UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Competition between Coral and Algal Holobionts

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biology

by

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2012

The Dissertation of Katie Lynn Barott is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
San Diego State University
2012

DEDICATION

To my grandmother, Agnes Pearthree Barott, that I may appreciate the many opportunities before me.

EPIGRAPH

Success is not final, failure is not fatal: it is the courage to continue that counts.
- *Winston Churchill*

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ACKNOWLEDGEMENTS

None of this work would have been possible without the support and guidance of my advisor, Dr. Forest Rohwer. The opportunities he has given me are beyond compare; the top of the list being the chance to see some of the most remote and untouched coral reefs in the world. I am not convinced that he has not tried to do me in on these very same reefs, suggesting dives to collect samples on murky shark-infested reefs near sunset and pushing intense work hours in the tropical heat. Despite this, he has been a great friend and mentor through it all, never slow to taunt and always eager to talk about science. I would not be the scientist I am today without everything he has taught me.

I am extremely fortunate to have worked with such a supportive, and despite Forest's best efforts, positive group of people. My greatest thanks to the women in the lab over the years: Becky Vega Thurber, Linda Wegley Kelly, Yanwei Lim, Allison Gregg, Dana Willner, Tracey McDole, and Alejandra Prieto Davo. Their support throughout the dissertation process, from teaching me laboratory techniques to providing psychological support, has been invaluable. To the guys in the lab, clean up your mess! Your humor and reliable happy hour companionship have made the process a joy, even when the science seemed to drag.

Completing this dissertation has been a collaborative effort on all fronts. While doing research in the field, I have relied greatly on the assistance and expertise of other more seasoned coral reef ecologists. These people have taught me more that I probably recognize, and have become great friends along the way. Kristen Marhaver

first brought me to Curacao, where she taught me the local coral species, how to care for corals, and to truly appreciate what the reef has to offer. Her unflagging enthusiasm for all things coral and the value of tiny beers made long days and nights working in the lab amongst the heat and mosquitoes not only bearable but also fun. Thanks are also due to Mark Vermeij, Aaron Hartman, and Allison Gregg for all of their help and making Curacao a great place to learn about corals and talk about science.

I was fortunate enough to sneak onto two research cruises to the Line Islands during my thesis. These trips were the culmination of the hard work and dedication of many people. Many thanks are due to Enric Sala, Stuart Sandin, and Brian Zgliczynski for their part in making these expeditions possible. Thank you also to Jen Smith, Liz Dinsdale and Gareth Williams for teaching me to identify corals and algae of the Line Islands (though I have yet to master them all).

I owe special thanks to Beltran Rodriguez-Mueller. In addition to helping me with some traditional bioinformatics, he turned my many questions and novice attempts to compare metagenomic libraries into novel statistical tools that helped me figure out what might be happening with the microbes when corals and algae compete. I must also thank Dana Willner and Bahador Nosrat for their help with bioinformatics.

Lastly, I thank my family. My parents, for never letting anyone slow me down, and Byron, for putting up with me through the ups and downs, the time away from home doing research in the field, for never letting me take myself too seriously, and for giving in to his better judgment to let me get our puppy Duke, whose effusive

unconditional love and goofiness was a major source of stress relief as I wrote this dissertation.

Chapter 1 is currently being prepared for submission of publication of the material. Katie L. Barott and Forest L. Rohwer. The dissertation author was the primary investigator and author of this paper.

Chapter 2, in full, has been accepted for publication of the material in Marine Ecology Progress Series, 2012, Katie L. Barott, Gareth J. Williams, Mark J. A. Vermeij, Jill Harris, Jennifer E. Smith, Forest L. Rohwer, and Stuart A. Sandin. The dissertation author was the primary investigator and author of this paper.

Chapter 3, in full, is a reprint of the material as it appears in PloS ONE. Katie L. Barott, Jennifer E. Smith, Elizabeth Dinsdale, Mark Hatay, Stuart Sandin, and Forest L. Rohwer; 2009. The dissertation author was the primary investigator and author of this paper.

Chapter 4, in full, is a reprint of the material as it appears in Environmental Microbiology. Katie L. Barott, Beltran Rodriguez-Brito, Jan Janouškovec, Kristen Marhaver, Jennifer E. Smith, Patrick Keeling, and Forest L. Rohwer; 2011. The dissertation author was the primary investigator and author of this paper.

Chapter 5, in full, is a reprint of the material as it appears in Proceedings of the Royal Society B. Katie L. Barott, Beltran Rodriguez-Mueller, Merry Youle, Kristen L. Marhaver, Mark J. A. Vermeij, Jennifer E. Smith, and Forest L. Rohwer; 2011. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Competition between Coral and Algal Holobionts

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2012
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Coral reefs around the world have suffered devastating losses of reef building corals with a concomitant increase in benthic algae. While it is clear that a variety of local and global disturbances play a role in the replacement of corals by algae, the mechanisms behind this transition are not. Space is limited on coral reefs, and competition between corals and benthic algae plays a major role in shaping the composition of the benthos. The objective of this dissertation was to investigate the dynamics of coral-algae competition, with a focus on how different algae affect coral physiology and their associated microbes, and how these small-scale dynamics

influence the distribution of corals and algae on pristine to degraded coral reefs. I found significant differences in the composition and outcomes of coral-algae interactions across reefs; corals were consistently better competitors against crustose coralline algae (CCA), but were damaged by turf algae on inhabited but not uninhabited reefs, suggesting that competition dynamics are affected by human activity. Physiological investigations of coral interactions with four common types of benthic algae (CCA, calcareous macroalgae, fleshy macroalgae, and turf algae) demonstrated that all algae except CCA cause net heterotrophy and disruption of coral tissue and pigments along the interaction border. These effects were negated by antibiotics, indicating that disruption of coral health during algal competition is mediated by microbes. These same algae were found to harbor highly diverse bacterial and eukaryotic microbial communities, but their competitive interfaces with corals hosted a community of microbes distinct from either side. Turf algae borders had a large proportion of potential pathogens, while fleshy macroalgae led to an increase of bacterial carbohydrate utilization metabolisms. This suggests that turf and fleshy macroalgae compete with corals by stimulating bacterial growth and respiration and promoting the invasion of opportunistic pathogens on corals, leading to coral mortality and freeing space for the alga. This dynamic appears to be amplified by human disturbances such as overfishing and eutrophication, which remove the top-down and bottom-up controls on algae.

CHAPTER 1

Introduction: The invisible players shape benthic competition dynamics on coral reefs

Abstract

Organisms are space-limited in benthic ecosystems like coral reefs, resulting in intense competition. Battles are constantly at play between holobionts (the macro-organisms plus their associated microbes) and biofilms trying to "crawl" over each other. Worldwide corals have been increasingly losing these benthic battles with a corresponding increase in the occurrence of coral-algae interactions. The mechanisms by which algae overgrow corals were originally thought to be relatively simple physical interactions like abrasion and shading. Now it has been shown that dissolved organic matter (DOM) and pathogens released by algae can create microbial mats that cause coral morbidity and death. There is also evidence that the complex flow patterns on reefs create "packets-of-water" that may actively deliver pathogens, DOM, and hydrophilic compounds to removed locations. Direct contact between corals and algae also delivers these, as well as hydrophobic chemicals, all of which alter the holobiont to the detriment of the coral. Human activities that release controls on algae and increase coral-algal contact exacerbate these dynamics, and the net effect is an increased microbialization of the reef, where algal-fed biofilms crawl along the coral, rotting away tissue and freeing space for the algae to advance in a positive feedback loop termed DDAM (DOM, Disease, Algae, and Microbes). In this review, we discuss

the current state of knowledge about competition between corals, benthic algae, and microbes and the implications for future reef health.

INTRODUCTION

Competition for space plays a major role in structuring benthic coral reef communities (1). On coral reefs, the two major groups of competing benthic organisms are corals and algae. While a shift from coral to algal dominance has been documented on reefs around the world, the dynamics of individual coral-algal interactions in these situations are poorly understood. Algae are known to replace corals after some major die-off events (e.g. mass bleaching, epidemics, etc.), yet how algae come to dominate over live adult corals and the roles of microbes (Bacteria and Archaea) and viruses in these interactions has only recently been studied. Environmental context plays a major role in these competitions and local impacts such as fishing and eutrophication have significant impacts on algal biomass (2,3), abundance (2,4), and species composition (2,5–7). These factors alter existing coral-algal interactions (2,3,6–11) and create new ones. The ramifications of global stressors, including changing sea surface temperature and ocean acidification, also change coral-algal interactions. Here we argue that a better understanding of competitive interactions on the reef benthos helps predict the direction that a reef is headed (coral vs. algal dominated) and the most important factors driving these changes. By understanding these dynamics it will be easier to identify strategies for stopping and reversing coral decline.

Shifting macro- and microbial baselines.

Scleractinian corals have dominated coral reefs for hundreds of thousands of

years (12). Along with other benthic calcifying organisms (e.g. crustose coralline algae [CCA] and *Halimeda* spp.), corals build the structural framework that supports the most diverse marine ecosystems on the planet (13,14). Over the last four decades, there have been documented changes in the benthic composition of coral reefs with a shift from coral to algal dominance. This shift is reflected in the focus of benthic competition research from coral-coral to coral-algae competition (Figure 1), and no doubt future work will begin to focus on alga-alga interactions (16), as corals become a more minor component of some reefs.

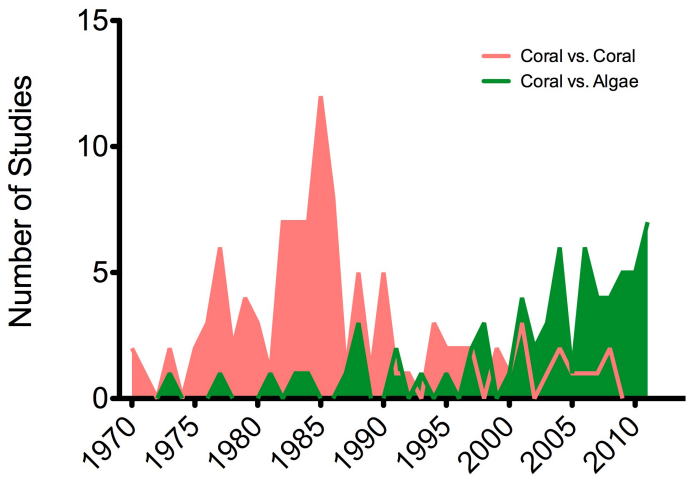

Figure 1.1. The number of studies published that directly investigate coral-coral and coral-algal competition by year over the last 40 years (1970 – 2011).

The historical dominance of corals shows that they are not easily overwhelmed by algae and that new disturbances in coral reef ecosystems must be changing benthic dynamics. This switch has been harder to study because the vast majority of studies have taken place on reefs that have already experienced significant local human impacts (15), leaving us with a distinct lack of information on how benthic

interactions play out in an intact system. The immense biodiversity of coral reefs around the world further complicates this research, since each different species of coral or alga likely has a unique response to any particular stressor. A final complicating issue is that coral- and algal-associated microbial and viral communities have largely been ignored in the majority of coral-algal competition studies. Since microbes and viruses play major roles in the health of all macro-organisms, the dearth of knowledge about this component of the ecosystem leaves a significant hole in our understanding of coral reef dynamics.

DESCRIPTIONS OF CORAL-ALGAE INTERACTION DYNAMICS

Coral-algae competition types.

Benthic algae on coral reefs are conventionally grouped into functional groups, including the crustose coralline algae (CCA), macroalgae, and turf algae. Each of these groups contains many different species, each with their own species-specific subtleties. The current knowledge of coral interactions, broken down into these three algal functional groups, is discussed below. However, we are just starting to scratch the surface of this subject.

Crustose Coralline Algae (CCA) are commonly associated with healthy reefs and generally have positive interactions with corals. These encrusting, calcifying, red algae help cement the reef structure and play an important role in maintaining net reef accretion (14). They also play an important role on the reef through their interactions

with corals. CCA facilitate coral settlement and survival (16,17), and some species of CCA directly stimulate coral metamorphosis and are preferentially selected by coral larvae as a settlement substrate (17,18), while more generally CCA are associated positively with coral recruit survival (16). Corals may also benefit from close associations with CCA since these algae can prevent colonization by turf algae and macroalgae that would otherwise harm adult corals, as well as inhibit coral settlement and survival (19,20). In addition, adult corals are not negatively affected by neighboring CCA to the same degree that they are by turf algae or macroalgae (3,21,22), suggesting that maintenance of high CCA cover on a reef will promote coral success at multiple life stages.

Macroalgae are the most commonly studied type of algae when it comes to coral-algal competition (23,24). A variety of different macroalgae species (mostly fleshy algae) have been shown to inhibit coral growth (11,25,26) and cause bleaching (21,26,27), hypoxia (21,22,28), and lower photosynthetic efficiency and chlorophyll *a* content of symbiotic zooxanthellae (26) along the edge of the coral colony. Competition with macroalgae can also reduce coral fecundity (29–31), with the most significant decline along the edge of the colony in contact with the alga (30). Macroalgae also prevent coral recruitment by occupying available substrate, inhibiting larval settlement, or lowering recruit growth and survival (31–35). Macroalgae are not always harmful to corals; shading by some macroalgae can protect corals from bleaching (36) and some seasonal algae do not visibly damage intact coral tissue (36).

Turf algae (i.e., "turfs") are heterogeneous assemblages of short filamentous algae, juvenile macroalgae, and cyanobacteria (37). Turfs are also home to diverse and essentially uncharacterized eukaryotic and prokaryotic microbial communities, as well as viruses (38). The heterogeneity of turf algal assemblages means that they have different effects on corals (36), although the majority of interactions studied have been negative. Turf algae inhibit coral growth and negatively influence adjacent coral tissue integrity (3,21,39–41), physiology (3,21,22,39,42), and fecundity (30). In addition, turf algae inhibit coral settlement and recruit survival (31–34,43).

***Distribution of coral-algae interactions*.**

Surveys of coral-algae interactions show that there are significant changes in the composition and outcome of interactions across different reefs. On uninhabited islands and protected regions of inhabited reefs, for example, corals consistently resist damage from turf algae. In contrast, on inhabited islands without protections, corals primarily lose to turf algae (Chapter 2). This is particularly important since turf algae are consistently the most abundant type of algae interacting with corals around the world (e.g. the Central Pacific (21), Red Sea (40), Caribbean (11,22), and Indonesia (41)), suggesting that coral-turf algae dynamics are a significant driver in coral reef ecology. Turf algae also have different impacts on corals than other algal types; when followed over time, turf algae have been shown to advance over corals on reefs where CCA did not (3). Furthermore, in contrast to turf algae, CCA interactions with corals

are positively correlated with coral cover (22), and even on islands influenced by human activity corals appear to be successfully competing against CCA (Chapter 2).

MECHANISMS OF CORAL-ALGAE COMPETITION

Initial studies of coral-algal competition were focused on the physical mechanisms corals and algae use to damage each other (reviewed by McCook in 2001 (23)). Algae employ tactics such as shading and abrasion, and corals respond with mesentery and nematocyst attack (44). More recent studies have shown that other biological factors change the relative competitive advantage in specific ways.

Geometry of the interaction

Metabolic resources are needed in order to defend or mount an attack against a competitor. In coral-algae competition, the availability of resources depends on the size and shape of the two organisms and the interaction zone between them. The perimeter of a coral colony, for example, is the region most often engaged in algal competition. By shunting resources gained from photosynthesis and heterotrophic feeding from the center of the colony, where no competition is occurring, to tissue along the perimeter, a coral could presumably support a strong defense against an algal attack. Furthermore, the perimeter of a colony grows much more slowly than the area of the colony (Figure 2), so a large coral colony would have a much greater pool of resources to defend its perimeter than a small colony. The shape of the area of the colony will also make a difference, since an oblong coral will have a greater

perimeter:area ratio, leaving more of the colony susceptible to attack and requiring more internal resources to defend itself. Conversely, algae must defend against/recover from herbivory and coral attack. The same perimeter:area dynamics occur here as well; algal stands that are grazed specifically along the edges could be maintained at their current size by one level of herbivory, whereas the same level of herbivory distributed haphazardly within the area of the algal stand might have little effect on algal advance over a coral (Figure 2).

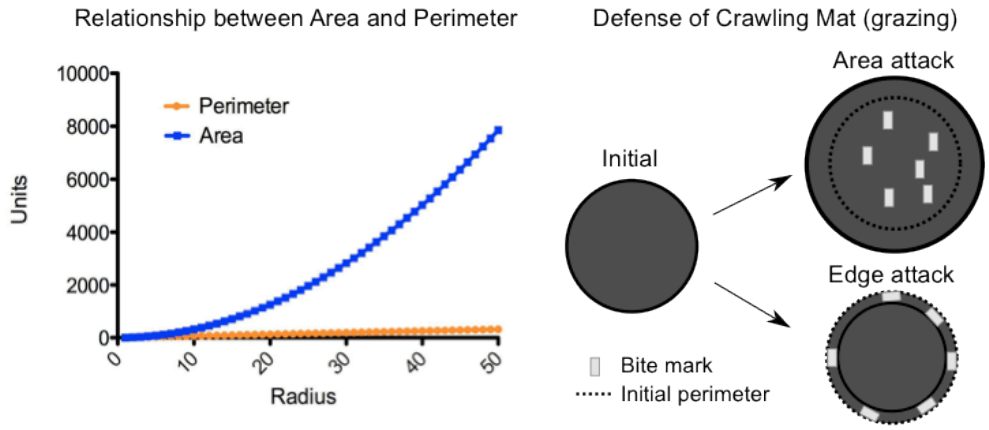

Figure 1.2. Relationship between perimeter and area and the ecological importance of this relationship.

Microbes and Viruses

The holobiont: Corals and benthic algae host diverse consortia of microorganisms, including protists (e.g. zooxanthellae, apicomplexans, diatoms), Bacteria, Archaea, fungi, and viruses, which together with the macro-organism form a holobiont (38,45–47). These holobionts are diverse and distinct from the microbiota in the surrounding seawater and other benthic biofilms (38,48,49), and are commonly species specific and conserved across space (38,45,50–52), but can show some

seasonal variation (53–55). Some coral species host a more general ‘coral’ community that is not specific to species but to site (52,56,57). Spatial heterogeneity of the holobiont can be high, with different compartments within a coral colony (e.g. skeleton, tissue, mucus) or alga (rhizoid, cauloid, meristem) housing different microbes (22,51,55,58,59).

Holobiont microbes play a variety of roles. Surface-associated microbes on corals and algae protect from invasion by pathogens and fouling organisms by outcompeting pathogens and occupying space, as well as producing inhibitory compounds (60–64). Associated microbes can affect development (e.g. *Ulva* spp.; (65)), settlement, and metamorphosis (e.g. invertebrate larvae) (18,66). Microbes also play a physiological role within the holobiont. Nitrogen fixing bacteria are associated with some coral species, providing fixed nitrogen to the host (67), while other coral-associated bacteria are involved in metabolizing abundant organic sulfur compounds (e.g. DMSP) released by zooxanthellae (68). These sulfur compounds also makes coral mucus a more selective environment that not all microbes can survive.

Disruption of the holobiont leads to changes in host physiology, including disease (69) and death (70,71). Experimental evidence shows that a variety of stressors affect the bacterial composition of holobionts, including temperature, inorganic nutrients, dissolved organic matter (DOM) and pH. Temperature stress shifts coral and algal holobionts towards pathogenicity (72,73), induces viruses (72,74), and is followed by bacterial invasion of the tissue (75), possibly due to loss of antibiotic activity in the mucus of corals (60,69) or chemical defenses of algae (73,76). Lowered

pH also causes an increase in potential pathogens and virulence genes in the bacterial communities associated with corals (72,77). Holobiont alterations also affect ecological interactions; for example, temperature-stressed CCA lose their stimulation of coral larvae settlement (78).

Microbial communities change with coral-algae competition. Benthic algae harbor rich microbiota, including a large number of potential pathogens and coral-disease associated microbes (38). These pathogens maybe transmitted to corals during competitive interactions (Thurber in review and (79)), but different groups of algae have distinct effects on coral-associated bacteria. Turf algae, for example, are associated with major shifts in the bacterial communities along the coral border, including more potential pathogens and virulence genes (22). These bacterial communities also lack several metabolisms found on a healthy holobionts, such as organic sulfur metabolism and antibiotic resistance (22). In contrast, coral interactions with CCA have a distinct community of bacteria at the interface, but are not pathogen-like. One common physiological signature that separates coral-CCA interactions from coral-turf and coral-macroalgae interactions is hypoxia. Both experimentally-initiated and naturally-occurring interactions between corals and turf or macroalgae are hypoxic (21,22,28), whereas coral tissues in contact with CCA remain superoxic (21,22). Low oxygen along the coral-algal interaction zone can be alleviated by removal of the alga (22) or by treatment with antibiotics (28) (and Barott

unpublished), suggesting that at least in all observed cases hypoxia is the result of microbial activity.

Specific versus opportunistic coral pathogens. The vast majority of coral diseases have no known etiological agent despite a rich research field (70). This lack of identification of a single causative agent for many coral diseases may be because corals are succumbing to infection by opportunistic pathogens and/or polymicrobial infections. For example, transmission of white band disease type I (WBDI), can be prevented by antibiotics or filtering microbes from the inoculum (53), but the search for a specific pathogen has come up empty. The symbiosis continuum maybe a more fruitful view of the system, since one species of microbe may interact with a macro-organism as anything from a mutualist to a pathogen. In the case of corals, changing environmental conditions are leading to an increase in the prevalence of coral diseases. This increase does not follow the typical etiology for most infectious agents, which capitalize on high host densities for transmission. Instead, coral diseases are increasing on degraded reefs where coral density has dramatically decreased. This strongly suggests opportunistic infections of a compromised coral host and not specific pathogens. The rise of compromised hosts and opportunistic infections is almost certainly due to warmer temperatures, increased algal competition, low water clarity, eutrophication, etc., as well as an increase in potential pathogens in the surrounding water column (80–82).

The DDAM model – DOM, Disease, Algae and Microbes: The DDAM model was proposed based on studies of microbial dynamics on coral reefs ranging from near-pristine to degraded (83,84). Algae (particularly turf algae and fleshy macroalgae) induce coral death by releasing DOM that stimulates microbial activity and leads to coral disease and death. Treatment of corals with algal DOM stimulates microbial activity and leads to disease and death (85,86), and increases coral bleaching and tissue necrosis along coral-algal interactions (42); however, antibiotics eliminate these symptoms ((28) and Barott unpublished). DOM is a product of photosynthesis that is exuded into the water column by many different types of algae (87). These exudates are highly labile, and fuel bacterial growth and respiration in mesocosms and over algal beds *in situ* (87,88). Algae-dominated reefs also have more microbes in the water column, which are comprised primarily of heterotrophs and potential pathogens (80). Experiments have shown that both adult and larval corals experience greater mortality in the presence of algae, but not when antibiotics are added, indicating that coral death in the presence of algae and DOM is microbe-mediated (28,89). This body of experimental and ecological evidence clearly indicates that algae 1) release DOM, 2) facilitate microbial growth and respiration on the benthos and the water column, particularly that of opportunistic pathogens, and 3) cause morbidity and mortality of corals that can be mitigated by antibiotics.

Direct Contact

Physical interactions between corals and algae can inflict damage directly on the competitor, potentially freeing space for the attacker to advance. However, physical mechanisms alone, typically tested through the use of plastic mimics, have been found to play a relatively minor role in coral-algae competition when compared to the effects live organisms (27,28). This difference is due to the transfer of chemicals and microbes to the competitor. Direct contact between algae and corals, for example, delivers DOM, potential pathogens, and hydrophobic metabolites (i.e. allelochemicals) (55) to the tissue of the competitor. For example, direct contact with some species of macroalgae, as well as their hydrophobic extracts, has been shown to cause bleaching and tissue necrosis of some corals. These responses vary depending on the species involved (13,15), and the mechanisms are not known. Direct toxicity to corals and their symbiotic algae and stimulation of microbes are both possibilities (90). Physical damage to the coral resulting from direct contact may also facilitate the delivery of these other biological mechanisms, such as by creating tissue lesions that allow chemicals and microbes to invade the otherwise defended coral epithelium. So far, studies of algal allelochemicals have identified a few hydrophobic compounds, all of which are assumed to require contact for transmission to the coral. Large macroalgae swaying in the current will be able to transfer hydrophobic compounds to neighboring corals. Similarly, smaller algae (e.g. turf algae) may directly contact corals along their perimeter; however, in the boundary layer hydrophobic molecules will not be able to

move unless they are encased in something like exosomes (91). So far, these vesicles have not been observed on reefs.

Comparisons of coral-algae competition models

Interactions between benthic organisms involve all of the main mechanisms discussed above (physical, microbial, and chemical) and are not mutually exclusive of each other. However, the current evidence for the biological significance of each factor differs. Physical interactions have been shown many times to cause less damage to corals than chemical or biological interactions. The significance of hydrophobic allelochemicals suffers from a lack of evidence on several levels: 1) antibiotics prevent most of the damage done by algae to corals in all cases tested to date, and 2) the ecological relevance of these compounds (i.e. their abundance and dynamics over time under natural circumstances) has not been established. To address this problem, we need to know the concentrations of these types of compounds on reefs, as well as quantify the extent contact occurs between corals and all types of algae; and then how much damage is done to corals at those contacts, preferably over time. Furthermore, the only data currently available that comprehensively quantify the types of algae interacting with corals show that macroalgae, the producers of most characterized hydrophobic allelochemicals, are only a minor component of coral-algae interactions, even on degraded reefs (22,92). Most of these interactions are actually dominated by turf algae on reefs near people or even CCA on some uninhabited reefs, yet no

hydrophobic compounds have been looked for or tested for toxicity against corals in either of these groups of coral reef algae.

Perhaps the most parsimonious model of coral-algae interactions is the expansion of the DDAM model to the ^{3}DAM model: DOM, Disease, Direct contact, Algae, and Microbes. All algae produce DOM that can fuel the ^{3}DAM feedback loop, and all algae and coral that have been tested harbor potential pathogens (38). Both of these things are mobile in the water column, making water-mediated interactions a universal and far-reaching competitive mechanism. Direct contact can also facilitate the delivery of DOM and microbes, as well as transfer hydrophobic compounds to the coral holobiont, but it is not necessary for antagonistic coral-algae interactions. One important phenomenon that will be important to document in future is the importance of contact versus water-mediated interactions on reefs of different health statuses. We expect that degraded reefs where macroalgae have become dominant will see an increase of direct coral-macroalgae contacts. At the same time, interactions with turf algae (the most abundant type of coral-algal interaction) will likely involve more direct contact on degraded reefs. For example, in the Line Islands, inhabited islands with intense fishing pressure have turf algae that form tall, dense carpets, whereas uninhabited atolls are populated by short and sparse turf algal stands (personal observation). The turf algae on the inhabited islands tend to do more damage to competing corals (Chapter 2), and shifts within turf algal communities brought on by human activity are likely promoting the competitive dominance of the turfs, including but not limited to: 1) a shift in the species composition of the turf algae to species that

are better competitors against corals (release more DOM, harbor more opportunistic pathogens, etc.), 2) increased height and density of resident turfs, leading to thicker boundary layers and higher concentrations of DOM, microbes, etc. within the boundary layer, and/or 3) increased contact with coral tissue due to taller algal fronds, leading to more transfer of DOM, microbes, and hydrophobic compounds to the coral. All of these differences amplify the ^{3}DAM dynamics and facilitate algal ability to kill and overgrow live coral colonies.

Envisioning the Invisible Reef

If direct contact by algae is not necessary to negatively influence corals, then it is imperative to envision how dissolved compounds and microbes move between the two holobionts. This is most easily explained by a basic understanding of flow dynamics. Coral reefs are complex physical structures that have a significant influence on the movement of water. Despite often high flow and wave action on coral reefs, net water transport is slow within and directly above the reef (93,94), and it is within these water masses that most coral-algae interaction dynamics occur.

Seawater has structure

There is a misconception that flow and advection homogenize the reef water landscape. In fact, the water over a coral reef is a varying, complex landscape that is shaped by the structure of the benthos and the flow of the water interacting with it. From the microbial perspective, every drop of seawater is a heterogeneous mix of gels,

strings of organic matter, microscopic particles, and discrete hotspots of microbial and viral activity (95). This layer of connectivity on coral reefs is only just beginning to be described and visualized.

Boundary layers. Like any object submerged in water, corals and other benthic organisms alter local flow conditions, creating shear and boundary layers. On a coral reef, this interaction between the benthos and surrounding water leads to the formation of three types of boundary layers: 1) benthic (BBL), 2) momentum (MBL), and 3) diffusive (DBL) (Figure 3 (94)). Each of these boundary layers works on a different scale (meters, centimeters, and millimeters to microns, respectively), and all are affected by the shape and porosity of the benthos plus the rate, type (turbulent, laminar, etc.) and direction of water flow. For example, strong currents can create very thin boundary layers, but immediately below the rapidly moving water will be a stagnant DBL. On reefs with lower flow or wave exposure, the boundary layers can be much thicker. In all cases, the structure of the reef limits flow and creates diffusion-limited environments in direct contact with benthic organisms where hydrophilic compounds such as DOM and microbes can accumulate. It is within these boundary layers that corals and algae interact.

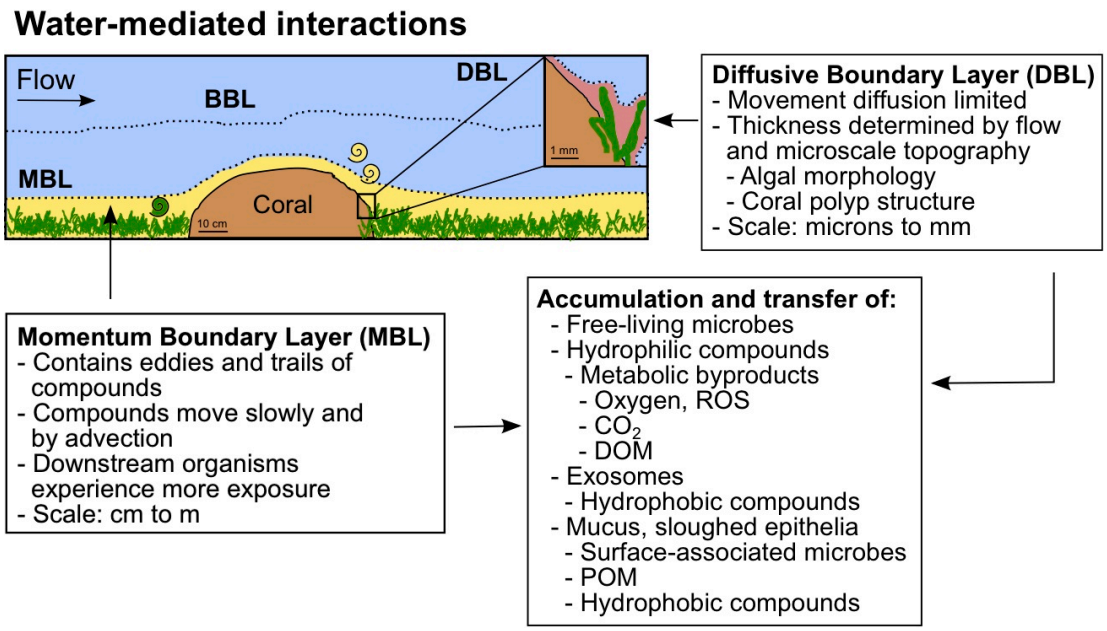

Figure 1.3. Boundary layers and water-mediated transport of hydrophilic compounds across a coral reef. BBL, benthic boundary layer; MBL, momentum boundary layer; DBL, diffusive boundary layer.

Ecological consequences. Parcels of water above the reef have different biological and chemical characteristics. At the smallest scale, the accumulation of metabolic compounds within the DBL affects the physiology of the organism the compounds originated from (e.g. build up of oxygen, carbon dioxide, or acetylene in the boundary later inhibits photosynthesis, respiration, and nitrogen fixation, respectively (96–98)), and removal of these compounds via flow both lessens their effect on the host physiology, as well as exposes downstream neighbors to them. Empirical studies indicate that these downstream effects occur on coral reefs. For example, water collected downstream of conspecific coral adults increases larval mortality, while water surrounding heterospecific corals does not (99). This response is due to the presence of microbes released or sloughed by conspecific adults, as

evidenced by loss of effect in the presence of antibiotics or when microbes are filtered out prior to larval treatment (99). These parcels of water also affect juvenile coral mortality *in situ*, causing higher mortality downstream of conspecific adults than upstream of conspecific adults or downstream of heterospecific adults (99). In another example, planktonic larval stages of benthic invertebrates (e.g. nudibrancs) and vertebrates (e.g. fish) respond behaviorally to spatially patchy hydrophilic cues, using them to find specific desirable habitats for recruitment (93,100); no simple task when one considers the diverse and complex landscape of the reef benthos.

These studies support the hypothesis that soluble compounds (DOM, microbes, viruses, etc.), while they may diffuse or be removed from the source via flow, can persist at a high enough concentration within a parcel of water to have significant ecological effects at great distances (cm to meters) without the need for direct contact or transmission via diffusion (93,99,100). As a consequence, corals that are downstream of aggressive benthic competitors are expected to more frequently to encounter harmful microbes, DOM, or hydrophilic toxins at higher concentrations in the water column. There is evidence that the direction of water flow affects coral-algae interactions, such that coral-turf algae interactions that face into the flow fair better (for the coral) than those facing away (downstream) from the flow (A. Brown, personal communication). This is presumably due to removal of harmful hydrophilic compounds from the boundary layer of above the interaction border.

Micro-vision: crawling mats.

The reefs surface is a vast landscape of holobionts with characteristic microbial and viral communities living on the surfaces of benthic macro-organisms. These holobionts crawl over each other in competition for space. In some cases these crawling assemblages are strictly microbial mats (e.g., black band disease (48)), whereas others are predicted to involve a combination of microbes plus physical and chemical intermediaries (e.g. DOM, allelochemicals). It is difficult to distinguish between microbial and toxin-mediated interactions on a reef, since the two likely feed upon each other and the phenotypes observed along coral-algae interactions appear the same with either mechanism. If DOM is the primary mechanism, microbial growth and respiration is promoted at the interface, leading to coral death through opportunistic infections and/or by creating zones of hypoxia that suffocate the coral holobiont (Figure 4). If hydrophobic molecules transferred by direct contact are involved, toxicity to the coral holobiont may facilitate invasion of the compromised tissue by opportunistic microbes, which crawl over the surface killing the tissue beneath and clearing the way for the alga to advance. Alternatively, hydrophobic compounds can also directly stimulate microbial growth and invasion of opportunistic pathogens and/or suffocation of the coral by residents of the holobiont (90) (Figure 4). With either competitive mechanism, a crawling edge is initiated where algal compounds promote the invasion of opportunistic microbes that damage the coral holobiont and clear space for the alga to advance (Figure 4). As mentioned earlier

(section 3.4), the occurrence of direct contacts between corals and algae likely increases on degraded coral reefs (Figure 4).

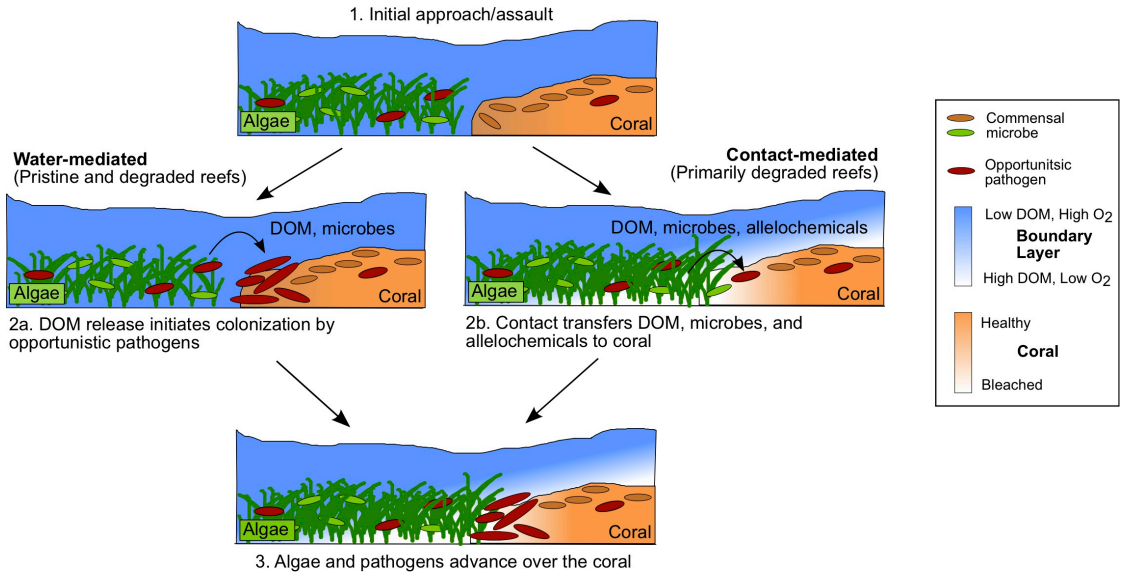

Figure 1.4. Benthic battles between corals and algae and examples of the mechanisms involved.

Implications for testing hypotheses.

The microstructure of a coral reef (parcels-of-water, crawling mats, boundary layers, etc.) is incredibly important to take into consideration when designing an experiment to test a mechanistic hypothesis. First, it is important to perform controlled experiments where parameters like light, water flow, etc. are known. In some cases, it is desirable to control diffusion across the boundary layers. This is most easily done by controlling the concentrations of the treatment in the surrounding water with a rapid change over to avoid bottle effects (e.g., Kline (85) and Kuntz (86)). In other cases, it is desirable to remove flow effects by moving to closed systems (e.g., aquaria,

enclosures on the reef); however, these types of experiments are only good for short-term studies because of bottle effects. In other cases, it might be desirable to measure the influence of flow, in which case a flow chamber may be used where flow rates and the type of flow are more controlled. Once a phenomenon has been experimentally documented under controlled experimental conditions, one may then go out to the field/reef and measure/observe if the same dynamics are occurring in the field. The effect of flow on benthic interactions becomes particularly important to understand since flow regimes are likely to change as reefs degrade. The flattening of reefs as corals die, for example, may actually contribute to resilience by increasing turbulence and creating thinner boundary layers. As any aquarist knows, bad water effects can partially be mediated by increasing flow, which more rapidly removes waste products, microbes, etc. from the boundary layer.

The third type of experimental design used extensively in the coral reef field is a "natural experiment" where corals, settlement plates, etc. are set out on the reef. These types of treatments are often raised off the benthos on cinderblocks or some other structure, which completely changes the local flow dynamics in unpredictable ways and is not representative of the types of flows that native benthic organisms (actually attached to the benthos and not raised above it) actually experience. Therefore, if flow is not controlled or measured within a ‘natural’ experiment, the results will be difficult to interpret and nearly impossible to compare to other experiments. Results from these ‘natural’ experiments are important to coral reef studies, but their limitations must always be kept in mind. Non-equivalent study

designs are one of the reasons that so many contradictory studies are found on coral reefs.

Conclusions

Coral reefs around the world are being lost at an alarming rate, and the pressure on these ecosystems is not likely to ease in the foreseeable future. Human population is booming, inevitably increasing coastal development and fishing pressure, while projections of fossil fuel usage indicate that carbon dioxide levels in the atmosphere will continue to rise, leading to more frequent and severe cycles of sea surface temperature anomalies that cause coral bleaching and death, and greater ocean acidification that may inhibit or slow calcification of reef organisms. In order to protect coral reefs and possibly restore those that are degraded, we hope that understanding the mechanisms and dynamics behind phase shifts provides useful insights.

Identifying the coral-algal competition dynamics on a coral reef has the advantage of providing an instantaneous snapshot of the state of the benthos. Unlike benthic cover, the cumulative outcomes of coral-algal interactions on a reef provide information on the health of the reef and the direction that it is headed. Furthermore, by surveying large areas of a reef and looking at all of the different combinations of players, one can get information from the reef itself about which types of interactions are most important, and over time which types of interactions are the fastest to change under different situations (increased sedimentation or nutrient load, decreased

herbivory, etc.). This allows for the rapid assessment of the impacts of both environmental restoration (e.g. marine protected areas) and damage (e.g. coastal development) without having to wait decades for coral cover to change. These data could also guide restoration strategies by prioritizing protection of areas of reef where corals are successfully competing against algae or areas with more competitively dominant species of corals, as determined *in situ* for the very reef in concern. By increasing our grasp of benthic reef dynamics through studying how they function across a range of circumstances and locations, we hope to better predict how reefs will respond to a changing world, and be better prepared to prevent the loss of these fascinating ecosystems.

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Acknowledgements

Chapter 1 is currently being prepared for submission of publication of the material. Katie L. Barott and Forest L. Rohwer. The dissertation author was the primary investigator and author of this paper.

CHAPTER 2

Natural history of coral-algal competition in the Line Islands

Abstract

Competition between corals and benthic algae is prevalent on coral reefs worldwide and has the potential to influence the structure of the reef benthos. Human activities may influence the outcome of these interactions by favoring algae to become the superior competitor, and this type of change in competitive dynamics is one potential mechanism driving coral-algal phase shifts. Here we surveyed the types and outcomes of coral interactions with benthic algae in the Line Islands of the Central Pacific. Islands ranged from nearly pristine to heavily fished. We observed major differences in the dominant groups of algae interacting with corals between sites, and the outcomes of coral-algal interactions varied across the different islands. Corals were generally better competitors against CCA regardless of location, and were superior competitors against turf algae on uninhabited islands. On inhabited islands, however, turf algae were generally the superior competitors. When corals were broken down by size class, we found that the smallest and the largest coral colonies were the best competitors against algae; the former successfully fighting off algae while being completely surrounded, and the latter generally avoiding algal overgrowth by growing up above the benthos. Our data suggest that human disruption of the reef ecosystem may lead to a building pattern of competitive disadvantage for corals against

encroaching algae, particularly turf algae, potentially initiating a transition towards algal dominance.

Introduction

Coral reefs are areas of intense competition between sessile benthic organisms. Sufficient access to space and light is crucial for survival on the reef, and the ability to establish, maintain and extend territory (i.e. to outcompete fellow benthic organisms) can affect the composition, size and distribution of organisms on the benthos (1). Corals and benthic algae are two of the main groups that compete for space on a coral reef, and inter-specific interactions can have major effects on the growth and reproduction of benthic competitors (2). Corals, for example, can inhibit the growth algae, with the strength of inhibition determined by species identity and environmental conditions (3–6). The ability of corals to fight off their algal competitors becomes increasingly important in the face of local stressors (eutrophication, sedimentation, fishing) and global climate change (rising sea surface temperature, ocean acidification), particularly since algae are becoming more dominant on coral reefs around the world (7–10).

The effects of algae on corals can vary widely by the type of alga involved in the competition. Macroalgae, for example, have been shown to have a range of detrimental effects on corals, which include inhibition of coral recruitment, growth and fecundity (4,11–15). Many macroalgae have been found to produce secondary metabolites (i.e. allelochemicals) that cause some of these negative effects on corals (16–18). Turf algae, a diverse assemblage of filamentous algae, also have a variety of effects on corals. Turf algae can lead to hypoxia along competitive borders with corals (19,20), cause tissue damage and bleaching along the coral border (19,21), lower coral

fecundity (11), and can inhibit coral recruitment (7,14,22,23). Some algal assemblages, however, have little effect on neighboring corals (24) or coral recruitment (14), indicating that the composition of the turf community likely plays an important role in the interaction with corals. Turf algae are among the most abundant algal competitors that corals face (19–21), and as such likely play an important role in initiating algal phase shifts on disturbed coral reefs. Crustose coralline algae (CCA), in contrast, are generally less detrimental to corals than are other types of algae (6,19,20). CCA can even be beneficial for corals by providing settlement cues and substrate for coral larvae (25–27) while limiting colonization of some types of potentially harmful macroalgae (28).

Changing environmental factors such as eutrophication, reduced herbivory, or ocean acidification can shift the dynamics of interactions on the reef. For example, decreased herbivory leads to a decrease in CCA abundance (29), an increase in turf and macroalgae (30), and a shift towards algal dominance (7), whereas high herbivory is associated with more CCA and less turf and macroalgae (31–33). The types of herbivores present affects the distribution of algae on the reef (34), and selective removal of urchins versus herbivorous fish, for example, can have a major impact on the types of algae along coral borders (35). Nutrient enrichment can also alter competitive outcomes by both inhibiting coral growth and stimulating algal growth, although the effect of nutrients tends to be less than that of herbivores (9,29,31,33,36), though see (6). Ocean acidification (i.e. CO_2 enrichment) has also been found to increase macroalgal damage on corals (37) and inhibit the calcification of CCA (38),

potentially leading to a competitive advantage of turf and macroalgae over both corals and CCA.

Overall, local to global anthropogenic disturbances appear to be shifting the competitive advantage towards turf and macroalgal dominance with the concomitant loss of reef-accreting calcifiers such as corals and CCA. Here we surveyed the abundance, composition, and apparent outcome of different types of coral-algal competitive interactions in the Line Islands of the Central Pacific. Survey sites included two nearly pristine uninhabited islands, and spanned a gradient of human activity, inorganic and organic nutrient regimes, and microbial communities (39,40).

Methods

Site descriptions: This investigation was conducted during an expedition to the Line Islands in October-November 2010. The islands visited for this study (followed by the abbreviations used throughout the text) included Kingman Reef (KIN; 6.390 N, 162.360 W), Teraina (TER; 4.686 N, 160.420 W), Tabuaeran (TAB; 3.825 N, 162.349 W), Kiritimati (KIR; 2.008 N, 157.489 W), and Jarvis (JAR; 0.369 S, 160.008 W) (Figure 2.1). Surveys were grouped by the region within each island and are labeled by island abbreviation and location within the island (N, north; S, south; etc.). Kingman and Jarvis are uninhabited US protectorates that are managed by the US Fish and Wildlife Service as part of the Pacific Remote Islands Marine National Monument. Teraina (a.k.a. Washington), Tabuaeran (a.k.a. Tabuaeran), and Kiritimati (a.k.a. Christmas) belong to the Republic of Kiribati and are inhabited (approximately

1000, 3000, and 10,000 people per island, respectively; (39,41)). Kiritimati and Tabuaeran have previously been shown to contain higher abundances of fleshy algae, bacteria and viruses (40), while Kingman and Jarvis have a greater abundance of predatory fish and reef-building corals and CCA (39,42). Tabuaeran, Kiritimati and Jarvis have higher inorganic nutrient concentrations in the water column due to their location within the equatorial countercurrent with concomitant elevation of nearshore upwelling (39). All surveys for this study were conducted on the forereef with the exception of Kingman, where surveys were conducted on a patch reef in the large lagoon.

Benthic cover: The composition of the benthos was determined using the photoquadrat method (43). At each site, two 25 m transects were deployed at a constant depth of 10-12 m. A total of five quadrats placed at 5 m intervals were photographed per transect using a Canon G9 camera connected to a quadpod and frame (0.63 m^2 total area within each image). Image analysis of the photoquads was completed using Photogrid 1.0 (www.photogridnetfirms.com). A total of 100 points were placed in a stratified random design over each image, with the substrate under each point identified to the finest resolution possible (genus for corals, macroalgae and invertebrates when possible, and functional group for turf algae and CCA). When no biological cover was noted under a point, the non-biological substrate (e.g., sand) was recorded. Benthic cover data were complemented from comparable collections in May

2010 by scientists with NOAA's Coral Reef Ecosystem Division using similar methods (44).

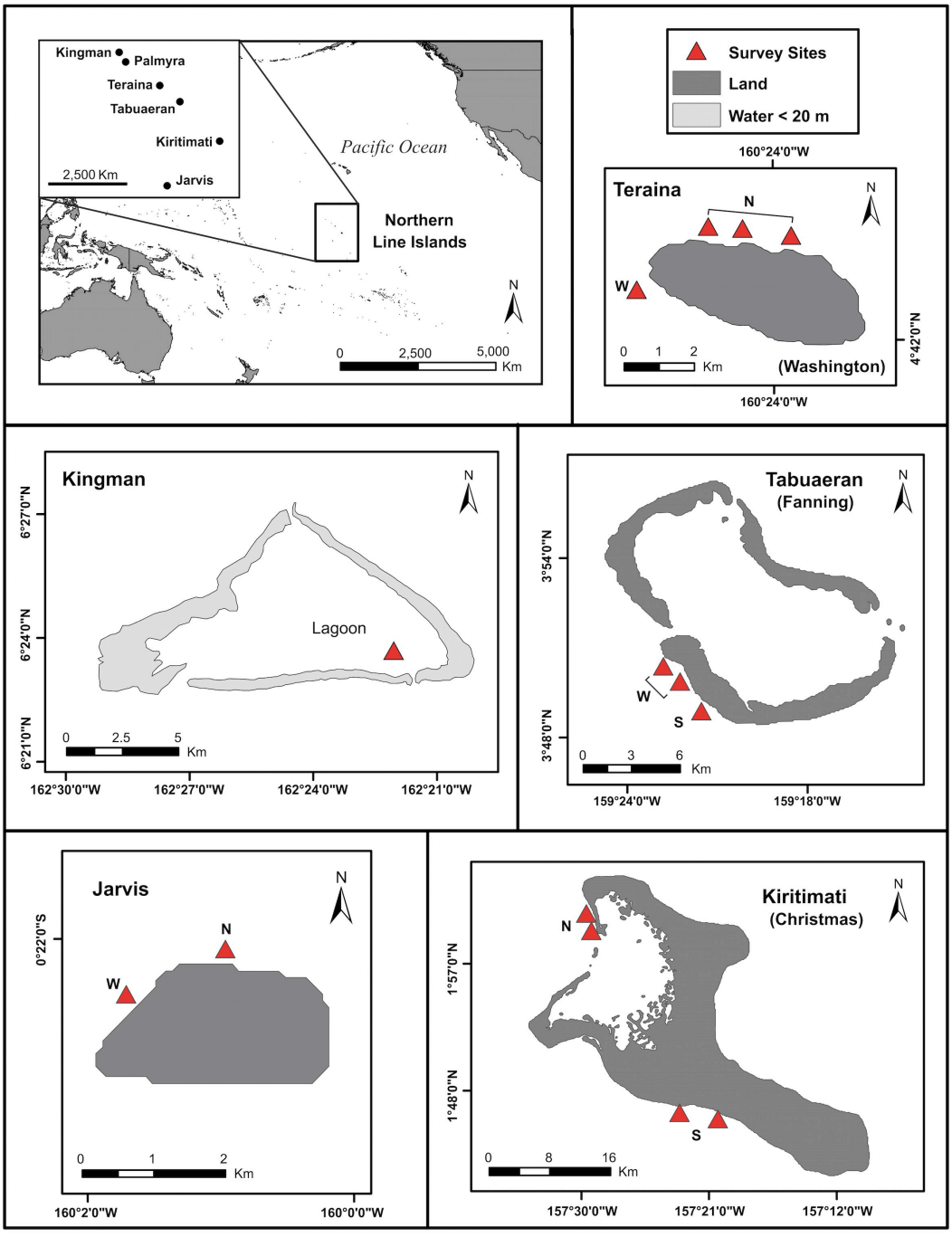

Figure 2.1. Maps of islands surveyed. Triangles indicate survey locations. Maps were generated using ArcGIS.

Surveys of coral-algal interactions: In order to quantify the abundance of coral-algal interactions, a line point intercept survey approach was used as previously described (19). All surveys were conducted at a constant depth of 10 m along a 10 m transect line, and at least two transects were conducted per site. For each coral colony intercepting the transect line, the identity (to genus level) and maximal colony diameter were recorded. Any alga in contact with the coral colony was identified to genus for macroalgae or functional group for CCA and turf algae. The proportion of the coral colony's edge involved in each type of coral-algal interaction was recorded, as well as the outcome of each interaction. Three outcomes of interactions were defined: coral overgrowing algae, algae overgrowing coral, and apparently neutral (Figure 2.2).

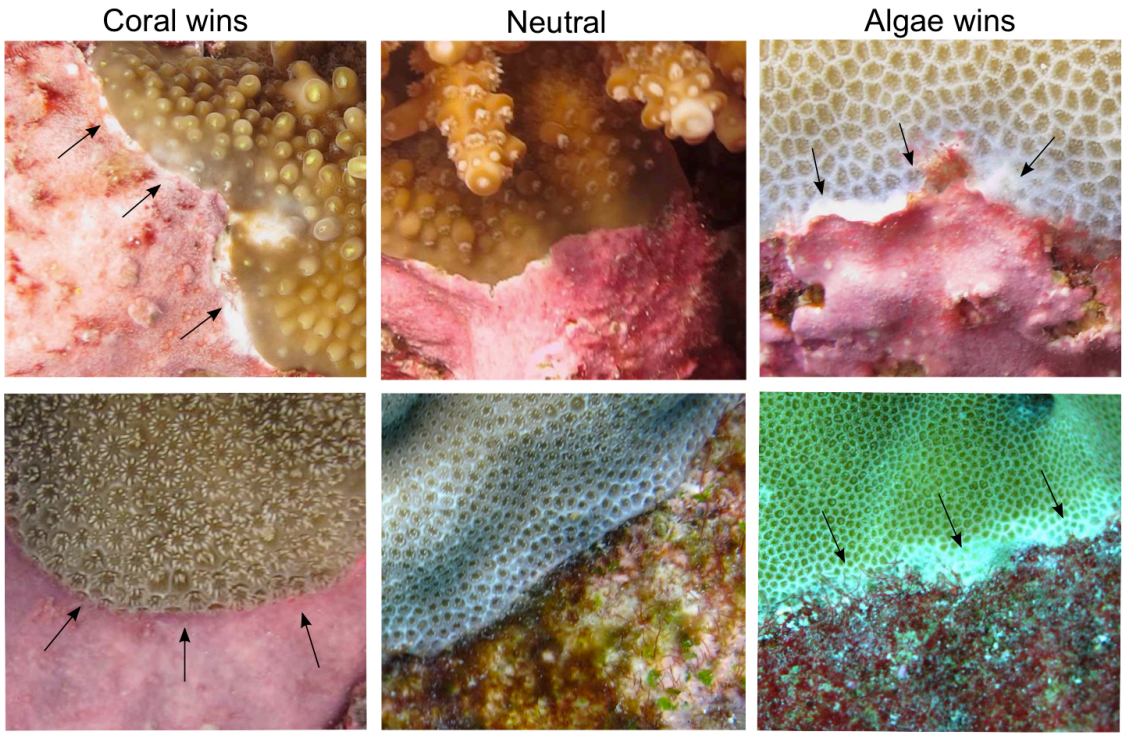

Figure 2.2. Coral-algal interaction examples. Left column, coral overgrowing/damaging algae; middle column, neutral; right column, algae overgrowing/damaging coral. Arrows indicate areas of tissue damage/overgrowth.

Statistical analyses: In order to test whether the proportion of algal types bordering corals was purely a function of their relative abundance on the reef benthos at the site we developed a novel statistical approach to deal with the multinomial nature of the data. In summary, the four-dimensional data (with the multinomial data being the relative abundance of CCA, turf, *Halimeda* spp. and fleshy algae) across the two groups (coral edge and reef benthos) were first visualized in a three-dimensional manner (the fourth variable is always a function of the proportion of the other three). This gave a visualization of the overlap between the two groups in three-dimensional space and a first indication as to whether the two groups differed (i.e. limited overlap in dispersion around each group centroid). To formally test the null hypothesis that the two groups did not differ (i.e. there was sufficient overlap between the two groups to suggest that the edge and benthos algal communities did not differ) we employed a resampling approach to estimate the probability of group membership affecting the distribution of distances from each group centroid (analogous to ANOVA logic). The null distribution of deviations was generated using a randomization procedure and the null deviations then compared to the actual deviations. When $P \leq 0.05$ we rejected the null hypothesis, indicating that the coral edge and reef benthos algal communities differed.

In order to determine if the proportion of coral borders with ‘no algae’ differed by size class, we used a non-parametric Kruskal-Wallis test with subsequent Dunn's procedure for pairwise comparisons, and applied a Bonferroni alpha of $P = 0.02$ to compensate for the multiple comparisons. We further determined if the number of

colonies with a greater proportion of their edge winning to algae versus losing to algae were statistically different from random (0.5) using a two-tailed binomial distribution test. The differences between sites based upon the algal proportions and outcomes along coral borders (e.g. percent coral border losing to turf algae) were determined using the Bray-Curtis index. A non-metric multidimensional scaling (nMDS) ordination was performed on the Bray-Curtis similarity matrix to visualize the separation of the sites based on coral-algal competition, and statistical clustering of sites was determined using a similarity profile test. All statistical analyses were completed using R (45).

Results

Composition of the reef benthos: Hard coral cover was greatest at Kingman (67%), Jarvis-W (58%) and Kiritimati-S (48%), and lowest at Teraina-W (9%) and Jarvis-N (11%; Figure 2.3). CCA cover was highest at Teraina-W (45%), followed by Kiritimati-S (33%) and Tabuaeran-S (30%; Figure 2.3). CCA was lowest at Kiritimati-N (3%) and Jarvis-N (8%), the two sites that also had the greatest abundance of turf algae (59% and 78% of the benthos, respectively; Figure 2.3). Turf algae cover was lowest at Tabuaeran-S (4%), but this site also had the highest abundance of macroalgae (43%), but these were primarily calcifying *Halimeda spp.* Macroalgal cover was also high at Teraina-N (32%; Figure 2.3), and was about half calcareous *Halimeda* spp. and half fleshy macroalgae.

Composition of algae interacting with corals: The types of algae that corals were interacting with varied by site. Kiritimati-N and Jarvis-N had the greatest proportion of the coral edge interacting with turf algae (>75% of each coral border), followed by Teraina (N&W), Kiritmati-S, and Jarvis-W (34-46%; Figure 2.3). The highest amount of edge occupied by CCA occurred at Kingman and Tabuaeran-S (35-40%; Figure 2.3). *Halimeda* spp. were most abundant along the coral edge at Teraina-N and both Tabuaeran sites (8-20%; Figure 2.3). Kingman, Tabuaeran-W, and Jarvis-W had the greatest proportion of coral edges that were not interacting with any algae (47-61%).

At all sites, with the exception of Tabuaeran-W, the abundance and composition of algae along the coral edge was not purely a function of the relative abundance of the algae found on the benthos (Figure 2.4, Supplementary Table 1). For example, coral borders at Kingman were comprised of a greater abundance of CCA than would be expected by chance alone based on the relative abundance of CCA on Kingman's benthos. Similarly, Teraina-W and Kiritimati-S had less CCA bordering corals than expected by chance alone (Figure 2.4). These two sites also had a greater proportion of turf algae interacting with corals than expected by chance alone; this was also true for Jarvis-W, Teraina-N, and Tabuaeran-S. In addition, Tabuaeran-S had less macroalgae, particularly *Halimeda* spp., interacting with corals than expected by chance alone given the relative abundance of macroalgae at the site (Figure 2.4).

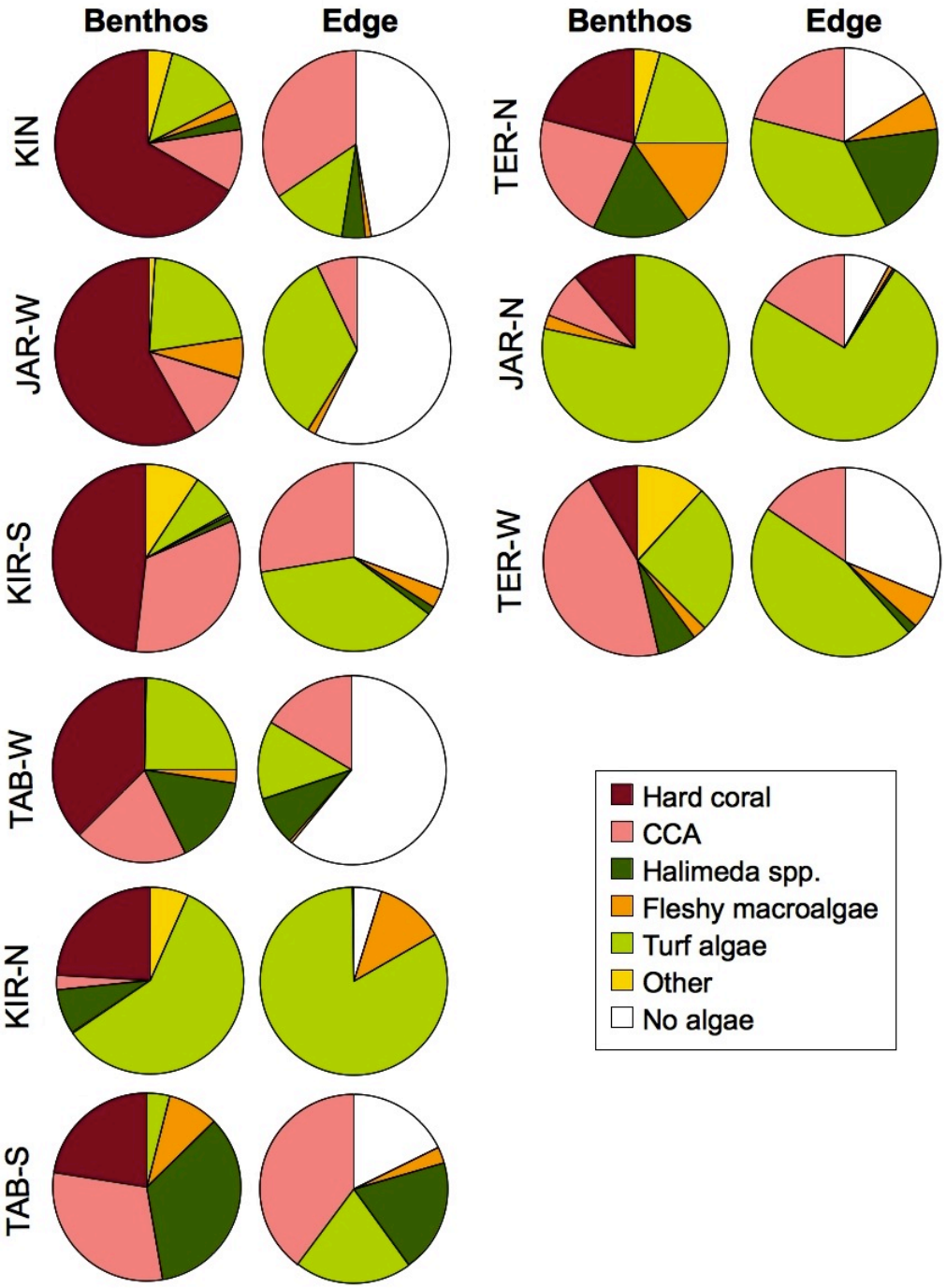

Figure 2.3. Composition of the reef benthos (left columns) and composition of algae along coral borders (right columns) at each site. Sites are listed by decreasing hard coral cover.

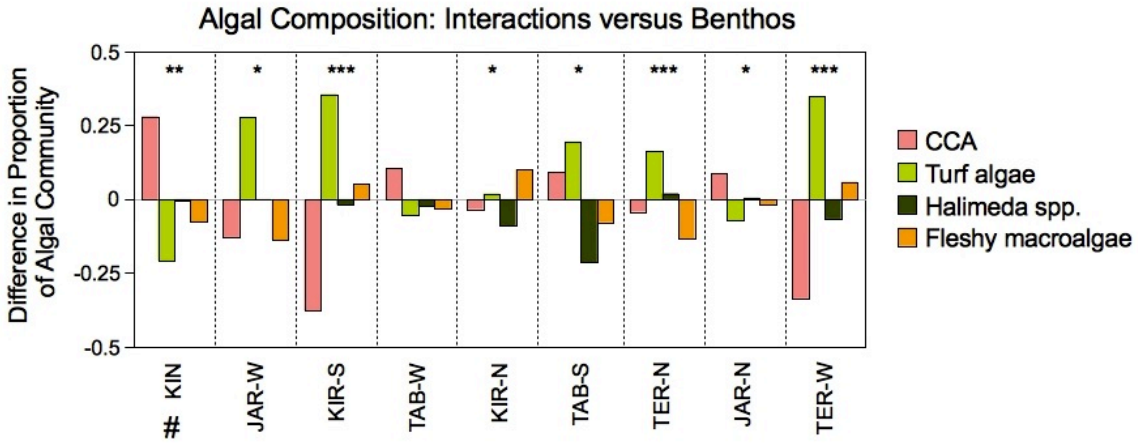

Figure 2.4. Difference between the composition of coral-algal interactions (i.e. algae along coral borders) and algal composition of the benthos. Greater than zero indicates enriched along the coral border. Sites are listed by decreasing hard coral cover. $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; lagoon habitat, #.

Coral colony size and algal interaction outcomes: Both Teraina sites and Kiritimati-S were dominated by small corals <40 cm in diameter (Supplementary Figure 1). Kiritimati-S differed from Teraina in that there were a greater number of corals per meter; this site had high coral cover (Figure 2.3) but all were small to mid-size coral colonies (Supplementary Figure 1). Kingman and Tabuaeran had the largest coral colonies present, with many corals reaching over a meter in diameter, primarily *Porites* spp. on Kingman and *Acropora* spp. on Tabuaeran (Supplementary Figure 1, 2). Jarvis-N had a low density of mostly small coral colonies (<20 cm), while Jarvis-W had a high density of coral colonies, including many large corals >80 cm that were almost entirely *Montipora* spp. (Supplementary Figure 1, 2).

The total proportions of coral colony borders that were not in contact with algae ("no algae") differed significantly across coral size classes (KW, $H = 87.96$, df =

5, p = <0.0001; Figure 2.5). For example, 5 cm and 10 cm classes had less "no algae" (i.e. more algae) than all larger size classes (Dunn's, p = < 0.0001 and p = < 0.0009,

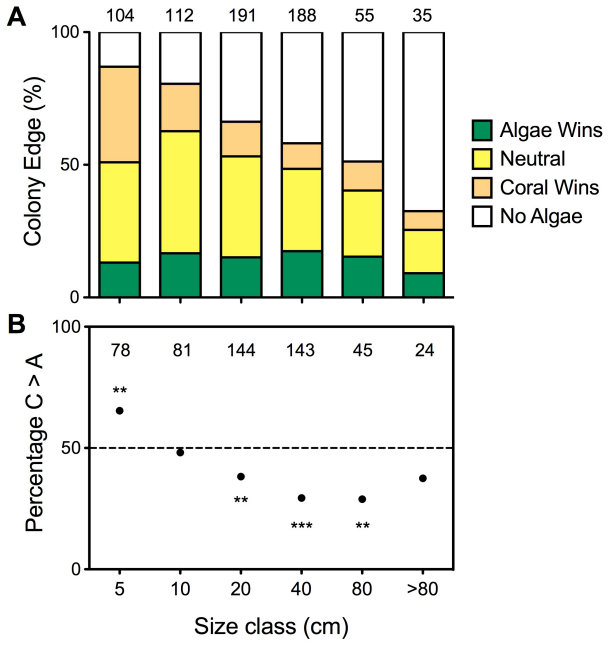

Figure 2.5. Coral-algal interactions by coral size class. A) Algal competition outcomes by size class. Numbers above columns indicate the number of colonies observed. B). Percentage of coral colonies where corals are winning against algae along a greater proportion of the colony edge than they are losing to algae. Numbers indicate the number of colonies included in the analysis (i.e. colonies with at least one non-neutral algal interaction). $p < 0.01$, **; $p < 0.001$, ***.

respectively), and the 20 cm class had less "no algae" than the two largest classes (80 cm and >80 cm; p = 0.0064 and p = 0.0008, respectively). The largest colonies (>80 cm diameter) on average had ~30% of their border in contact with algae, whereas the smallest colonies (<10 cm in diameter) were almost completely surrounded by algae (~80% of the perimeter, Figure 2.5a). The outcome of coral-algal interactions varied depending on coral size class. The smallest coral colonies (<5 cm) were the only coral size class to have a greater proportion of their border winning against algae than losing ($p < 0.001$, Figure 2.5b). A total of 104 corals fell into this class, and included 12

different genera. Both the 10 cm and >80 cm size classes (112 individuals from 12 genera and 35 individuals from 6 genera, respectively) showed no bias in the proportions of their edge winning and losing to algal competition (Figure 2.5b), indicating that for these coral classes the two are equal. Mid-sized coral colonies (20, 40, and 80 cm; including 434 individuals from 16 genera), in contrast, lost a greater proportion of their border to algae than they won (Figure 2.5b). Each size class included a wide variety of coral genera and morphologies, indicating that the patterns observed were size- and not necessarily species- or morphology-dependent.

Coral-algal interaction outcomes by site: Coral-algal interaction outcomes varied by site. Algae were winning the greatest proportion of competitive interactions along the coral edge at Kiritimati-N&S, Tabuaeran-S, and Teraina-W (Figure 2.6a). Jarvis-N, in contrast, had the greatest proportion of corals winning versus losing along the coral edges (Figure 2.6a). Within a site, coral-algal interaction outcomes varied by the type of algae. Corals tended to be superior competitors (i.e. coral winning more of the competitive edge than algae) against CCA ($p < 0.01$) except on Kingman and Teraina (N and W) where the two were not significantly different (Figure 2.6b). However, when corals were interacting with turf algae, the only site where corals were superior competitors was Jarvis-N ($p < 0.05$; Figure 2.6c). Competitive outcomes between corals and turf algae did not differ at Kingman, Jarvis-W, Kiritimati-N, or Teraina-N, but corals were losing a greater proportion of competitive interactions

along their border to turf algae at Kiritimati-S, Teraina-W, Tabaueran-S and Tabuaeran-W ($p < 0.01$; Figure 2.6c).

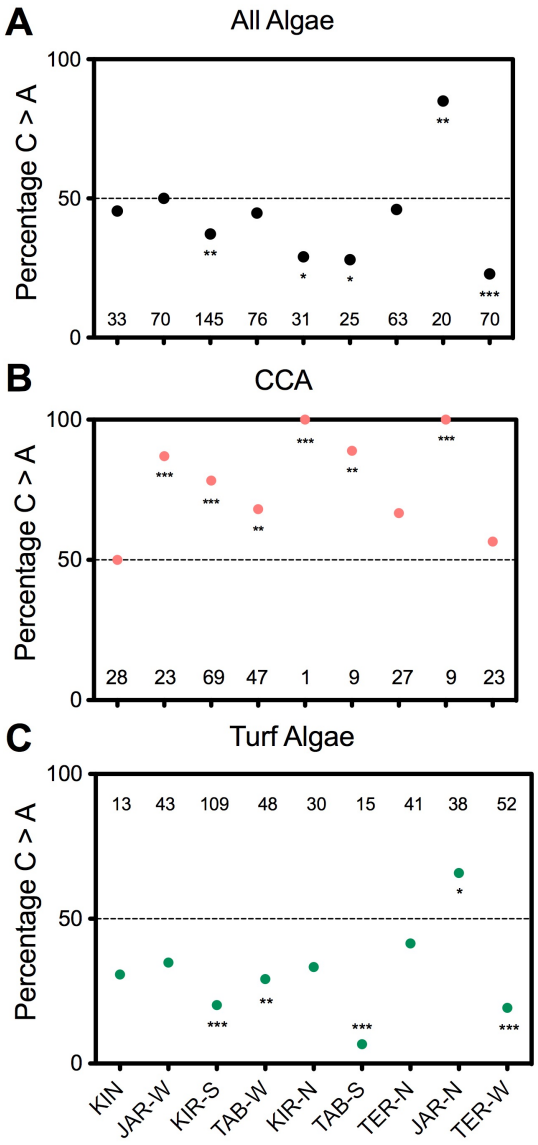

Figure 2.6. Percentage of coral colonies where corals are winning against all algae types (A), CCA (B) or turf algae (C) along a greater proportion of the colony edge than they are losing to that type of algae. Numbers indicate the number of colonies included in the analysis (i.e. colonies with at least one non-neutral algal interaction). Sites are listed by decreasing hard coral cover. $P < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; lagoon habitat, #.

A similarity profile test indicated that the sites formed four significant clusters based upon the types and outcomes of coral-algal interactions (Figure 2.7). The first cluster included Kingman, Tabuaeran-W, and Jarvis-W, and a non-metric multidimensional scaling (nMDS) ordination indicated a correlation of these sites with a high proportion of edge not in contact with algae. Jarvis-N and Kiritimati-N formed another cluster, correlated with a high proportion of turf algae along the coral edges at these sites. While forming a single cluster, however, Jarvis-N appeared to be correlated with corals mostly winning against turf algae while at Kiritimati-N they were losing (Figure 2.7). Teraina-N and Tabuaeran-S clustered together, and were likely correlated with a high proportion of the coral edges interacting with *Halimeda* spp (calcified macroalgae). Teraina–W and Kiritimati-S also clustered together, and were correlated with a high proportion of the coral edge interacting with other types of fleshy algae (e.g. *Caulerpa* spp., *Lobophora* spp., etc.) (Figure 2.7). The clustering of the different sites by coral-algal competitive types and outcomes did not appear to be purely a function of the relative abundance and composition of the coral genera present at the sites since sites with different dominant coral genera (e.g. Jarvis-W, Kingman, and Tabuaeran-W; Supplementary Figure 2) still clustered together based on competitive outcomes (Figure 2.7).

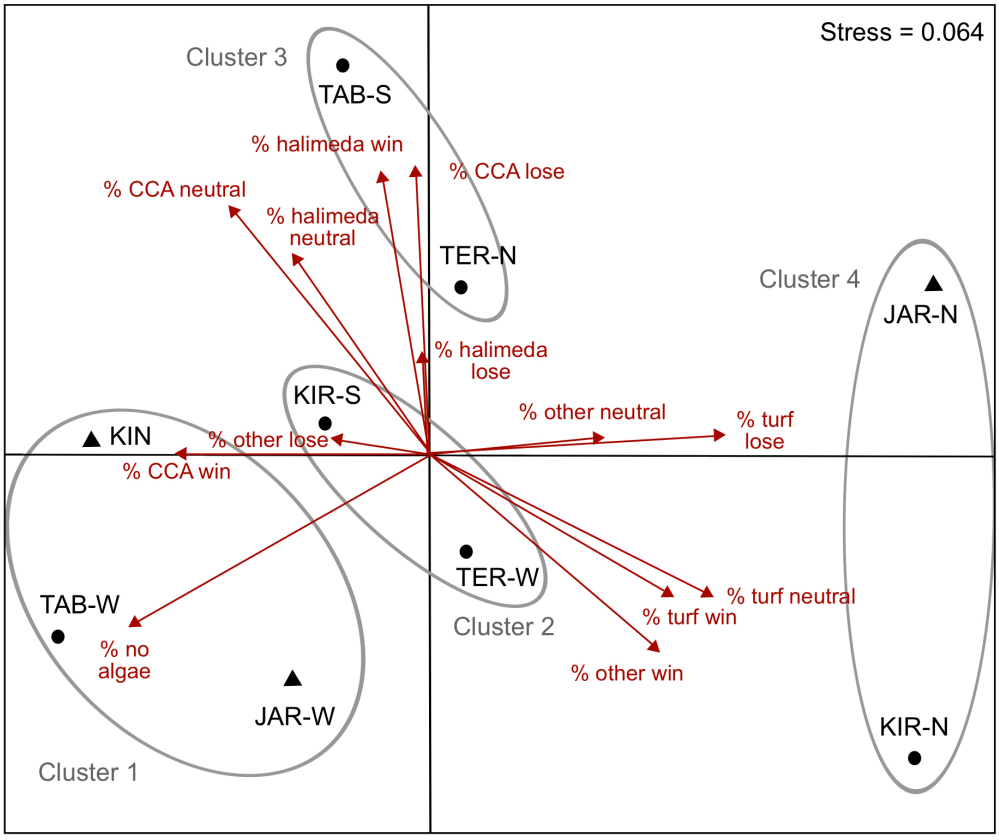

Figure 2.7. Non-metric multidimensional scaling plot of outcomes of coral-algal interactions by site using Bray-Curtis similarity index. Circles, inhabited islands; triangles, uninhabited islands. Clusters were determined by a similarity profile (SIMPROF) test (alpha=0.05).

Discussion

Coral colony size and possible strategies for competition with benthic algae: Small coral colonies were typically surrounded by algal competitors along most of their perimeter, yet these small colonies tended to be better competitors against algae. Partial coral mortality most often occurs from bottom related processes (e.g. algal competition) for all coral size classes, and these types of partial mortality events often result in total mortality for small colonies (46,48). It is possible then that small colonies that were not good competitors against algae may not have been observed due

to their high rates of mortality, leaving only the small colonies that were the competitively dominant survivors to be observed. This dynamic suggests that size escape may be an important strategy employed by corals for surviving algal competition (46,48), making growth a particularly important investment for small colonies. This investment usually requires an energetic tradeoff, however, indicated by observations that young corals of the smallest size classes grow quickly but are not reproductively active (49–51), saving their limited resources to grow and defend their borders. This strategy may explain why the small colonies observed here were winning against algae, since much of their available energy is likely spent on growth and competition.

Mid-sized corals, in contrast, lost to algae more than they won. It is likely that these adult coral colonies invest less energy into fighting off algae at their borders, particularly since they are likely at a reproductive age and thus may allocate a significant amount of energy towards reproduction (49–51). In addition, small losses along the colony edge are not as significant for these colonies, since a loss of 1 cm to an algal competitor is only a small proportion of the entire colony. While it is still important to maintain tissue health and growth along the colony edge, less of the entire colony's energy resources are likely diverted to this area as more energy is invested in reproduction. Competition and growth are not sacrificed, however, and previous observations that the edges of larger colonies contain few to no reproductive polyps (11,50) suggest that the energy of these polyps is allocated towards growth and competition in lieu of reproduction. If reproduction does affect competition, the time

of year these types of surveys are done may influence the outcomes observed along coral borders with algae since many corals reproduce on seasonal cycles. Due to the single time-point nature of the current study, this hypothesis remains to be tested.

The largest coral colonies observed (>80 cm), like small colonies, appeared to be better competitors against algae than their mid-sized counterparts. However, in contrast to small colonies, the proportion of the perimeter of large colonies interacting with algae was low (~30%; Figure 2.5a). Large corals appeared to use an "escape in height" strategy (46), growing up off of the benthos and avoiding algal competition all together. Since relatively little of the colony border was interacting with algae, less energy would be needed to defend against the algae than if the entire perimeter were in contact with algae, like for smaller colonies. Furthermore, the area from which a large coral colony can draw energy (e.g. from symbiotic zooxanthellae photosynthesis (52) or heterotrophic feeding (53)) is much greater than for a small colony, since the area of a colony increases much more rapidly than the perimeter. Therefore, large colonies likely have more energy to draw from that can be used to fight algae along the border, while still having enough energy to invest in reproduction.

Coral-algal interactions change with human habitation: The composition and outcome of coral-algal competition varied depending on the site. In general, coral competition with CCA did not appear to be detrimental for corals, regardless of the site or level of human habitation. On the other hand, corals appeared to lose more often to turf algae at inhabited sites, while being equal or superior at uninhabited sites.

Corals at inhabited sites may be weaker competitors due to an increased abundance of potentially pathogenic bacteria and a higher prevalence of certain coral diseases (40). In addition, algae may become better competitors at the inhabited islands due to increased inorganic nutrient concentrations that may increase fleshy algal growth or increase the abundance of pathogenic bacteria. On inhabited islands increases in the success of turf algal competition over corals may be a result of a shift in reef fish community structure due to fishing pressure (39,41,54). These changes in the fish community likely alter herbivore consumption rates, and may allow turf algae to increase in abundance and/or change the composition of the turf assemblage entirely. These changes may affect the production of dissolved organic carbon (55) or allelochemicals by the turf algae, possibly increasing the release of compounds that are detrimental to corals (17,20,56) and may therefore affect the outcomes of coral-algal competition over time.

Differences in latitude and biogeography (e.g. nutrient levels) are not likely the driving factor for changes in coral-algal competition outcomes observed here. Jarvis, for example, experiences significant upwelling of nutrients and has a high abundance of coral interactions with turf algae, yet the corals here are winning the majority of the competitive interactions against turf algae (Jarvis-N) or even (Jarvis-W). Kiritimati, on the other hand, also experiences equatorial nutrient upwelling but is inhabited (41), and the corals here are primarily losing ground to algae. Similarly, on the oligotrophic (i.e. non-upwelling, low nutrient) islands of Kingman and Teraina, we still find that on the inhabited island of Teraina the corals are losing to the turfs more often than not,

while on the uninhabited island of Kingman the corals are winning more often than not. The common thread that appears to influence the outcome of coral-algal competition on both nutrient-rich and oligotrophic islands is human habitation. Fishing pressure is high on many of the inhabited reefs, which has resulted in major shifts in the reef fish and benthic community structure (39–41,54), and this may be a primary cause behind the differences between coral-algal competition outcomes.

Future work will require following coral-algal interactions at different sites over time in order to determine if the instantaneous observations described here are consistent through time or indicative of long-term outcomes (i.e. increases or decreases in coral cover). Factors such as the seasonal variability of algal and coral growth and reproduction could not be taken into account in this study given the remote nature and limited access to these islands. The morphology of different coral species should be recorded in future studies, since this feature may influence the importance of algal competition. For example, it is possible that some coral species grow above the benthos faster than their basal edges get overgrown by algae (e.g. branching or plating growth forms) while others may be restricted in their upward growth (e.g. encrusting growth forms), making success in algal competition more important for colony survival.

Turf algae and CCA as indicators of coral reef health: The dynamics of coral interactions with turf algae could be indicative of a reef's future development. Both Tabuaeran-W and Kiritimati-S, for example, had high coral cover, but corals here

were losing to turfs more than they were winning. It is possible that these reefs could be transitioning from the current coral dominated state to one dominated by turf algae. An analysis of similar surveys that were conducted on the inhabited Caribbean island of Curacao (20) showed that turf algae were winning more than losing against corals at each of the eight sites surveyed, supporting the hypothesis that human habitation plays a role in coral-algal competitive dynamics. Furthermore, these data indicate that corals are losing ground to turf algae on Curacao, and this island has seen a decline in coral cover over the last several decades (57). Coral reefs in the Line Islands are remote and far removed from the main population centers of the world, yet our work here shows how human activities like subsistence fishing can affect the dynamics of coral-algal interactions, which may be important for the long-term stability of the coral reef ecosystem as a whole.

Acknowledgements

This expedition would not have been possible without the support of the captain and crew of the M/Y Hanse Explorer. Many thanks are due to Nichole Price for field assistance and to Brian Zgliczynski for managing the logistics of the research cruise. A Special Use Permit from the US Fish and Wildlife Service was obtained for research on Kingman and Jarvis, part of the Pacific Remote Islands Marine National Monument. We also thank the NOAA Coral Reef Ecosystem Division for providing benthic cover data for Jarvis-N.

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Acknowledgements

Chapter 2, in full, is a reprint of the material as it has been accepted for publication in Marine Ecology Progress Series. Katie Barott, Gareth Williams, Mark Vermeij, Jill Harris, Jennifer Smith, Forest Rohwer, and Stuart Sandin. The dissertation author was the primary investigator and author of this paper.

Appendix

Supplemental Figures

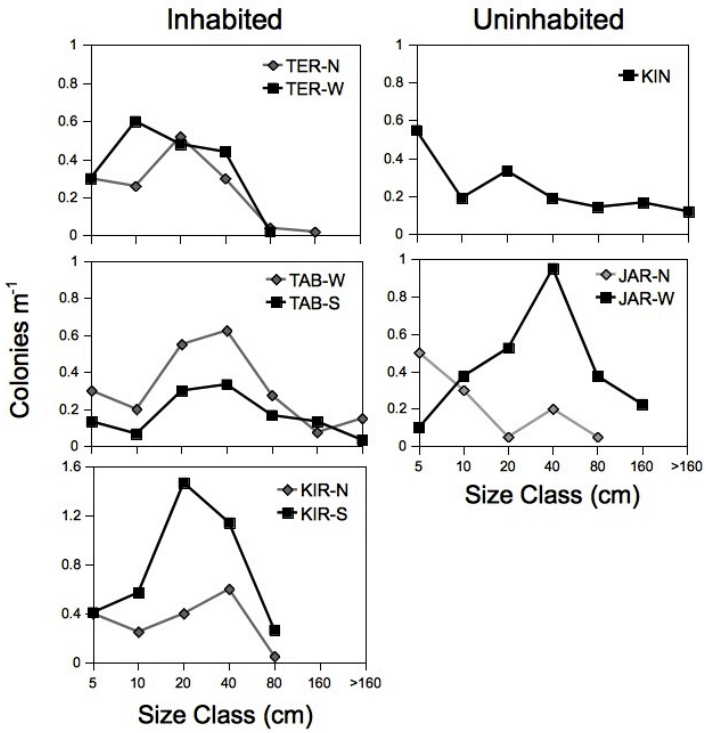

Figure S1. Coral size class distribution by site. Sites are shown here grouped by island.

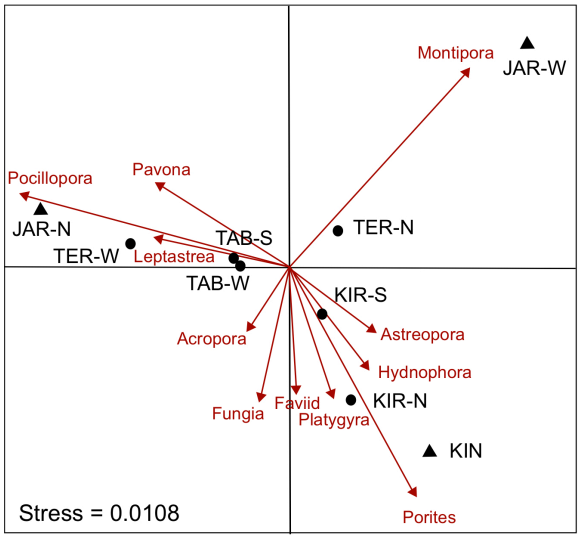

Figure S2. Non-metric multidimensional scaling plot of coral genus composition by site. Circles, inhabited islands; triangles, uninhabited islands.

Supplemental Tables

Table S1. Statistical comparison of algal composition along the coral edge versus benthos. Significance cutoff of p=0.05.

		Null distribution								
Site	**Actual deviation**	**0.10%**	**1%**	**5%**	**10%**	**50%**	**90%**	**95%**	**99%**	**99.9%**
KIN	0.407	0.407	**0.420**	0.431	0.435	0.444	0.446	0.447	0.448	0.449
TER_N	0.464	**0.466**	0.471	0.476	0.478	0.482	0.484	0.484	0.484	0.485
TER_W	0.470	**0.480**	0.490	0.497	0.500	0.505	0.507	0.507	0.508	0.508
TAB_W	0.509	0.493	0.499	0.503	0.504	0.508	**0.509**	0.509	0.510	0.510
TAB_S	0.548	0.528	0.548	**0.560**	0.564	0.575	0.579	0.580	0.581	0.582
KIR_N	0.166	0.159	0.164	**0.168**	0.170	0.173	0.175	0.175	0.176	0.177
KIR_S	0.460	**0.510**	0.517	0.521	0.523	0.526	0.527	0.527	0.527	0.528
JAR_N	0.334	0.310	0.329	**0.346**	0.354	0.371	0.375	0.376	0.378	0.381
JAR_W	0.166	0.158	0.163	**0.168**	0.170	0.173	0.175	0.175	0.176	0.177

CHAPTER 3

Hyperspectral and physiological analyses of coral-algal interactions

Abstract

Space limitation leads to competition between benthic, sessile organisms on coral reefs. As a primary example, reef-building corals are in direct contact with each other and many different species and functional groups of algae. Here we characterize interactions between three coral genera and three algal functional groups using a combination of hyperspectral imaging and oxygen microprofiling. We also performed *in situ* interaction transects to quantify the relative occurrence of these interaction on coral reefs. These studies were conducted in the Southern Line Islands, home to some of the most remote and near-pristine reefs in the world. Our goal was to determine if different types of coral-coral and coral-algal interactions were characterized by unique fine-scale physiological signatures. This is the first report using hyperspectral imaging for characterization of marine benthic organisms at the micron scale and proved to be a valuable tool for discriminating among different photosynthetic organisms. Consistent patterns emerged in physiology across different types of competitive interactions. In cases where corals were in direct contact with turf or macroalgae, there was a zone of hypoxia and altered pigmentation on the coral. In contrast, interaction zones between corals and crustose coralline algae (CCA) were not hypoxic and the coral tissue was consistent across the colony. Our results suggest that at least two main characteristic coral interaction phenotypes exist: 1) hypoxia and coral tissue

disruption, seen with interactions between corals and fleshy turf and/or some species of macroalgae, and 2) no hypoxia or tissue disruption, seen with interactions between corals and some species of CCA. Hyperspectral imaging in combination with oxygen profiling provided useful information on competitive interactions between benthic reef organisms, and demonstrated that some turf and fleshy macroalgae can be a constant source of stress for corals, while CCA are not.

Introduction

Coral reef ecosystems are among the most diverse and threatened ecosystems on the planet. Estimates suggest that 20 % of the world's coral reefs have already been lost, with another 50 % likely to be lost in the near future [1] due to a variety of human influences [1-3]. Local factors such as overfishing, habitat destruction and pollution from terrestrial runoff (e.g. eutrophication) are causing direct destruction of reefs. Global threats such as rising sea surface temperatures have led to widespread bleaching events [4] and coral disease has emerged as a critical problem [5,6]. Other climate related stressors such as ocean acidification may lead to loss of reef structure [2,7]. While the impacts of local threats may be reduced through management action, global threats to coral reefs are likely to increase in severity in the coming years [2,8].

Disturbed coral reefs are typically characterized by loss of coral cover followed by an increase in the abundance of fleshy algae (turf and macroalgae), a phenomenon that has been termed the coral-algal phase shift [9,10]. There are a wide variety of factors that can work in concert to lead to a coral-algal phase shift. In the Caribbean, a combination of release from top-down control due to loss of the sea urchin *Diadema antillarum* to disease, coupled with overfishing, eutrophication, and destruction of the physical structure of the reef due to hurricanes, led to nearly complete loss of Caribbean corals [3,11-13]. Loss of coral cover due to bleaching or disease can also lead to a coral-algal phase shift [14] since coral death results in available substrate for fast-growing algal species to colonize and eventually dominate the substratum. Loss of herbivorous fish due to overfishing also facilitates this process

by allowing algal growth to proceed unchecked, and this can be further exacerbated by addition of nutrients [10].

Benthic coral reef communities are areas of constant competition for space and light [15,16]. For sessile organisms these battles are a matter of survival and have fundamental consequences for the physical and biological structure of a coral reef community [17]. Despite the abundance of interactions between and among corals and algae, few studies have directly addressed the mechanisms or detailed characteristics of these interactions [15]. It is important to understand how corals and algae compete in order to understand what happens to tip the scales in one direction or another when the ecosystem is perturbed; especially when considering coral-algal phase shifts and possible conservation and restoration strategies.

Corals and algae utilize different physical (sweeper tentacles, messentarial filaments, overtopping, abrasion) and chemical (allelopathy) strategies to compete for and maintain space [16,18,19]. Several indirect mechanisms may also exist whereby microbes mediate these competitive interactions. Previous work has shown that dissolved compounds from algae can cause coral death indirectly by enhancing microbial activity [20,21] and that the addition of dissolved organic carbon (DOC) compounds including those found in algal photosynthates is sufficient to cause coral mortality due to microbial overgrowth [22,23]. On a reef-wide scale, it has been found that increasing abundance of benthic algae coincides with an increase in the abundance of microbes, including many potential pathogens, which may cause stress to corals and lead to the higher prevalence of coral disease [24]. In addition, algae

serve as reservoirs of coral disease [25] and can lead to disease transmission when the two are in direct contact. Despite the various competitive mechanisms that exist there is little information known about the details of these interactions. Does one competitor physically or chemically kill the other and if so how? Are algae able to overgrow live coral? Are microbes involved in these interactions? Experimental evidence has shown that hypoxia can occur on coral surfaces in the presence of algae, suggesting that microbial growth is stimulated [20], however, more data are needed to determine how common hypoxic zones are on the reef.

Hyperspectral imagery is a potentially informative tool for exploring coral-algal interactions. Hyperspectral images are produced by imaging spectrometers, which use an optical dispersing element (e.g. grating or prism) that splits the light into many wavelength bands, which are then detected using 100-1000s of detectors (e.g. across a CCD chip). In this way, an imaging spectrometer can make spectral measurements of a line. By stepping through one line to the next, a hyperspectral image is built. Hyperspectral imaging is a very active area of research and development, particularly in the area of remote sensing. Airborne hyperspectral imaging combined with Global Information Systems (GIS) is used for agricultural mapping [26,27], mineral exploration [28], and aerial monitoring of coral reef benthic habitats [29,30] but has not been used on the scales at which coral reef organisms interact. Multi-spectral imaging has been used to non-invasively monitor diseases in humans [31,32], suggesting that hyperspectral images may be informative for monitoring tissue changes in corals and algae.

All photosynthetic organisms have absorbance/reflectance spectra that are directly related to their light harvesting pigments. While a significant amount of photophysiology has been conducted on benthic marine algae, almost all photo documentation of corals to date has been done in the visible light range, yet there remains a wealth of information outside of this range that can be captured by hyperspectral imaging. Corals possess characteristic fluorescence profiles [33-36] generated by a wide diversity of pigments from the coral animal, their symbiotic dinoflagellates and associated microbes [37,38]. Despite the prevalence of fluorescent pigments in corals, their role remains an outstanding question. Hypothesized functions include photoprotection [33,39,40], enhancement of photosynthesis [41], and quenching of reactive oxygen species [38,42]. Previous studies show that corals undergoing bleaching have different pigment profiles due to loss of zooxanthellae [43] and changes in coral-associated pigments [44], and that these changes are predictive of coral survival [44]. Hyperspectral imagery can capture changes in host and symbiont pigments at interaction zones and facilitate identification of different organisms at the interaction zone based upon their unique reflectance spectra.

The goals of this study were to determine the prevalence of different coral-coral and coral-algal interactions *in situ*, quantify the oxygen profiles at the boundary layers across interaction zones for a variety of coral and algae, and characterize the hyperspectral signatures of these interaction zones from coral reefs in the uninhabited Southern Line Islands. We identified three main categories of benthic interactions occurring on these healthy reefs: coral vs. coral, coral vs. alga, and alga vs. alga. Upon

close examination of different interactions, we identified commonalities and characteristics that were specific to the type of interaction involved. Coral interactions with fleshy algae (turf and macroalgae) were consistently hypoxic, while borders with CCA or other corals were not. Disrupted coral tissue was clearly distinguishable from healthy tissue, algal tissue, cyanobacterial colonization, and bare skeleton. Finally, based on field observations, we found that coral-algal interactions are a constant and widespread feature of healthy coral-dominated reefs.

Materials and Methods

Study site and specimen collections: This study was performed during an expedition to the Southern Line Islands, central Pacific in March-April 2009. The islands visited were Vostok (10.1°S, 152.38°W), Starbuck (5.62°S, 155.93°W), Malden (4.017°S, 154.93°W), Flint (11.43°S, 151.82°W), and Millennium (9.94°S, 150.21°W). A variety of corals, algae, and coral-algal and coral-coral interaction zones were collected via SCUBA using a hammer and chisel (see details below). Samples were collected under a Scientific Research Permit issued by the Republic of Kiribati for the period of March 24 – May 5, 2009.

Underwater interaction surveys: Surveys were conducted at 10 m depth on SCUBA to determine the abundance of different types (species, genus or functional groups) of coral-algal interactions and to quantify the outcome of these interactions. A total of five 10 m transects were assessed on the leeward side of Millennium Atoll.

Along each of the transect lines a point intercept approach was used whereby every coral that was within contact of the transect line was examined and all algal interactions that occurred on that colony were recorded. The algal species and/or functional group (for turf and CCA) was recorded for every interaction. Additionally divers determined the outcome of the interaction by noting whether the algae was overgrowing the coral, the coral was overgrowing the algae, or if the interaction appeared to be neutral.

Hyperspectral imaging: Several coral-algal interaction zones were collected in the field over the duration of the research cruise. All samples were collected with a hammer and chisel, placed in a Ziploc bag, and upon return to the surface were placed in buckets with ambient seawater and returned to the ship. Once shipboard all specimens were kept in 10 liter aquaria with continuous aeration at ambient seawater temperature (~30°C) in shaded natural light and were imaged within 2-4 hours of return to the ship using the methods described below. The following pairs were imaged: 1) Coral-algae: *Pocillopora verrucosa* vs. *Gracilaria* sp. (n = 4), *Pocillopora verrucosa* vs. *Bryopsis pennata* (n = 6), *Montipora* sp. vs. mixed red turf algae (n = 4), *Pocillopora verrucosa* vs. cyanobacteria (n = 4), and various coral genera (*Favia* sp., *Montipora* sp. or *Pocillopora* sp.) vs. CCA (n = 6); and 2) Coral-coral: *Pocillopora verrucosa* vs. *Montipora* sp. (n = 1) and *Acropora* sp. vs. *Montipora* sp. (n = 1) (Table 3.1).

The Resonon PIKA II imaging spectrometer and its associated software, SpectrononPro v.1.15, was used to gather and analyze multibeam images of coral, algae and the interaction zones between them. Light exposure was set using a sheet of white Teflon placed on top of a Petri dish (which eventually held the specimen). After Spectral Focusing and Dark Adjustment the same piece of Teflon was used to record the Response Curve. Once an image was obtained, the mean spectrum of an area of sample was determined using the "Make ROI and Mean" tool in the accompanying SpectrononPro software. In order to compare spectra between samples, several normalization techniques were tested to eliminate small differences in the absolute relative reflectance of similar spectra. The simplest and most straightforward was to calculate the slope between every pair of wavelengths across the spectrum and plot (i.e. the first derivative of the reflectance). A number of image processing algorithms contained in the SpectrononPro software were tested for applicability to the corals and algae that were imaged. Many of these tools were developed for agricultural use, so were tested for usefulness when comparing marine photosynthetic organisms. The algorithms were: Green-orange-chlorophyll (GOC), Color Infrared (Color IR), Simple Ratio (SR) [45], Normalized Difference Vegetative Index (NDVI) [46] and Atmospherically Resistant Vegetative Index (ARVI) [47], in addition to the default True Color rendering. True Color, GOC, and Color IR yield false-color images. True color uses the three bands red (640 nm), green (550 nm), and blue (460 nm), GOC uses the three bands green (515 nm), orange (575 nm), and chlorophyll (685 nm), and Color IR uses the three bands green (550 nm), red (650 nm), and infrared (IR; 860 nm)

with a 2 % Stretch Contrast Enhancement. SR, NDVI, and ARVI are black-and-white mono (1-layer) images from a data cube with a 2 % Stretch Contrast Enhancement (i.e. set the darkest 2 % of pixels to black, the brightest 2 % of pixels to white, and stretch the remaining 96 % of values between black and white). In order to determine pixel values, SR uses the ratio between IR (800 nm) and red (680 nm), NDVI uses IR (800 nm) and red (680 nm) as input for the formula (IR - red) / (IR + red), and ARVI uses the bands IR (800 nm), red (680 nm), and blue (450 nm) as input for the formula (IR - 2*red + blue) / (IR+ 2*red – blue).

Dissolved oxygen measurements: To determine how dissolved oxygen concentration changed across several different types of coral-algal interaction zones, dissolved oxygen (DO) levels at the boundary layer were measured using an oxygen microprobe (Unisense; Aarhus C, Denmark). The microprobe was calibrated before each interaction zone was measured using aerated seawater to obtain the atmospheric saturation level of dissolved oxygen (100 % DO), followed by a solution of 0.1 M sodium hydroxide and 0.1 M sodium ascorbate for the 0 % DO reference point. Once calibrated, the probe was lowered to the boundary layer above the surface of the algae, coral, or interface zone under a Leica MZFLIII dissecting microscope. Five random points from within each zone were measured for a total of at least 10 seconds per point, and a measurement of aerated seawater was taken between each point. Data were recorded using the Unisense SensorTrace Basic v.1.13 software.

Dissolved oxygen data analysis: Ten recordings at each point were averaged to obtain the minimum or maximum level of dissolved oxygen (DO) for each point probed. The percent DO was calculated relative to the measurement of aerated seawater (100 % DO) prior to each measurement of the sample point. Normalization was carried out to account for drift of the instrument signal over the course of the measurements. The average of the percent DO from the 5 points taken within a zone was calculated to get the average maximum or minimum percent DO for the given sample zone (i.e. coral, algae, or interface). Several different coral-algal species (or functional groups for turf algae and CCA) interactions were examined including: 1) Coral-algae: *Pocillopora verrucosa* vs. *Gracilaria* sp. (n = 6), *Montipora* sp. vs. red turf algae (n = 4), *Pocillopora verrucosa* vs. cyanobacteria (n = 7); various coral genera (*Favia* sp., *Montipora* sp. or *Pocillopora* sp.) vs. CCA (n = 6), and *Montipora* sp. vs. white band disease (n = 2), and 2) Coral-coral: *Pocillopora verrucosa* vs. *Montipora* sp. (n = 1) (Table 3.1). As with the hyperspectral images, dissolved oxygen measurements of interfaces were taken within 2 - 4 hours of removal of samples from the reef. The non-parametric Man-Whitney test was used to compare DO levels because of uneven sample sizes. Significance was assessed by an asymptotic 2-tailed test with $p < 0.05$.

Results and Discussion

We identified a wide variety of interactions between benthic organisms on the reefs of the Southern Line Islands. Overall, coral vs. coral (Figure 3.1A), coral vs.

algae (Figure 3.1B, C), and algae vs. algae (Figure 3.1C, D) were the most common. This island archipelago has relatively low biodiversity with some 50 species of coral, 10 common species of macroalgae, many species of turf algae, and at least 5 species of crustose coralline algae (CCA).

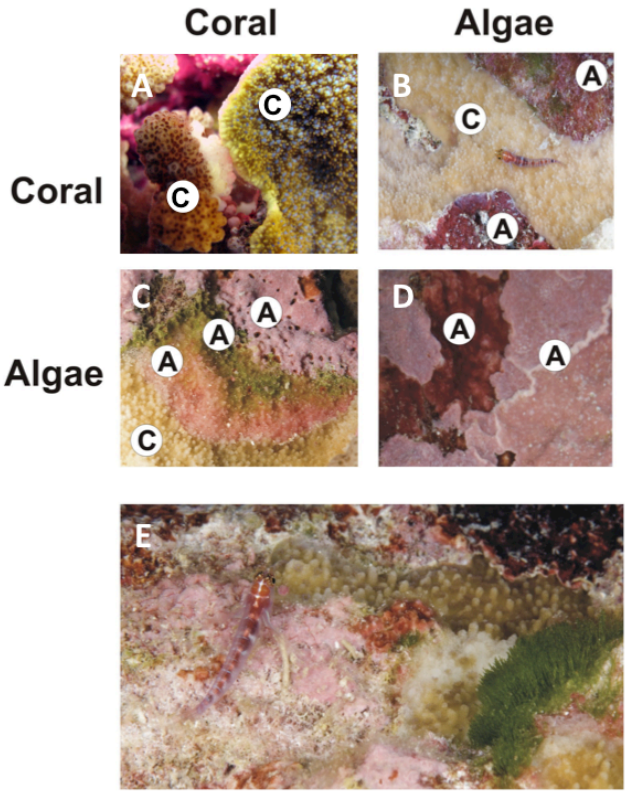

Figure 3.1. Examples of boundaries between coral and algae. A) *Pocillopora* sp. vs. *Montipora* sp., B) algae vs. *Montipora* sp., C) *Montipora* sp. and various algae, D) crustose coralline algae vs. crustose coralline algae, and E) diverse interactions including coral, fleshy algae, crustose coralline algae, and other invertebrates.

The low level of diversity still provides for an enormous number of possible different paired interactions between groups of benthic organisms, which is further complicated by the fact that most interaction zones include multiple organisms. For example, in Figure 3.1C the algal interface actually consists of at least 3 algal types

(e.g. a green alga, a red alga, and a CCA) and Figure 3.1E shows how extremely complex these zones can be with multiple species of algae and corals intermingled. Benthic transects to quantify coral-algal interactions found on average 4.57 interactions per linear meter (Figure 3.2A). Of these, over half of the interactions were either neutral or were designated as coral-dominated (Figure 3.2A), while in other cases different genera/functional groups of algae appeared to out-compete the coral.

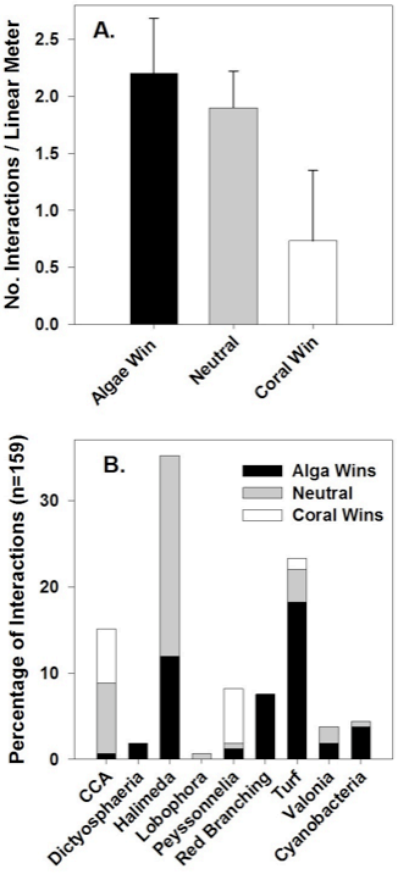

Figure 3.2. Summary of interactions between corals and algae from surveys of Millennium Atoll. A) Total number of interactions between corals and algae, B) outcome of coral-algal interactions by algal group.

For example, the fleshy red macroalga *Gracilaria* sp. was observed at every stage of overgrowth of *Pocillopora* colonies, from initial algal growth up from the base of the coral head over live coral tissue to dead colonies completely overtaken by the alga. The remoteness of the study site prohibited time series examinations of these interactions, yet the presence of *Gracilaria* sp. at various stages of overgrowth of living coral colonies was a clear indication of competitive dominance of this alga. Furthermore, close up observations revealed several stages of algal advance, including fronds directly in contact with live coral tissue and fronds surrounded by areas where the coral tissue had died. In contrast, other types of algae such as CCA or *Peyssonnelia* sp. were found being overgrown by corals in many cases (Figure 3.2B). These observations demonstrate the variability between different coral-algal species/functional group interactions.

Spectral analysis of corals and algae: The spectral signatures of corals and algae were contrasted to determine the consistency of the spectra between individuals from the same group, and whether hyperspectral images were therefore useful for characterization of interaction zones. While hyperspectral imaging has been used in other studies to ascertain organisms over large spatial scales, it has never been used to discriminate among organisms on the scale examined here. Furthermore, this is the first study to use hyperspectral imaging as a tool for characterizing competitive interactions between different photosynthetic reef organisms (Table 3.1). As a test case, several crustose coralline algal (CCA) specimens were imaged and the mean

spectrum across each was calculated. The CCA spectra were very similar to each other (Figure S1A).

Table 3.1. Summary of oxygen microprobing and symptoms from hyperspectral images from coral-algae and coral-coral boundaries in the Southern Line Islands.

	Oxygen at interface (% of seawater background)	**Symptoms (moving from algae to coral)**
Coral-algae		
Pocillopora verrucosa ***Gracilaria* sp.**	17-95 %; average 68.1 % (n = 6)	Algae is proceeded by a cyanobacteria Bleached coral tissue and some bare skeleton at interface Disruption of coral tissue nearest interface (n = 4)
Pocillopora verrucosa ***Bryopsis* sp.**	nd	Bare skeleton between algae and coral Distinct white band at interface Coral tissue is peeling off skeleton in patches (n = 6)
***Montipora* spp.** **Red turf algae**	18-74 %; average 44.8 % (n = 4)	Algae is proceeded by a cyanobacteria Algal filaments extending over interaction zone Bleached coral tissue (but no bare skeleton) at interface Disruption of coral tissue nearest interface (n = 4)
Damsel fish territories ***Pocillopora verrucosa*** **Red cyanobacteria**	31-49 %; average 40.6 % (n = 7)	Algae is proceeded by an unknown green algae At interaction zone, polyps remain intact, calicoblastic tissue is peeling off in patches (n = 4)
Coral - CCA ***Favia* sp.** ***Montipora* sp.** ***Pocillopora verrucosa*** **CCA**	85-132 %; average 107 % (n = 6)	CCA and coral interaction very tight association No coral tissue disruption (n = 6)
White band disease ***Montipora* spp.**	30-47 %; average 38.3 % (n = 2)	nd
Coral-Coral		
Pocillopora verrucosa ***Montipora* sp.**	132.8 % (n = 1)	Disruption of tissue on *Montipora* spp. (Figure S2; n = 1)
Coral-Algae-Coral		
***Acropora* sp.** ***Montipora* sp.**	nd	Disruption of *Acropora* spp. tissue next to algae (n = 1)

A number of normalization techniques were tested to eliminate differences in absolute reflectance values. The simplest and most straightforward was to calculate the slope between every pair of wavelengths across the spectrum and plot (i.e. the first derivative; Figure S1B). This transformation normalized the data and highlighted the most important components (i.e. differences) of the spectra.

The main groups of reef algae encountered (e.g. encrusting CCA, fleshy reds, fleshy greens (*Bryopsis* sp.), and turf algae) were imaged and compared to ground truth this novel method (Figure 3.3A). Each group had a characteristic spectrum, clearly distinguishable using the hyperspectral data. This result was expected given that different types of algae contain a variety of pigments, which would lead to clear differences in reflectance spectra among taxa. Different coral genera were then imaged with the hyperspectrometer. The expected maximum reflectance wavelengths for corals were 575 and 685 nm [35,36], and were readily seen in the coral spectra generated by the hyperspectrometer (Figure 3.3B). The reflectance curves collected were similar to the spectra described in previous studies for various coral species around the world [29]. When compared, the reflectance spectra from different coral genera were similar and were not clearly distinguishable from each other (Figure 3.3B), unlike the different algal groups (Figure 3.3A).

To test if spectra varied between corals and algae, two selected coral spectra (*Pocillopora* sp. and *Porites* sp.) were compared to the different reef algae. As shown in Figure 3.3C, it is easy to distinguish between these major groups at the fine scale using the spectra alone. While corals are spectrally very similar to each other due to

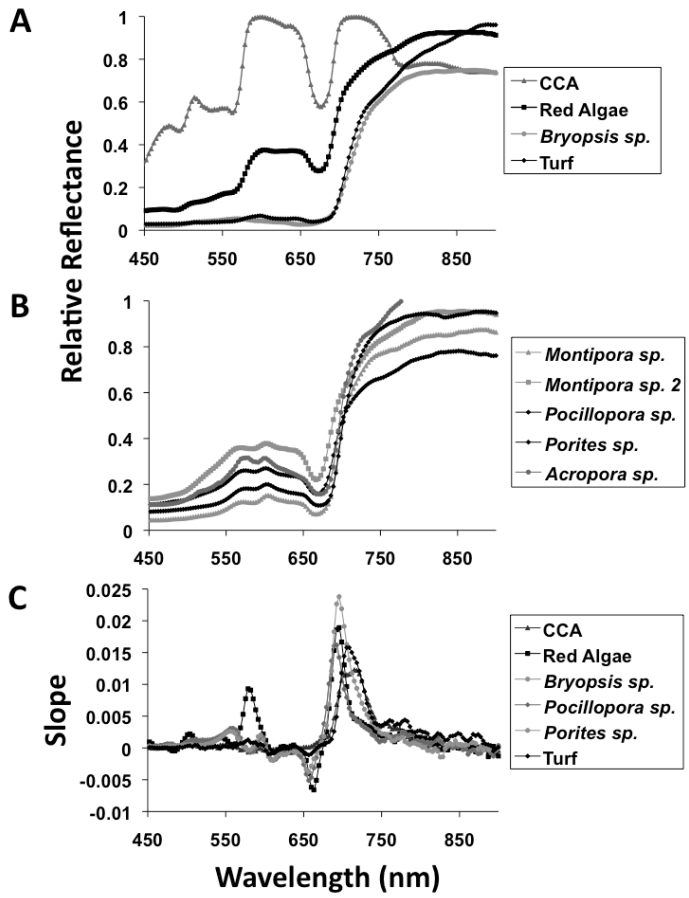

Figure 3.3. Comparison of spectra from different algal and coral groups. A) Relative reflectance of different algae: CCA, red alga, *Bryopsis* sp., and turf algae. B) Relative reflectance of 4 different corals: *Montipora* sp. (2), *Pocillopora* sp., *Porites* sp., and *Acropora* sp. C) Slope of coral and algal reflectance spectra, including CCA, red alga, *Bryopsis* sp., *Pocillopora* sp., *Porites* sp., and turf algae.

their symbiotic dinoflagellate and host pigments, they are distinct from the major algal groups. Together these results show that hyperspectral signatures are sufficient to broadly differentiate between different types of algae and corals. Furthermore, we can now compare spectra from healthy corals and algae to determine if the spectra (e.g. pigment presence or patterning) changes or breaks down when the two organisms are in direct contact. Changes in pigmentation or tissue structure revealed by

hyperspectral images along interaction zones may be a symptom of stress due to competition between the two groups.

Corals versus algae: Interactions between corals and algae were highly abundant across the coral reefs surveyed (Figure 3.2). A series of these interactions involving different species were imaged with the hyperspectral camera and dissolved oxygen levels were measured across the surfaces of the interaction zones (Table 3.1). Comparisons of hyperspectral images of coral-algal borders indicated that interaction zones shared common characteristics. Interactions with fleshy algae (e.g. *Gracilaria* sp., *Bryopsis* sp., and various turf algae) were typically characterized by bleached or disrupted coral tissue near (mm scale) the interface (Table 3.1). These areas were often pale, indicating loss of zooxanthellae from live coral tissue, and in many cases the characteristic patterning of coral pigments and polyps was altered and the tissue appeared damaged. In areas where skeleton was revealed following coral tissue death, cyanobacteria were observed (Figure 3.4A, Table 3.1). We found that at the point of contact for all of the interactions between corals and fleshy turf algae or macroalgae there was a zone of hypoxia, but the degree of hypoxia varied depending on the type of alga involved (Figure 3.5). A Mann-Whitney test recognized four groups in the data. For example, coral interaction zones with *Gracilaria* sp. were less hypoxic ($p < 0.05$) on average than corals interaction zones with turf algae (Figure 3.5). The cause of the observed hypoxia remains to be determined, and may be due to respiration of the coral tissue itself, microbial respiration on the surface of the coral, or a

combination of the two. The degree of coral mortality likely affects the level of hypoxia, and the degree of coral tissue mortality appears to be related to the functional group and most likely the species of algae with which it comes in contact (Table 3.1).

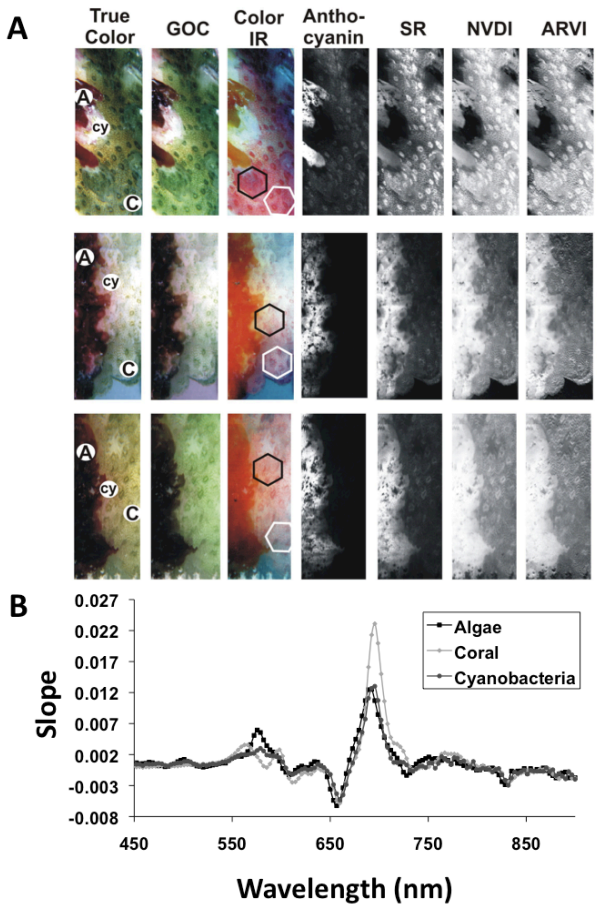

Figure 3.4. Different renderings of hyperspectral images of the interaction zones between the coral *Pocillopora* sp. and red alga *Gracilaria* sp. A) The location of algae (**A**), coral (**C**) and cyanobacteria (**cy**) are indicated in the True Color image. The color coding is determined for **True Color** using the three bands red (640nm), green (550nm), and blue (460nm); **GOC** using the three bands green (515 nm), orange (575 nm), and chlorophyll (685 nm); **Color IR** using the three bands green (550 nm), red (650 nm), and infrared (IR; 860 nm); **SR** (Simple Ratio) using the ration between 800 nm and 680 nm; **NVDI** (Normalized Vegetative Density Index) using IR (800 nm) and red (680 nm) in the formula (IR - red) / (IR + red); and **ARVI** (Atmospherically Resistant Vegetative Index) using the bands IR (800 nm), red (680 nm), and blue (450 nm) entered in the formula (IR - 2*red + blue) / (IR+ 2*red – blue). Each rendering uses a 2 % Stretch Contrast Enhancement. B) The slope of the average relative reflectance for the algae, coral, and cyanobacteria imaged in A. Areas used to determine the average reflectance are indicated by hexagons in the IR rendering.

In contrast, the interactions between corals and crustose coralline algae (CCA) did not show any evidence of hypoxia (Figure 3.5). Hyperspectral images of coral-CCA interactions showed that corals and CCA were in close association, yet there were no areas of cleared coral skeleton and coral tissue was not disrupted or visibly stressed near the interface (Table 3.1). This may be because CCA do not stimulate or alter microbial communities associated with the coral tissue, or do not directly damage or kill the coral tissue through release of allelochemicals. These data indicate that there are at least two distinct mechanisms of interaction between corals and algae. In the case of at least some fleshy turf and macroalgae, coral tissue structure and pigmentation were clearly disrupted and dominated by respiration, indicating microbial overgrowth and clear stress to the coral animal, while corals interacting with CCA showed no signs of stress.

As a test case, interaction zones between the coral *Pocillopora* sp. and a red alga *Gracilaria* sp. were characterized in detail using hyperspectral imaging. These images are data rich and there is a vast literature of different techniques for processing hyperspectral images. Here we used renders and utilities built into the supporting software Spectronon Pro to identify a process that best displayed the differences in the interaction zones. Figure 3.4A shows some of the more visually informative processing. In general, the advancing *Gracilaria* sp. branches were preceded by a thin line of cyanobacteria, followed by an area of bare skeleton and then disrupted coral tissue (Figure 3.4A). The oxygen levels at the interface were somewhat variable. Regions of apparently bare skeleton following coral tissue death had oxygen levels

typically near or just below ambient (data not shown), while areas of disrupted coral tissue were hypoxic (Figure 3.5).

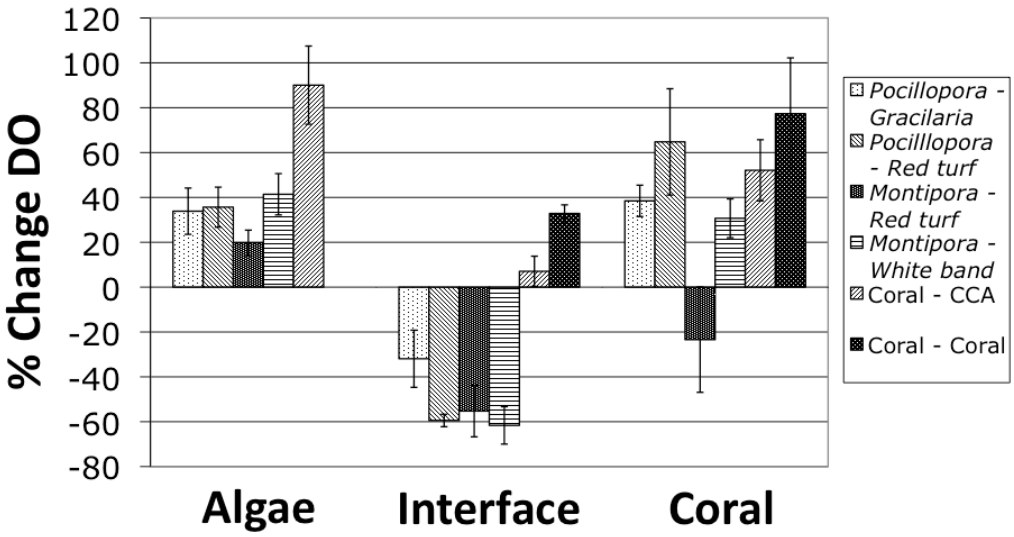

Figure 3.5. Dissolved oxygen profiles of coral interaction zones. Interactions measured were *Pocillopora verrucosa* vs. *Gracilaria* sp. (n = 6), *Pocillopora verrucosa* vs. red turf algae (n = 7), *Montipora* spp. vs. red turf algae (n = 4), *Montipora* sp. vs. white band (n = 2), coral vs. CCA (n = 6), and coral vs. coral (n = 1).

Because both components of coral-algal interactions are photosynthetic, a number of image processing algorithms normally used for agriculture were also tested. Of these, specific pigments such as anthocyanin (similar reflectance peaks as the red algal phycobilin pigments, Figure 3.4A) and carotenoids (not shown) were useful for distinguishing changes along the interaction zone and between the coral and the algae. The GOC and Color IR renderings helped visualize the different components of the interaction zone (Figure 3.4A). Colonization of exposed skeleton at the interface by cyanobacteria was seen by hyperspectral imagery, and the reflectance spectra for cyanobacteria were clearly distinguished from the spectra of the *Gracilaria* sp. and

Pocillopora sp. (Figure 3.4B). Renderings of Simple Ratio (SR), Normalized Vegetative Density Index (NVDI), and Atmospherically Resistant Vegetative Index (ARVI) all showed similar patterns (Figure 3.4A). The algal component had the highest values (white coloring), while the coral showed patterning likely due to the distribution of zooxanthellae. Except for anthocyanin, all renderings clearly showed that advancing algal fronds in direct contact with coral tissue cause a clear disruption of the natural patterns of the coral polyps and pigmentation in the area surrounding the point of contact (Figure 3.4A, top row).

Corals versus corals: Two types of coral-coral interaction zones were found. In an active border region, one coral was disrupting the tissue of the other (**dt** = disrupted tissue, Figure S2). The spectra of these boundaries showed no evidence of algal colonization, and these borders were not hypoxic (Figure 3.5). Competition between corals is known to involve mesenterial filaments, sweeper tentacles and nematocysts [48,49], and so would be expected show a distinct oxygen profile from coral-algal competition. One note of interest, in the *Pocillopora* sp., individual polyps appear to be releasing large quantities of mucus (labeled as **m** in Figure S2A). Constant activation of a stress response by competition is likely affecting the overall health of the coral, and previous work has shown that competition between corals can reduce growth and fitness [50].

The second type of coral-coral interaction zone can actually be defined as a coral-algae-coral zone. In this case algae have colonized the area between the two

corals. On Millennium Atoll, the majority of apparent coral-coral interactions were actually found to be coral-algae-coral interactions upon close examination (Table 3.1, Figure S2B). It appears that as corals compete with one another, an area between the two competitors is cleared of live tissue, which is then colonized by opportunistic algae and likely microbes. Previous work has found that competing corals constantly advance and retreat, leaving areas of cleared space in between the two colonies as they recover from competitive interactions [51]. It is unknown if the presence of algae between the two colonies is detrimental, beneficial, or neutral for the competing corals.

Conclusions: Interactions between corals and algae were a widespread feature of the near-pristine coral reefs of the Southern Line Islands. Hyperspectral imagery and oxygen profiles of coral-algal interaction zones demonstrated that these interactions have characteristic profiles that depend on the species and functional group of algae involved. Coral interaction zones with fleshy algae (e.g. red and green macroalgae and turf algae) were characterized by disrupted coral tissue near the interface, and were consistently hypoxic. Hypoxia suggests that respiration by microbial activity may dominate these areas. For example, human wounds are often hypoxic as a result of microbial respiration, which hinders the host immune response and slows or prevents wound healing [52]. While we cannot rule out coral respiration as a cause of hypoxia, in experimental manipulations, algae placed near corals led to hypoxia on the coral surface and mortality, which was eliminated by the addition of

antibiotics, indicating that coral death was microbially mediated [20]. In contrast to fleshy algae, we found that coral interactions zones with CCA were not hypoxic, and coral tissue at the interface appeared normal. These findings indicate that competitive interactions between reef building corals and fleshy algae or CCA likely have fundamentally different consequences for corals and reef communities as a whole. CCA have been found to be beneficial for corals in many cases by providing a settlement substrate and metamorphosis cue for coral larvae [53,54] and by helping maintain the structural stability of reefs [55]. On the other hand, fleshy algae may be a constant source of stress for corals. On reefs where algae are released from grazing pressure and/or nutrient limitation, fleshy algae dominate [3,9-11], and their ability to disrupt live coral tissue, as observed in this study, likely plays an important role. More data are needed to determine how these patterns vary between different fleshy and calcified algal taxa.

The complexity of coral interactions was further revealed upon close examination with hyperspectral imagery. For example, the majority of apparent coral-coral interactions were actually coral-algae-coral interaction zones. In addition, the red alga *Gracilaria* sp. appears to rapidly advance over some species of coral, directly disrupting the tissue and clearing areas of skeleton that are subsequently colonized by cyanobacteria. These observations demonstrate that some algae (e.g. cyanobacteria) will opportunistically colonize available space, while others actively overgrow corals (e.g. *Gracilaria* sp.). Interactions with turf algae were not as dramatic and did not show areas of cleared coral skeleton, but turfs were still disruptive to adjacent coral

tissue although the advance appeared much more gradual and variable than that of *Gracilaria* sp. The advance of turf algae was likely limited by intense grazing pressure on these reefs as turf algae are readily consumed by reef herbivores. Although grazing rates of herbivores were not measured, the high abundance of herbivorous fish and general low abundance of algae on Millennium Atoll suggests that grazing pressure may be limiting algal growth (data not shown).

Hyperspectral imagery was confirmed as a useful tool to visualize the small scale interaction zones between corals and algae, and extends the spectral range encompassed in analysis from previous studies of coral spectra [34-36,29,56]. This technique clearly identified the players involved in various coral and algae interactions, and revealed changes in tissue patterning and pigmentation at the interaction zone. Hyperspectral imagery is currently being developed for remote monitoring of coral reef benthic communities, but could be expanded as a useful tool for future monitoring of coral reefs by rapidly characterizing the abundance of coral-algal competition borders *in situ* at the fine scale. Hyperspectral imaging technology is not currently available for underwater fine-scale analysis, but multi-band technology encompassing important wavelengths indicative of corals, algae and microbes (e.g. cyanobacteria) is a viable next step.

This is the first study to describe the physiological characteristics of different types of coral-algal interactions on a coral reef. The combination of hyperspectral imagery with dissolved oxygen measurements of these interactions indicate that coral-algal interfaces vary among species and in overall characteristics. Interaction zones

between corals and at least some fleshy algae appear to be detrimental to corals. On the other hand, CCA do not appear to disrupt corals in the same manner and in fact facilitate the maintenance of coral reefs by providing settlement substrate for corals and solidifying the reef structure. The results of our study show that some fleshy algae, a highly diverse group of benthic primary producers that includes macroalgae as well as turf algae, can have competitive advantages over slower growing reef building corals. These types of algae can be disruptive to live coral tissue, and are increasingly abundant on impacted coral reefs worldwide [1,3,9-11,14,57]. Understanding the drivers that shift competitive dominance towards fleshy algae remains an outstanding research question, the answers to which are important for developing effective management, conservation and restoration strategies for coral reefs.

Acknowledgements

We would like to thank Enric Sala, the National Geographic Society and the owner, captain and crew of the Hanse Explorer for organizing and coordinating the research expedition. We also thank Enric Sala for providing underwater images of coral-algal interactions, and David Zawada and Resonon Inc. for advice on hyperspectral imaging.

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Acknowledgements

Chapter 3, in full, is a reprint of the material as it appears in PLoS ONE. Katie Barott, Jennifer Smith, Elizabeth Dinsdale, Mark Hatay, Stuart Sandin, and Forest Rohwer; 2009. The dissertation author was the primary investigator and author of this paper.

Appendix

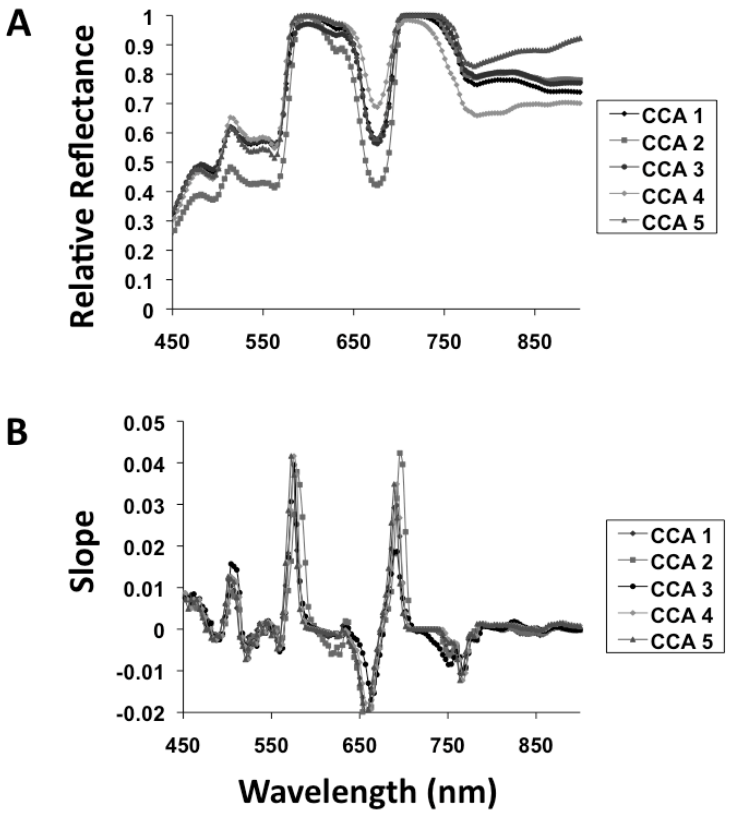

Figure S1. Average reflectance spectra from crustose coralline algae (CCA). A) Relative reflectance of 5 different CCA specimens. The CCA fragments were overexposed as evidenced by peaks that are cut off at 1 (630 nm; 730 nm). B) Slope (first derivative of reflectance spectrum) of the 5 CCA specimens in A.

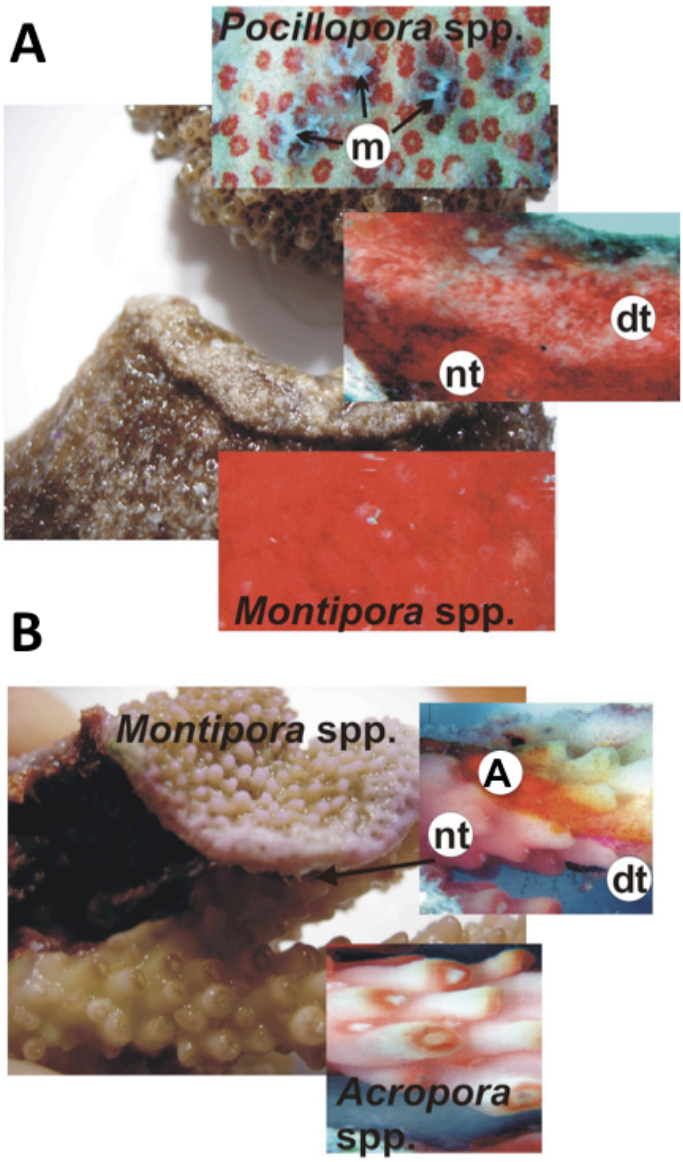

Figure S2. Two types of interaction zones between corals. A) Active coral interaction zone where one coral is attacking another and damaging the tissue with mesenterial filaments. B) Interaction zone between two corals where algae has established itself between the two competing corals.

CHAPTER 4

Microbial diversity associated with benthic algae and corals

Abstract

The coral reef benthos is primarily colonized by corals and algae, which are often in direct competition with one another for space. Numerous studies have shown that coral-associated Bacteria are different from the surrounding seawater and are at least partially species specific (i.e. the same bacterial species on the same coral species). Here we extend these microbial studies to four of the major ecological functional groups of algae found on coral reefs: upright and encrusting calcifying algae, fleshy algae, and turf algae, and compare the results to the communities found on the reef-building coral *Montastraea annularis*. It was found using 16S rDNA tag pyrosequencing that the different algal genera harbor characteristic bacterial communities, and these communities were generally more diverse than those found on corals. While the majority of coral-associated Bacteria were related to known heterotrophs, primarily consuming carbon-rich coral mucus, algal-associated communities harbored a high percentage of autotrophs. The majority of algal-associated autotrophic Bacteria were Cyanobacteria and may be important for nitrogen cycling on the algae. There was also a rich diversity of photosynthetic eukaryotes associated with the algae, including protists, diatoms, and other groups of microalgae. Together these observations support the hypothesis that coral reefs are a vast

landscape of distinctive microbial communities and extend the holobiont concept to benthic algae.

Introduction

Microbes are associated with a wide variety of organisms, and are increasingly recognized to play an important role in host health and metabolism (1–4). Corals, for example, are inhabited by a diverse and abundant array of microbes that are distinct from the surrounding seawater (5–12). These microbes produce antibiotics (13,14) and are involved in the biogeochemical cycling of nitrogen, carbon and sulfur on the holobiont (15–20). Despite the known and potential roles of microbes in coral health and metabolism, identification of the primary factors controlling the types of microbes associated with corals is an ongoing question. There is evidence that coral-associated microbial communities are species specific (7,12), but it has also been shown that the same species from different locations harbor distinct microbial communities (21). In addition, the composition of coral-associated microbial communities has been found to change when corals undergo bleaching (22), are housed in aquaria vs. natural environments (23), and when they are exposed to stressors (24). These observations are intriguing because they open the possibility that the coral holobiont may adapt to changing conditions by changing microbial associates in a manner similar to adaptive bleaching (7,25,26). Furthermore, the microbial communities themselves play a role in determining the types of microbes that colonize the coral surface through niche occupation and antagonism towards other bacteria (13,27). This suggests that a combination of host factors, microbial associates, and environmental conditions play an important role in shaping microbial associations, which then play a role in the health and function of the host coral.

Benthic algae are a major component of coral reef ecosystems and are increasingly abundant on coral reefs around the world (28–30). However, despite the significance of microbial associations recognized with other benthic reef organisms (e.g. corals and sponges), our knowledge of algal-associated microbial communities is limited. Benthic algae are often grouped together by form and ecological function (31). Turf algae, for example, are small filamentous algae and are among the most productive groups on the reef benthos (32), and this success has been attributed in part to the contribution of high nitrogen-fixation rates due to Cyanobacteria present among the turf community (33–35). In addition, many species of crustose coralline algae (CCA) promote settlement of coral and other invertebrate larvae (36–38). This effect is due primarily or in part to Bacteria associated with the CCA (39–41); however, little is know about the composition of these microbial communities as a whole or their interactions with the host CCA. Recently it has been shown that elevated temperature alters the composition of CCA-associated Bacteria, which in turn negatively affects the recruitment of coral larvae (42). A few studies of cultivable bacteria found that CCA harbor some unique isolates compared to other reef substrates (43,44), but cultivation techniques notoriously miss the vast majority of environmental microbes (45). Finally, despite these hints at their significance, very little is known about microbial associations with the diverse suite of macroalgal species on coral reefs.

Field observations have shown that benthic algae affect microbial communities in the surrounding seawater. For example, algal-dominated patches of reefs have been found to have lower levels of oxygen in the overlying seawater, indicating that

microbial activity is higher in these areas (46). Furthermore, Dinsdale et al. found that reefs dominated by algae had higher abundances of heterotrophic bacteria and potential pathogens than coral-dominated reefs (47). It has been proposed that these changes are the result of labile organic carbon released by benthic algae that is stimulating microbial activity, and indeed it has been found that microbes rapidly consume algal-derived organic matter (48). Increases in the release of algal exudates on a reef as a result of increased algal abundance on the benthos may be affecting reef health by altering the production, abundance and function of the surrounding microbial communities, thus potentially leading to increases in coral disease, coral death, and increased algal proliferation on the reef. Understanding the diversity and function of microbes associated with benthic reef algae will further our understanding of potential relationships between these two groups, as well as provide insight into how benthic algae interact with the microbial world, including on their surfaces, the surrounding water column, and organisms with which they come into contact (e.g. corals, herbivores, etc.).

Here we describe the composition of bacterial communities associated with four major ecological functional groups of benthic algae: encrusting calcifying algae (CCA), upright calcareous algae (*Halimeda opuntia*), fleshy macroalgae (*Dictyota* sp.), and turf algae. For comparison, the bacterial communities associated with the common reef-building coral *Montastraea annularis* were also analyzed using the same approach: high-throughput sequencing of the V1 - V3 region of the 16S rRNA gene.

We show that the algae host characteristic bacterial communities that are more diverse than those associated with corals.

Results

Each library contained between 33,321 – 107,917 reads, with an average read length between 305 - 439 base pairs after primer and barcode removal (Supplemental Table 2). Algal libraries had the highest abundance of chloroplast contamination, which ranged from 12 – 86 % of the sequences. The total number of bacterial sequences per library after chloroplast removal is listed in Supplemental Table 2, and ranged from 9,503 – 104,364 sequences. All sequences were submitted to the NCBI Sequence Read Archive (SRA023821.1).

Diversity of Bacteria associated with corals and algae: The values of the alpha diversity metrics (i.e. observed OTUs, predicted OTUs [Chao1] and Shannon Diversity [H']) varied depending on whether the RDP or QIIME pipeline was used and whether or not the data was error corrected by denoising; however, the relative differences between samples was consistent regardless of the method used (Supplemental Table 3). For ease of interpretation, only the QIIME analyses of the denoised sequences are discussed.

A range of 163 - 259 different OTUs were observed associated with coral tissue from Site 1, with a predicted range of 266 - 346 OTUs in the community (Chao1, Figure 4.1a). Richness was higher on corals at Site 2 (323 and 461 OTUs

observed and predicted, respectively; Figure 4.1a). Bacterial diversity as determined by the Shannon-Weiner index (H') ranged from 2.84 – 4.51 at Site 1, and was highest at Site 2 (5.03, Figure 4.1b). The richness associated with each of the different types of algae was higher than that observed on any of the coral samples. Of the different algae, *Dictyota* sp. had the highest number of predicted OTUs (2,375 - 3,300) followed by *Halimeda opuntia* (2,119 - 2,167), turf algae (1,725 - 1,961), and CCA (953 – 1,232; Figure 4.1a). Bacterial diversity (H') associated with algal tissue was also higher than that observed on corals, with H' ranging from 6.22 – 7.82 (Figure 4.1b). The highest observed diversity was associated with *H. opuntia*, but overall, bacterial diversity was similar for all algae except CCA, which was lower than the other three algal types (6.22 - 6.36 vs. 6.91 – 7.82, respectively; Figure 4.1b).

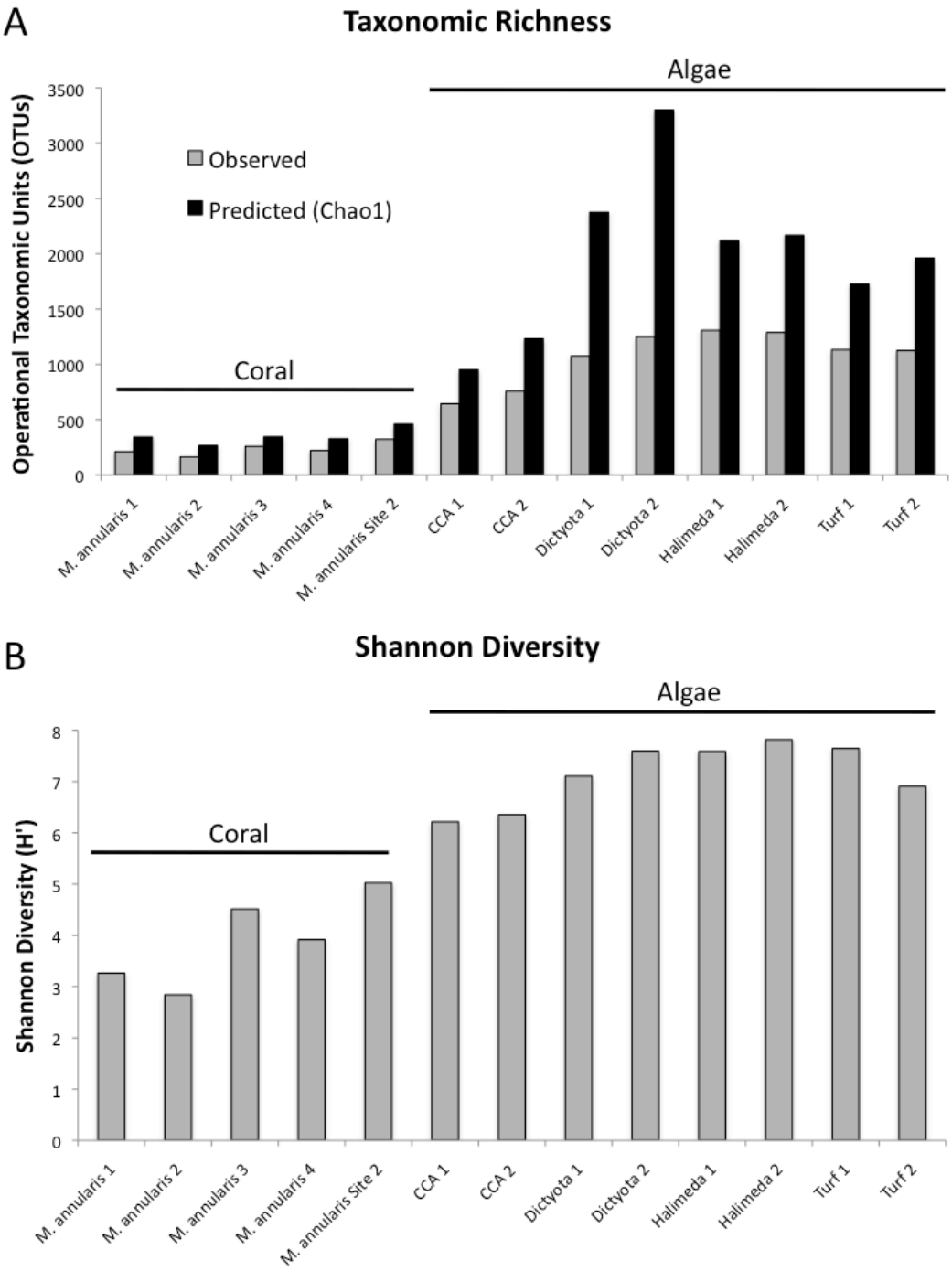

Figure 4.1. Alpha diversity of bacteria associated with the coral *M. annularis* and benthic algae. A) Number of OTUs observed and predicted (Chao1) and B) Shannon diversity of bacteria from corals and algae. OTUs were grouped at 97 % similarity.

Composition of Bacteria associated with corals and algae: A range of 14 – 21 unique phyla were found associated with corals. Coral-associated bacterial communities were dominated by sequences related to Proteobacteria (~ 75%), followed by Bacteroidetes, Firmicutes, and Actinobacteria (Figure 4.2). Coral from Site 2 had a greater abundance of sequences related to Actinobacteria than corals from Site 1 (23 % vs. 2.1 – 5.8 %; Figure 4.2).

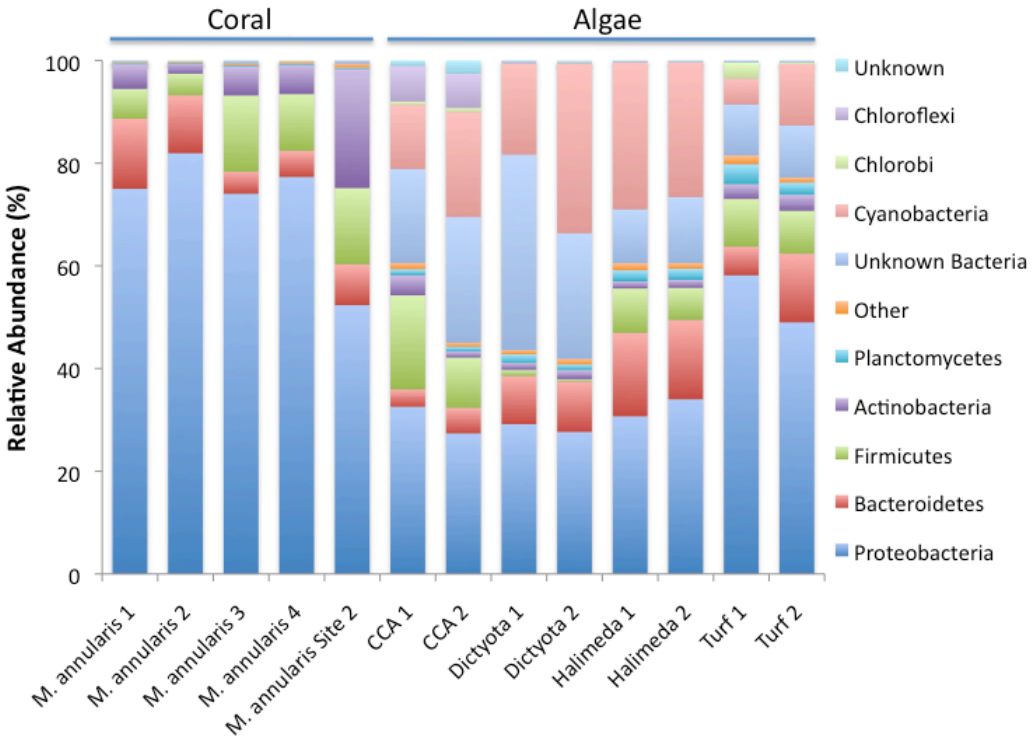

Figure 4.2. Relative abundance of phyla associated with the coral *M. annularis* and benthic algae. 'Unknown' sequences could not be classified into any known group. 'Unknown Bacteria' sequences classified as Bacteria but could not be further identified.

The most abundant genus from all coral samples was *Acidovorax* (43 %, Table 4.1), a member of the Comamonadaceae family. Other members of this family were also common on corals (e.g. *Diaphorobacter* [5.3 %], *Delftia* [2.3], and *Curvibacter*

[3.6]). Other genera common on coral tissue included *Lactobacillus* (6.6 %), *Aquabacterium* (8.2 %), *Cloacibacterium* (8.2 %) and *Propionibacterium* (3.7 %, Table 4.1).

Table 4.1. Classification of the 10 most abundant bacterial OTUs associated with the coral *Montastraea annularis* and benthic algae, listed from most to least abundant. Relative abundance (%) of each OTU is included in parentheses.

M. annularis	**Coralline Crustose Algae (CCA)**	***Dictyota* sp.**	***Halimeda opuntia***	**Turf Algae**
Acidovorax (43)	Bacteria (11)	Cyanobacteria (5.0)	Cyanobacteria Group I (8.0)	*Acidovorax* (11)
Cloacibacterium (8.2)	*Lactobacillus* (8.0)	Bacteria (4.2)	Cyanobacteria (6.0)	*Lactobacillus* (5.3)
Aquabacterium (8.2)	Chloroflexaceae (6.5)	*Acidovorax* (3.7)	*Lactobacillus* (5.0)	*Cloacibacterium* (4.9)
Lactobacillus (6.6)	Cyanobacteria Group I (5.6)	Cyanobacteria (2.8)	Cyanobacteria (4.9)	*Curvibacter* (3.8)
Diaphorobacter (5.3)	Cyanobacteria (5.1)	Bacteria (2.7)	*Curvibacter* (2.7)	Alphaproteobacteria (2.4)
Propionibacteria (3.7)	*Curvibacter* (4.2)	Cyanobacteria (2.1)	*Silicibacter* (2.0)	Cyanobacteria (2.1)
Curvibacter (3.6)	*Pseudomonas* (3.9)	Bacteria (2.0)	Rhodobacteraceae (2.0)	Rhodobacteraceae (2.0)
Pseudomonas (2.9)	*Delftia* (3.8)	Cyanobacteria (2.0)	*Delftia* (2.0)	*Silicibacter* (1.6)
Methylobacterium (2.3)	Bacteria (3.2)	Bacteria (1.7)	*Psuedomonas* (1.5)	Rhizobiales (1.3)
Novosphingobium (2.3)	Cyanobacteria Group VIII (2.0)	Cyanobacteria (1.5)	Cyanobacteria (1.2)	*Prosthecochloris* (1.1)

Between 18 - 22 unique phyla were associated with the different types of algae. Sequences similar to Proteobacteria and Cyanobacteria were abundant across all types of algae (Figure 4.2). Of the different algae, *Dictyota* sp. and *H. opuntia* had the highest abundance of sequences related to Cyanobacteria (33 % and 27 %, respectively; Figure 4.2). CCA communities also included an abundance of sequences similar to Firmicutes (18 %) and Chloroflexi (7 %), while *Dictyota* sp., *H. opuntia* and turf algae included an abundance of sequences related to Bacteroidetes (10 – 15 %). A

large proportion of both the CCA and *Dictyota* sp. libraries could not be classified beyond Bacteria (18 – 38 %; Figure 4.2). The most abundant OTU (97 % similarity) associated with algae varied by functional group. CCA libraries were dominated by sequences most closely related to various Cyanobacteria (5.1 – 5.6 %), *Lactobacillus* (8.0 %), Chloroflecaceae (6.5 %), *Curvibacter* (4.2 %), *Pseudomonas* (3.9 %), and *Delftia* (3.8 %, Table 4.1). The ten most abundant OTU associated with *Dictyota* sp. included groups related to various unclassified Bacteria and Cyanobactera (1.5 – 5.0 %), while *H. opuntia* libraries were dominated by sequences related to Cyanobacteria Group I (8.0 %), *Lactobacillus* (5.1 %), *Curvibacter* (2.7 %), *Silicibacter* (2.0 %), and Rhodobacteraceae (2.0 %, Table 4.1). Turf algae shared several OTU in common with corals, including those most similar to *Acidovorax* (11 %), *Lactobacillus* (5.3 %), *Cloacibacterium* (4.9 %), and *Curvibacter* (3.8 %, Table 4.1). The most abundant OTU associated with turf algae also included sequences most similar to an unknown Alphaproteobacteria (2.4 %), Rhodobacteraceae (2.0 %), Rhizobiales (1.3 %), and *Prosthecochloris* (1.1 %).

All of the algal-associated communities contained a low abundance of sequences similar to several genera of Cyanobacteria previously found associated with coral black band disease (BBD), including *Leptolyngbya, Geitlerinema, Oscillatoria, Phormidium* and Cyanobacterium SC-1 and OSC (Myers et al. 2007) (Table 4.2). None of these genera were found associated with any of the coral tissue samples. In addition, analysis of the libraries by BLASTn found a low abundance of hits to *Aurantimonas coralicida* 16S rDNA, the only known coral pathogen previously found

associated with algae (Nugues et al. 2004), in every library (Table 4.2). The abundance of sequences similar to coral disease associated bacteria was generally higher in the algal libraries with the exception of CCA (Table 4.2). Conversely, the abundance of sequences similar to general potential pathogens was highest on coral from Site 2 and turf algae (Table 4.2).

Table 4.2. Abundance of potential pathogens associated with corals and algae. Libraries were analyzed by BLASTn. Values listed are the percentage of the sequences in each library that were similar to the listed coral diseases. The top three most abundant of each pathogen are in bold. The list of coral disease-associated bacteria was obtained from Mouchka et al. 2010.

Library	White Plague (*A. coralicida*)	Black Band Disease	Coral disease associated	Potential pathogens
Coral 1	0.04	0	12.4	4.7
Coral 2	0.01	0	12.2	3.5
Coral 3	**0.05**	0	22.3	**7.3**
Coral 4	**0.10**	0	26.0	5.3
Coral Site 2	**0.08**	0	29.1	**14.6**
CCA 1	0.01	0.08	22.6	6.0
CCA 2	0.02	0.18	20.1	4.6
Dictyota 1	0.003	0.37	**39.6**	4.6
Dictyota 2	0.01	**1.7**	**40.8**	4.1
Halimeda 1	0.004	**1.2**	30.2	4.9
Halimeda 2	0.01	**0.87**	33.4	5.2
Turf 1	0.04	0.05	**51.1**	**9.3**
Turf 2	0.04	0.10	25.3	7.1

Phylogenetic distance between coral and algal-associated bacterial communities: Principal component analysis (PCA) of the weighted UniFrac distance showed that corals and algae harbor characteristic communities of Bacteria. All of the coral tissue samples clustered to the right of the graph along the primary axis (65 % of the variability) and away from the algal samples, but did not cluster in the second

dimension (12 % of the variability, Figure 4.3). The bacterial communities associated with the corals from Site 2 did not fall within the loose cluster of communities from Site 1, indicating that there may possibly be some differences in communities due to location. Algal-associated bacterial communities clustered separately from the corals and by the type of alga. *H. opuntia* communities were the most similar to each other, clustering tightly (Figure 4.3). The *Dictyota* sp. libraries also clustered together, although not as closely as the *H. opuntia* libraries. Turf algal communities had the greatest separation between the two libraries, and one turf library most closely clustered with one of the CCA libraries (Figure 4.3). Overall, the two PCA axes explained 76.8 % of the variation between the different communities.

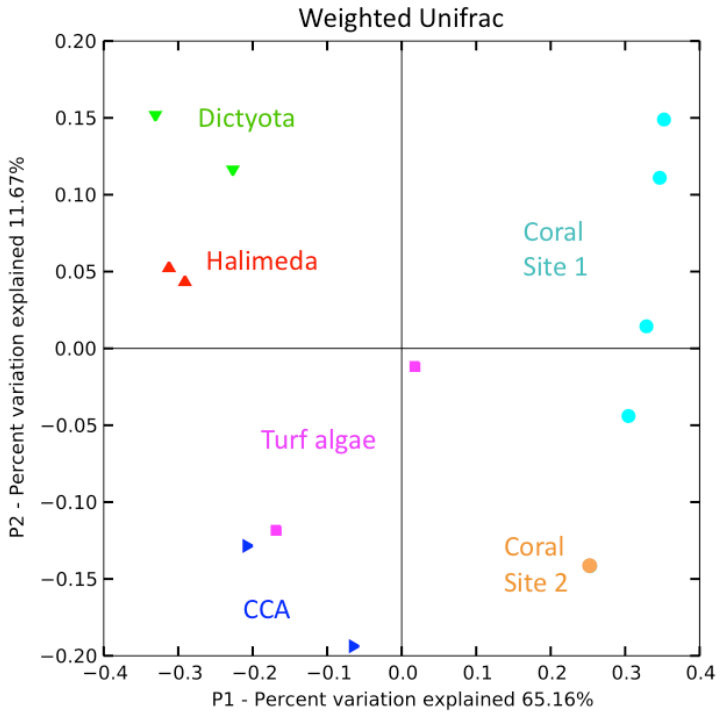

Figure 4.3. Principal component analysis of weighted UniFrac distance. Light blue = *M. annularis* from Site 1, orange = *M. annularis* from Site 2, dark blue = CCA, green = *Dictyota* sp., purple = turf algae, red = *Halimeda opuntia.*

General metabolic composition of coral and algal-associated communities:

Coral-associated bacterial libraries were dominated by sequences most similar to facultative anaerobes (55 – 83 %, Figure 4.4a). In contrast, the majority of classifiable Bacteria associated with the various algal tissues were most similar to obligate aerobes (54 – 93 %, Figure 4.4a). Coral bacterial communities were dominated by groups most closely related to known heterotrophs (>99 % for coral tissue; Figure 4.4b), while algal-associated bacterial communities contained more groups related to photoautotrophs, varying between 32 % of the community for *H. opuntia* to 5 % for turf algae (Figure 4.4b). Algal-associated communities also harbored a greater abundance of sequences that could not be classified to family or genus, and thus had more unknown metabolisms. The majority of the sequences were most closely related to gram-negative Bacteria for all coral and algal- associated bacterial libraries (data not shown).

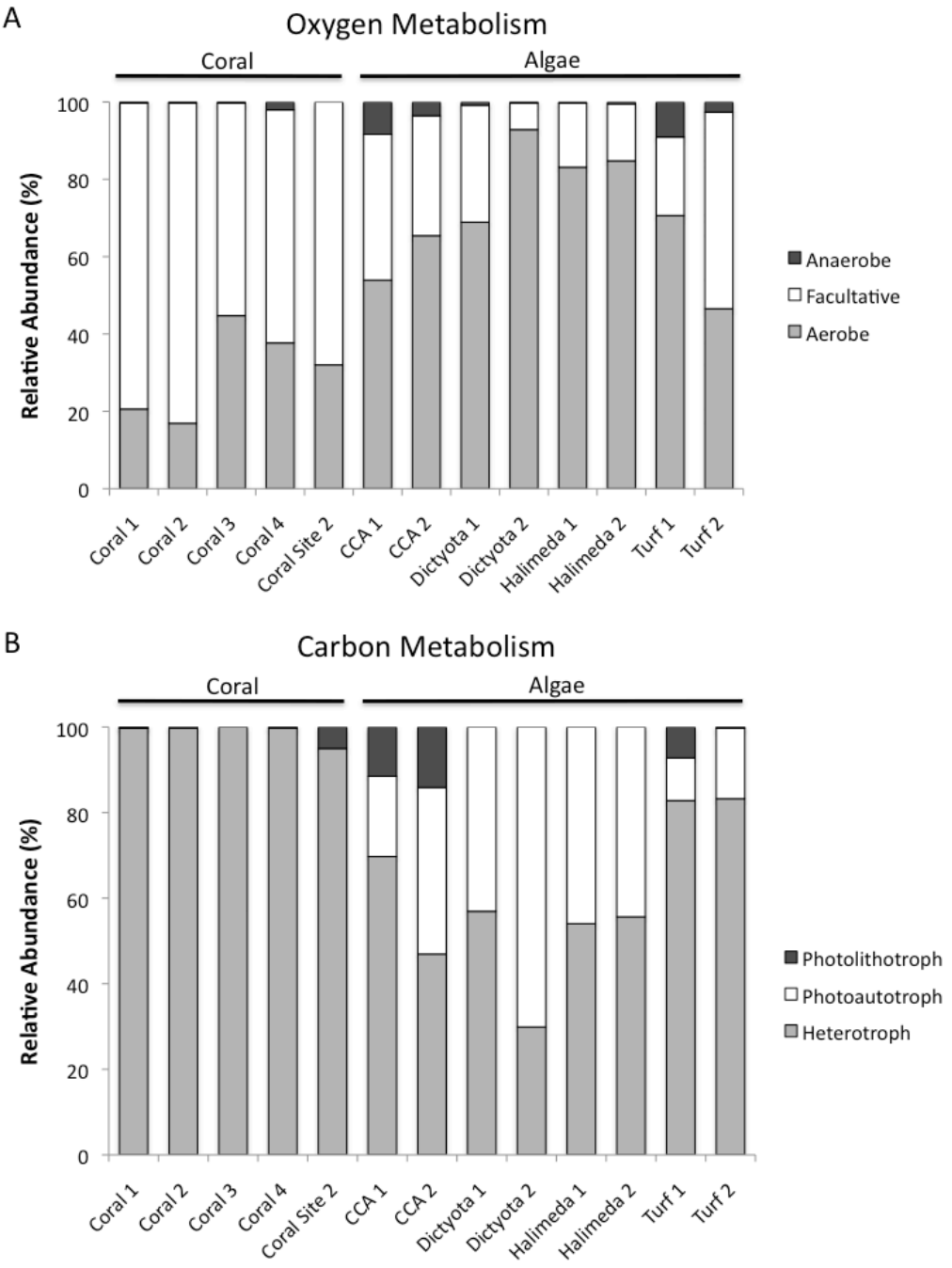

Figure 4.4. Relative abundance of classifiable bacterial metabolisms associated with the coral *M. annularis* and benthic algae. A) Oxygen metabolism and B) carbon metabolism.

Chloroplast diversity and taxonomy associated with benthic reef algae: The number of OTUs similar to chloroplasts was highest in the *H. opuntia* samples (115 - 123), followed by *Dictyota* sp. and turf algae (76 - 100), and was lowest associated with CCA (43 – 58; Figure 4.5a). There was little difference between the number of observed and predicted OTUs (Figure 4.5a), indicating high coverage of the communities. Again, the highest predicted richness was observed on *H. opuntia*, followed by *Dictyota* sp. and turf algae, and lastly CCA (Figure 4.5a). Shannon-Weiner diversity of algal-associated chloroplasts followed a different pattern than richness. *H. opuntia* and turf algae had the highest diversity (4.04 – 4.68), followed by CCA (2.56 – 3.10) and lastly *Dictyota* sp. (1.67 – 2.26) (Figure 4.5b).

The majority of chloroplast sequences associated with each algal library were likely from the host (Florideophyceae (red algae) for CCA, Phaeophyta (brown algae) for *Dictyota* sp., and both Phaeophyta and Florideophyceae for turf algae). The exception was the *H. opuntia* libraries, which were dominated by sequences most closely related to the diatom family Bacillariophyceae (40 – 70 %), followed by Florideophyceae (13 – 35 %; Figure 4.6). Florideophyceae were also present on *Dictyota* sp. (4.3 - 8.1 %; Figure 4.6). CCA libraries included sequences similar to both green and brown algae (Ulvophyceae [2.2 – 11 %] and Phaeophyta [6.8 – 21 %], respectively; Figure 4.6). All libraries contained a low abundance of sequences similar to a wide variety of unicellular algae (Figure 4.6).

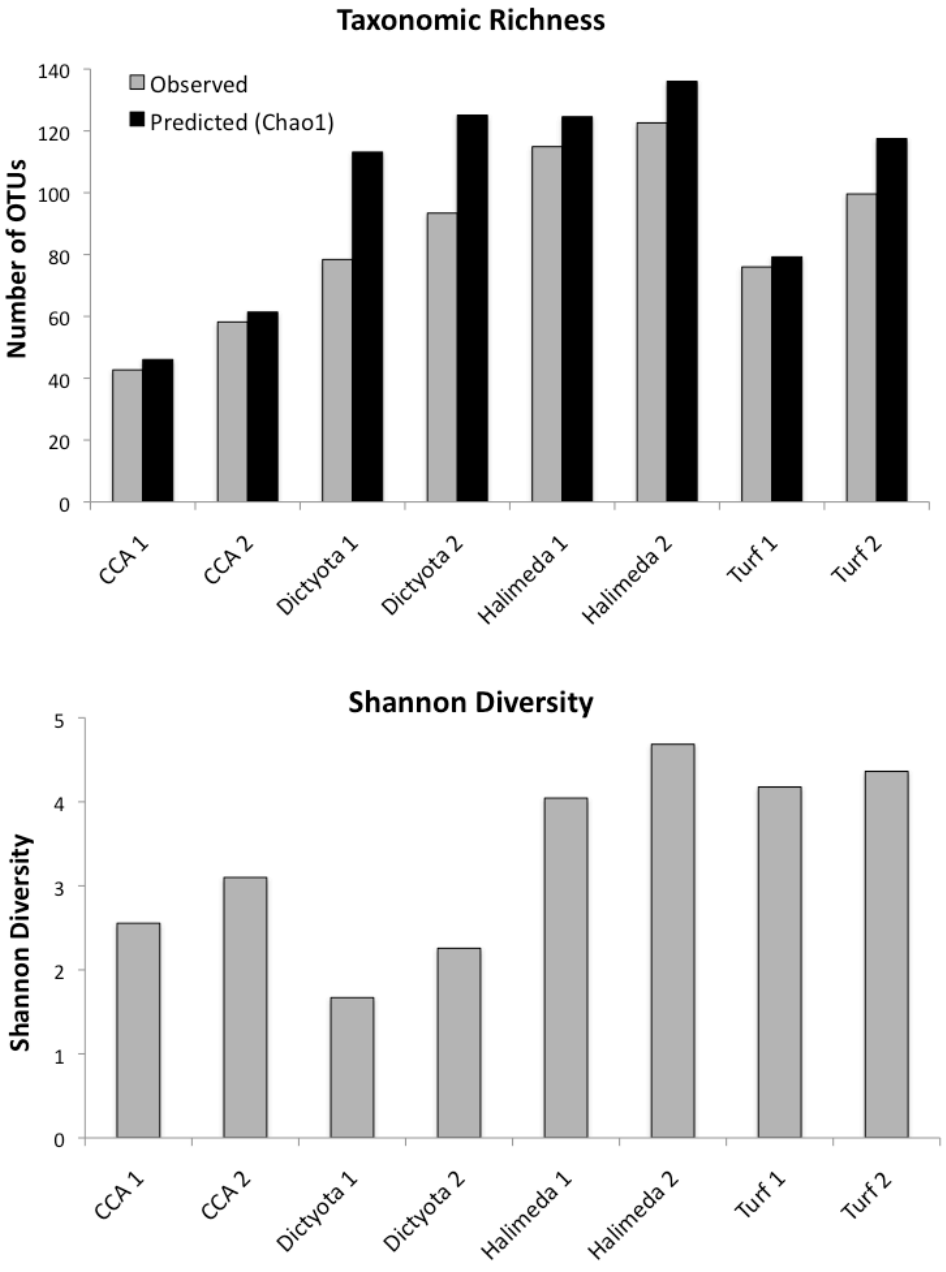

Figure 4.5. Diversity of chloroplasts associated with benthic algae. OTUs were grouped at 97 % similarity.

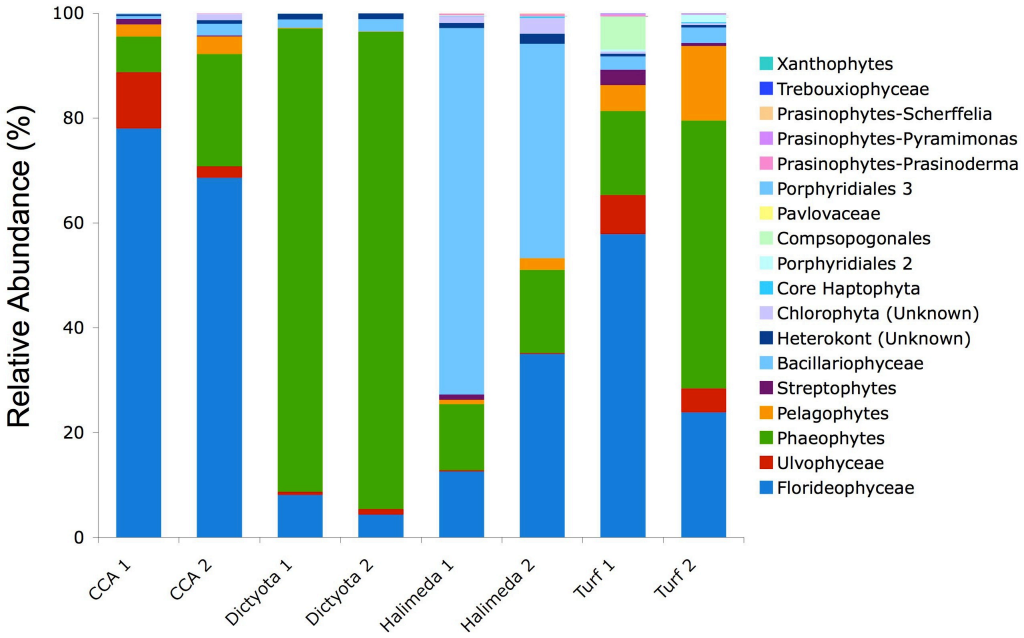

Figure 4.6. Relative abundance of chloroplast phyla associated with benthic algae. Taxa listed fall into the following groups: Green algae - Prasinophytes-Prasinoderma, Prasinophytes-Scherffelia, Prasinophytes-Pyramimonas, Streptophytes, Trebouxiophyceae, Ulvophyceae, and Chlorophyta; Haptophytes (unicellular algae) - Core Haptophytes and Pavlovaceae; Heterokonts - Bacillariophyta (diatoms), Pelagophytes (algae), Phaeophytes (brown algae), and Xanthophytes (yellow-green algae); and Red algae - Compsopogonales, Florideophyceae, Porphyridiales 2, and Porphyridiales 3.

Discussion

Expanding the holobiont concept to algae. The holobiont concept was first applied to corals after they were found to host abundant and species-specific microbial communities, and these microbes were hypothesized to provide some benefit to their host (7,25,26). Our results demonstrate that the holobiont concept may also be applicable to benthic algae. Bacteria are abundant on algal surfaces, ranging from 1 x 10^6 – 3 x 10^7 per cm^2 on various macroalgae (49) and 1.6 x 10^7 per gram wet weight on CCA (43). Here we have found that, much like coral-associated Bacteria, algal-

associated bacterial communities are specific to the type of algae examined. In addition, these characteristic bacterial communities associated with each of the different types of algae were more diverse than those found on corals, including up to 10 times more types of taxa. The data from each library in this study represent a pool of five different individuals, thereby providing an in-depth snapshot of the characteristic types of Bacteria that typically colonize these organisms. There is some variability between the two pools from the same species, indicating that there is variability between individuals; however we cannot assess the magnitude of this individual variation. Our data do suggest that *Halimeda opuntia,* for example, has the least variability and hence likely the greatest host specificity. Conversely, turf-associated Bacteria showed the most variability between libraries. It is likely that the diverse heterogeneous assemblage of algae that makes up turf algal communities increases the heterogeneity of the associated bacterial communities, leading to the differences observed in this study.

The characteristic nature of some of the algal-associated Bacteria support the hypothesis that algae influence the types of Bacteria that can survive on the algal surface, and there are a variety of mechanisms algae may employ to achieve this. For example, some algae produce secondary metabolites that are directly toxic to Bacteria (50,51) or inhibit quorum sensing (52,53), and physical mechanisms like mucus release and tissue sloughing likely affect the types of bacteria that survive on the algal surface (50,54). Furthermore, release of organic compounds by algae may selectively promote growth of certain groups of bacteria, and it has been shown that algal DOM

differentially stimulates bacterial growth based on the type of alga from which it originated (48).

Bacteria associated with algae are also likely providing some benefits to their algal host. Similar to coral-associated microbes, studies have found that Bacteria isolated from algae are antagonistic towards some types of fouling Bacteria (55), and in addition some isolates help prevent fouling by invertebrate larvae (56,57). It is possible that resident Bacteria on algal surfaces are excluding algal pathogens and resource competitors (i.e. fouling invertebrates and photosynthetic eukaryotes), thus indirectly promoting the health of the host. In addition, some groups of Bacteria associated with algae may be fulfilling multiple beneficial roles for the host. Cyanobacteria, for example, which were abundant on the various benthic algae, have been shown to protect algae from herbivory (58), and nitrogen fixed by this group may serve as an important nutrient source for the host alga (33,35).

Finally, the types of microbes associated with algae may have implications in coral reef health. First, all of the algal libraries examined contained sequences related to Bacteria found associated with coral disease states. While coral disease associated Bacteria are not necessarily pathogens and may be opportunistic colonizers of degraded coral tissue, the presence of these types of Bacteria suggest that benthic algae may be a potential source of coral pathogens and/or opportunistic colonizers that may lead to coral death in the wake of stress or disease. Secondly, Cyanobacteria most similar to those associated with coral black band disease were observed on all four groups of algae examined. *Halimeda opuntia* has previously been found to harbor and

transmit the coral pathogen *A. coralicida* (59). Given that the present study found bacteria closely related to *A. coralicida* as well as BBD, it is possible that various benthic reef algae serve as reservoirs for a variety of potential coral pathogens. It remains to be determined if the presence of these bacteria associated with CCA, *Dictyota* sp., or turf algae can lead to transmission of coral disease.

Benthic algae harbor diverse communities of photosynthetic eukaryotes. Abundant sequences related to chloroplasts in the libraries showed that there is a large diversity of photosynthetic eukaryotes associated with benthic reef algae. Over a 100 different OTUs were observed, most of which were novel. The presence of a high diversity of photosynthetic eukaryotes suggests that there may be intense competition on the surface of the algae for nutrients and light, a phenomenon that has been hypothesized with interactions between algae and benthic diatoms (50). In fact, many types of macroalgae release allelochemicals that target photosynthetic eukaryotes such as diatoms (50,51,54), yet despite these defenses it is clear that a wide variety of microalgae are capable of colonizing algal surfaces.

Coral-associated bacteria are primarily facultative anaerobes and show site-specificity. The diversity of the coral-associated bacterial communities in this study is similar to the diversity observed from previous 16S rDNA and metagenomic studies of coral-associated microbes (7,10,12,16,22,24). These communities were dominated by sequences similar to known facultative anaerobes (60 – 80 % total), with the

remainder comprised of sequences similar to strict aerobes. The capacity of the microbial community for oxygen-dependent and anaerobic respiration presumably reflects adaptations to the variations in oxygen saturation of coral tissues and the surrounding boundary layer, which range from superoxic during the day to anoxic at night (60). Previous work has demonstrated that the heterotrophic communities associated with corals are more productive than the surrounding seawater communities (61), and the dominance of heterotrophs and relatively rapid production rates of coral-associated microbes reflect the lability of the carbon-rich habitat of the coral mucus (62).

The composition of the coral-associated bacterial communities showed differences between the two sites studied, indicating that location plays a role in shaping coral-bacterial associations. While the phylogenetic composition of coral-associated bacteria from the two different sites, located approximately 40 km apart, was similar, the relative abundances of each of the different taxa present were different. This suggests that while the bacterial communities associated with *M. annularis* vary significantly between sites, likely due to environmental factors, the species of coral likely shapes the phylogenetic composition of the associated bacterial community regardless of location.

Microbial diversity associated with coral reefs. Benthic organisms are proving to be an enormous reservoir of microbial diversity. Given an average of ~ 300 bacterial OTUs estimated to be associated with any given coral species and that there

are ~ 50 coral species in the Caribbean, there are an estimated 15,000 different types of bacteria associated with Caribbean corals. If we now include the four different groups of algae in this study (fleshy macroalgae, encrusting calcareous algae, upright calcareous algae, and turf algae), there are an estimated 7700 OTUs associated with this very small sample of algal diversity in the Caribbean (out of ~ 500 spp.). If bacterial taxa associated with different species of algae within these four functional groups are as species specific as coral-associated bacteria appear to be, there are potentially tens of thousands of unique bacterial taxa associated with the reef benthos. On Curacao, for example, there are as many as 142 different algal species along one 115 m reef transect, and an average of 54 unique algal species per 25 m^2 (63). If each algal species has a characteristic bacterial community as diverse as those identified here, there are potentially 135,326 - 468,600 different bacterial taxa along one 115 m transect from the shoreline down the reef slope. Additionally, within one 25 m^2 reef plot, there are between 51,462 - 178,200 bacterial taxa associated with the algae alone. Given that a large proportion of all the algal-associated bacterial libraries were unclassifiable beyond Bacteria, this represents a huge proportion of unexplored microbial diversity in the world.

Conclusion

Benthic reef algae have characteristic microbial communities associated with their tissue. Very little is known about the role that this diversity plays in reef ecology, but there are likely both positive or facilitative interactions as well as negative or

antagonistic interactions between the micro and macrobiota. These microbial assemblages likely contribute to nutrient cycling and gas exchange and subsequent growth and abundance of corals and algae but may also include several potential pathogens. The specific interactions between algae and the microbial world have important implications for reef health. As reservoirs of coral pathogens, they have the potential to transmit disease across the reef, and as algae become increasingly abundant on coral reefs around the world, this may create a positive feedback loop whereby the more algae that are present, the greater the potential to transfer pathogens. In addition to their affects on corals, bacteria associated with benthic algae likely play a role in the proliferation of algae by fixing nitrogen, preventing herbivory, and possibly by exclusion of algal pathogens and competing primary producers. Photosynthetic eukaryotes associated with algae, on the other hand, may be competing with the host alga for nutrients, light, and inorganic carbon. It remains to be seen how changes in environmental conditions such as reduced herbivory, increased eutrophication, and elevated sea surface temperature influence the microbial communities associated with benthic reef algae and how these changes affect the physiology and success of algae on coral reefs around the world.

Experimental Procedures

Sample Collection: Samples were collected from the island of Curacao, Netherlands Antilles, with permission from the CARMABI research station. All algal samples and the majority of the coral samples were collected 8 - 10 m deep at Site 1

(Water Factory) on the southern side of the island (12° 06’ 35.10” N, 68° 57’ 22.91” W). An additional set of coral samples were collected from a similar depth at a distant site on the far western point of the island (Site 2; 12° 22’ 36.40” N, 69° 09’ 39.51” W).

Tissue samples were collected from the coral *Montastraea annularis* and four different types of algae: 1) crustose coralline algae (CCA), 2) *Halimeda opuntia*, 3) *Dictyota* sp. and 4) turf algae. Tissue punches were collected underwater using a hollow punch and hammer (diameter = 0.64 cm), with the exception of the *H. opuntia*, which was collected by hand. Each tissue sample was placed in an individual sterile whirl-pack underwater. Two tissue samples were taken from 5 different individuals for each of the four types of algae, 20 different coral colonies of *M. annularis* were sampled at Site 1, and 5 different colonies of *M. annularis* were sampled at Site 2. Samples were returned to the lab within 30 - 60 min, placed in a solution of 25 mM sodium citrate, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ammonium sulfate to preserve nucleic acids, and frozen at -20°C. All reagents were from Fisher Scientific unless otherwise noted.

DNA extraction: Coral tissue was removed from the skeleton using an airbrush with 0.2 µm filter-sterilized TE buffer (10 mMTris(hydroxymethyl)aminomethane hydrochloride [pH 8]/1 mM EDTA). An aliquot of the tissue slurry (500 µl) was centrifuged for 20 min at 14,000 x g and resuspended in lysis buffer (50 mM Tris•HCl, pH 8.3, Sigma-Aldrich; 40 mM EDTA, pH 8; 0.75 M sucrose, Sigma-

Aldrich). A lysozyme digestion (5 mg ml^{-1}, Sigma-Aldrich) was performed for 30 min at 37°C. This was followed by a second lysis with proteinase K (0.5 mg ml^{-1}) and sodium dodecyl sulfate (SDS, 1 %) at 55°C overnight. Following lysis the sample was incubated at 70°C for 10 min to inactivate the enzyme, and DNA was precipitated by adding sodium acetate (0.3 M final concentration, Sigma-Aldrich) and an equal volume of isopropanol. This was then incubated at -20°C for 4 - 5 hr. The DNA was then pelleted by centrifugation at 14,000 x g for 20 min at 4°C and resuspended in TE buffer. At this point a cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich) extraction was performed (1 % SDS, 0.7 M NaCl, 0.27 mM CTAB). The sample was incubated at 65°C for 10 min. The sample was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1; Sigma-Aldrich). The DNA was precipitated by adding 0.7 volumes isopropanol and incubating at -20°C overnight. DNA was pelleted by centrifugation at 14,000 x g for 15 min at 4°C. The pellet was washed with cold 70% ethanol, dried, and resuspended in 10 mM Tris (pH 8, Sigma-Aldrich). Algal tissue was homogenized with an epi-mortar. An aliquot of homogenate (250 µl) was used for DNA extraction with the Mo Bio UltraClean Soil Kit (Solana Beach, CA) according to the manufacturer's instructions. All DNA extracts were stored at -20°C.

PCR and sequencing preparation: A 526 base pair (bp) region of the 16S rRNA gene (16S rDNA) including the variable regions 1 - 3 was selected for tag pyrosequencing. This region was amplified using the bacterial forward primer 27F,

which also included the primer B adaptor for pyrosequencing on the 5' end (5'-GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3'). The bacterial reverse primer 534R was also used, and included the sequencing primer A and a unique 8 bp barcode on the 5' end (5'-GCCTCCCTCGCGCCATCAGNNNNNNNNCAATTACCGCGGCTGCTGG-3'). Barcodes were error-correcting Hamming sequences (64), and can be found in Supplementary Table 1. The length of the amplicon including barcode and 454 primers was 578 bp. Amplifications were run under the following conditions: 94°C for 5 min; 29 cycles of 94°C for 1 min, 60°C - 0.5°C/cycle for 1 min, and 72°C for 1 min; followed by 72°C for 10 min. Each DNA extract was amplified by four replicate PCR reactions, which were then combined. These PCR products were then purified using the Bioneer AccuPrep PCR Purification Kit (Alameda, CA) and the amount of DNA in each sample was quantified using the Quant-iT PicoGreen assay (Invitrogen, Carlsbad, CA). The algal PCR products were then pooled such that two pools were generated per algal type. To generate these pools, PCR amplicons from 5 different individuals of the same type of alga were combined in equimolar amounts. The same barcode was used for each of the five algal samples within one pool. Coral samples were also pooled in groups of five individuals for a total of four pools from Site 1 and one pool from Site 2. Each sample within a pool was amplified independently but with the same barcode, as done with the algal samples. The barcode used for each library is listed in Supplemental Table 2. Once this was complete, sample pools were combined

together in equimolar amounts and sequenced using the 454 Titanium platform at Engencore (University of South Carolina).

Sequence analysis: Sequences were first screened for quality using the following parameters: minimum quality score of 25, minimum sequence length of 200 bp, maximum length of 1000 bp, and no ambiguous bases in the entire sequence or mismatches in the primer sequence. Any sequences not meeting these parameters were excluded from downstream analyses. Sequences were then sorted by barcode into their respective samples and the barcode and primer sequences were removed. Sequences were then denoised (i.e. error corrected) (65). For comparison, denoised and non-denoised sequences were analyzed for diversity by two different methods: the Ribosomal Database Project (RDP) pyrosequencing pipeline (pyro.cme.msu.edu) and the Quantitative Insights into Microbial Ecology (QIIME) pipeline (66). Sequences were first grouped into operational taxonomic units (OTUs) with a 97 % identity threshold. Using the RDP pipeline, sequences were aligned with the Infernal aligner and then grouped using a complete linkage clustering method. Using QIIME the sequences were clustered by CD-HIT (67). Once clustered, a representative sequence from each OTU was selected and taxonomic identity was assigned to each representative sequence using the RDP taxonomic classifier at 80 % confidence (68). Sequences classified as chloroplasts by the RDP were removed from the bacterial libraries and analyzed separately. Sequences classified as unknown Cyanobacteria were suspected to include chloroplasts, and were further screened by BLASTn against

the Silva SSU rRNA database (E value = 10-20, minimum alignment length = 151 bp). Sequences with a best match to eukaryotes (i.e. chloroplasts) were separated from the bacterial libraries and analyzed with the other chloroplasts.

Chloroplast taxonomy was determined by aligning the representative sequences from each OTU to 185 organism-specific plastid and bacterial 16S rRNA genes downloaded from GenBank using MAFFT (69). A PHYML maximum likelihood (ML) tree (GTR+I+gamma 4) with aLRT branch supports was calculated from the total dataset (590 sequences; (70)). Short, low quality or divergent sequences were separated at this point and their phylogenetic affiliations to the organism specific sequences were determined individually. The remaining sequences from the total dataset were processed through multiple rounds of ML tree drawing and sequences with significant (> 0.95 aLRT supports) to known plastids were progressively excluded from the dataset. Narrowing the total dataset using this procedure allowed for determination of phylogenetic affiliations of most of the sequences to eukaryotic phyla or families. Where the phylogenetic position was not significantly resolved or the sampling of plastid lineages was not sufficient the classification was designated as such (e.g. unidentified heterokont).

In order to analyze alpha diversity, bacterial and chloroplast libraries were randomly sub-sampled using QIIME so that sequencing effort (i.e. the number of sequences in each library) did not affect diversity comparisons. Bacterial libraries were sub-sampled at a step size of 90 sequences from 1 – 9,500 sequences a total of 10 times. Chloroplast libraries were sub-sampled at a step size of 50 sequences from 1 –

5,500 sequences a total of 10 times. Once the libraries were rarified, the following alpha-diversity metrics were determined: total observed species (OTUs), predicted species (Chao1), and Shannon-Weiner diversity (H'). The same alpha diversity metrics were also determined using the RDP pyrosequencing pipeline. Beta-diversity of the bacterial communities was analyzed in QIIME using a weighted UniFrac analysis. Principal components for each sample library were generated from the UniFrac distances and plotted in two dimensions. Finally, individual bacterial libraries were analyzed by BLASTn against two different databases of 16S rDNA sequences of 1) potential pathogens and 2) coral disease-associated bacteria (Mouchka et al. 2010). BLASTn parameters for a significant hit required over 150 base pair alignment, E-value less than 1×10^{-10}, and greater than 95 % identity, and the number of sequences that hit each database was tallied.

Acknowledgements

We would like to thank Mark Vermeij and CARMABI for use of the laboratory in Curacao. We also thank Dana Willner, Bahador Nosrat, Alejandro Reyes Munoz and Alejandra Prieto Davo for bioinformatics support, and Nancy Knowlton for valuable discussions on benthic biodiversity.

Funding

Funding for this work was provided by the National Science Foundation Grant OCE-0927415 to FLR. KLB was supported by a National Science Foundation Graduate Research Fellowship.

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Acknowledgements

Chapter 4, in full, is a reprint of the material as it appears in Environmental Microbiology. Katie L. Barott, Beltran Rodriguez-Brito, Jan Janouškovec, Kristen Marhaver, Jennifer E. Smith, Patrick Keeling, and Forest L. Rohwer; 2011. The dissertation author was the primary investigator and author of this paper.

Appendix

Supplemental Table 1. List of barcode and primer sequences used for multiplex tag sequencing.

Barcode	Barcode sequence	Primer sequence (5'-PrimerA-Barcode-534R-3')
1	AACCAACC	GCCTCCCTCGCGCCATCAGAACCAACCCAATTACCGCGG CTGCTGG
2	AACCATCG	GCCTCCCTCGCGCCATCAGAACCATCGCAATTACCGCGGC TGCTGG
3	AACCATGC	GCCTCCCTCGCGCCATCAGAACCATGCCAATTACCGCGGC TGCTGG
4	AACCTACG	GCCTCCCTCGCGCCATCAGAACCTACGCAATTACCGCGGC TGCTGG
5	AACCTAGC	GCCTCCCTCGCGCCATCAGAACCTAGCCAATTACCGCGGC TGCTGG
6	AACCTTCC	GCCTCCCTCGCGCCATCAGAACCTTCCCAATTACCGCGGC TGCTGG
7	AACGAACG	GCCTCCCTCGCGCCATCAGAACGAACGCAATTACCGCGG CTGCTGG
8	AACGAAGC	GCCTCCCTCGCGCCATCAGAACGAAGCCAATTACCGCGG CTGCTGG
9	AACGATCC	GCCTCCCTCGCGCCATCAGAACGATCCCAATTACCGCGGC TGCTGG
10	AACGATGG	GCCTCCCTCGCGCCATCAGAACGATGGCAATTACCGCGG CTGCTGG

Supplemental Table 2. Summary of the number of reads and average read length per library following quality screen and primer removal. Reads analyzed refers to the number of reads following removal of chloroplast contamination. Barcode number corresponds to the barcode sequences found in Supplemental Table 1.

Library	Barcode	Total reads	% Choroplast	Reads analyzed	Avg. read length
Coral 1	3	95,542	0.18	95,368	308
Coral 2	10	33,329	0.26	33,244	439
Coral 3	6	57,160	1.53	56,285	436
Coral 4	1	63,341	0.15	63,244	438
Coral Site 2	10	44,205	0.56	43,959	305
CCA 1	4	48,022	17.38	39,677	438
CCA 2	5	47,487	23.01	36,559	439
Dictyota 1	7	69,931	86.41	9,503	319
Dictyota 2	8	107,917	84.92	16,279	318
Halimeda 1	8	66,434	21.55	52,117	439
Halimeda 2	9	55,548	19.34	44,807	438
Turf 1	3	39,686	14.23	34,039	438
Turf 2	2	69,474	12.24	60,969	313

Supplemental Table 3. Comparison of diversity results (Chao1 [H']) of bacterial 16S rDNA sequences. Libraries were analyzed for diversity before and after denoising using either the QIIME or RDP pyrosequencing pipeline with 97 % similarity clustering.

Library	Qiime	Qiime denoised	RDP	RDP denoised
Coral 1	1141 (6.39)	343 (3.26)	1257 (3.48)	622 (2.14)
Coral 2	692 (5.34)	266 (2.84)	612 (2.45)	451 (1.94)
Coral 3	808 (6.49)	345 (4.51)	631 (3.51)	417 (2.90)
Coral 4	884 (6.00)	328 (3.92)	841 (3.29)	534 (2.74)
Coral Site 2	1361 (7.42)	461 (5.03)	1151 (4.20)	724 (3.49)
CCA 1	1969 (7.67)	952 (6.22)	1986 (4.89)	1278 (4.34)
CCA 2	2721 (7.64)	1231 (6.36)	3545 (5.09)	2378 (4.49)
Dictyota 1	2485 (5.24)	2375 (7.11)	3823 (6.16)	2918 (5.13)
Dictyota 2	6190 (9.05)	3300 (7.60)	7279 (6.61)	5348 (5.69)
Halimeda 1	3844 (8.73)	2119 (7.59)	4960 (5.98)	3550 (5.43)
Halimeda 2	3954 (9.01)	2167 (7.82)	4991 (6.22)	3622 (5.64)
Turf 1	3511 (9.09)	1725 (7.64)	3566 (5.94)	2546 (5.56)
Turf 2	5065 (8.90)	1961 (6.91)	7529 (5.73)	5234 (4.86)

CHAPTER 5:

Microbial to reef scale interactions between corals and algae

Abstract

Competition between reef building corals and benthic algae is of key importance for reef dynamics. These interactions occur on many spatial scales, ranging from chemical to regional. Using microprobes, 16S rDNA pyrosequencing, and underwater surveys, we examined the interactions between the reef-building coral *Montastraea annularis* and four types of benthic algae. The macroalgae *Dictyota bartayresiana* and *Halimeda opuntia,* as well as a mixed consortium of turf algae, caused hypoxia on the adjacent coral tissue. Turf algae were also associated with major shifts in the bacterial communities at the interaction zones, including more pathogens and virulence genes. In contrast to turf algae, interactions with crustose coralline algae (CCA) and *M. annularis* did not appear to be antagonistic at any scale. These zones were not hypoxic, the microbes were not pathogen-like, and the abundance of coral-CCA interactions was positively correlated with percent coral cover. We propose a model in which fleshy algae (i.e., some species of turf and fleshy macroalgae) alter benthic competition dynamics by stimulating bacterial respiration and promoting invasion of virulent bacteria on corals. This gives fleshy algae a competitive advantage over corals when human activities, such as overfishing and eutrophication, remove controls on algal abundance. Together these results demonstrate the intricate connections and mechanisms that structure coral reefs.

Introduction

Coral reefs, the most biologically diverse marine ecosystems, are supported by the structural complexity provided by hermatypic corals (1). However, coral reefs around the world are becoming increasingly dominated by benthic algae, resulting in a loss of habitat and biodiversity (2–5). This trend is driven by algal overgrowth of live and recently dead corals, a trend facilitated by modern environmental changes such as decreased herbivory (6–9), eutrophication (8,10), increased coral bleaching associated with climate change (11–13), and coral disease (14–16). While this phenomenon has been well documented, the mechanisms by which algae overtake corals are not well understood. Competition between corals and benthic algae is common on coral reefs worldwide (17–20) and these interactions are frequently harmful to the coral, causing tissue damage and necrosis (3,20–23), reduced zooxanthellar function (23–26), and reduced coral fecundity (3,20,27). On the other hand, some algae have little effect on corals (28,29), such as certain species of crustose coralline algae (CCA) that promote coral settlement (30,31) and inhibit recruitment of macroalgae that would otherwise compete with corals (32,33).

Benthic algae also influence the microbes associated with corals, disrupting the complex community of the healthy coral holobiont—the symbiotic consortium including the coral animal, zooxanthellae, Bacteria, Archaea, fungi, and viruses (34). For example, algae can transmit pathogens to adjacent corals causing disease (35). Allelochemicals released by the algae may also stress the corals and disrupt the holobiont, causing loss of normal functions and coral mortality (36,37). Several lines

of evidence suggest that one mechanism for the deleterious effects of algae on corals is the release of organic carbon by the algae that fuels increased local activity of microbes. Coral-algal interactions in aquaria, for example, result in coral necrosis and hypoxia as a result of bacterial activity (38) and hypoxia has been observed in situ when turf and macroalgae border coral (17). Consistent with this hypothesis are findings that experimental addition of dissolved organic carbon alters the coral holobiont by increasing potential pathogens (39), leads to coral mortality and disease symptoms (40,41), and is deadly to corals whereas addition of inorganic nutrients is not (25,40,41).

We hypothesized that stressful coral-algal interactions compromise the normal function of the coral holobiont, allowing potentially pathogenic microbes to invade and the algae to overgrow the coral. In order to better understand these micro-scale dynamics and how they affect coral reef composition, we investigated the physiological and bacterial responses of the coral holobiont to interactions with different functional groups of benthic algae and quantified the prevalence of coral-algal interactions at reefs with different levels of human influence (figure S1, (42)). In situ interactions between the dominant reef-building Caribbean coral *Montastraea annularis* and four types of benthic algae were studied: encrusting calcified red algae (crustose coralline algae [CCA]); fleshy brown macroalgae (*Dictyota bartayresiana*); upright calcareous green algae (*Halimeda opuntia*), and a mixed assemblage of turf algae (figure 5.1). Physiological changes across these four types of coral-algal interfaces were compared by measuring the dissolved oxygen levels at the interaction

zones with and without algae removal. Algal-induced changes to the bacterial constituents of the holobiont were assessed by identifying the taxonomic composition of coral-associated bacteria across the same four types of interactions by pyrosequencing of the 16S rRNA gene. Our results demonstrate that each alga exerts its own characteristic suite of effects on the coral holobiont, and that these micro-scale dynamics have the potential to drive changes in reef community composition.

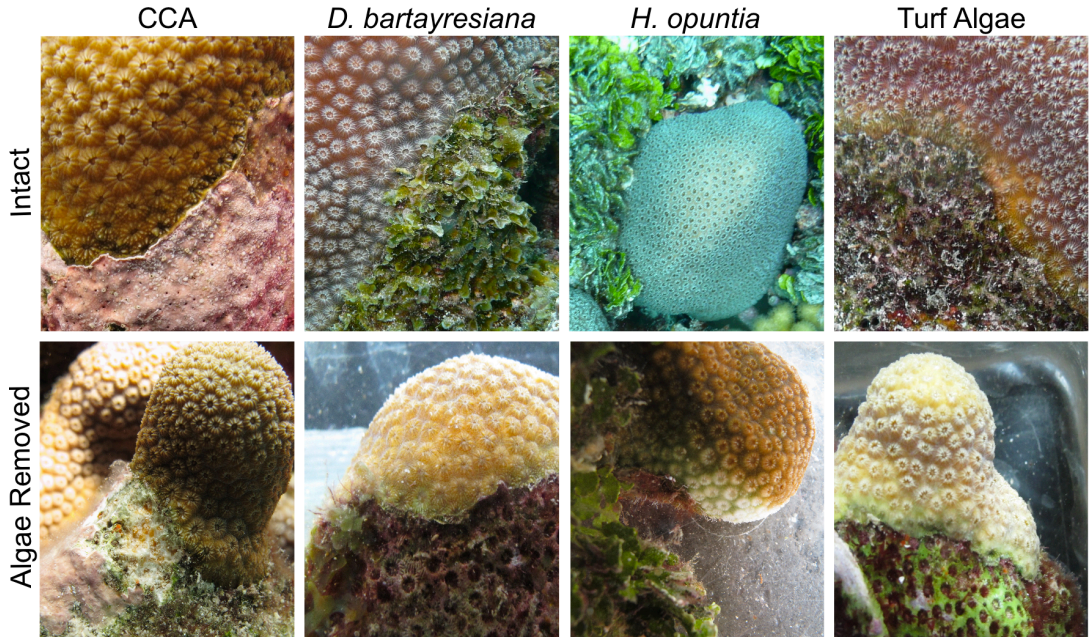

Figure 5.1. Typical interaction zones between the coral *Montastraea annularis* and the four types of algae examined. Shown are intact interactions (top row) and interactions after algal removal (bottom row).

Materials and Methods

Physiology of the coral holobiont at algal interaction zones: This study was conducted on the island of Curacao, former Netherlands Antilles, under the auspices of CARMABI. Interactions between the dominant reef-building coral *Montastraea*

annularis bordering one of four groups of algae: crustose coralline algae (CCA), *Dictyota bartayresiana, Halimeda opuntia*, and turf algae were studied (figure 5.1). Ten colonies of each interaction type (40 total) were identified on the reef (8-10 m deep, Water Factory; figure S1). The algae were removed from five of the ten coral-algal interactions of each type, taking care not to damage the adjacent coral tissue (figure 5.1). All colonies were removed from the reef 10–12 days later by breaking off columns below the live coral to avoid tissue damage. The concentration of dissolved oxygen (DO) was measured within 1 mm of the surface of each interaction using an oxygen microprobe (Unisense, Denmark) as previously described (electronic supplementary material [ESM], (17)). Four replicate readings were taken within each of three zones of the interaction: 1) coral tissue from the center of the colony, 2) coral tissue < 0.5 cm from the algae, and 3) algal tissue.

Microbial sampling: Tissue samples were collected from each of the four types of coral-algal interactions using a hollow punch (diameter = 0.64 cm) and hammer (8-10 m deep, Water Factory; figure S1). Tissue punches were collected from five different zones: 1) coral tissue from the center of the colony (>10 cm away from algae), 2) coral tissue adjacent to the algae, 3) the interaction zone, 4) algal tissue adjacent to the coral, and 5) algal tissue >10 cm away from the interface. Five different interactions of each type were sampled, for a total of 5 replicate tissue samples per zone per coral-algal interaction type. DNA was extracted from each sample and the bacterial 16S rRNA genes were amplified and pyrosequenced (see

ESM, table S2, (43)). Sequences were screened for quality, sorted by barcode, grouped into operational taxonomic unit (OTU, 97% similarity), and classified as previously described (ESM, (43)). A resampling-based rank comparison was employed to identify the taxa that were over- or under-represented in the five libraries from across each type of interaction (ESM).

Metabolic reconstruction: The metabolic profiles of the bacterial communities present in coral tissue away from algal interactions and those over-represented in coral tissue near or at each algal interface were estimated. For each taxon the closest relative with a sequenced genome was selected and the metabolic profile from that genome (determined by the SEED database) was included and weighted by the taxon's relative abundance. The metabolic profile for each community was then calculated as the linear combination of the metabolic profiles of each included taxon, weighted by its relative abundance, and XIPE was used to determine which metabolic subsystems were statistically different at the interfaces (90% confidence level, 5000 iterations; (44)). Statistical ranking was again performed to determine which metabolic subsystems were over-represented at the different coral-algal interaction zones relative to each other. The metabolic shifts observed at algal interfaces were then compared with those previously observed in corals subjected to abiotic stress (39) by principal component analysis.

Surveys of coral-algal interactions: Survey sites spanned the leeward side of Curacao and included different levels of human impact (e.g. adjacent population and sewage signature, (42)) that declined with increasing distance from the capital, Willemstad (figure S1). Surveys to quantify the types and abundances of interactions between corals and algae were conducted at 10 m depth as previously described (ESM, (17)). Percent cover of benthic organisms was determined from photoquadrats at 10 m depth (ESM).

Results

Physiological changes of the coral holobiont due to algal interactions: Dissolved oxygen (DO) concentrations in the boundary layer above *M. annularis* tissue distant from the site of algal interaction were hyperoxic relative to ambient seawater (192 - 282 mmol l^{-1} above ambient; ambient = 212 mmol l^{-1}; figure 5.2*a*). Likewise, the algal boundary layer was hyperoxic for all four types of algae examined, ranging from 418 - 775 mmol l^{-1} above ambient, figure 5.2*a*. However, when *M. annularis* was interacting with any of the four types of algae, the DO concentration at the interaction zone was decreased (paired *t*-test: $p < 0.02$ for each interaction type; figure 5.2*a*). These decreases resulted in DO levels below ambient for corals bordering *H. opuntia, D. bartayresiana,* or turf algae (95, 12, and 5.2 mmol l^{-1} below ambient, respectively) while corals adjacent to CCA maintained hyperoxia (184 mmol l^{-1} above ambient). Algal removal resulted in significant DO increase for *H. opuntia* (70%; *t*-test, $p = 0.003$) and *D. bartayresiana* (52%; $p = 0.03$), restoring hyperoxia at these

interfaces (figure 5.2*b*). Removal of turf algae restored hyperoxia but recovery was not statistically significant (36%, p = 0.21); coral-CCA interfaces remained hyperoxic after algal removal (figure 5.2*b*).

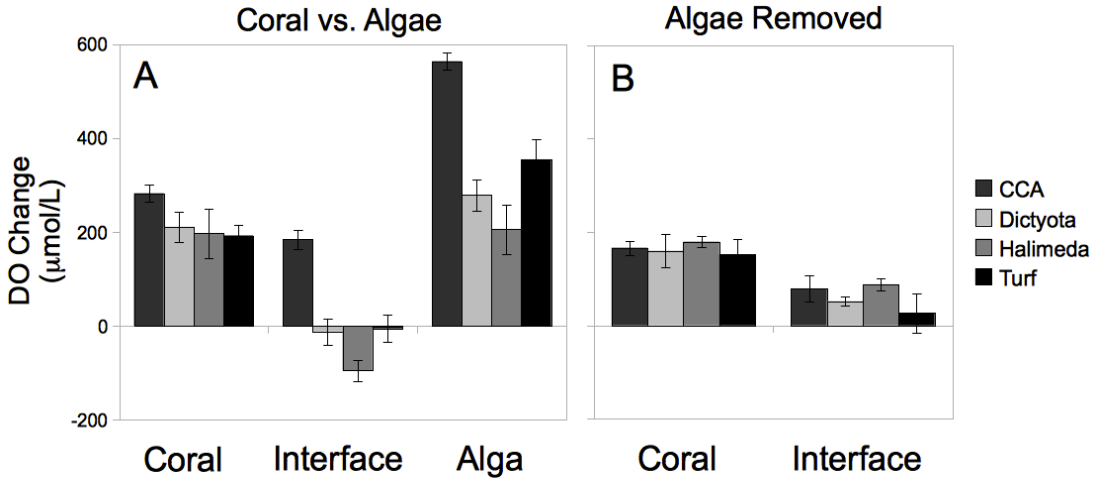

Figure 5.2. Dissolved oxygen (DO) concentration changes at the zone of interaction between the coral *M. annularis* and benthic algae. (*a*) Unaltered coral-algal interactions. (*b*) Coral-algal interactions 10 days after removal of the algae. DO concentrations are shown relative to atmospheric saturation of seawater (212 mmol L^{-1}). CCA, crustose coralline algae; Dictyota, *Dictyota bartayresiana*; and Halimeda, *Halimeda opuntia*. N=5 for all treatments; +/- SEM.

Changes to coral-associated bacteria due to algal interactions: The number of observed and predicted (Chao1) bacterial OTUs increased in coral tissue near all types of algae except *H. opuntia* relative to coral tissue distant from algae (table S1). In addition, the Shannon-Weiner diversity (H') of the coral-associated bacterial communities increased in tissues near CCA (from 3.26 to 4.72) and *D. bartayresiana* (from 2.84 to 3.28), but decreased for coral tissue adjacent to *H. opuntia* or turf algae (table S1). Three of the four coral-algal interfaces showed high diversity (5.70 – 7.64) comparable to that observed for the corresponding algal tissues (6.22 – 7.82), the

exception being the *H. opuntia* interface (4.58, table S1). When the phylogenetic distance between the 16S rDNA libraries was analyzed by principal component analysis (PCA), the coral-associated bacteria distant from algae clustered together along with those from coral tissue adjacent to *H. opuntia*, while those adjacent to CCA, *D. bartayresiana*, and turf algae were distant from the coral-associated communities and also from each other (figure S2). Some taxa were over-represented at or near the algal interfaces and the number varied depending on the type of algae involved: near CCA, 20 taxa or 38% relative abundance; near *D. bartayresiana*, 19 taxa or 21% relative abundance; near turf algae, 14 taxa or 13% relative abundance; or near *H. opuntia*, 12 taxa or 11% relative abundance (figure 5.3).

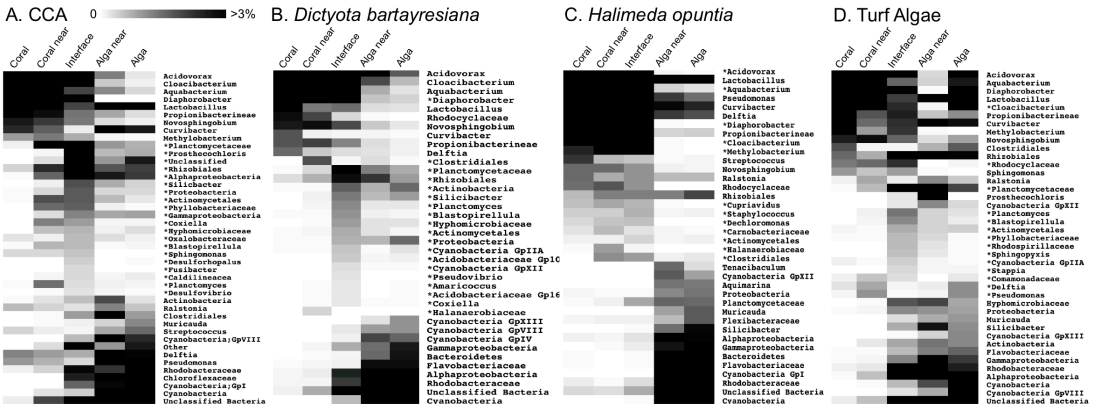

Figure 5.3. Heat map of relative abundance of Bacteria associated with coral-algal interfaces. Each panel shows the relative abundances across all five zones of interaction with one type of algae. Bacterial taxa are listed at the highest classifiable level; taxa listed above genus (e.g., family or order) include only members that could not be classified at a lower level. OTUs at the top of each list are those most abundant in coral tissue, those at the bottom the most abundant in the algal tissue. Scale bar represents relative abundance (%) of each taxon within each library. Asterisks indicate taxa over-represented in coral tissue at or near the coral-algal interface.

A majority (30/45) of over-represented taxa were enriched at only one type of coral-algal interface. Of the remaining 15 taxa, 11 were over-represented at two interfaces, three at three interfaces (all members of the Planctomycetaceae), and one (Actinomycetales) at all four interfaces.

The metabolic capabilities of the coral-associated bacterial communities were also altered by proximity to algal interfaces. For example, coral-associated 16S rDNA libraries were dominated by sequences related to facultative anaerobes (43). In contrast, we found sequences related to strict anaerobes present in coral tissue near or at interfaces with three of the four groups of algae: 8.5% relative abundance at CCA interfaces; 2.2% relative abundance near *D. bartayresiana* interfaces; 2% relative abundance near *H. opuntia* interfaces; but absent near and at interfaces with turf algae. The number of metabolic pathways (from the SEED database) that were over- or under-represented within these over-represented taxa also varied depending on the alga present (turf algae, 29; CCA, 22; *D. bartayresiana,* 13; and *H. opuntia*, 2). Interfaces with three of the types of algae (turf algae, CCA, and *D. bartayresiana*) shared several metabolic trends. Specifically, several pathways were under-represented at all three interfaces: membrane transport (including Type III and Type IV secretion systems), stress response, aromatic catabolism, and flagellar motility (figure 5.4). Likewise, all three showed an increased abundance of pathways for metabolism of single-carbon compounds, fatty acids, potassium, and purines. Coral-turf interfaces uniquely showed a reduction in organic sulfur assimilation as well as iron acquisition and metabolism. The two significant changes at *H. opuntia*

interactions were decreased abundance of genes for gram-positive cell wall components and di- and oligosaccharide metabolism (figure 5.4).

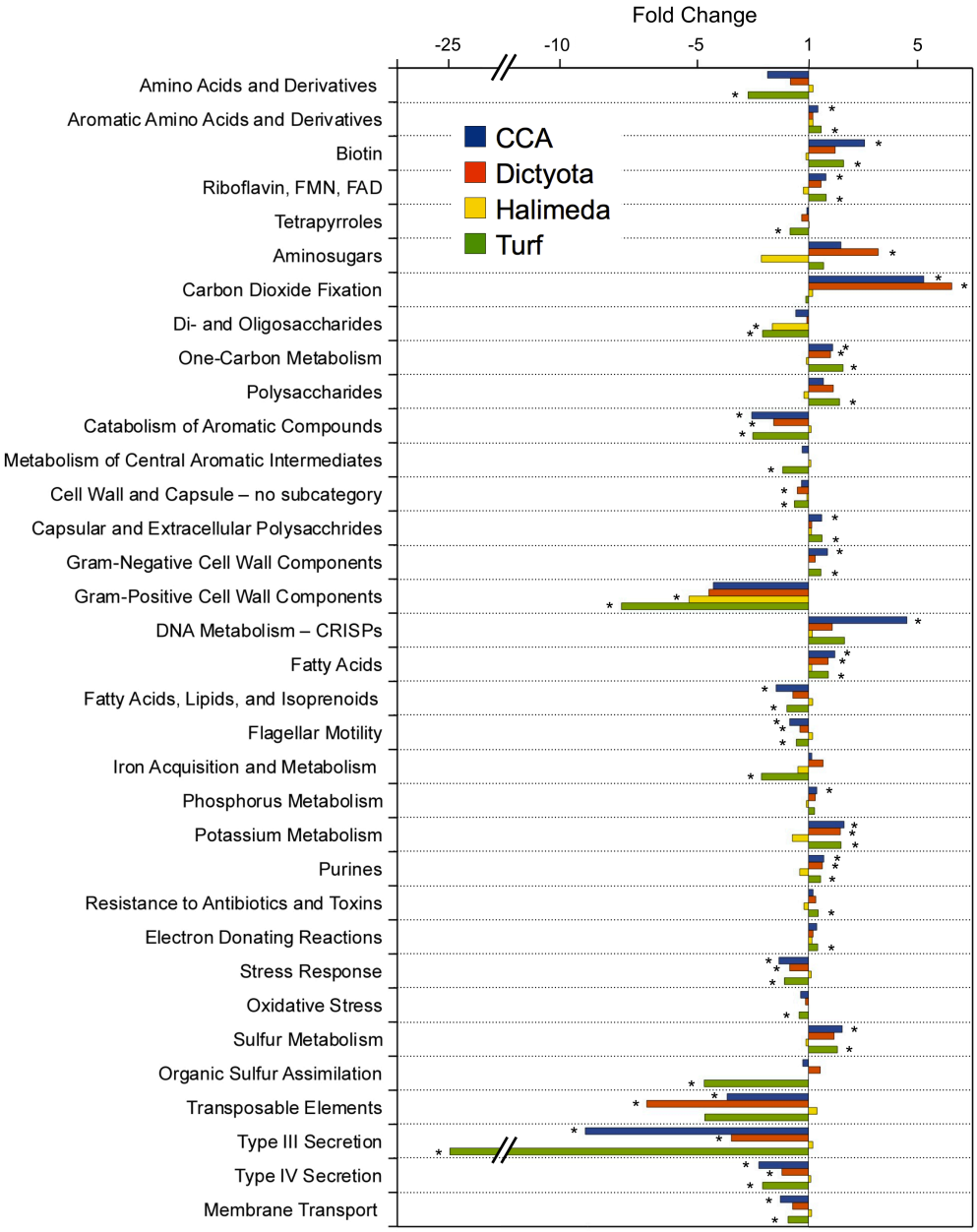

Figure 5.4. Altered metabolic subsystem abundances in coral-associated Bacteria at coral-algal interfaces. Shown are subsystems that were significantly increased or decreased in the Bacteria over-represented in coral tissue at or near at least one type of algal interface. The fold change is relative to corals distant from the interface. Asterisks indicate significant differences (90% confidence with 5000 iterations).

Comparison of the metabolic subsystems in the coral-associated bacterial communities near or at the interfaces with each other showed more virulence and potassium metabolism genes at interactions with turf algae and more carbohydrate metabolism genes at *D. bartayresiana* interactions (table 5.1). The communities near or at CCA interactions had more metabolic genes related to cell maintenance than the other interfaces, while those near or at *H. opuntia* interfaces were similar to coral-associated communities. Principal component analysis (PCA) of the reconstructed metabolic subsystems showed that coral-associated communities clustered closely with the *H. opuntia* interface community, while CCA, *D. bartayresiana*, and turf algae interface communities were distant from the corals and from each other (figure S3), mirroring the taxonomic clustering (figure S2). A PCA was also performed to compare these metabolic changes at coral-algal interactions with previously collected data on the coral holobiont's response to abiotic stress treatments (nutrient addition, temperature increase, decreased pH, and DOC addition; (39)). The metabolic changes associated with all four types of algal interactions clustered together with the DOC treatment (figure S4).

Reef-scale changes in coral-algal interactions: Every coral colony observed was interacting with at least one type of alga, with an average of 61– 80% of the coral perimeter involved in any type of algal interaction. Interactions with turf algae were the most abundant, accounting for 32 – 58% of the coral edge (figure 5.5*a*). The percentage of the coral edge bordered by CCA showed the most obvious trend,

Table 5.1. Influence of different algal interactions on corals across multiple spatial scales.

	Measured Attribute	CCA (Encrusting)	*Halimeda* spp. (Upright calcareous)	Turf Algae	Fleshy Macroalgae	References
Reef Scale	Interactions on healthy reef[1]	↑↑	↓↓	↓↓	↓↓	This study
	Coral recruitment[2]	↑↑	↓↓	0 / ↓↓	↓↓	45-47,67,68
	Coral fecundity[2]	no data	no data	↓↓	↓↓	3,20,27
Colony Scale[7]	Shading and abrasion[3]	0	+++	0	+++	69
	Tissue damage[3]	0 / +++	0 / +++	+++	+++	17,18, 36,38
	Bleaching[3]	0	+++	+++	+++	17,25,36, this study
	Photosynthesis inhibition (Expt)[4]	no data	med	no data	low-high	36,38
	Photosynthesis inhibition (Natural)[4]	none	no data	low	no data	26
Microbial Scale	No. of over-represented bacterial taxa at interface	20	12	14	19	This study
	Predicted bacterial metabolic subsystems enriched at interface	Cell wall, Cofactors, Nucleotides, Photosynthesis, Respiration	Membrane transport, Aromatics, Motility, Stress response	Virulence, Potassium	Carbo-hydrates	This study
Molecular Scale	Allelochemical impact on coral[4]	no data	high	no data	high	36
	DOC release[5]	med	none - low	high	med - high	57, Haas unpublished
	Oxygen change at interface[6]	↑↑	↓↓	↓↓	↓↓	17,38, this study

[1] Coral-algal interactions: ↓↓, decrease; ↑↑, increase; [2]Algal impacts on coral reproduction: ↑↑, promotes; ↓↓, inhibits; [3]Physical impacts of algae on corals: +++, present; 0, absent; [4]Range of algal impacts on holobiont photosynthesis (quantum yield inhibition [Fv/Fm]): 0.67, none; 0.5 - 0.65, low; 0.25 - 0.5, med; 0 – 0.25, high; expt, experiments; [5]Dissolved organic carbon release by algae (DOC, mM m^{-2} h^{-1}): 0 – 150, low; 151 – 300, med; >300, high; [6]Boundary layer oxygen conditions at interface: ↑↑, hyperoxic; ↓↓, below ambient. [7]For a comprehensive review of physical interaction mechanisms please see McCook et al. 2001.

averaging 12 – 13% at the eastern and western ends of the island and declining to ~0% at sites near the center of the island where human influence is greatest (figure 5.5*a*, (42)). Herbivore biomass was also lowest at sites nearest the center of the island (13.5

– 22.6 g m^{-2}) versus the eastern and western points (27.1 – 47.3 g m^{-2}; Carmabi, unpublished data). The number of coral-algal interactions did not correlate with changing percent cover for either CCA or turf algae (figure S5*a,b*). Coral cover, however, was higher at sites where a larger percentage of the coral edge interacted with CCA (p=0.026, figure 5.5*b*), but was not correlated with the percentage of coral edges interacting with turf algae (figure S5*c*).

Discussion

Reef to colony-scale responses to algal interactions: Every coral colony observed in this study was interacting with at least one alga and the frequency of interactions was unrelated to the local percentage of benthic coral or algal cover. The most common coral-algal interactions observed were between corals and turf algae. These interactions were found to negatively affect the physiology of the coral holobiont by eliminating net oxygen production along the interface (figure 5.2; (17)). While algal removal and coral recovery occurred in situ, DO measurements were taken in an aquarium. This eliminated the effects of local hydrodynamics, permitting measurement of the net flux of oxygen at the interaction zone. The two species of macroalgae examined here also caused DO levels to decrease below ambient, but the magnitudes of their effects differed. Turf algae and many macroalgae have been shown to limit coral growth and negatively impact the bordering coral tissue (17,18,23,24,26), lower coral fecundity (27), and inhibit larval settlement (45–47), thereby impacting corals on multiple scales in time and space (table 5.1). Given the

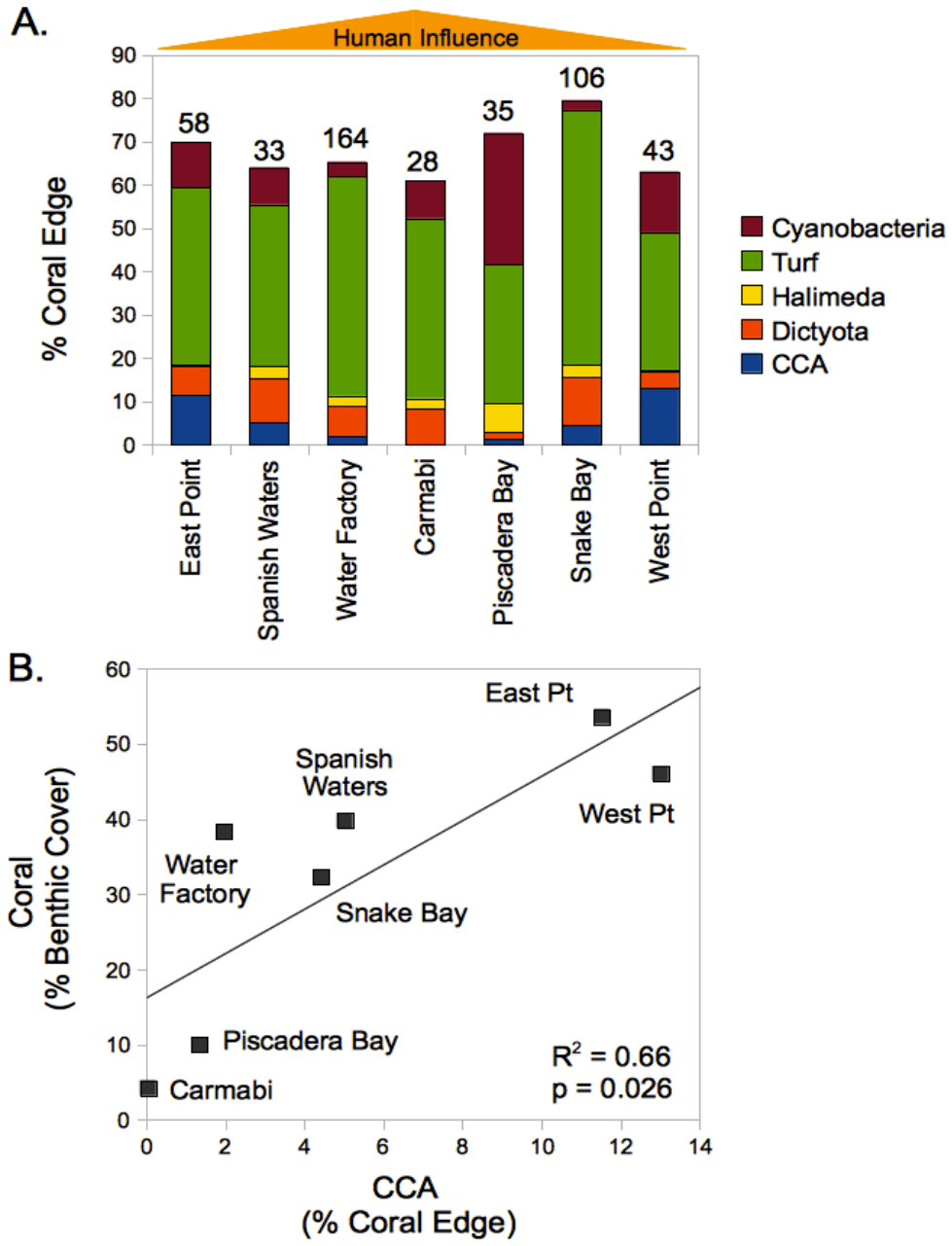

Figure 5.5. Abundance of coral-algal interactions across a range of human impact and coral cover. (*a*) The average percent of coral colony edge interacting with the indicated type of algae at the seven surveyed sites east to west across Curacao; the remainder of the perimeter not interacting with algae included interactions with sand, sponges, and other corals. Numbers above bars indicate the number of coral colonies observed at each site. (*b*) Relationship between benthic coral coverage and the average percent of each colony interacting with CCA at each site.

greater abundance of coral interactions with turf algae relative to other functional groups of algae around the world (figure 5.5; (17,18)), coral-turf interactions are likely important in influencing the structure of benthic coral reef communities.

In contrast to the algae discussed above, interactions with CCA did not exhibit hypoxia (figure 5.2, (17)). Since CCA appear to cause little stress to coral adults and can also benefit corals by preventing colonization of the coral border by other algae (33), we hypothesize that corals interacting with CCA are more successful on the reef. While some species of CCA can harm corals (48), our hypothesis is supported by the observation that the proportion of an individual coral colony edge interacting with CCA at a given site, regardless of CCA species, correlated positively with benthic coral cover (figure 5.5). Previous studies have also demonstrated that CCA are generally less detrimental to the health, growth, and photosynthetic efficiency of adjacent coral tissue than turf algae (17,26). Since some species of CCA also promote coral settlement (30,31), their influence on corals is counter to that of turf algae examined here across multiple spatial scales.

Micro-scale interactions between corals and algae: The coral holobiont is a selective environment for bacteria, as evidenced by the variety of stressors residents must counteract: host antibiotics (49,50), bacteria-bacteria antagonism (49,51), and dimethylsulfide (DMS), dimethylsulfoniopropionate (DMSP) (52,53) and free radicals (54) released by the zooxanthellae. We hypothesize that the holobiont becomes compromised when stressed by competition with certain algae, allowing microbes to

invade that do not possess the suite of metabolisms necessary to survive the normal holobiont landscape and that disproportionately capitalize on DOC released by the algae. This is the first study to identify the types of bacteria present along coral-algal interactions, and we find that bacterial stress response pathways were reduced at coral interfaces with CCA, *D. bartayresiana*, and turf algae (figure 5.4). Type III and IV secretion pathways, hallmarks of pathogenesis but important for some symbiotic interactions (55,56), were also lower at these three interface types, potentially indicating a breakdown of symbiosis. Carbohydrate metabolisms were enriched along these same three interfaces (figure 5.4, table 5.1) and bacterial communities at all coral-algal interfaces showed changes similar to DOC stressed corals (figure S4), together suggesting that bacteria present at some coral-algal interfaces may be consuming carbohydrates released from the neighboring algae (57).

Despite the above similarities, the different types of algae examined here have characteristic impacts on the bacterial component of the neighboring coral holobiont. CCA presence did not affect holobiont physiology, but did alter the holobiont composition, while *H. opuntia* had little effect on holobiont composition despite its impact on physiology (DO). Turf algae, on the other hand, affected holobiont physiology and had the most distinct influence on its bacterial community. The coral-turf interface was the only one to show increased bacterial virulence pathways (table 5.1), suggesting that coral-bacterial symbiosis may be breaking down further here and shifting toward a more pathogenic state compared to the other coral-algal interfaces. Additional support for this is evident in the decrease in organic sulfur assimilation at

the coral-turf interface. Organic sulfur compounds, particularly DMS and DMSP, are important for structuring coral-associated bacterial communities (52,53), and loss of this bacterial metabolism at coral-turf interfaces suggests that turf algae may have facilitated invasion of the holobiont by bacteria lacking these pathways. Further investigations are needed to determine the direct effects of these interface-associated microbial communities on coral health. Recent studies have shown that different species of algae alter the growth of coral bacteria (58), supporting the hypothesis that algae may directly alter the structure of the coral holobiont.

Coral-algal interaction mechanisms: This is the first time that algae have been shown to cause lower oxygen levels on corals in naturally occurring interactions; however, the mechanism remains in question. Loss of zooxanthellae due to shading and possibly allelopathy is the main cause of hypoxia at coral-*H. opuntia* interaction zones, since coral tissue was bleached but showed little change in the bacterial community. Alternative mechanisms are likely causing hypoxia at coral-*D. bartayresiana* and coral-turf interaction zones since these algae cause little to no shading. Possibilities include algal photosynthates (i.e., dissolved organic carbon [DOC]) that stimulate microbial respiration and pathogen invasion (38,40,41), algal allelochemicals that inhibit photosynthesis by the zooxanthellae and cause bleaching at the site of contact (36), direct physical damage, or some combination of these (table 5.1). Physical effects such as abrasion are often minimal compared to the effects of live algae (36,59), and while lipid-soluble extracts (i.e. allelochemicals) from some

algae have been shown to damage corals, these compounds are highly specific to the algal species and require direct contact for effect (36). In contrast, DOC is a water-soluble product of photosynthesis that is potentially released by many algae (57,60) and does not require contact to affect the coral holobiont. Various forms of DOC released by algae have been shown to kill corals and increase microbial growth rates (40,41), while some algae cause coral death and hypoxia which is mediated by microbes (38). Coral exposure to DOC also induces coral-associated viruses (61) and increases the proportion of pathogens on corals (39), and algae that release more DOC likely show a stronger effect (39). Since this study demonstrates similar patterns in oxygen levels and microbial composition on corals at some in situ coral-algal interaction zones, DOC is a likely candidate stimulating these changes. The significance of DOC in these interactions does not preclude the action of other mechanisms (e.g. allelochemistry). Indirect interactions within this complex system may also play an as yet unknown but important role (62), such as microinvertebrates associated with the algae that can draw down local oxygen levels or herbivory which may affect algal morphology (63).

Ecological implications: Micro-scale interactions between benthic algae and the coral holobiont have far-reaching implications for the composition of the reef. We propose a model whereby some fleshy algae (e.g. turf algae and fleshy macroalgae) act at the micro scale to stress corals, leading to macro-scale changes in the ecology of the reef (figure 5.5, table 5.1). On reefs approaching a phase-shift from the coral-

dominated to the algae-dominated state, the impacts of fleshy algae on the coral holobiont are worsened by increased fleshy algal cover and more abundant interactions with corals (64). These negative impacts span the range from micro-scale changes in microbial communities and oxygen drawdown to coral colony-scale effects such as damage to adjacent polyps and lowered fecundity of the adjacent coral colony, likely leading to reef-scale effects on coral abundance and distribution (table 5.1). Conversely, on healthy coral reefs where CCA and calcified macroalgae (*Halimeda* spp.) are more abundant, coral-algal interactions have less impact on the holobiont composition and physiology. CCA, in particular, promote coral proliferation through interactions at micro, colony, and reef scales (figure 5.5, table 5.1).

Various disturbances on the reef (herbivore removal via overfishing, eutrophication, elevated sea surface temperature, etc.) undoubtedly influence these micro-scale interactions, affecting benthic composition at the reef scale. One prominent factor likely affecting the distribution of the different types of coral-algal interactions is herbivory. Many herbivores preferentially feed on turf algae, lowering algal biomass (26,65,66). If highly grazed (i.e., short, low density) patches of turf algae are less detrimental to corals than less grazed (i.e., tall, dense) stands, then it is possible that herbivores attenuate the micro- and macro-scale effects of turf algae on corals. High herbivory also preferentially removes algae that compete with CCA (65), thus increasing the proportion of the benthos occupied by CCA, which can in turn lower recruitment of macroalgae to the reef (33). Environmental disturbances, by affecting the micro- and colony-scale interactions occurring between certain types of

algae and the coral holobiont, should manifest at the reef scale by influencing the distribution and outcomes of these interactions and ultimately the composition of the reef benthos.

Acknowledgements: We thank CARMABI in Curacao for use of their facilities, and Dana Willner, Matthew Haynes, Alejandro Reyes and Bahador Nosrat for technical and bioinformatics support. We also thank Andreas Haas for sharing data on algal DOC release.

Funding: Funding for this work was provided by the National Science Foundation Grant OCE-0927415 to FLR. KLB was supported by a National Science Foundation Graduate Research Fellowship.

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Acknowledgements

Chapter 5, in full, is a reprint of the material as it appears in Proceedings of the Royal Society B. Katie L. Barott, Beltran Rodriguez-Mueller, Merry Youle, Kristen L. Marhaver, Mark J. A. Vermeij, Jennifer E. Smith, and Forest L. Rohwer; 2012. The dissertation author was the primary investigator and author of this paper.

Appendix

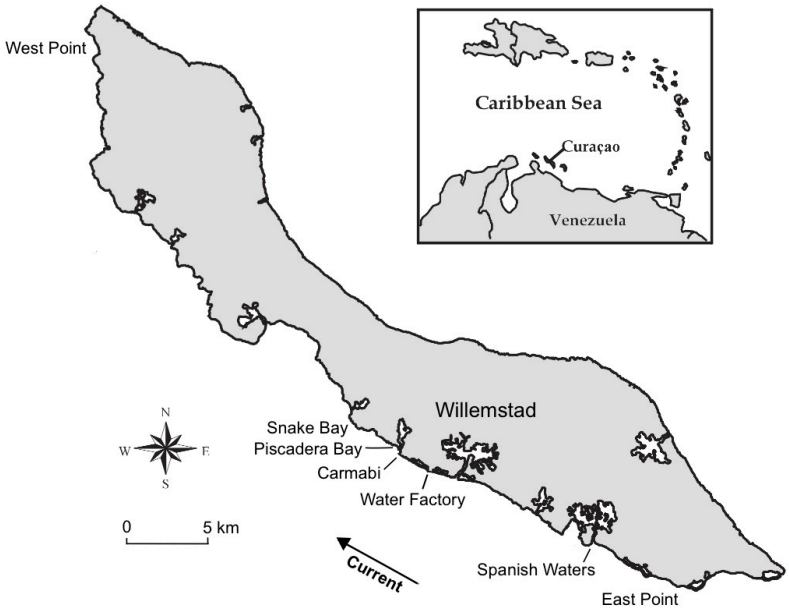

Figure S1. Map of survey and sample sites on Curacao. Surveys were conducted at all sites. Coral samples for physiological and microbiological analyses were collected from the Water Factory site. Willemstad is an industrial city and population center.

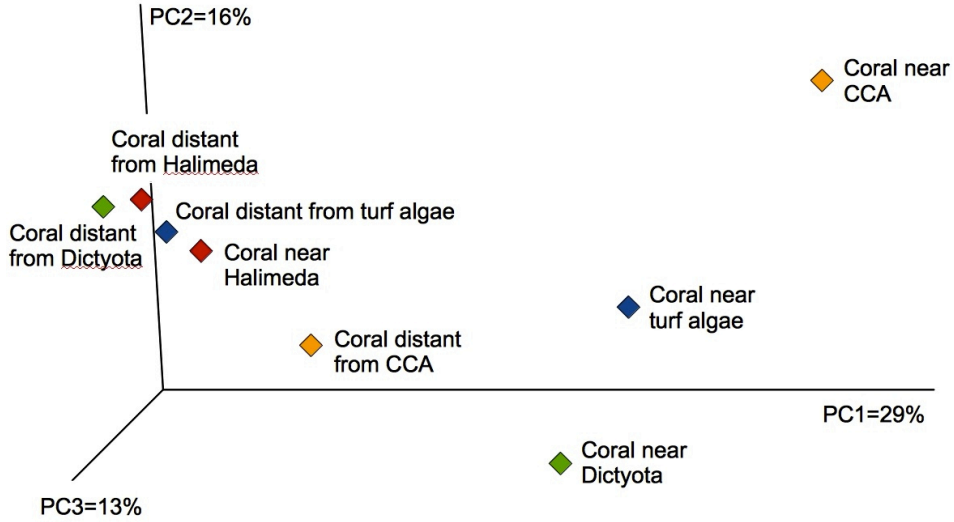

Figure S2. Principal component analysis of the phylogenetic distance (determined by unweighted UniFrac) between bacterial communities associated with *M. annularis*. Data shown includes coral distant from and near/at interfaces with four types of algae: CCA (crustose coralline algae), Dictyota (*Dictyota bartayresiana*), Halimeda (*Halimeda opuntia*), and turf algae.

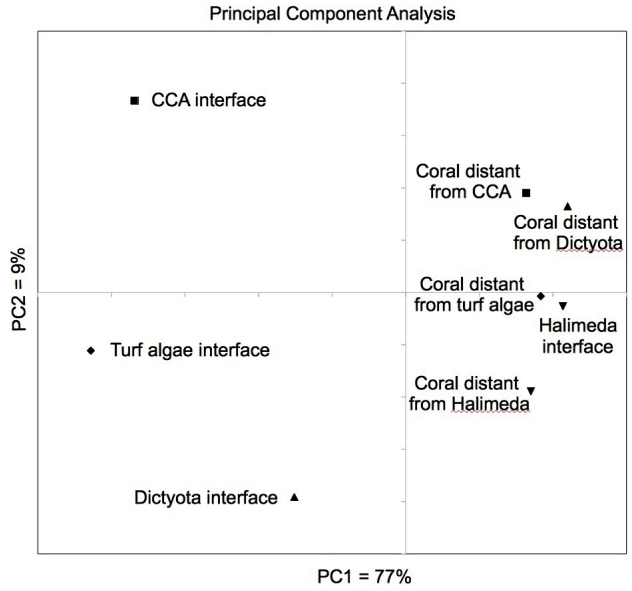

Figure S3. Principal component analysis of metabolic subsystems of coral-associated bacterial communities. Data shown includes the coral-associated bacterial communities and the Bacteria over-represented near/at interfaces with four types of algae: CCA (crustose coralline algae), Dictyota (*Dictyota bartayresiana*), Halimeda (*Halimeda opuntia*), and turf algae. Metabolic subsystems explain 86% of the variation between communities.

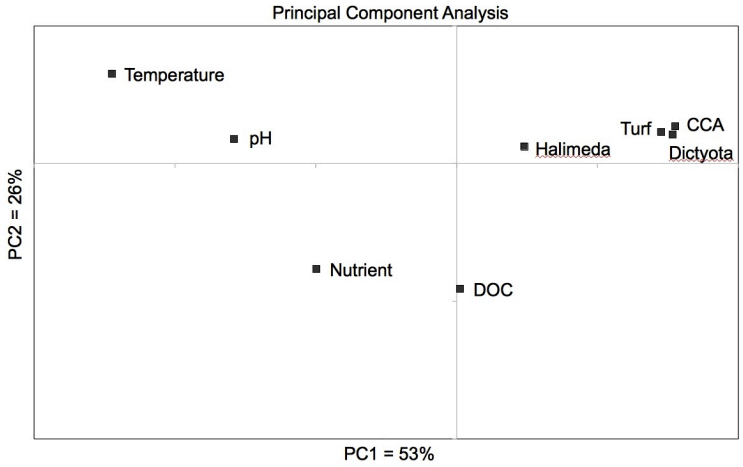

Figure S4. Principal component analysis of the effects of coral stress treatments and algal interactions on metabolic subsystems of coral-associated bacterial communities. For stress treatments, analysis used the fold change between each treatment (elevated temperature, lowered pH, elevated nutrients, or elevated dissolved organic carbon [DOC]) versus untreated coral. For algal interactions, the analysis represents the fold change of the over-represented interface communities versus that associated with coral tissue away from the interface. Axes are weighted; total variance explained is 79%.

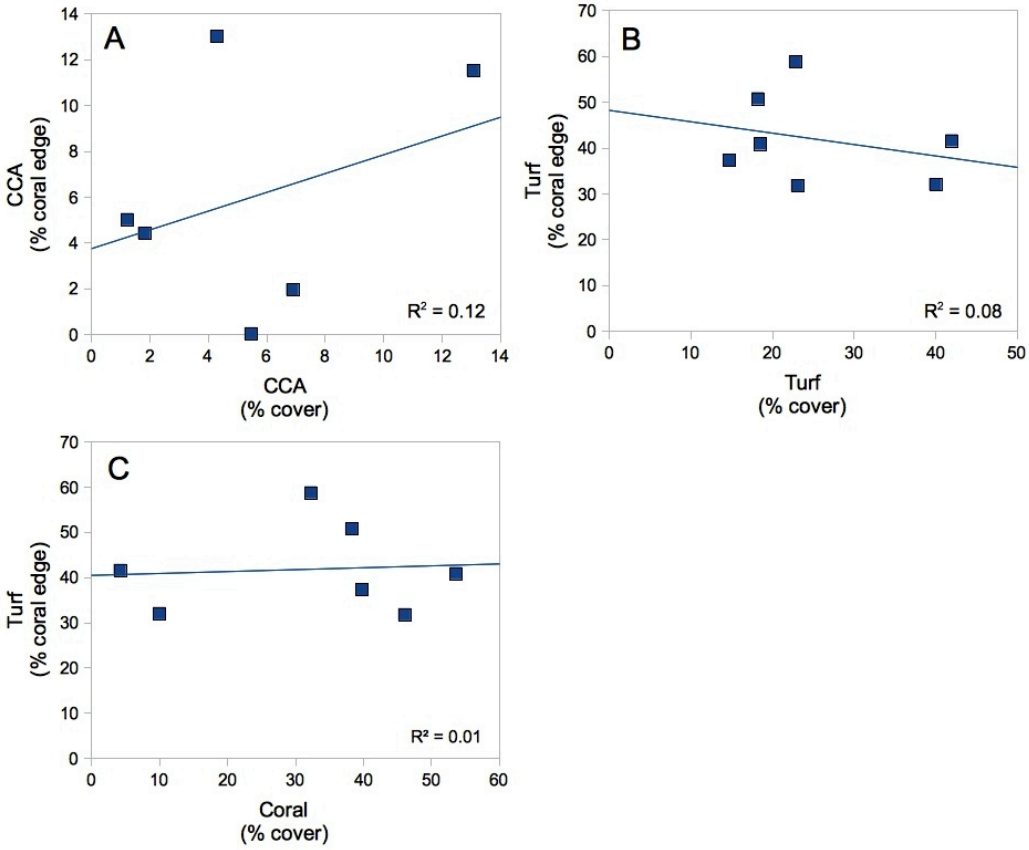

Figure S5. Relationships between benthic cover and coral-algal interactions. (*a*) percent of coral edge occupied by CCA versus CCA benthic percent cover; (*b*) percent of coral edge occupied by turf algae versus turf algae benthic percent cover; and (*c*) percent of coral edge occupied by turf algae versus coral benthic percent cover.

Table S1. Summary of taxonomic data for coral-associated bacteria 16S rDNA libraries from coral-algal interactions. OTU, operational taxonomic units defined at 97% similarity; H', Shannon-Weiner Diversity; CCA, crustose coralline algae; Dictyota, *Dictyota bartayresiana;* Halimeda, *Halimeda opuntia*.

Algal Inter-action	Zone	Bar-code	Avg. read length	Bacterial sequences	No. of OTUs	Chao1	Diversity (H')	Publication
CCA	Coral	3	308	95,368	211	344	3.26	Barott et al. 2011
	Coral near	4	309	80,224	516	939	4.72	This study
	Inter-face	5	311	70,085	751	1186	6.58	This study
	Alga near	4	438	39,677	644	952	6.22	Barott et al. 2011
	Alga	5	439	36,559	759	1232	6.36	Barott et al. 2011
Dictyota	Coral	10	439	33,244	164	266	2.84	Barott et al. 2011
	Coral near	6	305	85,547	267	476	3.28	This study
	Inter-face	11	310	59,581	807	1407	5.70	This study
	Alga near	7	319	9,503	1076	2375	7.12	Barott et al. 2011
	Alga	8	318	16,279	1250	3300	7.60	Barott et al. 2011
Halimeda	Coral	6	436	56,285	259	346	4.51	Barott et al. 2011
	Coral near	11	437	44,762	245	387	4.38	This study
	Inter-face	7	437	50,341	367	609	4.58	This study
	Alga near	8	439	52,117	1308	2119	7.59	Barott et al. 2011
	Alga	9	438	44,807	1289	2167	7.82	Barott et al. 2011
Turf Algae	Coral	1	438	63,244	222	329	3.92	Barott et al. 2011
	Coral near	1	304	104,364	298	512	3.55	This study
	Inter-face	2	441	46,944	1071	1756	7.64	This study
	Alga near	3	438	34,039	1133	1725	7.64	Barott et al. 2011
	Alga	2	313	60,969	1125	1961	6.91	Barott et al. 2011

Table S2. Barcode and primer sequences used for multiplex tag sequencing.

Barcode #	Barcode sequence	Primer sequence (5'-PrimerA-Barcode-534R-3')
1	AACCAACC	GCCTCCCTCGCGCCATCAGAACCAACCCAATTACCGC GGCTGCTGG
2	AACCATCG	GCCTCCCTCGCGCCATCAGAACCATCGCAATTACCGC GGCTGCTGG
3	AACCATGC	GCCTCCCTCGCGCCATCAGAACCATGCCAATTACCGC GGCTGCTGG
4	AACCTACG	GCCTCCCTCGCGCCATCAGAACCTACGCAATTACCGC GGCTGCTGG
5	AACCTAGC	GCCTCCCTCGCGCCATCAGAACCTAGCCAATTACCGC GGCTGCTGG
6	AACCTTCC	GCCTCCCTCGCGCCATCAGAACCTTCCCAATTACCGC GGCTGCTGG
7	AACGAACG	GCCTCCCTCGCGCCATCAGAACGAACGCAATTACCGC GGCTGCTGG
8	AACGAAGC	GCCTCCCTCGCGCCATCAGAACGAAGCCAATTACCGC GGCTGCTGG
9	AACGATCC	GCCTCCCTCGCGCCATCAGAACGATCCCAATTACCGC GGCTGCTGG
10	AACGATGG	GCCTCCCTCGCGCCATCAGAACGATGGCAATTACCGC GGCTGCTGG
11	AACCTTGG	GCCTCCCTCGCGCCATCAGAACCTTGGCAATTACCGC GGCTGCTGG

Supplementary Methods

Surveys of coral-algal interactions: All surveys were conducted at 10 m deep along a 10 m transect line, and at least two surveys were conducted per site. For each coral colony intercepting the transect line, the proportion of the colony's edge involved in an algal interaction was recorded, the coral was identified to the species level, and the alga was identified to species, genus or functional group for CCA, turf algae, and cyanobacteria. Percent cover of benthic organisms was determined from three transects per site at 10 m depth. Twenty photoquadrats of 0.5 m^2 were taken per transect. A simple linear regression model was used to compare benthic cover with algal interaction abundance. Significance was determined using the R statistical software.

Oxygen microprobe measurements: Coral colonies were transported to the lab within 20 minutes of collection and maintained in flow-through aquaria. Physiological measurements were taken within 1 - 24 h of removal from the reef. All measurements were conducted under a dissecting microscope with the aid of a micromanipulater (UniSense, Denmark). The oxygen probe (OX50; UniSense, Denmark) was calibrated using an anoxic solution of 0.1 M sodium hydroxide and 0.1 M sodium ascorbate as the 0% DO reference point and aerated seawater as the atmospheric DO saturation reference. Readings were recorded using the Unisense SensorTrace BASIC (version 3.0.2) software. All DO measurements were taken during the day and at least 1 hour before sunset. Afterwards, the coral colonies were returned to their original location on the reef. A paired t-test was used to determine if DO levels were lower at the interaction zone compared to the center of the coral colony. A Student's t-test was used to determine if the DO levels at intact interaction zones were significantly different from interaction zones where algae had been removal.

Tissue collection and DNA extraction: Tissue samples were placed in individual sterile whirlpacks underwater, returned to the lab in 30–60 min, then submerged in a solution of 25 mM sodium citrate, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ammonium sulfate to preserve nucleic acids, and stored at –20 °C. Coral tissue was later removed from the skeleton using an airbrush with 0.2 mm filter-sterilized TE buffer. An aliquot of the tissue slurry (500 ml) was centrifuged

for 20 min at 14,000 x g and resuspended in lysis buffer (50 mM Tris HCl, 40 mM EDTA, 0.75 M sucrose). A lysozyme digestion (5 mg/ml) was performed for 30 min at 37 °C, followed by a second cell lysis with protinase K (0.5 mg/ml) and SDS (1%) at 55 °C overnight. Following lysis the sample was incubated at 70 °C for 10 min to inactivate the enzyme, and DNA was precipitated by the addition of sodium acetate (0.3 M final concentration) and an equal volume of isopropanol. This mixture was then incubated at –20 °C for 4–5 hr and the DNA was pelleted by centrifugation at 14,000 x g for 20 min at 4 °C. The pellet was resuspended in TE and a CTAB extraction was then performed (1% SDS, 0.7 M NaCl, 0.27 mM CTAB). The sample was incubated at 65 °C for 10 min, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated by adding 0.7 volumes isopropanol and incubating at -20 °C overnight, then pelleted by centrifugation at 14,000 x g for 15 min at 4 °C. The pellet was washed with cold 70% ethanol, dried, and resuspended in 10 mM Tris (pH 8). Algal tissue was homogenized with an epi-mortar. An aliquot of homogenate (250 ml) was used for DNA extraction with the MoBio UltraClean Soil Kit (Solana Beach, CA) according to the manufacturer's instructions. DNA was stored at –20°C until processing.

PCR and sequencing preparation: A 534 base pair (bp) region of the 16S rRNA gene (16S rDNA) including variable regions 1–4 was selected for tag pyrosequencing. This region was amplified using the bacterial forward primer 27F, which also included the primer B adaptor for pyrosequencing on the 5' end (5'-

GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3'). The bacterial reverse primer 534R was also used, and included the sequencing primer A and a unique 8 bp barcode on the 5' end (5'-GCCTCCCTCGCGCCATCAGNNNNNNNNCAATTACCGCGGCTGCTGG-3'). Barcodes (Table S1) were error-correcting Hamming sequences (1). PCR amplifications were carried out under the following conditions: 94 °C for 5 min; 29 cycles of 94 °C for 1 min, 60 °C touchdown (- 0.5 °C/cycle) for 1 min, and 72 °C for 1 min; followed by 72 °C for 10 min. Each DNA extract was amplified by four replicate PCR reactions, which were then pooled. Replicate samples from each interaction zone were amplified using primers with the same identifying barcode. PCR products were purified using the Bioneer PCR Cleanup Kit (Alameda, CA). The amount of DNA in each sample was then quantified using the Quant-iT PicoGreen Assay (Invitrogen). Replicate samples from each zone (each with the same identifying barcode) were then pooled in equimolar amounts and then the different zone pools (each with a unique barcode) were pooled together in equimolar amounts. Amplicons were sequenced using the 454 Titanium platform at Engencore (University of South Carolina).

Sequence analysis: Barcode and primer sequences were removed and sequences were denoised using Pyronoise (2). Diversity was analyzed using the QIIME pipeline version 1.1.0 (3). Sequences were grouped into using UCLUST (4) with a 97% identity threshold; taxonomic identity for each OTU was determined by the RDP taxonomic classifier at 80% confidence (5). Chloroplast sequences were

identified and removed as described previously (6). To eliminate bias due to differences in sequencing effort (i.e., the number of sequences in each library), libraries were rarefied (step size, 90 sequences; 9,500 sequences total, repeated 10 times (6)). Once the libraries were rarified, the following alpha-diversity metrics were determined: number of OTUs, estimated number of OTUs (Chao1), and Shannon-Weiner diversity (H'). Beta-diversity was analyzed by the UniFrac distance metric.

In order to identify the taxa that were over- or under-represented in the five libraries from across each type of interaction a rank comparison was performed. For this, 2000 sequences were sampled from each library with replacement. Each sequence was classified by taxon (e.g., genus) and the relative abundance of the taxa in that library was determined. For each taxon, the five libraries were then compared and ranked 1st through 5th based on the abundance of that taxon in each. This sampling and ranking process was repeated 500 times to identify libraries that were consistently at the top or the bottom of the rank list for a given taxon (confidence level > 70%). These taxa over-represented in coral tissue near or at each algal interface and bacteria associated with coral tissue away from algal interactions were further examined by reconstructing the potential metabolisms of these bacterial communities. Metabolic reconstructions were performed using the 10 most abundant taxa for the coral libraries, while all over-represented taxa were included for each type of interface. For taxa that could only be identified down to the order or family level, multiple representatives within that family were used to estimate the average metabolism for that taxonomic group.

Supplementary References

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CHAPTER 6: Conclusions

Competition between benthic organisms is an important driver of coral reef ecology. As demonstrated in Chapter 2, the composition and outcome of coral-algae interactions varies significantly between reefs even within one island. Surveys of benthic interactions have the potential to provide useful insights to the health of the reef, and can easily be expanded to include all types of benthic organisms (sponges, soft corals, etc.), giving the observer information on the most relevant types of interactions present in their particular reef habitat. In Chapters 3 – 5, microbial dynamics are clearly demonstrated to influence interactions between macro-organisms on coral reefs, yet there are many outstanding questions remaining as we attempt to identify the exact mechanisms involved and the ecological role of these dynamics on reefs around the world. This final chapter outlines some of the present challenges that remain for studying this system, and proposes approaches to address them.

Challenges of studying coral reef competition

Coral reefs are incredibly diverse. The sheer number of possible species combinations between corals and algae on one reef, compounded by the differences between reefs around the world, presents a significant challenge to characterizing coral-algae competitive dynamics. Individual coral species respond differently to different species of algae, and different coral species can have distinct responses to a single species of alga (1,2). Further complexity arises when considering environmental

conditions (depth, flow, nutrients, etc.), which may influence the outcome of each different pair of species. For corals, the variation in response to different types of algae is likely a result of differences in coral morphologies, leading to different physical interactions (e.g. shading and abrasion, boundary layer thickness), the ability of the individual to defend against or attack algae (e.g. mesentery filaments (2,3) and development of sweeper tentacles (4)), the immunological state of the coral at a given point in time (stressed vs. not), the energetic resources of the colony, and variations in coral tolerances to algal attack due to unidentified genetic variation. From the algal side, differences in competitive ability may be the result of differences in the types and amount of dissolved organic matter (DOM) and/or allelochemicals released (5,6). Both of these factors vary by species (5) and most likely vary with environmental conditions (e.g. light and nutrient availability).

The size of each competitor is another factor influencing competition. Small coral colonies, for example, are highly susceptible to mortality (7,8). They have less energetic resources for defense and repair than large colonies, particularly since most algal interactions occur along the colony perimeter, which is relatively large compared to the area of a small colony. Large colonies, on the other hand, have the advantage of small perimeter to area ratios and the ability to avoid algal contact and shading altogether by growing up above the benthos, whereas small colonies are stuck close to the substrate (Chapter 2, (7,9)). Similar dynamics likely apply to algae. A large alga may be a stronger competitor against a coral colony than a small heavily grazed alga, possibly by releasing more DOM and/or allelochemicals and creating more physical

contact. However, while ecological data suggests that size affects coral-algae competition (Chapter 2), this has not been tested experimentally or investigated beyond the Central Pacific.

Conditions in situ alter micro-scale dynamics and impede measurements. Factors including flow and light influence the release of DOM and allelochemicals while changing their transport rate to competitors, thus either enhancing or diminishing the effects of these compounds on competing holobionts. These factors can be highly variable, and make it technically challenging to study small-scale dynamics along coral-algal interactions *in situ*. For example, measuring micro-scale oxygen dynamics on the reef has proven impossible on reefs with much water motion since the available micro-sensors (~50 micron diameter glass electrodes) are extremely fragile. Even in the lab they require the use of micromanipulators, and on the reef they would need to be secured to the benthos for data acquisition, yet remain portable enough so more than one interaction could be measured on a dive. Another challenge is the technical inability to measure microbial dynamics, such as growth and respiration, *in situ* and at the micro-scale. Common methods for quantifying microbial activity involve the use of radioisotopes (e.g. incorporation of tritiated leucine or thymidine (10,11)), which are restricted from use on coral reefs by many governments. Furthermore, the surface- and boundary layer-associated nature of competitive interaction borders make these methods, even if they were available for use in the

field, inadequate. Future research will require new tools to directly investigate microbial dynamics along coral-algal interactions *in situ*.

Benthic dynamics vary over time. Coral and algal benthic cover do not predict the types of coral-algae interactions that are occurring on the reef (Chapter 2), nor do they describe the directionality of the reef (coral vs. algae advancing) and therefore the factors influencing these dynamics are difficult to identify. Few studies have comprehensively described the abundance, distribution, or outcomes of natural interactions between corals and algae on a coral reef (12–15), and these have mostly been snapshots in time. We need to characterize the temporal dynamics of benthic interactions in order to confirm if the 'snapshot' nature of current coral-algae competition surveys is a valid approach. Studies that have monitored natural interactions over time have been limited to a small number of interaction pairs (2,16) or do not quantify how much of the coral is involved in the interaction or the phenotype (i.e. where tissue damage is observed) (14). Many algae are seasonal, and corals may be able to recover from intermittent damage due to ephemeral algal blooms (17), so losses to these types of algae may be less significant for the overall health of the corals on the reef. Furthermore, in coral-coral competition, there are often reversals in the competitively dominant colony over months to years (18), and the same may be true for coral-algae competition. Finally, corals may divert more resources to reproduction at certain times of year, and so may yield more to algal competition during those times. Only by studying how coral-algae interactions

progress over time will any of these dynamics become clear. Finally, in addition to quantifying the outcomes of interactions on the reef over time, we need to identify the differences between interactions that are advancing or retreating at the molecular and microbial scale and how these factors change with environmental context (e.g. flow, nutrients, herbivory) in order to understand the mechanisms driving competitive outcomes.

New conceptual approaches are needed.

Recent work by has made strides in understanding the mechanisms of coral-algal interactions at the small scale, although what determines if a reef is overgrown by algae or if corals will recover from a disturbance remains an ongoing question. Unfortunately, coral biologists are often limited to describing symptoms, and it has been difficult to determine the causes of apparent diseases or disentangle the variety of possible factors that are causing the symptoms we observe. The majority of research on coral disease has not been able to identify a single pathogen associated with corals presenting similar symptoms (19), and benthic competition research has not been able to separate the cause and effect relationships between DOM, allelochemicals, and coral mortality. Stress to the coral from allelochemicals might allow for invasion of opportunistic microbes that cause infection and coral tissue necrosis, allowing for algal overgrowth of the coral tissue, while local enrichment of labile DOM may lead to microbial overgrowth of the coral, again leading to opportunistic infections and tissue necrosis. However, some corals are less susceptible to algal-induced mortality.

On the whole we do not know enough about coral biology, immunology, or stress response to address the subtleties that may underlie a microbial infection or toxin response. This is partly due to the problem of shifting baselines (20); most corals reside on reefs where there are people altering the dynamics of the system, and what may appear to be a healthy coral could actually be a coral experiencing a variety of chronic physiological stressors. This makes it much more difficult to identify what is 'normal' for a coral, as well as pinpoint what cellular pathway changes during acute stress are significant since many may already be activated due to chronic stress.

Human disease has been studied in much more detail than coral disease, and may provide us with some useful insights. Bacterial infections are a significant cause of death in many different clinical situations, but while the infection may be the immediate cause of death, it may be the result of a variety of other factors that do not involve an infectious agent. In these cases, if we do manage to treat the infection, it will likely return since the underlying cause was not addressed. Cystic fibrosis (CF) is one such example; here a mutation in one protein leads to misfolding and diminished activity, causing dysfunctional ion transport across the epithelium and thus diminished activity of epithelial cilia and dehydrated mucus that is difficult to expel. Patients with CF eventually die of polymicrobial infections due to colonization by opportunistic bacteria that would normally be cleared from the body by the action of cilia (21). Another example is the human immunodeficiency virus (HIV). In this case, the virus does not directly cause death, but by damaging the immune system, indirectly leads to secondary infections that are ultimately the cause of death (22). In both of these cases,

the immediate cause of death (microbial infection) would never lead one to discover the true nature of the disease. In coral biology, there are likely a similar variety of direct and indirect killers of corals, and we must keep these in mind when trying to determine the causes underlying coral mortality. Instead of searching for specific pathogens, it may be more useful to look for other factors that may cause an individual coral to become more susceptible to opportunistic or polymicrobial infections.

Potential tools for moving forward.

In order to address the above challenges, there are two complementary approaches that could be undertaken: 1) development of high-throughput techniques to survey a large number of competitive interactions *in situ*, and 2) selection of representative species pairs to investigate competitive mechanisms in detail that may then be compared to high throughput observations, possibly allowing for generalization of some mechanisms to functional groups, genera, growth forms, etc. Representative groups may be used in experiments and manipulations in order to determine how environmental conditions influence benthic interactions and the health of each holobiont. This could allow for predictions of reef resistance and resilience to particular disturbances and restoration efforts. Furthermore, advances in coral biology and the tools we have may increase our ability to understand specific mechanisms that regulate coral responses to stressors from temperature to polymicrobial infections. Below are several new approaches that may prove informative and help address many of the challenges to studying benthic competition dynamics on coral reefs.

Hyperspectral imagery. All photosynthetic organisms have characteristic absorbance spectra due to their light harvesting pigments, and changes in these pigments are often indicative of health. For example, hyperspectral imagery is used in agriculture to measure changes in absorbance of crops at pigment-specific wavelengths, and this information is used to determine the health of the plants, which may determine changes in the management of the field, such as watering, fertilization, or pest treatment (23). Hyperspectral imagery has also been used previously in coral reef ecology (24–28). This technology can be used to remotely identify community composition, and advanced applications could be developed to use this technology to assess the physiological state of photosynthetic organisms as well, much like is done in agriculture. In addition to photosynthetic pigments from their endosymbionts, corals also have a variety of pigments that can be characteristic of a species and indicative of health. For example, fluorescence can be predictive of larval settlement success (29). In adults, changes in pigments are predictive of bleaching and mortality in response to temperature stress (24), suggesting that they may be informative for identifying stressed areas of coral tissue (e.g. along competitive boundaries).

Coral fluorescent proteins (FPs) make up a significant proportion of total cellular protein (>50%), and this energy investment suggests that these proteins play an important role in coral biology. Coral pigments can vary within a species and at different life stages (29,30), yet their function is not well understood. Hypothesized roles include photoprotection, enhancement of photosynthesis, and antioxidant

activity. Green fluorescence measured *in situ* along coral-algal interactions is significantly different depending upon the type of algae. Borders in contact with turf algae have significantly lower GFP than the center of the colony, while borders in contact with CCA have higher GFP fluorescence (Barott, unpublished). While the significance of these changes is not known, it provides further evidence that turf algae and CCA interactions with corals are substantially different and that analysis of FPs may be informative for competition studies. Hyperspectral imagery has been used previously to characterize coral-algal interactions (Chapter 3). Bleaching of coral tissue (loss of chlorophyll signal) can be detected by this method, and has been observed along coral borders with fleshy macroalgae as well as turf algae (Chapter 3). Colonization of the coral surface by outside organisms (e.g. Cyanobacteria) following tissue necrosis due to algal competition has also been observed with this method. Coupled with excitation lights, hyperspectral cameras could be employed to study fluorescence as well as photosynthetic pigments, increasing the information gathered about the health of the organism. This method is also beneficial since it can be deployed underwater (31), and advances in this technology are continuously increasing the speed of image acquisition.

Reef-deployable imaging systems. More rapid, quantifiable, and less subjective assessments of benthic interactions would be extremely useful, facilitating comparisons between sites and data from different observers and allowing for surveys to be repeated more often while covering a greater number of interactions with less

effort and more consistency. One way to accomplish this would be an imaging system that could rapidly image large continuous areas of the benthos at a high resolution. Coupling this with hyperspectral imagery would allow for: 1) species identification, 2) quantification of the number and types of species interactions (including length of each interaction, phenotype along each edge, and percent of the colony perimeter involved in each interaction), 3) photosystem capacity of each organism (e.g. photosynthetic yield), and 4) organism health (pigments and fluorescent proteins), across large swaths of reef.

One example of a system that could be modified to achieve this is ATRIS, which takes high-resolution geo-referenced images underwater and is currently being used for benthic habitat mapping, and organisms in the images are identified manually (32–34). If this system were also equipped with a hyper- or multi-spectral imaging system, designed for the appropriate wavelengths for coral and algal species identification and physiological markers, these images could provide all of the above information. The data obtained from these images would integrate the responses of corals to allelochemicals and microbial stress by measuring the physiology of both interacting partners, in addition to rapidly surveying the abundance and distribution of coral-algae interactions. Traditional ecological metrics, such as benthic cover, would also be obtained from these images. Furthermore, the processing of these images could be automated, making both surveying and data analysis more rapid, which would allow for surveying of larger areas, more sites, and more time points. Generating mosaics of underwater images of coral reefs is already automated and has been used to

monitor large areas of reefs (35–37). This type of large-scale imaging would also provide information of the geometry of coral-algae interactions. Coral morphology, perimeter to area relationships, and other physical parameters could be determined used to address the hypothesis that colony size and shape affect competitive ability. Corals with a long perimeter interacting with algae relative to the area of the coral colony may be worse off than a coral with a large area to perimeter ratio, possibly achieved by growing up above the benthos, giving the coral enough area to draw energy from so that they can successfully defend their basal borders.

Multi-dimensional sensors. There is a pressing need to measure microbial dynamics *in situ* and in multiple dimensions over the surface of the reef. Hypoxia has been observed along many types of coral-algal interactions, but these measurements were taken in aquaria after coral-algal interactions were removed from the reef (Chapters 3 and 5, (1)). Current methodologies make it difficult to avoid this, since the small spatial scales of boundary layers require microprobes that are too fragile to use on corals underwater with the surge and currents present on many reefs. Wangpraseurt et al. 2012 managed to measure oxygen above coral interactions with turf algae and CCA, and while hypoxia was not found at either interface, oxygen flux at the interface was significantly lower than either side (16). Unfortunately, we do not know which direction the interactions were moving of if there was any visible damage to either side at the time the measurements were taken. The difficulty of using microprobes to measure coral boundary layers also limits the number of interactions surveyed of each

type. This method is further limited because it only provides information at one point in space and time, restricting our ability to survey the dynamics along and across heterogeneous interfaces.

New methods are needed to directly and rapidly determine microbial activity and net oxygen dynamics along different coral-algae competition boundaries. One promising option is optodes, which are a tool to visualize the concentration of a selected analyte (e.g. oxygen, glucose, pH). They involve the immobilization of an analyte-specific indicator on a surface, which changes color or luminescence in response to the concentration of the analyte. These indicators can be immobilized on two-dimensional (2D) sheets (38) or beads, which allow for visualization of analyte concentrations in 3 dimensions (3D) (39). A simple point-and-shoot camera with an emission filter attached records the response of the indicator following excitation by the appropriate wavelength of light. Since the response time of optode indicators is typically microseconds, measurements can be taken across a surface or coral-algae interface in the time it takes to place the optode and take a picture. Optodes can also be left in place for time-lapse images, allowing for short temporal dynamics (several hours to days) to be determined without the need for a diver to be present. One drawback of this method is that it may interfere with local flow conditions.

One benefit of optodes is their potential for measuring microbial dynamics *in situ*. Oxygen concentration, while useful as a cumulative measure of respiration and photosynthesis, is not a direct measurement of microbial activity. Low oxygen levels along coral-algae interaction borders could be due to loss of photosynthesis, increased

respiration by the coral animal or the associated microbial community, or any combination of all three. In order to tease apart the role of microbial activity in this melee, we need direct measurements of microbial activity across coral-algae interfaces. One promising tool is biological oxygen demand (BOD) sensors (40–42). These are oxygen optodes coated with microbial cultures immobilized on the surface (40–42), and have been used for both point and 2D measurements in other systems. These sensors measure the microbial respiration response to a solution (e.g. seawater), and on a coral reef we would expect high BOD within the boundary layer above algae that are releasing labile DOM. These sensors, like other optode systems, have the benefit of being deployable both in a laboratory setting and *in situ*, as well as measuring microbial dynamics in multiple dimensions and over time. They are also adaptable, such that different types of microbes can be immobilized on the optode. For example, potential pathogens or non-pathogenic 'healthy' reef microbes could be used to test the hypothesis that different groups of microbes respond differently to algal and coral boundary layer conditions (e.g. turf algae selectively stimulate the growth of pathogenic bacteria). Finally, optodes allow for measurement of many different types of analytes, making them a versatile tool for understanding microbial dynamics at coral-algal interfaces. For example, pH sensitive optodes could be used to determine the redox state of the interface, which could be used along with oxygen and microbial respiration data to estimate relative rates of photosynthesis and respiration from the different components of the interface (microbes versus macrobes).

High-throughput sequencing. Molecular analysis of holobionts is challenging due in part to their diversity. Targeted gene studies, such as the 16S rRNA gene sequencing employed in this dissertation, are useful because they allow us to increase the signal of a desired group (in this case Bacteria), while avoiding contamination from other organisms (like macroalgae and corals). However, this approach is subject to several biases. 'Universal' primers used for amplifying select genes are never comprehensive of all taxa (43), differences between primer sets can confound comparisons between studies, and you will not find anything you do already know is there. Furthermore, while taxonomy may hint at function, it in no way defines it (especially for microbes) (44). Functional metagenomics, on the other hand, avoids the bias of primers and instead involves random sequencing the nucleic acids of the entire holobiont community. Unfortunately, the signal from a bacterial cell or a virus in the context of a holobiont is swamped by that of a multi-cellular eukaryote like a coral. Past functional studies of the microbial component of coral holobionts have used enrichments (e.g. Percoll density gradients) to remove much of the host contamination (45,46), but as in any enrichment some of the desired target is lost along the way while much of the contamination remains. This limits the quantitativeness of the results. Also, when investigating something like a holobiont, it may actually be desirable to know what the host is doing as well as its microbial members. Fortunately, sequencing technologies are advancing rapidly, allowing us to sequence more base pairs from less and less template. The advantage of increased sequencing depth is that one could potentially skip purification and amplification steps, giving data about the host

(dominant signal), while also obtaining enough sequence data from the less abundant microbial members of the holobiont.

Conclusion

The primary goal of this dissertation was to obtain a more detailed mechanistic understanding of how corals live and die. If we are to come up with new strategies to slow the continuing decline of coral reefs around the world, we need to go beyond broad generalized dynamics. Chapter 2 establishes the utility of large-scale surveys of competition on the reef benthos, and future work on this front will benefit from integrating the use of large-scale high-resolution camera systems. These systems will greatly increase the area of reef (and number of interactions) covered in a dive (from 10 m belt transects to 100 m^2 photomosaics or more), while making assessments of holobiont physiology quantitative and more detailed (Chapter 3) than inherently subjective and variable diver observations. Chapters 4 and 5 demonstrate how diverse microbial communities associated with benthic organisms can change in specific ways when two holobionts interact. Future work will benefit from advances in sequencing technologies, which will increase our ability to identify functional changes in microbial and viral communities on the benthos, while also looking at the molecular changes within the host macro-organism. By deploying large-scale ecological surveys in conjunction with molecular surveys, we should be able to link microbial processes with macro-organism dynamics, providing novel insights into how coral reefs function. Through applying this knowledge, it is my hope that we may keep reefs

thriving on this planet for generations to come.

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