoides* colonies (see Chapter 3). SML Sequences from corals near or interacting with *Dictyota menstrualis* were most related to gammaProteobacteria in the genus *Oceanospirillales* (n = 2), and *Actinobacteria* (n = 4). Sequences from corals near or interacting with *Halimeda opuntia* were more diverse and related to uncultured *Cyanobacteria*, betaProteobacteria, *Edwardsiella* sp. (n = 1) and *Escherichia coli* (n = 1) within the gammaProteobacteria, *Stappia* sp. (n = 1) within the Rhodobacterales in the alphaProteobacteria, and finally *Actinobacteria* (n = 2). *Actinobacteria* taxa were only found associated with macroalgae in this study.

Discussion

Most eukaryotes have been documented to associate with assemblages of microbial symbionts that aid in their development, health, and adaption to environmental conditions (Zilber-Rosenberg & Rosenberg 2008). Numerous studies have examined coral-microbial associates, particularly those related to coral disease, but few have examined healthy coral microbes and whether natural environmental interactions shift these assemblages. Our study demonstrates that two common Caribbean macroalgae appear to shift the microbial assemblages of contacted corals along their interaction zone, and within 5 cm of the coral-algal interface. Microorganisms associated with the coral species *P. astreoides* were more resistant to change than those associated with *M. faveolata,* and microorganisms associated with both coral species in the Florida Keys were more susceptible to change than those found in Belize or St. Thomas. The ability of a coral species to maintain a stable microbial assemblage regardless of environmental fluctuations and ambient stress levels may indicate which coral species and coral reefs are more robust and resilient. Here we define resilience as the capacity to absorb, reorganize, and adapt to change resulting from stressors or disturbances (Nystrom & Folke 2001, Mumby et al. 2007). These and previous results imply that *P. astreoides* is more robust and resilient than *M. faveolata,* from a holobiont perspective. This pattern is maintained across the

large geographic scale that we examined (>1000 km between sample sites), providing information to elucidate why *P. astreoides* continues to proliferate and resist disease on present-day coral reefs, whilst *M. faveolata* does not (Garzón-Ferreira et al. 2001; Lafferty et al. 2004).

Macroalgae are becoming increasingly prolific on coral reefs, thus their interaction with corals and other benthic organisms have also increased. For example, in the Florida Keys as coral cover declined by 38% between 1996 and 2000, brown macroalgae in the genus *Dictyota* sp. grew to cover nearly 56% of the benthos during the summer months and have now become dominant members of most Caribbean reefs between 0-25 m (Porter et al. 2002; Lirman & Biber 2000; Edmunds 2002; Beach et al. 2003). In the present study, *Dictyota* sp. interacted with up to ~ 70% of the corals surveyed and *Halimeda* sp. up to ~ 40%, and their release of both high levels of DOC that stimulate microbial activity, and potent secondary metabolites, may serve as the mechanisms that negatively affect competing corals and/or their microbial symbionts (Rashar & Hay 2010). Although two studies have documented the ability of macroalgae to cause microbially-driven hypoxic zones at the coral-algal interface (Smith et al. 2006; Barott et al. 2009), our study is the first to quantify changes in the microbial community structure along gradients of coral-algal interaction, and to show how these changes differ among three regions of the Caribbean.

Several hypotheses may explain why *M. faveolata*- associated microorganisms were more susceptible to the effects of encroaching macroalgae than were *P. astreoides* microbiota. Both *M. faveolata* and *P. astreoides* (Hexacorallia:Scleractinian) are commonly found throughout the Caribbean Sea and form mounding morphologies. Both species composed a large majority of the corals surveyed at all 3 locations during this study: *Montastrea* sp. composed ~10-40% and *Porites* sp. composed ~ 20-30%. Although they are both common, these coral genera evolved via two distinct phylogenetic lineages (Romano & Palumbi 1996; Medina et al. 2006), and vary in their reproductive life history strategies (Brooder vs. Spawner), which may play a role in microbial symbiont acquisition (Sunagawa et al. 2010). Because *P. astreoides* is a brooding coral and vertically transmits *Symbiodinium* from parent colony to planulae, it is possible that microbial symbionts are also transferred during spawning events (Harrison & Wallace 1990, Richmond & Hunter 1990). Koty Sharp and colleagues (*in press*) have provided evidence that bacterial cells are present in newly released larvae from *P. astreoides* colonies. Vertical transmission is common and often important to invertebrates, as it ensures the longevity

of host-microbial partnerships (Smith & Douglas 1987), and provides an opportunity for co-evolution and co-diversification of host and symbionts (Munson et al. 1991). The occurrence of vertically transmitted microbial symbionts within *P. astreoides* planulae might indicate a more evolved and stable relationship between host coral and microbial symbionts than for *M. faveolata.*. Thus, environmental changes and competitive interactions may be less likely to alter this symbiosis in comparison to a broadcasting coral (e.g. *M. faveolata*), that requires coral recruits to horizontally acquire microbial symbionts from the environment. Horizontal transmission is also very common in invertebrates, but the onset of the association depends on the availability of microorganisms in the environment, and the host requires a specific mechanism for acquisition (Moran & Baumann 2000; Nussbaumer et al. 2006; Kikuchi et al. 2007; Apprill et al. 2009). Therefore, *M. faveolata* may be at a disadvantage to *P. astreoides* in terms of its ability to maintain a specific and stable microbial symbiosis across generations and environmental gradients.

Another indication as to why *M. faveolata* and *P.astreoides* differ in their reactions to encroaching macroalgae may be disease susceptibility; *M. faveolata* can be afflicted by a host of bacterial diseases, including but not limited to White Plague, Dark Spot, Black Band, Yellow Blotch/Band, and Red Band, whilst *P. astreoides* has been associated with only two diseases thus far: White Plague and Yellow Blotch/Band (Garzón-Ferreira et al. 2001). In general massive reef building corals such as *Montastraea* species tend to be more susceptible to diseases than are many other types of corals (Lafferty et al. 2004). Members of the genus *Porites* also are able to fight fungal invasion by laying impenetrable walls of calcium carbonate (Le Champion-Alsumard et al. 1995; Ravindran et al. 2001), and some of their tissues are completely devoid of adhering microbes, suggesting the presence of strong mechanisms for microbial mediation (Johnston & Rohwer 2007). Colonies of *Porites* sp. thus may possess more host factors that permit the manipulation of microbial symbionts than do those of *M. faveolata.*

Our results revealed a large amount of inter-site variability, particularly between St. Thomas and the other two sites. Our previous studies also found that the highest diversity of coral-associated bacteria occurred in the SML of St. Thomas corals in comparison to Belize and Florida (see Chapter 3). Here, we documented few shifts in the microbial assemblages of either coral-algal species interaction studied in St. Thomas. We hypothesize that greater bacterial loads in the water column at St. Thomas may have caused an increase in "environmental noise" and

thus a failure to detect impacts of coral-algal competition on their microbial assemblages. DGGE excels at economically and efficiently revealing clear shifts in community structure across large numbers of samples, but may not be sensitive enough to quantify small-scale shifts, such as those possibly occurring on corals at St. Thomas, particularly if they are diluted with background noise. In St. Thomas, high levels of shoreline development and urban runoff combined with steep mountain slopes increase water turbidity and sedimentation onto the surrounding reefs (Brooks et al. 2007, Nemeth & Nowlis 2001). Heavy rainfall can also overload existing sewage systems, resulting in intermediate and severe pollution of coastal waters. High levels of rainfall during the week prior to sample collection may have exacerbated these point-source disturbances (July 19-29, 2009; weatherunderground.com/history/), causing lasting effects on the coral-associated microbial assemblages in St. Thomas, particularly because our study site was < 2.5 km from shore and near the Cyril E. King international airport in St. Thomas. Thus, impacts of competition with macroalgae on corals at St. Thomas may have been masked by larger-scale environmental disturbances on the reefs in comparison to Belize and the Florida Keys. Alternately, St. Thomas reefs may experience relatively large microbial fluctuations, but still remain healthy in comparison to Florida reefs.

The Florida Keys reef tract and adjoining Bays support extensive recreational and commercial harvesting activities (Ault et al. 2005), and are downstream from one of the world's largest water management systems, which has caused major alteration to the hydrology and quality of coastal habitats (Browder & Ogden 1999). Nutrient- and phytoplankton-rich water flows across tidal channels and through groundwater supplies onto Florida reefs, raising major concerns for surrounding reef health (Hu et al. 2003; 2004). Furthermore, Florida reefs are exposed to a massive number of snorkelers (2.9 million/yr) and divers (0.8 million/yr) each year (NOAA 2000 Census) that cause additional damage and stress to reef structures. The clearest microbial assemblage shifts that we observed on both *M. faveolata* and *P. astreoides* corals occurred in the Florida Keys, possibly because these corals are exposed chronically to multiple anthropogenic stressors (e.g. nutrient loading, sedimentation, diver damage etc.). Corals on Florida reefs therefore may be unable to maintain stable microbial assemblages when faced with the additional challenge of encroaching macroalgae or other competitive assaults.

This study is one of the first to examine coral-macroalgal competition from a microbial perspective. Our results likely are conservative, because they are based on field surveys of the

study organisms in their natural environment without manipulation, and the DGGE method that we used was not able to detect extremely fine-scale microbial differences in this system. However, using these methods we still detected a clear difference between the microorganisms associated with isolated coral controls and those associated with corals engaged in competitive interactions with macroalgae. In future studies we hope to continue to broaden our understanding of coral-algal-microbial relationships. It will be important to consider how coral species vary in the mechanisms they employ to mediate their consortia of microbial symbionts, including antimicrobial compounds from the host or microbial symbionts and/or host excretion of fixed carbon and soluble lipids that encourages microbial growth (Crossland et al. 1980; Brown & Bythell 2005). Corals exist in a diverse and dynamic environment of fluctuating conditions and interactions with other organisms. Based on the results of this study, the impacts of encroaching benthic organisms on the resident microbial assemblages of corals must be considered when collecting samples and drawing conclusions about coral-microbial relationships. Thus, previous evidence that coral-associated microbial assemblages from the same species vary when collected from different locations on the colony (Daniels et al. 2011), and/or from different geographic locations (Littman et al. 2009), may at least in part, be attributed to unreported or unidentified interactions with adjacent organisms. Determination of the microbial consequences of coral-macroalgal phase-shifts is another essential step toward determining which coral species are the most stable when exposed to stressful environmental conditions and changes. In particular, coral-microbial assemblages that shift markedly when their host colonies interact with encroaching or competing macroalgae (e.g. *M. faveolata*), may indicate that these coral host species, and the reefs on which they are dominant reef-builders, require enhanced protection and further conservation than those reefs composed of more robust and resilient coral species (e.g. *P. astreoides*).

Table 4.1: PERMANOVA results for all coral-algal interactions at each of three sites in the Caribbean (df = 4). Treatment variables were: coral control, macroalgal control, coral near macroalgae, coral-algal interaction zone, and macroalgae near coral.

PERMANOVA Results			F	R^2	P	*
M. faveolata	Belize	*D. menstrualis*	1.49	0.46	0.059	
		H. opuntia	1.43	0.4	0.034	*
	St. Thomas	*D. menstrualis*	3.41	0.53	**0.001**	**
		H. opuntia	2.9	0.51	**0.001**	**
	Florida	*D. menstrualis*	1.55	0.55	**0.034**	*
		H. opuntia	1.13	0.39	0.244	
P. astreoides	Belize	*D. menstrualis*	1.54	0.41	**0.019**	*
		H. opuntia	1.65	0.42	**0.008**	**
	St. Thomas	*D. menstrualis*	2.88	0.49	**0.001**	**
		H. opuntia	3.42	0.58	**0.001**	**
	Florida	*D. menstrualis*	1.56	0.51	**0.042**	*
		H. opuntia	2.29	0.57	**0.001**	**

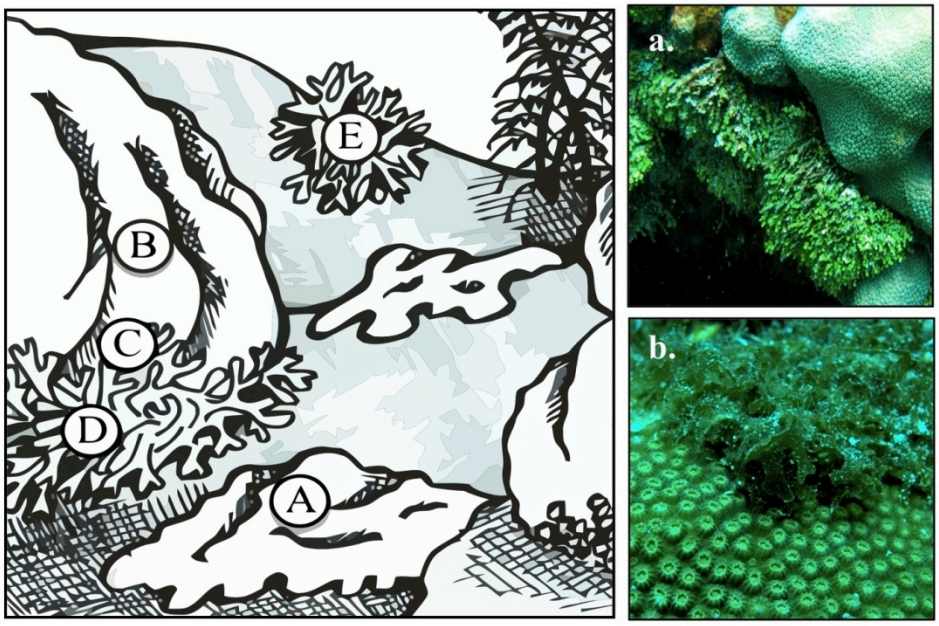


Figure 4.1: Depiction of the microbial sampling regime used to examine natural coral-algal interaction gradients (A-E; Morrow Chapter 4) and the effect of macroalgal extracts applied to coral surfaces (F-H; Morrow Chapter 5). Plastic tipped syringes (5 ml) were used to a sample 5 x 5 cm area for microbial analysis from A) Coral controls (*Montastraea faveolata* or *Porites astreoides*) not interacting with macroalgae or other benthic invertebrates, B) Coral surface mucus layer (SML) 5-cm from the coral-algal interface, C) Coral SML directly in contact with macroalgae, D) Macroalgal surface 5-cm from coral-algal interface, and E) Macroalgal control (*Dictyota menstrualis* or *Halimeda opuntia*) not interacting with corals or benthic invertebrates.

Photos depict: a) *Halimeda* macroalgae interacting with M*ontastraea* sp., b) *Dictyota* macroalgae interacting with *Montastraea* sp. *Photo credit: K. Morrow*

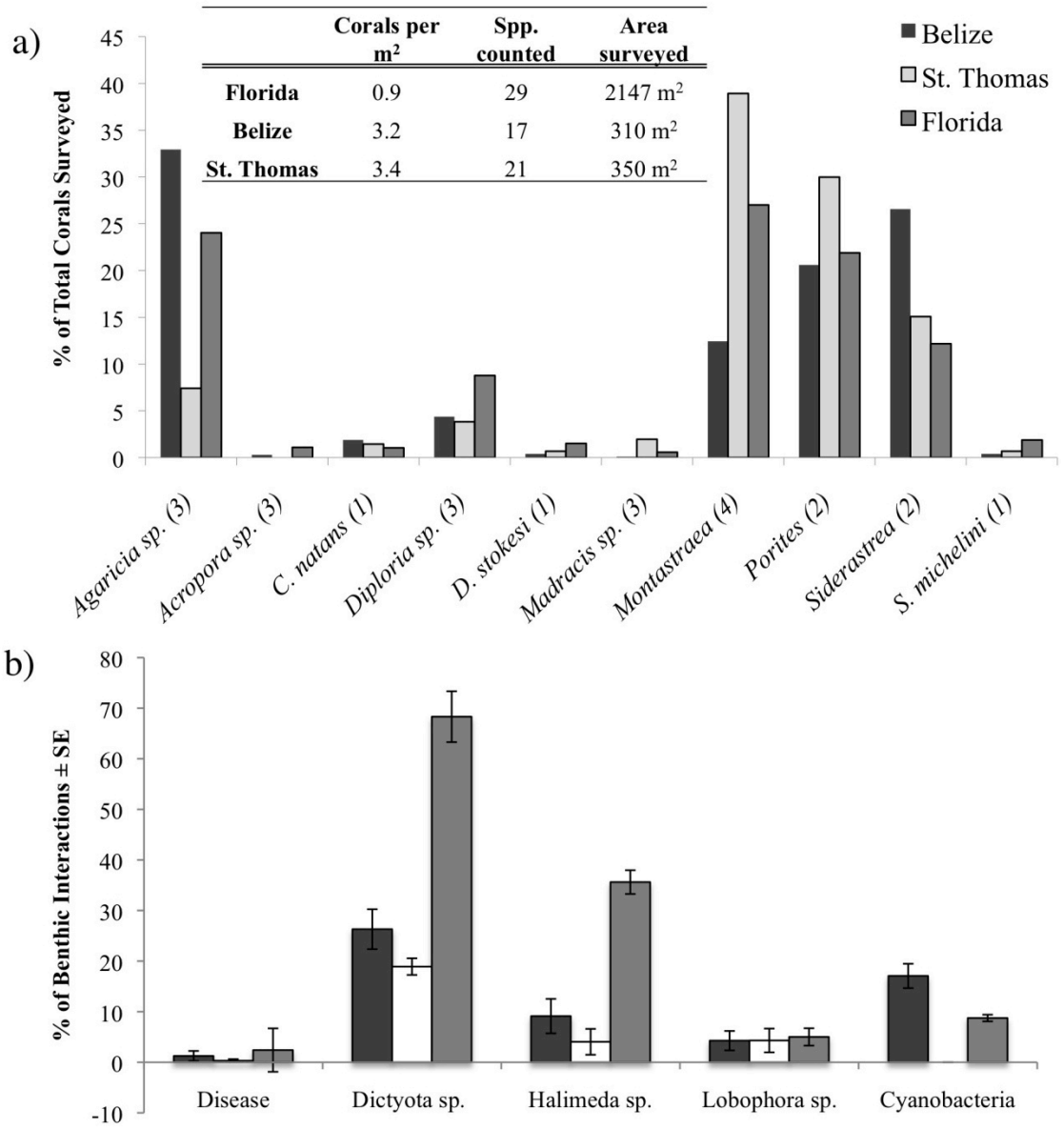


Figure 4.2: a) Coral diversity at all three sites (percent of total corals surveyed at each site). Florida data was collected during the 2009 NOAA Florida Keys National Marine Sanctuary Coral Health and Diversity Survey Cruise. Table depicts the number of corals per m^2, the number of species observed, and the total area surveyed over 2008 and 2009 in Belize and St. Thomas and in 2009 in Florida. The number in parentheses corresponds to the number of species within each genus. b) Percent of total corals ± SE surveyed that were diseased and/or interacting with macroalgae or cyanobacteria.

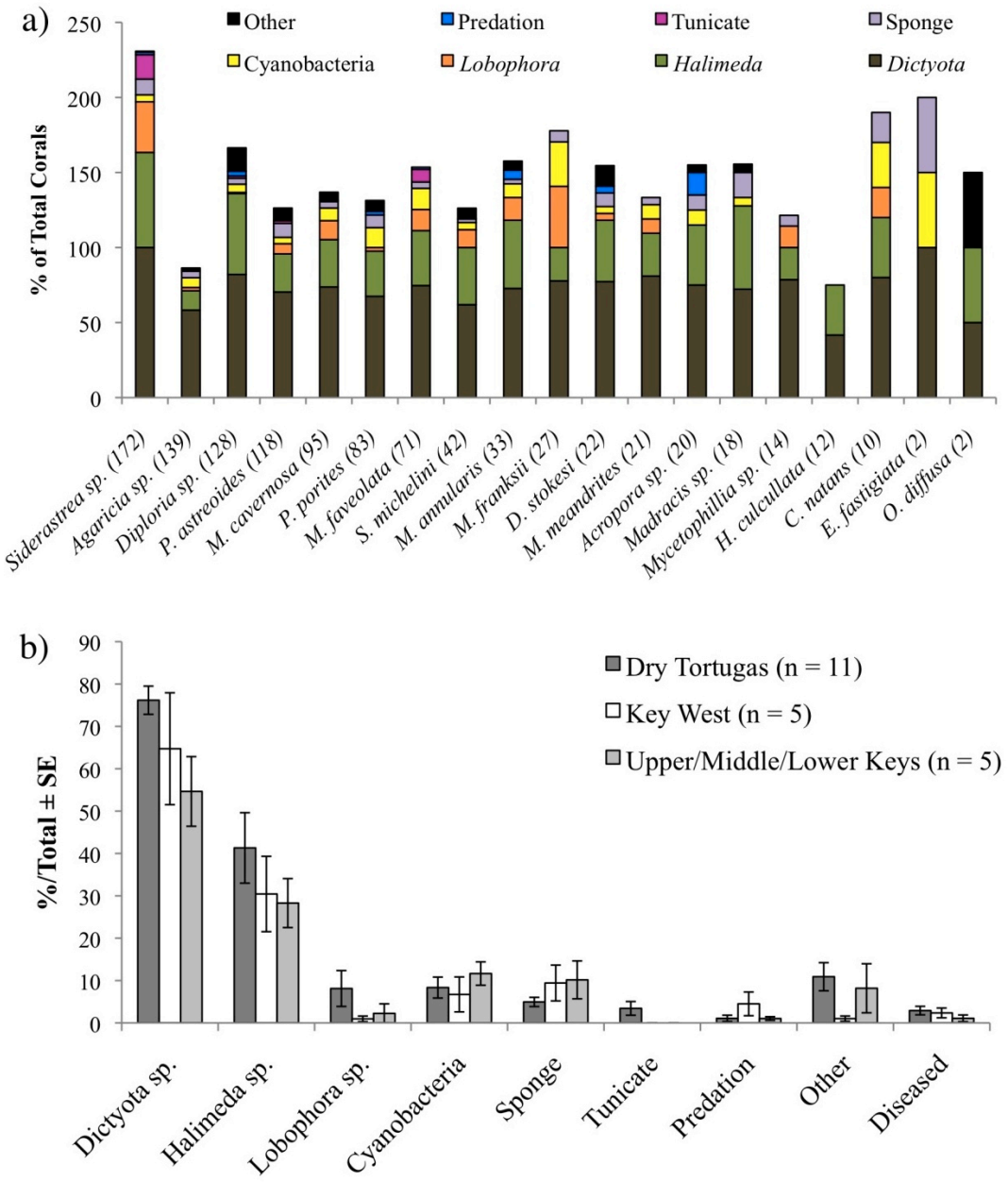


Figure 4.3: a) Most common corals across 21 sites surveyed during the September 2009 NOAA FKNMS Coral Healthy and Diversity Cruise and the percentage of those that were interacting with 8 different benthic components: Macroalgae within the genera *Dictyota, Halimeda,* or *Lobophora*; benthic cyanobacteria, sponges, tunicates, predation such as from corallivorous snails or fire worms, any other interacting benthic component and whether the coral exhibits symptoms of disease. The number within parentheses corresponds to the actual number of colonies surveyed from most to least abundant. b) Percentage ± SE of total corals that were interacting with macroalgae and/or invertebrates in the Dry Tortugas National Park, Key West region, and Upper/Middle/Lower Keys region, which includes Carysfort, Molasses, Sombrero, and Looe Key reefs. Also includes the percentage ± SE of diseased corals within the census (< 2% of total corals).

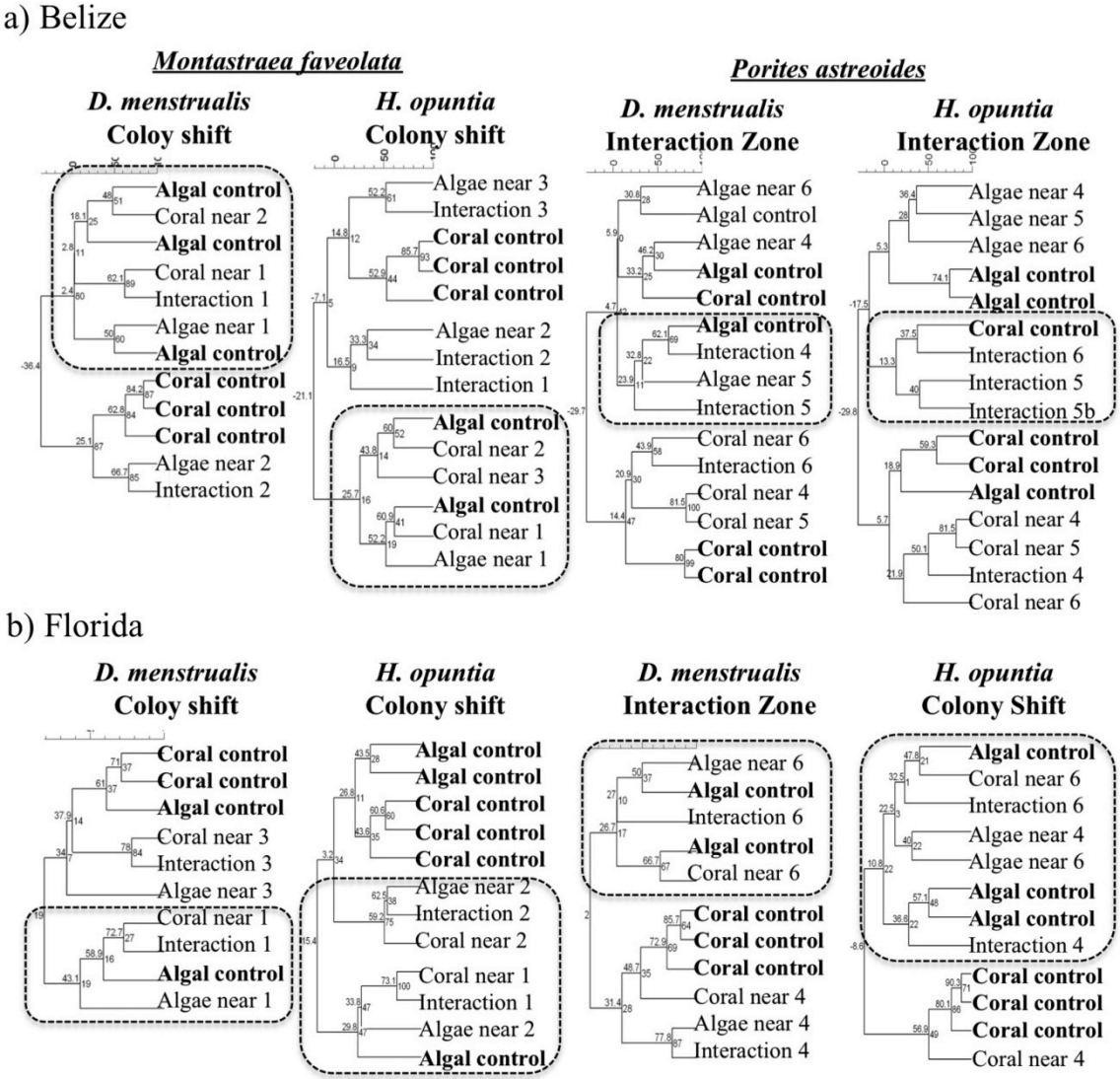
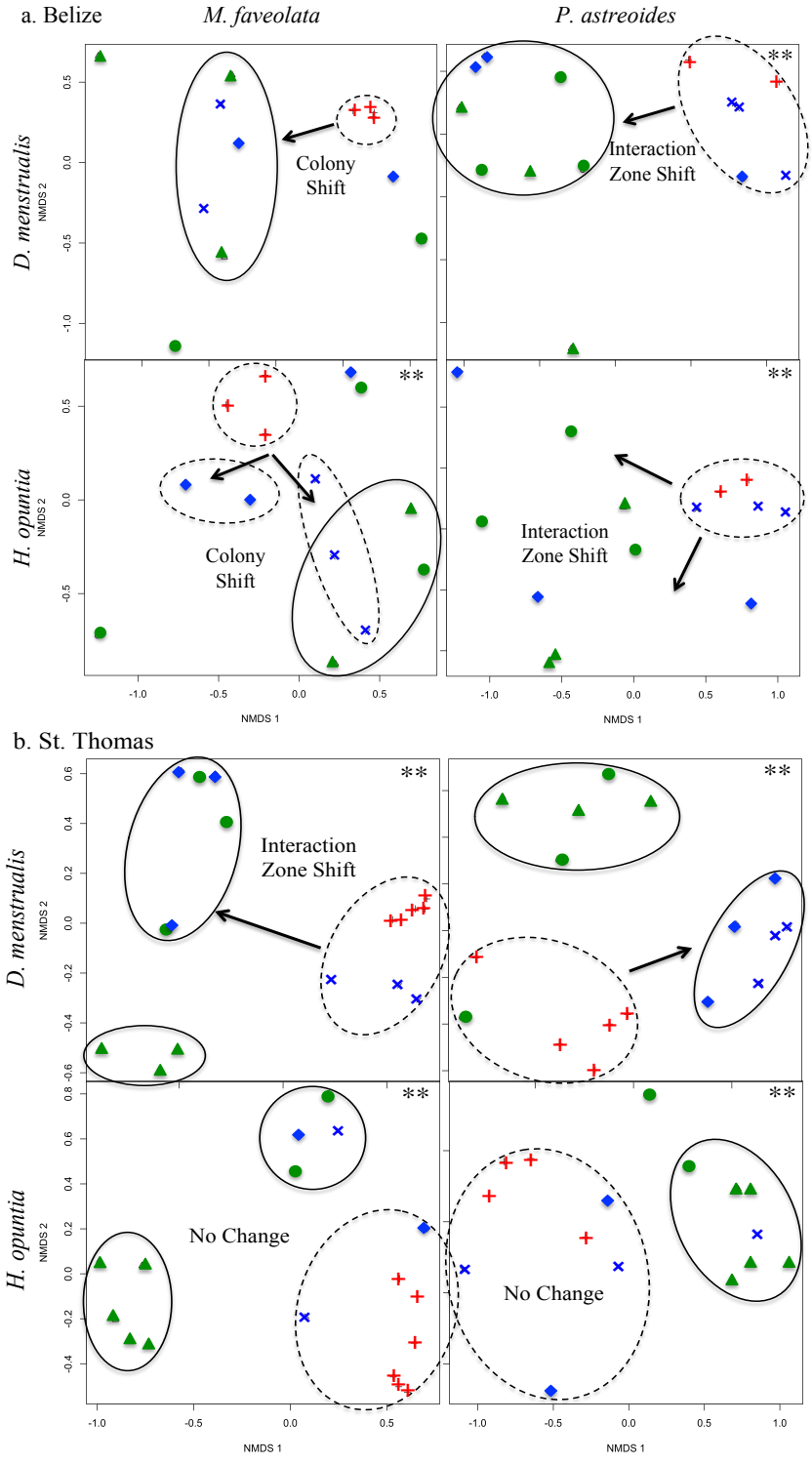


Figure 4.4: Hierarchical cluster analyses using WARD algorithms and DICE coefficients based on band presence/absence from DGGE profiles. Bacterial assemblages from *M. faveolata* and *P. astreoides* interacting with *Dictyota menstrualis* and *Halimeda opuntia* macroalgae in Belize and Florida were analyzed separately. No pattern was shown in St. Thomas and interactions are not represented. Dotted lines enclose microbial shifts toward macroalgal-associated microbial communities. Coral and algal controls are in bold for easier reference. Exterior tree numbers are percent similarity and interior tree numbers are bootstrap support based on 1000 iterations.


a. Belize
M. faveolata
P. astreoides
D. menstrualis
H. opuntia
NMDS 1
NMDS 2
Colony Shift
Interaction Zone Shift
**
b. St. Thomas
No Change

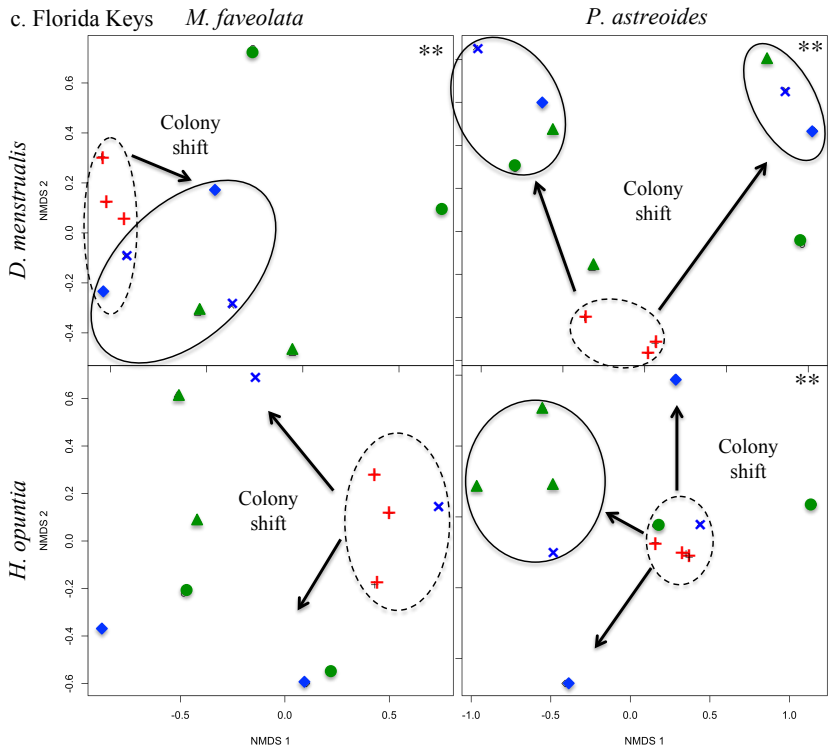


Figure 4.5: nMDS plots based on DGGE profiles for each coral-algal interaction (n = 4) between *M. faveolata* and *P.astreoides* corals and *D.menstrualis* and *H. opuntia* macroalgae at each sites: a) Belize, b) St. Thomas, and c) Florida. Solid circles enclose clusters related to macroalgal-associated microbes and dashed circles enclose clusters related to coral control-associated microbes. Arrows indicate potential shifts in microbial populations. ** indicates a significant PERMANOVA test ($P < 0.05$).

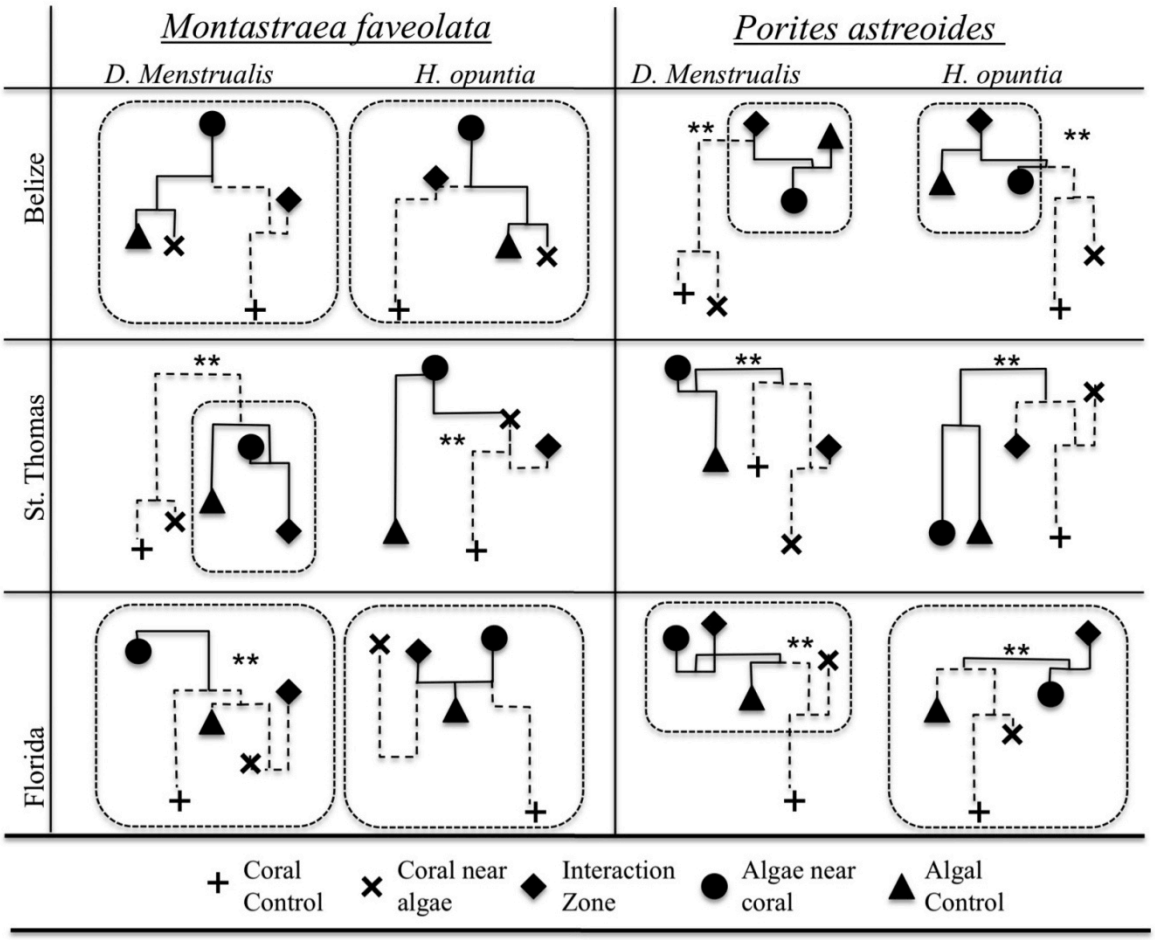


Figure 4.6: Meandist dendrograms of the relationship among microbial assemblage DGGE profiles from the 5 sampling regions (coral control, coral 5-cm from algae, interaction zone, algae 5-cm from coral, and algal control) at all sampling sites (Belize, St. Thomas, and Florida). Dashed lines connect coral samples and solid lines connect algal samples. Coral-algal pairs that are entirely enclosed by a dotted line indicate coral colony microbial shifts to macroalgal bacteria. Dotted lines that partially enclose regions indicate a shift to macroalgal bacteria at the interaction zone but not on the entire colony.

Chapter 5

Allelochemicals produced by Caribbean macroalgae can alter coral-microbial assemblages *in situ.*

Introduction

Reef-building corals harbor eukaryotic and prokaryotic microorganisms that form dynamic mutualistic, parasitic, and commensal associations with the coral host, and exhibit substantial genetic and ecological diversity (Rohwer et al. 2002; Rosenberg et al. 2007, Ainsworth et al. 2009). Although many of the roles that microorganisms play in coral physiology and immune function remain unknown, recent evidence suggests that corals harbor specific and beneficial microbial assemblages (Kvennefors et al. 2010; Sunagawa et al. 2010; Morrow Chapter 3), that likely provide the first line of defense against bacterial or fungal infection (Banin et al. 2001; Shnit-Orland and Kushmaro 2009). Protection may be provided by the production of antibiotics and/or by microbial symbionts filling a niche that otherwise would be open to infection by opportunistic pathogens (Ritchie 2006). Detecting when and why shifts occur in healthy coral-microbial assemblages, prior to visual signs of disease, could be an informative indicator of negative environmental changes.

Macroalgae are becoming increasingly abundant competitors of present-day corals, especially on reefs where rates of herbivory are low and/or dissolved nutrients are high (reviewed in Chadwick & Morrow 2011; Fong & Paul 2011). Macroalgae maintain an arsenal of competitive mechanisms to physically and biochemically compete with corals and other reef invertebrates (reviewed in McCook et al. 2001; Fong & Paul 2010), and many have the ability to mediate the surrounding microbial landscape. Large amounts of organic carbon exuded by macroalgae into the surrounding environment naturally stimulates microbial colonization and reproduction near algal surfaces and within the water column (Steinberg et al. 2002; Lane & Kubanek 2008). Thus, macroalgae are naturally challenged by microbial fouling and disease, which sometimes causes mass mortalities (Harvell et al. 1999), but interestingly the majority of macroalgae remain healthy and devoid of heavy biofouling. Recent evidence suggests that

macroalgae may even facilitate a diverse and oftentimes specific assemblage of beneficial microorganisms that can utilize algal-derived sugars and assist in the decay process of algal fronds (Goecke et al. 2010; Barott et al. 2011).

These observations coupled with recent antibacterial assays have revealed that macroalgae can often mediate deleterious microorganisms through the production of surface-associated chemical defenses (Engel et al. 2002; Puglisi et al. 2004; Paul et al. 2006; Jiang et al. 2008). Macroalgae can actively respond to microbial challenge by releasing reactive oxygen species (ROS), and producing defensive secondary metabolites that prevent bacterial communication, swarming, and attachment to surfaces (Engel et al. 2006; Lane & Kubanek 2008, Maximilien et al. 1998, Manfield 1999). One of the largest groups of algal-derived antifungal chemical defenses, which included Bromophycolides and callophycoic acids, was recently discovered within the red macroalga *Callophycus serratus* (Lane et al. 2009). Another red macroalga, *Bonnemaisonia hamifera,* was found to naturally mediate epibiotic bacteria by producing broad-spectrum growth inhibiting secondary metabolites (Nylund et al. 2008). Finally, one of the brown macroalgae examined in the present study, *Lobophora variegata*, was previously shown to produce a 22-membered lactone, lobophorolide, which demonstrated activity against pathogenic and saprophytic marine fungi (Kubanek et al. 2003). Numerous studies have revealed that macroalgae are a rich source of antifouling and antibacterial compounds (reviewed in Goecke et al. 2010), and these are just a few examples of the thousands of marine secondary metabolites that have been identified (Hay 1996), many of which play an important and dynamic role in ecological interactions.

Few studies, however, have examined whether algal-derived compounds, including both secondary metabolites and other dissolved organic compounds, can influence coral physiology and microbial assemblages. On the organismal scale, allelopathy has been implicated in the reduction of coral larval settlement (Birrell et al. 2008, Paul et al. 2011), and reduced coral growth rate and fecundity (McCook et al. 2001; Foster et al. 2008). Common Pacific macroalgae and their lipophilic extracts caused bleaching and tissue mortality in ~ 40-70% of colonies when placed in direct contact with coral tissues and herbivores were removed (Rashar & Hay 2010). Coral reefs dominated by macroalgae experience lower levels of oxygen and higher abundances of heterotrophic bacteria, including potential pathogens, in the overlying seawater (Haas et al. 2010; Dinsdale & Rohwer 2008). Excess labile carbon excreted by macroalgae into the water

column is believed to stimulate microbial activity (Haas et al. 2010), and is correlated with zones of hypoxia and tissue mortality at coral-algal interfaces (Smith et al. 2006; Barott et al. 2009). Coral contact with the green alga, *Halimeda opuntia*, appeared to trigger white plague type II disease, and the causative bacterium, *Aurantimonas coralicida* was found on *H. opuntia* thalli (Nugues et al. 2004). One culture-dependent study found that lipophilic and hydrophilic extracts from common Caribbean macroalgae could stimulate and inhibit coral-associated microorganisms, but activity varied markedly by algal and bacterial species (Morrow et al. 2011). The most consistent result was that hydrophilic (polar) compounds produced by the brown alga, *L. variegata,* inhibited > 90% of the coral-associated bacteria assayed. These studies caution that abundant macroalgae and the compounds they produce are liable to undermine overall reef health and stability by altering the healthy microbial assemblages associated with corals and act as reservoirs for harmful epizootics. However, many of these studies are preliminary, and the general importance of coral-algal-microbial associations requires much additional research.

The goal of the present study was to examine the effect of macroalgal extracts on coral-microbial assemblages *in situ* using culture-independent methods. We incorporated crude extracts from 3 common Caribbean macroalgae into stable gels at natural concentrations and applied them directly to *Montastraea faveolata* and *Porites astreoides* corals on reefs in both Florida and Belize for ~72 hrs. Denaturing gradient gel electrophoresis (DGGE) was used to examine changes in the microbiota associated with the surface mucus layer (SML) of both coral species. Macroalgal extracts had no visible impact on experimental coral colonies, but most extracts caused a detectable shift in coral-microbial assemblages. Some extracts were more potent, causing the assemblages on the entire colony to shift to a new microbial state, whereas others had little to no impact. Both coral species were affected by extracts, but the microbiota within the SML of *P. astreoides* corals were slightly more susceptible to the effects of macroalgal compounds, particularly in Florida.

Methods

Macroalgal Collection and Extraction

Macroalgae were collected by hand on SCUBA at depths of 8-15 m between 2005-2008 in the Florida Keys (*D. pulchella*), Belize (*Dictyota sp., H. tuna*), and St. Thomas, U.S. Virgin

Islands (*L. variegata*; Table 5.1). All samples were placed in plastic zip-lock bags at depth and brought to the surface, then placed in seawater-filled coolers and transported back to the laboratory (< 3 h). Clean seaweeds, free of substantial epiphyte growth or other macroscopic material, were frozen at -20°C. Samples of the green alga *H. tuna* were flash-frozen in liquid nitrogen to prevent degradation of the diterpenoid compounds (Paul & Van Alstyne 1992). All frozen algal samples were transported on ice to the Smithsonian Marine Station in Fort Pierce, FL for chemical extraction and analysis. These macroalgae were chosen based on their previously identified chemical activity against coral reef microorganisms (Morrow et al. 2011).

Extraction of compounds from macroalgae was conducted at the Smithsonian Marine Station in Fort Pierce, FL. Frozen bulk samples of macroalgae were first wet-weighed and then lyophilized over several days. Lyophilized samples were weighed and exhaustively extracted with a 1:1 ethyl acetate:methanol (lipophilic or non-polar extract) solvent solution over 3 consecutive 24-h periods, followed by three 24-h extractions in 1:1 ethanol:deionized water (hydrophilic or polar extract). Extracts were filtered to remove large fragments of organic material and the solvents removed via rotary evaporation at 35°C in a Thermo-Savant speed-vac concentrator. After which, concentrated extracts were frozen (-20°C) and transported to field laboratories for extract assay experiments. We tested extracts at natural concentrations based on algal wet weight (g) (Table 5.1), similar to methods used by Puglisi et al. 2007. Extract concentrations were also determined as g dried extract per g of sample tissue.

Algal Extract Assay

Assay techniques were adapted from previous studies that examined the effect of sponge metabolites on coral photosymbionts (Pawlik et al. 2007). Assay experiments were conducted on both *Montastraea faveolata* and *Porites astreoides* corals at 10-15 m depth on Wonderland Reef adjacent to the Mote Marine Laboratory in Summerland Key, FL (May 2009) and on South Water Cay Reef adjacent to the Smithsonian's Carrie Bow Cay Field Station in Belize (August 2009). Extracts were re-suspended in 1-ml of 95% ethanol and incorporated into polysaccharide gels (Phytagel plus freshwater) at natural concentrations then poured into prepared 5-cm plastic petri dishes leaving the gel surface 2-3 mm below the edge of the petri plate. Each dish was prepared by covering the outside with duct-tape to maintain consistent light levels when applied to the coral surface. Holes were drilled into opposing sides of the petri dish and a zip-tie

threaded through, to which a bungee cord was attached. The metal hooks on each bungee cord were bent to 90° angles to allow optimal attachment to corals.

On SCUBA, after extracts were poured and solidified, divers inverted each petri plate so that the gel surface faced the coral colony. The gel remained 2-3 mm above the colony surface once attached, so as not to smother the coral tissues. Petri plates were fixed in place by stretching the attached bungee cord around coral colonies and tapping each bungee cord hook into the coral skeleton (Figure 5.1a & 1b). Subsequent monitoring showed that corals quickly recovered (< 1 wk) from bungee cord attachment. For each experimental colony, we attached three inverted petri plates: 1) shading/plate control with no gel, 2) solvent control with gel but no extract, and 3) treatment gel containing macroalgal extract. Immediately prior to petri-plate attachment 5-ml plastic tip syringes were used to collect triplicate samples from the coral SML (5 x 5 cm area) as an initial microbial assemblage control. Each syringe was capped pre- and post-sample collection to prevent additional seawater contamination and sterile nitrile gloves were worn to reduce human bacterial contamination. This process was replicated on five healthy coral colonies of each coral species and each extract type in Florida (n = 4) and Belize (n = 4; Table 5.1).

Deployed petri plates were monitored when possible to ensure they were undisturbed and remained in place. After 72 hrs of exposure, microbial samples were collected, as described above, from areas of the coral SML not exposed to petri-plates. A 5-ml microbial sample was also collected from the SML directly under each petri-plate treatment (Figure 5.1). Additionally, after syringe samples were taken, a small (1x1 cm^2) sample of coral tissue was chiseled off of the colony from under each petri-plate treatment, and from a location on the colony that was not exposed to treatments. Tissue samples were immediately returned to the field laboratory and flash frozen in liquid nitrogen for subsequent biomarker and gene expression analyses by collaborators at the University of North Florida. Tissue samples were only taken in Belize during the summer of 2009, and not in Florida. Florida Keys experiments were conducted under the Florida Keys National Marine Sanctuary research permit #FKNMS-2008-019.

After samples were brought to the surface, syringes were placed in seawater-filled coolers and transported back to the laboratory (< 3 hrs), where they were immediately processed for transport and subsequent culture-independent analyses. Syringes were placed tip down in test-tube racks for ~15 minutes to allow the mucus to settle to the bottom, then 2 ml of

concentrated mucus was transferred to cryovials and centrifuged at 10,000 x *g* for 10 min. The seawater supernatant was poured off and the remaining mucus pellet frozen at -20°C. Microbial samples were transported back to Auburn University on ice and thawed at 4°C prior to DNA extraction using the MOBIO Ultraclean® Microbial DNA Isolation kit (Carlsbad, CA), according to the manufacturers instructions, with an additional (10 min) heating step to 64°C to increase DNA yield. Extracted DNA was stored at -80°C until PCR amplification.

Live Algal Application

Live samples of the brown macroalgae *L. variegata* and *Dictyota* sp. were collected at South Water Cay reef in Belize and kept in running seawater tables overnight. Thalli of approximately the same weight (see results for means) were gathered and attached to bungee cords with small zip-ties. Fake aquarium plants of similar size and shape to live macroalgae were also attached to bungee cords with zip-ties and left in running seawater tables overnight. Using methods similar to those outlined above, live macroalgae and fake plant controls were attached to both *M. faveolata* and *P. astreoides* corals at ~8-15 m depth on South Water Cay Reef, Belize (Figure 5.1c & 1d). As above, triplicate samples were taken from the coral SML prior to algal application to serve as initial microbial assemblage controls. After 72 hrs, another set of triplicate SML samples were taken in addition to a microbial sample from under the live macroalgae and fake aquarium plants. Samples were transported and processed as outline above. All macroalgae and aquarium plants were weighed after the experiment was completed. Unfortunately, the majority of samples collected from *P. astreoides* corals were lost, thus we will only report results from the *M. faveolata*-algal interaction experiment.

PCR Amplification and DGGE Analysis

Universal bacterial primers 27F-GC (5' -CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG CAG AGT TTG ATC MTG GCT CAG-3') and 518R (5' -ATT ACC GCG GCT GCT GG-3'), were used to amplify the 16S rRNA gene from bacterial isolated genomic DNA from coral mucus. The forward primer was modified to incorporate a 40-bp GC *clamp* for resolution on a denaturing gradient gel electrophoresis (DGGE) system (Muyzer et al. 1993; Ferris et al. 1996). These primers amplify a 491-bp section of the 16S rRNA gene of members of the domain *Bacteria*, including the highly variable V1-V3 regions (Ashelford et al.

2005; Huse et al. 2008). All PCR was performed on a thermalcycler (model: Master cycler epgradient, Eppendorf, Hauppauge, NY) as follows: 12.5 µl EconoTaq PLUS GREEN 2X Master Mix (Lucigen), 0.5 ul of each 20 uM primer, and adjusted to a final volume of 25 µl with nuclease-free water. Strip tubes, master mix and nuclease free water were UV-irradiated for 20 minutes prior to the addition of primers under sterile conditions in a laminar flow hood to reduce contamination (Millar et al. 2002). DNA template was added during an initial 'hotstart' of 3 min at 94°C, followed by a 'touchdown' PCR protocol, in which the annealing temperature was decreased from 65°C by 1°C every cycle until reaching a touchdown temperature of 54°C, at which temperature 35 additional cycles were performed as follows; 94°C for 45 sec, 54°C for 45 sec, and 72°C for 1.5 min; and 1 final cycle at 94°C for 45 sec, 54°C for 45 sec, and 72°C for 7 min followed by cooling to 4°C. PCR products were analyzed by agarose gel electrophoresis (1% w/v agarose) stained with ethidium bromide and visualized using a UV transilluminator.

Samples were separated using a Hoefer SG50 (Hoefer Inc.) DGGE system. PCR products were loaded onto an 8% acrylamide gel and run with 0.5 X TAE buffer (Tris base, acetic acid, EDTA) and a 35-60% linear denaturing gradient of formamide and urea. Gels were electrophoresed at 60°C for 15 min at 50 V, and subsequently for 10 hrs at 100 V (or 1000 V·hrs) on the DGGE system. After electrophoresis the gels were stained for 30 min with 1X SYBR-Gold nucleic acid stain (Invitrogen) in 0.5 X TAE buffer, rinsed, and photographed using a UV transilluminator (AlphaImager, Cell Biosciences). Images were saved as 8-bit TIFF files followed by alignment, normalization, band class identification, and statistical analysis using Bionumerics V. 5.0 (Applied Maths).

Band Excision and Sequencing

Uniquely dominant and distinct bands were dabbed with a sterile pipette tip and placed directly into PCR strip tubes containing UV-sterilized nuclease free water. Bands were re-amplified with the previously described touchdown protocol using the 27F/518R primer set without the -GC clamp. PCR products were analyzed with agarose gel electrophoresis (1% w/v agarose) stained with ethidium bromide and visualised using a UV transilluminator. An ammonium acetate-ethanol precipitation was performed and the resulting product amplified using the BigDye® sequencing reaction; 1.0 µl of BigDye®, 1.5 µl of 5x Buffer, 0.5 µl of 10 µM 27F, 4 µl of nuclease free water, 3 µl of Template. The following thermocycler conditions

were used; 95°C for 30 s, 50°C for 30 s, and 60°C for 4 min, at which temperature 30 additional cycles were performed. PCR products were purified using the BigDye® XTerminator Purification Kit (Applied Biosciences) and shipped to the Smithsonian Institution Laboratories of Analytical Biology in Suitland, MD for sequencing. The Smithsonian Institution performed high-throughput (96-well) sequencing on an ABI sequencer. Sequences were trimmed using CLC Genomics Workbench (CLC Bio) and blasted against the NCBI Blastn database and those sequences with > 96% identity and E-values < 1×10^{-20} were accepted for downstream analysis.

DGGE Profile Analysis

DGGE images were imported into Bionumerics V 5.0 (Applied Maths) and subjected to a series of steps to allow multiple gel images to be reliably compared, these were: identify sample lanes, apply a background subtraction, normalize to the reference standards run on each gel, and identify and quantify bands. Sample comparison and band matching was initially conducted in Bionumerics, in which band classes were constructed based on optimal position tolerance and optimization settings. A bifurcating hierarchical dendrogram and similarity matrix representing sample clusters was initially constructed for each coral-algal interaction at each site using the WARD algorithm and DICE coefficients derived from the band alignment. A binary matrix based on band presence/absence was exported from Bionumerics, converted to a distance matrix and analyzed using the R statistical package (Ihaka & Gentleman 1996).

Kruskal's nonmetric multidimensional scaling (nMDS) analysis and permutational multivariate analysis of variance (PERMANOVA) were used to assess the multivariate relationships among and between binary DGGE profiles for each coral-extract species pair at each site (Florida & Belize). A highly significant PERMANOVA result suggests that initial coral microbial assemblages are significantly different from extract- and/or control-associated microbial samples. The Euclidean distances (we used Jaccard) between points in an nMDS plot are inversely proportional to the similarity of the samples. The number of dimensions (k) was determined by first running a scree plot to determine stress (i.e. an inverse measure of fit to the data) as a function of dimensionality. Kruskal's stress formula was used as an informal method of determining the appropriate number of dimensions (McCune & Grace 2002). These data were analyzed using the metaMDS and Adonis utilities within the Vegan package in R. metaMDS is unique in that it calls on isoMDS to perform nMDS, but also searches for the most stable

solution by performing several random starts (we used 20; Ihaka & Gentleman 1996). The relationship among samples is represented in a plot of the first two nMDS dimensions (Figure 5.2).

A final analysis of the data was completed using Multiple Response Permutation Procedure (MRPP) and related Meandist functions within the Vegan package in R. We combined the three post-treatment controls (shading, solvent, and post SML) into a 'treatment controls' group. Thus, MRPP determined whether the 3 sampling treatments (initial control, treatment controls, and extract treatment) formed distinct groupings based on their DGGE profiles. In an MRPP analysis samples are *a priori* assigned to 2 or more groups (in our case 3 groups) and used to calculate a distribution of the average intra-group distance by randomly permutating the data *n* times (in our case n=5000), which determines a *p*-value for the observed intra-group distance in the data. Additionally, an *A* parameter is calculated that relates the observed intra-group average distance to the mean of the calculated distribution. When the value of *A* is close to 1 it is indicative of very tight groupings. We performed MRPP separately on each of 6 coral-extracts pairs, *M. faveolata* and *P. astreoides* corals paired with lipophilic extracts *H. tuna* and both lipo- and hydrophilic extracts of *L. variegata* at both sites. We did not analyze *Dictyota* sp. extracts using MRPP because of the negative-result illustrated with nMDS (Figure 5.2a). Additionally, the function, Meandist, illustrates a matrix of mean within-cluster dissimilarities (diagonal) and between cluster dissimilarities (off diagonal elements) based on MRPP analysis. Meandist was used to construct dendrograms of result matrices based on the within-group and between group dissimilarities (Figure 5.2). Horizontal lines within the histogram are drawn at the level of mean between-cluster dissimilarity and vertical lines connect within-cluster dissimilarities to this line (Van Sickle 1997).

Results

DGGE Results

We examined the effect of four macroalgal extracts on the microbial assemblages associated with the SML of *M. faveolata* and *P. astreoides* corals in Belize and Florida using PCR-DGGE of the 16S rDNA gene. DGGE profiles based on 16S rRNA universal bacterial primers (27F/518R) revealed diverse bacterial assemblages for all samples with intraspecific similarity among initial controls. Extracts caused a range of effects on coral bacteria, but

generally increased in activity from *Dictyota* sp. to *H. tuna* to *L. variegata* (lipophilic) to *L. variegata* (hydrophilic), respectively. Due to technical issues during collection and extraction all samples exposed to *Dictyota pulchella* extracts in Florida were lost, but we did analyze the impact of extracts from *Dictyota* sp. on corals in Belize. DGGE analysis suggests that neither *Dictyota* sp. (lipophilic) extract nor treatment controls had a significant effect on microbial assemblages in either coral species in Belize (Figure 5.2a). Thus, we are confident that our experimental controls for shading and solvent did not significantly alter coral microbial assemblages on either coral species.

Based on permutational MANOVA results (Table 5.2) and nMDS plots (Figure 5.2b), lipophilic extracts from the green calcareous alga, *Halimeda tuna,* did cause a shift in the microbial assemblages of *P. astreoides* corals ($P < 0.001$) in Florida. However, the same extracts did not significantly shift the microbial assemblages associated with either coral in Belize (Figure 5.2b and Figure 5.4). The effect may not have been detected in Belize because only two of the five replicates proved to be usable sample sets after collection and extraction. Hierarchical cluster dendrograms compared *P. astreoides* microbial samples exposed to *H. tuna* in Belize (Figure 5.3a) and Florida (Figure 5.3b) and illustrated the significant differences between initial SML samples collected prior to extract exposure and all treatment samples collected 72 hrs after exposure in Florida; again there were no differences detected among treatment types in Belize.

Microbial samples from *M. faveolata* corals exposed to lipophilic extracts from the brown alga *L. variegata* were significantly altered in Belize (PERMANOVA; $P = 0.004$), but not in Florida ($P = 0.738$; Table 5.2), when compared to initial controls. The opposite was true for *P. astreoides* corals; a significant change was detected on Florida corals (PERMANOVA; $P < 0.001$), but not in Belize ($P = 0.126$; Table 5.2). Based on nMDS plots (Figure 5.2c) and Meandist dendrograms (Figure 5.4), the entire colony microbial assemblages were shifted by *L. variegata* (lipophilic) extracts on *M. faveolata* corals in Florida and on *P. astreoides* corals in Belize. This is not the case for *M.faveolata-L.variegata* (lipophilic) interactions in Florida, where the extract primarily had a point-source effect, directly under the applied extract, and not on the surrounding colony microbiota ($P = 0.738$; Table 5.2). There was no effect of *L. variegata* (lipophilic) extracts on *P. astreoides* in Belize, which is particularly evident in meandist dendrogram results (Figure 5.4).

Finally, *L. variegata* (hydrophilic) extracts had the most extensive effect on all coral-microbial assemblages at both sites. Results suggest that *L. variegata* (hydrophilic) extracts altered the entire coral colony microbiota, including all treatment controls (shade & solvent) and post-SML samples, to a specific assemblage that is significantly different in structure from initial SML microbial samples (PERMANOVA; $P < 0.01$; Table 5.2; Figure 5.2d). Similarity results based on hierarchical cluster analyses showed that *P. astreoides* post treatment samples are approx. -36% similar to initial samples from the same colonies, and *M. faveolata* post treatment samples are approx. -7% similar to initial samples from the same colonies. Overall, hydrophilic extracts from *L. variegata* had the most significant impact on coral SML microbial assemblages. From a coral species perspective, *P. astreoides* microbial assemblages were slightly more susceptible (4:7 interactions were significant) to change as a result of extract application in comparison to *M. faveolata* corals (3:7 interactions), particularly in Florida (Figure 5.4).

Results from the DGGE analysis of microbial samples from *M. faveolata* corals exposed to *L. variegata* and *Dictyota* sp. live macroalgae were complimentary to the extract application results. On average, we applied thalli of *L. variegata* macroalgae that weighed 1.5 g ± 0.3 SE and 1.4 g ± 0.2 SE of the corresponding fake aquarium plants to *M. faveolata* colonies in Belize. Hierarchical cluster analyses (Figure 5.5a) illustrate that initial microbial control samples cluster separately from microbial samples taken after *L. variegata* application. nMDS plots (Figure 5.5b) and PERMANOVA results ($P = 0.006$; Table 5.2) illustrate that samples taken from under *L. variegata* macroalgae and fake aquarium plants were significantly different from initial and post controls. On average, we applied thalli of *Dictyota* sp. macroalgae that weighed 0.5 g ± 0.1 SE of and 1.2 g ± 0.2 SE of the corresponding fake aquarium plants to *M. faveolata* colonies in Belize. Hierarchical cluster analyses did not illustrate a discernable effect of macroalgae to coral microbial assemblages. Most microbial samples (initial control, post control, macroalgae, and fake plant) clustered by coral colony (Figure 6a). These data were analogous to previously reported extract results and supported by nMDS plots ($P = 0.896$; Figure 5.2a) and PERMANOVA results (Table 5.2), which showed little effect of *Dictyota* sp. macroalgae on *M. faveolata* coral-microbial assemblages.

Sequencing Results

Of the 150 bands excised and sequenced from DGGE gels only 18 sequences were sufficiently identified. This difficulty was presumably due to contamination from adjacent bands during band excision. Sequences from initial SML samples prior to extract application closely matched the common bacteria found in a previous 454-pyrosequencing study (see Chapter 3) that examined microbial assemblages associated with natural coral-algal interactions.

Sequences with > 96% identity within initial SML control samples of *M. faveolata* were most similar to the alphaProteobacteria within the genus *Rhodobacter* (n=1) and within the gammaProteobacteria in the genus *Edwardsiella* sp. (n=2) within the Enterobacteriales and also in the genus *Halomonas* sp. (n =1) within the Oceanospirillales. Sequences from *M. faveolata* treatment controls (post, shade, and solvent) were most related to members of the Oceanospirillales (n=1) within the gammaProteobacteria. Finally, sequences from *M. faveolata* SML samples collected from under *L. variegata* (lipophilic) extracts were most related to *Firmicutes* in the genus *Bacillus* sp. (n=1), *Actinobacteria* (n=1), and *Cyanobacteria* in the genus *Synechococcus* (n = 2). *Actinobacteria* taxa were found associated with macroalgae in a previous study as well (see Chapter 4). We were unable to identify sequences from samples under the other three extracts (*Dictyota sp., H. tuna, L. variegata* hydrophilic).

Sequences with >96% identity within initial SML control samples of *P. astreoides* were most related to *Firmicutes* in the genus *Bacillus* sp. (n=1), and gammaProteobacteria in the genus *Edwardsiella* sp. (n=2) within the Enterobacteriales, and in the genus *Halomonas* sp. (n =1) within the Oceanospirillales. Sequences from *P.astreoides* treatment controls (post, shade, and solvent) were most closely related to members of the gammaProteobacteria within the Oceanospirillales (n=1), within the Methylococcales in the genus *Methylomonas* sp (n=1), and within the Oceanospirillales in the genus *Halomonas* sp. (n=1). Finally, sequences from *P. astreoides* SML samples collected from under *L. variegata* lipo- and hydrophilic extracts were most related to gammaProteobacteria in the genus *Edwardsiella* sp. (n=2) within the Enterobacteriales, and members of the Oceanospirillales (n=1). We were unable to identify sequences from samples under the remaining two extracts (*Dictyota sp., H. tuna*.).

Discussion

The study of coral-algal competitive interactions can be physical or chemical in nature, but we demonstrate here that macroalgal-allelopathy is correlated with shifts in coral-microbial assemblages to a different assemblage entirely. Whether these shifts are advantageous to competing macroalgae and/or detrimental to the coral host is not yet known and requires further research. We examined the effect of four different macroalgal extracts on both *M. faveolata* and *P. astreoides* coral microbial assemblages on Florida and Belize reefs. Concurrently, we studied the effect of two different species of macroalgae applied directly to *M. faveolata* coral colonies in Belize. We did not record any visual signs of degradation or bleaching, suggesting that the experimental gels and algal thalli were not smothering the coral tissues and that the photosynthetic potential of coral tissues was not significantly affected. We found that extracts from different species of macroalgae had variable and sometimes widespread effects on coral-associated microbiota and that these effects were not always consistent at both sampling sites. A series of studies have shown that the release of organic carbon by benthic macroalgae can be detrimental by enhancing microbial colonization of live corals (Kline et al. 2006; Smith et al. 2006), which creates areas of hypoxia and subsequent coral tissue mortality (Barott et al. 2009). However, this is the first study to provide additional evidence of changes in the structure of coral-associated microbial assemblages as a result of macroalgal-allelochemical activity *in situ*.

Macroalgal extracts and live thalli from *Dictyota* sp. had the least effect on coral microbial assemblages using PCR-DGGE. These negative results were important because they established that our treatment controls for both experiments did not have detectable effects on the coral microbiota. Thus, we make the assumption that the changes identified in the microbial assemblages during concurrent experimental treatments were a result of the applied extracts. We chose *Dictyota* sp. brown macroalgae because members of this genus are dominant members of the benthos throughout the Caribbean (Rogers et al. 1997, Edmunds 2002, see Chapter 4), they produce dictyol compounds active against herbivory and biofouling (Cronin & Hay 1996; Schmitt et al. 1995), , in addition to producing lipophilic metabolites that damage corals when in direct contact (Rashar and Hay 2010). However, our study did not detect an effect of *Dictyota* sp. on coral-microbial associates. This may be due to a lack of activity against coral-associated microbes or because the concentration of compounds that are exuded onto the algal surface and/or to particular parts of the thalli is too low to cause an effect.

Green calcareous macroalgae in the genus *Halimeda* are also some of the most common seaweeds found in the Caribbean (Rogers et al. 1997, Edmunds 2002, see Chapter 4). They are known to produce diterpenoid feeding deterrents that are activated by fish herbivory and/or injury (Paul & Van Alstyne 1992). Because *Halimeda* sp. can quickly convert one secondary metabolite (halimedatetraacetate) to a more potent defensive compound (halimedatrial) after breakage, live *Halimeda* thalli were not used in this study. Furthermore, we tested all *Halimeda* extracts used in this study and found that they only contained halimedatetraacetate, not halimedatrial, thus our results are conservative compared to the possible effects of live macroalgae in the field. The only significant effects of lipophilic extracts from *H. tuna* were seen on *P.astreoides* corals in Florida. And, previous antibacterial assays with coral reef bacteria showed that extracts from *H. tuna* varied widely in their activity against specific taxa, some bacteria were stimulated and others inhibited (Morrow et al. 2011). It is possible that the bacteria associated with *M. faveolata* are less susceptible to *H. tuna* extracts than those associated with *P. astreoides,* but further research is needed to confirm these differences.

The most extreme changes to coral microbial assemblages were in response to hydrophilic compounds and live thalli from the brown macroalga *L. variegata.* Microbial samples from both *M. faveolata* and *P. astreoides* coral colonies were dramatically affected by *L. variegata.* All samples collected after 72 hrs of exposure to extracts were shifted to a completely different and distinct assemblage of microorganisms in comparison to initial controls. This unpredicted result is quite significant because it suggests that a relatively small area of exposure (5-cm diam. gel) to this macroalga may have a colony-wide effect on coral microbes. A similar result occurred when live *L. variegata* thalli were applied to corals, although the effect was not as widespread. Lipophilic extracts from *L. variegata* also caused shifts in both coral species, but it was not consistent at both sites. It appears that *L. variegata* (lipophilic) extracts had extremely variable effects on *P. astreoides* in Belize in comparison to Florida, and a larger sample size may have allowed use to interpret these results better. The non-significant results for *M. faveolata-L.variegata* (lipophilic) interactions in Florida are likely due to more variable microbial assemblages in comparison to Belize. But, meandist dendrogram results for *M. faveolata* do suggest that the microbial assemblages collected after extract exposure are more similar to one another than to initial microbial assemblages, even though the result is not significant (Figure 5.4).

We chose *L. variegata* for this study because it commonly overgrows the edges and competes for space with the two study corals on deeper Caribbean reefs (> 15 m; Morrow pers. obs.; see Chapter 4). In addition to overgrowing corals (Jompa & McCook 2002a), causing bleaching (Rashar & Hay 2010), and tissue mortality (Jompa & McCook 2002b), previous laboratory assays demonstrated that hydrophilic compounds from *L. variegata* have broad-spectrum antibacterial activity against coral reef microorganisms (Morrow et al. 2011). Recent evidence also shows that brown macroalgae often produce hydrophilic sugar compounds such as dulcitol that inhibit bacterial quorum sensing (Dobretsov et al. *in* press). Dulcitol is present in *L. variegata* polar extracts based on bioassay-guided fractionation and may be responsible for some of the microbial mediation seen during this study (Morrow et al. 2011, Dobretsov & Paul *unpubl. results*). Thus, previous studies coupled with the evidence presented here, show that *L. variegata* has significant potential to alter microorganisms associated with competing corals and that future research is needed to characterize the active compounds within extracts of the hydrophilic fractions.

Many examples of antibacterial activity in marine macroalgae have been demonstrated and it is believed that secondary metabolites act as a fundamental mechanism for microbial deterrence and control of pathogens or epiphytes on macroalgae (Engel et al. 2002). They produce a host of different compounds to combat microbial attack, including fatty acids, phenols, acetylenes, terpenes, coumarins, carbonyls, and polysaccharides (reviewed in Goecke et al. 2010). We also know that macroalgae host a diverse and specific assemblage of microorganisms (Barott et al. 2011) and that antimicrobial activity is widespread among alga-associated bacteria (Burgess et al. 1999; Wiese et al. 2009). Thus some of the activity attributed to macroalgal chemical defenses in this study may actually be microbial in nature. For example, members of the genera *Bacillus* and *Pseudoalteromonas* are often successful colonizers of macroalgal surfaces and are efficient producers of antimicrobial and antifouling compounds (Burgess et al. 2003, Kanagasabhapathy et al. 2006, Wang et al. 2008). And we did sequence some members of these taxa from under algal extract plates in this study. Regardless, only a few studies have examined the ecological function of these algal- or microbial-derived compounds (Engel et al. 2002), but it is believed that extracts from marine macroalgae selectively target marine microorganisms (Engel et al. 2006), and that compound concentrations may even increase during microbial-attack (Vairappan et al. 2010).

The present study is an important addition to the study of macroalgal-allelopathy and is a first step toward examining the effect of macroalgal compounds on coral reef microorganisms. We realize that these experiments would have benefited from deep-sequencing technology such as with 454-pyrosequencing. However, the costs were prohibitive due to the large number of samples and interacting coral-algal pairs. We realize that although DGGE has significant drawbacks, it was the appropriate method in this instance to survey large numbers of samples in order to identify significant patterns for further study. These patterns provide new perspectives on coral-algal competition and provide support for additional questions about the consequences of altering the natural state of coral microbial communities. As present-day reefs undergo phase-shifts to alternative dominants that often have potent biochemical defense mechanisms (Norström et al. 2009; McManus & Polsenberg 2004), we should question what effect it will have on overall reef health and physiology.

Table 5.1: When and where each of the four algal extracts were originally collected. Extract were incorporated into experimental gels at the listed natural concentrations, based on wet weight. Dates of experiment deployment and retrieval are listed for each site and extract type.

Natural Concentrations of algal extract (mg) per algal wet wt. (g)

Florida 2009	Algae From	Concen. (mg/g)	Deployed	Retrieved
D. pulchella (lipophilic)	Florida Keys (2008)	14.01	5/23/09	5/26/09
H. tuna (lipophilic)	Florida Keys (2008)	12.02	5/23/09	5/26/09
L. variegata (lipophilic)	St. Thomas (2008)	16.51	5/23/09	5/26/09
L. variegata (hydrophilic)	St. Thomas (2008)	29.9	5/23/09	5/26/09
Belize 2009				
Dictyota sp. (lipophilic)	Belize (2008)	7.53	8/7/09	8/10/09
H. tuna (lipophilic)	Belize (2006)	24.4	8/7/09	8/10/09
L. variegata (lipophilic)	St. Thomas (2008)	16.51	8/14/09	8/17/09
L. variegata (hydrophilic)	St. Thomas (2008)	29.9	8/14/09	8/17/09

Table 5.2: a) Permutational multivariate analysis of variance (PERMANOVA) and b) Multi-response permutation procedure (MRPP) results for each coral-extract pair sampled in Belize and Florida 2009 as a function of the following variables: initial controls, treatment controls (solvent, shade, and post SML samples combined), and extracts. c) Results for each *M. faveolata* coral-live alga pair were reported as a function of the following variables: initial control, post control, fake algae, live algae.

a. PERMANOVA

Belize		***Dictyota* sp. (lipo)**	***H. tuna* (lipo)**	***L. variegata* (lipo)**	***L. variegata* (hydro)**
M. faveolata	R^2	0.106	0.07	**0.242**	**0.302**
	P	0.804	0.887	**0.004****	**0.003****
P. astreoides	R^2	0.102	0.151	0.133	**0.223**
	P	0.98	0.492	0.126	**< 0.001****
Florida					
M. faveolata	R^2	-	0.158	0.089	**0.499**
	P	-	0.233	0.738	**0.014***
P. astreoides	R^2	-	**0.229**	**0.253**	**0.231**
	P	-	**<0.001****	**<0.001****	**<0.001****

b. MRPP

Belize		***Dictyota* sp. (lipo)**	***H. tuna* (lipo)**	***L. variegata* (lipo)**	***L. variegata* (hydro)**
M. faveolata	A	-0.033	-0.064	**0.076**	**0.093**
	Δ	0.863	0.996	**0.006****	**0.002****
P. astreoides	A	-0.073	-0.011	0.022	**0.099**
	Δ	0.967	0.669	0.114	**< 0.001****
Florida					
M. faveolata	A	-	0.004	-0.013	**0.167**
	Δ	-	0.35	0.709	**0.024***
P. astreoides	A	-	**0.092**	**0.098**	**0.087**
	Δ	-	**<0.001****	**<0.001****	**<0.001****

c. Live Algae Application in Belize

M. faveolata		***Dictyota* sp. (LIVE)**			***L. variegata* (LIVE)**
PERMANOVA	R^2	0.135	-	-	**0.236**
	P	0.896	-	-	**0.006****
MRPP	A	0.039	-	-	**0.048**
	Δ	0.926	-	-	**0.005****

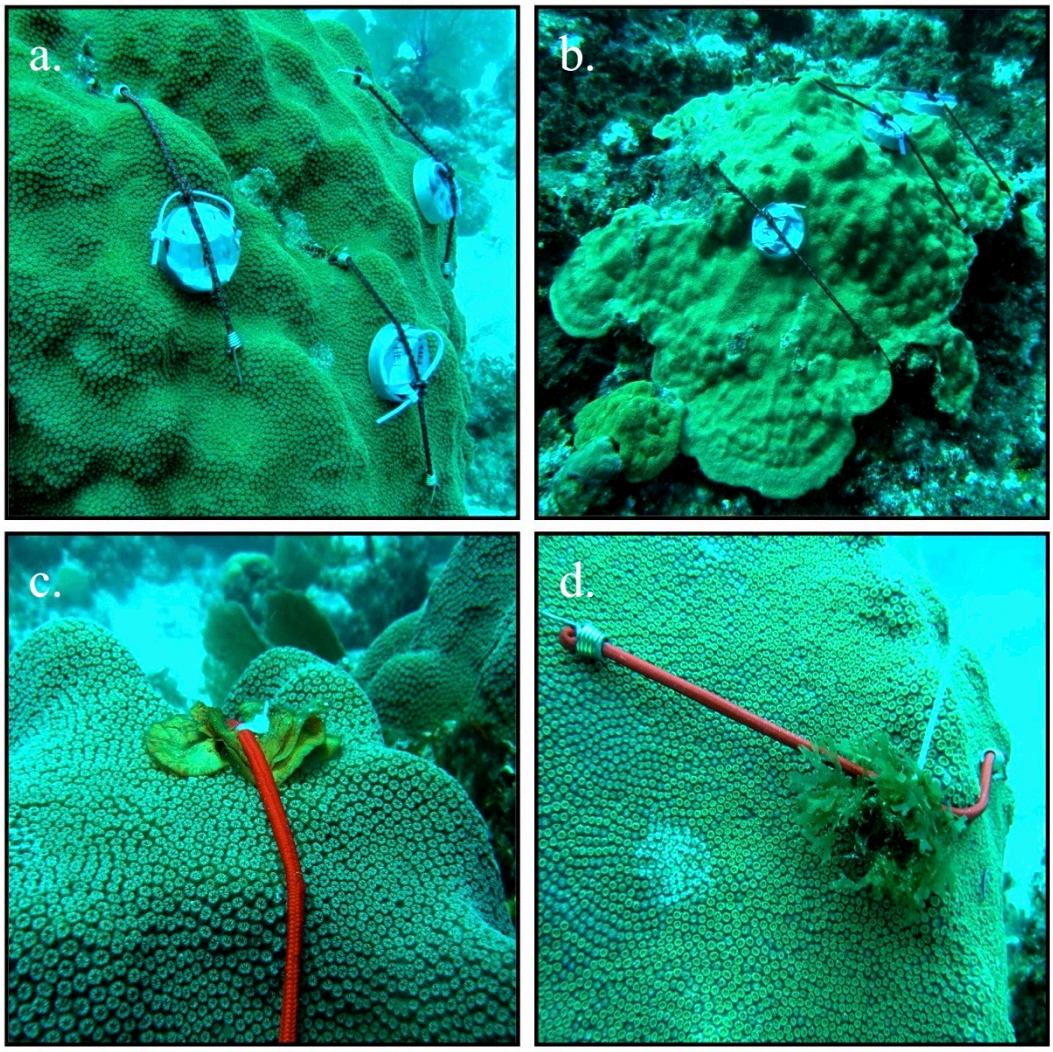
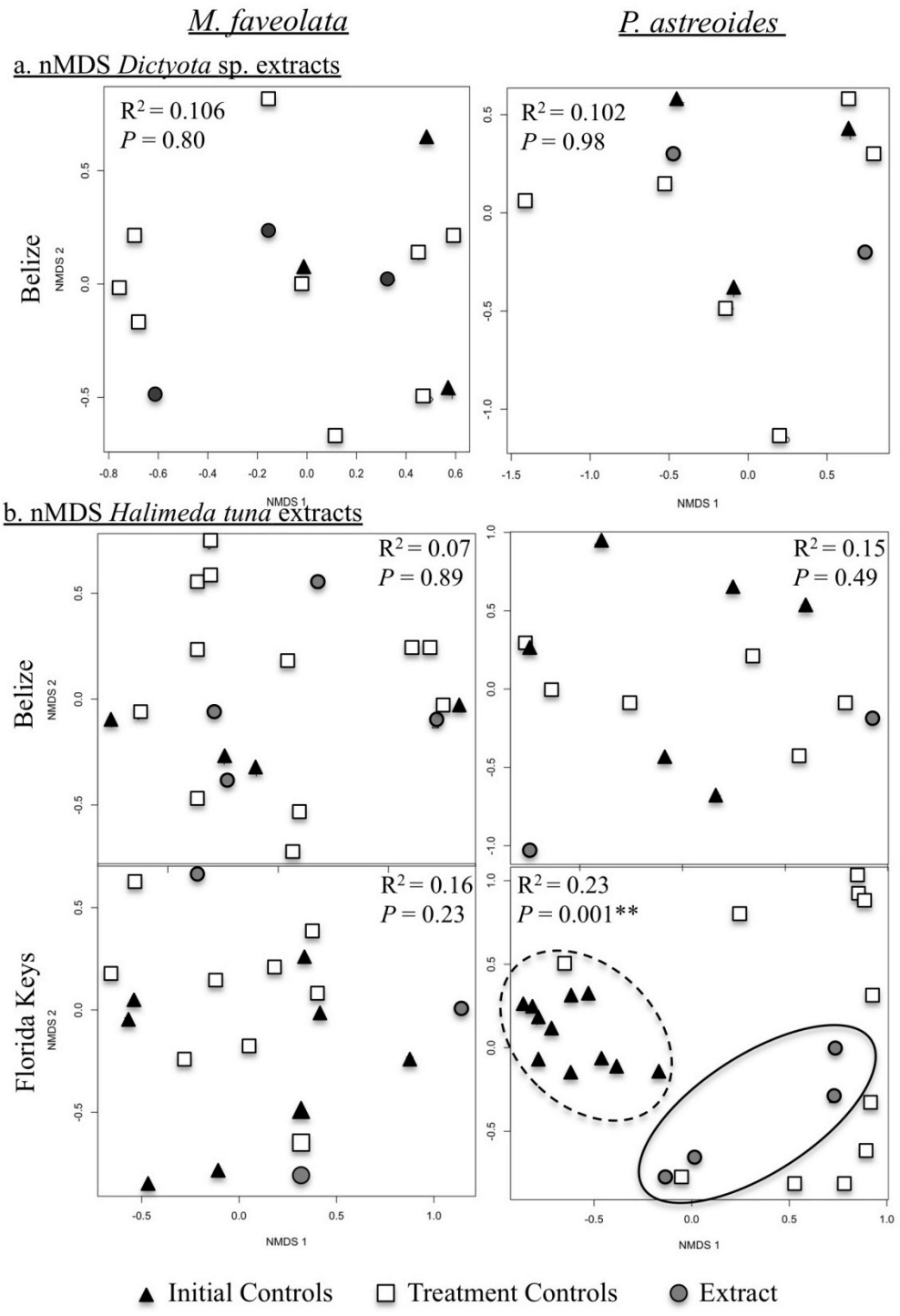
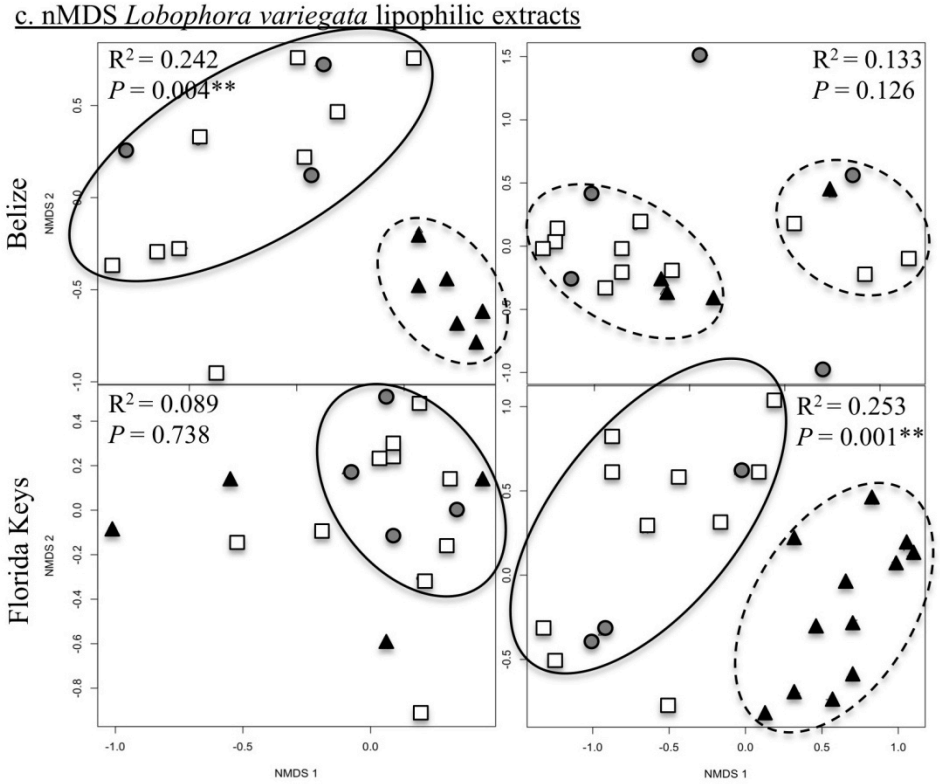


Figure 5.1: Photos illustrate three experimental plates (shade control, solvent control, and macroalgal extract) on: a.) *M. faveolata* and b.) *P. astreoides* corals. Experimental application of c.) *Lobophora variegata* decumbant brown macroalgae and d.) *Dictyota* sp. foliose brown macroalgae to *M. faveolata* coral colonies in Belize 2009.


M. faveolata
P. astreoides
a. nMDS Dictyota sp. extracts
R² = 0.106
P = 0.80
R² = 0.102
P = 0.98
Belize
NMDS 2
NMDS 1
b. nMDS Halimeda tuna extracts
R² = 0.07
P = 0.89
R² = 0.15
P = 0.49
R² = 0.16
P = 0.23
R² = 0.23
P = 0.001**
Belize
Florida Keys
NMDS 2
NMDS 1
Initial Controls
Treatment Controls
Extract

M. faveolata *P. astreoides*

c. nMDS *Lobophora variegata* lipophilic extracts



d. nMDS *Lobophora variegata* hydrophilic extracts

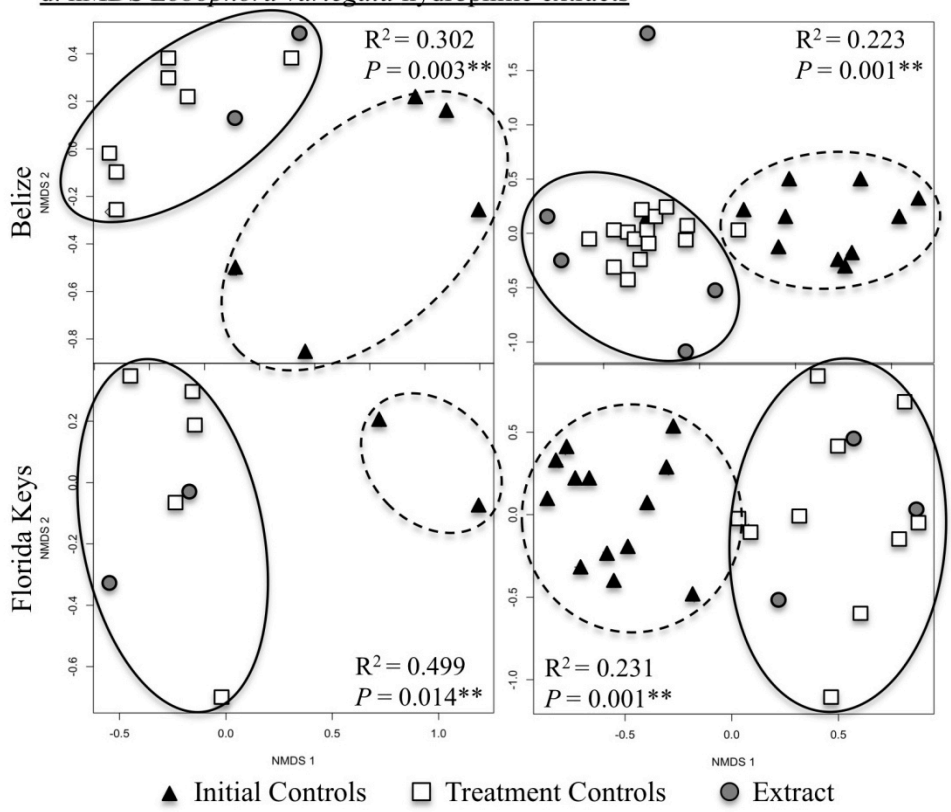


Figure 2 (previous two pages): nMDS plots based on DGGE profiles for each coral-extract interaction pair at each site (Belize & Florida) between *M. faveolata* or *P.astreoides* corals and extracts of: a) *Dictyota* sp. (lipophilic), b) *H. tuna* (lipophilic), c) *L. variegate* (lipophilic), and d) *L. variegata* (hydrophilic). Solid circles enclose clusters related to extract-associated microbial samples and dashed circles enclose clusters related to initial coral control-associated microbial samples. PERMANOVA results are reported in the corner of each interaction pair. ** indicates a significance of $P < 0.05$.

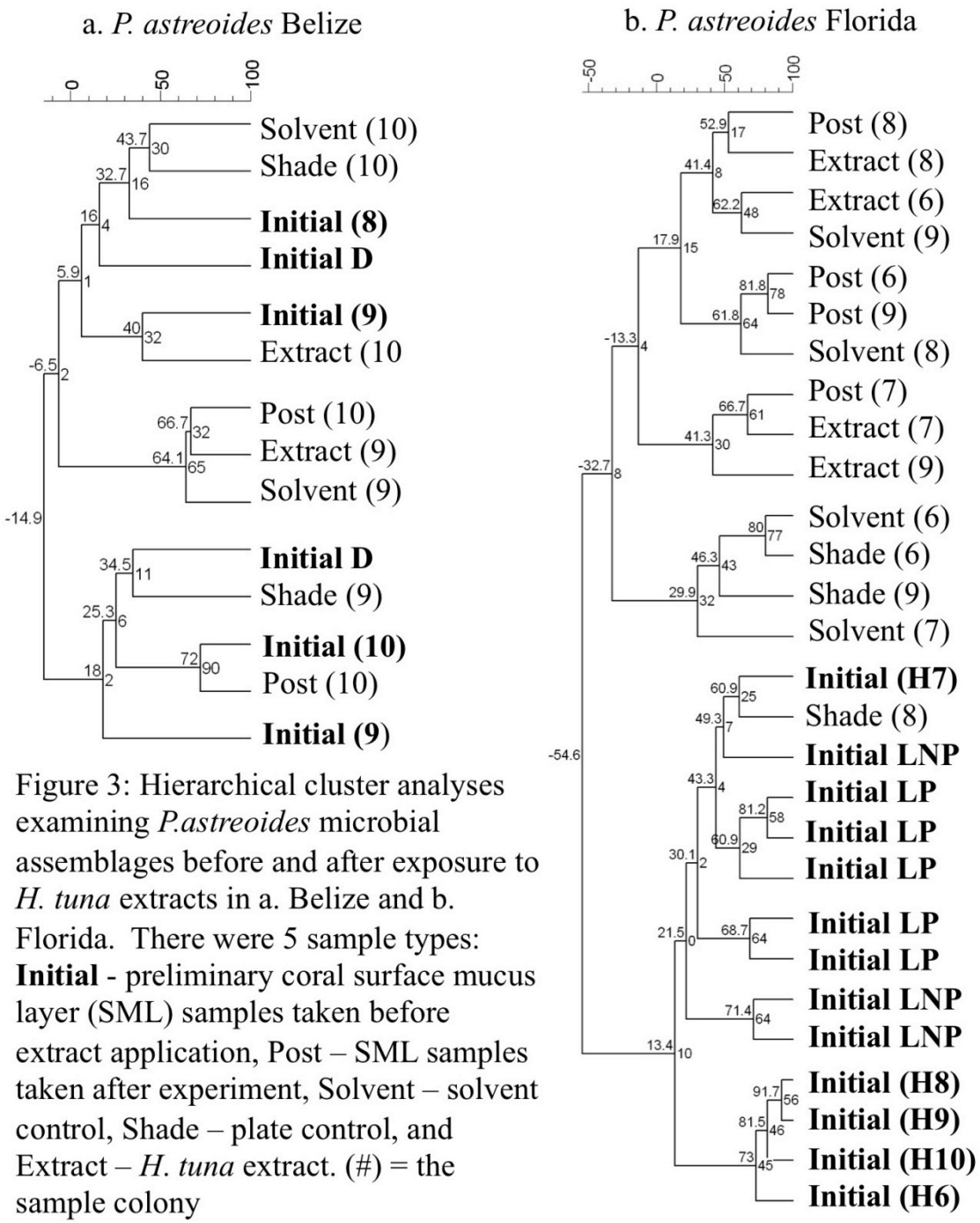


Figure 3: Hierarchical cluster analyses examining *P.astreoides* microbial assemblages before and after exposure to *H. tuna* extracts in a. Belize and b. Florida. There were 5 sample types: **Initial** - preliminary coral surface mucus layer (SML) samples taken before extract application, Post – SML samples taken after experiment, Solvent – solvent control, Shade – plate control, and Extract – *H. tuna* extract. (#) = the sample colony

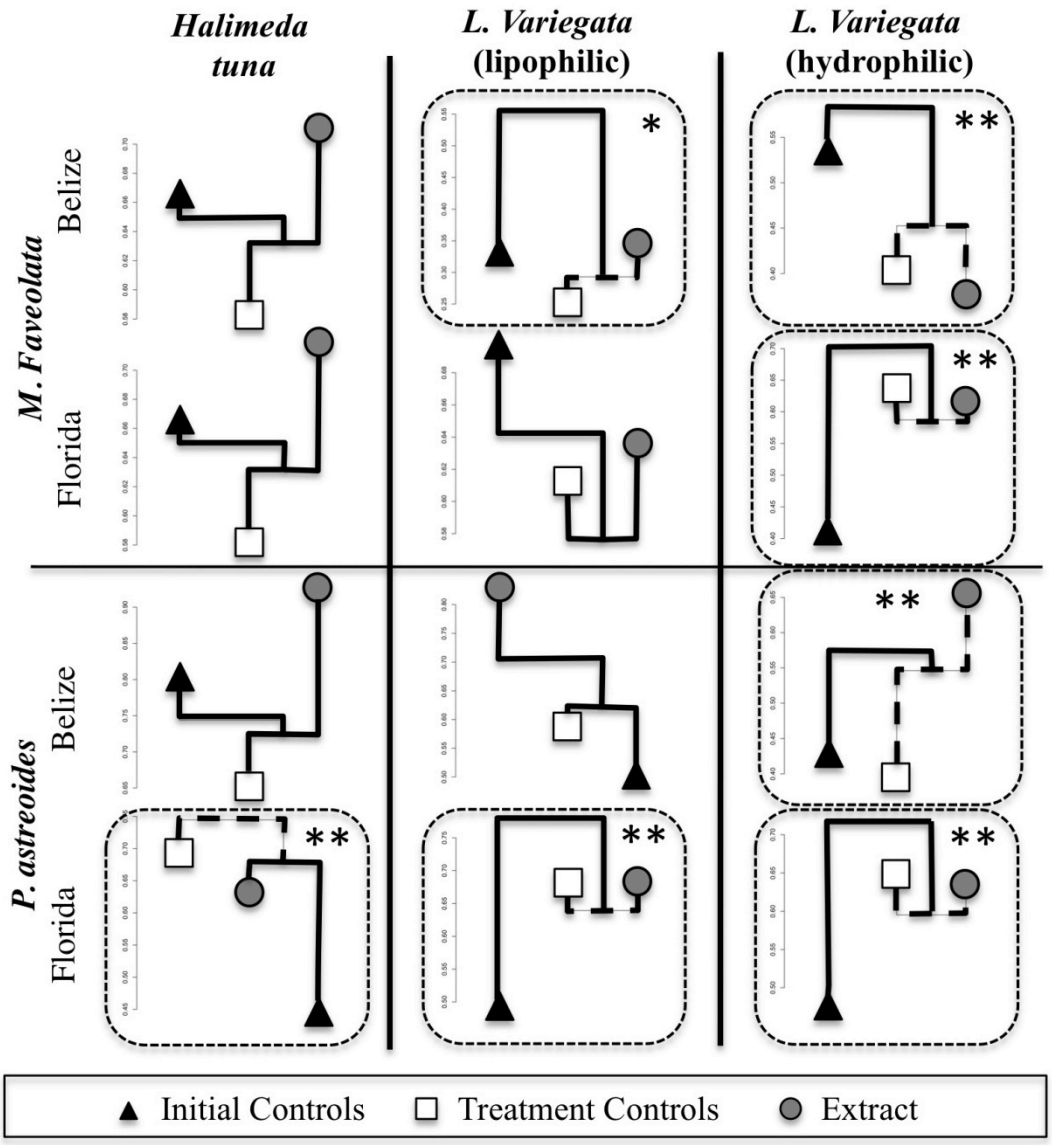


Figure 4: Dendrograms illustrating the effect of extracts on *M. faveolata* and *P. astreoides* coral microbial assemblages in Belize and Florida based on a 'meandist' matrix of within-group and between-group dissimilarities using Ward's minimum variance algorithm. Dashed circles around interaction groups represent a significant effect of extracts on the microbial assemblage (*P < 0.05, **P < 0.01).

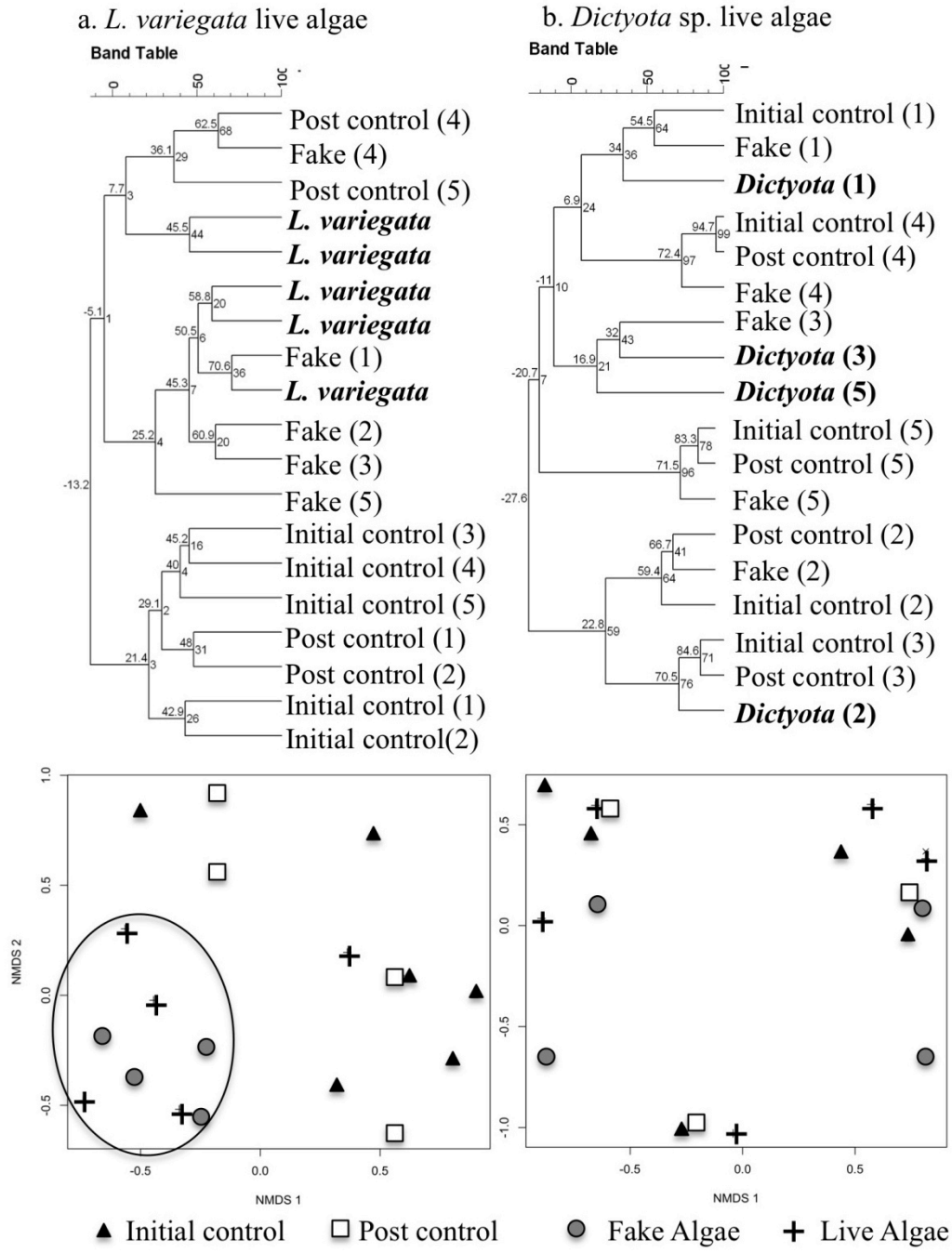


Figure 5: Hierarchical cluster analyses showing similarity among microbial samples (%; inner tree) with bootstrap support (1000 iterations, outer tree) to examine the effect of live macroalgae in the genera *L. variegata* and *Dictyota* sp. applied to *M. faveolata* coral colonies in Belize 2009. Numbers in parantheses correlate to the experimental colony (#1-5). Results are further illustrated by nMDS plots of each coral-algal pair.

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