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EFFECTS OF NITROGEN-FIXING SYMBIOTIC CYANOBACTERIA ON THE MICROBIAL ECOLOGY OF THE CORAL, MONTASTRAEA CAVERNOSA

BY

JESSICA K JARETT

BS, Southampton College, 2005

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Microbiology

May, 2012

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ACKNOWLEDGEMENTS

This Ph.D. has been the most challenging and rewarding undertaking of my life so far, and I have been very fortunate to have a great number of people who have helped me in this journey. First and foremost, my advisor Michael Lesser. I am grateful to him for his patience, guidance, support, and encouragement over these last five years, and for always being in my corner. Thank you for starting me on this project and trusting me enough to let me take some new approaches. I know we haven't always seen eye to eye but I think we've come to understand each other! I respect him tremendously and I have grown so much as a scientist under his guidance.

I would like to thank my committee members, Marc Slattery, Ruth Gates, Lou Tisa, and Marian Litvaitis for their guidance and expertise. Special thanks to Marc Slattery from the University of Mississippi for help with experiments and collections in the field and our many edifying conversations. Ruth Gates from the University of Hawaii made me think more deeply about symbiosis and many of my underlying assumptions, which has certainly improved my writing and my work. Lou Tisa has brought to the table his truly encyclopedic knowledge of prokaryotic biology and physiology. Marian Litvaitis's expertise in invertebrate biology was invaluable.

Many people at the University of New Hampshire Genome Center have helped me with the bioinformatic aspects of this project. I gleaned a great deal of invaluable information and insight from conversations with Kelley Thomas and Holly Bik. I am very grateful to Holly for helpful advice and assistance with 454 sequencing and data analysis, and for teaching me how to use ARB and QIIME. Many hours were spent in the

computer room at Gregg Hall with Linda van Diepen and Kazu Okamoto, both of whom were always willing to talk over problems and give help. Steve Vollmer at Northeastern performed the differential expression analysis for the metatranscriptomic data, I am looking forward to our continued collaboration with him and his graduate student Stefan Kaluziak.

I am indebted to Anne Cohen and Kathryn Rose at the Woods Hole Oceanographic Institution, who scanned the coral cores and instructed me in the complex process of measuring growth from CT scans. Dan Brazeau at the University of New England performed the AFLP analysis and helped me interpret the results. He also enabled Cara Fiore and I to be trained on the new 454 GS Junior pyrosequencer at UNE, and very graciously opened his home to us while we were there. Ruth Gates and Hollie Putnam kindly provided a curated database of *Symbiodinium* ITS2 sequences that saved me a great deal of time and effort.

I am grateful to Bill Fitt at the University of Georgia for allowing me and my lab mates to work out of his small field station on Key Largo, and Dustin Kemp for his assistance with boating, diving, and coring while we were there. Marc Slattery, Deb Gochfeld, Liz Kintzing, Erica Hunkin, Cole Easson, and Sly Lee were all willing to offer excellent advice and a helping hand when needed, whether it be with diving, boating, experiments in the wet lab, or just generally making things work on a small island. Liz Kintzing deserves a special commendation for tolerating my whining with admirable grace while I was earning my scientific diver certification in cold, cold New Hampshire waters. Thanks also the Aquarius aquanauts, including Cara Fiore, Marc Slattery, and Cole Easson, who collected some of my samples from Florida, and to the topside crew who processed them. I thank the staff and interns at the Caribbean Marine Research Center on Lee Stocking Island, and the Little Cayman Research Centre on Little Cayman Island for logistical support. Miss Emily at LCRC earned my undying gratitude by taking excellent care of me when I was very sick during one field trip there.

I am extremely grateful for the financial support of the National Science Foundation Graduate Research Fellowship Program, a luxury that enabled me to focus full-time on my research for three years. The University of New Hampshire generously provided travel funding, the COLSA summer fellowship, a summer teaching assistant fellowship, and most importantly the Dissertation Year Fellowship that gave me the freedom to write without worrying about other commitments. The UNH Marine Program, the PADI Foundation, and a Lerner-Gray Marine Research Grant from the American Museum of Natural History helped to fund my research.

In the Microbiology program at UNH, I must thank Bob Mooney who was always happy to help with any number of odd and obscure tasks. Dan Merselis and Seana Fraser worked in the Lesser lab as undergraduates and helped with several aspects of this project. It was a pleasure getting to know both of them and I wish them all the best in their future endeavors. Cara Fiore and Nate Olson joined the Lesser lab in my second year and we quickly became fast friends. Nate's meticulousness encouraged me to improve my laboratory technique and I greatly miss my thought-provoking conversations with him. Cara has been my lab mate, dive buddy, field trip comrade, voice of reason, shoulder to cry on, and all around awesome friend. She has helped me out countless times and I don't think I would have made it this far without her. Cara, I quadrat you.

Special thanks to Elissa Ford and the Ford family, my "foster family" here in New England. Crystal Ellis, Brian Schuster, Christine Chapman, Cintia Felix, and Lauren Petersen are some of the best friends anyone could ask for and helped me stay more or less sane for the past five years. The NHSPCA and all the wonderful staff and volunteers there were my second home on Saturday afternoons, and took great care of my cat Squishy while she was there. My deepest gratitude to all of my family for all their love, patience, support and understanding. Mom, you've encouraged me and cheered for me every step of the way. Every day I am thankful that I get to have you as a mom. I love you and I hope I've made you proud.

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ABSTRACT

EFFECTS OF NITROGEN-FIXING SYMBIOTIC CYANOBACTERIA ON THE MICROBIAL ECOLOGY OF THE CORAL, MONTASTRAEA CAVERNOSA

By
Jessica K. Jarett
University of New Hampshire, May, 2012

Corals form the physical structure of coral reefs, one of the most ecologically and economically important ecosystems in the world. The abundant and broadly distributed Caribbean coral *Montastraea cavernosa* forms a symbiosis with intracellular nitrogen-fixing cyanobacteria in some, but not all colonies, which make up approximately 30% of the population and display a characteristic orange fluorescence. Diverse and functionally important microbial communities of dinoflagellates, bacteria, Archaea, viruses, fungi, and other organisms are also associated with corals and together with the host compose what is termed the coral holobiont. Whether the cyanobacteria are mutualists, commensals, or parasites, and their effects on the coral holobiont, are unknown. The influence of the cyanobacteria on the microbial ecology of *M. cavernosa* and overall holobiont fitness were investigated using sequencing of ribosomal RNA PCR amplicons, metatranscriptomic sequencing of rRNA and mRNA from the holobiont, and experimental tests of various fitness metrics. The cyanobacterial symbionts appear to be diverse, but many are *Pleurocapsa*-like and related to nitrogen-fixing cyanobacterial symbionts of diatoms. Their presence does not affect the taxonomic composition of the diverse coral-associated prokaryotic community, but such communities do exhibit significant geographic differences. Metatranscriptomic analysis of mRNA indicates that coral-associated prokaryotes, including cyanobacteria, are transcriptionally active, although few transcripts related to nitrogen fixation were recovered. There were no significant fitness differences between colonies with and without cyanobacteria for any of the metrics tested, including coral growth, response to thermal stress, and the ability to deter predators and produce cyanobacterial toxins. Genotyping of coral hosts revealed that colonies with and without cyanobacteria form two genetically distinct populations at small spatial scales, providing evidence for selection. The cyanobacteria do not appear to be parasitic to the coral host, but any potential benefits they may convey remain unknown. Analysis of metatranscriptomic data to investigate differences in the functional activity of coral hosts and associated microbial communities is ongoing.

INTRODUCTION

The coral *Montastraea cavernosa* is abundant on Caribbean and Eastern Atlantic coral reefs and has an extremely broad depth range from shallow waters (3 m) to the mesophotic zone (100 m) (Reed, 1985, Lesser, *et al.*, 2010). A high degree of polymorphism exists in skeletal morphology (Goodbody-Gringley, *et al.*, 2012) colony color (Kao, *et al.*, 2007), and polyp behavior (Lasker, 1981), but molecular markers have confirmed that *M. cavernosa* is a single species across its range (Budd, *et al.*, 2012). This coral sometimes forms a symbiosis with nitrogen-fixing cyanobacteria (Lesser, *et al.*, 2004) in addition to the typical dinoflagellate photosymbionts, *Symbioindium* (Freudenthal, 1962). These colonies are distinguished by a high density (10^7 cells cm^{-2}) of intracellular cyanobacteria and a characteristic orange fluorescence. This color is attributed to autofluoresence of phycoerythrin, a cyanobcaterial photosynthetic pigment, although other host associated fluorescent pigments may also contribute to this color (Kao, *et al.*, 2007). Most of the light-harvesting Photosystem II (PSII) units of the cyanobacteria are uncoupled from photochemistry, so the cyanobacteria are presumed to be living heterotrophically, likely utilizing glycerol which is present in high concentrations in coral tissue (Lesser, *et al.*, 2004). The slightly higher respiration rates and significantly lower net photosynthesis rates of colonies with cyanobacteria support this hypothesis (Lesser, *et al.*, 2007).

Orange colonies fix nitrogen during the evening and early morning hours, when oxygen concentrations in the coral tissue are low (Lesser, *et al.*, 2007). Although nitrogen fixation has not been conclusively attributed to the cyanobacteria, these cells do contain nitrogenase (Lesser, *et al.*, 2004), and only orange colonies reduce acetylene in controlled experiments (Lesser, *et al.*, 2007). The principal enzyme involved in nitrogen fixation, nitrogenase, is highly sensitive to oxygen (Tripplett, 2000), so times of low oxygen are likely optimal for nitrogen fixation in this system. The cyanobacteria are thought to respire glycerol in order to provide energy for nitrogen fixation. Orange colonies are most abundant below 15 m, where they make up about a third of the total population. The lower irradiance and photosynthesis rates at depth result in a lower concentration of oxygen and are hypothesized to increase the number of hours in the day when nitrogen fixation is possible, explaining the distribution of these colonies (Lesser, *et al.*, 2007).

Ratios of naturally occurring stable isotopes indicate that the resident *Symbiodinium* populations are incorporating fixed nitrogen (Lesser, *et al.*, 2007). This apparent transfer from the cyanobacteria to the dinoflagellates must be mediated by the coral host, because the two symbiont populations live in different tissue layers (the epidermis and gastrodermis, respectively) and have no direct contact with each other (Lesser, *et al.*, 2004). Coral tissue does not have a significantly depleted $\delta^{15}N$ ratio, so the coral host does not utilize the fixed nitrogen directly. *Symbiodinium* densities are not significantly different in brown and orange colonies, but the DNA content per cell is increased in dinoflagellates from orange colonies (Lesser, *et al.*, 2007). This indicates that more cells in this population are in the DNA synthesis (S), gap (G_2) and mitosis (M) phases of the cell cycle, and thus have a higher rate of division (i.e., a higher growth rate).

Coral reefs are thought to be nitrogen-limited environments, so the input of fixed nitrogen from cyanobacteria seems likely to affect coral holobiont fitness in some way. However, if cyanobacteria confer a significant fitness benefit, it is curious that orange colonies are not more common, particularly at deeper depths where the increased potential for nitrogen fixation could make them even more advantageous. The fact that not all colonies host cyanobacteria implies that they may also impose fitness costs, perhaps under specific environmental conditions; or that not all colonies are equally capable of hosting cyanobacteria. At the same time, the relatively high frequency of orange colonies in the population implies that any deleterious effects on fitness must not be large, or these colonies would not persist.

The presence of nitrogen-fixing cyanobacteria in a coral raises fundamental questions about the role of nitrogen in this system and the effects of an input of fixed nitrogen. The control of symbiotic algae by invertebrate hosts has been proposed to follow a chemostat model (Falkowski, *et al.*, 1993), whereby the density and growth rate of the symbionts is controlled by the concentration of a limiting nutrient and the dilution rate at which cells are removed from the chemostat. *Symbiodinium* have a much higher potential growth rate than their host, and it has been believed that they are prevented from overgrowing their host by nitrogen limitation (Falkowski, *et al.*, 1993). In the *M. cavernosa* system, *Symbiodinium* are supplemented with fixed nitrogen, yet their density remains constant. According to the chemostat model, there must be an increase in the dilution rate to compensate for the extra nutrient addition; increased dilution would also account for the observed increase in *Symbiodinium* growth rates in orange colonies. How the host controls symbiont density is not known, but the lack of an isotopic signal for nitrogen fixation in coral host tissue implies that dinoflagellates are expelled rather than digested.

An internal source of fixed nitrogen could provide several advantages to different organisms associated with the coral, and could also impact overall holobiont fitness. The dinoflagellates could benefit from an increased ability to turn over damaged photosynthetic enzymes, and may be able to translocate more fixed carbon to their host. The increased availability of nitrogen may change the nutritional composition of the coral mucus (Ducklow & Mitchell, 1979, Meikle, *et al.*, 1988), or the ability to produce different antimicrobial compounds that are present in mucus (Ritchie, 2006, Geffen, *et al.*, 2009), which could affect the diverse microbes associated with the coral mucus, tissue, and skeleton. Factors directly related to the presence of cyanobacteria could also affect the organisms associated with the coral colony. The consumption of oxygen by the symbiotic cyanobacteria while respiring glycerol may have an effect on oxygen concentration in coral tissue and mucus. Because coral tissues undergo large diel fluctuations in oxygen concentrations (Dykens & Shick, 1982, Kuhl, *et al.*, 1995), this effect could be more pronounced during certain times of day. Cyanobacteria might also produce bioactive compounds (e.g., microcystin) that could affect the behavior, abundance, and distribution of microbes associated with the coral (Richardson, *et al.*, 2007). Even if only a few community members are directly affected by the cyanobacteria, they may be key species that interact with and affect many other species, possibly resulting in large indirect effects of the cyanobacteria.

In this dissertation, I address outstanding questions about the *M. cavernosa*-cyanobacteria symbiosis from multiple perspectives ranging from the molecular level to the holobiont. I first review what is known about nitrogen-fixing symbioses in the marine environment, to place the *M. cavernosa* system in a broader context. A metagenetic survey of 16S rRNA PCR amplicons provides a taxonomic profile of the coral-associated prokaryotic community, attempts to identify the symbiotic cyanobacteria, and describes the effect of cyanobacteria on other members of the prokaryotic community. Metatranscriptomic analysis of expressed genes in brown and orange colonies explores the functions and activity of the coral host, *Symbioindium*, prokaryotes, and other members of the holobiont. In the final chapter, the effects of cyanobacteria on holobiont fitness are considered, to determine the possible ecological impacts of this symbiosis.

CHAPTER I

NITROGEN-FIXING SYMBIOTIC ASSOCIATIONS IN THE MARINE ENVIRONMENT[1]

Introduction

The ability to convert atmospheric nitrogen into ammonia is restricted to members of the Bacterial and Archaeal domains in a process known as nitrogen fixation. Biological nitrogen fixation (BNF) has a long evolutionary history (Raymond, *et al.*, 2004) and contributes significantly to the amount of "new" nitrogen available to a wide variety of terrestrial, aquatic and marine organisms (Galloway, *et al.*, 1995, Falkowski, 1997). The availability of nutrients, especially nitrogen, influences the trophic biology and ecology of all organisms and ultimately their ecological distribution and abundance. In the marine environment, a ratio of 106:16:1 of carbon:nitrogen:phosphorus has been described for the open ocean planktonic primary producers. Known as the Redfield ratio, this ratio was long believed to reflect the absolute requirements for phytoplankton growth (Redfield, 1934) with nitrogen often cited as a limiting macronutrient. The role of nitrogen as a limiting nutrient in the oceans over ecological timescales has generally been accepted as a paradigm. However, the oceanographic community has recently begun to grapple with the idea of multiple resource co-limitation on primary productivity in the worlds oceans instead of a simple Liebig's Law of the Minimum approach where only a single resource limits productivity (Arrigo, 2005). This is a fundamental shift in thinking about nutrient biogeochemistry in the world's oceans and has initiated many new studies on the physiology of phytoplankton that are essential for making accurate carbon flux calculations (Karl, *et al.*, 2002, Zehr & Ward, 2002, Arrigo, 2005). Recent research has also found that the Redfield ratio more accurately represents a global average of the planktonic community, rather than a specific requirement for the growth of phytoplankton (Klausmeier, *et al.*, 2004). Physical factors such as local oceanography (Li & Hansell, 2008, Church, *et al.*, 2009), exogenous input from nutrient runoff, and aeolian deposition (Fanning, 1989, Dong, *et al.*, 2000) are now known to influence deviations from this ratio which still supports the growth of primary producers. Additionally, biological factors such as the microbial transformation of nitrogen by nitrogen fixation, nitrification and denitrification have recently become more appreciated as processes influencing nutrient stoichiometry (Arrigo, 2005, Ward, *et al.*, 2007).

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[1] An excerpt of this chapter has been published as: Fiore CL, Jarett JK, Olson ND, Lesser MP (2010) Nitrogen fixation and nitrogen transformations in marine symbioses. Trends in Microbiology 18 (10): 455-463

In the marine environment, nitrogen fixation was underestimated by early studies (Capone & Carpenter, 1982), but recent studies estimate fixation rates closer to that of terrestrial environments (90-130 Tg N yr^{-1}) (Galloway, *et al.*, 1995) or higher (Karl, *et al.*, 2002, Quigg, *et al.*, 2003). Several findings have helped to close this gap in nitrogen budgets, beginning with the upward correction of nitrogen fixation estimates for the cosmopolitan genus of free-living marine nitrogen-fixing bacteria *Trichodesmium* spp. (Capone, *et al.*, 1997), followed by the discovery of large numbers of oceanic unicellular cyanobacteria including *Crocosphaera* and *Cyanothece* using molecular approaches (Montoya, *et al.*, 2004, Zehr, *et al.*, 2007). More recently, a unique group of picoplanktonic cyanobacteria called UCYN-A was described and its genome sequenced (Tripp, *et al.*, 2010). The reduced genome of these cells lacks the oxygen-evolving Photosystem II and several key metabolic pathways, and thus they can fix nitrogen during daylight hours. Although they cannot fix carbon, they can generate energy and reducing power from light and are thought to be photoheterotrophs that may depend on other organisms for critical nutrients (Bothe, *et al.*, 2010). The abundance of UCYN-A in colder, deeper waters than either *Trichodesmium* or *Crocosphaera* also expands the geographic range in which oceanic nitrogen fixation is known to occur (Moisander, *et al.*, 2010). These discoveries, and the realization of the importance of marine sources of nitrogen in the global nitrogen budget has highlighted the need for continuing research into the complex cycling of nitrogen in the marine environment (Zehr & Ward, 2002). While nitrogen cycling is also influenced by anthropogenic impacts (Vitousek, *et al.*, 2002) and physical forcing (Zehr & Ward, 2002), microbial transformations of nutrients provide the fundamental underpinning to understand these processes and to improve our knowledge of the dynamics of microbial nitrogen cycling (Ward, *et al.*, 2007). The implications of nutrient transformations by marine microbes on seawater nutrient composition, global nutrient cycling, and plankton population distributions, were recently reviewed, highlighting the complexity and importance of microbes in nitrogen cycling (Arrigo, 2005, Ward, *et al.*, 2007). Many studies have focused on free-living diazotrophs, which constitute a large proportion of nitrogen transformation; however, there are many gaps in our knowledge of which bacteria contribute to nitrogen fixation.

For example, nitrogen fixation by symbiotic bacteria has been described in zooplankton and phytoplankton (Braun, *et al.*, 1999, Zehr, *et al.*, 2000) and non-planktonic organisms (Carpenter & Culliney, 1975, Mohamed, *et al.*, 2008) which could potentially contribute to filling in the unknowns for nitrogen budgets in specific habitats. Newly discovered symbiotic nitrogen fixers now appear to occur frequently, and these symbioses influence host ecology and may have potentially large impacts on global nutrient cycling.

As a result of environmental nitrogen limitation in habitats such as mid-ocean gyres and shallow tropical waters, or nutrient poor food sources such as wood (Carpenter & Culliney, 1975), organisms in these habitats often experience nitrogen limitation. A number of organisms have coevolved into symbiotic relationships with diverse group of prokaryotes that can perform BNF to overcome this limitation. Nitrogen fixation is the transformation of dinitrogen gas (N_2), the most abundant but also biologically unavailable form of nitrogen on the planet, into biologically available ammonia (NH_3). Symbiotic bacteria that can fix nitrogen are not limited to the marine environment and many terrestrial nitrogen-fixing symbioses are often utilized in agronomy for their ability to replenish nutrients in the soil. Due to the important agricultural role nitrogen-fixing plant symbioses play around the world, these provide some of the best-understood models of bacterial symbiosis. The processes of initiation, recognition, infection and biochemical communication between the host plant and nitrogen-fixing bacteria are well described (Garg & Geetanjali, 2007). In freshwater systems, aquatic plants such as *Azolla*, which host heterocystous cyanobacteria as symbionts, as well as free-living cyanobacteria, are major sources of fixed nitrogen and are well-documented participants in nitrogen cycling in aquatic ecosystems (Kalff, 2002). Additionally, a majority of the early work on the physiology and genetics of nitrogen fixation was conducted on culturable free-living terrestrial bacteria in the genera *Klebsiella*, *Azotobacter* and *Clostridium* (Klipp, *et al.*, 2004). Conversely, diversity and physiological ecology of marine diazotrophic symbioses are not as well understood. This review encompasses historical and recent studies on the nitrogen fixation process with a particular focus on marine symbiotic associations. I also address where significant gaps exist in our knowledge of nitrogen fixation in these unique symbioses.

Biological Nitrogen Fixation

Nitrogen fixation is an energetically expensive process in which the two nitrogen atoms of a dinitrogen molecule are each reduced to NH_3. This process requires two enzymes, which form the nitrogenase complex. The Fe protein is a homo-dimer encoded by the *nifH* gene, and has a 4Fe:4S core. The Fe protein is reduced by the electron mediator, ferredoxin, and then reduces the second enzyme involved in the fixation process, the Mo-Fe protein. The Mo-Fe protein is a four-subunit $\alpha_2\beta_2$ protein with $\alpha\beta$ dimers that are coded for by the *nifD* and *nifK* genes respectively, and contains a unique Fe/Mo-cofactor. The Mo-Fe protein reduces the N_2 molecule and is then subsequently reduced by the Fe-protein, completing the cyclic process.

In addition to the *nifHDK* genes, 17 other genes have been discovered in the nitrogenase gene cluster; the function of a number of these still remains unknown. Some of the additional genes of known function encode for protein cofactors or products necessary for the biosynthesis of inorganic cofactors, or proteins involved in electron transport (Dixon, 2004). The rest of the suite of *nif* genes with known functions include those that code for regulators of transcription and translation of the nitrogenase complex, as well as the overall fixation process. The reduction of the dinitrogen molecules is an energetically costly process, requiring 16 ATP molecules and 8 reduced electron carriers per molecule of dinitrogen reduced, necessitating tight regulation of this process and the biosynthesis of associated enzymes (Tripplett, 2000). Expression of nitrogen regulating genes is tightly controlled at the transcriptional and post-transcriptional levels, and varies from species to species, often involving numerous genes on multiple operons (Merrick, 2004). Global regulation of nitrogen metabolism is controlled by nitrogen regulatory proteins (Ntr) such as NtrC, B, L, rpoN (σ^{54}), GlnK and GlnD that interact either directly or indirectly with the *nif* operon. Additionally, both negative (*nifL*) and positive (*nifA*) regulators of transcription are present within the nif operon itself (Klipp, *et al.*, 2004).

Oxygen (O_2) and NH_3 are two main factors influencing the expression of *nif* genes and nitrogen fixation (Tripplett, 2000). O_2 irreversibly inactivates both the Fe and MoFe proteins, by oxidizing the metal-S cores of the proteins, releasing two sulfhydryl groups and one S^{2-} in the case of the Fe protein and S^{2-}, Mo, and Fe from the MoFe protein. In addition to inactivating the metal cofactor, this reduction of O_2 also produces reactive oxygen species including hydrogen peroxide, superoxide radicals, and possibly singlet O_2, which contribute to oxidative damage of the protein and other cellular components. Several electron carriers involved in nitrogen fixation such as ferredoxin can also be oxidized by O_2 and produce superoxide radicals. O_2 down regulates nitrogenase transcription when present, and is sensed by a flavin cofactor (FAD) on the NifL protein (Merrick, 2004). When O_2 levels are high, the NifL protein suppresses NifA activity so that the synthesis of O_2-sensitive nitrogenase is shut down at transcription.

Sufficient concentrations of NH_3 obviate the need for nitrogen fixation. When NH_3 is low, the NtrC protein is activated and promotes transcription of *nif*A, which in turn promotes transcription of the *nif* operon, enabling nitrogen fixation to occur. When NH_3 is in excess and therefore nitrogen fixation is unnecessary, the activity of NtrC and NtrL proteins is inhibited, resulting in inhibition of *nif* transcription. The NifL protein is regulated by *gln*D and *gln*K gene products, while NtrC activity is regulated by NtrB (an enzyme that is both a kinase and phosphatase), which in turn, is regulated by the nitrogen status of the cell (Merrick, 2004). Regulation of the *nif* operon and other operons involved in nitrogen fixation is complex and can vary among species, and research into the genetics of nitrogen fixation has predominantly been conducted in free-living bacteria (Klipp, *et al.*, 2004), but there may be differences in symbiotic bacterial gene expression. To illustrate this point, a recent study on the well-known *Azolla*-cyanobacteria symbiosis, collected the first molecular data on post-translational modification of NifH in a symbiotic system (Ekman, *et al.*, 2008). They found two forms of NifH, a modified form which was likely inactive in the cyanobiont, and an unmodified active form. The concentration of the active form of the protein NifH was over 9 times higher in the cyanobiont than the free-living cyanobacteria, and NifK was also 2.5 times more abundant. This correlated very well with the observation of increased rates of fixation when the bacteria are *in hospite* as compared to free-living (Watanabe, 1982). The mechanism for this post-translational modification appears to differ between symbiotic and free-living systems; the mechanism and the identity of the modification remain to be determined.

Protection of nitrogenase from oxygen

The transcriptional and translational regulation of nitrogenase synthesis described above is important in regulating the process of nitrogen fixation. Many diazotrophs, however, currently live and have evolved in environments where O_2 is present at atmospheric concentrations or under conditions where oxygenic phototrophs, such as cyanobacteria, evolve O_2. Under such conditions nitrogenase can be quickly degraded which decreases, or stops, nitrogen fixation and requires the energetically expensive replacement of the proteins, so these organisms have evolved several strategies for protecting nitrogenase from O_2. Broadly speaking these strategies involve reducing O_2 concentrations in the cell, conformational changes of the nitrogenase protein, or spatial or temporal separation of the fixation process from O_2. In symbioses, the host, the symbiont, or both partners may contribute to the protection of nitrogenase from O_2, often using the same basic mechanisms utilized by free-living diazotrophs or adapting them to life *in hospite*. Additionally, some hosts create protected microaerobic or anaerobic environments for their nitrogen-fixing symbionts, such as root nodules in legumes or the gut in termites (Breznak, 1982, Udvardi & Day, 1997).

Reducing the concentration of O_2 in the cell is a common O_2 protection strategy and can be achieved by a variety of mechanisms. Aerobic respiration both scavenges O_2 and produces ATP and reductant that can be used in nitrogen fixation (Hochman & Burris, 1981). Hydrogenase enzymes are present in all aerobic nitrogen-fixing organisms (Fay, 1992), and oxidize the H_2 produced by nitrogenase in the oxyhydrogen or Knalgas reaction, thereby removing an inhibitor of nitrogen fixation (H_2) (Mortenson, 1978), conserving reducing power, and consuming O_2. Antioxidants such as catalase, peroxidase, and particularly superoxide dismutase play important roles in reducing the concentration of reactive oxygen species (ROS) that are produced during photosynthesis and aerobic respiration, and can inactivate nitrogenase in addition to damaging other cellular components (Fay, 1992).

Nitrogenase can be protected from the damaging effects of O_2 by temporarily "shutting off" and associating with a 2Fe-2S protein (Shetna's protein), (Scherings, *et al.*, 1977, Scherings, *et al.*, 1983). This mechanism has been studied in *Azotobacter* spp.; in this system the 2Fe-2S protein oxidizes the Fe protein (encoded by *nifH*), and an oxygen-tolerant complex containing the oxidized Fe protein, the Mo-Fe protein, and the 2Fe-2S protein is formed. Although this complex has not been directly observed in diazotrophs other than *Azotobacter,* results from other studies imply that a similar mechanism may exist in the cyanobacteria *Oscillatoria* sp. (Stal & Krumbein, 1985) and *Anabaena* sp. (Pienkos, *et al.*, 1983).

Spatial separation of nitrogen fixation from O_2 is accomplished in several ways. Organisms may live in environments such as sediments that are anoxic or microaerobic. Additionally, some filamentous cyanobacteria form specialized cells (heterocysts) for nitrogen fixation, which have thick glycolipid layers that limit the diffusion of O_2 into the cells (Thiel, 2004). Differentiation of heterocysts is a highly complex and tightly regulated process, and only occurs during nitrogen starvation (Wolk, *et al.*, 1994). One of the drawbacks of heterocysts is their high metabolic cost; their maximum frequency in a filament of cells is only about 25%, and it is estimated that about 4-5 vegetative cells are required to support each heterocyst with reductant and carbon skeletons for assimilation of nitrogen (Thiel, 2004). Some symbiotic diazotrophs, such as the cyanobiont of *Azolla,* also form heterocysts (Gebhardt & Nierzwicki-Baauer, 1991). It is presumed that the host provides some metabolic support or regulatory cues, or both, because heterocysts occur with much greater frequency in symbiosis than in the free-living state (Adams, 2000).

Other diazotrophs utilize a temporal separation of nitrogen fixation from oxygenic photosynthesis. In free-living cyanobacteria, nitrogen fixation typically occurs at night in non-specialized, photosynthetically competent cells (Bergman, *et al.*, 1997). For most of the cyanobacteria in which these patterns have been studied the cycles of photosynthesis and nitrogen fixation are endogenous and persist even under constant environmental regimes (Bergman, *et al.*, 1997). Temporal separation was also documented in the symbiotic diazotrophs of a scleractinian coral. Cyanobacterial symbionts of the coral *Montastraea cavernosa* display higher fixation rates in early morning and in the evening, when O_2 concentrations in coral host tissue are lower, and are likely utilizing products of photosynthesis as an energy source (Lesser, *et al.*, 2007). Because of the complexity of the processes of nitrogen fixation and photosynthesis, many possible mechanisms exist for control and separation; in symbiotic lifestyles, this gives both host and symbiont many methods of regulation and cooperation.

A small number of free-living cyanobacterial species use a combination of spatial and temporal separation to protect nitrogenase from O_2. The most notable of these is *Trichodesmium,* a non-heterocystous marine filamentous cyanobacterium that fixes nitrogen during daylight hours in a small number of cells that contain nitrogenase (Bergman & Carpenter, 1991, Janson, *et al.*, 1994). Although nitrogen fixation is localized, these cells differ from heterocysts in that they appear to contain all the necessary photosynthetic machinery and are otherwise undifferentiated. Maximal nitrogen fixation occurs in the middle of the light period, when quantum yields of chlorophyll fluorescence are low and carbon fixation decreases transiently, effectively separating the periods of highest nitrogen fixation and O_2 evolution (Berman-Frank, *et al.*, 2001). Although neither the spatial nor temporal separation in *Trichodesmium* is very efficient independently, in combination they allow nitrogen fixation to take place. The use of both temporal and spatial separation as in *Trichodesmium* presents another mechanism that could be at work in symbiotic nitrogen fixation associations, though it has yet to be reported.

Newly discovered UCYN-A cyanobacteria have eliminated the conflict between photosynthesis and nitrogen fixation by living photoheterotrophically (Bothe, *et al.*, 2010). These diazotrophs do not possess the O2-evolving Photosystem II but are able to generate energy from light through cyclic photophosphorylation, enabling them to have maximal nifH transcript expression during daylight hours. UCYN-A is the only group currently known with this lifestyle, and they are found exclusively in the open ocean; however, other undiscovered diazotrophs in other environments may use the same strategy.

When are nitrogen-fixing symbionts advantageous?

The ability to fix nitrogen, either endogenously or via a symbiont, allows organisms to escape ecological limitations in a variety of habitats. Organisms may be able to expand their spatial niches into low-nutrient environments, or their dietary niches to resources that have low combined nitrogen content, and can thus reduce competition or gain a competitive advantage. Organisms harboring symbiotic diazotrophs in mutualisms with highly evolved mechanisms for the efficient transfer of fixed nitrogen products are potentially more likely to gain these benefits.

Nitrogen-fixing organisms are ubiquitous in aquatic and terrestrial environments with limited inorganic nitrogen resources (Zehr, *et al.*, 2000, Vitousek, *et al.*, 2002). Lichens may be the best terrestrial example; they are common not only in desert soil crusts (Eskew & Ting, 1978, Belnap, 2002), but also on rocks (Seneviratne & Indrasena, 2006) and lava flows, where they help create soil and begin the process of primary ecological succession (Crews, *et al.*, 2001, Kurina & Vitousek, 2001). In all of these environments, both competitors and predators are scarce or absent. Similarly, the associations between higher plants and rhizobia (e.g., in legumes) allow these plants to thrive even in nitrogen-poor soils, a clear advantage over their competitors. Coral reefs are a marine ecosystem where it is advantageous to host nitrogen-fixing symbionts. The growth and high productivity of coral reefs in these oligotrophic waters has been seen as a paradox since Darwin's voyage on the *Beagle.* It is now believed that both predation on plankton (Hamner, 1995, Hamner, 2007), which transforms the nitrogen content of the prey into predator biomass; and nitrogen fixation, coupled with efficient nutrient cycling, on and around reefs contribute significantly to the nitrogen requirements of reefs (Webb, *et al.*, 1975). Diazotrophs are common in the water column (Hewson, *et al.*, 2007), on the substrate (Larkum, *et al.*, 1988, Charpy, *et al.*, 2007), and in associations with invertebrates such as sponges (Mohamed, *et al.*, 2008), corals (Lesser, *et al.*, 2004) and possibly tunicates (Paerl, 1984, Odinstov, 1991). Other classically nitrogen-limited habitats include mid-ocean gyres; areas with low rates of water exchange, such as the Red Sea and the Persian Gulf; and places where there is strong seasonal stratification of the water column. In these habitats, high C:N ratios limit the growth of phytoplankton, with the exception of nitrogen-fixing species, such as *Trichodesmium* spp., which commonly form large blooms (Sellner, 1997), and various diatoms which host nitrogen-fixing symbionts (Foster & Zehr, 2006, Foster, *et al.*, 2009). The abundance of these organisms and the large contribution they make to the nitrogen budget (Carpenter & Romans, 1991, Zehr, *et al.*, 2000) of the ocean clearly illustrate the ecological advantages of nitrogen fixation in an open ocean environment.

Environments that are nitrogen sufficient overall may still contain food sources that are nitrogen-poor and organisms that can subsist on these resources occupy a less competitive dietary niche. Wood-eating termites, which harbor symbiotic diazotrophs in their guts, are a typical terrestrial example (Breznak, 1982). Nitrogen-fixing microbes of various types are present in the guts of many other arthropods as well (Kim, *et al.*, 2001, Lilburn, *et al.*, 2001, Nardi, *et al.*, 2002). Like termites, marine shipworms also consume wood, which has a very high C:N ratio, as a primary food source. They harbor dense cultures of nitrogen-fixing bacteria in a specialized organ called the gland of Deshayes (Waterbury, *et al.*, 1983), which provide the host with fixed nitrogen and allow the shipworm to survive on a wood diet (Luyten, *et al.*, 2006, Lechene, *et al.*, 2007). Diazotrophic symbionts can also allow their hosts to adapt to fluctuating food resources. Herbivorous sea urchins in temperate regions consume kelps and other seaweeds in which nitrogen content varies seasonally, and the diazotrophic bacteria in their guts regulate nitrogen fixation accordingly to supply sufficient nitrogen resources to the urchin year round (Guerinot & Patriquin, 1981).

Marine nitrogen-fixing symbioses

The advantages of hosting diazotrophic symbionts are clear, but specifics such as the degree of integration between host and symbiont physiologies, transmission of symbionts, and significance of nutrient fluxes to hosts and/or symbionts vary from species to species. For some of the more well studied marine symbiotic systems such as shipworms, sea urchins, diatoms and dinoflagellates, as well as reef building corals and sponges some or all of these aspects of the symbiotic system are known.

Shipworms

The wood boring mollusks of the family Teredinidae, commonly known as shipworms, have caused significant destruction of man-made wooden structures placed in the ocean. Referred to as the termites of the sea, these mollusks bore holes in wood and utilize the cellulose as their sole nutrient source (Distel, 2003). Woody plants contain only 0.03% to 0.1% nitrogen, thus these shipworms require an additional source of nitrogen to supplement their diet. Acetylene reduction assays of three species, *Psiloteredo megotar*, *Lyodrus pedicellatus*, and *Teredo navalis*, revealed that the shipworms benefit from microbially fixed nitrogen (Carpenter & Culliney, 1975). For these three species, fixation rates were inversely correlated with dry weight, with higher fixation rates for juveniles when compared to the adults. This difference was attributed to juveniles having small gills making them inefficient filter feeders, and *T. navalis* collected from the oligotrophic Sargasso Sea exhibited a fixation rate 20 times higher compared to the other species which were found only in coastal habitats. The higher fixation rates for the juveniles and Sargasso Sea samples indicates that the symbiotic bacteria are able to adjust their nitrogen contribution to the system depending on the host's nitrogen deficiency (Carpenter & Culliney, 1975). A recent study utilizing multi-isotope imaging mass spectrometry (MIMS) in *Lyrodus pedicellatus* directly demonstrated that the symbiotic bacteria were fixing nitrogen, which was transferred to the host cells (Lechene, *et al.*, 2007).

Characterization of the symbionts was first conducted on cultured isolates. Axenic cultures of nitrogen-fixing bacteria (family Spirillaceae) isolated from the cecum of *L. pedicellatus* fix nitrogen anaerobically, but are also capable of aerobic growth (Carpenter & Culliney, 1975). Further analysis of this symbiosis was based on isolates obtained from five additional species of shipworms: *Bankia gouldi, T. navalis, Teredo furcifera, Teredo bartischi,*and *Psiloteredo healdi.* The symbionts identified were endosymbiotic, residing within bacteriocytes in the host gill tissue. The same isolate was obtained from all six species and was not only capable of nitrogen fixation but also possessed cellulolytic activity. The ability of the symbiotic bacteria to digest cellulose indicates an additional host derived benefit of the symbiosis: the bacteria assist in the digestion of the host's sole food source (Waterbury, *et al.*, 1983).

Ribotype analysis of the symbionts resulted in the description of a novel genus of g-proteobacteria named *Teredinibacter,* with the original isolate given the species name *turnerae* (Distel, *et al.*, 1991, Distel, *et al.*, 2002). Additional symbionts within this genus have been identified and all host bacteriocytes contain a single dominant symbiont ribotype, as well as a second less abundant ribotype (Distel, *et al.*, 1991, Distel, *et al.*, 2002, Luyten, *et al.*, 2006). Although multiple symbionts were identified in this work the functional role of each is unknown. The nitrogen fixation rate was found to vary between bacterial types within the bacteriocytes, indicating the potential for individual ribotypes to contribute different amounts to the total fixed nitrogen (Lechene, *et al.*, 2007). It is also speculated that the presence of multiple symbionts allows the host to digest multiple wood types or produce various celluolytic enymes (Distel, *et al.*, 2002).

Investigations into the transmission of symbionts in teredinids indicate they may have the ability to acquire symbionts both vertically and horizontally (Sipe, *et al.*, 2000). Symbionts have yet to be cultured from juvenile shipworms, and the bacteria appear well suited to a free-living life style, implying a horizontal mode of transmission for a number of species (Greene & Freer, 1986, Imam, *et al.*, 1990). However, a PCR based investigation of the species *Bankia setacea* revealed a novel species within the *Teredinibacter* genus as well as its presence in host eggs and ovaries, indicating vertical transmission (Sipe, *et al.*, 2000).

It is unclear if the bacterial associates of shipworms are obligately symbiotic. The genome of *T. turnerae* does not have the features typically seen in obligate symbionts such as reduced genome size or loss of % G + C content, and in fact has many features in common with genomes of free-living bacteria, such as a large repertoire of genes for the production of secondary metabolites and protection from bacteriophage (Yang, *et al.*, 2009). This would suggest that *T. turnerae* is a facultative endosymbiont that was recently or is currently existing in a free-living state. Yet, recent molecular evidence strongly suggests that wood-eating bivalves are monophyletic and arose at approximately the same time that bacterial symbionts were acquired, then subsequently diversified into shallow and deep water lineages (Distel, *et al.*, 2011). Co-evolution of nitrogen-fixing bacteria with shipworm hosts would tend to suggest that this relationship is closer and more obligate than previously assumed, and has certainly opened niches to shipworms and facilitated their evolutionary success.

Sea Urchins

Another type of marine organism that has evolved a symbiotic association with nitrogen-fixing bacteria due to nitrogen limitation in its diet are some species of sea urchins. Nitrogen-fixing bacteria associated with sea urchins occur worldwide and in multiple urchin species, though the presence and abundance of the symbionts varies depending on season, and food being consumed by the host. The urchin *Strongylocentrotus droebachiensis* contains nitrogen-fixing symbionts in its intestinal tract, but only in individuals that are fed on kelp, which is a nitrogen deficient food source in the late spring and summer when it is primarily consumed (Guerinot, *et al.*, 1977). Further investigation using multiple species or urchins from different habitats (Nova Scotia kelp bed; Barbados eelgrass, rocks, and shallow reef; and Canada Northwest territory shallow water) found a significant inverse relationship between kelp nitrogen content and urchin nitrogenase activity (Guerinot & Patriquin, 1981), and a similar relationship for eelgrass. Nitrogen fixation in tropical urchins was highly variable, probably as a result of the differences in diet; *Tripneustes ventricosus* had the highest fixation rates and feeds on eelgrass which has low nitrogen content, while *Diadema antillarum* and *Echinometra lacunter* had much lower fixation rates and feed on coral polyps, encrusting algae, diatoms and macroalgae, which have comparatively higher levels of nitrogen. Variation within the temperate urchins was also observed and appeared to fluctuate in response to the level of nitrogen in the food source. Up to 5-fold seasonal fluctuations in the nitrogen content of kelp were documented. Correspondingly, seasonal variation in fixation rates in urchins suggested that in the summer and fall when nitrogen content of kelp is low, nitrogen fixation could be an important source of nutrients. Nitrogenase may be suppressed by metabolic products, because some urchins that tested negative for nitrogen fixation still contained nitrogen-fixing bacteria (Guerinot & Patriquin, 1981). The nitrogen-fixing bacteria appeared to reside specifically in the gut and were identified as *Vibrio spp.* based on cultures, but were not molecularly identified.

Nitrogen-fixing bacteria may be environmentally acquired by sea urchins, but their normal mode of transmission is currently unknown. Transmission of nitrogen fixing bacteria was initially investigated using feeding experiments in *S. droebachiensis* (Guerinot & Patriquin, 1981, Guerinot & Patriquin, 1981). Groups of urchins were starved for three months and nitrogen fixation rates and bacterial counts were quantified. These urchins were then fed kelp or placed in tanks of filtered seawater with antibiotics, followed by putting the urchins in unfiltered seawater and providing kelp again. The kelp-fed group of urchins was initially negative for nitrogen fixation activity and only low numbers of nitrogen-fixing bacteria were present in the gut, however, after two weeks of being fed excess kelp, nitrogen fixation was observed and these urchins contained significantly higher numbers of nitrogen-fixing bacteria. Urchins exposed to antibiotics did not exhibit measurable rates of nitrogen fixation and no nitrogen-fixing bacteria were detected. Urchins from the antibiotic treated seawater that were placed in seawater with no antibiotics and fed kelp started to exhibit nitrogen fixation activity, and nitrogen-fixing bacteria were detected after 18 days. While the described experiments indicate there is environmental acquisition of nitrogen-fixing bacteria by the urchin *S. droebachiensis,* there has been no documentation of vertical transmission for this system. Additionally, there is little information on the diversity of these symbionts, which could easily be assessed using molecular techniques, and is thus a potential topic for future investigations into this system, particularly given the commercial and ecological importance of many urchin species.

Dinoflagellates

The unicellular protists, diatoms and dinoflagellates, often inhabit nutrient poor ecosystems such as mid ocean gyres and thus symbiotic associations with nitrogen-fixing bacteria are potentially advantageous. Blooms of dinoflagellates and diatoms can often be detected remotely from space and due to their high abundance they play an important role in nutrient cycling in oceanic and freshwater systems. Many of these planktonic protists harbor symbiotic cyanobacteria and the association can vary from endosymbiont to epibiont. The heterotrophic dinoflagellates *Ornithocercus*, *Histioneis* and *Citharistes* have cyanobacterial symbionts of the genera *Synechococcus* and *Synechocystis*, which reside within the horizontal groove in *Ornithocercus* and *Histioneis* and in a special chamber within the cells in *Citharistes* (Gordon, *et al.*, 1994). *Ornithocercus* sp. also commonly host large unpigmented bacterial cells as epibionts.

Gordon et al. (1994) proposed that the hosts are likely receiving fixed nitrogen and fixed carbon from the symbionts, providing the host species with an advantage over other heterotrophic plankton during times of nutrient limitation. This hypothesis is supported by the spatial and temporal distribution of these dinoflagellates, which is correlated with ambient nutrient levels. They are common in surface waters of tropical and subtropical seas, and their abundance fluctuates seasonally in the Gulf of Aqaba's surface waters, peaking during the autumn when stratification and nutrient limitation are high (Gordon, *et al.*, 1994). Populations are homogeneous throughout their depth of occurrence in early spring following winter mixing, but their numbers increase in surface waters when summer stratification begins and peak in the fall when nitrate levels at the surface reach their lowest concentration (Gordon, *et al.*, 1994). Similarly, symbiotic associations of cyanobacteria and dinoflagellates in the Bay of Bengal were more common in the spring intermonsoon period than during the summer and winter monsoon; the spring intermonsoon is also a period of nitrate limitation in surface waters (Jyothibabu, *et al.*, 2006). O_2 levels in the seawater are also high at this time, thus the host may provide a unique low O_2 environment for nitrogen fixation (Gordon, *et al.*, 1994, Jyothibabu, *et al.*, 2006).

Early investigations of *Ornithocercus magnificus* and *O. steinii* detected no nitrogen fixation using the acetylene reduction method, but noted that carbon transport between the symbionts and host was still possible (Janson, *et al.*, 1995). Foster and others found several different morphotypes of cyanobacteria and other bacteria associated with *Ornithocercus* and *Histioneis depressa,* some of which were expressing nitrogenase as indicated by immunolabeling (Foster, *et al.*, 2006). Recent work by Farnelid and others suggests that non-cyanobacterial symbionts are likely responsible for most of the nitrogen fixation observed in intact symbioses of *Ornithocercus* and *Amphisolenia* (which hosts photosynthetic eukaryotic endosymbionts) (Farnelid, *et al.*, 2010). Nitrogenase (nifH) sequences were amplified from 62% of single-cell specimens, but only 2 of these 21 sequences were cyanobacterial in origin; the rest were from diverse heterotrophic bacteria and included representatives from *nif*H Clusters I, II, and III, as well as a putative new cluster. Thus, they proposed that the cyanobacteria are photobionts, and perform a photosynthetic rather than a diazotrophic function. The presence of cyanobiont-like particles in food vacuoles of *Ornithocercus* may mean that hosts derive nutrition from symbionts by digesting them (Tarangkoon, *et al.*, 2010).

Diatoms

Diatoms, another common member of the phytoplankton community, are well documented hosts of cyanobacterial symbionts in both aquatic and marine habitats, but nitrogen fixation has only been investigated in a few species. The best known of these is *Rhopalodia gibba,* found in freshwater, in which each cell contains 1 to 10 vertically transmitted spheroid bodies capable of nitrogen fixation (Geitler, 1977, DeYoe, *et al.*, 1992, Prechtl, *et al.*, 2004). The spheroid bodies are cyanobacteria-like but do not contain chlorophyll or phycyoerythrin, so they are incapable of performing photosynthesis and are obligately dependent on the host. They are closely related to UCYN-A, a newly discovered group of free-living photoheterotrophic cyanobacteria that fix nitrogen but lack the oxygenic Photosystem II (Bothe, *et al.*, 2010). Several studies have demonstrated that the assocation of spheroid bodies with *Rhopalodia* is a highly developed and evolutionarily old symbiosis. The genome of the spheroid bodies is greatly reduced in size compared to free-living cyanobacterial relatives, and shares many features with the genomes of mitochondria and chloroplasts (Kneip, *et al.*, 2008). Analysis of the small subunit ribosomal DNA of the host and spheroid bodies showed parallel phylogenies and indicated that symbionts were acquired by a common ancestor approximately 12 million years ago and retained as the host lineage diversified (Nakayama, *et al.*, 2011). The spheroid bodies are now classified as organelles and commonly used as a model system to study the evolution and acquisition of organelles, as they are evolutionarily much younger than plastids or mitochondria (Bothe, *et al.*, 2010, Nakayama, *et al.*, 2011).

Several genera of marine diatoms also harbor cyanobacterial symbionts. *Hemiaulus* sp. and *Rhizosolenia* host the heterocystous *Richelia intracellularis*, *Chaetoceros* associates with extracelluar *Calothrix* cyanobacteria, and the symbiont of *Climacodium frauenfeldianum* has been indentified as *Crocosphaera watsonii* (Janson, *et al.*, 1999, Carpenter & Janson, 2000, Foster, *et al.*, 2011). Nitrogen fixation was demonstrated for *Richelia* associated with *Hemiaulus* via acetylene reduction (Carpenter, *et al.*, 1999), and precise fixation and growth rates for both intact associations and free-living symbionts were determined by Foster and others for the hosts *Hemiaulus, Chaetoceros,* and *Climacodium;* and the symbionts *Calothrix, Richelia,* and *Crocosphaera* (Foster, *et al.*, 2011). These symbioses appear to be both specific and mutually beneficial. *Richelia* spp. are monophyletic and only distantly related to other cyanobacterial endosymbionts, but the hetR gene (involved in heterocyst differentiation) sequences differed in *Richelia* associated with different host genera, indicating a high degree of host specificity (Janson, *et al.*, 1999). This specificity also suggests that symbionts may be vertically transmitted. Diatoms and cyanobacteria have higher growth rates in symbiosis than apart, and diatom hosts appear to influence the metabolism and growth of the symbionts, because *Richelia* fixed 81-744% more N than required for its own growth when grown with a host. Fixed nitrogen is transferred to the host on a timescale of minutes to hours, but the mechanism of transfer and the identity of the product transferred remain unknown.

Diatom symbioses make large contributions to the nitrogen budget of the ecosystems in which they are found, particularly under bloom conditions. Blooms of *Richelia intracellularis* associated with *Hemiaulus hauckii* are responsible for 89-100% of nitrogen fixation in the western tropical North Atlantic when they are present (Foster, *et al.*, 2007), and the same symbiont hosted by *Rhizosolenia* contributes 35 – 48% of nitrogen fixation in the Gulf of California , and 40-70% in the Mediterranean Sea. Foster et al estimated that symbiotic diatom populations may be an equally important source of fixed nitrogen as *Trichodesmium* on an ocean basin scale (Foster, *et al.*, 2011).

Corals

Coral reefs provide an opportunity to study relatively newly discovered associations between nitrogen-fixing bacteria and hosts such as corals and sponges that provide important ecosystem services (Moberg & Folke, 1999). These symbioses have allowed hosts to be ecologically successful despite the low combined nitrogen of these tropical coastal environments. Nitrogen-fixing bacteria have been hypothesized to be symbionts of reef building corals since Williams et al. identified nitrogen fixation in the skeleton of *Acropora variabilis* (Williams, *et al.*, 1987). This preliminary research did not investigate the diversity of symbionts or whether the host or symbiont gained any benefit from the association. However, based on the results from light/dark acetylene reduction assays it was hypothesized that the symbionts were photosynthetic cyanobacteria (Williams, *et al.*, 1987). Similarly, Odinstov observed nitrogenase activity in the hydrocoral *Millepora* and zooxanthellae, unicellular green algae and bacteria were noted in the skeleton as well as the tissue (Odinstov, *et al.*, 1987). It was not clear, however, which member of the consortium was fixing nitrogen or how similar this association might be to that of hermatypic corals. Further investigation into the coral-diazotroph symbiosis identified nitrogen-fixing bacteria of the class γ-proteobacteria associated with the skelton of *Favia favus* (Shashar, *et al.*, 1994), which displayed higher rates of fixation under illumination, and with exposure to glucose-enriched seawater. These results indicate that the nitrogen-fixing bacteria are utilizing glucose from either the host or algal co-symbiont as an energy source, implicating a potential benefit for the symbionts in this relationship. *Vibrio* spp. capable of nitrogen fixation (as measured by acetylene reduction) have also been cultured on nitrogen-free media from the mucus of *Mussimilia hispida* from Brazil (Chimetto, *et al.*, 2008).

Microbial communities associated with corals are highly diverse and dynamic, and include many possible nitrogen-fixing bacteria (Rohwer, *et al.*, 2002, Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2009). Metagenomic analyses found representatives from nitrogen-fixing bacterial lineages, as well as sequences coding for nitrogenase proteins (Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2009). However, it was not until 2004 that an endosymbiotic nitrogen-fixing bacteria symbiont was conclusively identified (Lesser, *et al.*, 2004). The cyanobacterial symbionts transfer fixed nitrogen to the coral's algal symbionts, and fixed carbon in the form of glycerol has been hypothesized to be respired by the cyanobacteria to provide energy for nitrogen fixation (Lesser, *et al.*, 2007). The cyanobacteria cannot meet their energetic requirements with photosynthesis as more than 80% of Photosystem II units are uncoupled from photochemistry. Thus, the cyanobacteria likely respire the glycerol to meet their energetic requirements, thereby increasing the total respiration of the coral colony, which may account for observed lower net photosynthesis rates in colonies with cyanobacteria (Lesser, *et al.*, 2007). Initial analysis of the bacterial diversity for this system indicated that this symbiosis is comprised of a single cyanobacterial species (Lesser *et al.* 2004), but newer results from deep sequencing of 16s rRNA genes show that the cyanobacterial population is diverse (Jarett, unpublished). Morphologically similar cyanobacteria have been observed in multiple samples of *Acropora cytherea* from the Great Barrier Reef, but were not investigated further (Kvennefors & Roff, 2009). Nitrogen-fixing cyanobacteria and proteobacteria have been observed in close association with tissue of the Hawaiian corals *Montipora capitata* and *Montipora flabellata.* The benefits to each partner in these symbioses as well as whether the bacteria are endo- or epibionts remains unknown; however, a correlation was found between Vibrionaceae *nifH* transcript number and algal symbiont abundance for *M. capitata,* suggesting a close relationship (Olson, *et al.*, 2009). A diverse community of bacteria were identified but a conserved phylogenetic cluster of bacteria in the Vibrionacea family were found only in association with *M. capitata,* and a less conserved cluster of g-proteobacteria were only associated with *M. flabellata.* This symbiont specificity may indicate coevolved, highly specific, symbiotic associations.

For most of these associations, it is unknown if there is direct transfer of fixed nitrogen to the coral, if the symbiosis is a mutualism, parasitism, or neither, or even how widespread this symbiosis is among coral taxa and how this impacts the health and growth of the coral host. Whether symbionts are acquired by horizontal or vertical transmission is also largely unknown. For tissue-associated microbes in particular, the transmission of photosynthetic dinoflagellate symbionts of reef-building corals may offer clues. *Symbiodinium* are transmitted both vertically and horizontally, but vertical transmission appears to create or facilitate more specific symbioses (Stat, *et al.*, 2008), and is more common in areas geographically isolated from other reefs, such as Hawai'i (LaJeunesse, *et al.*, 2004). The diversity of nitrogen-fixing bacteria that have been identified with different coral species indicates the potential for a range of types of symbiotic associations between the coral host, its alga, and nitrogen-fixing symbionts.

Sponges

Microbial nitrogen transformations have also been observed in sponges, another prominent coral reef organism. Sponges are an important component in reef ecosystems as they consolidate rubble, have high biodiversity and biomass, and influence constituents of the surrounding water (Reiswig, 1973, Diaz & Rutzler, 2001, Taylor, *et al.*, 2007). Many sponges, termed bacteriosponges or high microbial abundance (HMA) sponges, contain high densities of diverse bacteria, a large fraction of which are metabolically active (Kamke, *et al.*, 2010) and have been shown to be important in nitrogen cycling.

All nitrogen transformations have been observed in sponges, although not all occur in the same sponge. The ability of sponges to control their pumping rates allows them to control the oxygen content of their tissues and influence the metabolism of associated bacteria, such that both aerobic and anaerobic processes can occur in the same sponge. Denitrification, anammox and nitrification were conclusively observed in the cold water sponge *Geodia baretti* (Hoffmann, *et al.*, 2009), while nitrification and nitrogen fixation have been documented in multiple studies on sponges (Wilkinson & Fay, 1979, Southwell, *et al.*, 2008). Surprisingly, even low microbial abundance (LMA) sponges have been shown to have high rates of nitrification and denitrification, despite their much smaller bacterial populations (Schläppy, *et al.*, 2009).

The association of nitrogen-fixing bacteria with sponges was first noted by Wilkinson and Fay (1979) using the acetylene reduction assay on sponges from the Red Sea. This activity was attributed to cyanobacteria because the sponges that tested positive all contained cyanobacteria, while the sponge that tested negative contained no cyanobacteria. The acetylene reduction assay was also used to detect nitrogen fixation in the sponge *Halichondria* sp. from the coast of Taiwan (Shieh & Lin, 1994). However, it was found that $^{15}N_2$ tracer studies were more reliable in the detection of nitrogen fixation (Wilkinson, *et al.*, 1999) and stable isotope signatures ($\delta^{15}N_2$) have since been used to screen sponges that may harbor nitrogen-fixing bacteria (Mohamed, *et al.*, 2008). Mohamed et al (2008) used molecular genetic technqiues to examine nitrogen fixation in sponges from the Florida Keys. Diverse *nifH* sequences from α- and γ-proteobacteria, cyanobacteria, and *Desulfovibrio* spp. were detected in sponges that exhibited low $d^{15}N_2$ values indicative of nitrogen fixation (*Ircinia strobilina* and *Mycale laxissma*). While nitrogen fixation in sponges was first attributed to cyanobacteria, heterotrophic bacteria such as Vibronaceae species are also associated with this process (Shieh & Lin, 1994). Expression of *nifH* was only detected from cyanobacteria by Mohamed et al, which was attributed to sampling during daylight hours; expression of *nifH* by other bacteria may take place at other times. Most of the sequences obtained from the sponge samples were novel and were not observed in the water column.

There are many indications that the association of microbes, including diazotrophs, with sponges is a highly evolved relationship. Many of the microbial lineages occur only in sponges (Simister, *et al.*, 2011), and some groups are genus- or species-specific (Montalvo & Hill, 2011), with closely related sponges hosting very similar communities even if they are geographically very distant (Montalvo & Hill, 2011). This specificity may be enabled by vertical transmission. Some sponge gametes or larvae harbor a single bacterial species or a complex assemblage, including Cyanobacteria, Proteobacteria, Actinobacteria, Bacteroidetes, and Planctomycetes (Oren, *et al.*, 2005, Usher, *et al.*, 2005). Much of this information has been based on electron microscopy, which provides visual confirmation of the microbial community but generally does not provide phylogenetic or metabolic information. Recent studies using 16s rRNA identified bacteria in the larvae and gametes of sponges (Steindler, *et al.*, 2005), however, analysis of functional genes such as *nifH* is needed to determine if nitrogen-fixing bacteria are transmitted vertically and if fixation is occurring in the larvae. The morphological and physiological differences between LMA and HMA sponges indicate that symbiotic microbes may have influenced the evolution of sponges (Weisz, *et al.*, 2008). LMA sponges are adapted to filter large quantities of water in order to acquire as much particulate organic matter as possible. HMA sponges are more dense, and have more complex internal morphology that increases the surface area inside the sponge and maximizes the contact time with water. Along with their slower pumping rates, this allows HMA sponges to create steep concentration gradients within their tissues and fosters the growth of dense and diverse microbial communities (Weisz, *et al.*, 2008). The bacteria in these symbioses presumably benefit from the availability of a suitable habitat, whereas the sponges benefit from the transfer of fixed nitrogen (Freeman & Thacker, 2011). Little is known about how nitrogen is transferred to the host or in what form (Wilkinson, *et al.*, 1999). Despite the wealth of knowledge about sponge-associated bacteria, relatively little is known about which bacteria are fixing nitrogen, how they are transmitted, how prevalent this symbiosis is, and how this affects nutrient cycling within the sponge and on the reef.

The data from studying urchins as well as some of the other well studied symbiotic systems such as dinoflagellates, shipworms and plants indicate the main benefit to the host is receiving a source of fixed nitrogen, while the benefit to the symbionts, if these are mutualisms, is often less clear. As suggested by various studies the biggest advantage for the symbionts may be that the host provides a suitable microaerobic environment for the symbionts to fix nitrogen (Gordon, *et al.*, 1994, Jyothibabu, *et al.*, 2006). Many of the nitrogen-fixing bacteria are also photosynthetic and so the host may provide a suitable environment for this process as well, depending on where the bacteria are found on or within the host. In the corals, it has been speculated that the symbiotic cyanobacteria, because they are capable of minimal photosynthesis, are living heterotrophically and gain carbon in the form of glycerol from the host (Lesser, *et al.*, 2007). Hosts appear to provide a safe refuge from predation, which may be an added benefit to these symbionts, however, more research into the specificity and evolution of these associations is needed before these questions can be answered.

Evolution and ecology of marine nitrogen fixing symbiosis

Symbiotic associations between nitrogen-fixing bacteria and their eukaryotic hosts cover a continuum of symbiotic associations. These continua may be characterized in a number of ways three of which are: (1) spatial and temporal aspects of the association, (2) specificity of members involved, and (3) the necessity of the association to the individual members (Starr, 1975).

The temporal and spatial continuum is apparent when looking at nitrogen fixing symbiosis involving diatoms and dinoflagellates. In the summer months when environmental biologically available nitrogen levels are low, diatoms and dinoflagellates are more abundant and more often associated with nitrogen-fixing symbionts (Jyothibabu, *et al.*, 2006). There is also variation in symbiont location on and in the host in these symbiosis, wherein symbionts are sometimes found as epibionts and sometimes as endobionts as with the diatoms *Chaetoceros spp.* and *Rhodophilia gibba* respectively (Prechtl, *et al.*, 2004, Foster & Zehr, 2006).

The host and symbiont specificity of the association also varies among host species and may also fall along the commensal - mutualistic - parasitic continuum. Highly specific symbioses are generally considered more evolved symbiotic associations and are largely mutualistic in nature (Douglas, 1995). A high diversity of nitrogen-fixing bacteria are associated with tropical marine sponges with bacterial symbiont representatives from all major nitrogen-fixing bacterial taxonomic groups (Mohamed, *et al.*, 2008). In contrast to this diverse symbiosis only a few bacterial symbiont types are present in marine shipworms (Distel, *et al.*, 2002). For these symbioses it is clear that the symbiosis is more evolved for shipworms when compared to that of sponges.

The third continuum is the necessity of the symbiosis to the members involved. For some members the association is obligatory in that they are unable to survive aposymbiotically or in a free-living state. When examining the *Montastraea cavernosa* symbiosis a colony of an aposymbiotic host can be found neighboring a symbiotic host, indicating that this association, although perhaps beneficial to both host and/ or symbiont, is not obligatory (Lesser, *et al.*, 2004). On the other end of the continuum is the intracellular spheroid body symbiont of the diatom *Rhopalodia gibba* where neither host nor symbiont has been observed or cultured without the presence of the other member of the association (Prechtl, *et al.*, 2004). Where each of the individual marine nitrogen-fixing symbioses falls along these continua can reveal insight into the ecological and evolutionary aspects of these associations. Because of this, it is necessary to address gaps in our understanding of these symbioses in regards to their placement along these continua.

Future research directions

The paucity of knowledge about most marine nitrogen-fixing symbioses is important to recognize, and address, because we do not know the impact that these associations have on the ecology of either the hosts or of the larger ecosystems of which they are a part. The presence of symbiotic diazotrophs can raise fundamental questions about our understanding of their hosts. It is thought that the photosynthetic dinoflagellate symbionts of corals are nitrogen limited, and that this limitation controls their growth and prevents them from multiplying faster than the host cells (Falkowski, *et al.*, 1993). However, in the *Montastraea cavernosa*/cyanobacterial symbiosis, nitrogen fixed by the cyanobacteria is transferred to the dinoflagellates in quantities sufficient to generate a stable isotope signal consistent with the utilization of nitrogen fixation, but without causing a significant increase in zooxanthellae growth rates (Lesser, *et al.*, 2007), challenging this classical understanding.

On a larger scale, nitrogen budgets have been calculated for many ecosystems, but marine nitrogen budgets seldom take into account diazotrophic symbioses that appear to be uncommon. We do not yet know the magnitude of their contribution, but it may be substantial, particularly considering that many symbioses may remain undiscovered. For instance, sponges have not typically been considered major players in nitrogen cycling on coral reefs, but in light of recent studies (Mohamed, *et al.*, 2008, Hoffmann, *et al.*, 2009) this should be reconsidered. Similarly, the contributions of symbionts of dinoflagellates and diatoms have not always been considered in the estimation of global and oceanic nitrogen budgets, but new estimates of their nitrogen fixation rates clearly indicate that they should be included (Foster, *et al.*, 2011).

The potential impact of changing environmental conditions, such as eutrophication, ocean acidification, and climate change on these symbioses is also unknown. Biologically available nitrogen is often abundant in eutrophic areas; this could obviate the need for nitrogen fixation and make these symbioses between nitrogen fixing bacteria and a variety of hosts less common. Symbioses that are obligate for each partner, such as the *Rhopalodia gibba* and spheroid body partnership, might have greatly reduced fitness in such environments and perhaps be extirpated from these areas. High atmospheric CO_2 concentrations that cause ocean acidification increase nitrogen fixation rates in the free-living cyanobacterium *Trichodesmium* (Hutchins, *et al.*, 2007, Levitan, *et al.*, 2007), but living inside a host-mediated environment may impose different challenges and restrictions on symbionts, or none at all if the host buffers against these changes. Ocean acidification poses threats to calcifying organisms (Jokiel, *et al.*, 2008, Doney, *et al.*, 2009), some of which (shipworms, corals, sea urchins, and some sponges) are hosts to diazotrophic symbionts. Reductions in abundance or species richness of these hosts may lead to a loss of diversity in symbionts as well. Rising sea surface temperatures due to climate change also affect many of the host organisms discussed above or their other symbionts, particularly corals, sponges, and plankton (Hays, *et al.*, 2005, Hoegh-Guldberg, *et al.*, 2007, Webster, *et al.*, 2008). Future research should focus on three broad goals: (1) characterization of known symbioses, particularly with respect to nutrient and energy exchange between partners, to determine if relationships are mutualisms; (2) discovery of new diazotrophic symbioses; (3) describing the impacts of these symbioses on their respective hosts and the ecosystems of which they are a part. As our understanding of the ecology and physiology of these system increases so will our understanding of their global importance.

CHAPTER II

METAGENETIC ANALYSIS OF THE PROKARYOTIC COMMUNITIES ASSOCIATED WITH BROWN AND ORANGE COLONIES OF THE CORAL MONTASTRAEA CAVERNOSA

Introduction

Microbial Communities Associated with Corals and Coral Reefs

Microbes, both eukaryotes and prokaryotes, are responsible for many key processes and nutrient transformations on coral reefs, from photosynthesis to nitrogen cycling (Dinsdale, *et al.*, 2008). While many of these microbes are free living in the water column or the benthos, others are associated with various host organisms such as algae (Barott, *et al.*, 2011), sponges (Webster & Taylor, 2011), or corals (Rohwer, *et al.*, 2002, Wegley, *et al.*, 2007). Some are only loosely associated with their hosts, whereas others are integrated into obligate or facultative symbioses. Among the most important of these symbionts on coral reefs are the dinoflagellates *Symbiodinium* sp. that are associated with scleractinian corals and some other cnidarians (Freudenthal, 1962). The dinoflagellates perform photosynthesis and translocate much of the fixed carbon to the host, supporting growth, metabolism, and reproduction (Muscatine, 1967, Falkowski, *et al.*, 1984). Although once considered a single panmictic species (Freudenthal, 1962), *Symbiodinium* are now known to be genetically diverse and the impact of different 'clades' or genotypes of *Symbiodinium* on the coral holobiont has been studied extensively. The presence of different clades in symbiosis with corals can affect their response to thermal and light stress (Rowan, *et al.*, 1997, Sampayo, *et al.*, 2008, Howells, *et al.*, 2012), growth (Little, *et al.*, 2004), and possibly resistance to disease (Stat, *et al.*, 2008, Correa, *et al.*, 2009), .

The study of other microbes, including Bacteria, Archaea, fungi, and viruses, associated with corals is a relatively new field. Initial studies revealed abundant and diverse communities of microbes (Rohwer, *et al.*, 2002, Wegley, *et al.*, 2004, Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2009) that are distinct from that of the water column (Rohwer, *et al.*, 2002, Wegley, *et al.*, 2004, Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2009, Sunagawa, *et al.*, 2010). Much of the initial research in this area focused on cataloging the full extent of diversity within these communities and describing biogeographic and temporal patterns. Using clone libraries and rarefaction curves, Rohwer *et al.* (2002) proposed that three species of Caribbean corals had approximately 6,000 different bacterial operational taxonomic units (OTUs, roughly equivalent to species) associated with them. Later studies utilizing high-throughput sequencing technology (e.g., 454 pyrosequencing) yielded somewhat lower diversity estimates (Sunagawa, *et al.*, 2010), although the absolute numbers in any study should be taken with some caution as PCR and sequencing error can greatly inflate diversity estimates in pyrosequencing data sets in particular (Quince, *et al.*, 2009, Behnke, *et al.*, 2011). Corals from different families have distinct prokaryotic communities; some associates are apparently species-specific, and taxa that are shared are often present at different levels of abundance (Rohwer, *et al.*, 2002, Sunagawa, *et al.*, 2010). More closely related coral species, however, have very similar prokaryotic communities, as Littman *et al.* (2009) found in three species of the genus *Acropora* from the Great Barrier Reef (GBR). Spatial differences have been investigated from the small scale, within a single coral colony, to a regional scale, comparing geographic patterns. Corals are often described as being composed of several 'compartments,' namely the skeleton, coral tissue, and surface mucus layer, and the prokaryotes found in each compartment can differ (Sweet, *et al.*, 2010). Communities on outer tips can differ from those on the inner areas of branches in corals with diffuse morphology (Rohwer, *et al.*, 2002), and even very closely spaced samples from colonies with less complex mounding morphologies show significant variability (Daniels, *et al.*, 2011). Studies at larger spatial scales have found that corals have generally consistent bacterial communities, but the types that dominate can vary at different locations. Rohwer, *et al.* (2002) found very similar communities in the same coral species separated by 3000 km, whereas Littman, *et al.* (2009) found that the species dominating coral-associated communities were different in two locations on the GBR. Temporal stability in bacterial communities has been found in some studies but not others. Communities associated with *Isopora palifera* and in the water column in Taiwan change rapidly with the seasons (Chen, *et al.*, 2011) and all but one of the dominant bacterial groups on *Oculina patagonica* in the Mediterranean are different in the summer and winter (Koren & Rosenberg, 2006). However, *Acropora millepora* bacterial assemblages on the central GBR are consistent throughout the year (Littman, *et al.*, 2009). These seemingly conflicting results may be explained by the magnitude of seasonal changes at the different study sites. Both the Mediterranean Sea and Taiwan are at higher latitudes than the central GBR, and experience large changes in temperature and rainfall in different seasons, which likely contributed to the seasonal differences in water column communities observed by Chen *et al* (2011). Variation in the water column community may also affect the coral-associated community, but with the exception of Chen *et al* (2011) the water column was not sampled in these studies.

The function of coral-associated microbial communities is also beginning to be explored, although much remains speculative or unknown. Many possible functions have been proposed based on what is known about the microenvironment of the coral holobiont, and the chemical and biological resources available there. Coral tissue and mucus layers experience large diel changes in oxygen concentrations, with hyperoxic conditions during the day and hypoxic or anoxic conditions during darkness (Dykens & Shick, 1982, Kuhl, *et al.*, 1995). This enables both oxic and anoxic processes to take place in this environment, broadening the possible range of bacterial metabolism. Coral associated bacteria are thought to perform several steps in nitrogen cycling, including nitrogen fixation (Lesser, *et al.*, 2004, Lesser, *et al.*, 2007), and ammonia oxidation (Wegley, *et al.*, 2007). Cycling of sulfur, particularly in the forms of dimethylsulfoniopropionate (DMSP) and dimethyl sulfide (DMS), is also an important process in coral microbial communities (Raina, *et al.*, 2010). DMS and DMSP have been used as sole carbon sources in media to culture dominant bacterial types associated with *Montipora* and *Acropora* (Raina, *et al.*, 2009). Coral-associated bacteria are also known to be prolific producers of antimicrobial compounds, which are postulated to play key roles in structuring the microbial community and protecting the coral host from invading or endogenous opportunistic pathogens (Ritchie, 2006, Rypien, *et al.*, 2010). Multiple genes involved in the production of and resistance to antimicrobial compounds and toxins, as well as genes involved in virulence and the oxidative stress response, have been found in metagenomes from healthy and stressed corals (Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2009).

More recent research into coral-associated microbial communities has investigated the response to environmental stressors, disease, and the physiological condition of the coral host. Bacterial communities respond to several different environmental conditions, including water depth (a proxy for light) (Klaus, *et al.*, 2007), nutrient and dissolved organic carbon concentrations (Klaus, *et al.*, 2007, Vega Thurber, *et al.*, 2009), pH (Meron, *et al.*, 2011), and temperature (Ritchie, 2006, Vega Thurber, *et al.*, 2009, Littman, *et al.*, 2010). In addition to changing the composition of the community, there is evidence that the activity of the bacteria also changes with environmental conditions. Culturable bacteria associated with *Montastraea annularis* changed both their production of and resistance to antimicrobial compounds at 25° and 31° C (Rypien, *et al.*, 2010). Under environmental conditions that are considered stressful or detrimental for the coral host, the universal pattern of change in bacterial communities is increased abundance and activity of *Vibrio* spp. and other “disease-associated” groups, and an increase in genes associated with virulence (Bourne, *et al.*, 2008, Vega Thurber, *et al.*, 2009, Littman, *et al.*, 2011). A key factor that seems to drive these changes is the physiological condition of the coral host. For example, *Acropora millepora* colonies that experience seasonal changes in water temperature of more than 10° C have stable bacterial communities throughout the year (Littman, *et al.*, 2009). Despite this adaptive capacity, above-average temperatures, even if they are below the threshold for bleaching, stress the host and shift the microbial assemblages (Ritchie, 2006, Vega Thurber, *et al.*, 2009). Bacterial communities associated with corals harboring opportunistic or ‘weedy’

Symbiodinium types that may not contribute as much fixed carbon to host metabolism (Stat, *et al.*, 2008), particularly clade D, also exhibit much larger changes in response to thermal stress and greater abundances of *Vibrio* species (Littman, *et al.*, 2009, Littman, *et al.*, 2010). Interestingly, bacterial communities very similar to those found in diseased or stressed corals (Sunagawa, *et al.*, 2009, Kimes, *et al.*, 2010) are also found in sponges subjected to thermal stress, even though their normal microbial communities are different from those of corals (Webster, *et al.*, 2008). It is clear that microbial communities in corals are structured by external environmental conditions, the physiological state of the host, and interactions with other members of the holobiont.

Methods for Studying Microbial Communities

In the past, all studies of environmental prokaryotes were culture-based and could enumerate only those bacteria that were able to grow on traditional media. Direct microscope counts indicated that a vast number of bacteria, as much as 99% of the cells in the environment, were present and viable but non-culturable (Staley & Konopka, 1985, Rappe & Giovannoni, 2003). Host-associated and symbiotic microbes in particular remain difficult to culture because of their specific metabolic and environmental requirements, although new methods such as transcriptomics are useful for directing culturing attempts. For example, a metatranscriptome from the medicinal leech *Hirudo verbana* revealed that a *Rikenella*-like bacterium in the gut utilizes mucin glycans, and a culture medium containing mucin was designed that allowed the cultivation of this organism for the first time (Bomar, *et al.*, 2011). It was not until the advent of DNA sequencing technology that information about the "uncultured majority" became accessible. Until very recently, the majority of studies involved amplification, restriction mapping or sequencing of a phylogenetically informative gene, usually the small subunit of the ribosomal RNA gene (16S rRNA). This is still a very popular approach, and a variety of different techniques and tools are available, with varying degrees of specificity, coverage per sample, and cost per sequence.

Terminal restriction fragment length polymorphism (T-RFLP) is a PCR based approach wherein the gene of interest is amplified using fluorescently labeled primers and the PCR amplicons are digested with a restriction enzyme. The length of the resulting fragments is determined by capillary electrophoresis, yielding a “fingerprint” of the community. This is a low cost method that delivers consistent results and thus can be applied to many different samples (Osborn, *et al.*, 2000). However, it may not reveal the full diversity of the community because terminal restriction fragments (TRFs) from different organisms may be the same length, and fragments that are very similar in size may not be distinguishable depending on the sequencing instrument that is used. This method is also subject to the inherent biases of PCR, and can only supply presence/absence data, not information on the abundance of the groups represented by each TRF. A related method is automated ribosomal intergenic spacer analysis (ARISA), where the internal transcribed spacer region between ribosomal RNA genes is PCR amplified with fluorescently labeled primers and the length of the amplicons is determined. ARISA is less frequently used than T-RFLP but has many of the same benefits and drawbacks.

Denaturing gradient gel electrophoresis (DGGE) is also PCR based, but primers include a GC-rich “clamp” sequence on the 5’ end of the amplicons, which prevents the complete dissociation of PCR products when they are separated by DGGE. The bands on the gel are visualized and the banding patterns are compared; bands of interest can be excised and sequenced to determine their identity. DGGE results can be variable and thus internal standards on each gel are necessary for the accurate comparison of different gels. DGGE has the same issues as TRFLP in that bands of the same size may represent multiple sequences, and data are limited to presence/absence. This technique, combined with sequencing of the dominant bands, has been particularly common in studies of *Symbioindium* diversity using the ITS2 genetic marker (LaJeunesse, 2002, LaJeunesse, *et al.*, 2004), although it is being replaced by the amplification, cloning and sequencing of other informative markers with less intragenomic variation such as *cp*23S (Apprill & Gates, 2007, Stat, *et al.*, 2008, Correa & Baker, 2009).

Newer methods of prokaryotic community analysis rely on DNA sequencing rather than fingerprinting. The first of these methods to come into common use was clone library sequencing. A gene of interest is amplified with PCR, and single sequences are inserted into vectors, which are then transformed into bacterial cells and grown on agar plates. Transformed colonies are selected and grown in broth, the plasmids are purified and the inserts are sequenced. This technique has the highest cost per sequence of any of these methods and is time-intensive, but it can yield full-length 16s rRNA sequences. Because of the costs, a significant trade-off exists between increasing the number of samples or increasing the depth of sequencing on each sample. Additionally, if the gene of interest codes for a product that is lethal to the host cell or otherwise restricts its growth, clone libraries may not be not technically feasible.

All of the methods discussed above produce a relatively small amount of data that can easily be analyzed on a standard laptop computer with traditional analysis programs. However, capturing most of the diversity present in samples (i.e., producing saturated rarefaction curves) with these methods is typically prohibitively expensive, time-consuming, or both, and conclusions drawn from such under-sampled communities are uncertain. Advancements in sequencing technology such as pyrosequencing and Illumina/Solexa have enabled the rapid generation of orders of magnitude more data at a much lower cost per base, and the resulting flood of data has moved the bottleneck from the production of sequence data to its analysis and interpretation (Glenn, 2011). Many high-throughput sequencing data sets require special analysis programs and much more extensive computational resources that may cost more than the sequencing itself. Despite these issues, high-throughput sequencing has revealed the diversity and biological patterns of even the most complex microbial communities in unprecedented detail, and new platforms that yield even more data at even lower cost are continually being developed.

High-throughput sequencing is available from several platforms, with varying costs per base, bases per run, read length, and error rates (Glenn, 2011). The most commonly used are Illumina and 454 pyrosequencing, both of which allow sample multiplexing to reduce costs (Hamady, *et al.*, 2008). Shotgun or amplicon-based sequencing can be performed on any platform. Thousands to millions of reads are produced per run, allowing many more samples to be analyzed in much greater depth than was previously possible. Read lengths are shorter than traditional Sanger sequencing, with Illumina currently at 200 bp (paired-end), and 454 reads near 700 bp, although newer chemistry can produce about 3000 bp reads with a high error rate (Glenn, 2011). These shorter reads are less phylogenetically informative than full-length sequences, although with careful selection of the amplified gene region even short reads can contain adequate taxonomic and phylogenetic information (Quince, *et al.*, 2009, Youssef, *et al.*, 2009, Kunin, *et al.*, 2010, Schloss, 2010, Jeraldo, *et al.*, 2011). However, some 16S rRNA gene regions may be more prone to sequencing error, and thus less informative, due primarily to the presence of long homopolymers (Behnke, *et al.*, 2011). Overall error rates, particularly for pyrosequencing, remain problematic but several computational approaches have been devised to ameliorate this (Quince, *et al.*, 2009, Behnke, *et al.*, 2011, Quince, *et al.*, 2011).

All of these methods, from T-RFLP to pyrosequencing, share a critical step: the amplification of the gene of interest with PCR. The quality and reliability of results is therefore dependent on how well the PCR amplicons reflect the organisms present in the sample. The template (i.e., sample), reaction conditions, and primer sequences can all be sources of bias and error in PCR. The mixture of DNA molecules in the template may be biased due to the sample preservation technique (Sekar, *et al.*, 2009) and/or DNA extraction method (Wilson, 1997, Hong, *et al.*, 2009), which may not lyse all cells or may fragment nucleic acids (von Wintzingerode, *et al.*, 1997). Such bias within samples is problematic for all types of downstream analysis. Inhibitors of PCR such as polysaccharides, enzymes, and proteins are frequently present and may have different concentrations in different samples, biasing comparisons (Wilson, 1997). Differential amplification of mixtures of template molecules is a common issue with PCR and is influenced by the primer sequences, template G+C content, annealing temperature, and cycle number (von Wintzingerode, *et al.*, 1997, Wilson, 1997, Sipos, *et al.*, 2007). Ideally all templates would have equal hybridization efficiency with the chosen primers, but this is difficult to achieve, especially with universal primers that often contain degeneracies. Higher annealing temperatures can increase preferential amplification of templates perfectly matching the primer sequence over those with one or more mismatches (Sipos, *et al.*, 2007). Restricting the number of cycles may reduce this bias, but probably only in low-diversity template mixtures (Suzuki & Giovannoni, 1996).

PCR reactions can also generate artifacts, or sequences that did not exist in the original sample, including chimeric sequences. Chimeras are hybrid products of multiple parent sequences formed when a truncated extension product from an earlier cycle of PCR anneals to a different template and is extended. Chimeras can appear to be novel, genuine sequences, leading to the 'discovery' of organisms that do not actually exist and artificially inflating diversity estimates, particularly in high-throughput sequencing data sets (Quince, *et al.*, 2009, Edgar, *et al.*, 2011). Damaged template DNA and mixtures of highly similar sequences are more likely to generate chimeras, but increasing elongation times and limiting cycle number during PCR can reduce their formation (von Wintzingerode, *et al.*, 1997). The same chimeras can form reproducibly in independent PCR reactions, and can make up a large percentage (up to 45%) of the sequences in a library (Haas, *et al.*, 2011). Several programs now exist for the detection of chimeric molecules either *de novo* or utilizing a reference database of potential parent sequences (Huber, *et al.*, 2004, Edgar, *et al.*, 2011, Haas, *et al.*, 2011).

Primer sequence may be the most critical factor in PCR, particularly when attempting to amplify a variety of template sequences. Ideal primers match perfectly to the sequence of interest and are not complementary to other sequences, i.e., they are highly specific. The 16S rRNA gene is most commonly targeted in surveys of prokaryotes, and it produces a transcript with a complex secondary structure of conserved functional loops and variable stems that mostly provide structural support. Even within this highly conserved gene, it is not possible to design truly universal primers that match perfectly to all known taxa, and thus mismatches are inevitable within at least some taxonomic groups, particularly those that contain unique or unusual base changes or motifs (Baker, *et al.*, 2003, Teske & Sorensen, 2008). Mismatches to the primer result in preferential amplification of templates that match the primer more completely, or perfectly, and even high-abundance taxa can be missed if they anneal poorly to the primer (Sipos, *et al.*, 2007). Increased sequencing depth is unable to compensate for mismatches or non-matches to PCR primers (Hong, *et al.*, 2009). The task of primer design becomes even more difficult when the length of the amplicons is critical, as for most high-throughput sequencing platforms that can only produce short reads, because the regions of the 16S rRNA gene are variably informative (Schloss, 2010). The optimal combination of primers, gene region, and amplicon length will also vary depending on the environment being studied, as demonstrated by Soergel *et al.* in a recent comprehensive survey of all viable combinations of these parameters (Soergel, *et al.*, 2012). Different environments are not equally represented in reference databases, and even 'universal' primers will match different proportions of the total diversity in different environments. Thus, a primer pair or gene region that is optimal for studying the human gut may not accurately reflect the diversity of the termite gut, or ocean water, and vice versa. In this study, the coral associated community was shown to be particularly under-represented in reference databases (Soergel, *et al.*, 2012). The ultimate weakness of primers is that they can only be designed based on information that is already known. Sequences from organisms in the environment with no representatives in databases cannot be used to design primers, and if they do not match existing primers they will be missed. Moreover, there is no way to know using PCR if these organisms exist at all, or how diverse or abundant they may be. All primers are biased, so it is important to select primers based on what is known about their coverage and about the organisms present in the environment of interest. Because of this bias, even studies with the best experimental design and sequencing can yield seriously flawed or incomplete results if the wrong primers are chosen. These issues are driving the ongoing shift from PCR-based approaches to "shotgun" approaches such as metagenomics and metatranscriptomics.

The goals of this chapter were to characterize the prokaryotic communities associated with brown/green and orange colonies of *Montastraea cavernosa*, and determine if they differ in the two colony types. Spatial differences in these communities were investigated by sampling corals from three different locations around the Caribbean and comparing the coral associated communities with those found in the water column. We also aimed to identify the symbiotic cyanobacteria, and investigate their relationship to other cyanobacteria.

Methods and Materials

Sample Collection and Processing

Samples from three orange and three brown/green colonies of the coral *Montastraea cavernosa* were collected with a hammer and chisel on SCUBA from a depth of 15 m at each of three locations in the Caribbean. Corals were sampled in August of 2010 and 2011 at North Perry Reef, Lee Stocking Island, Bahamas (LSI, 23°47'0.03" N, 76°6'5.14" W); in July of 2011 at Conch Reef, Key Largo, Florida (FL, 25°0'7.73" N, 80°22'48.68" W); and in May of 2008 at Rock Bottom Wall, Little Cayman, Cayman Islands (LC, 19°42'6.32" N, 80°3'25.11" W). Three replicate water samples (4 L) were collected from 1 m above the reef substrate at each location in 2011, contemporaneously with coral collections. An additional water sample was collected from a depth of 30m at Little Cayman in 2008. Coral and water samples were transported back to laboratories in covered, seawater-filled coolers and processed within 1 hr of collection.

Coral samples were gently airbrushed while being held upside down with 0.2 μm filtered sea water from a distance of approximately 15 cm to remove mucus and loosely associated bacteria then placed in saline DMSO buffer (Seutin et al. 1991) for the preservation of DNA, frozen at -20° C or below, and transported to the University of New Hampshire. Water samples were vacuum filtered onto 47 mm 0.2 μm pore size filter membranes (Millipore), preserved and transported as described above. Genomic DNA was extracted from corals and water filters using a PowerSoil DNA extraction kit (MoBio) and stored at -20° C. A CTAB protocol was used to extract DNA from water filters. Briefly, samples were minced with a razor blade and homogenized with a plastic pestle in 600 μl CTAB buffer (cetyltrimethyl ammonium bromide 2% w/v, EDTA 0.744 % w/v, sodium chloride 8.18% w/v, Tris base 1.21% w/v, β-mercaptoethanol 0.2% v/v), incubated with 5μl 20 mg ml^{-1} Proteinase K at 65° C for 2 to 3 hours, and extracted with an equal volume of chloroform. DNA was precipitated with cold 100% ethanol, washed twice with 70% ethanol, the pellet was air-dried and re-suspended in molecular grade water, and stored at -20° C. DNA samples were quantitated with a Nanodrop 2000c spectrophotometer (Thermo Scientific) and CTAB and PowerSoil extractions of water filters were pooled in an equimolar fashion.

Primer Testing

Several different PCR primer sets were tested to determine the best approach for amplifying both Bacterial and Archaeal 16s rRNA genes. Primers were designed to be complementary to either Eubacteria or Archaea, or were 'universal' and had good theoretical coverage in both domains. First, a eubacterial primer set was sequenced on a pilot scale. Pyrosequencing fusion primers consisting of Roche Titanium A (forward) or B (reverse) adapter sequences and a 10-base molecular identifier (MID) tag unique to each sample prepended to PCR primers 968-F (5'-AACGCGAAGAACCTTAC-3') and 1401-R (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (S. Minocha, University of New Hampshire). The final amplicon size produced by these primers was 503 bp. DNA from two samples of *M. cavernosa*, one brown and one orange collected from LSI in 2010, was used as template in triplicate PCR reactions containing 0.5μl Titanium *Taq* polymerase (Clontech), 1 × Titanium Taq PCR buffer, 0.2 mM dNTPs (Promega), 1 μM of each primer, and 7 or 6.2 ng total template (*M. cavernosa* brown and orange, respectively). Cycling conditions were 95° for 2 min, followed by 30 cycles of 95° for 1 min, 53° for 45 sec, 72° for 3 min, and a final extension at 72° for 10 min. Five μl of each PCR product was visualized on a 1% agarose gel to check for amplification, then PCR products were pooled, separated on a gel, and excised bands were purified using a QIAquick Gel Extraction kit (Qiagen). PCR products were quantitated with a Nanodrop

2000c spectrophotometer (ThermoScientific) and pooled in an equimolar fashion, further purified using AMPure XP beads (Agencourt), and quantitated with a DynaQuant 200 fluorometer (GE Healthcare). The final pooled products were sent to the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign where bidirectional multiplex pyrosequencing was carried out on 1/8 of a picotiter plate.

Primers specific to Archaea were also tested (Baker, *et al.*, 2003, Teske & Sorensen, 2008, Wang & Qian, 2009, Lee, *et al.*, 2010, Porat, *et al.*, 2010) (Table 2.1). For each primer pair, multiple samples of brown and orange *Montastraea cavernosa* DNA were tested in PCR reactions containing 0.25μl Titanium *Taq* polymerase (Clontech), 1 × Titanium Taq PCR buffer, 0.2 mM dNTPs (Promega), 1.25 μM of each primer, and 25 ng total template. Reactions were incubated at 95° for 5 min, followed by 30 cycles of 95° for 1 min, x° for 1 min, and 72° for 1 min, where x is given for each primer pair tested in Table 2.2. PCR products were visualized and if present, bands of the correct size were excised and gel purified as described above, then ligated overnight into pGEM-T Easy vectors and transformed into JM109 high-efficiency competent cells with a Promega cloning kit according to the manufacturer's directions. Cells were incubated at 37° C for 24 hr on Luria-Bertani (LB) agar plates with 100 μg ml^{-1} ampicillin, spread with 100 μl of 100 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and 20 μl of 50 mg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). White colonies were transferred to LB broth with ampicillin and grown overnight at 37° C, then plasmids were isolated using a PureYield Plasmid Miniprep kit (Promega) and inserts were Sanger sequenced from primer SP6 at the University of New Hampshire Hubbard Center for

Genome Studies DNA sequencing core facility. For the U789F/U1068R primer set, frozen cultures were sent to Functional Biosciences for plasmid isolation and Sanger sequencing from the T7 primer. Sequences were trimmed in Geneious, aligned to the SILVA reference database (version 108), and classified to the least common ancestor (LCA) against the SILVA taxonomy. The closest sequence match in the SILVA database was also recorded.

Pyrosequencing with Universal Primers

The universal U789F/U1068R primer set was selected for full-scale sequencing due to its performance in the previously described primer testing, its ability to capture 94.8 – 97.7 % of publically available bacterial and Archaeal 16s rRNA sequences (Wang & Qian, 2009), and its proven success in sponge and marine water samples (Lee, *et al.*, 2010). The V5-V6 hypervariable regions of the 16s rRNA bracketed by these primers were amplified with PCR and sequenced by bidirectional multiplex pyrosequencing using barcoded primers (Table 2.3). Fusion primers were designed as described above using the PCR primers U789F (5'-TAGATACCCSSGTAGTCC-3') and U1068R (5'-CTGACGRCRGCCATGC-3'), then analyzed with OligoAnalyzer 3.1 (Integrated DNA Technologies) to identify primers and primer pairs with strong interactions. These primers were re-designed with different MID tags and re-tested until strong interactions were minimized. The final amplicon size including the MID tag and 454 adapters was 349 bp. Triplicate 25μl PCR reactions containing 2.5 – 78 ng total template and reagents as for Archaea primer testing were incubated under the following conditions: 95° for 5 min, followed by 30 cycles of 95° for 30 sec, 53° for 30 sec, 72° for 45 sec, and a final elongation at 72° for 6 min. PCR products were checked and purified as for the eubacterial primer pilot pyrosequencing, except that samples were pooled after AMPure XP bead purification and quantitation by fluorometry. The final pooled sample was sent to the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign where bidirectional pyrosequencing was carried out first on 1/16 of a picotiter plate to test for an equal distribution of MID tags. Based on these results, the individual samples were re-quantitated with a Qubit dsDNA HS assay kit (Invitrogen) and pooled into a new sample, which was sequenced on a full picotiter plate.

Pyrosequencing Data Analysis

Sequences from the 968F/1401R (eubacterial) and U789F/U1068R (universal) primer sets were analyzed separately, using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso, *et al.*, 2010) on the Amazon Elastic Compute Cloud (EC2) except where noted, with appropriate modifications for each primer set. For both data sets, sequences from *Xestospongia muta* samples were included in clustering along with coral samples, but were not included in downstream analyses. Raw sequence reads less than 300 (eubacterial) or 200 (universal) bp in length, with more than two mismatches with the primer sequence, with ambiguous nucleotides, or with an average quality score less than 25 were discarded. Primers were trimmed from the sequences, reads were assigned to their samples of origin based on MID tags, and reads originating from the B adapter (i.e., reverse reads) were reverse complemented. Reads were clustered with trie, which collapses reads that are prefixes of each other into clusters (Qiime team, unpublished), and singleton reads that were not part of any cluster were discarded.

Singleton reads are almost universally erroneous, and their removal is a rapid and effective alternative to computationally intensive denoising for reducing error within a population of reads (Behnke, *et al.*, 2011). Remaining reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using the uclust algorithm (Edgar, 2010) and settings --max_accepts 20, --max_rejects 500, --stepwords 20, and --word_length 12, and the most abundant sequence in each cluster was selected as the representative sequence. Representative sequences were imported into ARB (Ludwig, *et al.*, 2004) and aligned to the SILVA non-redundant reference database, release 108 (Pruesse, *et al.*, 2007) using the SINA plug-in. Aligned sequences exported from ARB were re-formatted for use in the QIIME pipeline using QIIME and custom Perl scripts. Sequences that were poorly aligned (defined as sequences with a run of at least 50 nucleotides without gaps), flagged as possibly chimeric by UCHIME (Haas, *et al.*, 2011), or with significant BLAST (Altschul, *et al.*, 1990) matches to a custom database of likely contaminants were removed from further analysis. The contaminants database was built from the SILVA reference database and contained 18s rRNA sequences for alveolates (including *Symbiodinium),* Faviid corals, and Demospongiae. Sequences were searched with a word size of 50 and were considered significant matches if the *E*-value was less than 1×10^{-10} and percent identity was at least 97%. The identity of OTUs was determined by assigning taxonomy to each representative sequence using the RDP classifier and a minimum confidence cutoff of 0.8 (Wang, *et al.*, 2007) within QIIME. OTUs assigned as "Root" or "Root:Bacteria" were considered suspect and BLASTed against the NCBI nr database, and removed from further analysis if the top hit was not 16s rRNA. OTUs with "Chloroplast" in the RDP assigned taxonomy were also removed.

OTU tables were rarefied to equalize sampling depth across all samples, then observed species were counted and rarefaction curves were drawn. Eubacterial OTU tables were subsampled at a depth of 100 to 7,002 sequences with 30 steps in between, and rarefaction was repeated a total of 10 times. Universal OTU tables were rarefied in the same manner from 100 to 26,518 sequences with 50 steps in between. An approximately-maximum-likelihood phylogenetic tree was created for each dataset with Fasttree 2 (Price, *et al.*, 2010) in QIIME and used to calculate weighted UniFrac distance values and perform the UniFrac Monte Carlo significance test (Hamady, *et al.*, 2010). These distance values were used to generate principal coordinates which were plotted in 2 dimensions. Analyses of variance and G-tests were used to determine if any OTUs were significantly more abundant or more commonly present in a given sample type. Read count data for OTUs were square root transformed and the Bray-Curtis similarity index was calculated in PRIMER; the resulting similarity matrix was used in multidimensional scaling (MDS) and analysis of similarities (ANOSIM).

OTUs assigned as Cyanobacteria from sequencing with Eubacterial primers were analyzed separately. Sequences of cyanobacterial OTUs were aligned to the SILVA reference database using the SINA aligner, the closest match in the SILVA database was determined at a minimum identity of 0.95. Query sequences were classified with the lowest common ancestor algorithm against the SILVA and RDP taxonomies, with the top 90% of the search results used in LCA classification. OTUs were then imported to ARB, aligned, and added to the reference tree using the parsimony method. The reference tree was pruned, retaining imported OTUs and their closest matches from the database search, as well as other taxa of interest.

In-silico primer coverage comparison

Cyanobacteria are a group of primary interest to this study, so an *in silico* comparison of primer coverage within this group was performed using the ProbeMatch tool on the Ribosomal Database Project (RDP) website (Cole, *et al.*, 2009). Coverage of the eubacterial and universal PCR primer pairs was determined and compared for 0, 1, and 2 mismatches at various levels of the cyanobacterial phylogeny.

Results

Eubacterial Primer Testing

Pyrosequencing of eubacterial PCR amplicons resulted in 49,885 total reads, 43,359 of which passed quality filters and 21,242 of which were non-singletons. Clustering at a similarity threshold of 97% produced 848 OTUs. Screening with UCHIME resulted in 9.3% of OTUs (n=79) being flagged as chimeras and removed. OTUs that did not align to the reference database (n=20) or had best BLAST matches that were not prokaryotic 16S rRNA genes (n=14) were also removed from analysis; 735 OTUs remained and were used in all downstream analyses. Rarefaction curves of OTUs (Figure 2.1) reached asymptotes well before the maximum sequencing depth, indicating that the sequence recovery for these communities was saturated.

OTUs were assigned to 14 different phyla by RDP, but 22.1% of reads in orange and 54.3 % of reads in brown *M. cavernosa* were classified only as bacteria and could not be assigned to a phylum (Figure 2.2). The most abundant phyla in orange *M. cavernosa* were Cyanobacteria (34%), Proteobacteria (29%), unassigned bacteria (22.1%), and

Actinobacteria (5.8%) whereas brown *M. cavernosa* was markedly different, with unassigned bacteria most abundant (54.3%), followed by Proteobacteria (27.5%), Bacteriodetes (6.2%), and Actinobacteria (4.3%). Within the Proteobacteria, α-proteobacteria were more abundant in orange than brown *M. cavernosa* (18.8% vs 7.8%, respectively), while γ-proteobacteria were more common in brown than orange (12.4% and 5.2%).

Putative Cyanobacterial Symbionts

A diverse and abundant putative cyanobacterial symbiont group was found exclusively in the orange colony. The cyanobacterial community was dominated by only a few OTUs, but a tail of low-abundance OTUs was also present (Figure 2.3). Lowest common ancestor classification with the SILVA reference database classified three of the top five most abundant cyanobacterial OTUs as *Pleurocapsa* (OTUs 86, 761, and 421), one OTU as *Planktothrix* (OTU 0), but the most abundant OTU (OTU 793) could not be assigned at the given similarity cutoff (Table 2.4). When they were added to the SILVA reference tree, OTUs 86, 761, and 421 grouped together with *Pleurocapsa* and closely related groups, OTU 793 clustered with *Gloeocapsopsis crepidinium*, *Nostoc,* and *Trichormus azollae,* and OTU 0 was most closely related to *Trichodesmium erythraeum, Planktothrix rubescens,* and other planktonic cyanobacteria (Figure 2.4). The first two groups, although separate from each other, both include known symbionts, such as the spheroid bodies of *Rhopalodia gibba* (AJ582391), the symbiont of *Climacodium frauenfeldianum* (AF193247), and *Trichormus azollae* (AJ630454). Also found in both groups are sequences isolated from or known to be found in extreme environments, such as thermal springs (*Pleurocapsa* DQ293994, unicellular thermophilic cyanobacterium DQ471449) and desert hypoliths (*Myxosarcina* AJ344561, *Chrococcidiopsis polansiana* AJ344556, uncultured bacterium HM241058), as well as known nitrogen fixers (*Pleurocapsa, Rhopalodia gibba,* and *Trichormus azollae*). These two groups may represent a mixed, diverse population of nitrogen-fixing cyanobacterial symbionts.

OTU 0 was most closely related to planktonic cyanobacteria such as *Trichodesmium* (Figure 2.4). This clade does not include any known symbionts and may represent microbes from the water column that were trapped in the mucus of the coral and not completely removed by airbrushing. Similarly, many other low-abundance OTUs also group with other cyanobacteria that are known to be planktonic, such as OTUs 469 and 239, which are closely related to WH7803 and other strains of *Synechococcus.*

The cyanobacterial sequence originally amplified from *M. cavernosa* using cyanobacteria-specific primers when the symbiosis was first described was also added to the tree. This sequence clustered with an OTU from the brown colony and Candidatus *Synechococcus spongiarum* from the sponge *Xestospongia muta*, but was not closely related to any of the high-abundance OTUs from the orange colony in this study.

Archaea and Universal Primer Testing

Testing of Archaea-specific primers yielded five primer pairs that produced clear, bright bands (amplicons) of the anticipated size. These amplicons were cloned and a small number (n = 2 - 4) of clones were sequenced for each primer pair. These sequences were primarily 18S rRNA, Eubacterial 16S rRNA, or had no matches of prokaryotic origin in the NCBI nr database. A small clone library was constructed from the universal primers U789F and U1068R, which recovered 16 different Eubacterial sequences from 17 16S rRNA clones (Table 2.5).

Pyrosequencing with Universal Primers

The full-scale pyrosequencing of universal primer amplicons produced 804,513 total reads, 711,780 of which passed quality filters. Singleton reads (n= 175,880) were discarded, for a final total of 535,900 reads. From these, 4,609 OTUs clustering at 97% similarity were identified. 1,080 of these OTUs were discarded because they did not align to the reference alignment (791), were flagged as chimeras (94) were contaminants (7), or had a best BLAST match to a non-16S rRNA sequence in the NCBI nr database (189). All water samples were sequenced to saturation (representative samples shown, Figure 2.5). Rarefaction curves of observed OTUs did not reach an asymptote for some coral samples (Figure 2.5).

Analyses of β-diversity were performed using several different diversity metrics to describe both community diversity and the factors influencing community structure, an approach that has been advocated by several investigators (Kuczynski, *et al.*, 2010, Anderson, *et al.*, 2011). Multidimensional scaling (MDS) of the Bray-Curtis similarity index on square-root transformed abundance data showed that communities in corals and water are distinct, but coral-associated prokaryotes are not different in brown and orange *M. cavernosa* (Figure 2.6). Principal coordinates analysis (PCoA) of the weighted UniFrac distance, a phylogenetic diversity metric that takes into account the evolutionary distance between communities and the abundance of sequences (Lozupone, *et al.*, 2006, Lozupone, *et al.*, 2007), had similar results (Figure 2.7). The first two principal coordinates explained 37.12% and 25.23% of the variation between samples. Significance testing using the same weighted UniFrac metric found that few samples were significantly different from each other (Table 2.6). Clustering based on Bray-Curtis similarity separated water samples from corals, and brown and orange coral samples from LSI were grouped into a single cluster (Figure 2.8).

Analyses of similarity (ANOSIM) were performed in PRIMER 5.0 to test for differences between groups of samples. Water samples were significantly different from coral samples (P= 0.01, Global R = 0.846), as suggested by their groupings on MDS and PCoA plots (Figures 2.6, 2.7). A two-way ANOSIM on coral samples found that brown and orange coral samples were not significantly different (P= 0.683, Global R = -0.062), but samples from all three different locations were (P=0.02, global R =0.543; FL vs. LC: R statistic = 0.407, P = 0.01; FL vs. LSI: R statistic = 0.444, P = 0.05; LC vs. LSI: R statistic = 0.796, P = 0.01). ANOVA and the G test of independence were used to determine which OTUs, if any, were significantly associated with a sample type. A number of OTUs were significantly more abundant or more commonly present in water samples (ANOVA and G test, respectively, Bonferroni corrected $P < 0.05$), but no OTUs were significantly different between brown and orange coral samples or between coral samples from different locations.

Taxonomic classification of OTUs at the phylum level by RDP revealed the patterns in diversity of prokaryotic communities possibly contributing to the observed differences between water and coral samples (Figure 2.9). Water samples from all locations contained a similar diversity of prokaryotes, with differences in the relative abundance of some taxa at different sampling locations. In water samples from LSI, unassigned bacteria were more abundant than at other locations, but cyanobacteria were less abundant. Euryarchaeota and other Archaea were more abundant in water from FL and water from 30 m depth at LC. Communities in coral samples were more diverse than water samples and were highly variable, even within replicate samples of the same color morph from the same location. Coral samples contained several phyla that were rare or absent in water samples, including Acidobacteria, Chlamydiae, Chlorobi, Chloroflexi, Firmicutes, and Candidatus Poribacteria which is a group of bacteria usually identified as symbionts of sponges. Bacteria that could not be assigned to a phylum were also more abundant in coral samples, indicating that these communities are not well represented in reference databases. Although the total percentage of reads assigned to Archaea was low, some patterns in the diversity of this group were apparent. Water samples contained mostly Euryarchaeota, while coral samples hosted primarily Crenarchaeota (Figure 2.9). Among coral samples from LSI, brown colonies consistently had more Archaea than orange colonies; Archaeal communities were similar in brown and orange samples from FL and LC.

Proteobacteria dominated both water and coral samples, but the composition of proteobacterial orders was very different. Water samples were primarily composed of α-proteobacteria, including Rickettsiales, Rhodospirillales, and Rhodobacterales, and the γ-proteobacteria Oceanospirillales were also prominent (Figure 2.10). The proteobacterial community in the water sample from 30 m at LC clearly differentiated this sample from other water samples, with an increased abundance of Alteromonadales, Rhodobacterales, and a decrease in Rhodospirillales. Communities in coral samples were more variable and diverse than those in water, similar to the pattern seen for other prokaryotic phyla (Figure

2.10). Rhizobiales and Vibrionales were often more abundant in coral samples than water samples. Vibrionales and Alteromonadales both contain known or suspected coral pathogens and were consistently less abundant in samples from LSI than from FL or LC. Desulfovibrionales were prominent in a single orange colony from LC; 95% of these reads originated from a single OTU in this sample, which shares 100% identity with the corresponding region of the 16s rRNA sequence of *Desulfovibrio marinisediminis* (Takii, *et al.*, 2008). Very similar sulfate-reducing bacteria have also been repeatedly isolated from corals infected with black band disease (Viehman, *et al.*, 2006).

Many of the reads assigned as cyanobacteria, particularly in coral samples, originated from chloroplasts (Figure 2.11). Cyanobacterial reads in water samples were mostly classified as Synechococcales, whereas those in coral samples were primarily chloroplasts. The true cyanobacteria that were present in coral samples included groups such as the Pseudoanabaenales, Chroococcales, and Oscillatoriales that were absent from the water column. Chloroplasts from candidate division CAB-I and Chlorophyta dominated coral libraries, although stramenopile and Rhodophyta chloroplasts were abundant in some samples. Several of the most abundant Chlorophyta OTUs were highly similar to *Ostreobium,* a green alga commonly found living in coral skeletons (Lukas, 1974). A cyanobacterial OTU represented by 4801 reads in a single coral sample (McBr.LC.1) was most closely related to the chloroplast of *Fucus vesiculosus* (94% sequence similarity to FM957154.1, $e = 10^{-100}$) and was suspected to be a contaminant, perhaps representing benthic algae that were inadvertently sampled. This OTU and all other chloroplast OTUs were excluded from analyses.

The universal primer set did not appear to re-capture the putative cyanobacterial symbionts that were seen from pyrosequencing with eubacterial primers. True cyanobacterial reads made up only a small proportion of coral sample libraries, and no clear increase in cyanobacterial reads was observed in orange coral samples using the universal primer set. While 34% of the reads were cyanobacterial in the orange sample of *M. cavernosa* sequenced with eubacterial primers (Figure 2.2), the greatest abundance of cyanobacterial reads from sequencing with universal primers was just 4%, found in an orange coral sample from the same location, LSI. Non-chloroplast cyanobacterial reads made up on average 8.2% of the reads in water samples, an unusually low proportion that led to the investigation of differences in primer coverage discussed below (In-silico primer comparison). Given that coral-associated cyanobacteria appear to be poorly represented in the universal primer data set, the community analyses above address only the non-cyanobacterial portion of the prokaryotic communities in orange and brown *M. cavernosa* samples.

Generally speaking, β-diversity analyses did not reveal clear differences between prokaryotic communities in brown and orange *M. cavernosa*, although coral and water samples were significantly different by all metrics. The absence of reads from the putative cyanobacterial symbionts in the universal primer data set likely contributed to the apparent close similarity of the captured brown and orange communities. If the symbionts had been captured, these sequences might have differentiated the two colony types.

In-Silico Primer Coverage Comparison

Coverage of the cyanobacteria sequences in the RDP database was compared for the universal and eubacterial primer sets. The percentage of total sequences that matched primers perfectly (0 mismatches), and with 1 and 2 mismatches was determined for each family of cyanobacteria.

While the total coverage of the universal primer set was higher for most families, the eubacterial primers perfectly matched a higher percentage of sequences than the universal primers for all families (Figure 2.12). Total coverage of each primer set was less than 50% for most families.

Discussion

Eubacterial Primer Sequencing

Pyrosequencing with Eubacterial primers captured a diverse coral-associated bacterial community, as well as putative cyanobacterial symbionts (Figure 2.2). The dominant bacterial phyla in brown and orange *Montastraea cavernosa* samples were different, with the brown sample dominated by unassigned bacteria, and the orange sample by cyanobacteria. Cyanobacterial OTUs found exclusively in the orange colony were designated as putative symbionts, but it must be noted that without replicate samples of each colony type to demonstrate that these cyanobacteria are consistently present in orange samples and absent in brown samples, it is not possible to conclusively identify these cyanobacteria as symbionts. However, the large differences in abundance of all cyanobacterial OTUs between the orange and brown sample (Figure 2.2), in combination with the high density (10^7 per cm^2) of cyanobacterial symbionts in coral tissue (Lesser, *et al.*, 2004), suggests that these sequences likely do represent the symbionts.

The most abundant putative symbiont OTUs grouped into three clades, with three of the top five OTUs similar to *Pleurocapsa*, one closely related to *Gloeocapsopsis*, and one affiliated with *Trichodesmium* and *Planktothrix* (Figure 2.4). The *Pleurocapsa* group contains sequences from nitrogen-fixing symbionts of the diatoms *Rhopaloida gibba* (Prechtl, *et al.*, 2004) and *Climacodium frauenfeldianum* (Foster, *et al.*, 2011), and the *Gloeocapsopsis* group includes *Trichormus azollae,* a diazotroph associated with the water fern *Azolla* (Baker, *et al.*, 2003). Interestingly, both of these groups also include taxa that are found in extreme environments; *Pleurocapsa* and the unicellular thermophilic cyanobacterium (DQ471449) can be found in thermal springs (Ward & Castenholz, 2002), and *Chrococcoidiopsis, Myxosarcina,* and an uncultured bacterium (HM241058) exist as hypoliths on the undersides of rocks in desert environments (López-Cortés, *et al.*, 2001). The affiliation of bacteria from corals with those from extreme environments may seem unusual, but the high redox potential often found in hot springs (Segerer, *et al.*, 1993) and coral tissues (Dykens & Shick, 1982) may create similar selective forces in both environments. The presence of multiple phylogenetically distinct and abundant groups of cyanobacteria associated with *M. cavernosa* suggests that the symbionts may exist as a mixed population, perhaps serving different ecological or functional roles in the coral holobiont. The *Planktothrix* group and other low-abundance OTUs are likely to represent bacteria from the water column that were trapped in the coral mucus and not completely removed by airbrushing, because the *Planktothrix* group does not include any known symbionts. The presence of a cyanobacterial OTU from the orange colony in this group supports this hypothesis.

A cyanobacterial sequence isolated from orange *M. cavernosa* when the symbiosis was initially described (Lesser, *et al.*, 2004) was not recaptured from the orange colony in this study. Colonies in both studies were sampled from the same location, LSI, so genetic differences in cyanobacteria due to biogeographic differences seem unlikely. The cyanobacteria-specific 16s rRNA primers used in the 2004 study may have preferentially amplified a low-abundance bacterial population, perhaps explaining why a similar OTU was only found in the brown colony in this study. Both sequences were closely related to Candidatus *Synechococcus spongiarum*, which has previously been reported exclusively in association with sponges, including the giant barrel sponge *Xestospongia muta* (Erwin & Thacker, 2007, Erwin & Thacker, 2008). Like *M. cavernosa, X. muta* is abundant on Caribbean coral reefs and has a very broad depth range. Microbes associated with sponges and corals experience similar frequent fluctuations in oxygen concentrations, so these hosts may share some members of their microbial communities (Fiore, *et al.*, 2010). Although the results of this sequencing suggest that these sequences are not the cyanobacterial symbionts, they may represent generally coral-associated cyanobacteria, which have been found in many other studies (Rohwer, *et al.*, 2002, Hong, *et al.*, 2009, Sunagawa, *et al.*, 2010, Ceh, *et al.*, 2011, Chen, *et al.*, 2011, Meron, *et al.*, 2011).

Although the results from eubacterial primers were promising, we also wanted to characterize the Archaeal community associated with *M. cavernosa.* Archaea living on corals are diverse, novel, and highly abundant, making up nearly 50% of the total prokaryotic population in *Porites astreoides* (Wegley, *et al.*, 2004), they do not appear to form host species-specific associations with corals (Kellogg, 2004, Wegley, *et al.*, 2004). In this study and others, Archaea were found to make up only a small fraction of the reads in 16S rRNA libraries. However, a recent study in the sponge *Geodia barretti* found low representation of Archaea in 16S clone libraries despite the high abundance and transcriptional activity of these cells (Radax, *et al.*, 2012). The low ribosome content of most Archaea may account for this bias (Valentine, 2007), and suggests that Archaea may be more important members of the holobiont than previously thought. None of the Archaea-specific primers tested gave satisfactory results, so a universal primer set that amplified both eubacteria and Archaea was selected. This primer set had previously been used successfully with several species of sponges from the Red Sea, as well as corresponding water samples (Lee, *et al.*, 2010). It was known *a priori* that Archaea were likely to make up only a small proportion of the total reads, as in the previous study.

Universal Primer Sequencing

A diverse prokaryotic community of both Eubacteria and Archaea was recovered using universal primers. Unfortunately, the putative symbiotic cyanobacteria identified in sequencing with universal primers were not recaptured. Most OTUs assigned as cyanobacteria originated from chloroplasts, and after these OTUs were excluded from analysis, very few cyanobacterial OTUs and reads remained. The maximum abundance of cyanobacteria in any coral sample was just 4%, in an orange sample from LSI. Given that the symbiotic cyanobacteria are present in high densities and that cyanobacterial reads and OTUs were abundant in the orange colony sequenced with eubacterial primers, a much greater proportion of cyanobacterial reads would be expected if the symbionts were captured with the universal primers. Other lines of evidence including flow cytometry, electron microscopy, and acetylene reduction assays support that the symbiotic cyanobacteria are indeed present, but were not captured by this primer set. Cyanobacteria made up on average just 8.2% of reads from water samples, an unusually low proportion compared to other studies, where cyanobacteria typically compose approximately 30-40% of reads (Frias-Lopez, *et al.*, 2002, Sunagawa, *et al.*, 2010), so primer bias against cyanobacterial sequences, discussed below, is probably responsible.

Without the cyanobacterial OTUs, this study is essentially comparing the prokaryotic communities less the cyanobacteria, addressing the question of whether the cyanobacteria or the fixed nitrogen they provide influence the coral-associated prokaryotic community. No significant differences were observed between brown and orange coral samples at any location, or as a whole, for any metric tested (Figures 2.6, 2.7). The highly abundant putative symbiotic cyanobacteria found in sequencing with eubacterial primers are likely to be primarily responsible for distinguishing the prokaryotic communities in the two colony types, so their absence in sequences obtained with the universal primers makes these samples seem more similar than if the entire prokaryotic community had been captured. The universal primers may also have been biased against other taxonomic groups whose presence could have distinguished the colony types, although this cannot be confirmed with these data.

Prokaryotic communities in coral samples were significantly different from those in water samples, and contained taxa not present in the water column. One group of particular interest is the Rhizobiales, an abundant part of the proteobacterial fraction of reads in some but not all coral samples (Figure 2.10). Rhizobiales have been repeatedly implicated as nitrogen fixers associated with corals, and similar *Bradyrhizobium*-like sequences for the nitrogenase enzyme (*nifH*) have been recovered from *M. cavernosa* and other corals in Hawai'i and Australia (Olson, *et al.*, 2009, Olson, 2010, Lema, *et al.*, 2012). These reads may represent a generally coral-associated group with functional importance in nitrogen cycling within the holobiont.

The overall communities in water samples were relatively consistent among replicate samples and locations, but communities associated with corals were highly variable, even within replicate samples of the same colony color from the same location (Figure 2.9). There is disagreement as to the degree of variability that can be expected between replicate coral samples, perhaps because of differences in spatial scale, sampling methodology, and the method used to characterize the microbial community. Using clone libraries, DGGE, and T-RFLP, Littman *et al.* (2009) found that replicate samples of three species of branching *Acropora* were highly similar, and samples of the massive coral *Montastraea annularis* that were pyrosequenced by Barott *et al.* (2011) were also very similar to each other. However, other studies have indicated that prokaryotic communities associated with corals may be much more variable, even at small spatial scales (i.e., within a single colony) (Hong, *et al.*, 2009, Kvennefors, *et al.*, 2010, Chen, *et al.*, 2011, Daniels, *et al.*, 2011). This may be due to the temporary dominance of a particular group of bacteria (Chen, *et al.*, 2011); such variation is greatest in the summer (Hong, *et al.*, 2009), which was when samples for this study were collected. It has been suggested that samples from at least six replicate colonies are required to capture all of the prokaryotic diversity associated with a particular species of coral at a particular site (using DGGE, (Kvennefors, *et al.*, 2010), but this level of replication is rarely achieved.

Prokaryotic communities associated with *M. cavernosa* were significantly different at all three sampling locations, despite the variability between replicate coral samples. Differences in coral-associated prokaryotic communities at different sites are commonly observed at spatial scales from less than 1 km to hundreds of km (Reia & John, 2006, Littman, *et al.*, 2009, Kvennefors, *et al.*, 2010, Barott, *et al.*, 2011). Interestingly, water from FL and LC contained more reads assigned to the order Vibrionales, and reads from coral disease-associated taxa were more abundant in coral samples from these locations (Figure 2.10). A similar correspondence between communities in the water column and in corals was observed at a much smaller scale, at sites near Heron Island on the GBR, indicating that local environmental conditions are likely to influence both free-living and host-associated prokaryotic communities (Kvennefors, *et al.*, 2010). Given the much larger distance between locations in this study, differences between locations are expected to be even more distinct.

In some samples of *M. cavernosa*, proteobacterial taxa that have been associated with diseased or stressed corals in previous studies were abundant or even dominant members of the prokaryotic community (Figure 2.10). Other studies have characterized various species within Rhodobacterales, Clostridiales, Camplyobacterales, Alteromonadaceae, Vibrionales, and Desulfovibironales as being associated with corals affected with several different diseases or syndromes (Mitchell & Chet, 1975, Frias-Lopez, *et al.*, 2002, Frias-Lopez, *et al.*, 2003, Sunagawa, *et al.*, 2009) as well as seemingly healthy corals that have been subjected to stress (Ritchie, 2006, Garren, *et al.*, 2009, Vega Thurber, *et al.*, 2009, Littman, *et al.*, 2010). However, members of the genus *Vibrio* associated with *Montipora* in Hawai'i have also been found to contain functional *nifH* sequences (Olson, *et al.*, 2009), and most of the culturable nitrogen-fixing bacteria from the coral *Mussismilia* in Brazil were *Vibrios* (Chimetto, *et al.*, 2008), suggesting that this genus may be beneficial to the holobiont. The γ-proteobacterial orders Vibrionales and Alteromonadales were the most common stress- or disease-associated taxa in this study, and seemed to be more frequently associated with corals from LC and FL. The water column at both of these locations also contained more reads assigned to Vibrionales than water from LSI. There was no clear association between the presence of cyanobacteria and the abundance of stress-associated groups. However, one orange colony from LC did contain many reads from *Desulfovibrio marinisediminis* (Takii, *et al.*, 2008), which is free-living in sediments and genetically very similar to sulfate-reducing bacteria that have been repeatedly isolated from colonies affected by black band disease (Viehman, *et al.*, 2006). This colony appeared to be healthy when visually inspected before sampling, but the presence of this OTU could indicate that this colony was in the preliminary stages of black band disease. Alternatively, this OTU could have arisen from sediment that was inadvertently collected along with the coral.

Comparison of Results from Eubacterial and Universal Primers

The eubacterial and universal primer sets yielded conflicting results, particularly about the key cyanobacterial members of the prokaryotic community. The eubacterial primers recovered a diverse set of OTUs from putative symbiotic cyanobacteria, whereas the universal primers produced mainly chloroplast sequences and did not recover the cyanobacterial symbionts. Marked differences in the coverage of each family of cyanobacteria for the two primer sets were suspected to be responsible for the lack of cyanobacterial reads from sequencing with universal primers (Figure 2.12). The largest difference between the coverage of the two primer sets was in the number of sequences with perfect matches to the primers (i.e., 0 mismatches). Universal primers did not match any sequences perfectly in Family III or Families V – XIII. Based on the number of perfect matches to the primer in each cyanobacterial family, the cyanobacterial group most effectively captured by the universal primers is chloroplasts, a prediction that is confirmed by the prevalence of reads originating from chloroplasts in this dataset. Sequences perfectly matching the primers are known to be preferentially amplified over sequences with mismatches; even sequences making up a high proportion of a mixed community may be nearly absent in the amplicon pool due to this phenomenon (Sipos, *et al.*, 2007). Thus, it appears that strong bias against most cyanobacterial families in the universal primer set is responsible for the failure to capture the cyanobacterial symbionts. When an abundant and likely functionally important member of the prokaryotic community is known to be missing from the results, it is difficult to draw robust conclusions about differences or patterns in these communities.

The contrasting results of the two primer sets used in this study illustrate the seriousness of primer bias. While bias against rare taxa or groups poorly represented in reference databases, such as the Nanoarchaeota (Baker, *et al.*, 2003), is widely known, it has been believed that different primer sets would reveal similar patterns in a given prokaryotic community, and that similar overall conclusions could be reached. The failure of biased primers to recover numerically and functionally significant members of the community, and the incorrect or incomplete conclusions resulting from this, has only recently been appreciated (Hong, *et al.*, 2009). A recent comprehensive analysis of the taxonomic informativeness of thousands of combinations of primers, amplicon length, and environment showed that the selection of 16S rRNA gene region, primers, and amplicon length is critical to generate the most informative data set for a given level of sequencing effort (Soergel, *et al.*, 2012)Different regions of the 16S gene varied in informativeness, but contrary to what many other studies have found, it was not necessary to target hypervariable regions. Most importantly, different primers will amplify different proportions of the total diversity in different environments; this was shown to be particularly problematic in environments such as coral reefs whose diversity is under-represented in reference databases used to assign taxonomy. In the case of this study, the universal primer set did not recover the symbiotic cyanobacteria observed in the initial analysis using eubacterial primers. If sequencing had been the only method used to investigate the prokaryotic community of *M. cavernosa*, this symbiosis might never have been discovered. Soergel *et al.* (2012) recommend *a priori* validation of the combination of primers, read length, environment, and reference database for all studies; while this may not be feasible for all investigators, the coverage of different taxonomic groups by a primer set is easy to determine using tools like the RPD Probe Match utility (Cole, *et al.*, 2009). However, even with the most thorough optimization of these parameters, the coverage of taxa that are remaining to be discovered and are thus absent from reference databases cannot be determined.

Shotgun approaches to characterizing microbial diversity are able to overcome the inherent biases of PCR-based approaches, and are becoming increasingly popular for this reason. These approaches also provide a great deal of functional information in addition to the taxonomic information provided by classical PCR-based amplification of ribosomal genes. Shotgun sequencing of DNA or RNA (metagenomics and metatranscriptomics, respectively) from an environmental sample can be used to characterize the function of the entire community. If RNA samples are used, the abundance of a given rRNA or transcript can be used as a proxy for the activity level of the given species or gene, respectively (Gaidos, *et al.*, 2011, Helbling, *et al.*, 2011). A major drawback of shotgun approaches is that a usually only a small fraction of reads can be annotated functionally or taxonomically, and the increasing volume of reads produced as sequencing costs drop has created a computational bottleneck. However, the discovery of novel sequences and the depth and breadth of information that are now easily attainable are also key factors driving the shift towards these approaches.

TABLE 2.1 Archaea-specific and universal primer sequences.

Primer	Sequence (3'-5')	Reference
U341-F	CCTACGGGRSGCAGCAG	Baker et al. 2003
A346-F	GGGGYGCAGCAGGCG	Wang and Qian 2009
A519-F	CAGCMGCCGCGGTAA	Wang and Qian 2009
U519-F	CAGCMGCCGCGGTAATWC	Baker et al. 2003
U779-R	GGGTATCTAATCCSSTTAGC	Baker et al. 2003
U789-F	TAGATACCCSSGTAGTCC	Baket et al. 2003
U789-R	GGACTACSSGGGTATCTA	Baker et al. 2003
Arch806-R	GGACTACNSGGGTMTCTAAT	Porat et al. 2010
A884-R	CGDMCGTACTYCCCA	Wang and Qian 2009
A958-F	AATTGGABTCAACGCC	Wang and Qian 2009
A1052-R	GARCTGRCGRCGGCCATGCA	Wang and Qian 2009
U1053-R	CTGACGRCRGCCATGC	Baker et al. 2003
U1068-R	CTGACGRCRGCCATGC	Baker et al. 2003
UA1406-R	ACGGGCGGTGWGTRCAA	Baker et al. 2003

TABLE 2.2 Archaea-specific PCR primer testing

Forward primer	**Reverse primer**	**Annealing temperature (°C)**	**Cloned**
U341-F	U779-R	50, 53	No
U341-F	A884-R	50, 54	No
A346-F	A884-R	55, 56, 57, 58, 60, 62, 64	No
A346-F	U779-R	50, 53	No
A346-F	U789-R	54	Yes
A519-F	A958-R	55	No
A519-F	A958-R	54, 56, 58, 60	No
A519-F	A1052-R	60	No
A519-F	U1053-R	55, 60	No
U519-F	A1052-R	55	Yes
U519-F	U1053-R	55	No
U519-F	A958-R	50	Yes
U519F	Arch806-R	55	Yes
U789-F	U1068-R	53	Yes
A958-F	UA1406-R	55	No

TABLE 2.3 Universal pyrosequencing fusion primer elements

Primer element	Sequence
MID-1	ACGAGTGCGT
MID-2	ACGCTCGACA
MID3	AGACGCACTC
MID-4	AGCACTGTAG
MID-5	ATCAGACACG
MID-6	ATATCGCGAG
MID-7	CGTGTCTCTA
MID-8	CTCGCGTGTC
MID-9	TAGTATCAGC
MID-10	TCTCTATGCG
MID-11	TGATACGTCT
MID-13	CATAGTAGTG
MID-14	CGAGAGATAC
MID-15	ATACGACGTA
MID-16	TCACGTACTA
MID-17	CGTCTAGTAC
MID-18	TCTACGTAGC
MID-19	TGTACTACTC
MID-20	ACGACTACAG
MID-21	CGTAGACTAG
MID-22	TACGAGTATG
MID-23	TACTCTCGTG
MID-24	TAGAGACGAG
MID-25	TCGTCGCTCG
MID-26	ACATACGCGT
MID-27	ACGCGAGTAT
MID-28	ACTACTATGT
MID-30	AGACTATACT
MID-32	AGTACGCTAT
MID-33	ATAGAGTACT
MID-34	CACGCTACGT
MID-35	CAGTAGACGT
MID-36	CGACGTGACT
MID-37	TACACACACT
MID-38	TACACGTGAT
MID-39	TACAGATCGT
MID-41	TAGTGTAGAT
primer F-789	TAGATACCCSSGTAGTCC

primer R-1068	CTGACGRCRGCCATGC
TitaniumA	CGTATCGCCTCCCTCGCGCCATCAG
TitaniumB	CTATGCGCCTTGCCAGCCCGCTCAG

TABLE 2.4 Taxonomic assignment of putative symbiotic cyanobacterial OTUs.

OTU	% of reads	SILVA lowest common ancestor	Closest match
793	23.7	Unclassified;	
86	21.6	Bacteria;Cyanobacteria;SubsectionII; SubgroupII; Pleurocapsa;	AJ344559
0	16.3	Bacteria;Cyanobacteria;SubsectionIII; Planktothrix;	HM126903
761	12.5	Bacteria;Cyanobacteria;SubsectionII; SubgroupII; Pleurocapsa;	HQ832924
421	5.4	Bacteria;Cyanobacteria;SubsectionII; SubgroupII; Pleurocapsa;	JF344064
208	3.4	Bacteria;Cyanobacteria;SubsectionIII; Leptolyngbya;	AB275351
385	3.1	Bacteria;Cyanobacteria;uncultured;	HM241058
535	2.4	Bacteria;Cyanobacteria;SubsectionIV; SubgroupI;	AY328898
828	1.9	Bacteria;Cyanobacteria;SubsectionII; SubgroupII; Chroococcidiopsis;	DQ532167
773	1.6	Bacteria;Cyanobacteria;SubsectionI; Synechococcus;	HM585026
94	1.3	Bacteria;Cyanobacteria;SubsectionIII; Leptolyngbya;	FJ203552
551	1.0	Bacteria;Cyanobacteria;SubsectionI;	AF132771
632	1.0	Bacteria;Cyanobacteria;	AB275351
347	0.8	Bacteria;Cyanobacteria;	FJ589716
812	0.6	Bacteria;Cyanobacteria;	HM241066
469	0.5	Bacteria;Cyanobacteria;SubsectionI; Synechococcus;	AY172803
817	0.5	Bacteria;Cyanobacteria;SubsectionI; Cyanobacterium;	EU259177
698	0.3	Bacteria;Cyanobacteria;SubsectionIII; Leptolyngbya;	AY493590
322	0.3	Unclassified;	
642	0.2	Bacteria;Cyanobacteria;SubsectionIII;	EF654035
239	0.2	Bacteria;Cyanobacteria;SubsectionI; Synechococcus;	HM129360
223	0.2	Unclassified;	
170	0.2	Unclassified;	
113	0.1	Unclassified;	
234	0.1	Unclassified;	
522	0.1	Bacteria;Cyanobacteria;SubsectionII; SubgroupII; Chroococcidiopsis;	DQ532167
594	0.1	Bacteria;Cyanobacteria;	FJ230792
727	0.1	Unclassified;	
747	0.1	Bacteria;Cyanobacteria;SubsectionI; Cyanobacterium;	EU259177
231	0.1	Bacteria;Cyanobacteria;SubsectionII; SubgroupII; Pleurocapsa;	HQ832924

TABLE 2.5 Affiliation of cloned sequences from universal primers.

Sample	Lowest common ancestor (SILVA)	Closest match

McBr	Bacteria;Acidobacteria;Holophagae	HQ153882
	Bacteria;Actinobacteria;Acidimicrobiia; Acidimicrobiales	FJ229965
	Bacteria;Actinobacteria;Acidimicrobiia; Acidimicrobiales; Sva0996 marine group	FJ229958
	Bacteria;Cyanobacteria;Chloroplast	GU119715
	Bacteria;Cyanobacteria;Chloroplast	GU119621
	Bacteria;Cyanobacteria;SubsectionI; uncultured	EF159853
	Bacteria;Nitrospirae;Nitrospira; Nitrospirales; Nitrospiraceae; Nitrospira;	GU118615
	Bacteria;Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Methylobacterium	DQ471331
	Bacteria;Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Pseudovibrio	AY372904
	Bacteria;Proteobacteria; Alphaproteobacteria; Rhodospirillales; Rhodospirillaceae	FJ202655
	Bacteria;Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Ralstonia	FJ193819
	Bacteria;Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Delftia	FJ192309
	Bacteria;Proteobacteria; Deltaproteobacteria; Sh765B-TzT-29	FJ203559
McOr	Bacteria;"Proteobacteria"; Alphaproteobacteria; unclassified_ Alphaproteobacteria;	GU118192
	Bacteria;"Planctomycetes"; "Planctomycetacia"; Planctomycetales; Planctomycetaceae;	GU118069
	Bacteria;"Proteobacteria"; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia;	AY178059

TABLE 2.6 Comparison of samples using weighted UniFrac metric. Only

comparisons with a significant Bonferroni-corrected P-value are shown.

Sample comparison		P-value
H2O.FL.1	McOr.LSI.2	< 0.01
H2O.FL.3	McOr.LSI.2	< 0.01
H2O.LC.2	McOr.LSI.3	< 0.01
H2O.LC.3	McOr.LSI.2	< 0.01
H2O.LSI.2	McBr.LSI.3	< 0.01
H2O.LSI.2	McOr.LSI.2	< 0.01
H2O.LSI.3	McBr.LSI.3	< 0.01
H2O.LSI.3	McOr.LSI.2	< 0.01
McBr.FL.2	McBr.LC.2	< 0.01
McBr.LC.2	McOr.LSI.3	< 0.01
McBr.LSI.3	McOr.LSI.3	< 0.01
McOr.FL.3	McOr.LSI.2	< 0.01
McOr.FL.3	McOr.LSI.3	< 0.01

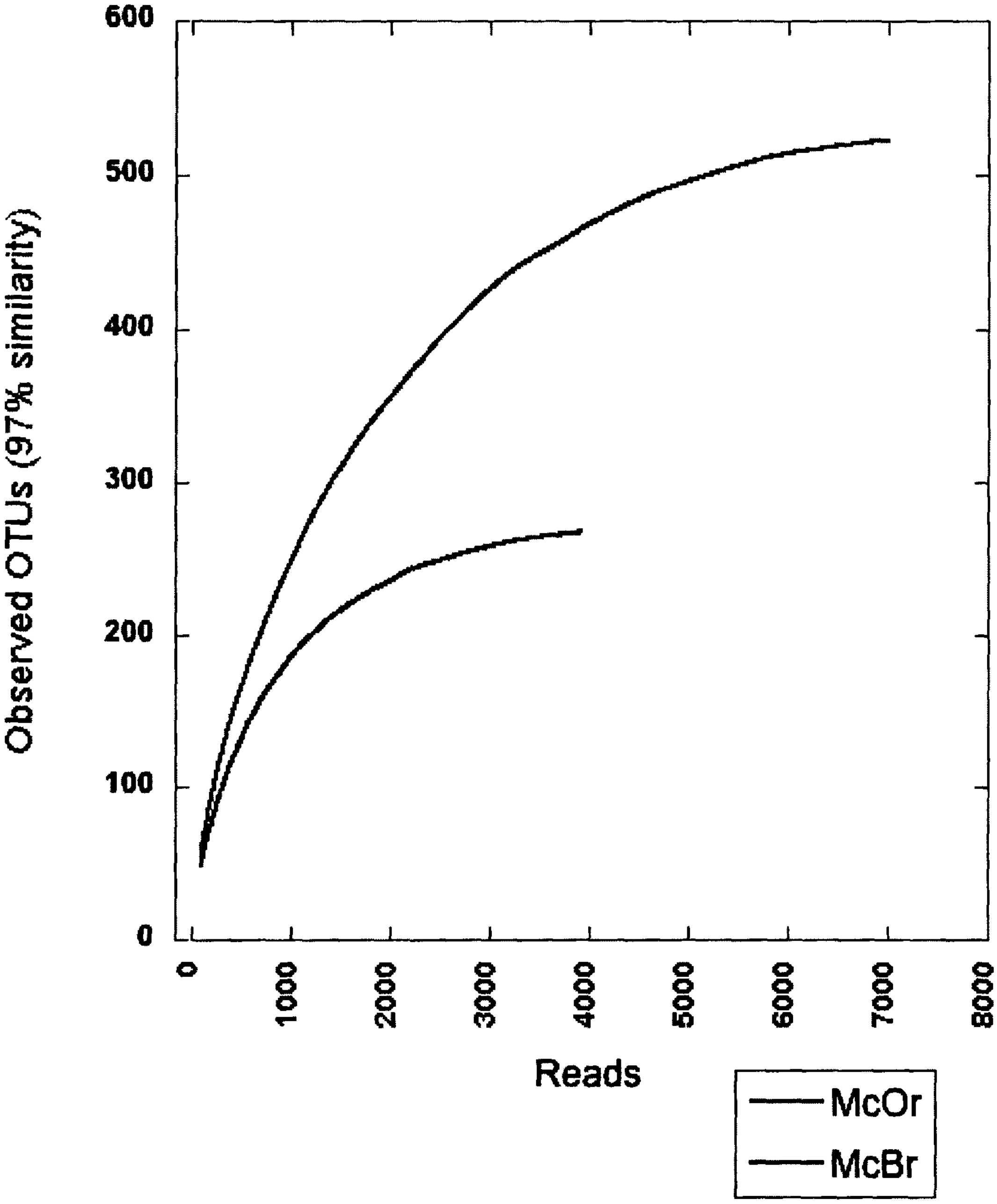


Figure 2.1. Rarefaction curves of observed OTUs (clustered at 97% similarity) for pyrosequencing of a brown and an orange colony of *Montastraea cavernosa* with Eubacterial primers.

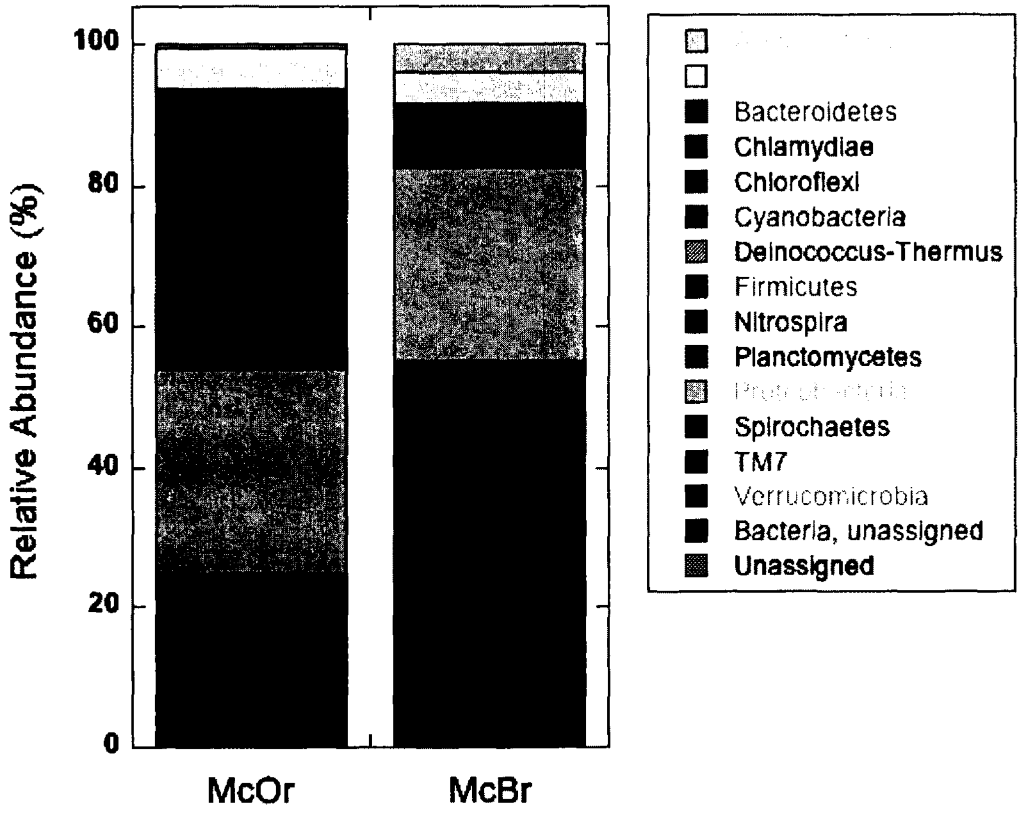


Figure 2.2. Relative abundance of reads assigned to prokaryotic phyla from sequencing of a brown and an orange colony of *Montastraea cavernosa* with Eubacterial primers. Sequences were classified at a confidence level of 0.8 using the RDP classifier. 'Unassigned' reads could not be assigned to any known taxonomic group, and 'Bacteria, unassigned' reads were classified as Bacteria but could not be identified further. A total of 7174 reads from the orange colony and 4061 reads from the brown colony were analyzed.

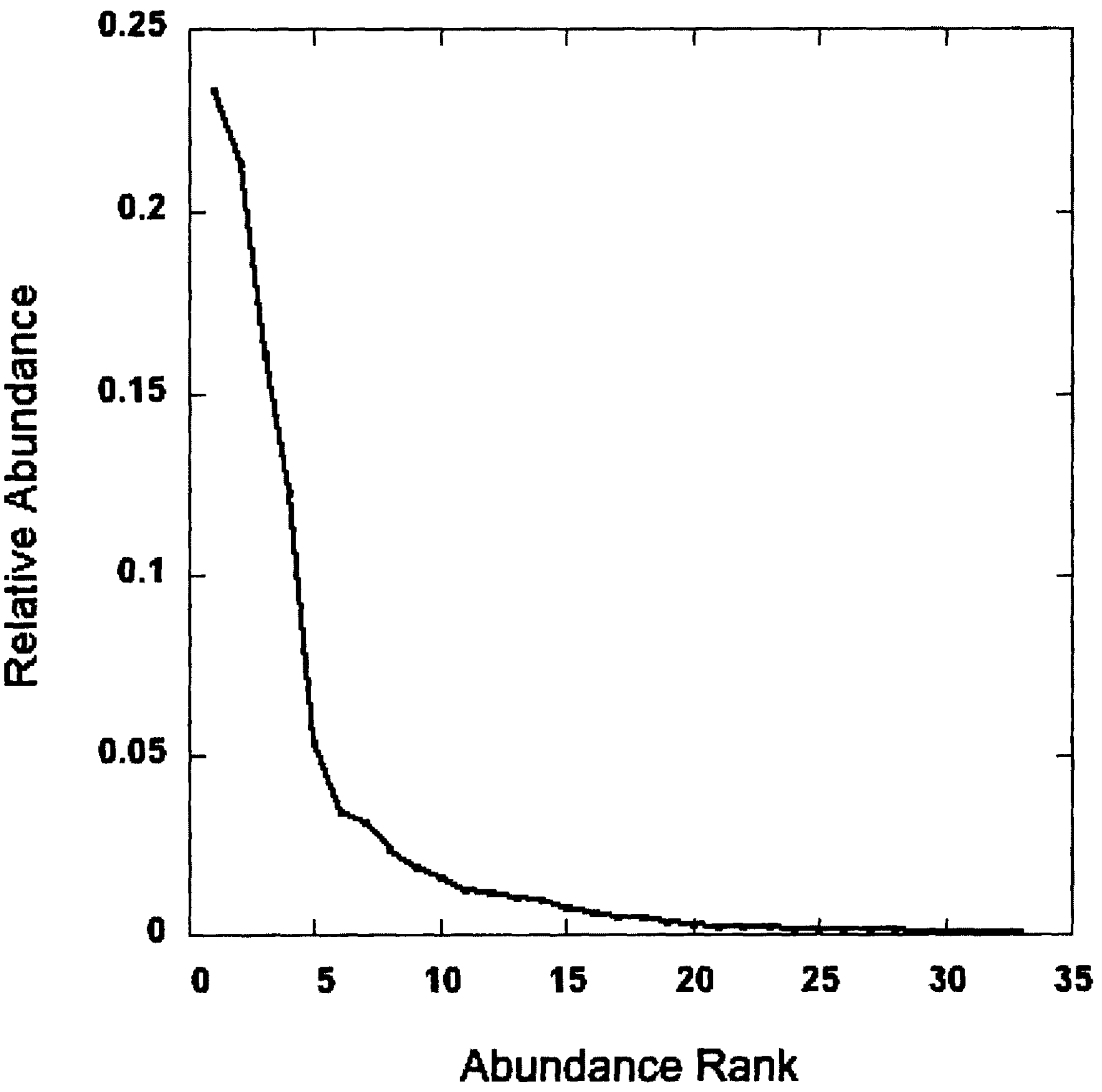


Figure 2.3. Rank abundance curve for putative cyanobacterial symbionts, OTUs classified as Phylum Cyanobacteria at a confidence level of 0.8 by the RDP classifier and found exclusively in the orange colony of *M. cavernosa*. Relative abundance is given as the proportion of cyanobacterial reads, not the total reads per sample.

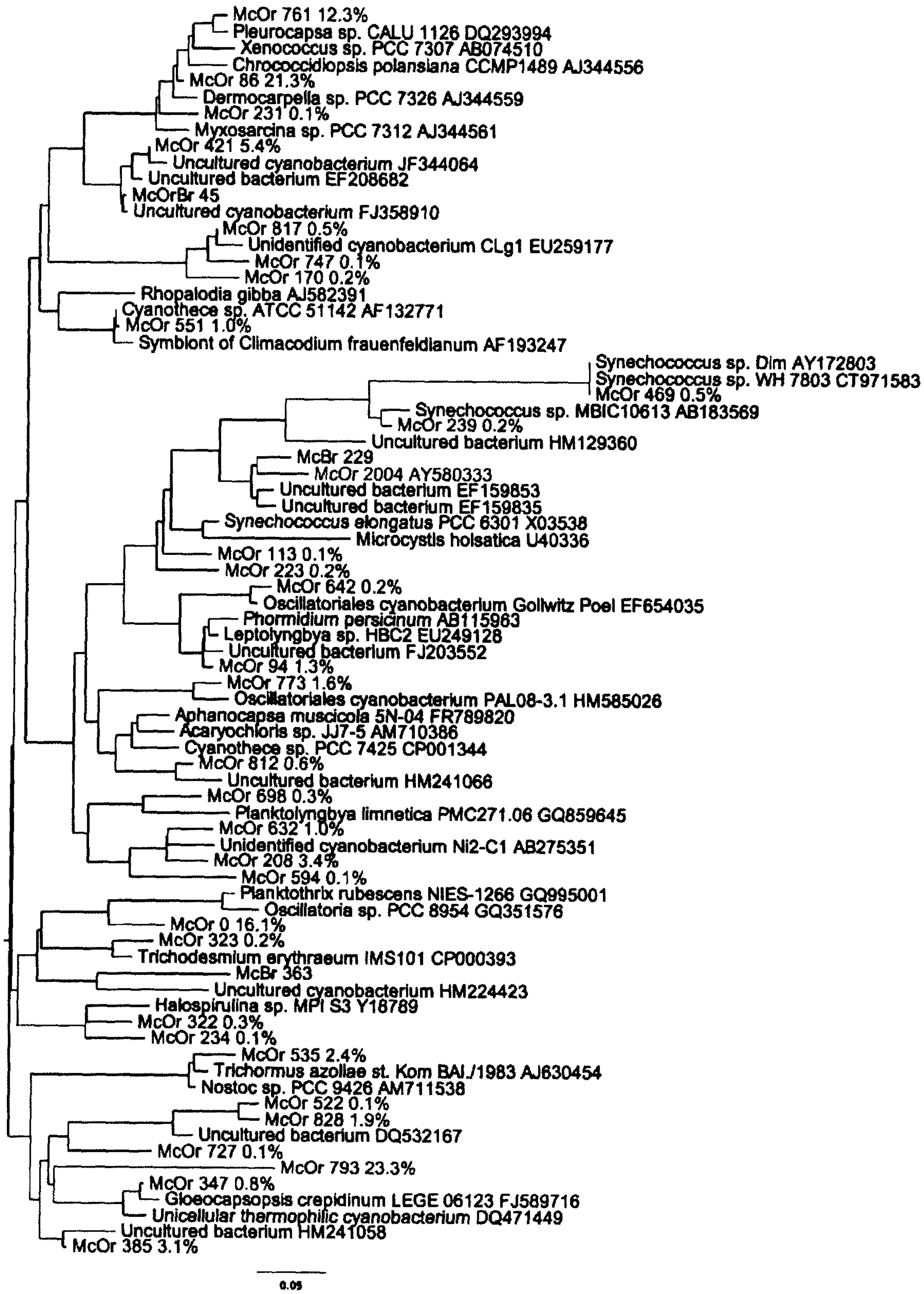

McOr 761 12.3%
Pleurocapsa sp. CALU 1126 DQ293994
Xenococcus sp. PCC 7307 AB074510
Chrococcidiopsis polansiana CCMP1489 AJ344556
McOr 86 21.3%
Dermocarpella sp. PCC 7326 AJ344559
McOr 231 0.1%
Myxosarcina sp. PCC 7312 AJ344561
McOr 421 5.4%
Uncultured cyanobacterium JF344064
Uncultured bacterium EF208682
McOrBr 45
Uncultured cyanobacterium FJ358910
McOr 817 0.5%
Unidentified cyanobacterium CLg1 EU259177
McOr 747 0.1%
McOr 170 0.2%
Rhopalodia gibba AJ582391
Cyanothece sp. ATCC 51142 AF132771
McOr 551 1.0%
Symbiont of Climacodium frauenfeldianum AF193247
Synechococcus sp. Dim AY172803
Synechococcus sp. WH 7803 CT971583
McOr 469 0.5%
Synechococcus sp. MBIC10613 AB183569
McOr 239 0.2%
Uncultured bacterium HM129360
McBr 229
McOr 2004 AY580333
Uncultured bacterium EF159853
Uncultured bacterium EF159835
Synechococcus elongatus PCC 6301 X03538
Microcystis holsatica U40336
McOr 113 0.1%
McOr 223 0.2%
McOr 642 0.2%
Oscillatoriales cyanobacterium Gollwitz Poel EF654035
Phormidium persicinum AB115963
Leptolyngbya sp. HBC2 EU249128
Uncultured bacterium FJ203552
McOr 94 1.3%
McOr 773 1.6%
Oscillatoriales cyanobacterium PAL08-3.1 HM585026
Aphanocapsa muscicola 5N-04 FR789820
Acaryochloris sp. JJ7-5 AM710386
Cyanothece sp. PCC 7425 CP001344
McOr 812 0.6%
Uncultured bacterium HM241066
McOr 698 0.3%
Planktolyngbya limnetica PMC271.06 GQ859645
McOr 632 1.0%
Unidentified cyanobacterium Ni2-C1 AB275351
McOr 208 3.4%
McOr 594 0.1%
Planktothrix rubescens NIES-1266 GQ995001
Oscillatoria sp. PCC 8954 GQ351576
McOr 0 16.1%
McOr 323 0.2%
Trichodesmium erythraeum IMS101 CP000393
McBr 363
Uncultured cyanobacterium HM224423
Halospirulina sp. MPI S3 Y18789
McOr 322 0.3%
McOr 234 0.1%
McOr 535 2.4%
Trichormus azollae st. Kom BAI/1983 AJ630454
Nostoc sp. PCC 9426 AM711538
McOr 522 0.1%
McOr 828 1.9%
Uncultured bacterium DQ532167
McOr 727 0.1%
McOr 793 23.3%
McOr 347 0.8%
Gloeocapsopsis crepidinum LEGE 06123 FJ589716
Unicellular thermophilic cyanobacterium DQ471449
Uncultured bacterium HM241058
McOr 385 3.1%
0.05

Figure 2.4 (previous page). 16S rDNA phylogeny trimmed from the SILVA reference tree, with cyanobacterial OTUs from sequencing with Eubacterial primers added with parsimony tool in ARB. OTUs found exclusively in the orange colony are labeled 'McOr' and shown in red; OTUs found exclusively in the brown colony or shared between samples are labeled 'McBr' and 'McBrOr' respectively, and shown in green; sequence from Lesser *et al.* 2004 is labeled 'McOr 2004' and shown in orange.

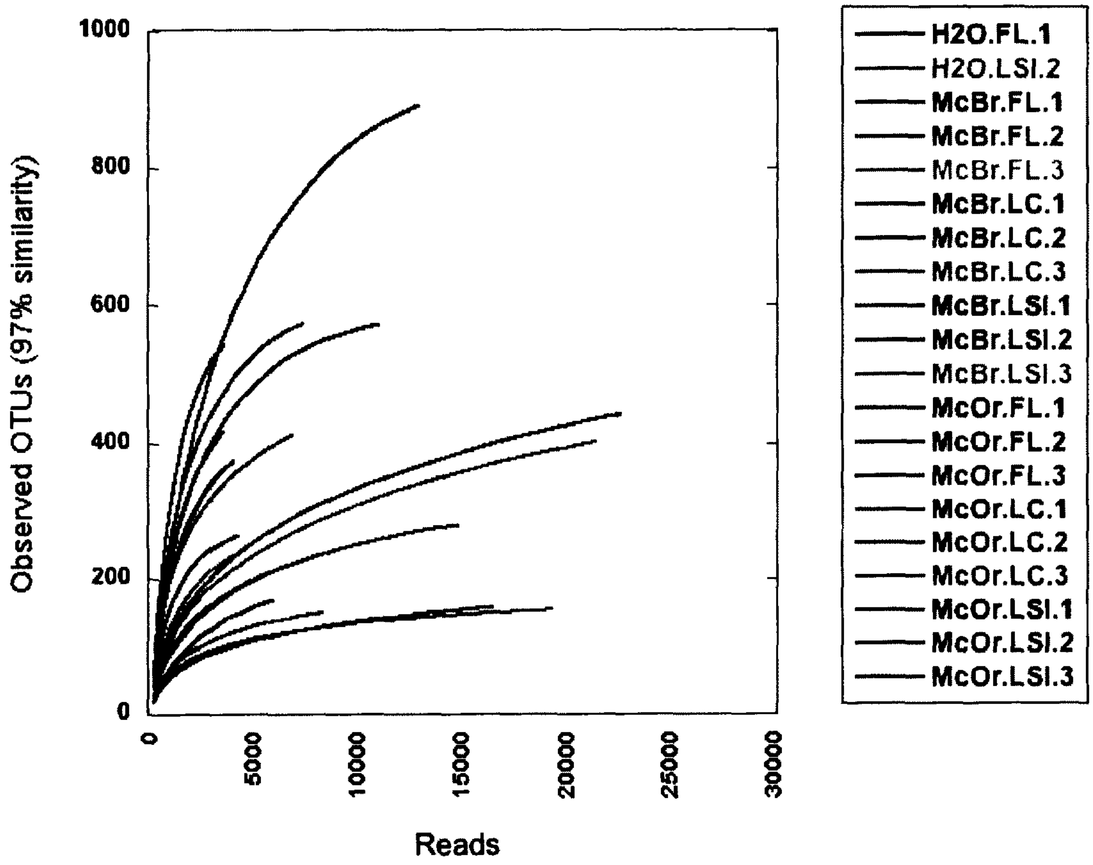


Figure 2.5. Rarefaction curves of observed OTUs (clustered at 97% similarity) for pyrosequencing of brown (McBr) and orange (McOr) *M. cavernosa* samples and selected water samples with Universal primers. Water sample rarefaction curves were all similar and the samples displayed are representative.

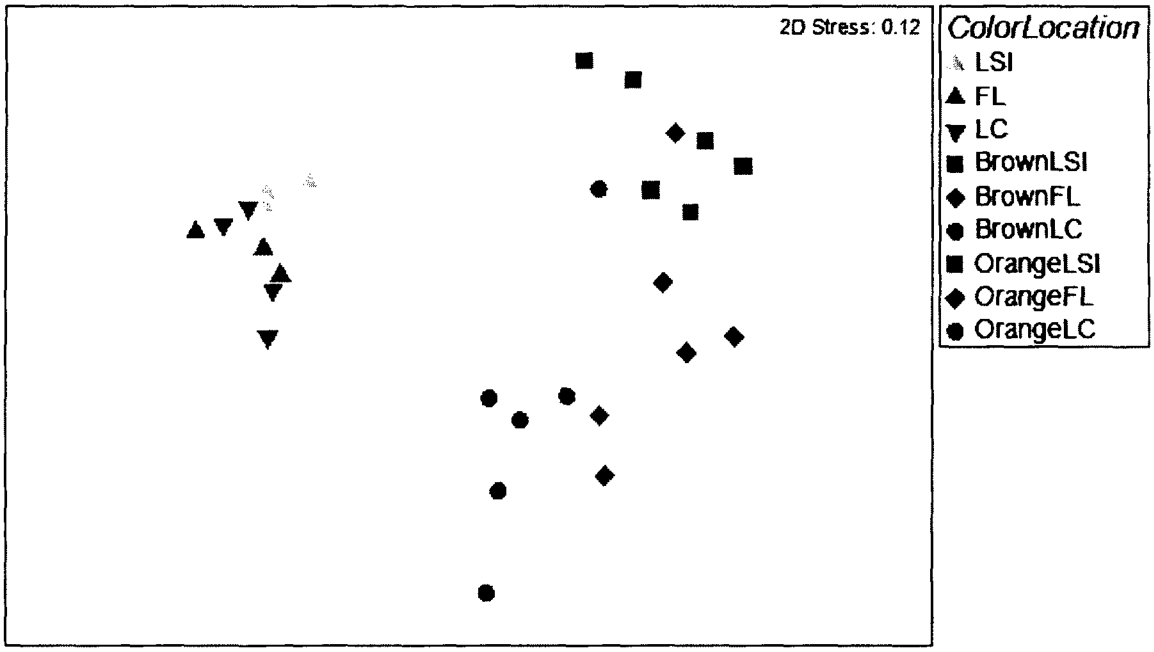


Figure 2.6. Multidimensional scaling (MDS) plot of differences in prokaryotic communities associated with brown and orange samples of *M. cavernosa* and water samples from the Florida Keys (FL), Little Cayman Island (LC), and Lee Stocking Island (LSI). Plotted from Bray-Curtis similarity of square-root transformed read counts for each OTU.

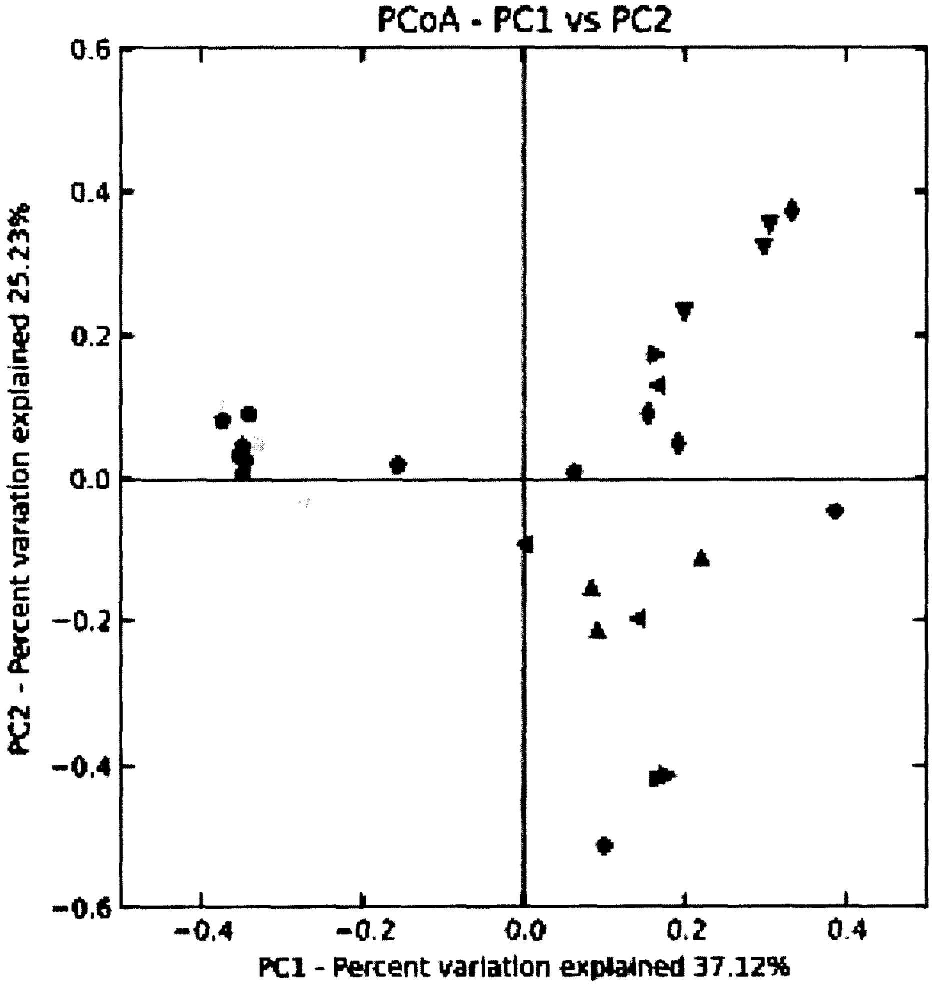


Figure 2.7. Principal coordinates analysis of weighted UniFrac distance from pyrosequencing of prokaryotic communities associated with *M. cavernosa* and water samples with Universal primers. Color key: Brown *M. cavernosa* from LC: lime green circles; FL: dark green triangles; LSI: Olive green diamonds. Orange *M. cavernosa* from LC: orange triangles; FL: red triangles; LSI: brown triangles. Water from LC: light blue squares; FL: dark blue pentagons; LSI: medium blue circles.

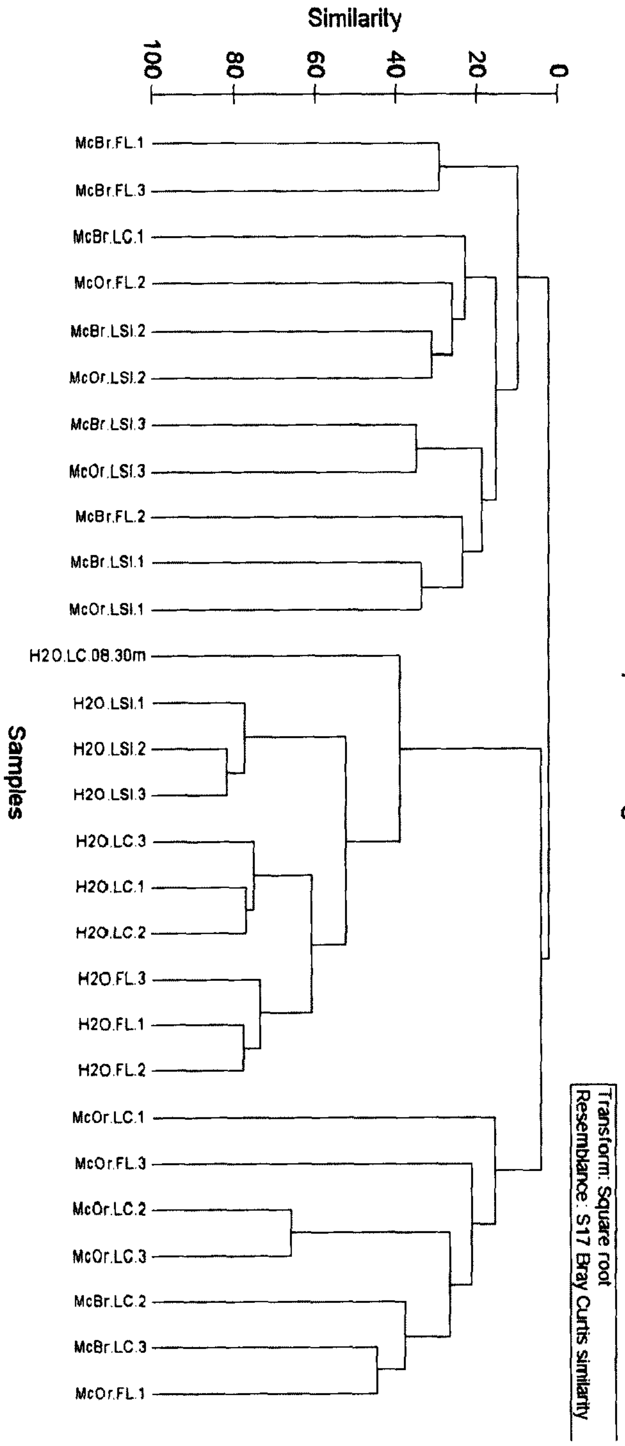


Figure 2.8. Complete linkage clustering of prokaryotic communities in coral and water samples sequenced with Universal primers, based on Bray-Curtis similarity of square-root transformed read counts for each OTU.

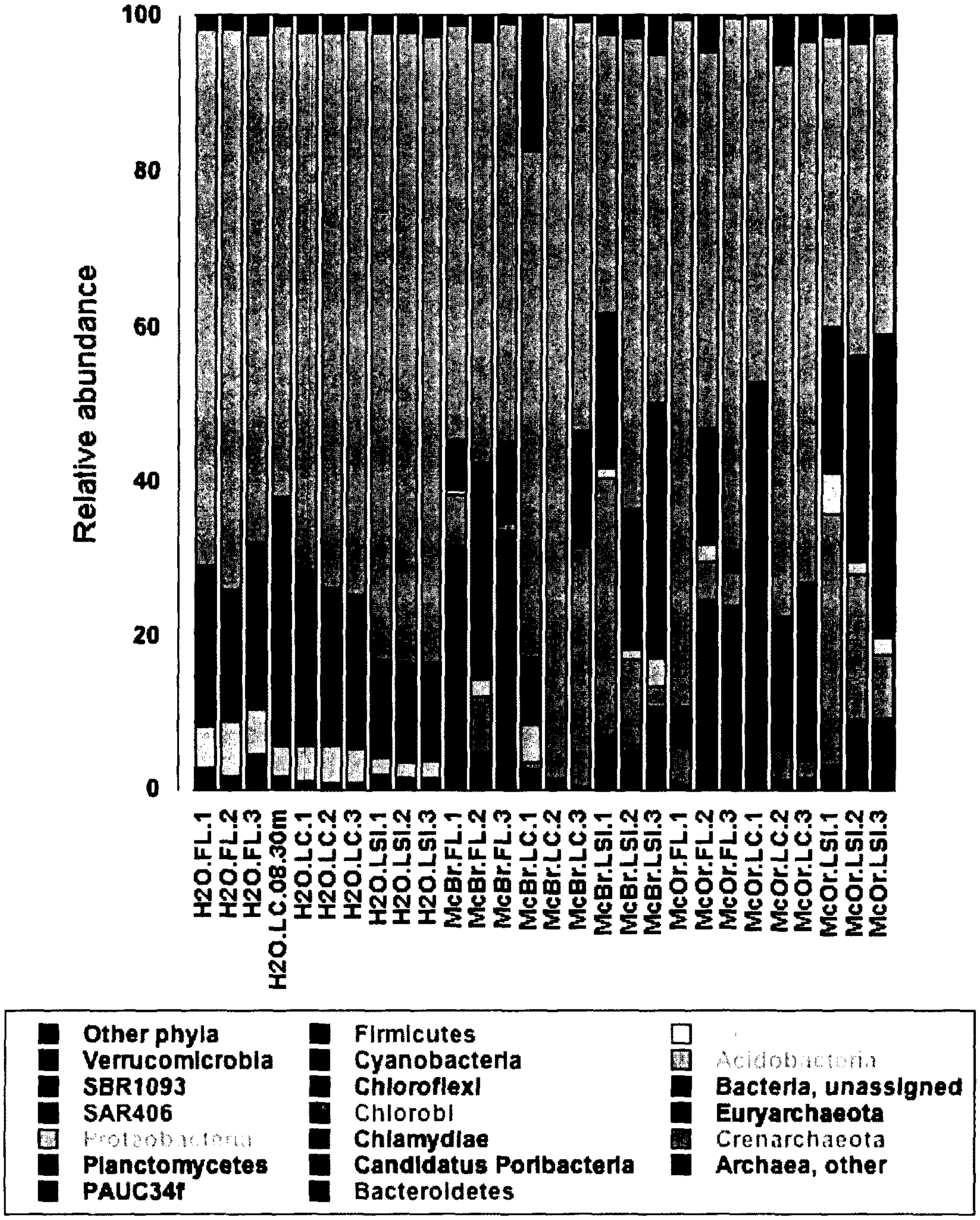


Figure 2.9. Relative abundance of prokaryotic phyla in the water column and associated with brown (McBr) and orange (McOr) *M. cavernosa* from the Florida Keys (FL), Little Cayman Island (LC), and Lee Stocking Island, Bahamas (LSI). Numbers indicate replicate samples. Sequences were classified at a confidence level of 0.8 using the RDP classifier. 'Unassigned' reads could not be assigned to any known taxonomic group, and 'Bacteria, unassigned' reads were classified as Bacteria but could not be identified further. 'Other phyla' include Armatimonadetes, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospirae, Spirochaetes, Synergistetes, Tenericutes, ABY1, GN02, MSBL6, NKB19, OP3, TM6, TM7, WPS-2, WS3, ZB2, and ZB3.

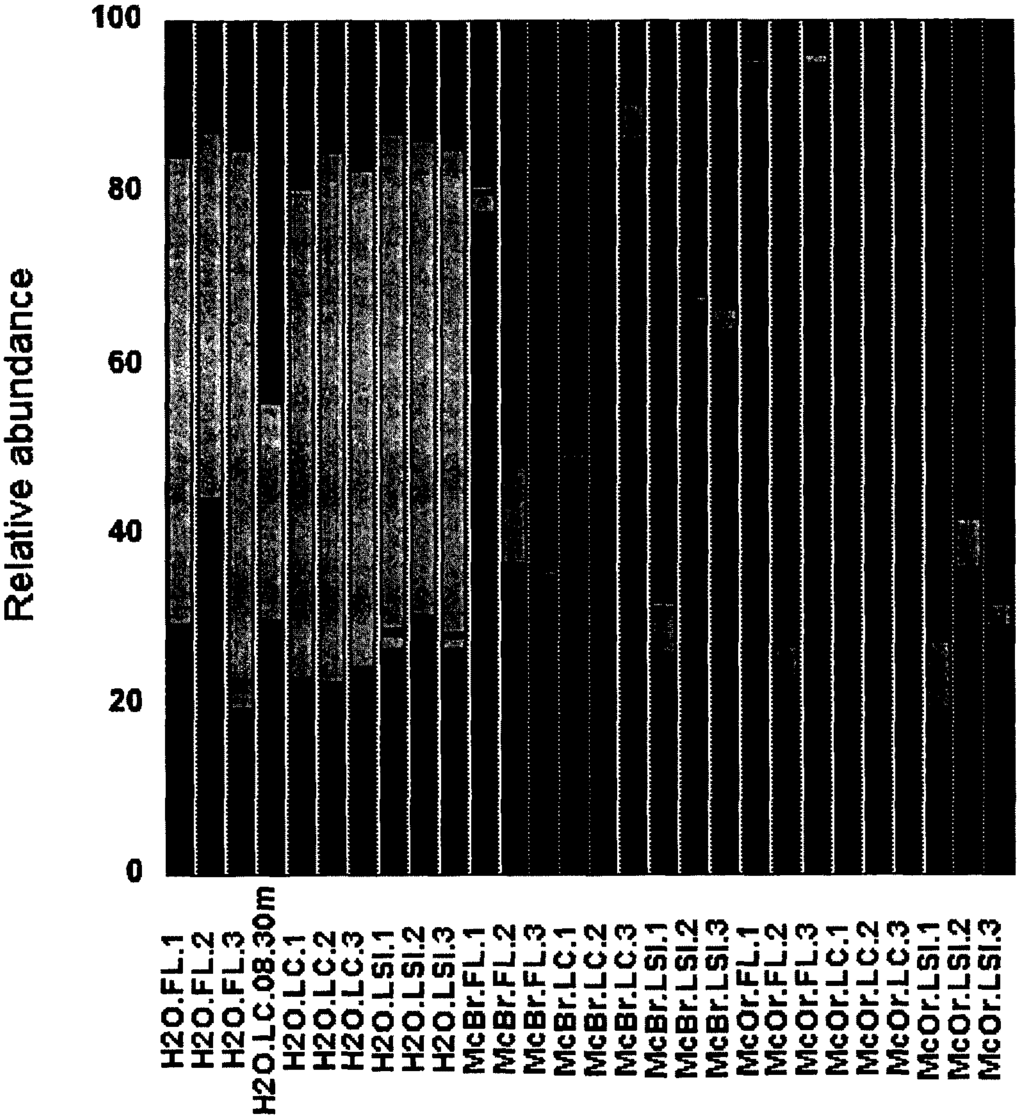


Figure 2.10. Relative abundance of Proteobacterial taxa in the water column and associated with brown (McBr) and orange (McOr) *M. cavernosa* from the Florida Keys (FL), Little Cayman Island (LC), and Lee Stocking Island, Bahamas (LSI). Numbers indicate replicate samples. Sequences were classified at a confidence level of 0.8 using the RDP classifier. Key: see next page.

■ Proteobacteria, Other
■ Alphaproteobacteria, Other
■ Alphaproteobacteria, Rhizobiales
■ Alphaproteobacteria, Rhodobacterales
■ Alphaproteobacteria, Rhodospirillales
▨ Alphaproteobacteria, Rickettsiales
■ Alphaproteobacteria, Sphingomonadales
■ Betaproteobacteria, Other
▨ Betaproteobacteria, Burkholderiales
■ Betaproteobacteria, Rhodocyclales
■ Deltaproteobacteria, Other
■ Deltaproteobacteria, Desulfovibrionales
■ Epsilonproteobacteria, Other
■ Gammaproteobacteria, Other
■ Gammaproteobacteria, Alteromonadales
■ Gammaproteobacteria, Chromatiales
■ Gammaproteobacteria, Oceanospirillales
■ Gammaproteobacteria, Pseudomonadales
■ Gammaproteobacteria, Vibrionales
■ Gammaproteobacteria, Xanthomonadales

Key for Figure 2.14.

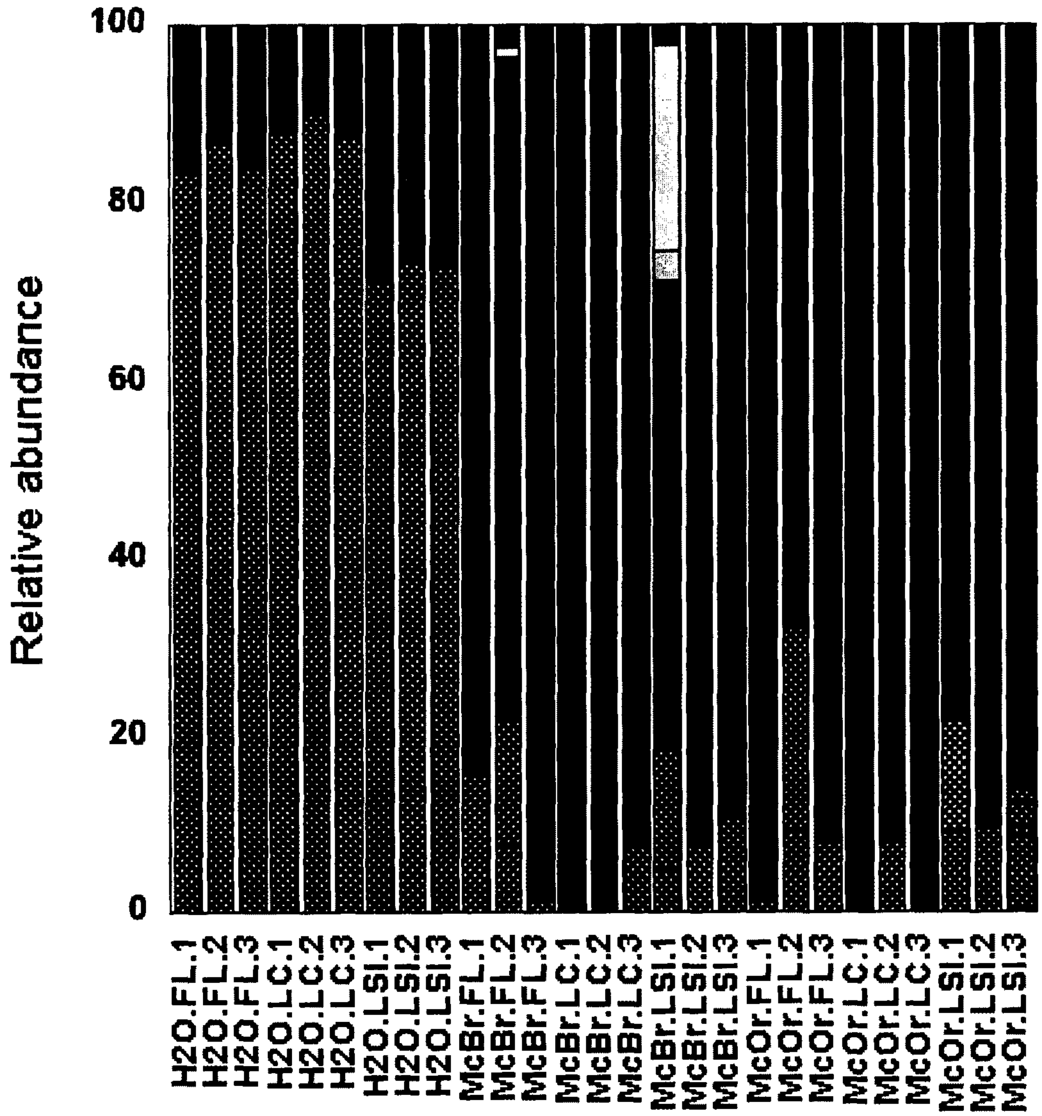


Figure 2.11. Relative abundance of reads assigned to the phylum "Cyanobacteria" in the water column and associated with brown (McBr) and orange (McOr) *M. cavernosa* from the Florida Keys (FL), Little Cayman Island (LC), and Lee Stocking Island, Bahamas (LSI). Numbers indicate replicate samples. Sequences were classified at a confidence level of 0.8 using the RDP classifier. Key: see next page.

■ Other
□
□
■ **Chloroplast, other**
■ **Chloroplast, unassigned**
■ **Chloroplast, CAB-I**
■ **Chloroplast, Chlorophyta**
■ **Chloroplast, Cryptophyta**
■ **Chloroplast, Haptophyceae**
■ Chloroplast, Rhodophyta
▩ Chloroplast, Stramenopiles
■ **Chloroplast, Streptophyta**
▩ **Nostocophycideae, Nostocales**
▩ Oscillatoriophycideae, other
▩ **Oscillatoriophycideae, Chroococcales**
▩ **Oscillatoriophycideae, Oscillatoriales**
▩ Synechococcophycideae, other
▩ Synechococcophycideae, unassigned
▩ **Synechococcophycideae, Pseudanabaenales**
▩ **Synechococcophycideae, Synechococcales**

Key for Figure 2.11 (previous page).

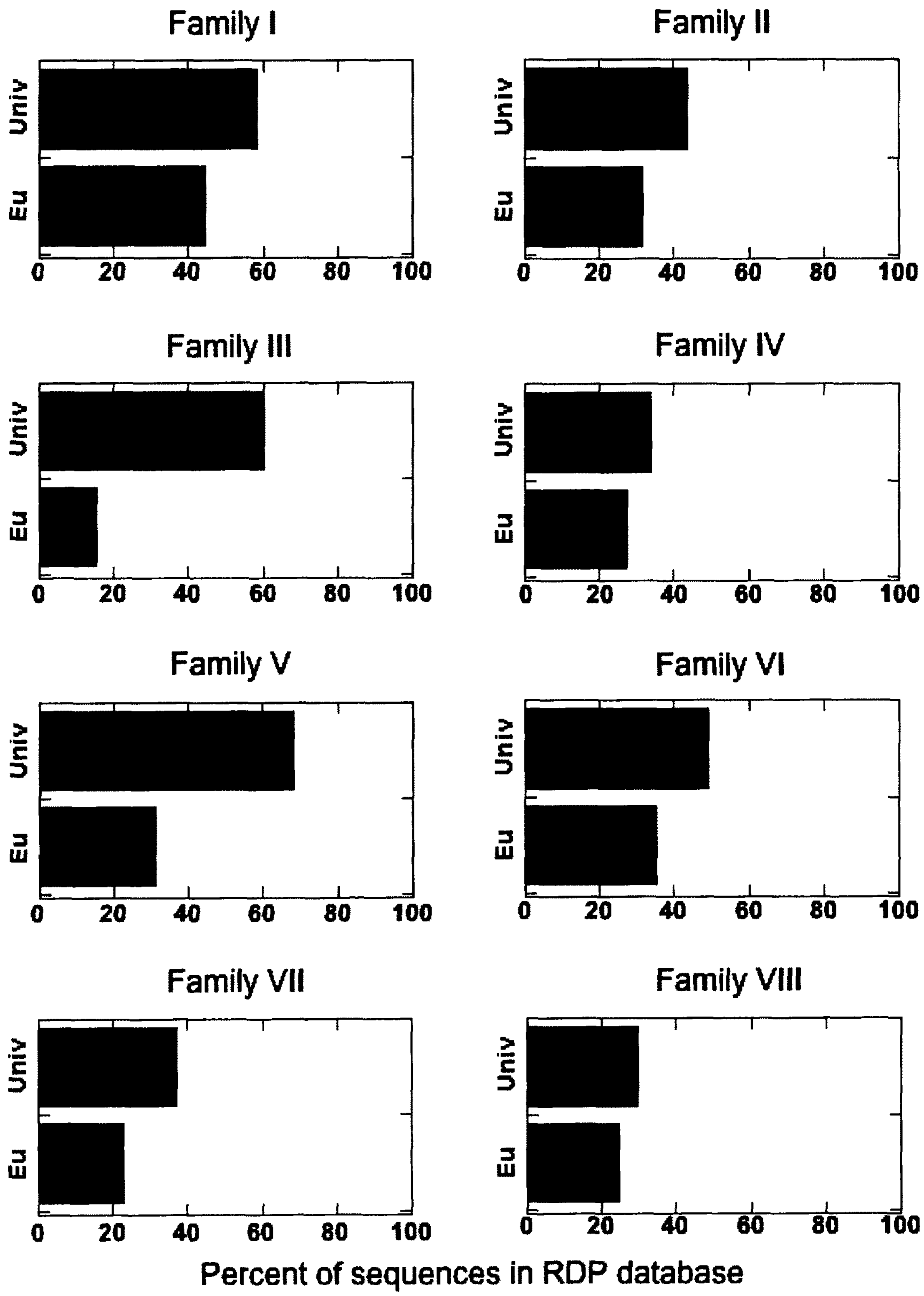


Figure 2.12a. Coverage of cyanobacterial families I - VIII in the RDP database by Universal and Eubacterial primers with 0, 1, and 2 mismatches with primer. Key: see next page.

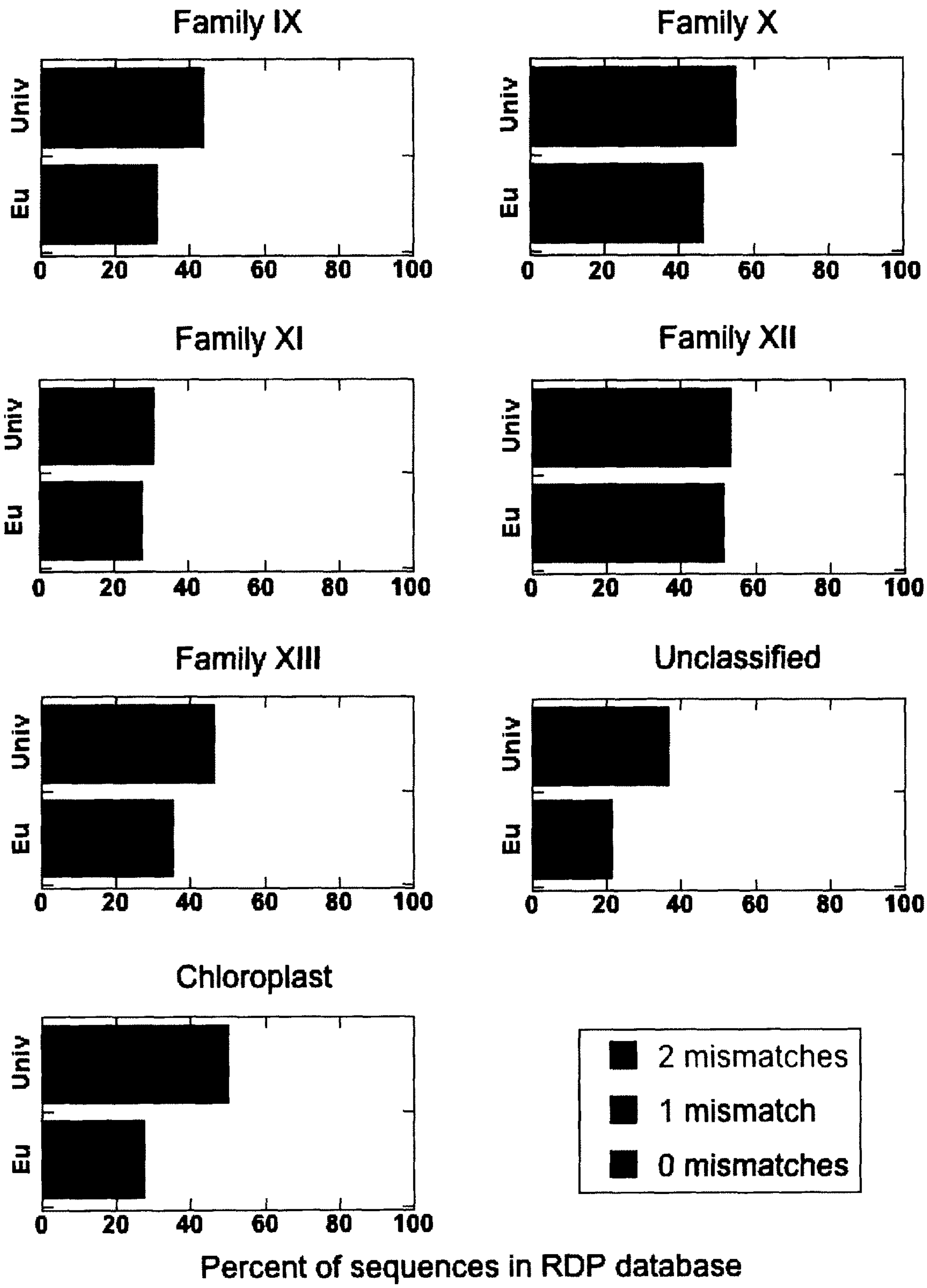


Figure 2.12b. Coverage of cyanobacterial families IX – XIII, chloroplasts, and unclassified cyanobacteria in the RDP database by Universal and Eubacterial primers with 0, 1, and 2 mismatches with primer.

CHAPTER III

METATRANSCRIPTOMIC ANALYSIS OF MONTASTREA CAVERNOSA HOLOBIONTS

Introduction

Development of Metatranscriptomics

The study of bacteria in the natural environment has a long history. For many decades this involved isolating bacteria from the environment, and conducting experiments using pure cultures in the laboratory. However, comparisons of microscope counts of cells from natural samples to the number of colonies and colony types that were cultured from such samples revealed that more than 99% of the bacteria present did not grow on traditional culture media, a phenomenon known as the "great plate count anomaly" (Staley & Konopka, 1985, Rappe & Giovannoni, 2003). Many of these bacteria remain uncultured today, due to nutritional or environmental requirements that are unknown or difficult to meet with traditional culture media and conditions. This shortcoming of traditional culturing led to the development and widespread use of DNA-based surveys of environmental prokaryotes using phylogenetic markers, most commonly the small subunit of the ribosomal RNA gene (16S or SSU rRNA) (Schmidt, *et al.*, 1991).

Many studies successfully explored "who is there" in the environment with this approach, resulting in the discovery of many new phylotypes of bacteria, some of which, such as *Candidatus Pelagibacter ubique,* are dominant members of their respective communities (Morris, *et al.*, 2002, Rappe, *et al.*, 2002). The main drawback of phylogenetic markers is that they cannot provide reliable information about the genomes, function, and physiology of the bacteria they represent, because even strains within a single species can have significant differences in their genome content and phenotype (Rasko, *et al.*, 2008).

Newer approaches have focused on the complete genomes of prokaryotic communities as a way of revealing processes and functions in their natural environments (DeLong, 2005). Metagenomics, the sequencing of DNA from environmental samples, is a way to study the genomic repertoire of all the organisms present in a sample. DNA is isolated from a sample and either cloned into bacteria and sequenced or sequenced directly, without the bias or difficulties of PCR. A landmark metagenomic study of the microbes in the Sargasso Sea was conducted by Venter *et al.* in 2004 (Venter, *et al.*, 2004), discovering new bacterial phylotypes, many previously unknown genes, and the widespread distribution of proteorhodopsin (Beja , *et al.*, 2000). Other groups investigated the communities in acid mine drainage (Tyson, *et al.*, 2004), soil, and whale falls in the deep sea (Tringe, *et al.*, 2005). These early studies sequenced DNA using clone libraries, but most investigators have transitioned to high-throughput sequencing methods such as pyrosequencing, Illumina, SOLiD, or other short-read sequencers, due to the greatly decreased cost per base (Poretsky, *et al.*, 2009, Glenn, 2011, Qi, *et al.*, 2011, Marchetti, *et al.*, 2012).

Comparison of metagenomes to reference databases allows function and taxonomy to be assigned to those reads that have significant similarity to sequences in the database, creating a catalog of the functional potential of the microbes in an environment, as well as the taxa that are present. The number of reads that can be annotated and the reliability of the annotation depend on the quantity, quality, and diversity of information in the reference databases. The inability to annotate a large proportion of sequences in a metagenome is a perennial problem; most environmental DNA is either not similar to anything in the database, or its matches have no known function or taxonomic classification (Tyson, *et al.*, 2004, Venter, *et al.*, 2004, Tringe, *et al.*, 2005). Even if a sequence is similar to that of an annotated protein, functions within a protein family can be so diverse that the characterized representatives of that family may not share the same function as the protein sequence from the metagenome (e.g., (Howard, *et al.*, 2006)). Because metagenomes are derived from DNA rather than RNA, unannotated sequences can also represent pseudogenes or non-functional copies of genes that are present in the genome but are not expressed. A high proportion of annotated sequences is particularly hard to achieve in data sets from environments that are poorly represented in reference databases, including coral reefs (Soergel, *et al.*, 2012). Characterizing and confirming the function of genes is a time-consuming process, so the lack of information about environmental sequences is likely to remain a problem well into the future.

The drawbacks of metagenomes are related to the fact that they are sequenced from genomic DNA. Even with very deep sequencing, most metagenomes are not well sampled because the DNA pool is very large (Riesenfeld, *et al.*, 2004). Significant sequencing effort is required to cover a single genome, but an environmental sample includes genomes from every organism present in the sample, so adequate coverage is difficult to achieve. DNA-based approaches also reveal only the functional potential of the community, not which genes are actually being expressed, or how they change in response to the environment.

Metatranscriptomics has become popular as a way to overcome many of the disadvantages of metagenomics, but is not without drawbacks of its own. The power of this approach comes from sequencing of RNA rather than DNA, to reveal the actual function of a community at a given point in time, not just its functional potential. Enzyme activity levels have been shown to be closely correlated with the expression of their transcripts in the metatranscriptome, allowing predictions to be made about processes occurring in the environment (Helbling, *et al.*, 2011). Metatranscriptomes can be used to compare environments or time points, or measure responses to experimental treatments (Poretsky, *et al.*, 2009, Gilbert, *et al.*, 2010, Hewson, *et al.*, 2010, Marchetti, *et al.*, 2012). In addition to ability to measure rapid or short-term changes in expression of genes, detection of alternative splicing and different gene isoforms is a significant potential advantage of metatranscriptomics over metagenomics (Trapnell, *et al.*, 2009). Metatranscriptomes can be easier to deal with bioinformatically because they are by definition a subset of all the genomes in the sample, so good depth of coverage is easier to attain. However, in complex environments with diverse biota the transcriptome pool is large enough that even high-throughput sequencing (~1 million reads) may only achieve 1% coverage, or less (Gifford, *et al.*, 2011). Difficulties with pseudogenes are minimized because sequences in transcriptomes represent expressed genes that are assumed to be functional. Transcriptomes from a single organism (Kreps, *et al.*, 2002, Okazaki, *et al.*,

2002) have been used for over a decade, but metatranscriptomics with mixed or environmental samples has only recently become common (Frias-Lopez, *et al.*, 2008, Urich, *et al.*, 2008, Poretsky, *et al.*, 2009, Gifford, *et al.*, 2011, Radax, *et al.*, 2012). Whereas single-source transcriptomes can be mapped to reference genomes where they are available, reliable annotation of metatranscriptomes, like metagenomes, remains problematic and many transcripts cannot be assigned to a function or taxonomic origin. Sequencing the genomes of single cells is now possible and has been used to compare small numbers of cells from the same environment (Yoon, *et al.*, 2011), often in tandem with a metagenome from the same sample (Blainey, *et al.*, 2011). This technique may eventually replace metagenomics and metatranscriptomics in the future.

Ribosomal RNA in Transcriptomics

The goal of most RNA sequencing is to obtain transcripts, or messenger RNA (mRNA) sequences, so contamination with ribosomal RNA (rRNA) is a major issue in most transcriptomes, and particularly in metatranscriptomes that contain a mix of different rRNA molecules. Messenger RNAs make up just 1-5% of total cellular RNA in *Escherichia coli* (Neidhart & Umbarger, 1996), so if steps are not taken to reduce the amount of rRNA, ribosomal sequences can make up over 90% of the reads in a metatranscriptome (Urich, *et al.*, 2008, He, *et al.*, 2010, Stewart, *et al.*, 2010). Even when rRNA is depleted, it is extremely difficult to remove it completely. As sequencing costs decrease, it has become more common to remove rRNA reads in bioinformatics processing rather than removing rRNA molecules from the sample, but these methods are typically used in tandem. Several methods are available for removing rRNA from total

RNA samples, which vary in their specificity, effectiveness, and ability to preserve the original abundance distribution of mRNAs. If only eukaryotic transcripts are desired, an oligo-dT selection to capture polyadenylated mRNA is typically used (Bailly, *et al.*, 2007, Qi, *et al.*, 2011); this method is not effective for prokaryotic mRNA, most of which lacks poly-A tails (Dreyfus & Régnier, 2002).

Alternatively, removal of rRNAs may be performed. The use of duplex-specific nucleases takes advantage of the high abundance of rRNA in most RNA samples. Total RNA is reverse-transcribed into cDNA, denatured, and allowed to reassociate; the rRNA molecules will tend to form duplexes, which are then cleaved by the nuclease (Yi, *et al.*, 2011). This method can alter the relative abundances of mRNAs by depleting highly abundant transcripts, but has the advantage of working well even with partially degraded RNA. Exonucleases degrade RNAs possessing a 5' monophosphate, the majority of which are thought to be rRNAs (Bürgmann, *et al.*, 2007, Garbeva & de Boer, 2009). However, exonucleases are less efficient at rRNA removal than other methods, and also appear to target partially degraded mRNAs, resulting in skewed mRNA relative abundances (He, *et al.*, 2010). Subtractive hybridization uses oligonucleotide probes that hybridize with rRNAs and are then removed (Stewart, *et al.*, 2010, Chen & Duan, 2011). The effectiveness of this process depends on the degree of complementarity between the probe sequence and the target sequence(s); only rRNAs complementary to the probes will be removed. A variety of kits are commercially available with probes targeting commonly used experimental organisms, such as humans, mice, plants, and yeast (Chen & Duan, 2011). For environmental samples or non-model organisms, it is possible to create a custom probe mixture based on the sequences in an individual sample (Stewart, *et al.*, 2010), but this is a very labor-intensive process and best used only when the organisms in the sample are known to be poorly removed by other kits or methods (e.g., for many Archaea (He, *et al.*, 2010, Stewart, *et al.*, 2010)). All subtractive hybridizations require intact RNA, because degraded RNAs may not contain the target site for the probes. This method has the advantage of preserving transcript abundances with high fidelity (He, *et al.*, 2010). None of the rRNA reduction methods above will completely eliminate rRNA, so ribosomal sequences must be removed during bioinformatics processing. It is critical to do this before annotation because rRNA sequences can generate up to 90% false positive matches to proteins (Tripp, *et al.*, 2011).

When phylogenetic information about the community is desired, total RNA can be sequenced without any rRNA removal in what is sometimes called a "double-RNA" approach (Urich, *et al.*, 2008). The mRNA sequences are used to provide information about the function of the community, as in traditional transcriptomics. The rRNA sequences are used as a rough indicator of the abundance, metabolic activity, and taxonomy of the prokaryotic community. The abundance of rRNA within a cell is positively correlated with metabolic activity, so the abundance of rRNAs from a given taxon can be used to infer how active that taxon is in the environment (Campbell, *et al.*, 2011, Gaidos, *et al.*, 2011). Sequencing or amplification of environmental DNA often recovers many species or phylotypes of bacteria represented by only a few reads, termed the "rare biosphere" (Sogin, *et al.*, 2006, Pedros-Alio, 2007). However, when the same methods are applied to RNA, this long tail is absent or greatly reduced (Gaidos, *et al.*, 2011), indicating that some members of the rare biosphere are metabolically inactive at any given point in time. A study of oceanic bacteria at multiple time points showed that while abundance and activity were correlated in most bacteria, approximately 50% of the rare biosphere is metabolically active, and about half of the members of the prokaryotic community experience periodic cycles of rarity and abundance (Campbell, *et al.*, 2011). The tandem sequencing of mRNA can reveal what metabolic and biogeochemical processes these members of the rare biosphere are involved in. The double-RNA approach was pioneered in soil samples (Urich, *et al.*, 2008), and has also been used to investigate nitrogen transformations in the cold-water sponge *Geodia barretti* (Radax, *et al.*, 2012).

Coral Transcriptomes

Transcriptomics has only recently been applied to corals and their symbionts, and studies usually address the interactions of corals and dinoflagellate symbionts and the response to ecologically relevant stressors, particularly thermal stress. An early microarray study on the temperate anemone *Anthopleura elegantissima* revealed the transcriptional changes in cell cycle and apoptosis mediators that are necessary to maintain symbiosis with dinoflagellates (Rodriguez-Lanetty, *et al.*, 2006). Schwarz *et al.* (2008) created expressed sequence tag libraries from *Acropora palmata* and *Montastraea faveolata* at life history stages from egg to adult coral. More recent research has typically utilized high-throughput pyrosequencing (Meyer, *et al.*, 2009, Polato, *et al.*, 2011, Traylor-Knowles, *et al.*, 2011). There are only two published transcriptomic surveys of *Symbiodinium*. The first is an EST library created by Leggat *et al.* (2007, 2011) from symbionts *in hospite* and freshly isolated from the host, subjected to several thermal stress treatments, ammonium addition, and altered inorganic carbon concentrations

(Leggat, *et al.*, 2007, Leggat, *et al.*, 2011). The second is a large pyrosequenced data set from two cultures exposed to heat, cold, darkness, and high light (Bayer, *et al.*, 2012). The few studies that have addressed gene expression in the prokaryotic members of the coral holobiont have typically utilized microarray technology (Kimes, *et al.*, 2010). A newly released metatranscriptome from the cold water sponge *Geodia barretti* found abundant transcripts coding for key nutrient transformations mediated by microbes that are known to occur in this sponge (Radax, *et al.*, 2012). Corals and many types of sponges both possess diverse and abundant microbial communities (Rohwer, *et al.*, 2002, Littman, *et al.*, 2009, Sunagawa, *et al.*, 2010, Chen, *et al.*, 2011, Schmitt, *et al.*, 2012), so metatranscriptomics could be fruitful in studies of corals as well.

Previous transcriptomic studies of corals tend to suffer from the same set of problems, only some of which are due to logistical constraints related to the biology of corals. Many of these studies do not have replicated experimental designs (Leggat, *et al.*, 2007, Schwarz, *et al.*, 2008, Meyer, *et al.*, 2009), or if properly replicated, samples within a treatment or samples from multiple treatments were pooled prior to sequencing, obscuring variation (Polato, *et al.*, 2011, Traylor-Knowles, *et al.*, 2011). Lack of replication limits statistical analyses and reduces the ability to draw conclusions about biology, regardless of the depth of sequencing per sample (Prosser, 2010). Most of these studies utilize corals in the larval stage that have not yet acquired symbionts to reduce contamination with symbiont RNA, but the applicability of such studies to adult corals with dinoflagellate symbionts is questionable. The presence of photosymbionts is associated with dramatic transcriptional changes in temperate anemones related to reef-building corals (Rodriguez-Lanetty, *et al.*, 2006), although the initiation of symbiosis with compatible *Symbiodinium* in *Acropora palmata* and *Montastraea faveolata* occurs with only minimal changes to the host transcriptome (Voolstra, *et al.*, 2009). EST libraries from the same two corals at different developmental stages from unfertilized eggs to adult corals also provide evidence that expression signatures at different stages are distinct (Schwarz, *et al.*, 2008). Metagenomes from *Porites* and *Acropora* have included sequences from prokaryotes, viruses, and fungi associated with corals (Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2009, Littman, *et al.*, 2011), but with the exception of fungi (Amend, *et al.*, 2011), there are no published transcriptome data for these members of the holobiont, and no previous transcriptomic studies consider the holobiont as an intact system. Given the importance of these organisms to the functions and fitness of the coral holobiont (Ainsworth, *et al.*, 2010, Gates & Ainsworth, 2011), this is a significant gap in our knowledge.

The prokaryotic community of corals has frequently been characterized using marker genes such as the 16S ribosomal RNA (Rohwer, *et al.*, 2002, Littman, *et al.*, 2009, Sunagawa, *et al.*, 2010, Chen, *et al.*, 2011). Studies of sediment and ocean water comparing sequences derived from DNA and RNA have demonstrated that the most active fraction of the prokaryotic community is not always the most abundant, and vice versa (Campbell, *et al.*, 2011, Gaidos, *et al.*, 2011). The prokaryotic community associated with *M. cavernosa* has been profiled using high-throughput pyrosequencing (i.e., 454) of PCR amplicons derived from DNA (Chapter 2), and the sequence data from this chapter can be utilized in a "double-RNA" approach to compare community profiles from DNA and RNA (Urich, *et al.*, 2008). This will yield not only a robust characterization of the taxonomy of the community that is free of PCR bias, but also estimates of the functional diversity of different taxa. This study is the first metatranscriptome from coral holobionts, including the ecologically and functionally important prokaryotic community. In this chapter, the functional (mRNA) portion of the metatranscriptome will be analyzed for changes in gene expression in brown and orange colonies within the coral host, dinoflagellate, and prokaryotic compartments. Comparing the expression of genes in the two transcriptomes will yield important insights into the functional differences and ecological niches of brown and orange colonies.

Methods and Materials

Sample Collection, Preparation, and Sequencing

Samples from three orange and three brown/green colonies of the coral *Montastraea cavernosa* were collected with a hammer and chisel on SCUBA from North Perry Reef, Lee Stocking Island, Bahamas (23°47'0.03" N, 76°6'5.14" W) near midday in August of 2011. Coral samples were transported to the laboratory in covered coolers filled with seawater, then gently airbrushed while being held upside down with 0.2 µm filtered sea water from a distance of approximately 15 cm to remove mucus and loosely associated bacteria. Samples were placed in RNA-Later buffer (Ambion), frozen at -20° C or below, and transported to the University of New Hampshire.

Total RNA was extracted from coral samples using an RNAqueous extraction kit (Invitrogen) according to the manufacturer's instructions except for the following modifications: tissue was homogenized by bead beating in 750 µl of lysis buffer with 0.3g of 160 µm glass beads for 3 min at maximum speed in a Vortex Genie 2 with

Turbomix attachment (Scientific Industries, Inc.), and total RNA was eluted twice with 50 µl and 15 µl of elution solution. DNA was removed using the "rigorous" protocol for the TURBO DNA-free kit (Invitrogen), and complete digestion of DNA was confirmed by the absence of visible bands from PCRs amplifying cnidarian actin and Eubacterial 16S rRNA. RNA samples were diluted 1:10 and run on a Bioanalyzer using the RNA 6000 Pico Assay kit (Agilent). The RNA Integrity Number and concentration were recorded for each sample, and electropherograms were examined for the presence of the 18S and 28S rRNA peaks, a level baseline, and the absence of peaks smaller than the 18S or larger than the 28S which typically indicate RNA degradation and genomic DNA contamination, respectively. Samples were sent to the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois for the preparation of libraries and Illumina sequencing. Eukaryotic rRNA was removed from 1µg of total RNA from each sample using subtractive hybridization with a RiboMinus Eukaryote kit (Invitrogen) according to the manufacturer's instructions. Libraries were prepared with TruSeq RNAseq sample prep kits (Illumina), pooled in equimolar concentrations, and quantitated with qPCR. Multiplexed samples were sequenced on a HiSeq2000 using a TruSeq SBS sequencing kit (version 3), on a single lane for 100 cycles from each end of the fragments. The sequencing run was analyzed with Casava 1.8 (pipeline 1.8).

Data Analysis

Sequence analysis was conducted using CLC Genomics Workbench (CLCbio). Failed reads were removed and sequences from all coral samples were assembled *de novo* with a mismatch cost of 2, insertion and deletion cost of 3, length fraction of 0.5, similarity of 0.8, automatic selection of word size, and conflicts resolved by voting. Reads were mapped back to the assembly to check and refine the contigs. Contigs less than 300 nt in length were removed and the N50 was calculated using a custom perl script. Trimmed contigs were screened for rRNA against all available databases (SILVA, Greengenes, RDP-II, Rfam, and NCBI and HMP reference genomes) using riboPicker (Schmieder, *et al.*, 2012), with contigs aligning over 30 nt at a minimum of 20% coverage and 75% identity flagged as putative rRNA and excluded from further analysis. Cutoffs for the identification of rRNA were selected with guidance from the coverage-identity plot provided in riboPicker.

Expression levels were determined by mapping all of the reads for each coral sample to the list of putative mRNA contigs using the RNA-Seq utility in CLC. Reads were mapped with a minimum length fraction of 0.9, a minimum similarity fraction of 0.8, a maximum of 10 hits per read, and broken pairs were not included. Total read counts were summarized over all samples for each contig, then exported and analyzed in DESeq, an R package which utilizes a model based on the negative bionomial distribution to test for differential expression (DE) with high power and accuracy (Anders & Huber, 2010). *P*-values were Bonferroni corrected for multiple comparisons, and significantly differentially expressed contigs ($P \leq 0.05$) were sorted into two lists: those with a higher expression level in brown samples, and those with a higher expression level in orange samples. Each contig list was used to query the RefSeq protein database using BLASTX through the CAMERA web portal (Sun, *et al.*, 2011), with 100 hits per query, a minimum E-value of 1×10^{-3}, filtering of low-complexity sequences, a gap opening penalty of 11, and a gap extension penalty of 1. All non-rRNA contigs were annotated using the same database and search settings, and all BLAST results were imported into MEGAN4 (Huson, *et al.*, 2011), which uses a lowest common ancestor algorithm based on the taxonomic assignments of multiple BLAST hits to assign taxonomy to sequences.

In MEGAN4, contigs were divided into subsets based on their lowest common ancestor assignment and analyzed separately. "Prokaryote" contigs included those assigned to Archaea and Bacteria, "Zooxanthellae" contigs were those assigned to Alveolata or Viridiplantae, and remaining contigs were assigned to the "Coral host" category, except for Fungi contigs that were excluded from analysis. The SEED annotations and KEGG functions of contigs from the whole metatranscriptome and those that were significantly differentially expressed from each of the three subsets were compared in MEGAN.

Results

Illumina sequencing produced 34,011,854 to 45,494,072 paired-end reads per sample (average 40,840,612 reads per sample), for a total of 245,043,674 reads from fragments averaging 240 nt in length. Reads were assembled into 213,010 contigs, with an N50 for the assembly of 948 nt. Contigs less than 300 nt in length (n = 458) or flagged as rRNA by riboPicker (n = 895) were trimmed from the assembly, for a total of 211,657 putative mRNA contigs. On average, 34.4% of reads from each sample (± 8.6%) could be mapped back to mRNA contigs in RNA-seq analysis. 842 contigs were significantly differentially expressed (DE) between brown and orange samples; 314 of these had higher expression in brown samples and 528 had higher expression in orange samples (Figure 3.1).

Comparison of the metatranscriptome to the RefSeq database with BLASTX resulted in low annotation rates (Table 3.1). Based on taxonomic annotations, contigs were split into three groups based on their presumed compartment within the holobiont: prokaryote, zooxanthellae, and coral host. Almost 10 times more contigs were assigned to the coral host grouping than to the prokaryote or zooxanthellae grouping (Table 3.1). Twenty-three DE contigs originated from the prokaryote group, and the remainder were assigned to the coral host group.

Prokaryotic Contigs

Prokaryotic mRNA contigs included 5,066 assigned to Bacteria and 67 to Archaea. Contigs were assigned to diverse taxa, but the order Burkholderiales within the β-proteobacteria was clearly dominant (Figures 3.2, 3.3). Cyanobacteria had the second largest number of contigs, and were represented by three orders: Chroococcales, Nostocales, and Oscillatoriales (Figure 3.4). Archaeal contigs were primarily assigned to Halobacteria and Methanocella within Euryarchaeota, Nitrosopumiales within the Thaumarchaeota, and Thermoprotei and *Sulfolobus* within the Crenarchaeota (Figure 3.5).

The SEED functions assigned to prokaryotic contigs revealed a functionally diverse community with diverse metabolic capabilities and possible roles in the holobiont. Contigs consistent with both photoautotrophy and heterotrophy were present , and the housekeeping functions that would be expected in a complete transcriptome, such as those for transcription, translation, and the cell cycle, were represented (Figure 3.6). Many contigs that could be important to holobiont fitness and microbial interactions within the coral-associated community were present (Figure 3.7). Nitrogen cycling was represented by ammonia assimilation and *nifS*, which is a part of the nif gene cluster involved in nitrogen fixation. NifS catalyzes a reaction producing sulfide necessary to form the iron-sulfide core of the nitrogenase enzyme (Zheng & Dean, 1994) and is required for nitrogenase activity (Kennedy & Dean, 1992). The assimilation and oxidation of sulfur, and metabolism of aromatic compounds were also present. Contigs related to the oxidative stress response, including an iron superoxide dismutase and rubrerythrin, made up the majority of the stress response category, reflecting the hyperoxic conditions of coral tissue during daylight hours. The most abundant SEED category was virulence (Figure 3.6); this group was comprised primarily of contigs involved in resistance to antibiotics and the acquisition and uptake of iron (Figure 3.8). Phages and secondary metabolism were the least abundant categories.

Differentially expressed contigs in the prokaryote group were all assigned to either Bacteria or Phylum Proteobacteria. None could be annotated with SEED functions, but two could be assigned to KEGG pathways. One contig that was significantly more expressed in orange than brown colonies was annotated as *entF*, which is involved in the synthesis of enterobactin, a siderophore from Gram negative bacteria with the highest known affinity for iron. The second contig was also more expressed in orange colonies, and was annotated as *UBC*, which is involved in ubiquitination.

Zooxanthellae Contigs

Contigs assigned to both Alveolata (n = 4,942) and Viridiplantae (n = 2,057) were grouped into the zooxanthellae category, in an attempt to include the photosynthetic functions of *Symbiodinium*. There were no assignments to dinoflagellates specifically, but many of the Alveolata contigs were from related parasites and pathogens, including *Perkinsus* and the apicomplexans *Toxoplasma, Cryptosporidium, Babesia,* and *Plasmodium* (Figure 3.9). Viridiplantae contigs were assigned mostly to Chlorophyta, or to lower plants such as mosses.

SEED annotations of zooxanthellae contigs included functions necessary for a photoautotrophic lifestyle, such as photosynthesis, CO_2 fixation and metabolism, respiration, and response to oxidative stress (Figure 3.10). Contigs functioning in translation initiation and RNA processing and modification were more abundant than those involved in transcription (Figure 3.11). Several functional categories, including cell cycle and potassium homeostasis (within the membrane transport category) may contain contigs important in mediating interactions with the coral host (Figure 3.12). Ammonia transporters and flavohemoprotein comprised the nitrogen metabolism category. There were no DE contigs in the zooxanthellae category.

Coral Host Contigs

Taxonomic assignments of contigs in the coral host category were diverse, but were primarily Cnidaria (Figure 3.13). Some other taxa are known to be members of the holobiont, such as Fungi and stramenopiles, or are common coral reef inhabitants and could come from contamination of the sample during collection (e.g., Tunicata, Demospongiae, Echinodermata). Fungi contigs were excluded from functional analysis because Fungi are transcriptionally active members of the holobiont and thus these contigs are unlikely to originate from the coral host (Amend, *et al.*, 2011). Most DE contigs were classified as Cnidaria, but a few were assigned to Demospongiae and taxa within Coelomata (Figure 3.13).

Functional annotations of coral host contigs included some SEED categories that appear to originate from other members of the holobiont, because their functions are not biologically relevant to the host transcriptome. These categories included photosynthesis, CO_2 fixation, cell wall and capsule, dormancy and sporulation, and prokaryotic cell cycle (Figure 3.14). Housekeeping and general metabolic functions such as the metabolism of carbohydrates, DNA, and protein, made up the greatest number of contigs, but some categories of special interest to coral physiology were present. Stress response was the fourth most abundant functional category; most of these contigs were related to oxidative stress, but transcripts for osmotic stress and xenobiotic detoxification were also represented (Figure 3.15). Contigs related to potassium homeostasis were found in the coral host group as well as the zooxanthellae group of contigs, although those assigned to the coral host included genes such as KefC and TrkA that likely originate from mis-annotated prokaryotes (Jan & Jan, 1997) .

DE contigs in the coral host group were distributed across several functions and pathways (Table 3.2). Respiratory complex I, the first enzyme complex involved in oxidative phosphorylation, had several of its NADH dehydrogenase subunits more highly expressed in orange than brown (Figure 3.16). Most other DE contigs were part of cell signaling pathways (Table 3.2). In orange colonies, the MAPK, Notch, calcium signaling, and Toll-like/RIG-I-like receptor pathways and cell adhesion molecules had contigs that were significantly DE compared to brown morphs. Part of the cell cycle and p53 pathways and several cytochrome C oxidase subunits were more highly expressed in brown colonies.

Discussion

Taxonomic Groups in the Metatranscriptome

Contigs from the metatranscriptome of *Montastraea cavernosa* revealed the diverse communities of organisms composing the holobiont, and some of their functional capabilities that may contribute to overall holobiont fitness. Taxonomic assignments were made from BLASTX hits to putative mRNA contigs, but these may not be as reliable as assignments made from marker gene sequences such as the 16S rRNA. Protein coding genes vary in how phylogenetically informative they are, and while sequenced genomes are available for a growing number of taxa, many other taxa are represented by only a few functional genes or marker gene sequences. Taxonomic assignments are expected to be biased towards taxa that contain many representative sequences in the database, and against poorly characterized taxa. This problem is particularly acute for the wide diversity of prokaryotes and unicellular protists that remain uncultured (Behnke, *et al.*, 2011). Analysis of the rRNA fraction of the metatranscriptome will be used in the future to characterize the taxonomic composition of the community and confirm the results of mRNA annotation.

Based on putative mRNA sequences, a diverse prokaryotic community including bacteria and Archaea was present in *M. cavernosa* samples (Figure 3.1). Among the bacteria, Proteobacteria and in particular the Burkholderiales had the largest number of contigs (Figure 3.1, 3.3). Pyrosequencing of PCR amplicons of the 16S rRNA gene from the same samples also found that Proteobacteria were dominant members of the prokaryotic community, but Burkholderiales made up only a small fraction of these reads (Chapter 2), suggesting that this group is highly transcriptionally active. Cyanobacteria were the second most common source of prokaryotic mRNA contigs, and were mostly composed of Chroococcales and Oscillatoriales (Figure 3.4), supporting the results of pyrosequencing with eubacterial primers (Chapter 2) and cyanobacteria-specific primers (Lesser, *et al.*, 2004) that placed the putative cyanobacterial symbionts within the Chroococcales. Putative mRNA contigs were assigned to several cyanobacterial groups that were probably not present in the coral holobiont. Taxonomic groups with many full genome sequences tend to collect more mRNA annotations than groups with fewer sequenced representatives; this documented bias likely affected the taxonomic assignment of contigs in this study. Examples of taxa that may have collected annotations but are probably not present include *Acaryochloris* and *Microcystis aeruginosa*. *Acaryochloris* commonly lives in shaded habitats beneath ascidians and possesses a unique form of chlorophyll *d* that allows it to utilize far-red light for photosynthesis (Miyashita, *et al.*, 2003, Kuhl, *et al.*, 2005). However, far-red light is not present at the depth of sample collection due to attenuation by the water column, so it is unlikely that this group is genuinely present. While *Microcystis aeruginosa* is found exclusively in freshwater, it is a prolific producer of microcystins and other secondary metabolites, which ELISA assays of coral tissue extracts showed to be present in a small number of orange colonies of *M. cavernosa* (Chapter 4). Microcystins are also found in the microbial mat of black band disease of corals, as well as some free-living marine cyanobacteria on reefs (Richardson, *et al.*, 2007, Gantar, *et al.*, 2009, Stanić, *et al.*, 2011). Other groups are more likely to be present in the holobiont, and are also of special functional interest. The filamentous marine cyanobacterium *Moorea producta* is also known to produce secondary metabolites (Engene, *et al.*, 2011). Several taxa including *Nostoc, Trichodesmium,* and *Lyngbya* are capable of fixing nitrogen and might contribute to nitrogen cycling within the holobiont.

Previous studies have found that Archaea are abundant on some corals such as *Porites* but apparently are not associated with others, including Caribbean *Acropora* (Wegley, *et al.*, 2004). Here, contigs from three phyla of Archaea (Euryarchaeota, Thaumarchaeota, and Crenarchaeota) were observed (Figure 3.5). Similar taxa have been found in *Acanthastrea, Favia,* and *Fungia* from Australia and the Red Sea (Siboni, *et al.*, 2008). Communities in these corals were dominated by *Nitrosopumilus*, which oxidizes ammonia to nitrite (Konneke, *et al.*, 2005) and is thought to play an important role in nitrogen cycling in corals. *Nitrosopumilus* was recently re-classified in the new phylum Thaumarchaeota (Brochier-Armanet, *et al.*, 2008, Molloy, 2011), which contained many contig assignments in this study. In the sponge *Geodia barretti,* Archaea are numerically abundant and highly transcriptionally active cells, but were poorly represented in rRNA sequence counts from a metatranscriptome and in cloned 16S rRNA sequences (Radax, *et al.*, 2012). A low abundance of rRNA molecules appears to be a general Archaeal trait (Valentine, 2007), a hypothesis that can be tested using the ratio of mRNA and rRNA assigned to Archaea within this study. Combined with the finding of Crenarchaeotes in

16S rRNA sequencing (Chapter 2), it appears that Archaea are present and active in *M. cavernosa*.

The zooxanthellae group included contigs annotated as Alveolata and Viridiplantae (Figure 3.9), in an attempt to include the contigs related to photosynthesis and autotrophy in *Symbiodinium*. There were no dinoflagellate annotations, but sequence data on dinoflagellates is limited due to the immense size and unusual features of their genomes, none of which have yet been fully sequenced (Lin, 2011). Many of the assignments were to related apicomplexan parasites or pathogens, perhaps indicating that some mechanisms for associating with a host may be shared between parasites and symbionts (Figure 3.9) (Dale & Moran, 2006, Bright & Bulgheresi, 2010).

The coral host contigs were assigned primarily to Cnidaria, but a variety of other taxa were identified as well. Some of these may represent genes that are genuinely present in the host transcriptome but have not been previously described from cnidarians. Cnidarian genomes that are currently available include the hydrozoans *Hydractinia echinata* (Soza-Ried, *et al.*, 2010) and *Hydra magnipapillata* (Chapman, *et al.*, 2010), the scleractinian *Acropora digitifera* (Shinzato, *et al.*, 2011), and the actinarian *Nematostella vectensis* (Putnam, *et al.*, 2007); their genes often have high similarity to those of vertebrates, and many appear to be unique to cnidarians (Soza-Ried, *et al.*, 2010), so a finding of new sequences would not be unprecedented but merits further investigation. Other contigs not assigned to Cnidaria could represent other members of the holobiont that were present in the samples. Coral-associated fungi are diverse and metabolically active (Wegley, *et al.*, 2007, Amend, *et al.*, 2011, Littman, *et al.*, 2011), and stramenopiles have recently been reported to be embedded in the mucus and tissue of the massive coral *Favia* sp. in the Red Sea, forming a "white film" over the colony surface (Siboni, *et al.*, 2010). Similar films have also been observed on *M. cavernosa* (Lasker, 1981, Kao, *et al.*, 2007).

Expressed Genes in the Compartments of the Holobiont

Prokaryotic genes in the metatranscriptome reflected the diversity of taxonomic assignments, and coral-associated prokaryotes appear to have diverse modes of nutrition and express many genes that could play a role in holobiont fitness (Figure 3.6, 3.7). Indications of nitrogen cycling were present, including *nifS*, which plays an essential role in the production of the Fe-S metalloclusters of nitrogenase (Zheng & Dean, 1994). This gene is required for the full function of nitrogenases in *Azotobacter*, but may also be involved in the synthesis of Fe-S metalloclusters required for other enzymes (Johnson, *et al.*, 2005), as *nifS*-like genes have been found in organisms that do not fix nitrogen (Zheng, *et al.*, 1993). The presence of a contig annotated as rubrerythrin supports the hypothesis that NifS is involved in nitrogen fixation in this system, because the antioxidant activity of this protein has been shown to protect nitrogenase from reactive oxygen species in heterocystous cyanobacteria (Zhao, *et al.*, 2007). Multiple contigs involved in ammonia assimilation were found, allowing the prokaryotes to take up and utilize ammonia excreted by the coral host or fixed nitrogen (Figure 3.7). Genes for the metabolism of aromatic compounds and the stress response may help bacteria occupy unique nutritional niches and tolerate the fluctuating redox conditions in coral tissue and mucus (Dykens & Shick, 1982).

The most highly represented SEED system in the prokaryotic portion of the metatranscriptome was virulence (Figure 3.6) and these contigs were related primarily to resistance to antibiotics and the scavenging and uptake of iron (Figure 3.8a). Degradation rather than efflux of antimicrobial compounds seems to be the primary strategy of resistance (Figure 3.8b). A majority of the culturable bacteria associated with corals produce antimicrobial compounds (Ritchie, 2006, Rypien, *et al.*, 2010), so a large repertoire of genes for inactivating such compounds is not unexpected. Interestingly, the addition of nitrogen caused a significant increase in genes associated with antibiotic resistance in metagenomes of *Porites compressa*, perhaps indicating an additional effect of fixed nitrogen in orange colonies (Vega Thurber, *et al.*, 2009). The large number of contigs for iron acquisition could be involved in functions that are beneficial to the coral holobiont. Corals can ingest their mucus, so it has been hypothesized that iron scavenged by bacteria living in mucus may be utilized by the host (Knowlton & Rohwer, 2003). One of the two DE contigs in the prokaryotic fraction was EntF, which along with related genes catalyzes the formation of enterobactin (Raymond, *et al.*, 2003). This gene was more highly expressed in the orange morph. Orange colonies contain dense (10^7 cells cm^{-2}) populations of symbiotic cyanobacteria (Lesser, *et al.*, 2004), which require iron for their redox enzymes as well as the Fe-S metalloclusters in the nitrogenase enzyme required to fix nitrogen, so orange holobionts likely have a greater total requirement for iron, necessitating aggressive scavenging with this high-affinity siderophore.

There were no contigs taxonomically assigned to dinoflagellates within the zooxanthellae group and no contigs were significantly DE, but the unique features of the genome and gene expression in dinoflagellates may account for this. *Symbiodinium* has a relatively small genome, approximately the size of the human genome, but other dinoflagellates have genomes up to 50 times larger (LaJeunesse, *et al.*, 2005). Because of their extremely large genomes, there are no fully sequenced dinoflagellates, only EST libraries and similar data sets, so dinoflagellates are under-represented in sequence databases (Leggat, *et al.*, 2007, Lin, *et al.*, 2010, Jaeckisch, *et al.*, 2011, Bayer, *et al.*, 2012). Features that can complicate the analysis of dinoflagellate genomes include their high GC content, non-conventional bases, and many horizontally transferred genes. Genomic DNA in dinoflagellate cells exists as permanently condensed chromosomes, which appears to have necessitated unusual methods of gene regulation that are not well understood (Hackett, *et al.*, 2004). Almost all dinoflagellate mRNAs are capped with a conserved 22-bp spliced-leader sequence that may be involved in regulating translation (Zhang, *et al.*, 2007). This sequence has recently been utilized as a target for PCR primers to amplify cDNAs (Lin, *et al.*, 2010). The minimal changes in transcription observed in many studies of dinoflagellates suggest that either much of the gene regulation occurs post-transcriptionally (reviewed in (Leggat, *et al.*, 2011)), or that specific targeting of the spliced-leader sequences may be necessary to efficiently recover transcripts. Interestingly, exposure to bacteria provokes a relatively large transcriptional response, which has implications for dinoflagellates within the coral holobiont (Leggat, *et al.*, 2011).

SEED functions assigned to zooxanthellae contigs included the expected processes in a photosynthetic eukaryote, and reflected the environment of the coral holobiont. Photosynthesis, carbon fixation (including Rubisco), response to oxidative stress, heat shock proteins, and respiration were all represented (Figure 3.10). The contigs involved in potassium homeostasis may be important to osmoregulation and the maintenance of symbiosis with the coral host (Mayfield & Gates, 2007), as in rhizobial symbioses (Domi-nguez-Ferreras, *et al.*, 2009). The greater number of contigs assigned to translation initiation and RNA processing relative to transcription supports what is known about gene regulation in dinoflagellates (Figure 3.11).

Taxonomic and functional assignments of coral contigs were diverse and likely reflected both the actual taxa and functions as well as some mis-annotations. While most contigs were assigned to Cnidaria, assignments to Vertebrata and other higher taxa are not unexpected as many coral genes are more similar to those in vertebrates than other invertebrates (Putnam, *et al.*, 2007, Soza-Ried, *et al.*, 2010). Many of the expected SEED subsystems were represented, like oxidative stress (Figure 3.14). However, some contigs are clearly mis-categorized and do not originate from the coral host, such as those assigned to photosynthesis, cell wall and capsule, and dormancy and sporulation.

Potential Role of Osmotic Stress in Orange *M. cavernosa*

Many of the differentially expressed contigs found in orange colonies may be related to the demands of maintaining a large population of mostly heterotrophic intracellular symbionts, namely the cyanobacteria. Mayfield and Gates (2007) have proposed that osmoregulation is an important and dynamic process that helps to maintain the symbiosis between coral and dinoflagellate, and that osmotic stress is a key factor in the breakdown of this association during thermal stress (Mayfield & Gates, 2007). Functional zooxanthellae perform photosynthesis and translocate glycerol, which in addition to serving as a carbon source for the metabolism of the coral host, is also an osmolyte. When zooxanthellae are damaged by thermal stress, carbon fixation is impaired and the amount of glycerol translocated to the host is decreased, resulting in osmotic imbalance. In the proposed models, the host will attempt to produce more osmolytes, increasing its respiration rate and thereby producing more reactive oxygen species (ROS). If the condition persists, changes in cellular pH and volume can denature proteins, cause a loss of adhesion of cells, and eventually the loss or ejection of symbionts. In *M. cavernosa,* the majority of the Photosystem II complexes in the cyanobacteria are uncoupled from photochemistry and the cyanobacteria appear to be living heterotrophically, likely respiring glycerol (Lesser, *et al.*, 2007). Given the density of cyanobacteria populations, this depletion of the pool of glycerol could be significant, and might create chronic osmotic stress in these colonies.

All of the responses described above are predicated on the ability of the coral to sense osmotic stress and cue other cellular components to respond. The MAPK signaling pathway is involved in osmoregulation in eukaryotes (Cowan & Storey, 2003), and several components of this pathway were more expressed in orange colonies (Table 3.2). Parts of the respiratory complex I were also overexpressed (Table 3.2), which could be related to the energetic demands of correcting osmotic imbalances, or to the presumably increased need for membrane transport (e.g., of compounds containing newly fixed nitrogen from cyanobacteria to zooxanthellae) in orange colonies. Respiratory complex I is also a primary site of electron leakage to O_2 and thus generates ROS. The reason for higher expression in brown colonies of some parts of the p53 pathway is unclear, but could reflect significant down regulation in orange colonies, rather than upregulation in brown colonies.

Metatranscriptomic Sampling and Analysis

Many genes and functional categories that were of interest *a priori* based on knowledge of the *M. cavernosa* system were not recovered in this metatranscriptome. In particular, genes related to nitrogen fixation, assimilation, and other nitrogen transformations (e.g., ammonia oxidation) were poorly represented, and neither the cyanobacterial pigment phycoerythrin (PE) or any green fluorescent protein (GFP) homologs were found. For logistical reasons, the samples sequenced here were collected at midday, a time when nitrogen fixation is not occurring in this system (Lesser, *et al.*, 2007). The hyperoxic conditions in coral tissue during the daylight hours (Dykens & Shick, 1982) would irreversibly inactivate nitrogenase, so transcription of nitrogenase likely does not take place until the late afternoon or early evening, when oxygen concentrations are low and fixation begins. Respiration in the cyanobacteria may also aid in the protection of nitrogenase. Many of the other nitrogen-related genes would be required only when there is fixed nitrogen to be assimilated, metabolized or stored, so these genes are apparently not expressed during daylight hours. Phycoerythrin is a highly abundant protein in orange *M. cavernosa* and gives the colonies their characteristic fluorescence, so the lack of PE transcripts was unexpected. Although it is a light harvesting pigment in most cyanobacteria and thus might be expected to be expressed during the light period, PE can also serve as a nitrogen storage compound (Wyman, *et al.*, 1985) and may be transcribed only in the evening when fixed nitrogen is available. Metatranscriptomic sequencing of samples collected during nighttime hours would likely yield a significantly different array of processes and functions.

The choices made in data analysis of this metatranscriptome almost certainly affected the quality of the results. Although failed reads were discarded, reads were not trimmed for quality before they were assembled, which may explain why a relatively low percentage of the reads were successfully mapped back to the assembled contigs during RNA-Seq analysis (S. Vollmer, pers. comm.). The contributions of erroneous or poor-quality reads to the consensus sequences of contigs may have altered the true mRNA sequences sufficiently to reduce the number of significant BLASTX hits, despite the relatively high (i.e., permissive) E-value cutoff. Annotations discussed here are limited to hits to known and annotated genes, and do not include hits to hypothetical proteins, or to sequences recovered from other corals or similar organisms that may be of interest but whose function is unknown. The annotation rate of contigs in this study was approximately 32% for taxonomic annotations and less than 1% for functional SEED annotations, which is low compared to other marine metatranscriptomes (Poretsky, *et al.*, 2009, Marchetti, *et al.*, 2012, Radax, *et al.*, 2012).

Future Directions

Several hypotheses about the *M. cavernosa* holobiont are presented here, but this study is merely a preliminary analysis of a very large and complex data set, and the interpretations may change with further investigation. An alternative pipeline for re-analyzing the putative mRNA contigs is under development. Reads will be trimmed for quality with extremely high stringency, and assembled in Trinity, which is designed for the unique features of transcriptome data (Grabherr, *et al.*, 2011). New contigs will be annotated with a custom pipeline utilizing the SwissProt and UniRef 90 databases, and also compared directly to databases from genomic or transcriptomic sequencing of relevant organisms including *Nematostella, Pocillopora damicornis, Acropora digitifera, A. palmata,* and *Symbiodinium* (Putnam, *et al.*, 2007, Polato, *et al.*, 2011, Shinzato, *et al.*, 2011, Traylor-Knowles, *et al.*, 2011, Bayer, *et al.*, 2012). Differential expression analysis will be repeated, and specific taxonomic groups (Fungi, Archaea, viruses, etc.) will be investigated in more detail. Modification of the MEGAN program or its data inputs will be attempted, to display expression values rather than contig counts for more intuitive data visualization.

Analysis of the rRNA portion of the metatranscriptome is planned, to complete the "double-RNA" approach. This data will serve as a comparison to the mRNA data, as well as the pyrosequenced 16S rRNA PCR amplicon data (Chapter 2). A new iterative method utilizing mapping to a reference database of SSU sequences will be used to reconstruct full-length rRNA sequences from the total pool of reads. Reads will be mapped back to contigs with RNA-Seq and analyzed with the QIIME pipeline (Caporaso, *et al.*, 2010) to explore the relative abundance of different taxa.

TABLE 3.1. Annotation of putative mRNA contigs from each holobiont compartment.

	All contigs	**Prokaryotic**	**"Zooxanthellae"**	**"Coral host"**
Total contigs	211,657			
Taxonomy assigned	67,248	5133	6999	42661
SEED assigned	1897	618	179	509
KEGG assigned	16464	1087	1411	9947

TABLE 3.2 A. KEGG annotation of contigs with significantly higher expression in

brown colonies.

Contigs	gene	KO	Pathway	Function
2	CytC	8738	Apoptosis, p53 signaling pathway	cytochrome C
1	CDK5	2090	Axon guidance	cycylin-dependent kinase 5
1	Robo2	6754	Axon guidance	roundabout, axon guidance receptor 2
1	ACSM	1896	Butanoate metabolism	acyl-CoA synethtase
1	TTN	12567	Cardiovascular diseases	titin
3	NCAM	6491	Cell adhesion molecules (CAMs)	neural cell adhesion molecule
1	Chk1	2216	Cell cycle, p53 signaling pathway	serine/threonine-protein kinase
2	Chk2	6641	Cell cycle, p53 signaling pathway	serine/threonine-protein kinase
1	FOLR	13649	Endocytosis	folate receptor
2	NPC	12385	Lysosome	lysosome membrane protein
1	LIMP	6497	Lysosome	CD63 antigen, lysosome membrane protein
1	COX1	2256	Oxidative phosphorylation, Respiratory complex IV	cytochrome C oxidase
2	Cyto	411	Oxidative phosphorylation, Respiratory complex IV	ubiquinol-cytochrome C reductase iron-sulfur subunit
1	COX3	2262	Oxidative phosphorylation, Respiratory complex IV	cytochrome C oxidase subunit 3
3	SF3b	12828	Spliceosome	splicing factor 3B subunit 1

TABLE 3.2 B. KEGG annotation of contigs with significantly higher expression in

orange colonies.

Contigs	gene	KO	Pathway	Function
1	CHIA	1183	Amino sugar and nucleotide sugar metabolism	chitinase
2	CIITA	8060	Antigen processing and presentation	class II, major histocompatability complex, transactivator
1	CD38	1242	Calcium signaling pathway	NAD+ nucleosidase
1	TTN	12567	Cardiovascular diseases	titin
2	SELP	6496	Cell adhesion molecules (CAMs)	selectin, platele
2	VCAM1	6527	Cell adhesion molecules (CAMs)	vascular cell adhesion molecule
5	NCAM	6491	Cell adhesion molecules (CAMs)	neural cell adhesion molecule
1	Brn1	6676	Cell cycle, p53 signaling pathway	condensin complex subunit 2
1	HSPG2	6255	ECM-receptor interaction	Heparan sulfate proteoglycan 2 (perlecan)
1	FOLR	13649	Endocytosis	folate receptor
1	STI1	9553	Folding, sorting and degradation	stress-induced phosphoprotein 1
2	UBC	8770	Folding, sorting and degradation	ubiquitin C
1	Hh	6224	Hedgehog signaling pathway	Hedgehog, intercellular signaling
2	CD59	4008	Immune system, hematopoetic cell lineage	CD59 antigen
1	SRF	4378	MAPK signaling pathway	serum response factor
2	MEKK2 B	4420	MAPK signaling pathway	mitogen-activated protein kinase
1	MEKK	4461	MAPK signaling pathway	mitogen-activated protein kinase
4	Notch	2599	Notch signaling pathway	Notch transmembrane receptor protein
1	XPF	10848	Nucleotide excision repair	DNA excision repair protein
1	NuoA	330	Oxidative phosphorylation, Respiratory complex I	NADH dehydrogenase

1	NuoK	340	Oxidative phosphorylation, Respiratory complex I	NADH dehydrogenase
1	deaD	5592	RNA degradation	helicases
2	TRAF3	3174	Toll-like, RIG-I-like receptor signaling pathways	TNF receptor-associated factor 3
3	TBK1	5410	Toll-like, RIG-I-like receptor signaling pathways	TANK-binding kinase 1
1	Kca	4936	Vascular smooth muscle contraction	potassium large conductance Ca-activated channel

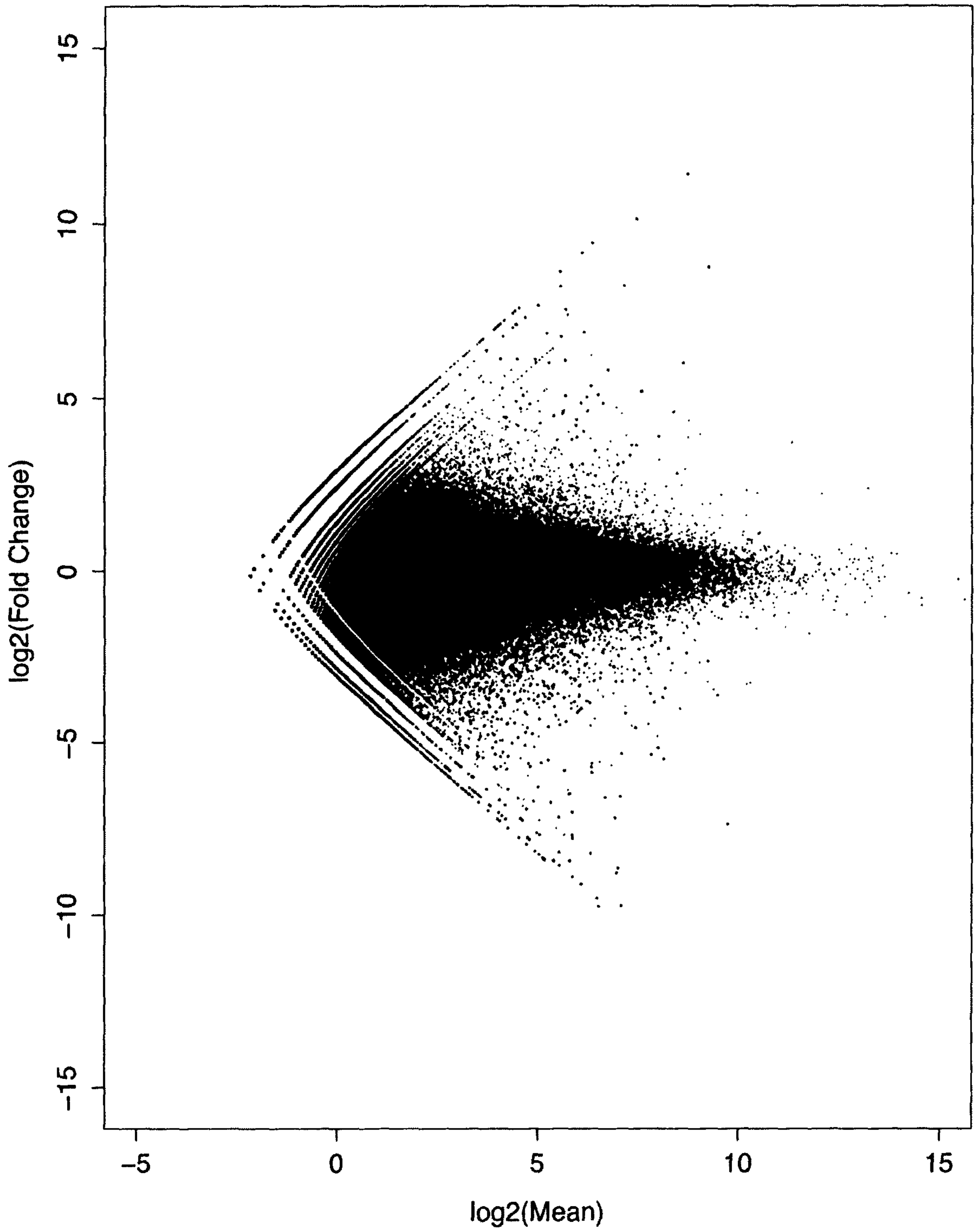


Figure 3.1. Volcano plot comparing mean expression values of putative mRNA contigs and fold change in expression between brown and orange colonies. Contigs with a negative value on the y-axis have higher expression in brown colonies; contigs with a positive value have higher expression in orange colonies. Contigs marked in red are significantly differentially expressed (Bonferroni corrected $P \leq 0.05$).

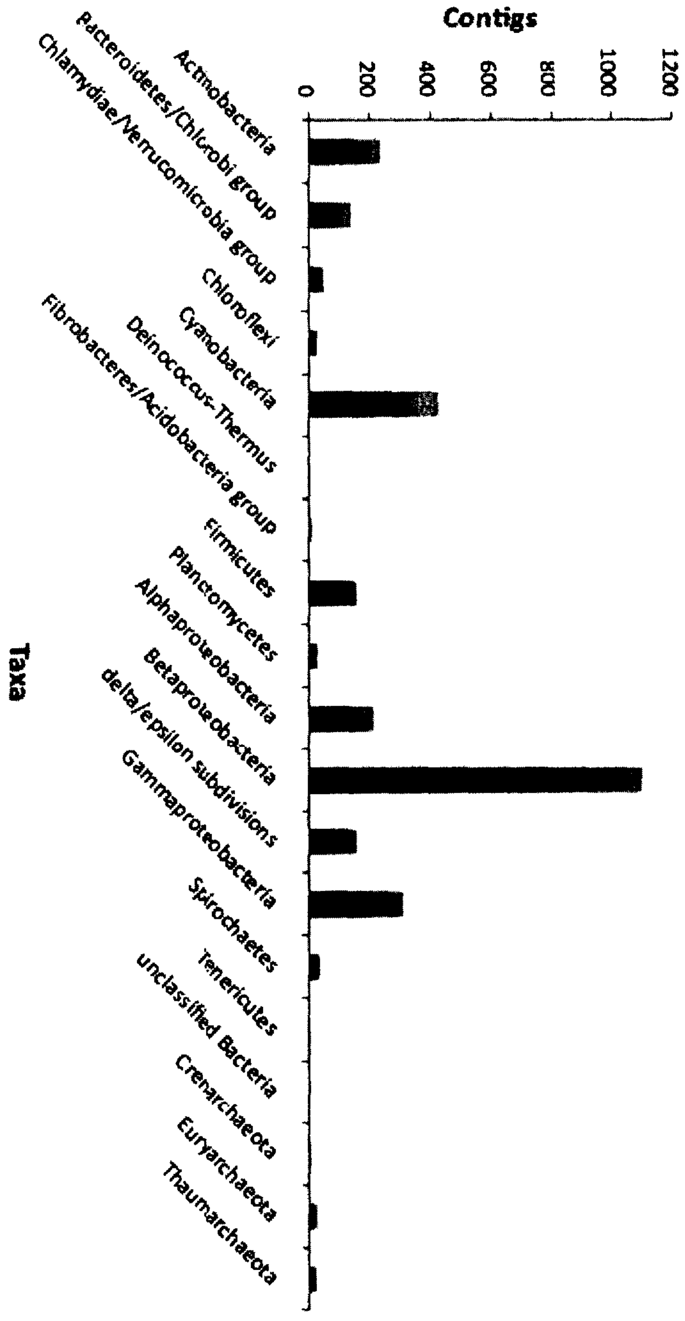


Figure 3.2. Taxonomic assignments of putative mRNA contigs annotated as Bacteria and Archaea (prokaryotic contigs). Note that values represent the number of different contigs, not their expression levels.

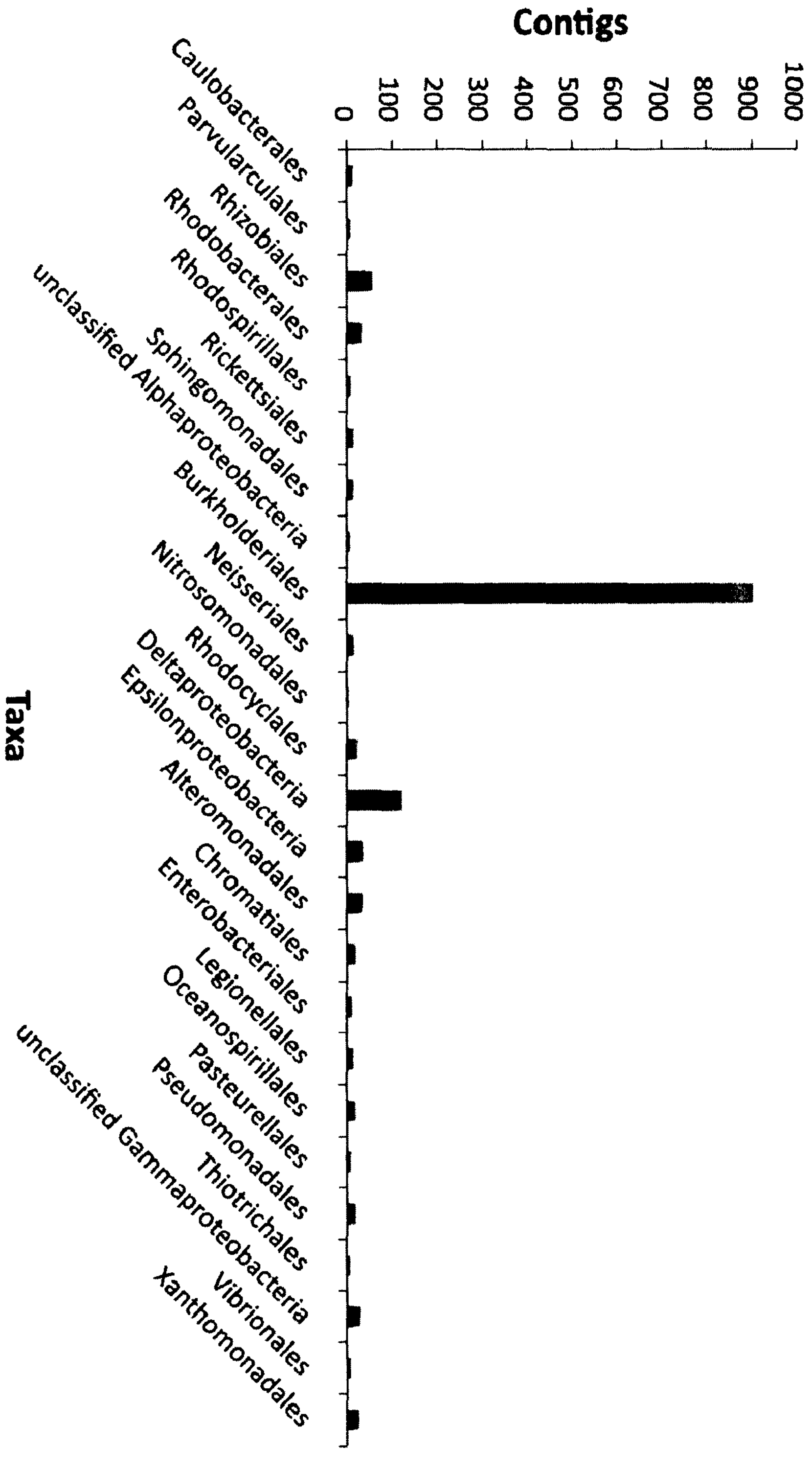


Figure 3.3. Taxonomic assignments of prokaryotic contigs annotated as Proteobacteria. Note that values represent the number of different contigs, not their expression levels.

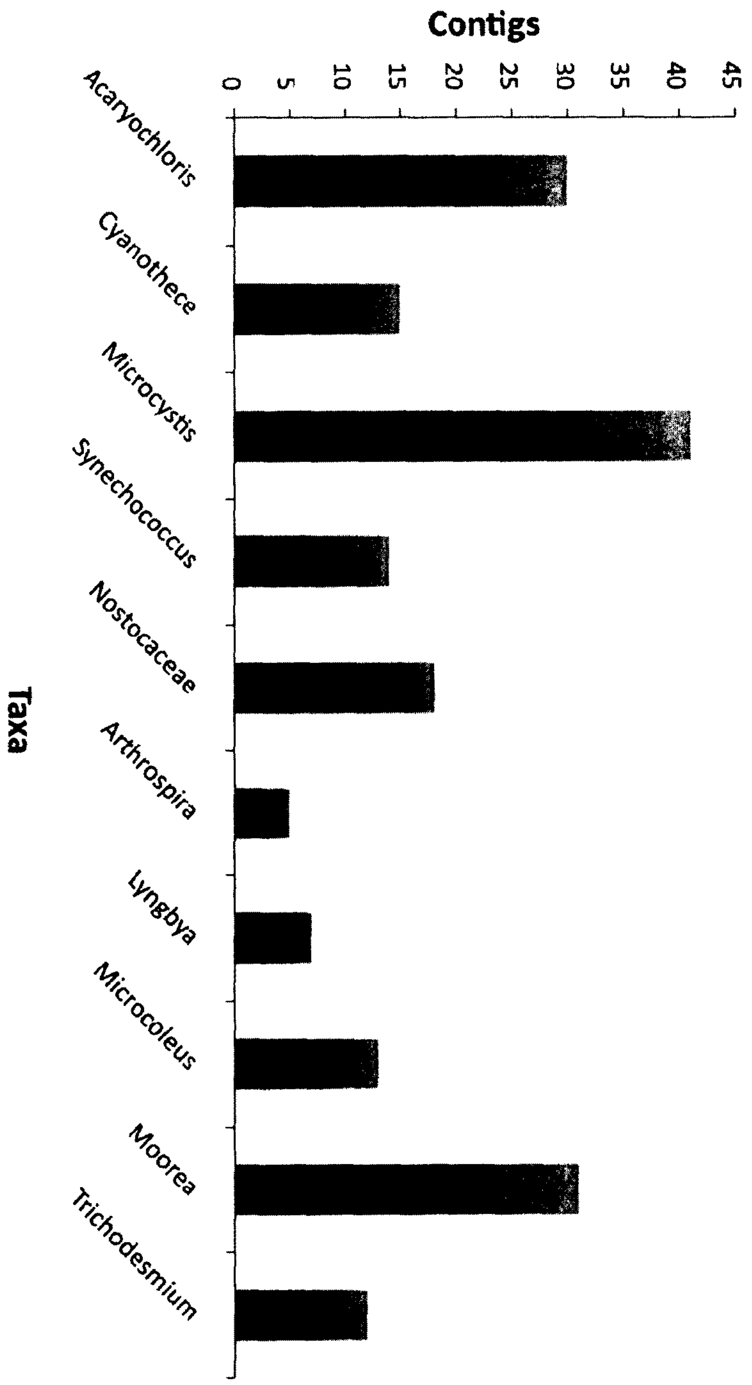


Figure 3.4. Taxonomic assignments of prokaryotic contigs annotated as Cyanobacteria. Note that values represent the number of different contigs, not their expression levels.

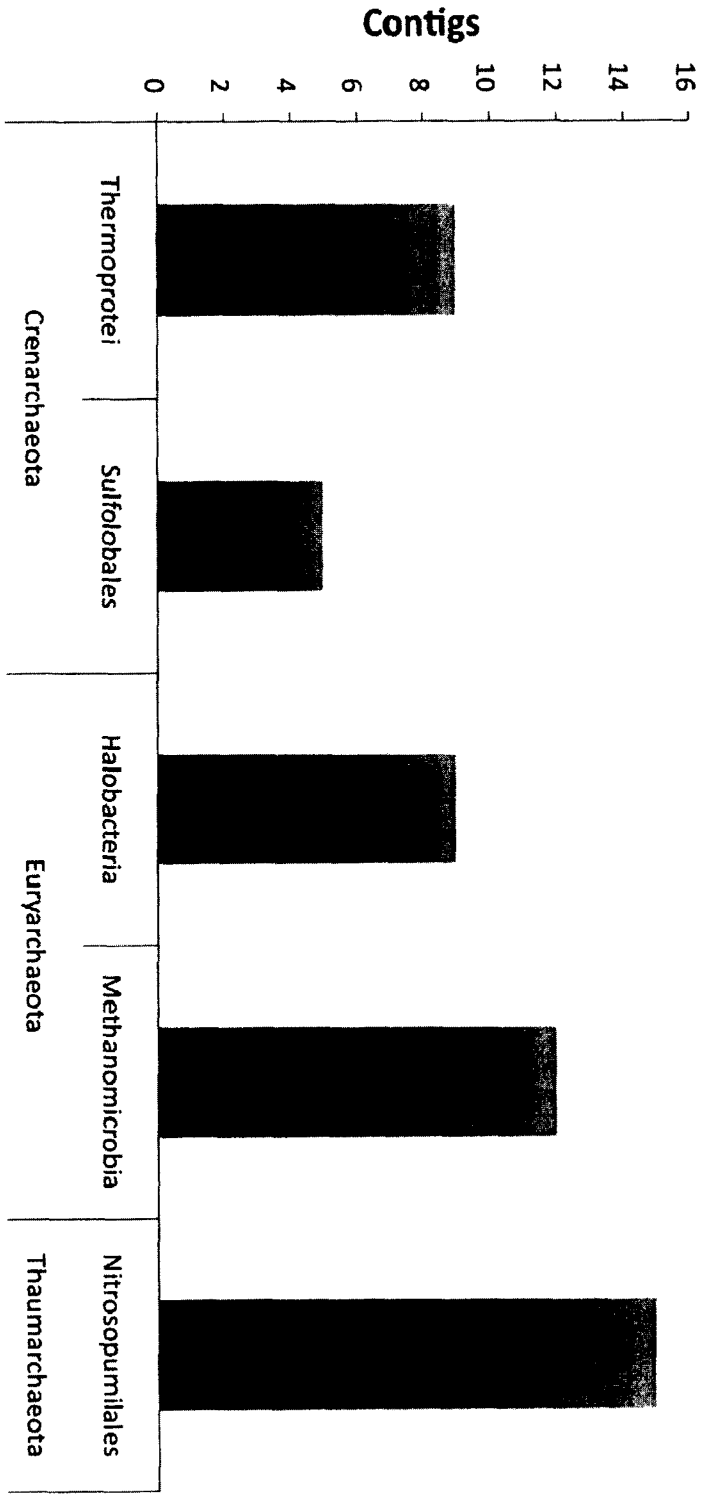


Figure 3.5. Taxonomic assignments of prokaryotic contigs annotated as Archaea. Note that values represent the number of different contigs, not their expression levels.

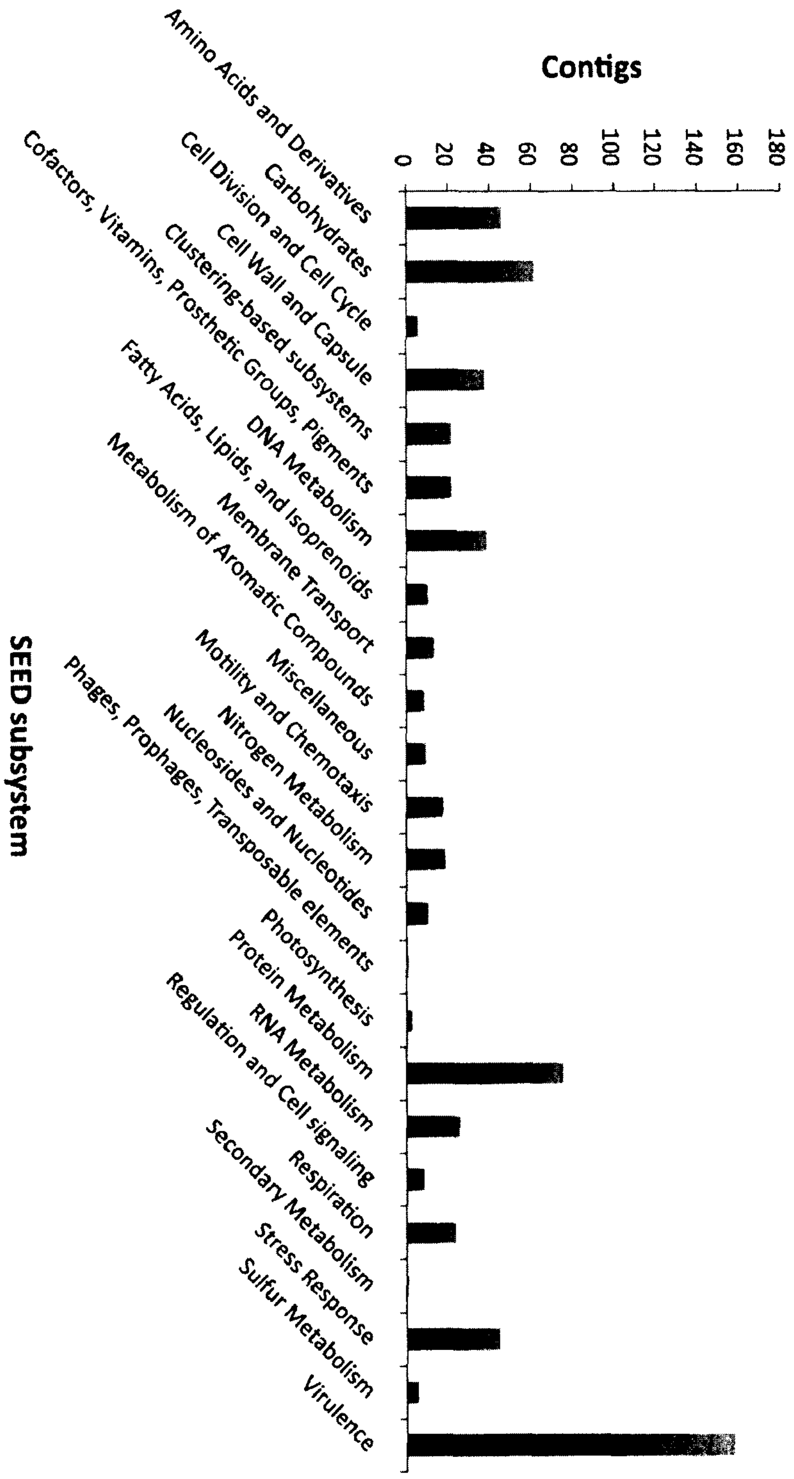


Figure 3.6. Functional assignment of prokaryotic contigs to SEED metabolic subsystems. Note that values represent the number of different contigs, not their expression levels.

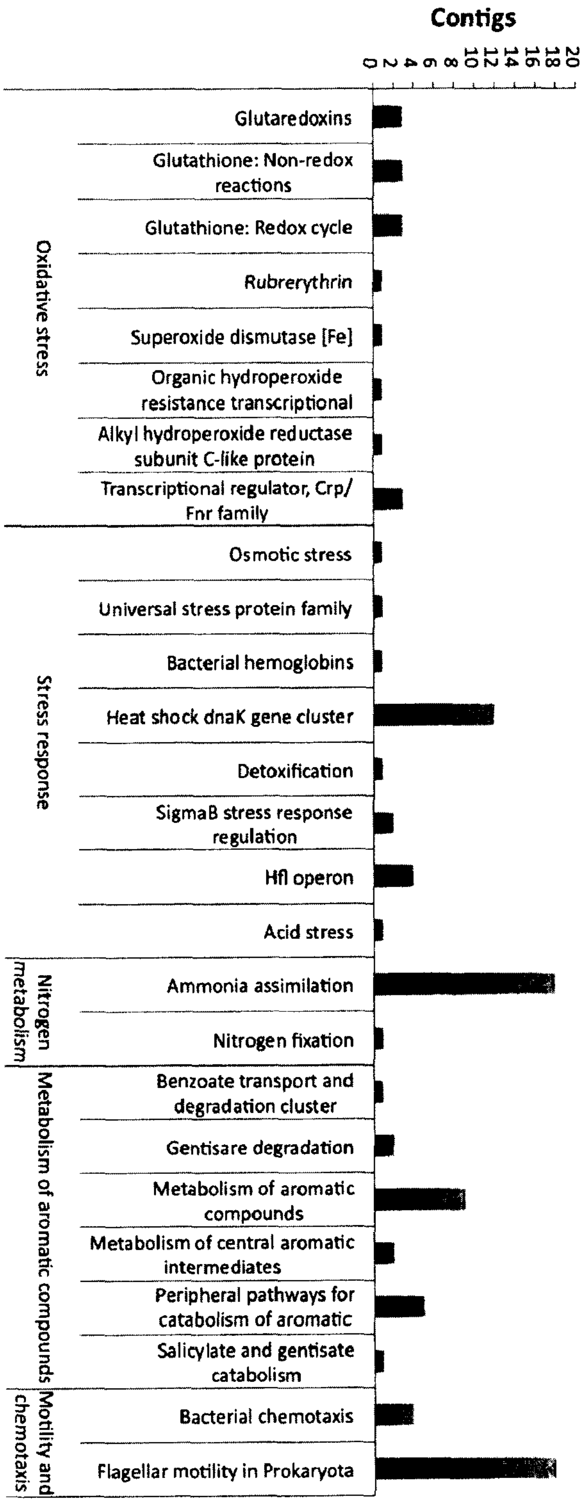
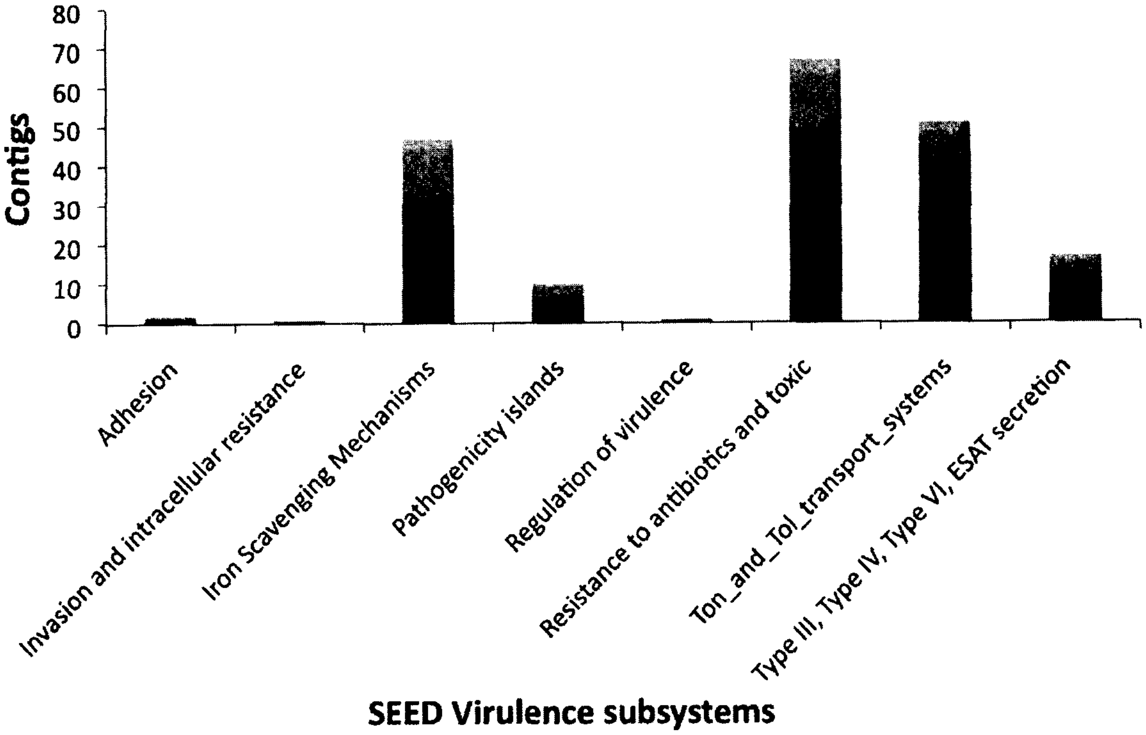


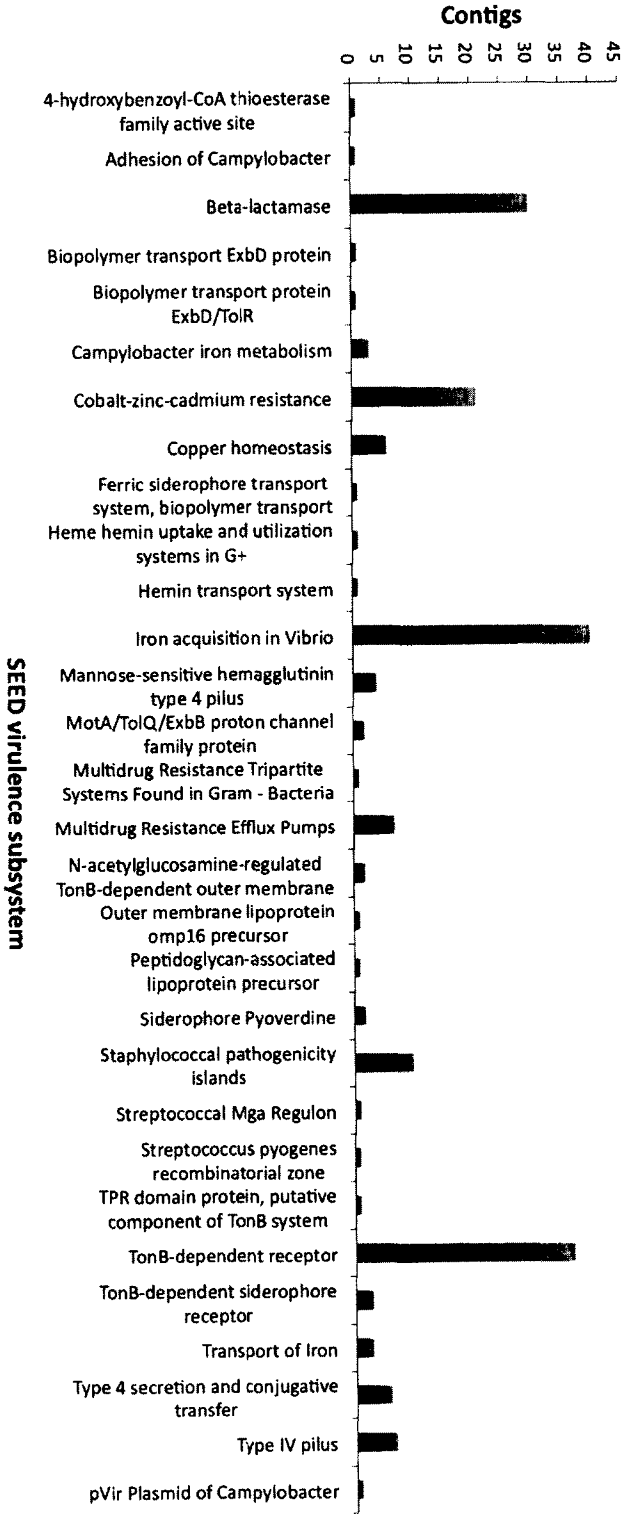
Figure 3.7. Functional assignment of prokaryotic contigs to SEED metabolic subsystems of special interest, presented in hierarchical format. Note that values represent the number of different contigs, not their expression levels.

A.



80
70
60
50
40
30
20
10
0
Contigs
Adhesion
Invasion and intracellular resistance
Iron Scavenging Mechanisms
Pathogenicity islands
Regulation of virulence
Resistance to antibiotics and toxic
Ton_and_Tol_transport_systems
Type III, Type IV, Type VI, ESAT secretion
SEED Virulence subsystems

B.


Contigs
0
5
10
15
20
25
30
35
40
45
SEED virulence subsystem
4-hydroxybenzoyl-CoA thioesterase family active site
Adhesion of Campylobacter
Beta-lactamase
Biopolymer transport ExbD protein
Biopolymer transport protein ExbD/TolR
Campylobacter iron metabolism
Cobalt-zinc-cadmium resistance
Copper homeostasis
Ferric siderophore transport system, biopolymer transport
Heme hemin uptake and utilization systems in G+
Hemin transport system
Iron acquisition in Vibrio
Mannose-sensitive hemagglutinin type 4 pilus
MotA/TolQ/ExbB proton channel family protein
Multidrug Resistance Tripartite Systems Found in Gram - Bacteria
Multidrug Resistance Efflux Pumps
N-acetylglucosamine-regulated TonB-dependent outer membrane
Outer membrane lipoprotein omp16 precursor
Peptidoglycan-associated lipoprotein precursor
Siderophore Pyoverdine
Staphylococcal pathogenicity islands
Streptococcal Mga Regulon
Streptococcus pyogenes recombinatorial zone
TPR domain protein, putative component of TonB system
TonB-dependent receptor
TonB-dependent siderophore receptor
Transport of Iron
Type 4 secretion and conjugative transfer
Type IV pilus
pVir Plasmid of Campylobacter

(Previous pages)
Figure 3.8. A) Prokaryotic contigs assigned to the virulence subsystem. B) Prokaryotic contigs assigned to the virulence subsystem, detailed view. Note that values represent the number of different contigs, not their expression levels.

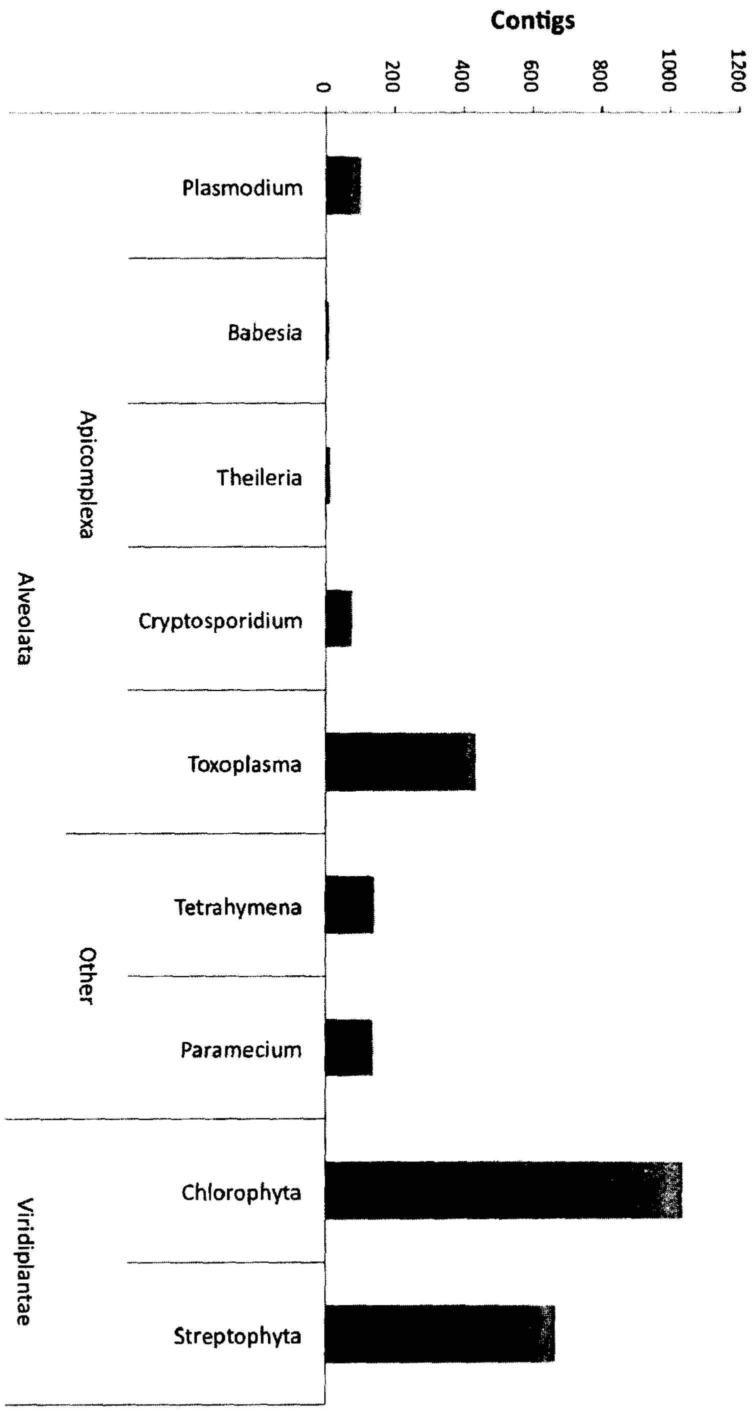


Figure 3.9. Taxonomic assignments of putative mRNA contigs annotated as Alveolata and Viridiplantae (zooxanthellae contigs), in hierarchical format. Note that values represent the number of different contigs, not their expression levels.

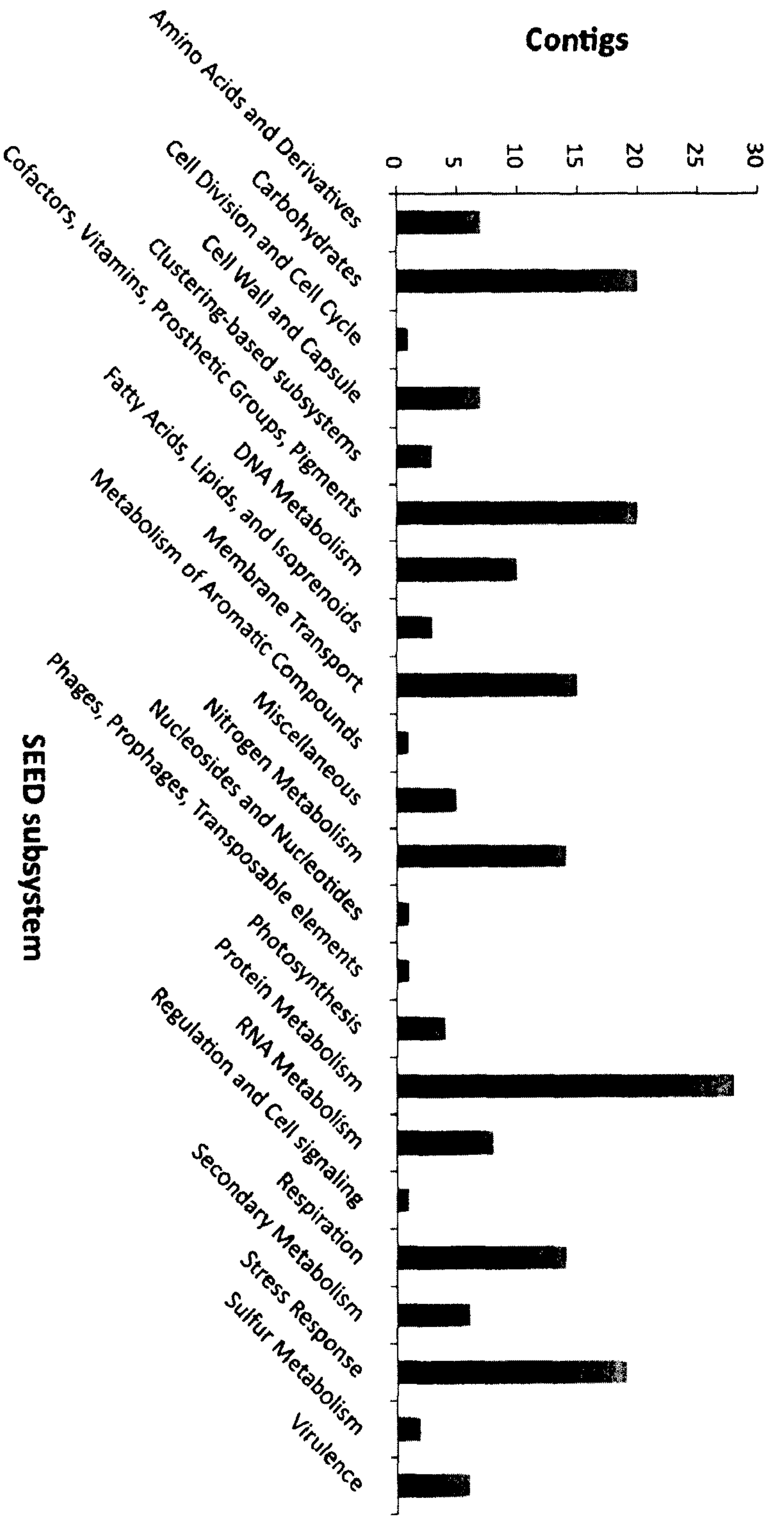


Figure 3.10. Functional assignment of zooxanthellae contigs to SEED metabolic subsystems. Note that values represent the number of different contigs, not their expression levels.

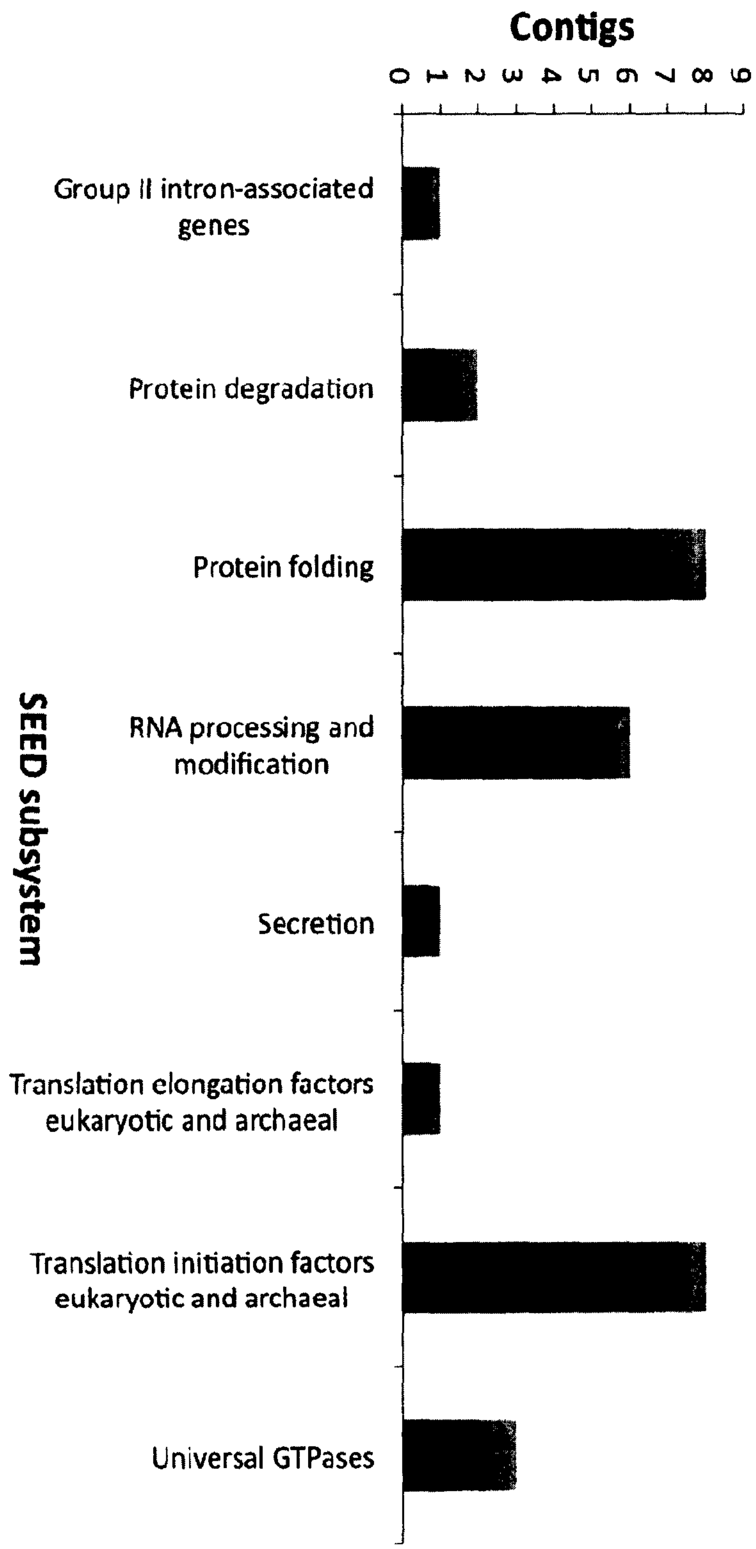


Figure 3.11. Functional assignment of zooxanthellae contigs within the SEED subsystems of protein metabolism, protein biosynthesis, and transcription. Selected subsystems and subcategories shown. Note that values represent the number of different contigs, not their expression levels.

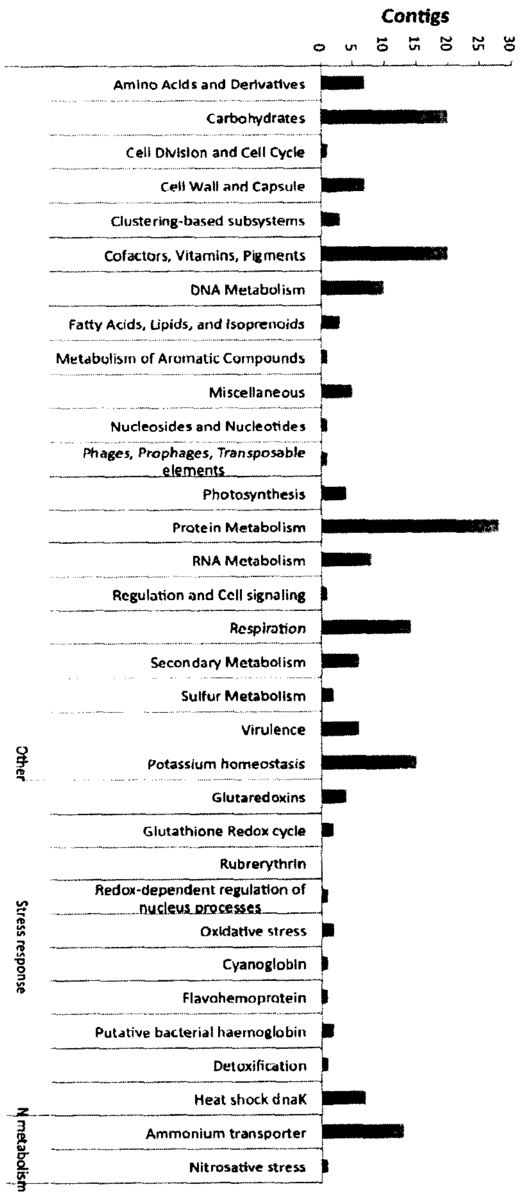


Figure 3.12. Functional assignment of zooxanthellae contigs to metabolic subsystems of special interest, presented in hierarchical format. Note that values represent the number of different contigs, not their expression levels.

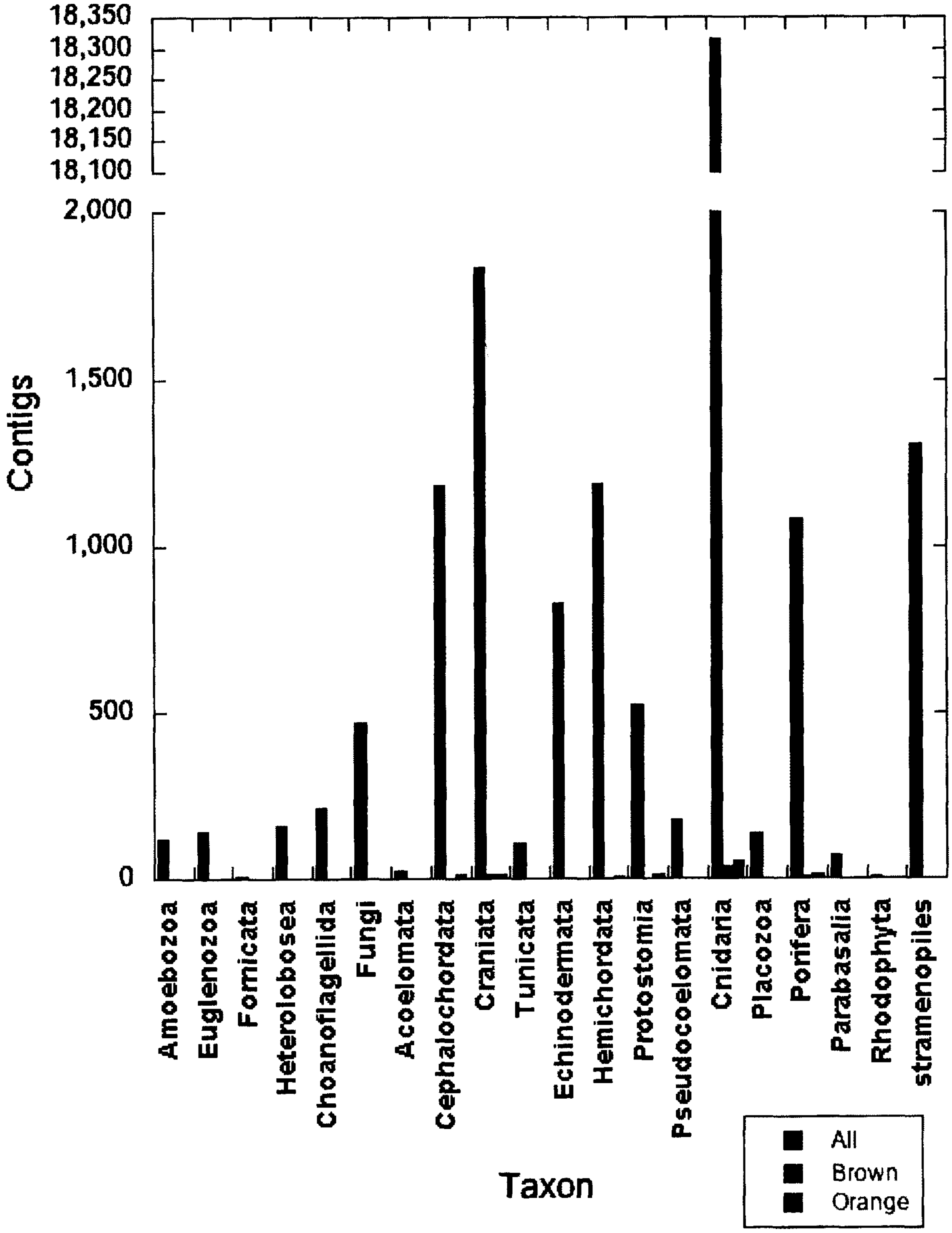


Figure 3.13. Taxonomic assignments of putative mRNA contigs annotated as "coral host." Note that values represent the number of different contigs, not their expression levels. "Brown" contigs had significantly higher expression levels in brown colonies, and "orange" contigs had significantly higher expression levels in orange colonies.

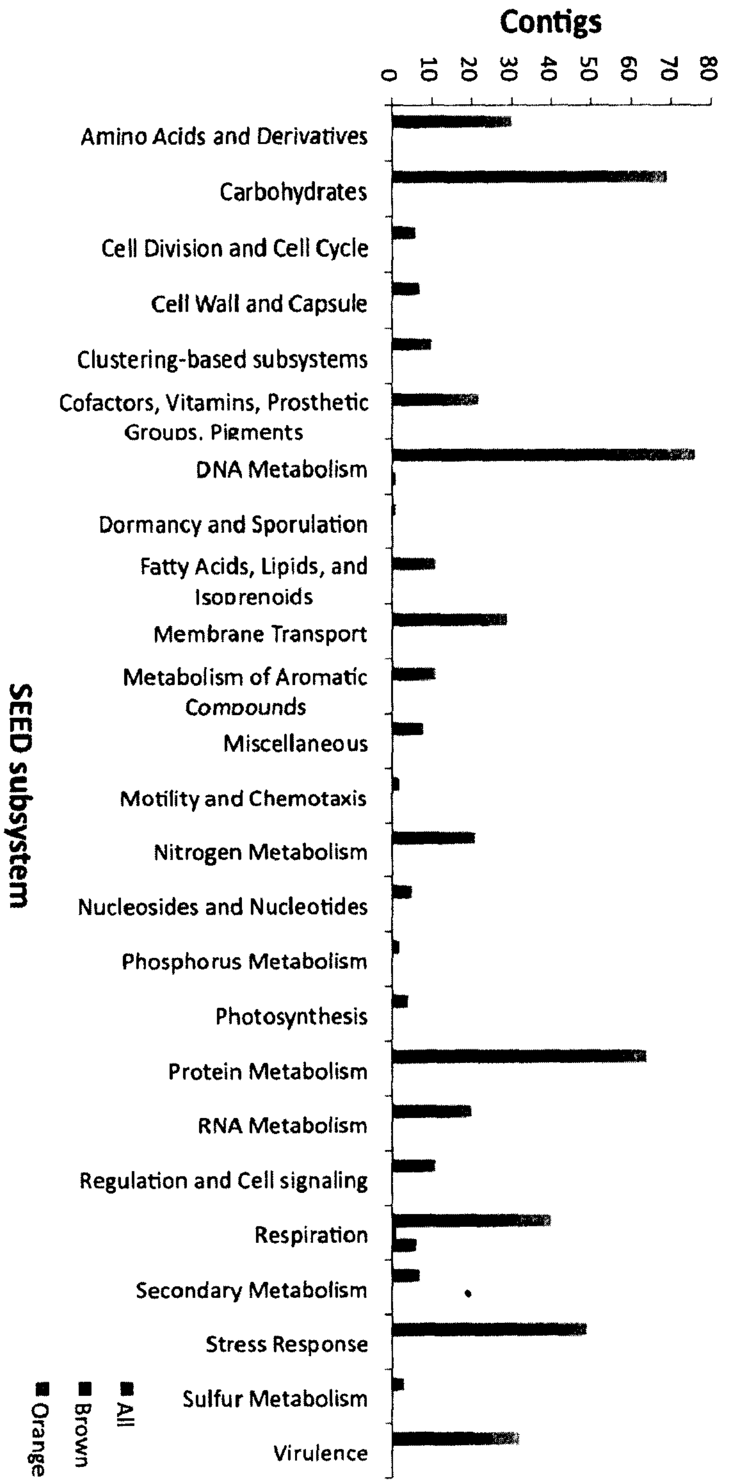


Figure 3.14. Functional assignment of coral host contigs to SEED metabolic subsystems. "Brown" contigs had significantly higher expression levels in brown colonies, and "orange" contigs had significantly higher expression levels in orange colonies. Note that values represent the number of different contigs, not their expression levels.

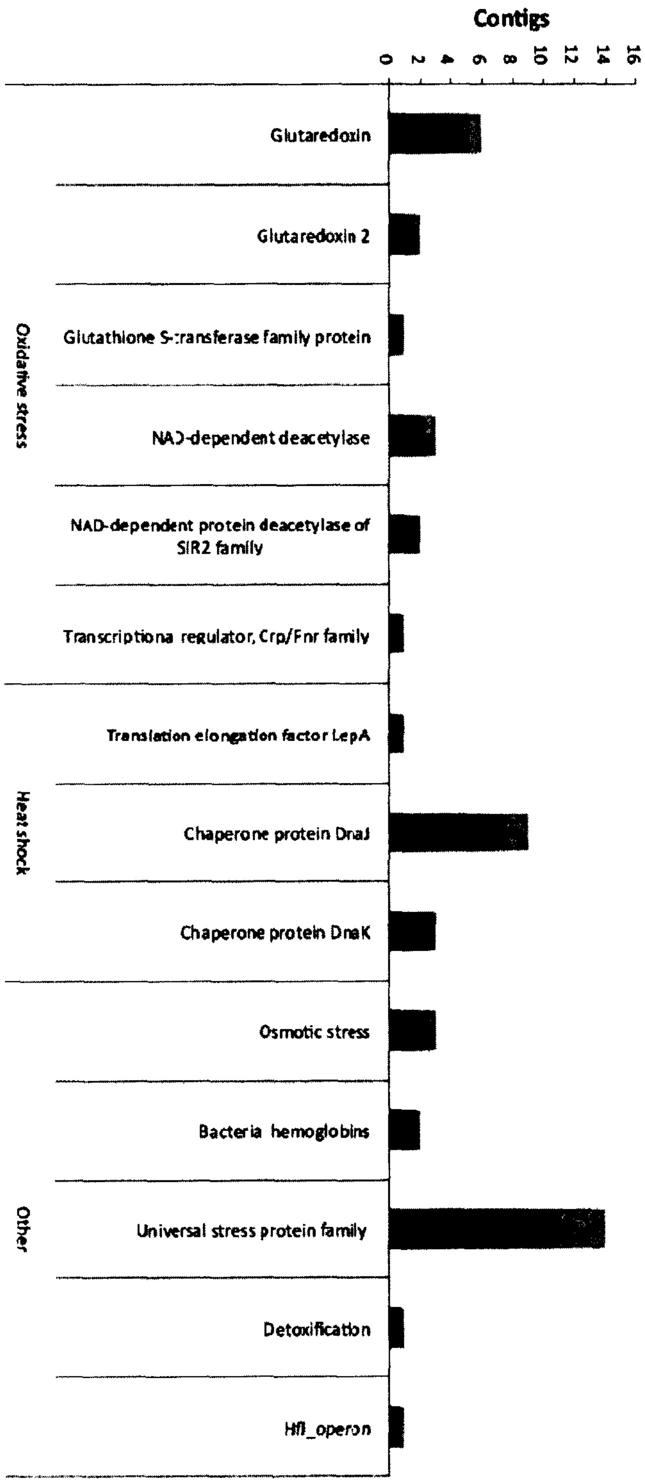


Figure 3.15. Functional assignment of coral host contigs to the SEED stress response subsystem, presented in hierarchical format. Note that values represent the number of different contigs, not their expression levels.

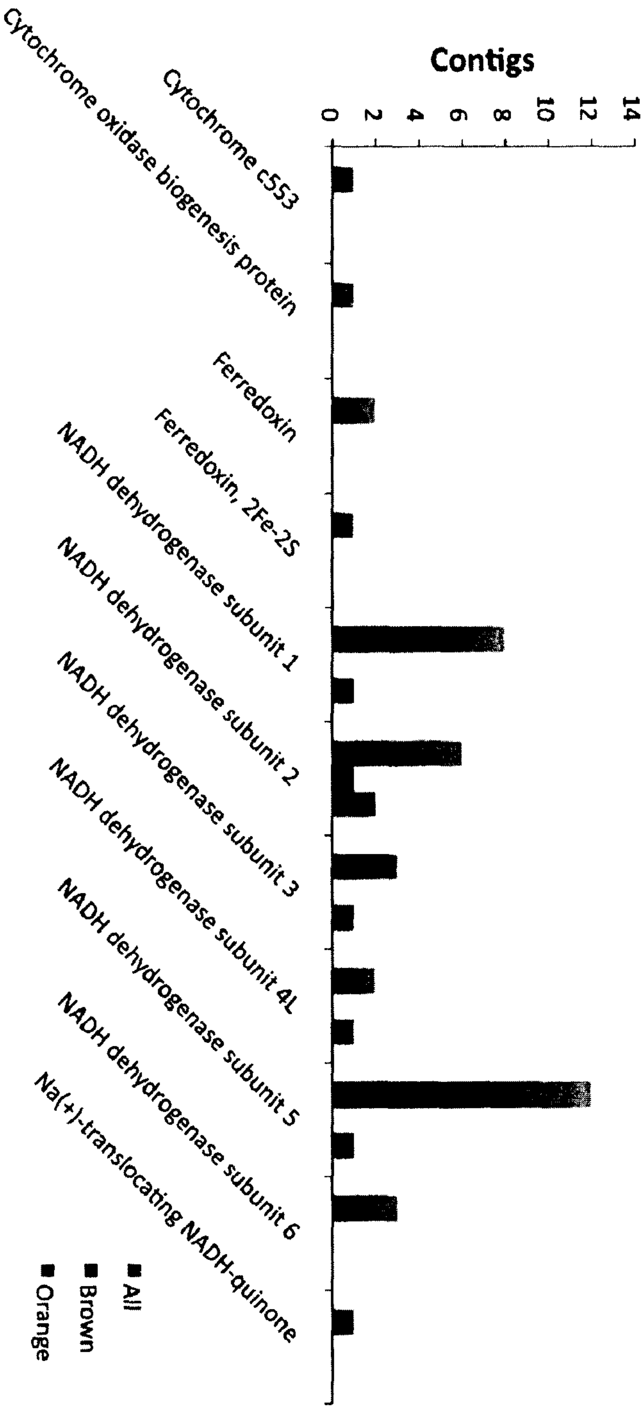


Figure 3.16. Coral host contigs assigned to the Respiratory complex I subsystem, within Respiration. "Brown" contigs had significantly higher expression levels in brown colonies, and "orange" contigs had significantly higher expression levels in orange colonies. Note that values represent the number of different contigs, not their expression levels.

CHAPTER IV

EFFECTS OF SYMBIOTIC NITROGEN-FIXING CYANOBACTERIA ON FITNESS OF THE HOST CORAL MONTASTRAEA CAVERNOSA

Introduction

Symbiosis

Symbioses, defined in the broadest sense as the close association of two or more organisms of different species, are ubiquitous and diverse in nature. Since Darwin's time, naturalists have been able to easily explain the existence of parasitism, but the evolution and persistence of mutualisms was more difficult to understand, as it seemed impossible for two different species to evolve to benefit each other without conflicts of interest occurring. Yet, mutualisms have arisen repeatedly and have persisted over evolutionary time. This can be explained by examining the genomes and evolutionary history of the organisms involved (Moran, 2007). Many organisms lack functional copies of genes coding for necessary products, either because they descended from ancestors that did not possess such genes, or through gene loss events. This loss of critical metabolic capabilities is common when organisms can obtain the necessary substances, such as vitamins, from their diet or environment. Animals are a particularly dramatic example of this; they lack the ability to synthesize many essential amino acids. Some organisms, particularly bacteria, are able to readily integrate foreign genes into their genomes, but the frequency of horizontal transfer events and their impacts on function and fitness are less understood in eukaryotes, and horizontal gene transfer appears to be less common in some lineages than others (Keeling & Palmer, 2008). Even in bacteria, the successful horizontal transfer of complex biosynthetic or metabolic pathways involving multiple genes is not common. Because of the differences in metabolic capabilities of different organisms, and the difficulty of acquiring new genes, mutually beneficial symbioses based on the exchange of needed products can arise readily (Moran, 2007). In this way, metabolic pathways are "acquired" through the symbiont. When one or both partners experience increased fitness through the association, traits that promote the symbiosis are selected for and the partnership is stabilized through evolution. Many symbioses involve nutritional exchanges, but "exchanges" can also include protection from predators. In the *Euprymna-Vibrio fischeri* symbiosis, the bacteria live in a specialized organ and produce light that camouflages the squid by counter-illumination (Jones & Nishiguchi, 2004). Some species of aphids contain *Rickettsiella* endosymbionts that change the body color of the insect from red to green, helping it avoid predators (Tsuchida, *et al.*, 2010).

Symbioses exist in many different forms, which fall along gradients from mutualism to parasitism, and from obligate to facultative. Mutualists and parasites were once thought to share few characteristics, but new research has shown that these types of associations have much in common, including many basic biological and evolutionary mechanisms (Sachs, *et al.*, 2011). Parasites are commonly thought to share several evolutionary patterns that help them to evade host defenses and maintain their fitness in the face of host adaptation. These patterns include negative frequency selection (i.e., rare phenotypes are more fit), high rates of sexual reproduction (which can produce new genotypes and phenotypes), arms races with hosts, and a rapid rate of evolution.

In contrast, evolution in mutualisms is constrained by the need to maintain the positive interactions between host and symbiont to maximize fitness for both partners. Positive frequency selection (i.e., common phenotypes are more fit), slow rates of evolution or even evolutionary stasis, and asexuality were thought to characterize the evolution of mutualists. Recent research demonstrates that the characteristics of mutualisms in particular are very diverse, and that the facultative symbionts involved in most mutualisms (Bright & Bulgheresi, 2010) share many features with pathogens. The molecular and genetic mechanisms necessary for association with a host are similar in mutualists and pathogens, including protein secretion systems (Dale & Moran, 2006), surface sugars and lectins for recognition and adhesion, and antioxidant enzymes for resisting host immune response (Bright & Bulgheresi, 2010). Negative frequency selection typically attributed to parasitic relationships has been observed in coral hosts at loci that are involved in interactions with beneficial symbiotic dinoflagellates (Schwarz, *et al.*, 2008). The large taxonomic diversity of symbiotic dinoflagellates (Rowan & Powers, 1992, Wilcox, 1998) and the similar evolutionary rates in symbiotic and free-living lineages (Wilcox, 1998) contradict the theory of evolutionary stasis in mutualists. In contrast to horizontally transmitted or facultative symbionts, obligate or "captured" mutualists such as the symbionts of insects (reviewed in (Moran, *et al.*, 2008) or the spheroid bodies of *Rhopalodia gibba* (Kneip, *et al.*, 2008) are unlike pathogens in many aspects of their genomes and interactions with hosts (Bright & Bulgheresi, 2010).

When the fitness of a host and symbiont are linked as they are in mutualisms, factors that strengthen or increase this linkage will tend to stabilize a symbiosis, while those that uncouple the fitness of the partners promote shifts to parasitic relationships or to a free-living state. Changes in the environment or evolution of the partners can affect the stability of symbioses (Sachs, *et al.*, 2011), but shifts to parasitism are more rare, and may be constrained by gene loss or pleiotropy (Sachs & Simms, 2006). Partner fidelity (e.g., vertical transmission), partner choice, and sanctions against "cheaters" (Kiers, *et al.*, 2003) can help to maintain mutualisms (Sachs & Simms, 2006). In some relationships, one partner provides a benefit to the other but incurs little or no fitness cost for doing so; for example, one partner may utilize a waste product or metabolic byproduct of the other. Such associations are particularly stable against shifts to parasitism, because there is little to be gained by "cheating" or withholding the benefit. Mutualism abandonment, in which one or both partners revert to a free-living state, has occurred multiple times in diverse lineages, with the important exception of obligately symbiotic lineages (Sachs & Simms, 2006). Abandonment appears to be more common in symbioses where one partner received only a small fitness benefit from the association, and in nutritional mutualisms.

Symbioses are integral to the fitness and ecological function of corals, a fact that has been acknowledged by the "holobiont" concept, in which the unit of selection includes the coral host, symbiotic dinoflagellates, and associated microbes (Rohwer, *et al.*, 2002). This idea has recently been expanded to the "symbiome," which includes all organisms within the sphere of the coral colony that share a common fate. The limits, capabilities, and tolerances of the symbiome reflect those of all the associated organisms that make up the whole (Gates & Ainsworth, 2011). In this study, this new concept is applied to the characterization of a novel symbiosis between *Montastraea cavernosa* and intracellular nitrogen-fixing cyanobacteria. I measure the impacts of symbiotic cyanobacteria on several measures of coral fitness to elucidate the potential costs and benefits of the association to the host. Fitness metrics were selected either because of their known importance to overall coral fitness, because they could plausibly be affected by the presence of cyanobacteria or the fixed nitrogen they provide, or for both reasons. The growth rates of colonies, their ability to deter predators, and their response to thermal stress were determined. In addition, *Symbiodinium* and coral host populations were genotyped to determine if genetic variation in the other partners of the holobiont could be responsible for any observed differences.

Growth

Coral growth is an integrated response to all contributions to holobiont fitness, both positive and negative, from the environment and from the members of the symbiome. Growth is important to both coral fitness and the formation and persistence of coral reefs. Rapid skeletal growth allows coral colonies to compete effectively for light and shade their competitors, while also providing the physical support for increasing biomass and surface area. Increased light collection provides additional energy that is then available to the coral for reproduction or other energetic demands. Many corals do not reproduce or allocate only minimal effort to reproduction until they have reached a minimum size (Szmant, 1991), demonstrating the importance of growth to reproduction as well as competition. Growth and disturbance also interact to influence coral fitness.

Larger coral colonies or fragments of colonies are more likely to survive storms (Highsmith, *et al.*, 1980), and this persistence and subsequent regrowth is a major driver of community structure on coral reefs (Wakeford, *et al.*, 2008).

Erosion from both living organisms and chemical and physical processes occurs continuously on coral reefs, and growth is necessary to compensate for this. External bioeroders include parrotfish (Labridae), pufferfish (Tetrodontidae), *Diadema,* and other grazers that remove part of the coral skeleton, whereas boring invertebrates such as sponges, bivalves, and polychaetes erode the skeleton internally. Many of these invertebrates preferentially bore in corals with dense skeletons, which offer greater protection from predators (Highsmith, 1981). Microbes including bacteria and fungi can also contribute to significant bioerosion. At a larger scale, when coral growth outpaces erosion, the physical structure of reefs is maintained and built, which allows reef ecosystems to persist in geological time.

Multiple environmental and physiological factors influence the rate of coral growth. Light and temperature have long been recognized as the most important of these factors for hermatypic corals (Weber & White, 1974, Baker & Weber, 1975, Jon, *et al.*, 1975). Light affects photosynthesis by *Symbioidinium* and thus the total amount of energy available for growth. In most coral species, growth decreases with increasing depth, although some species display maximum growth in the middle of their depth range, and the growth of a few species does not vary significantly with depth (Huston, 1985). Typically, faster growing coral species dominate the reef crest and shallow areas, whereas slower growing corals occupy deeper areas of the reef (Huston, 1985). Maximum growth rates occur within a relatively narrow temperature range for most corals. Cool water slows growth and is a key factor limiting the geographical range of coral reefs, and corals in warmer regions have higher average growth rates (Jon, *et al.*, 1975). However, temperatures slightly above average summer maxima can lead to thermal stress, bleaching, and reduced growth (Glynn, 1993). After corals recover from thermal stress, growth typically resumes at a normal rate, although this recovery can be protracted (Goreau & Macfarlane, 1990, Mendes & Woodley, 2002). Coral reefs are nutrient-limited environments, and elevated levels of both nitrogen and phosphorus can decrease coral growth by favoring the growth and division of *Symbiodinium* rather than calcification (Tomascik & Sander, 1987, Marubini & Davies, 1996).

As coral colonies become larger, the mass of both the living tissue and the skeleton increases, but in scleractinian corals "growth" typically refers to the growth of the skeleton rather than the animal itself. There are two main types of skeletal morphology, which are formed slightly differently. In imperforate corals (e.g. *Montastraea* spp.), the tissue overlays the outermost layer of the skeleton, and new layers of skeleton are formed when the tissue periodically uplifts. These horizontal layers are called dissepiments and form a "floor" for each polyp within the corallites. In imperforate corals, dissepiments cannot be thickened after they are formed because the living tissue is no longer in physical contact with previous layers of skeleton after it uplifts. Dissepiments are deposited in a similar way in perforate corals (e.g., *Porites* spp.), but the polyp tissue is interconnected through the upper part of the skeleton via holes in vertical skeletal elements, so previously deposited dissepiments may continue to thicken for several months after they are initially formed, as long as the living tissue remains in contact (Barnes & Lough, 1996).

Skeletal dissepiments contribute to banding patterns that record growth over time in the skeleton. In addition to very fine daily banding patterns, many corals display monthly or lunar banding and annual banding (Winter & Sammarco, 2010). Dissepiment formation in *Montastraea* spp. is related to the lunar cycle and this influence results in regularly spaced dissepiments, although the spacing varies between colonies. Water temperatures lower or higher than normal can suppress the formation of dissepiments (Mendes & Woodley, 2002, Winter & Sammarco, 2010), so even though dissepiment spacing is regular, the total linear extension of a coral can vary from year to year and between colonies (Dodge, *et al.*, 1992, Dávalos-Dehullu, *et al.*, 2008). The more prominent high-density and low-density banding patterns apparent in coral skeletons were shown to be annual with each year consisting of one high- and one low-density band, using radioactive fallout from nuclear arms testing that was incorporated into coral skeletons (Knutson, *et al.*, 1972). High-density bands are deposited during warm summer temperatures, whereas low-density bands are formed during the winter months (Cruz-Piñon, *et al.*, 2003). Thus, a pair of annual bands represents the time between annual thermal maxima (a "soft" year) rather than an exact calendar year (Carricart-Ganivet, 2011).

Variations in tissue thickness are primarily responsible for the width of banding patterns in the skeleton. In *Montastraea,* high density bands are formed by thickening of dissepiments, not an increase in their number or change in their spacing (Dodge, *et al.*, 1992). Thus, the more time that coral tissue remains on a given layer of the skeleton without uplifting to form a new dissepiment, the wider and denser the resulting band will be. Corals that are energetically replete have thicker tissue that grows more quickly

(Barnes & Lough, 1996), and uplifts more frequently, forming low-density skeletal bands. In contrast, when environmental conditions are not as favorable, tissue layers are thinner and corals must divide their energetic resources between skeletal accretion and tissue growth (Cohen, *et al.*, 2004). Because a similar amount of calcium carbonate is deposited over a thinner tissue layer, higher density bands are formed. Re-allocation of energy from skeletal to tissue growth can also occur when tissues are performing energetically expensive processes such as gametogenesis or recovery from bleaching (Anthony, *et al.*, 2002). Coral tissue thickness varies seasonally and is responsive to environmental conditions (Anthony, *et al.*, 2002), with lowest levels generally in the late summer when *Symbiodinium* populations are less dense and providing less fixed carbon, and higher levels in winter and spring when symbiont populations are at their largest and the most energy is available (Fitt, *et al.*, 2000). High and low density bands are generally widened when seasonal variation is exaggerated by warmer or colder than normal conditions, respectively. There is also evidence that suboptimal, eutrophic conditions can decrease calcification but increase the width of bands in what has been termed a "stretching" response (Carricart-Ganivet & Merino, 2001). Species with hemispherical colonies such as *M. cavernosa* allocate most of the energy available for growth to the skeleton rather than the tissue once they are larger than 5 – 14 cm radius (Anthony, *et al.*, 2002).

Colonial corals grow indeterminately and some massive corals can live for centuries, so coral skeletons represent an excellent source of information about the environment during these spans of time. Aside from density banding patterns, stable isotopes in the material of the skeleton and inclusions within it also reflect the environment surrounding the coral as it grew. Oxygen-18 (^{18}O) is typically measured to determine water temperature, although it can also record rainfall in equatorial regions where the annual variation in temperature is small (Barnes & Lough, 1996). Variations in ^{13}C are generally considered to be mediated by *Symbiodinium* and indicate light intensity and photosynthetic activity (Barnes & Lough, 1996). Strontium has also been used to investigate water temperature, and ^{15}N in the organic matrix of the skeleton can show past levels of this element (Marion, *et al.*, 2005). All of these factors ensure that corals will continue to be used as natural environmental recorders.

There are several different methods of measuring growth in corals, but the best method depends on the growth metric of interest. Skeletal growth in corals involves both extending the skeleton linearly and modulating its density; if both of these parameters are measured, calcification rate can be determined. These three metrics are non-redundant and at least two of them (from which the third can be calculated) are required for a complete description of growth. However, density, linear extension, and calcification rate are not independent of each other. Density and linear extension are known to be inversely correlated in *Montastraea* (Bosscher, 1993), and in *M. annularis* linear extension and calcification rate are positively correlated (Carricart-Ganivet & Merino, 2001). Both the frequency of tissue uplift and the deposition of calcium carbonate are related to the physiological condition of the coral animal, so calcification rate is the considered to most directly reflect the environmental conditions and the coral's response. Because calcification rate can be complex to measure, linear extension is a more commonly used metric. Caution must be used when interpreting patterns in linear extension, because corals experiencing poor environmental conditions can still maintain or even increase their rate of linear extension by decreasing their skeletal density (Carricart-Ganivet & Merino, 2001) or decreasing the mass or quality of their tissue (Anthony, *et al.*, 2002), so high extension rates do not necessarily indicate healthy corals or reefs.

Variation within and between coral colonies is also a source of error. Some skeletal elements display more informative variation than others; endothecal elements have frequently been considered to have little useful information (Dodge, *et al.*, 1992, Dávalos-Dehullu, *et al.*, 2008), but more advanced techniques can also reveal patterns that older methods cannot detect (Helmle, *et al.*, 2000). Some coral species have much more clear density banding patterns than others and are therefore used more commonly in studies; these are almost exclusively massive type corals, including *Montastraea* spp. and *Porites* spp. Within these species, some individual colonies may not have clear banding patterns, even if they are sampled from very similar, adjacent environments (Huston, 1985). Even within a single colony, the uplift process that forms new dissepiments can occur at different times and to different levels in different part of the colony, so records within a colony may not have consistent timing (Barnes & Lough, 1996). This extensive variation highlights the need for biological and technical replicates when measuring growth in corals.

Many classical methods of measuring coral growth can be labor intensive, imprecise, and have undesirable environmental impact, but sometimes they are the best way to definitively measure coral growth. Needle measurements, which involve fixing a nail into a colony and measuring the decrease in the length of the exposed portion of the nail over time, require multiple measurements but have minimal impact (Cruz-Piñon, *et al.*, 2003). Buoyant weighing is a non-destructive technique that allows an unlimited number of measurements, but it is typically limited to small colonies that can be easily transported to the balance for weighing (Davies, 1989). However, this is an excellent and frequently used technique for branching and foliose corals that are difficult to measure with other methods. A third method involves the collection of whole colonies for sectioning and X-ray imaging. Although the coral slabs obtained can yield samples for stable isotope measurements, as well as density and linear extension, this method is highly destructive and relies on sectioning the colony along the correct growth axis and creating slices of the optimal thickness for X-rays and other measurements. Staining living coral colonies with Alizarin red dye creates a red band in the skeleton that can be used to mark a known date in colonies that will be sampled later. In addition to the disadvantages of sectioning and X-ray, this method is labor intensive, colonies must be re-visited to be collected, and the dye itself can suppress growth for up to a week (Dodge, *et al.*, 1984).

Modern methods for measuring coral growth are somewhat less destructive and yield much more precise estimates. Samples are usually collected by coring rather than whole-colony sections, although cores can have similar issues as colony sectioning if they are not collected along the main growth axis of the colony. Coring holes can also be patched with underwater epoxy or similar materials to reduce impact. Cores can be scanned with computerized tomography (CT) scanners to create 3-D images that allow repeated virtual slicing to optimize the angle relative to the growth axis, or work with a curved growth axis (Bosscher, 1993, Cantin, *et al.*, 2010). Slice thickness and other properties can also be adjusted virtually to make features of interest easier to detect. The increased detail and resolution available from CT images have made measurements of some features and skeletal elements easier and more precise (Helmle, *et al.*, 2000, Cantin, *et al.*, 2010).

Predator Deterrence

Predation is an important structuring force on coral reefs for all organisms, including corals (Rotjan & Lewis, 2008). It can define the spatial range of organisms, limit their abundance, and affect the fitness of both predators and prey. Corals are preyed upon by fishes and invertebrates; these two groups have different patterns of predation and impacts. Invertebrate predators (e.g., *Drupella* snails) are typically uncommon but have periodic episodes of high abundance, when they have large impacts on coral mortality and cover (Rotjan & Lewis, 2008). Fish predators have different impacts depending on whether they consume the skeleton along with the tissue, but most fish are responsible for only partial mortality of adult coral colonies and represent a chronic stressor rather than a large disturbance and source of immediate mortality (Cole, *et al.*, 2010).

The major invertebrate predators of coral are molluscs, including the gastropods *Drupella* (Turner, 1994) and *Coralliophila* (Brawley & Adey, 1982); and echinoderms such as *Acanthaster planci*. Many other groups including nudibranchs, annelids, and crabs also prey on corals but have limited impact (reviewed in (Rotjan & Lewis, 2008)). *Acanthaster planci* is the best-known invertebrate corallivore, and is likely the species of greatest influence on Pacific coral reefs. Local population densities are highly variable but when they peak, these sea stars can destroy large areas of reef and cause high coral mortality. They feed preferentially on *Acropora, Montipora,* and *Pocillopora,* which can cause shifts in coral communities lasting for years. Snails of the genus *Drupella* also experience outbreaks, and are specialized feeders on acroporid corals (Turner, 1994). In the Caribbean, the snail *Coralliophila abbreviata* is responsible for high mortality of *Acropora, Agaricia,* and *Montastraea* in areas of outbreaks (Hayes, 1990), which tend to occur after hurricanes and other physical disturbances (Knowlton, *et al.*, 1990). In general, the pattern of high but localized coral mortality that is created by major invertebrate predators is similar to a disturbance event, and the recovery of the reef ecosystem follows a similar trajectory.

Corallivorous fish fall into two general categories: those that graze exclusively on coral tissue, and those that consume or damage the skeleton in addition to the tissue. Members of the latter group, which includes scarids, tetrodontids, and balistids, tend to be more generalist feeders, but they can still exert strong effects on the coral community (Rotjan & Lewis, 2008). There are many known examples where such predators limit the spatial and depth distribution of their preferred prey. *Pocillopora damicornis* is restricted to shallow lagoon habitats in Guam partly because of predation by balistid fishes (Neudecker, 1979), and in the Florida Keys *Madracis mirabilis* transplanted from 20 m to 13 m was highly damaged by scarids and tetrodontids (Grottoli-Everett & Wellington, 1997). Artificial lesions on *Montastraea annularis* similar to feeding scars caused by this group of fishes reduced coral growth for several months, so it is likely that their feeding activity has a similar effect (Meesters, *et al.*, 1994).

Small corallivores that remove only coral tissue and mucus, including chaetodontids (butterflyfish) and labrids (wrasses), are ecologically analogous to small herbivores in the terrestrial environment. They consume only small parts of the whole coral, very rarely cause colony mortality, and represent a chronic stressor on the colonies they feed on. Many of these fishes are obligate corallivores and feed very selectively; in the Pacific, tabular acroporids and other fast-growing corals make up the majority of their diet and are disproportionately consumed relative to their abundance (Cole, *et al.*, 2010). New estimates of grazing by butterflyfish on the Great Barrier Reef show that the impact of these fishes is much greater than previously appreciated. Just three species of butterflyfish consumed 8.9 – 13.5% of the total tissue biomass and 52 – 79% of the annual productivity of their preferred prey, tabular *Acropora* species (Cole, *et al.*, 2010). This seems likely to have a strong effect on coral fitness, reproduction, and community structure, but few studies have investigated these issues. Corallivores do appear to affect coral reproduction by selectively preying upon areas of colonies with the greatest reproductive effort and thus nutritional value (Rotjan & Lewis, 2008). These fish may also serve as vectors of coral disease, either through their feeding activity or fecal transmission. A variety of corallivorous fish have been observed to feed selectively on diseased areas of coral colonies (Chong-Seng, *et al.*, 2010), and in aquarium studies the presence of butterflyfish increased transmission of black band disease, even if the corals were caged (Aeby & Santavy, 2006).

The large impacts of predators on coral fitness have driven the evolution of several different strategies for deterring predation, which are not mutually exclusive. While many coral reef organisms have developed a cryptic habit to avoid encountering predators, this strategy is not feasible for hermatypic corals that require sunlight for their photosymbionts. Many species of fast-growing acroporid and pocilloporid corals host small symbiotic crabs and shrimp that can detect (Glynn, 1980) and help deter invertebrate predators, increasing survivorship of their hosts (Glynn, 1983). The coral *Porites compressa* changes the behavior of its polyps and increases its nematocyst density in response to predation, making it less palatable to butterflyfish (Gochfeld, 2004). Structural adaptations such as a calcified skeleton or hard spicules are common in many invertebrates on coral reefs and have been thought to provide a physical defense against predators, but they are often not deterrent in isolation, and appear to play only a supplementary role in deterring grazing (Waddell & Pawlik, 2000, O'Neal & Pawlik, 2002).

Noxious chemical compounds, sometimes called antifeedants, are perhaps the most important predator deterrence strategy used by many organisms on coral reefs, and their presence can have important indirect effects. Many species of macroalgae produce deterrent compounds that discourage grazing and can create refugia for other more palatable species of algae or small invertebrates (Littler, *et al.*, 1986, Hay, *et al.*, 1989). Many of the most conspicuous sponges on coral reefs are heavily chemically defended and avoided by fish predators (Pawlik, 2011). Few scleractians have been tested for antifeedants, but octocorals commonly contain them (Wylie & Paul, 1989, Sammarco & Coll, 1992). The distribution and concentration of these chemicals within the coral can be heterogeneous to maximize the protection of highly grazed or energetically costly structures, such as new growth (Wylie & Paul, 1989). Coral predators have been shown to respond to these chemical cues and have distinct feeding preferences for less-defended coral species (Alino, *et al.*, 1992). Although most chemical ecology studies have focused on octocorals or sponges rather than scleractinians, antifeedants may be more prevalent and more important in scleractians than currently appreciated. A recent review of the chemical ecology of Caribbean sponges defined groups of sponges based on their different adaptive strategies: a "palatable" group that lacks strong deterrent compounds, and allocates resources to growth, healing, and reproduction rather than defense; and a "defended" group that is avoided by predators, and grows, heals, and reproduces more slowly (Pawlik, 2011). A third group of "preferred" sponges are restricted to specific spatial refuges from predators. Scleractinian corals may also fall into similar groups based on their chemical defenses and life history traits. Although predator deterrence was not measured, an early study found that 91% of the 58 scleractinian species tested had at least one extract with bioactivity (e.g., cytotoxic, hemolytic, antimicrobial) (Gunthorpe & Cameron, 1990), indicating that many of these corals may be chemically defended. Many corallivores have strong feeding preferences for *Acropora, Pocillopora, Montipora, Agaricia,* and the *Montastraea annularis* species complex. These genera include fast-growing and highly productive species and the intensity upon which they are grazed suggests that they are not strongly chemically defended. Indeed, extracts from the fast-growing branching corals *Acropora, Pocillopora* and *Seriatopora* had little to no bioactivity, but other corals that are not preferred and typically grow more slowly, such as *Montastraea* and *Favites* species, had a higher frequency of extracts with bioactivity (Gunthorpe & Cameron, 1990).

Secondary metabolites are highly diverse chemically and serve equally diverse ecological functions in the organisms that produce them (Hay & Fenical, 1996, Nagle & Paul, 1999, Van Wagoner, *et al.*, 2007). Aside from being antifeedants, secondary metabolites can have antifouling or allelopathic properties that assist in competition for space (Engel & Pawlik, 2000, Morrow, *et al.*, 2011). They may attract gametes to each other (Coll, *et al.*, 1995, Slattery, *et al.*, 1999), or specialized consumers to their preferred prey (Nagle & Paul, 1999). Secondary metabolites can stimulate or inhibit the growth of bacterial populations of symbionts, pathogens, or fouling organisms in a bacterial species-specific manner (Morrow, *et al.*, 2011); similar effects can also be achieved by interference with quorum sensing (Givskov, *et al.*, 1996, Kwan, *et al.*, 2011). Many organisms produce multiple secondary metabolites, and a single compound can have multiple functions (Becerro, *et al.*, 1997, Kubanek, *et al.*, 2002). Additionally, the ecological functions of many compounds are unknown because so much research has focused on functions of potential pharmacologic importance such as cytotoxicity towards cancer cells. Secondary metabolites are often produced by the organism that they are isolated from, but they can also be obtained from dietary sources or microbial symbionts (Simmons, *et al.*, 2008). Nudibranchs that feed on *Sinularia* sequester antifeedants from their diet that make them unpalatable to their predators (Slattery, *et al.*, 1998). The structural resemblance of many secondary metabolites found in invertebrates to those found in bacteria first suggested that these chemicals were being synthesized by bacteria and subsequently transferred to the host (Konig, *et al.*, 2006). A well characterized example of this is the sponge *Theonella swinhoei*, which contains large populations of multiple bacterial symbionts. Filamentous α-proteobacteria *Candidatus Entotheonella palauensis* produce the peptide theopalauamide (Schmidt, *et al.*, 2000), and heterotrophic unicellular bacteria produce the macrolide swinholide A. The symbiotic cyanobacterium *Aphanocapsa feldmanni* and the sponge tissue do not, however, contain bioactive compounds (Bewley, *et al.*, 1996, Magnino, *et al.*, 1999). Symbiotic microbes can thus make significant contributions to host fitness by providing natural products that serve an adaptive purpose.

Cyanobacteria are important and prolific producers of bioactive chemicals in both a free-living and symbiotic state (Nagle & Paul, 1999, Van Wagoner, *et al.*, 2007). Several distinct chemical structures are utilized, and a single species can often produce multiple types of secondary metabolites (Kaebernick & Neilan, 2001, Oksanen, *et al.*, 2004). Although many of these metabolites are of health and economic concern due to their toxicity to animals, they are postulated to serve diverse functional roles in the natural environment (Kaebernick & Neilan, 2001). Some of the most commonly studied cyanobacterial toxins are microcystins, cyclic non-ribosomal peptides often found during cyanobacterial blooms in freshwater lakes (Paerl, *et al.*, 2001). On coral reefs, microcystin is produced by cyanobacterial pathogens that form part of the corsortium of microbes that cause black band disease (BBD) of corals (Frias-Lopez, *et al.*, 2003, Richardson, *et al.*, 2007, Miller & Richardson, 2011). Both sulfide and microcystin contribute to the etiology of this polymicrobial disease (Richardson, *et al.*, 2009). Microcystin in BBD appears to be produced primarily by strains of *Leptolyngbya, Geitlerinema,* and *Oscillatoria* (Myers, *et al.*, 2007, Richardson, *et al.*, 2007, Gantar, *et al.*, 2009), and includes both microcystin-LR and YR (Stanić, *et al.*, 2011). Different strains of cyanobacteria produce different toxins, and display maximal toxin synthesis rates under different environmental conditions (Stanić, *et al.*, 2011). Microcystins are also found in some free-living cyanobacteria from coral reef sediments and microbial mats (Gantar, *et al.*, 2009). The cyanobacteria associated with *Montastraea cavernosa* could produce microcystin or other secondary metabolites that could act as antifeedants, providing a benefit to the coral host.

A final strategy for deterring predators is aposematic coloration, the use of bright or distinctive colors or patterns to warn predators that an organism is chemically defended or unpalatable. This strategy is effective only against visual predators, which once conditioned typically do not sample, or release unharmed, these prey organisms. Some organisms that are not chemically defended have adapted to mimic the coloration of others that are, for example the North American viceroy butterfly (*Basilarchia archippus*) mimics the toxic monarch butterfly (*Danaus plexippus*). Marine examples of aposematic coloration include gastropods that feed on sponges (Becerro, *et al.*, 2006) and many species of nudibranchs (Faulkner & Ghiselin, 1983, Rudman, 1991). Colonies of *M. cavernosa* with and without cyanobacteria are distinctly different in color, and many fish have trichromatic vision that would be required to detect this difference. Reef fish can quickly learn to associate prey color with feeding deterrents and remember this association (Giménez-Casalduero, *et al.*, 1999), so while the orange color is derived from phycoerythrin fluorescence, it could also serve a secondary purpose as aposematic coloration.

Thermal Stress

The impact of warmer than normal temperatures on corals was first noted in the mid 1970s by Jokiel and Coles, who documented bleaching and mortality of corals near the site of coolant water discharge by a nuclear power plant (Jokiel & Coles, 1974). Bleaching is characterized by a pale or white appearance of the coral colony due to the loss of symbiotic dinoflagellates or their photosynthetic pigment; prolonged or severe bleaching often leads to mortality of the coral host. Bleaching is a generalized stress response of corals (Lesser, 2006, Weis, 2008, Lesser, 2011) and can occur after various stresses such as exposure to low temperatures, changes in salinity, or prolonged darkness, but only increased temperature is considered here, as it is the cause of the vast majority of bleaching events. The maximum thermal tolerances of coral holobionts vary widely depending on their location, but most corals live within about 1 - 2° C of their maximum thermal limits, regardless of the local temperature regime (Jokiel & Coles, 1990, Glynn, 1993, Hughes, *et al.*, 2003). When these thermal limits are exceeded for sustained periods of time, mass bleaching events occur, often leading to widespread coral mortality (Eakin, *et al.*, 2009, Eakin, *et al.*, 2010). Such events have increased in frequency and severity in recent decades due to anthropogenic warming of the climate, and threaten the continued existence of coral reefs (Hughes, *et al.*, 2003).

The earliest investigations of coral bleaching noted that not all corals are equally susceptible to thermal stress. Bleaching is conspicuously patchy at all spatial scales, from within a single colony to between reefs. High irradiance interacts with the effects of thermal stress (Lesser & Farrell, 2004), so shaded areas of colonies are frequently less bleached than non-shaded areas. Variable bleaching within a colony can also be due to the spatial distribution of different *Symbiodinium* types with different susceptibility (Rowan, *et al.*, 1997, Sampayo, *et al.*, 2008). Within a single coral species, some genotypes may be more sensitive than others (Edmunds, 1994). Corals with a branching morphology are known to be more vulnerable to thermal stress than corals with a massive growth form, and colonies living at deeper depths are often spared the worst effects of bleaching due to the lower irradiance and sometimes lower temperature at depth (Marshall & Baird, 2000). Different reefs may experience different levels of bleaching due to current or weather patterns; some reefs in the Society Islands that were under cloud cover during the peak of the 1998 bleaching event had much lower mortality than neighboring reefs which had been sunny (Mumby, *et al.*, 2001).

The ultimate cause of most mass bleaching events is thermal stress, which at the physiological level leads to the breakdown of photosynthetic processes in *Symbiodinium*, a lack of transfer of fixed carbon to the coral host, and eventually death of the *Symbiodinium* and/or their rejection by the host. In a typical healthy coral, the maximum daily light intensity exceeds the saturating irradiance of photosynthesis, i.e., there is more energy than the photosynthetic machinery of the dinoflagellates can absorb. This excess excitation energy (EEE) can lead to the generation of reactive oxygen species (ROS) and cellular damage, so dinoflagellates employ several photoprotective mechanisms to prevent this, including state transitions and non-photochemical quenching via xanthophyll cycling (Brown, *et al.*, 1999, Lesser, 2006, Lesser, 2011). In corals living in shallow water, photoprotection cannot completely compensate for EEE, and photodamage accumulates and is repaired on a daily basis, a phenomenon known as dynamic photoinhibition. This leads to fluctuations in photosynthetic parameters over the course of a typical day, that can reflect the light history of the coral (Jones & Hoegh-Guldberg, 2001, Winters, *et al.*, 2003).

The first site of damage from thermal stress is still debated, but three main hypotheses have been proposed. The first claims that the D1 protein subunit of Photosystem II (PSII) is damaged (Iglesias-Prieto, *et al.*, 1992). Damage to enzymes in the Calvin cycle, responsible for the fixation of CO_2, can reduce sinks for excitation energy, leading to a buildup of ROS (Lesser, 2006, Lesser, 2011). Finally, varying thermal stability of thylakoid membranes with different lipid profiles can lead to loss of membrane integrity, the dissipation of proton gradients, and a lack of ATP necessary for carbon fixation (Tchernov, *et al.*, 2004). Under this hypothesis, there is no direct damage to PSII so it continues to split water and generate O_2 and ROS, damaging the cell. New research demonstrating that the primary site of damage can vary depending on the genotype of the *Symbiodinium* has resolved some of this controversy (Buxton, *et al.*, 2012). Regardless of the first site of damage, the net result is the same: EEE generates ROS, creating oxidative stress (Lesser, 1997, Lesser, 2006, Lesser, 2011) leading to loss of functional PSII units. As the photosynthetic capacity declines, the same amount of light produces more EEE, and a positive feedback loop is created wherein the rate of damage to photosynethetic machinery exceeds the rate of repair. If there is inadequate time for the dinoflagellates to recover, bleaching and mortality results.

The photochemical processes upstream of carbon fixation can be measured easily and non-destructively with chlorophyll fluorometry. The most commonly used instrument for this purpose is a pulse amplitude modulated (PAM) fluorometer. Like other fluorometers, it measures the chlorophyll fluorescence emitted by PSII. However, in PAM instruments only fluorescence emitted in phase with a measuring light whose amplitude is continuously modulated is measured, allowing the detection of the weak fluorescence of PSII even in the presence of ambient light (Schreiber, *et al.*, 1986, Warner, *et al.*, 2010). The amount of PSII fluorescence indicates how many of these reaction centers are "open" or oxidized, and can therefore pass absorbed light energy onto the electron transport chain. “Closed” or reduced reaction centers cannot absorb the excitation energy and it is re-emitted as fluorescence. Based on the ambient light conditions, the light history of the coral, and what is known about photochemistry, many different parameters can be measured with a PAM (Schreiber, 2004, Warner, *et al.*, 2010). Specialized PAMs that operate underwater (Diving-PAM) (Winters, *et al.*, 2003) and integrate information into images (Imaging-PAM) (Ralph, *et al.*, 2005) are also used to study corals.

The most commonly measured parameter in studies of coral and *Symbiodinium* physiology is the quantum yield of PSII fluorescence, also called photochemical efficiency, and it reflects the number of functional PSII units. This is calculated as $(F_m-F)/F_m$, where F is the fluorescence emitted under ambient light, and F_m is the fluorescence emitted after a brief pulse of saturating light (~10,000 μmol photons m^{-2} s^{-1}), which closes all of the reaction centers (Schreiber, 2004). When the coral has been acclimated to darkness for at least 20 min, this parameter represents the maximum quantum yield (sometimes called the dark-acclimated yield), and is written as F_v/F_m; when the coral is illuminated, it is the effective, or steady state, quantum yield, F_v/F_m'. These parameters are a measure of how much of the excitation energy absorbed by PSII is being transferred to photochemical reactions. The effective quantum yield is affected by the light history of the coral, and in healthy corals it follows a typical cycle with maximal values at dawn and dusk and a minimum shortly after the solar maximum; the amplitude of the cycle is decreased in corals living in deeper water and exposed only to sub-saturating irradiances (Lesser & Gorbunov, 2001, Winters, *et al.*, 2003). If yields do not recover in the dark as expected, this indicates that photodamage accumulated over the course of the day has not been fully repaired (Jones & Hoegh-Guldberg, 2001). Thus, yields provide a sensitive and quantitative indicator of stress in *Symbiodinium* that can be measured before bleaching is visually apparent. Other metrics for measuring the effects of thermal stress on corals can be categorized as early or late indicators. Changes in gene expression levels and rates of respiration and oxygen evolution can detect stress before bleaching is visible, whereas cell counts performed with a hemocytometer or by flow cytometry, as well as changes in the color of the coral colony, are late indicators that show significant changes only after bleaching is underway or has already occurred.

Coral bleaching is a visual indication that the symbiosis between coral and *Symbiodinium* has broken down, an event that often results in mortality of the coral holobiont. Widespread coral mortality threatens the long term survival of reefs as well as the ecological services they provide (Hoegh-Guldberg, *et al.*, 2007), but even when corals survive and recover from bleaching, a variety of sub-lethal effects reduce their fitness. Many corals appear to consume their own structural components, gametes (if present at the time of bleaching) and lipid reserves to fulfill their energetic requirements when *Symbiodinium* are not providing sufficient nutrition (Szmant & Gassman, 1990, Ward, *et al.*, 2000, Grottoli, *et al.*, 2004). This results in reproductive failure or reduced reproductive output (Szmant & Gassman, 1990, Ward, *et al.*, 2000), reduced growth (Goreau & Macfarlane, 1990), reduced lipid stores and increased susceptibility to future stress (Ward, *et al.*, 2000, Grottoli, *et al.*, 2004), all of which have a negative impact on overall coral fitness.

Intrinsic differences

There are a wide variety of factors that can lead to changes in the metrics of fitness discussed above, some of which are intrinsic to the specific coral holobiont in question. The species and colony morphology of the coral host are important, with branching and massive type colonies having pronounced differences in fitness and response to environmental stressors (Loya, *et al.*, 2001, Baird & Marshall, 2002). Within a single coral species, differences in pigment (e.g., GFP-like proteins), stress-related gene expression, and other factors can affect the stress response (Edmunds, 1994, Dove, 2004, Bay, *et al.*, 2009, Polato, *et al.*, 2010). Clades and genotypes of *Symbiodinium* differ in their rates of carbon fixation and translocation to the host, as well as their resistance to thermal stress (Rowan, *et al.*, 1997, Robison & Warner, 2006, Stat, *et al.*, 2008, Buxton, *et al.*, 2012); these and perhaps other differences change their interactions with the coral-associated prokaryotic community (Littman, *et al.*, 2010). Variation in the composition or activity of the microbial community could lead to differences in the production of antifeedants, antimicrobials, and other secondary metabolites; the prevalence of stress- and disease-associated microbes; and the cycling of nitrogen, sulfur, and other important elements. To detect differences in fitness that are due to symbiosis with cyanobacteria, it is necessary to account for as many of these factors as possible. The composition of the prokaryotic community is investigated in Chapter 2 using pyrosequencing of 16S rRNA amplicons, and the gene expression and activity of the coral host, *Symbiodinium,* and microbial community are addressed in Chapter 3 using an Illumina-sequenced metatranscriptome. Here, genotyping of the coral host and *Symbioidinium* is used to determine if there are genetic differences in these members of the holobiont between brown and orange colonies. If the composition of genotypes is not significantly different in the two colony types, it is assumed that they are physiologically equivalent.

Population studies of most animals utilize markers based on mitochondrial genes, because their relatively rapid evolutionary rate makes them useful for distinguishing recently diverged populations, and their maternal inheritance simplifies analysis. However, mitochondrial genes evolve very slowly in anthozoans (Shearer, *et al.*, 2002) and are thus not useful for population-level studies. Microsatellite markers have been used by some coral researchers, but often suffer from low polymorphism and must be developed for each species of interest in an expensive and time-consuming process (Shearer & Coffroth, 2004, Wang, *et al.*, 2009). Amplified fragment length polymorphism (AFLP) has been used in coral research (Brazeau, *et al.*, 2005, Amar, *et al.*, 2008) because it includes markers from across the nuclear genome and requires no knowledge of DNA sequence. The protocol is nearly universal for all species, and a large number of markers can be developed very rapidly. The procedure utilizes a digest of genomic DNA with two different restriction enzymes, selective amplification of the resulting fragments, and gel electrophoresis to determine the length of all the fragments in the amplicon pool (Vos, *et al.*, 1995). The presence and absence of bands in different samples can be analyzed with several different statistical methods (Duchesne & Bernatchez, 2002). One of the drawbacks of AFLP is that the sequence of markers of interest cannot be determined without additional effort. The use of AFLP in *Montastraea cavernosa* from Little Cayman and Lee Stocking Island revealed significant population structure between locations and at different depths ranging from 3 to 90 m, illustrating the successful application of this technique to this species (Slattery, *et al.*, 2011, Brazeau, *et al.*, in press).

Genotyping of *Symbiodinium* came into frequent use after sequencing of the SSU rRNA genes revealed a diversity within the genus that was comparable to that typically observed between orders of dinoflagellates (Rowan & Powers, 1992). Previously, zooxanthellae were thought to comprise a single panmictic species in all corals, *Symbiodinium microadriaticum* (Freudenthal, 1962). The genus was subsequently divided into several lineages or clades (reviewed in (Rowan, 1998)), and currently 11 clades (A through I) and many types within each clade are recognized (Pochon & Gates, 2010). Clade-level differences roughly correspond to family-level differences, but the appropriate classification of types and subtypes is still debated.

The internal transcribed spacer unit between the 5.8S and 28S rRNA genes (ITS2) is almost universally used as the marker gene for typing *Symbiodinium*, because this noncoding region evolves more rapidly than genes whose function is selectively constrained, and thus it can reveal more fine-scale, sub-cladal differences that may be ecologically important (LaJeunesse, 2001). The best method for analyzing the resulting sequences is a point of contention. Classically, denaturing gradient gel electrophoresis (DGGE) has been used to distinguish types based on their migration through the gel, accompanied by sequencing of dominant bands (LaJeunesse, 2002). However, sequences with a high number of nucleotide differences (up to 15) may co-migrate on the gel, whereas those differing by only one nucleotide can form distinct bands (Apprill & Gates, 2007). These co-migrating sequences may not be detected in subsequent re-amplification and sequencing of excised bands, perhaps due to preferential amplification of abundant sequences in the PCR reaction (Sipos, *et al.*, 2007). Cloning and sequencing of ITS sequences has been utilized by some groups, and typically recovers greater diversity than DGGE, even discovering new types in well-studied corals (Apprill & Gates, 2007). While some researchers contend that cloning and sequencing introduces errors into the ITS sequences, cloning is a widely used technique to isolate sequences from environmental samples, and protocols for *Symbiodinium* ITS sequences have been established wherein putative new types must be present in multiple PCR reactions, and are checked for correct folding into the functional secondary structure (Apprill & Gates, 2007, Lesser, *et al.*, 2010, Stat, *et al.*, 2011). Additionally, most error in sequences is introduced in the PCR step (von Wintzingerode, *et al.*, 1997), which is common to both DGGE and cloning approaches.

The ITS marker is a multi-copy gene, and the variation between copies in a genome as well as the presence of pseudogenes can lead to difficulties in interpreting results, no matter what the sequencing approach (Thornhill, *et al.*, 2007). For this reason, some researchers use length polymorphisms of the chloroplast large subunit ribosomal RNA gene (cp23), although this marker is most useful for detecting fine scale, intracladal differences (Santos, *et al.*, 2003). The ecological significance of such fine-scale diversity in both the cp23 and ITS markers, where some types are distinguished by only one or two nucleotides, remains unclear. In a recent study, type C1 *Symbiodinium* with identical ITS sequences that were sampled from two locations on the Great Barrier Reef were shown to have significantly different responses to thermal stress. This suggests local adaptation is occurring that is not reflected in the ITS sequence (Howells, *et al.*, 2012).

Montastraea cavernosa colonies host diverse *Symbiodinium* communities containing unique C types ("Mcav") that appear to be adapted to variations in the light environment over this coral's broad depth range (Lesser, *et al.*, 2010). Brown colonies living in deep water (>45) have distinct communities from those in shallower water, but no significant differences have been detected between brown and orange colonies (Lesser, *et al.*, 2007).

Methods and Materials

Growth

Two brown and two orange colonies of *Montastraea cavernosa* were sampled in August 2008 at Conch Reef in Florida (25°0'7.73" N, 80°22'48.68" W), and five additional colonies of each type were sampled in March 2009. At North Perry, Lee Stocking Island, Bahamas (23°47'0.03" N, 76°6'5.14" W), one colony of each type was sampled in July 2009, and six orange and five brown colonies were sampled in July 2010. Cores 38 mm in diameter were removed from living colonies at a depth of 15 m using a pneumatic drill with compressed air supplied by SCUBA tanks. Cores were frozen intact for shipping back to the University of New Hampshire, then dried at 37° C for approximately 72 hours, with the exception of cores sampled from Florida, which had the tissue removed with a Water-Pik before freezing.

Cores were scanned using a Siemens Volume Zoom Helical Computerized Tomography (CT) scanner at the Woods Hole Oceanographic Institution. Scans were conducted at 350 mAs and 120 kV with a spiral acquisition protocol using a 0.5 mm width collimator, 0.5 mm slice thickness and a 0.5 mm table pitch. Cores were scanned along a transaxial plane and reconstructed using an ultra-high bone algorithm (u90u) at 0.1 mm increments. 3-D images were assembled and manipulated using Amira software (Visage Imaging). The use of CT imaging allows multiple virtual slices to be taken from cores to find the optimal thickness and angle for visualizing annual density bands in each core (Bosscher, 1993, Cantin, *et al.*, 2010). The annual linear extension rate was measured as the distance in mm between consecutive annual high-density bands. For each core, an image of the slice with the clearest bands beginning at the top of the core was exported from Amira. Bands in either exothecal (outside the corallite) or endothecal (inside the corallite) dissepiments were measured, depending on which skeletal element produced the clearest banding pattern for each core. In some very dense cores it was not possible to visualize exothecal banding patterns. Although it has been reported that endothecal dissepiments do not display clear banding patterns in *Montastraea,* the studies in which this was found utilized X-radiography on cut slabs of coral (Dodge, *et al.*, 1992, Dávalos-Dehullu, *et al.*, 2008). Fine details of the skeletal architecture are not always apparent in such X-rays. By using CT it is possible to optimize slice thickness to visualize either endothecal or exothecal elements, and image analysis software can detect clear banding patterns that are not readily apparent to the naked eye and likely would not be visible on traditional X-radiographs. This allowed the use of both exothecal and endothecal elements in this study, and in cores where both endothecal and exothecal bands were visible, a correspondence in banding patterns was clearly evident (Figure 4.1).

In each image, the distance between annual density bands was measured along replicate virtual transects drawn in ImageJ (NIH). At least three parallel transects were drawn from the top of the core and the grayscale variability plotted for each. The use of greyscale variability plots allows for more precise determination and measurement of density bands than examining images by eye. Transects varied in width from core to core as necessary to optimize peaks in greyscale plots. The correspondence of pixels to mm distance was determined using a scale saved in each image by Amira. The peaks in greyscale plots were compared to the marked transects in the corresponding image to determine the distance between each annual high-density band. A calendar year was assigned to each high-density band based on the number of bands from the top of the core and the date the core was sampled, and the linear extension for each year was averaged over the three replicate transects in each core. The linear extension over the calendar years 2010-2009 and 2009-2008 was averaged for each core, and a two-way ANOVA was used to compare the extension of cores from each colony type and sampling location. Cores with incomplete data for a given time window were excluded from that analysis.

Predator Deterrence

Sharpnose pufferfish (*Canthigaster rostrata*) were used to determine the response of fish predators to the coloration and chemical contents of brown and orange *M. cavernosa* colonies. Pufferfish are suitable model predators for this study because they feed on a wide variety of invertebrates in the field, have trichromatic vision, and acclimate readily to laboratory conditions (see (Slattery, *et al.*, 1998) for the use of congeners in the Pacific).

Aposematic Coloration

To determine if predators respond differently to *Montastraea cavernosa* colonies with and without cyanobacteria on the basis of colony color, red and green fluorescent proteins and phycoerythrin were tested in feeding assays. When red and green fluorescent proteins (Invitrogen) were mixed with squid paste as described below in concentrations of 2.46 $\mu l/cm^2$ and 24.6 $\mu l/cm^2$, red and green colored food pellets were produced that pufferfish would be able to visually distinguish. B-phycoerythrin from *Porphyridium cruentum* (Sigma) was tested in three concentrations: the estimated natural concentration (1x), and 10x and 100x the estimated natural concentration. An estimate of 1.49 μg phycoerythrin per cm^3 of coral tissue was determined assuming a tissue depth of 0.369 cm (D. Gochfeld, personal communication), 10^7 cyanobacterial cells per cm^2 of coral surface area (Lesser, *et al.*, 2004), and 55 fg of phycoerythrin per cyanobacterial cell (Wyman, *et al.*, 1985). These colored foods were presented to pufferfish in assays as described below.

Squid paste was used as a maintenance food for pufferfish and as a base for mixing with treatment substances (i.e., colored substances and chemical extracts). Equal masses of squid mantle flesh and distilled water were pureed in a kitchen blender with sodium alginate at 2% of the wet mass, and the resulting paste was frozen for transport to the field site. Once thawed, the squid paste was centrifuged for several minutes just before use to remove excess water. Phycoerythrin or fluorescent proteins were mixed with 300 μl of squid paste, collected in a 100 μl glass syringe, and extruded into a 0.25 M $CaCl_2$ solution to form a firm spaghetti-like strand of food. Plain squid paste for maintenance feeding and controls was prepared in the same manner but without the addition of extracts. If treatment foods containing extracts were colored, similarly colored controls were prepared with commercial food coloring to ensure that color did not influence fish behavior. All foods were presented to pufferfish in small pieces ("pellets") that could be consumed in a single bite.

Fish used in assays to test the deterrence of phycoerythrin were captured from Lee Stocking Island using hand nets, transported to the laboratory in a bucket, then maintained outdoors in individual 9 L tanks with flowing seawater covered with shade cloth. Fish were held for approximately 2 weeks and fed plain squid pellets three times a day. Each assay consisted of a maximum of three treatment foods (containing phycoerythrin) and corresponding controls, and assays were conducted at midday and mid-afternoon each day. Fish were pre-fed plain squid paste pellets before each assay to reduce hunger, then presented with treatment pellets one at a time. The different treatment foods were presented to each fish in a haphazard order. Treatment pellets were recorded as accepted if the fish swallowed and did not regurgitate the food, or rejected if the fish did not eat, "mouthed," or swallowed and then regurgitated the food. A control pellet was offered after each treatment pellet to control for satiation. If the fish rejected the control pellet, the response to the preceding extract was not included in the analysis. Each treatment food was tested on 10 – 14 fish. Fluorescent proteins were tested in the same manner at Little Cayman Island using locally caught pufferfish.

To determine if each individual treatment food was significantly deterrent, the number of fish that ate treatment pellets was compared to the number of fish that ate plain controls (always 100%) using a Fisher's exact test. Foods with P values less than or equal to 0.05 were scored as deterrent. To compare the deterrence of red and green fluorescent proteins, the proportion of fish that accepted each type was calculated, and then proportions were compared with *t*-tests.

Feeding Deterrence

Samples from brown and orange *Montastraea cavernosa* colonies were collected with hammer and chisel from a depth of 15 m at Little Cayman, Cayman Islands (n = 10 orange, n = 10 brown) and Lee Stocking Island, Bahamas (n = 5 orange, n = 5 brown). Samples were transported to the laboratory in seawater and immediately frozen, then transported to the University of Mississippi for processing.

Both organic and aqueous extracts were made from each sample from Little Cayman, because bioactive metabolites have been found in both of these fractions in other corals and invertebrates (Gochfeld & Aeby, 2008, Slattery & Paul, 2008). Each sample was placed in a covered beaker with 1:1 dichloromethane:methanol to produce an organic extract. After organic extraction, samples were placed in another beaker with Millipore water replaced daily for 3 days, to produce an aqueous extract. Both extracts were filtered, lyophilized, and weighed. Only organic extracts were made from samples from the Bahamas. The surface area of each coral sample was determined using the wax method (Gochfeld, 1991), and the tissue volume calculated using average tissue depth for this species, determined with decalcified replicate samples. Because predators perceive bioactive compounds on a per-volume basis, an amount of extract corresponding to 300 μl of coral tissue was used for each feeding assay.

Treatment foods for feeding assays were prepared as described above for phycoerythrin and fluorescent proteins, except that aqueous extracts were mixed with 300 µl of squid paste and sufficient sodium alginate to produce a firm paste (< 1 g per sample), not with $CaCl_2$. Because the addition of sodium alginate produces a different food texture, controls for aqueous extracts were also prepared with sodium alginate rather than $CaCl_2$. Assays were conducted on 10-14 fish at Lee Stocking Island as described above. Data were analyzed as described above, with extracts grouped by type and sampling location (i.e., Little Cayman organic, Little Cayman aqueous, Bahamas organic).

Microcystin Assays

To determine if any observed deterrence of pufferfish was due to toxins produced by the symbiotic cyanobacteria, assays for microcytin were conducted. Microcystins produced by various cyanobacteria including *Microcystis* spp, and can be detected by their inhibition of eukaryotic protein phosphatases (An & Carmichael, 1994). Preliminary mass spectrometry results had suggested but not confirmed the presence of microcystin in samples of *M. cavernosa* with cyanobacteria (M. Slattery, personal communication).

Samples from four orange and five brown colonies of *Montastraea cavernosa* were collected from a depth of 15m at North Perry, Lee Stocking Island, Bahamas. Samples were gently airbrushed with 0.2 µm filtered sea water (FSW) from a distance of approximately 15 cm to remove mucus and loosely associated bacteria, frozen at -50° C, and transported to the University of New Hampshire.

Pieces of coral tissue and skeleton approximately 1 cm^2 surface area were made with bone cutters and incubated with 1 mL of molecular-grade water in glass test tubes with rubber stoppers for 90 min at 65° C to remove all tissue from skeletons. Coral skeletons were removed and air-dried for later surface area determination with the aluminum foil method (Marsh, 1970). Methanol (Sigma) was added to a final concentration of 75% and samples were sonicated for 2 min at 50% duty cycle (Branson Sonifier 450). Samples were filtered through GF/F filters (Whatman) using vacuum filter flasks, and water was added to the filtrate to bring methanol concentration to < 5%. C18 columns (SepPak, Waters) were prepared by filtering two column volumes of 100% methanol followed by four column volumes of water, and then samples were filtered through the columns using a glass syringe. Columns were washed with two volumes of 20% methanol and excess liquid removed using a gentle vacuum. Columns were eluted into glass tubes with 2 mL 100% methanol and this eluate was stored at 4° C and used in ELISA assays. To ensure that this extraction procedure was effective, six coral samples were spiked with 5 ppm of microcystin-LR (Sigma-Aldrich) each before the incubation at 65° C, which would have yielded a final concentration of 5 ppb with 100% recovery.

A commercial ELISA kit for detecting microcystins (QuantiPlate kit for microcystins - EP 022, Envirologix) was used according the manufacturer's instructions. This assay detects several different types of microcystins, including LR, LA, RR, YR, as well as nodularin so it was expected that any microcystins present in the coral would be detected. A 1:1 dilution of each coral extract, and 1:40 and 1:80 dilutions of spiked samples were run concurrently. Microcystin concentrations were determined using the standard curves for each assay. Recovery rate was determined using the calculated concentration from the standard curve and a 100% recovery concentration of 50 ppb. Natural concentrations of microcystin were estimated using coral surface area and calculated recovery rate. Samples were scored as positive if the calculated microcystin concentration was higher than that of the lowest standard calibrator (0.16 ppb). A Fisher's exact test was used to compare the number of samples from brown and orange colonies that contained microcystin.

Thermal Stress

Five orange and five brown colonies of *Montastraea cavernosa* were collected from North Perry, Lee Stocking Island at a depth of 15 m. Colonies were transported to the lab in covered seawater-filled coolers and placed in large tanks with constantly running seawater, covered with two layers of neutral density screening. Each colony (genet) was split into 10 replicates (ramets) of approximately equal size using a hammer and chisel, and each ramet was marked on the underside with nontoxic colored modeling clay to distinguish ramets from the same genet (Figure 4.2). Ramets were allowed to heal for three days before the experiment began.

During the experiment, replicates were held in 56 L glass aquaria in water-filled tables with constantly running seawater. Treatment tanks were heated by 300 W aquarium heaters (MarineLand Visi-Therm). Treatment and control tanks were paired, and each pair of tanks contained ramets from a single brown genet and a single orange genet (i.e., tanks were blocked by genotype). Five brown and 5 orange ramets were arranged in an alternating fashion within each tank (Figure 4.2). A total of 5 pairs of treatment and control tanks were used. Each tank contained an airstone for circulation and was covered with two layers of neutral density screening, approximating the irradiance at the collection depth of the parent colonies. HOBO temperature loggers

(Onset, Bourne MA) were placed in four treatment tanks and two control tanks and recorded temperature every five minutes.

Coral replicates were held at ambient temperature (approximately 30 - 31° C) for sampling on day 0, then temperature was increased to approximately 0.5° C over ambient on day 1, and to approximately 1° C over ambient on day 2, then heaters were turned off and tanks allowed to cool to ambient temperature after sampling on day 7. Samples were collected on day 0, 4, 7, and 10. At each time point, one brown and one orange ramet were selected with a random number table and taken from each tank and processed as described below. On day 10, the experiment was terminated and all remaining ramets (two brown and two orange from each tank) were processed.

Ramets were gently airbrushed with FSW to remove mucus as described previously, and a small piece was removed with bone cutters, placed in RNALater (Ambion) and frozen at -50° C. On day 0, a small additional piece of each ramet was frozen in saline DMSO buffer (Seutin, *et al.*, 1991) for the preservation of DNA. The tissue on the remaining piece of each ramet was removed by airbrushing with FSW and the resulting slurry homogenized at moderate speed until the viscosity of the solution was similar to that of water. The total volume of slurry was recorded and 200 μl was preserved in 4% paraformaldehyde and frozen at -50° C for cell counts. Coral skeletons were air-dried and surface area determined using the tin foil method (Marsh, 1970).

Both *Symbiodinium* and cyanobacterial cells were analyzed by FACScan at the J.J. MacIsaac Aquatic Cytometry Facility (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine, USA). Cell abundances were measured with a Becton Dickinson FACScan flow cytometer (Franklin Lakes, New Jersey, USA) fitted with a 15-

mW, 488 nm, air-cooled Argon ion laser. Forward light scatter (FSC, or relative size), 90° light scatter (SSC), chlorophyll fluorescence (>650 nm), and phycoerythrin fluorescence (560-590 nm) were measured for all samples. Cell counts were normalized to surface area using the total volume of tissue slurry and the surface area of each ramet.

Beginning the night before the first sampling, all ramets were measured each night with a pulse amplitude modulated fluorometer (Diving-PAM, Walz, Germany) to determine their maximum quantum yield of Photosystem II (PSII). Damping was set at 2, measuring light intensity at 8, and gain at 2; settings were consistent each night with the exception of the gain, which was increased to 5 on the evening of day 2. An auto-zero correction was made each night immediately before measuring the first sample. Measurements were taken a minimum of 30 minutes after sunset, after corals acclimatized to the dark, and the manufacturer's plastic holder was used to hold the fiber optic probe.

Two-way repeated measures analyses of variance (ANOVA) were used to determine the statistical significance of observed differences in cell counts of *Symbiodinium* and cyanobacteria, and quantum yields of PSII. Each variable was tested for normal distribution using the Shapiro-Wilk test, and those that failed this test were natural log-transformed. Cell count data passed normality tests but no transformation achieved normality in the quantum yield data. Variables were also tested for sphericity using Mauchly's sphericity test, and data that violated this assumption are presented with Greenhouse-Geisser and Huynh-Feldt corrections. Time, treatment, colony color, and their interactions were used as factors in the ANOVA.

Coral host genotyping

Coral populations were genotyped using amplified fragment length polymorphism (AFLP). The three main steps of the protocol are: (1) digestion of genomic DNA and ligation of oligonucleotide adapters; (2) selective amplification of restriction fragments; and (3) analysis of amplified fragments on a gel (Vos, *et al.*, 1995). Modifications made for use in corals include the separation of *Symbiodinium* cells from coral host tissue prior to DNA isolation, and the addition of a pre-selective amplification step (Brazeau, *et al.*, in press).

Samples were collected with hammer and chisel across a depth gradient at Lee Stocking Island, Bahamas. Three brown colonies were sampled at 3, 10, 15, 22, 30, 45, 60, and 76 m. Six orange colonies were sampled at 10, 15, and 22 m, 5 colonies at 30 m, 4 colonies at 45 m, and one colony at 60 m. Samples were airbrushed to remove mucus as described previously, frozen in saline DMSO buffer (Seutin, *et al.*, 1991), and transported to the University of Buffalo. *Symbiodinium* cells were separated from coral tissue by macerating samples in saline DMSO buffer, then spun at 16,000 x *g* for 5 min. Genomic DNA was isolated from the homogenate using the Wizard SV Genomic DNA Purification System (Promega, Madison WI) according to the manufacturer's protocol for animal tissues. All samples were determined to be free of detectable *Symbioindium* DNA by a stringent *Symbiodinium*-specific PCR (Brazeau, *et al.*, 2005).

Because AFLP can be sensitive to PCR conditions, samples were processed at each step in large, random lots containing samples from multiple depths and from brown and orange colonies, to uniformly distribute any experimental error due to reaction conditions. All PCR reactions were done using a single thermal cycler, the final selective

PCR step was repeated three times for each sample, and bands were scored as present only if they appeared in all three replicates for the sample.

Genomic DNA was digested with *EcoRI* and *MseI* and ligated to corresponding adapters. These two enzymes represent a "frequent cutter" and a "rare cutter" respectively (Vos, *et al.*, 1995), where the use of the frequent cutter produces small DNA fragments ideal for separation on the gel, and the inclusion of the rare cutter reduces the number of fragments that are amplified. The reaction was carried out with both *EcoRI* and *Mse*I adapters as specified in Brazeau et al. (in press). A pre-selective PCR reaction using primers complementary to the adapters with the addition of a single nucleotide was used on diluted DNA fragments from the digestion/ligation reaction. A second selective PCR reaction contained primers with the same sequence, with the addition of 2 more selective nucleotides and a FAM tag. Final products were sequenced at the University of Florida's Interdisciplinary Center for Biotechnology Research, on an Amersham MegaBACE 1000 96 capillary sequencer, and electropherograms analyzed for bands 50 to 400 bp in size in 5 bp increments.

Two statistical analyses were used to determine if brown and orange colonies comprise a single population or multiple populations. Presence/absence banding patterns were used as the input for AFLPOP (Duchesne & Bernatchez, 2002), which allocates individuals to populations based on log-likelihood values. The log-likelihood threshold was set to 0, resulting in samples being assigned to the population with the highest likelihood for that genotype (banding pattern). Multiple discriminant function analysis (DFA) was used as a second method of classification. Unlike AFLPOP, which utilizes all markers, only markers that significantly contributed to the differentiation of populations were used in the DFA. These markers were identified using a forward stepwise analysis for model building, and added one at a time to the model, beginning with the marker with the largest contribution to differences between populations. A minimum tolerance-value of 0.01 was set to avoid adding redundant markers to the model.

Symbiodinium Genotyping

To ensure that physiological differences in *Symbiodinium* were not contributing to observed differences in fitness, *Symbiodinium* populations were genotyped in colonies used in the thermal stress experiment. Samples were prepared for DNA extraction by removing a small portion of coral tissue preserved in saline DMSO buffer (Seutin, *et al.*, 1991) from the skeleton and homogenizing tissue (Tissuemiser, Fisher Scientific) in 0.6 mL of 10X TE buffer (100 mM Tris, 10 mM EDTA, pH 8.0). Homogenates were centrifuged at 20,000 x *g* for 30 min and DNA was extracted from the resulting pellet using a Mo Bio PowerSoil DNA extraction kit according to the manufacturer's instructions, except that DNA was eluted in 40 µl of molecular water. DNA concentration and quality were measured using a Nanodrop 2000c spectrophotometer.

The internal transcribed spacer 2 region (ITS2) and flanking 5.8S and 28S regions of the rDNA were amplified using the primers ITSintfor2 (LaJeunesse, 2002) and ITS2rev (Apprill & Gates, 2007). Each 25 µl PCR reaction contained 0.5 µM of each primer, 200 µM of each dNTP, 0.25 µl of 50X Titanium *Taq* DNA polymerase (Clontech), 1X Titanium *Taq* buffer (Clontech), and 1 µl of template DNA. Amplification was performed using a touchdown PCR protocol with annealing temperatures from 62- 52° C (Apprill & Gates, 2007). PCR products were separated on a

1.0% agarose gel, excised and purified using a Qiagen QIAquick Gel Extraction kit and Silica-Gel columns (Denville Scientific) according to the manufacturer's instructions. Amplicons were ligated into the pGEM-T Easy Vector and transformed into *Escherichia coli* JM109 competent cells using a Promega cloning kit according to the manufacturer's instructions. Blue/white screening was performed by plating on Luria-Bertani (LB) agar plates supplemented with 100 μg ml^{-1} of ampicillin (Sigma), 100 μl of 100mM IPTG (isopropyl β-D-thiogalactopyranoside), and 20 μl of 50 mg ml^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) per plate. White colonies were inoculated into LB broth with ampicillin using sterile toothpicks, grown overnight with shaking at 37° C, and pelleted by centrifuging at 1500 rpm for 15 min. Supernatant was removed by inverting the plate, plates were covered with adhesive foil covers, frozen at -70°C and sent to Functional Biosciences (Madison, Wisconsin) for plasmid purification and sequencing using the plasmid-based T7 promoter primer.

Sequences were trimmed for quality and to remove cloning vectors, and sequences identified as *Symbiodinium* ITS2 using BLAST (GenBank) in Geneious (Drummond, *et al.*, 2010) were aligned in MEGA (Tamura, *et al.*, 2011). The alignment was compared to an existing non-redundant reference database of aligned *Symbiodinium* ITS2 sequences (R. Gates) and manually edited. The alignment was edited column by column, and non-consensus bases that did not occur in at least two independent clone libraries originating from different PCR reactions were in the case of sequences with single base changes, insertions, or deletions, edited back to the consensus for that position; or excluded from further analysis in the case of sequences with larger insertions or deletions. Sequences without an exact match in the reference base, but occurring in at least two independent PCR reactions, were considered to be genuine. This is a commonly used screening protocol for cloned ITS sequences to minimize the impact of PCR and sequencing errors (Lesser, *et al.*, 2010, Stat, *et al.*, 2011). Remaining sequences were compared to the *Symbiodinium* ITS2 reference database with BLAST to assign an identity. If a sequence matched exactly two sequences in the reference database equally well, a new category was created from both type names, but the sequence was counted only once. The abundances of each type in each coral were tallied and arranged in a matrix, the matrix was square root transformed, a Bray-Curtis similarity matrix was created, and non-metric multidimensional scaling was performed in PRIMER. Two-way analysis of similarities (ANOSIM) was performed to detect significant differences in *Symbiodinium* communities from different colony types or locations, and one-way ANOSIM was used to compare brown and orange samples at each location. The similarity percentages (SIMPER) tests was used to determine which types were contributing to the observed dissimilarity between the groups tested.

Results

Growth

Mean linear extension rates were highly variable, but the relative differences between brown and orange colonies at each location were consistent between 2008-2009 (Figure 4.3) and 2009-2010 (Figure 4.4). Brown colonies had mean extension rates that were slightly higher than, but not significantly different from, those of orange colonies at Lee Stocking Island, but brown and orange were indistinguishable in Florida. However, there was no significant effect of colony color or location on linear extension in either the 2008-2009 or the 2009-2010 period (Table 4.1). There was a high degree of morphological polymorphism (Figure 4.11) as well as variation in density (Figure 4.12) among samples, which may have contributed to the observed high variability in linear extension rates.

Predator Deterrence

Aposematic coloration

Red and green fluorescent proteins did not differ significantly in deterrence (paired t (8)= 2.306, P= 0.917), indicating that pufferfish do not discriminate between colonies of *M. cavernosa* based on color. On average, 65% (± 12.29%) of fish consumed red fluorescent protein foods, and 66% (± 3.59%) ate green fluorescent protein foods. All fish accepted phycoerythrin at all concentrations, thus phycoerythrin was judged to be not deterrent ($P = 1$ for all concentrations, Fisher's exact test). These results indicate that colony color does not affect the feeding behavior of sharpnose pufferfish.

Feeding Deterrence

There were no significant differences in deterrence between brown and orange colonies, for either aqueous (paired t (13)= 1.461, $P = 0.168$), or organic (paired t (18) = 1.448, $P = 0.165$) extracts from Little Cayman, or organic extracts from the Bahamas (Mann-Whitney test, median of 0.615 for orange colonies and 0.538 for brown colonies, U=5.000, n_1=n_2=5, P (exact) = 0.151). Organic extracts were generally more deterrent; 87% of extracts from orange colonies and 60% of extracts from brown colonies had P-

values less than or equal to 0.05 for Fisher's exact tests on individual extracts. Aqueous extracts were typically not deterrent; only 29% of extracts from brown colonies and 0% from orange colonies were significantly deterrent.

Microcystin Assays

An average of 33% (± 3.5%) of added microcystin was recovered from spiked samples (n=6). Microcystin concentrations were below the detection limit in samples from brown/green colonies, but two of five samples from orange colonies were positive for microcystin. Estimated natural concentrations of microcystin in these samples were 0.43 ppb cm^2 and 1.18 ppb cm^2, accounting for extraction recovery. The number of samples containing detectable amounts of microcystin was not significantly different in brown/green and orange colony types (Fisher's exact test, $P = 0.167$).

Thermal Stress

The temperature in treatment tanks was approximately 1° C higher than in control tanks on days 1 to 7, but due to a natural thermal stress event, temperatures in control tanks ranged from 30.5° C to over 32° C during this time, peaking on day 2 (Figure 4.5). Temperatures in control and treatment tanks equalized after heaters were turned off on day 7 to allow recovery, but remained over 30° C until days 9 and 10. Temperatures over 30° C are generally considered to be stressful for most corals (Fitt & Warner, 1995), so both treatment and control corals were assumed to be thermally stressed during this experiment.

Maximum quantum yields of PSII fluorescence were significantly different in orange and brown colonies and changed over time, but no other factors or interactions had a significant effect (Table 4.2). Yield data departed significantly from the assumption of sphericity, so Greenhouse-Giesser and Huynh-Feldt corrected *p* values are given. The effect of time is clearly apparent in the increased yields in all corals on days 1 and 2 (Figure 4.6). Maximum quantum yields increased from approximately 0.75 at the start of the experiment to near 0.8 on the evenings of day 1 and 2, then decreased and remained between approximately 0.7 and 0.6 for the remainder of the experiment. Differences between brown and orange colonies were most obvious on days 4, 5, 6, 7, and 9, but were subtle overall.

Samples were collected throughout the experiment and cyanobacteria and *Symbiodinium* cells were counted with flow cytometry. *Symbiodinium* counts passed the sphericity tests but cyanobacteria counts did not. As expected, cyanobacteria densities were significantly higher in orange than brown samples, and while time and treatment were not significant factors in isolation, their 3-way interaction with color was significant (Table 4.3). This appears to be due to a slight increase in cyanobacteria densities over the course of the experiment in all groups, but particularly in brown corals in control tanks (Figure 4.7). *Symbiodinium* densities were equal in brown and orange colonies at the beginning of the experiment, and there was no effect of colony color, time, treatment, or any interactions of these factors on cell densities (Figure 4.8, Table 4.4).

Symbiodinium Genotyping

Genotyping of the ITS region of the 18S rRNA of *Symbiodinium* recovered 15 distinct types, all within clade C (Figure 4.9). Some sequences did not have an exact match in the database but were equally similar to two types, both of which are included in the sequence names. Many sequences designated "Mcav" previously found in *M. cavernosa* were recovered (Lesser, *et al.*, 2010).

There was no significant difference between *Symbiodinium* communities in brown and orange colonies when samples from all locations were considered together (ANOSIM, Global R = 0.123, *P*= 0.24), and multidimensional scaling (MDS) did not separate brown and orange colonies (Figure 4.10). When locations were tested individually, R values were lowest for Little Cayman and Florida (Figure 4.9a, Figure 4.9b, ANOSIM, Global R = -0.037, *P* = 0.7 for both), and highest for Lee Stocking Island (Figure 4.9c, ANOSIM, Global R = 0.444, *P*= 0.10), indicating that brown and orange colonies were most dissimilar at Lee Stocking Island, although not significantly so. Several ITS types were restricted to one colony type; Mcav1, Mcav7, and C1182 were found exclusively in orange colonies, whereas Mcav9, C3/C1f, C1002/C3e, and C21/3d/C3k were found only in brown colonies. The effect of location on *Symbiodinium* populations was not significant (ANOSIM, Global R = 0.333, *P*=0.13), and sites were not well separated by MDS (Figure 4.10). Overall, colony color and location were not significant factors and no types were consistently or exclusively associated with either color at all locations.

Coral Host Genotyping

Brown and orange *M. cavernosa* from Lee Stocking Island formed two distinct populations based on colony color. Analysis with AFLPOP, which utilizes all of the markers to distinguish populations, correctly assigned orange samples to the "orange" population 87.5% of the time, whereas brown samples were correctly categorized just 50.5% of the time. However, discriminant function analysis that considers only markers that contribute significantly to differences between groups was able to correctly assign 100% of both brown and orange samples to their population of origin.

Discussion

Growth

Linear extension rates in Florida and Lee Stocking Island were comparable to the lower estimates from a previous study, which range from 2.0 to 10.9 mm/year for *M. cavernosa* living at depths of 6 to 25 m in Jamaica (Huston, 1985). Brown and orange colonies did not have significant differences in linear extension rates (Table 4.1), and neither did colonies at two locations, but genuine differences may have been obscured by the high variability observed both within and between colonies (Figures 4.3, 4.4). It is possible that a greater number of samples or longer time windows might have yielded different results. Extension rates in *M. cavernosa, M. annularis,* and four other Caribbean corals were shown to be variable by Huston (1985), and the many factors that influence growth, including light (Baker & Weber, 1975, Huston, 1985), temperature (Jon, *et al.*, 1975, Lough & Barnes, 2000), and water quality (Tomascik & Sander, 1987), likely interact with the genetics and physiology of the coral holobiont at multiple spatial and temporal scales to produce this variability, such that even colonies in very close proximity may have different linear extension rates. Internal waves transporting cold, nutrient-rich water from depth up to the reef may have had an additional influence on growth in Florida, where such events have been recorded at the Conch Reef sampling site (Leichter, *et al.*, 2003).

Some investigators advocate the use of calcification rate rather than linear extension for measuring growth, as the deposition of calcium carbonate is more closely linked to the physiological state of the coral and thus the environmental conditions. The extension of the skeleton depends on both the deposition of material and the skeletal architecture (Carricart-Ganivet, 2011). *Montastraea cavernosa* colonies are notoriously highly polymorphic (Lasker, 1981, Budd, *et al.*, 2012), and samples collected in this study reflected this morphological variability (Figure 4.11), as well as exhibiting great differences in density (Figure 4.12). The range and magnitude of differences between colonies sampled would have made the determination of calcification rate extremely difficult, so this was not undertaken.

A few researchers have cast doubt on the use of any skeletal growth parameters as indicators of coral health or environmental conditions (Anthony, *et al.*, 2002). In experiments with small colonies of *Goniastrea retiformis* and *Porites cylindrica* subjected to combinations of light limitation and sediment stress, growth of the skeleton and the tissue was largely uncoupled. Growth of the skeleton was insensitive to the experimental treatments, and energetic investment in skeletal growth was very small relative to that of tissue growth. However, these effects are likely to be different in larger colonies (>5 - 14 cm for hemispherical colony forms), which are predicted to allocate more of their energy to skeletal rather than tissue growth. Indeed, a recent documentation of a long-term decline in growth rates of *Diploastrea heliopora* in the Red Sea attributed to rising sea surface temperatures clearly demonstrates that skeletal growth rates respond to the environment (Cantin, *et al.*, 2010).

Predator Deterrence

M. cavernosa was found to be generally deterrent to an omnivorous fish predator, *Canthigaster rostrata*, but there were no differences in the deterrence of extracts from brown vs. orange colonies. Controls colored with red and green fluorescent proteins and phycoerythrin were readily consumed by fish, indicating that color differences do not affect predator preferences. Results from feeding and microcystin assays were congruent. Although ELISAs revealed detectable levels of microcystin in two of five orange colonies, differences between brown and orange colonies were not significant. If microcystin were present, the aqueous extracts would be expected to contain it, but fish were significantly deterred by just 29% of aqueous extracts from brown colonies, and 0% of extracts from orange colonies, so the observed differences in microcystin concentration do not appear to be ecologically relevant to predators. Together these results demonstrate that symbiotic cyanobacteria do not play a role in visually or chemically deterring predators.

Although commonly perceived as being limited to blooms of planktonic cyanobacteria in freshwater (Paerl, *et al.*, 2001), microcystins are appropriate toxins to test for in this system. They are present not only in free-living cyanobacteria on coral reefs (Gantar, *et al.*, 2009), but also in multiple types of cyanobacteria involved in black band disease (Myers, *et al.*, 2007, Richardson, *et al.*, 2007, Stanić, *et al.*, 2011), so it is feasible that the marine cyanobacteria associated with *M. cavernosa* might also produce toxins. Like antibiotics that are now appreciated for their signaling and other non-toxic environmental functions (reviewed in (Martinez, 2008) microcystin may act in other ways in cyanobacteria, and not just as a toxin. Microcystin synthetase genes are evolutionarily ancient and their origin predates the existence of metazoans, so it is unlikely that microcystins evolved to serve a defensive function against grazers (Rantala, *et al.*, 2004). Although its precise function is still unclear, microcystin may be involved in intra- and inter-specific cell signaling and communication and sensing the environment, all of which could be advantageous in a symbiosis (Dittmann, *et al.*, 2001, Babica, *et al.*, 2006, Schatz, *et al.*, 2007).

Thermal Stress

There were several differences in the response of brown and orange colonies to temperature treatment (Figures 4.6-8, Tables 4.2-4), most of which corresponded to what is known about the unique response of this coral to thermal stress. There was a slight but significant effect of color on maximum quantum yields of PSII, with orange colonies having higher values. A previous study found no differences in yield between colony types under typical environmental conditions (Lesser, *et al.*, 2007), so our finding suggests that cyanobacteria may enable orange colonies to maintain higher photochemical efficiency during thermal stress. This could confer a small fitness advantage. *Symbiodinium* densities were unchanged throughout the experiment (Figure

4.8), but cyanobacteria densities showed a significant interactive effect of time, treatment, and colony color. Cyanobacterial counts increased over the course of the experiment, particularly in brown colonies in control tanks (Figure 4.7). Because the cyanobacteria are likely living as heterotrophs rather than performing photosynthesis, they may not suffer from extensive photodamage as *Symbiodinium* does, and thus may exhibit a Q_{10} response to elevated temperature rather than a typical bleaching response. It is unclear how an increase in cyanobacterial densities would affect holobiont fitness.

M. cavernosa is known to be highly tolerant of thermal stress, and often remains visually healthy while other corals are undergoing bleaching (Fitt & Warner, 1995), which may account for the lack of a dramatic response to the temperature treatment. The increase in ambient seawater temperature that was ongoing during the experiment resulted in control corals being exposed to higher temperatures than anticipated, such that both control and treatment groups were likely exposed to significant thermal stress (Figure 4.5). Thus, the comparison of control and treatment corals is not comparing unstressed and stressed corals, but moderately stressed and highly stressed corals. Differences between the two groups were likely less pronounced than they would have been if control temperatures had been lower.

In addition to not visibly bleaching when exposed to high temperatures, many other physiological indicators in *M. cavernosa* show little response to short-term thermal stress. Gross photosynthesis, the gross photosynthesis/respiration ratio, and the ratio of chlorophyll fluorescence before and after exposure to the herbicide DCMU (a measure of how many PSII units are damaged and not contributing to photochemistry) of freshly isolated *Symbiodinium* show no significant change after 2 days *in hospite* at temperatures up to 32° C (Fitt & Warner, 1995). The density of *Symbiodinium* and their chlorophyll content per cell were unaffected by temperatures up to 34° C. Given the apparently robust nature of these symbionts, the lack of response to temperatures up to 33° C in this experiment (Figure 4.5) is not surprising.

Intrinsic Differences

Genotyping of coral hosts and *Symbiodinium* populations revealed significant differences in the coral host based on colony color, but not in *Symbiodinium*. The brown and orange colonies of *M. cavernosa* sampled at Lee Stocking Island each formed a distinct population that could be reliably distinguished based on AFLP profiles. It is important to note that the diagnostic bands in AFLP profiles are not sequenced, so the specific genetic differences between the two populations remain unknown. Another study using AFLP on samples of brown *M. cavernosa* from across broad depth gradients at Lee Stocking Island and Little Cayman Island found that the two locations were genetically distinct, and also revealed significant structuring of the population with depth, even though some colonies were only tens of meters apart (Brazeau, *et al.*, in press). These results illustrate that *M. cavernosa* is able to diverge into distinct populations at small spatial scales. This is surprising given that this coral is a broadcast spawner and thus has high dispersal potential (Szmant, 1991). The structuring of populations with depth and by colony color (i.e., symbiotic status) are both likely due to the forces of selection. The small spatial scale of sampling in both cases and the reproductive mode of *M. cavernosa* makes local recruitment or retention of larvae implausible, and if sweepstakes recruitment were occurring in *M. cavernosa*, the sampling of colonies of different sizes should have reduced any effect this would have by ensuring that several different cohorts were sampled (Flowers, *et al.*, 2002). Evidence that selection drives differences between colonies with and without cyanobacteria suggests that coral hosts may have adapted to cyanobacterial symbiosis over a long evolutionary history. It is possible that the genetic differences in orange colonies are responsible for their ability to host cyanobacteria, and perhaps brown colonies are unable to establish or maintain this association. These genetic changes could also contribute to reproductive isolation of brown and orange colonies, further promoting their differentiation despite their close proximity to each other.

Symbiodinium take up nitrogen fixed by the cyanobacteria (Lesser, *et al.*, 2007), and although this might be expected to influence the assortment of genotypes found in orange colonies, there was no overall difference between the communities in brown and orange colonies or between colonies from different locations. Some types were found exclusively in one colony type at a single location, but no types were consistently and specifically associated with brown or orange colonies across locations. This confirms previous results that found no differences in *Symbiodinium* populations (Lesser, *et al.*, 2007).

Most of the *Symbiodinium* sequences recovered in this study represent very closely related types, and the ecological and functional significance of these kinds of sub-cladal differences remains a point of contention. Several groups utilize a new cluster-based method that attempts to divide *Symbiondium* into ecotypes in which all the sequences in the group share an ecological niche and are more closely related to each other than they are to sequences in other groups (Correa & Baker, 2009, Stat, *et al.*, 2011). This analysis was not performed on this dataset, but given the lack of significant differences in classical ITS typing and in any fitness metrics tested in this study, it seems unlikely that any potentially different ecotypes in brown and orange colonies have an effect on growth, predator deterrence, or the response to thermal stress. However, it is possible that other aspects of fitness in *M. cavernosa* that were not measured are affected by the complement of *Symbioindium* genotypes. While differences in the ITS2 sequence are thought to correspond with differences in functional aspects of genome, new research has shown that the converse is not always true. Functional differences due to local adaptation are present in types with identical ITS2 sequences (Howells, *et al.*, 2012). The locations used in this study were separated by hundreds of km, equivalent to the spatial scale in Howells *et al.* (2011) so local adaptation may explain why sites with varying oceanographic conditions, and colonies with and without cyanobacteria, seem to share the same assortment of *Symbiodinium* genotypes.

Possible Factors Affecting Fitness in *Montastraea cavernosa*

Few significant fitness differences were detected between orange colonies with cyanobacteria and brown colonies without. Although there is no direct evidence that the cyanobacteria are beneficial symbionts to the coral, there is also no evidence to suggest that they are parasitic or pathogenic, because a significant fitness cost would be expected in such a condition. There are several possible scenarios that could explain this lack of differential fitness.

As the host animal, corals are typically thought of as exerting some control over their symbionts, and deriving the primary benefit from them; however, symbionts within a coral may interact with each other and derive costs and benefits that do not directly affect the host. It is possible that the primary benefit of the symbiotic cyanobacteria is conferred to the *Symbiodinium*.

The dinoflagellates in orange colonies display a strongly depleted $\delta^{15}N$ isotopic signal typical of nitrogen fixation, indicating that they take up most of the "new" nitrogen (Lesser, *et al.*, 2007), and they have a higher growth rate than dinoflagellates in brown colonies. But dinoflagellate densities are similar in brown and orange colonies, so part of the population in orange colonies must be diluted, as in a chemostat that maintains a constant cell density. The mechanism by which this occurs is unknown. Corals are capable of digesting their symbionts, but this would be expected to deplete the $\delta^{15}N$ ratio of the host tissue fraction, which has not been observed (Lesser, *et al.*, 2007), so it is more probable that the *Symbiodinium* are expelled (Hoegh-Guldberg, *et al.*, 1987). If symbionts are expelled from the colony intact, this increased 'seeding' of the reef environment from orange colonies may make these *Symbiodinium* more likely to be taken up by newly settled corals and thus increase their reproductive success. A similar phenomenon occurs when the squid *Euprymna scolopes* vent their light organs each day, increasing the abundance of their symbiont *Vibrio fischeri* in the local environment by as much as 30-fold, and ensuring a supply of symbionts for newly hatched aposymbiotic squid (Lee & Ruby, 1994). The benefits of cyanobacteria to the coral host could also be subtle and indirect, if the availability of nitrogen increases the ability of *Symbiodinium* to provide photosynthate to the coral, or increases its ability to resist or recover from environmental stress.

Colonies with cyanobacteria have a different depth distribution than colonies without, and appear to exist in a specific niche in deeper water. The relative abundance of colonies with cyanobacteria peaks at approximately 35% of the population at 15 m depth, then remains constant down to 46 m (Lesser, *et al.*, 2007). Presumably, the fitness benefits of hosting cyanobacteria are maximized in this range, and may only be apparent in the environmental conditions present at depth. Changes in the environment are known to alter the fitness of symbioses, and can shift them along the continuum from mutualism to parasitism, or cause abandonment of the partnership (Bronstein, 1994). The collection depth of 15 m represents the shallower edge of this distribution, and was selected partly because of the accessibility of sampling sites at this depth at all locations, and the reasonable amount of bottom time available for sampling using open-circuit SCUBA. However, fitness differences may have been more apparent if experiments had been conducted with colonies from 25 or 30 m depth, closer to the middle of the range. This sampling depth presents logistical problems, mostly because the no-decompression limit bottom times using SCUBA with air at such depth are quite limited.

The lack of observed differences in fitness could be due to the metrics chosen and how they were measured. The effects of cyanobacteria might be revealed by more sensitive measurements (e.g. tissue quality or growth, rather than skeletal growth), or in different aspects of fitness, such as reproduction. Gametes are energy-intensive to produce and contain high concentrations of nutrients (Ward, 1995, Leuzinger, *et al.*, 2003), so it is plausible that orange colonies might produce more gametes or gametes of higher quality. Sperm and eggs require different investments of energy and nutrients, and *M. cavernosa* is gonochoric (Szmant, 1991), so it is possible that the fitness effects of cyanobacteria are specific to male or female colonies. Tissue samples were collected in 2011 to determine the sex ratios of orange and brown colonies and determine their reproductive output, but no gametes were found. Other investigators reported that *M. cavernosa* around the Caribbean either failed to spawn or had minimal spawning of male colonies only, so this appears to be a general reproductive failure in 2011, rather than a sampling issue (A. Szmant, pers. comm.). Future work should include repeating these reproductive surveys, and repeating the thermal stress experiment with a greater difference between control and treatment temperatures and control tanks at a lower, non-stressful temperature might also yield different results. Ocean acidification is an important stressor that has already begun to affect coral reefs and will increased in the future, in combination with increased water temperatures (Doney, *et al.*, 2009). An experiment comparing the response of brown and orange colonies to increased temperature, decreased pH, and a combination of the two factors was conducted in 2011, and RNA and DNA samples could be used for a variety of analyses.

TABLE 4.1. Results of two-way analyses of variance on the effect of colony color and location on linear extension from 2008-2009 and 2009-2010.

2008-2009	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
	Color	0.60759	1	0.60759	0.50726	0.48498
	Location	1.99836	1	1.99836	1.66838	0.21197
	Color x Location	0.70398	1	0.70398	0.58774	0.45272
	Within Groups	22.75791	19	1.19778		
	Total	26.06785	22	1.1849		

2009-2010	**Source of variation**	**SS**	**df**	**MS**	**F**	**p**
	Color	0.52577	1	0.52577	0.51069	0.48454
	Location	1.44654	1	1.44654	1.40505	0.25219
	Color x Location	1.03894	1	1.03894	1.00914	0.32919
	Within Groups	17.50205	17	1.02953		
	Total	20.5133	20	1.02567		

TABLE 4.2. Results of two-way repeated measures analysis of variance on the effects of colony color, temperature treatment, and time on quantum yields of photochemistry. Mauchly's test of sphericity also shown. G-G, Greenhouse-Geisser and H-F, Huynh-Feldt epsilon corrections.

Mauchly's W	**Chi-Square**	**df**	***p***
0.03304	110.709	54	<0.00001*

Effect	**df**	**F**	***p***	**G-G**	**H-F**
Treatment	1	0.4926	0.487	-	-
Color	1	5.496	0.025*	-	-
Treatment x Color	1	0.0001	0.99	-	-
Time	10	26.9098	<0.0001*	<0.0001*	<0.0001*
Time x Treatment	10	1.0486	0.432	0.619	0.656
Time x Color	10	1.7025	0.132	0.084	0.062
Time x Treatment x Color	10	0.966	0.494	0.491	0.511

TABLE 4.3. Results of two-way repeated measures analysis of variance on the effects of colony color, temperature treatment, and time on densities of cyanobacterial cells. Mauchly's test of sphericity also shown. G-G, Greenhouse-Geisser and H-F, Huynh-Feldt epsilon corrections.

Mauchly's W	Chi-Square	df	*p*
0.32524	16.55355	5	0.0055*

Effect	df	F	*p*	G-G	H-F
Treatment	1	0.5419	0.472	-	-
Color	1	46.3839	<0.0001*	-	-
Treatment x Color	1	0.0093	0.924	-	-
Time	3	1.5319	0.25	0.261	0.259
Time x Treatment	3	3.1014	0.061	0.144	0.127
Time x Color	3	1.1459	0.365	0.251	0.247
Time x Treatment x Color	3	6.8061	0.0046*	0.0098*	0.0044*

TABLE 4.4. Results of two-way repeated measures analysis of variance on the effects of colony color, temperature treatment, and time on densities of *Symbiodinium* cells. Maunchly's test of sphericity also shown.

Mauchly's W	Chi-Square	df	*p*
0.51905	9.6541	5	0.0856

Effect	df	F	*p*
Treatment	1	0.1217	0.732
Color	1	0.0002	0.988
Treatment x Color	1	0.5296	0.477
Time	3	0.563	0.648
Time x Treatment	3	0.3893	0.763
Time x Color	3	0.6078	0.621
Time x Treatment x Color	3	1.6465	0.224

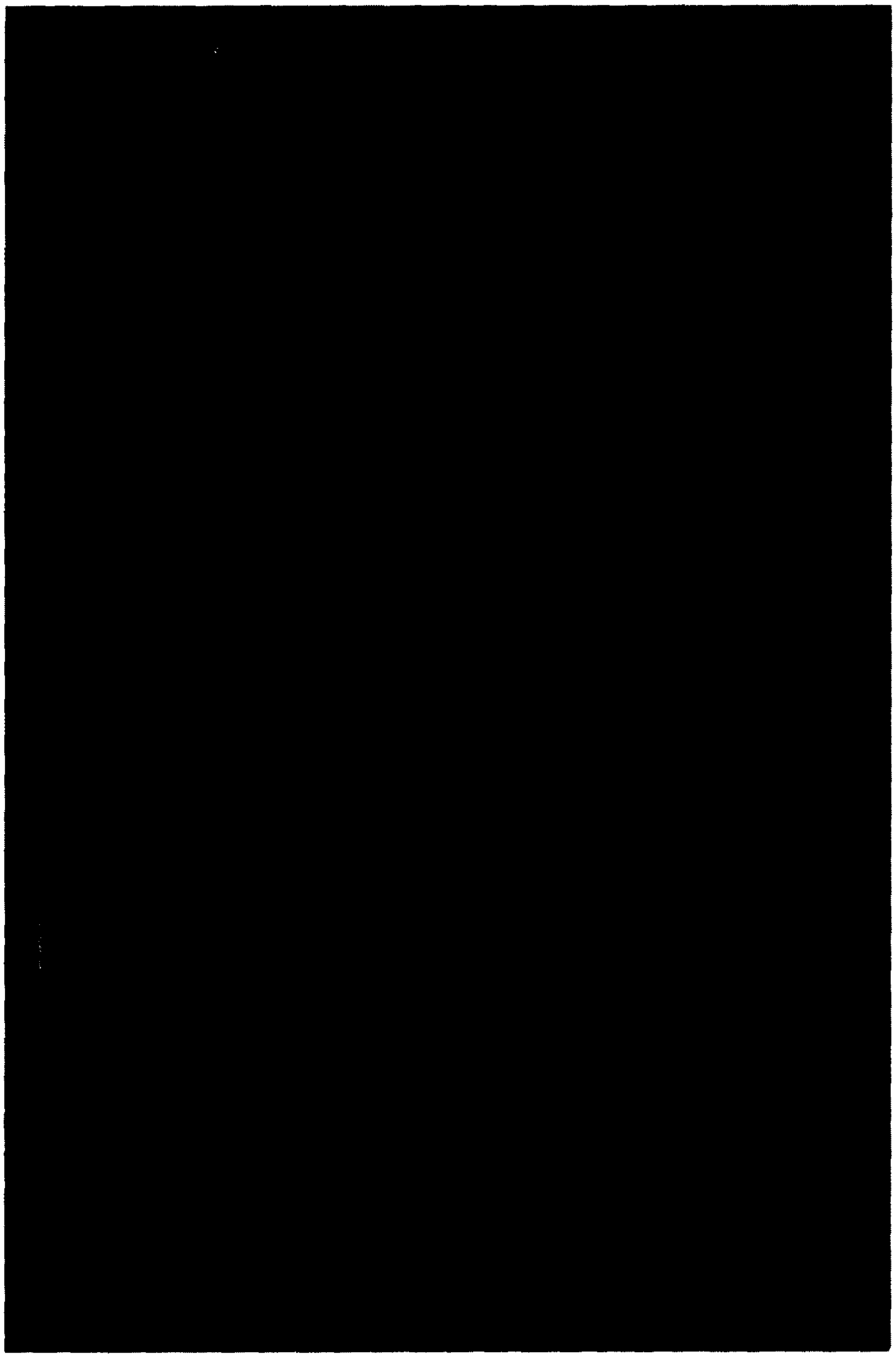


Figure 4.1. Virtual slice from a reconstructed computed tomography 3-D image of a core of *Montastraea cavernosa*, showing density banding patterns in both exothecal (red arrows) and endothecal (blue arrows) skeletal elements. Slice shown is roughly parallel to growth axis.

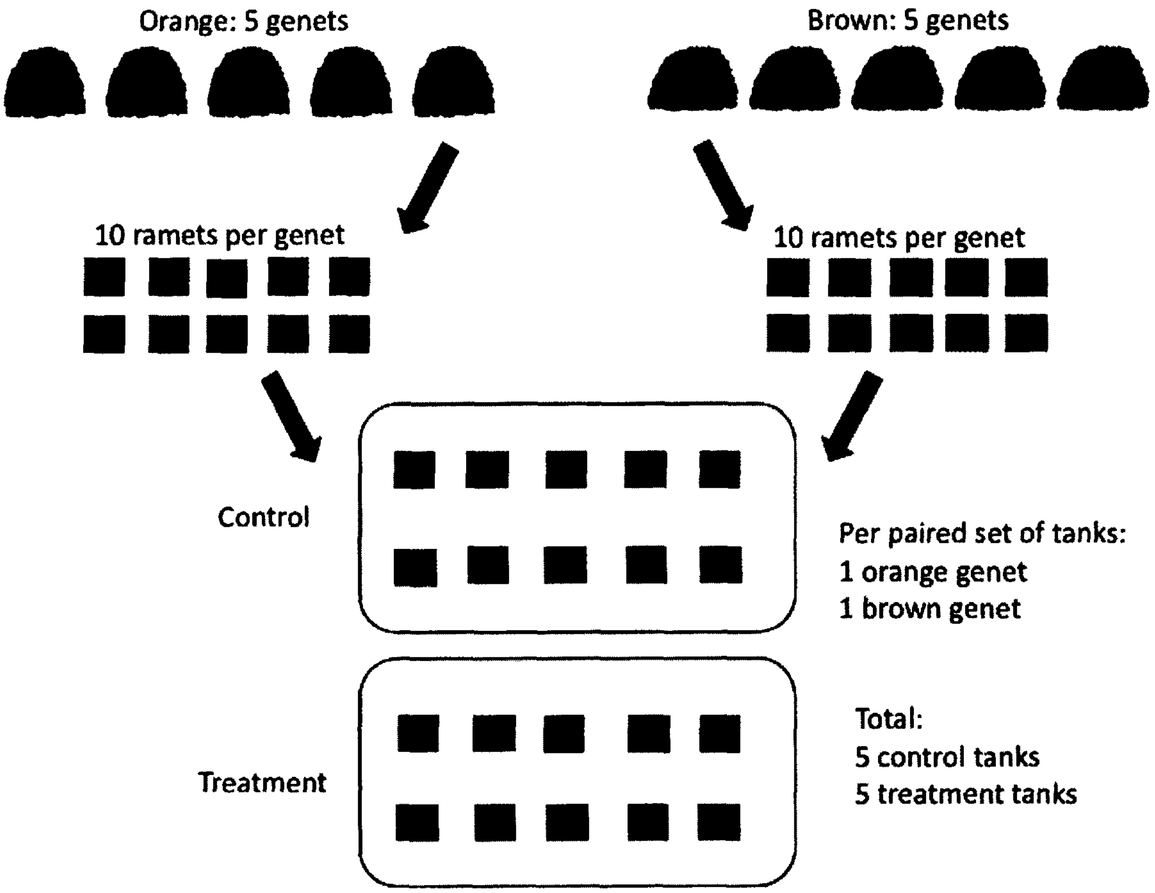

Orange: 5 genets
Brown: 5 genets
10 ramets per genet
10 ramets per genet
Control
Treatment
Per paired set of tanks:
1 orange genet
1 brown genet
Total:
5 control tanks
5 treatment tanks

Figure 4.2. Diagram of experimental design for thermal stress experiment. Five colonies (genets) of each type were collected and each was separated into 10 ramets. All of the ramets from one orange genet and one brown genet were distributed into paired treatment (heated) and control tanks. A total of 5 treatment and 5 control tanks were used.

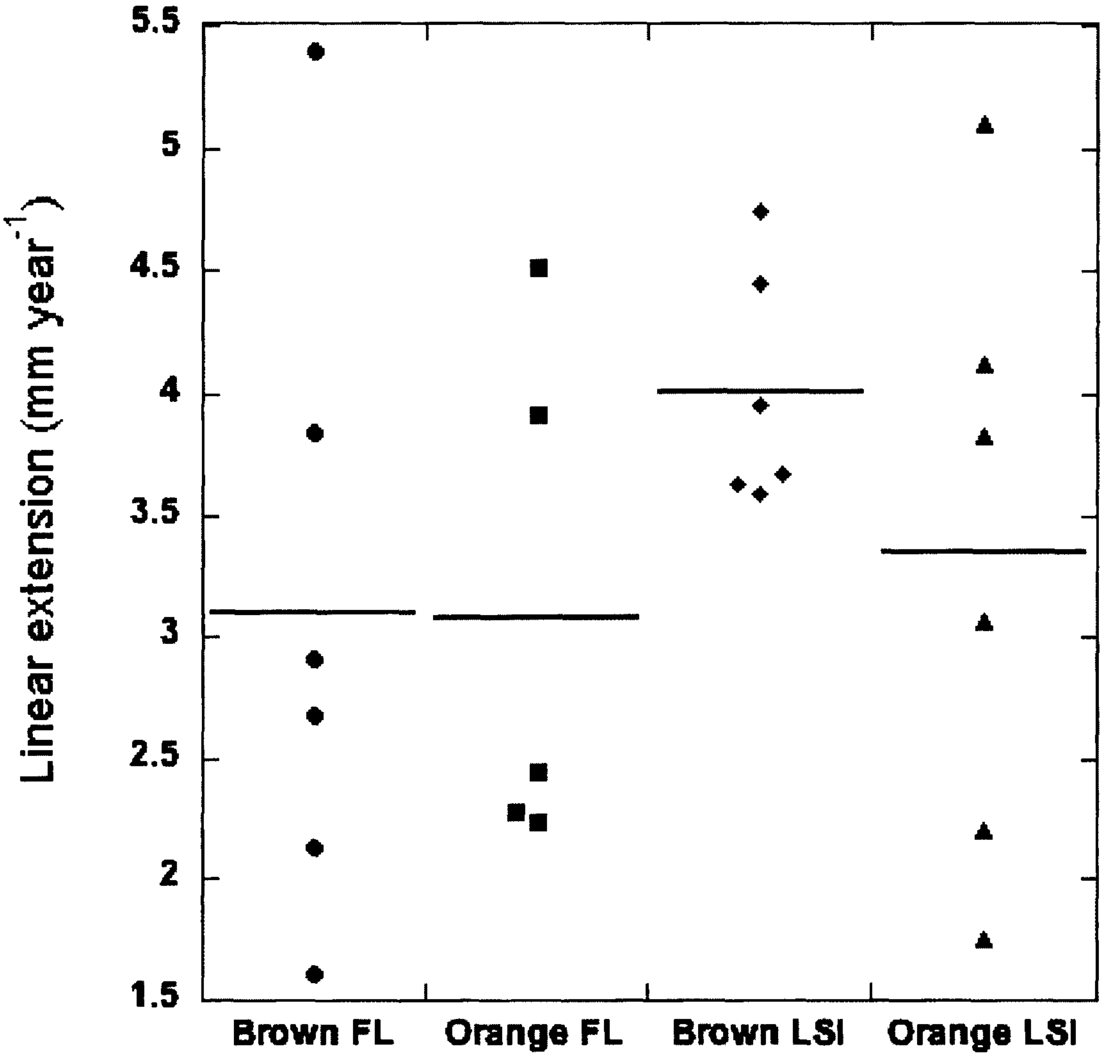


Figure 4.3. Dotplot of linear extension of brown and orange *Montastraea cavernosa* colonies from Lee Stocking Island, Bahamas (LSI) and the Florida Keys (FL) from 2008 – 2009. Each symbol represents a single colony, horizontal bars are the mean.

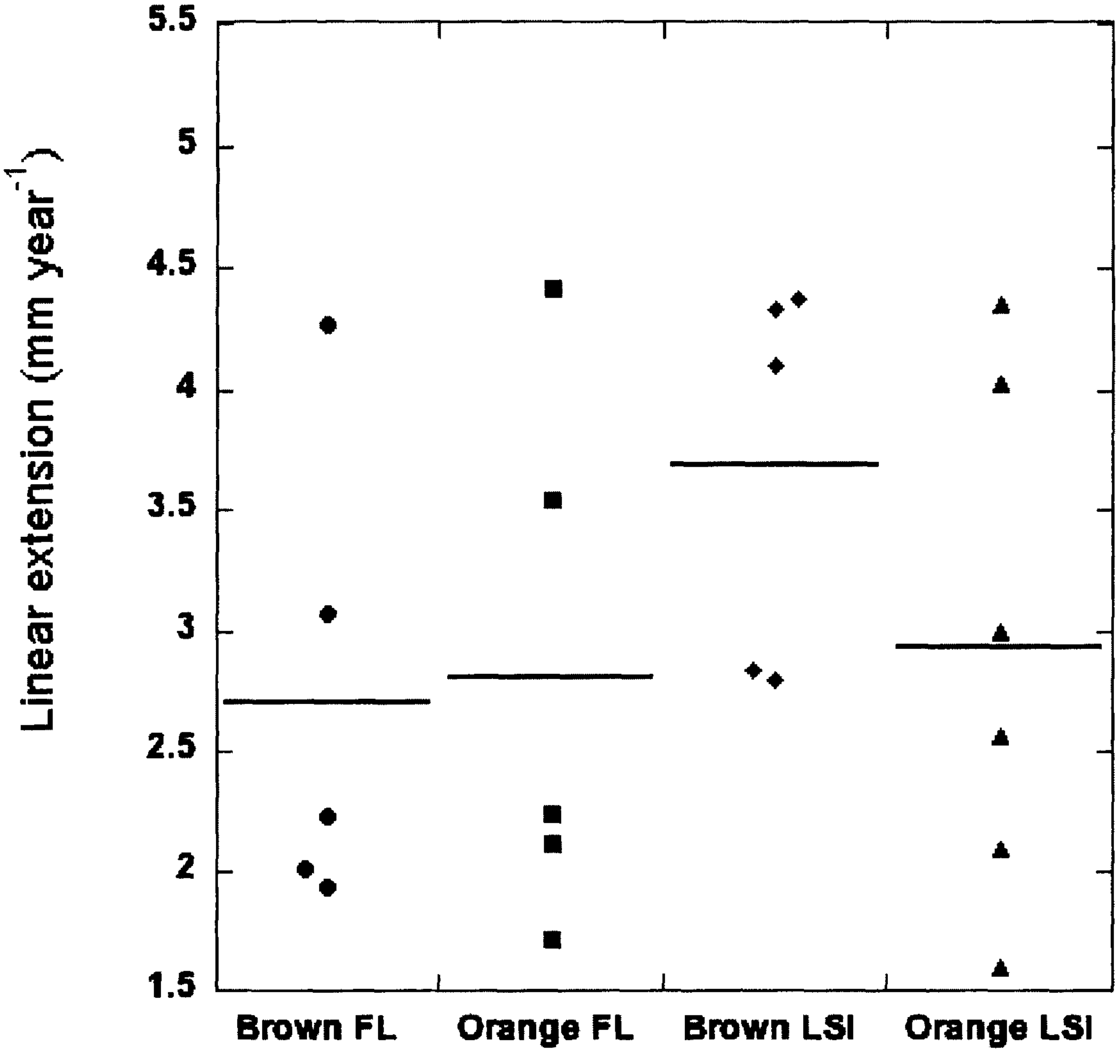


Figure 4.4. Dotplot of linear extension of brown and orange *Montastraea cavernosa* colonies from Lee Stocking Island, Bahamas (LSI) and the Florida Keys (FL) from 2009 - 2010. Each symbol represents a single colony, horizontal bars are the mean.

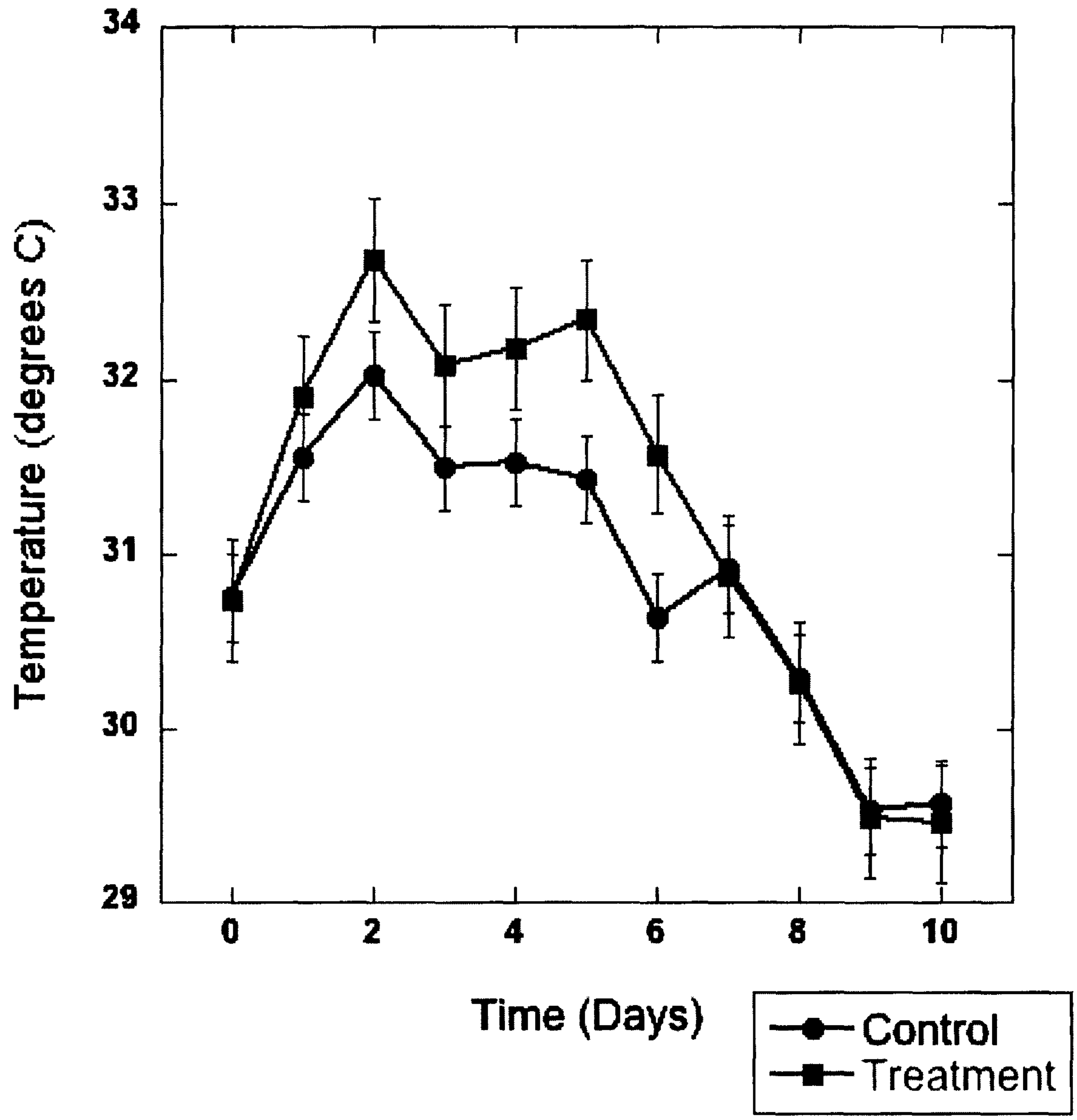


Figure 4.5. Temperature in control and treatment tanks during thermal stress experiment. Error bars are standard deviation.

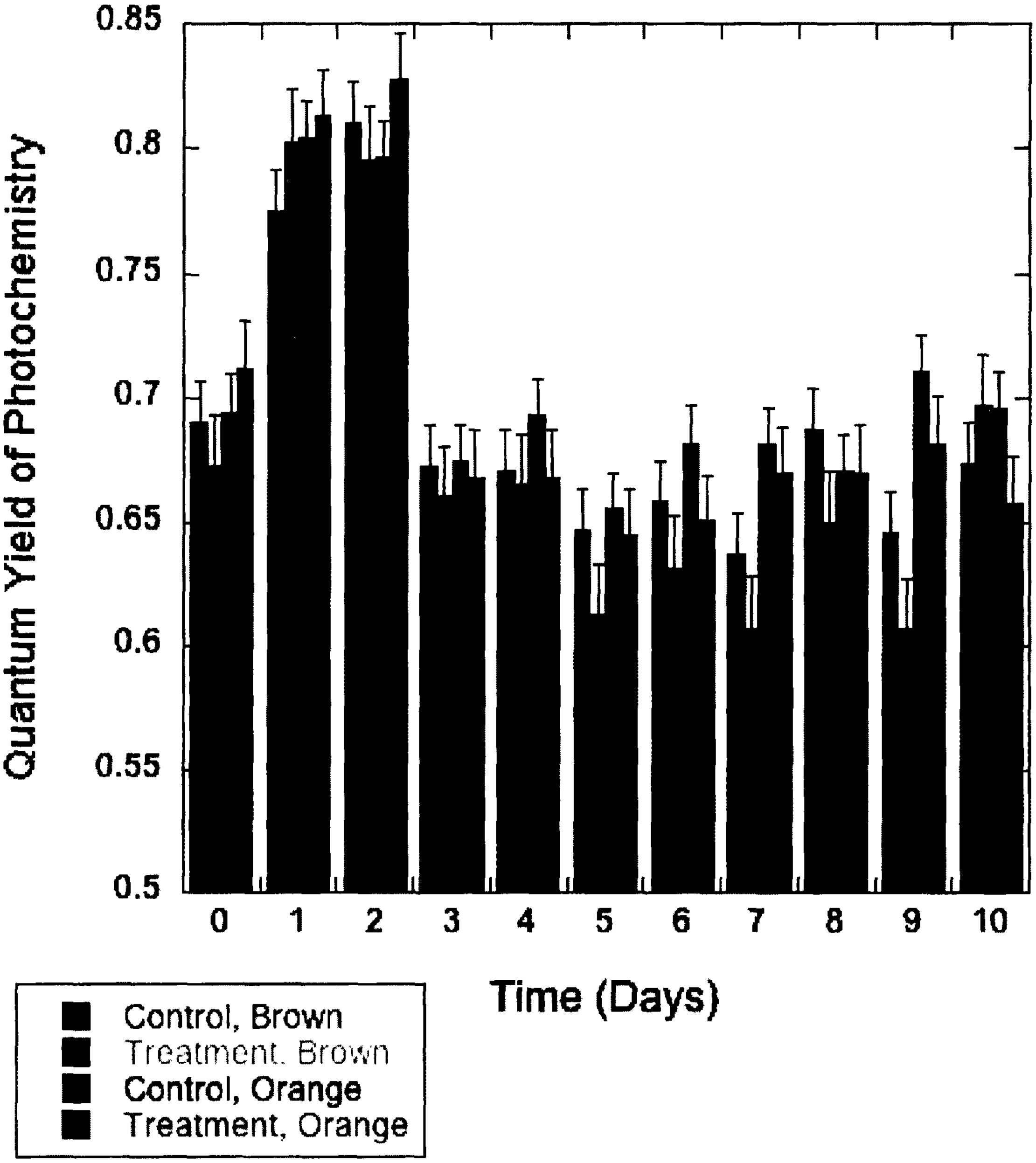


Figure 4.6. Maximum quantum yield of photochemistry (F_v/F_m) in brown and orange *Montastraea cavernosa* in control and treatment tanks during thermal stress experiment. Measurements were made on corals that had been dark-acclimated for a minimum of 30 min. Error bars are standard deviation.

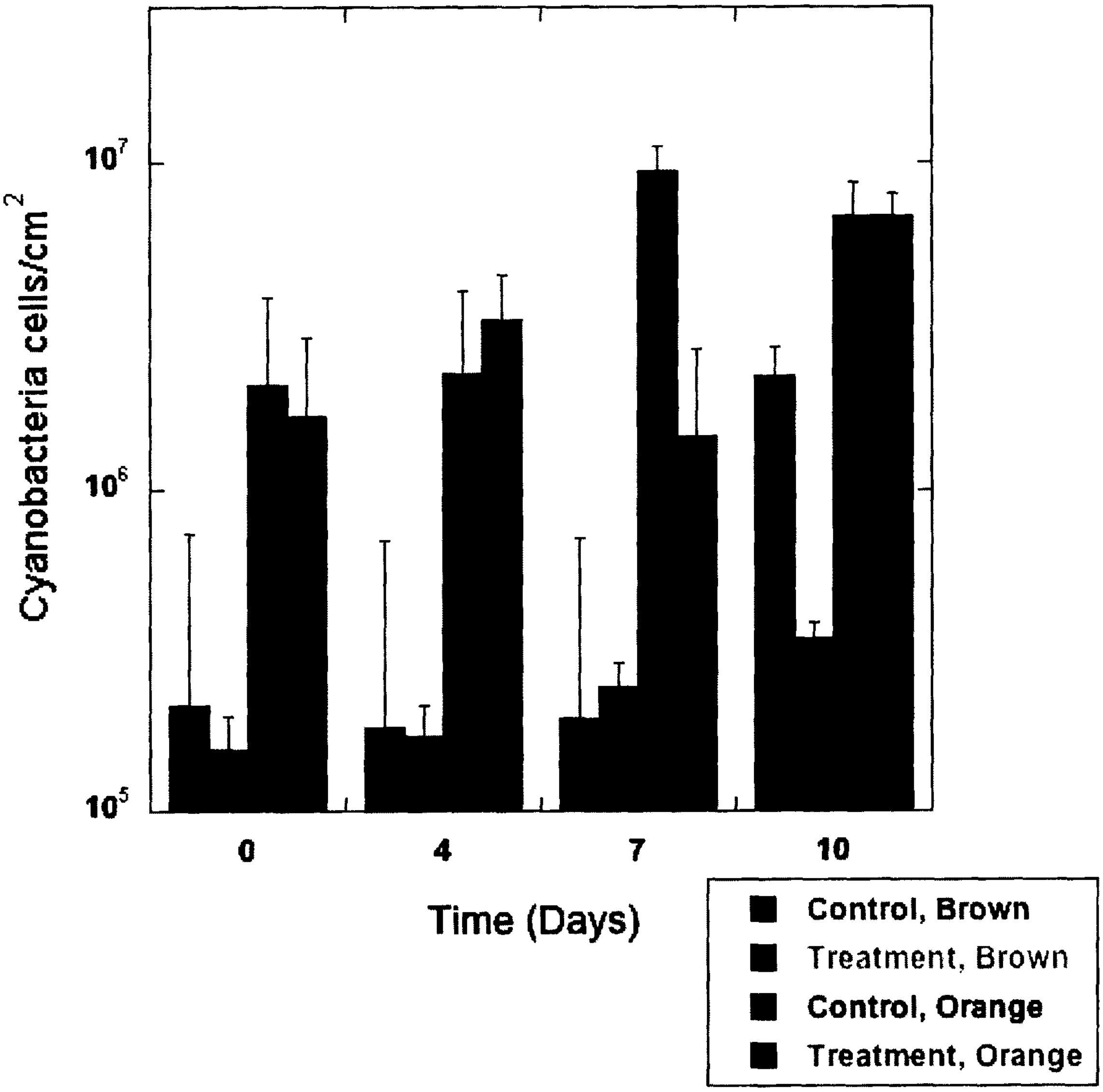


Figure 4.7. Density of cyanobacterial cells in tissue of brown and orange *Montastraea cavernosa* in control and treatment tanks during thermal stress experiment. Error bars are standard deviation.

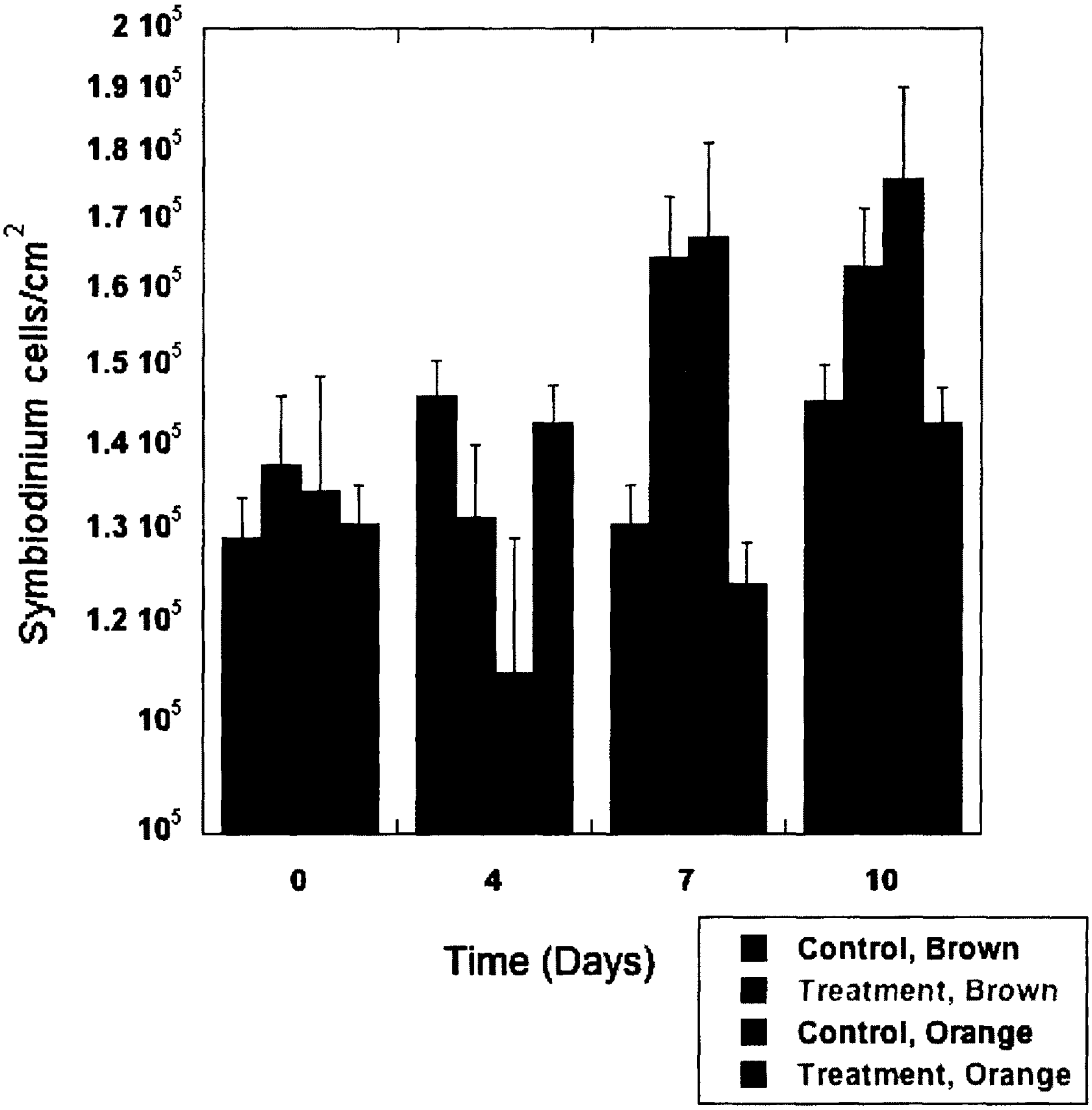
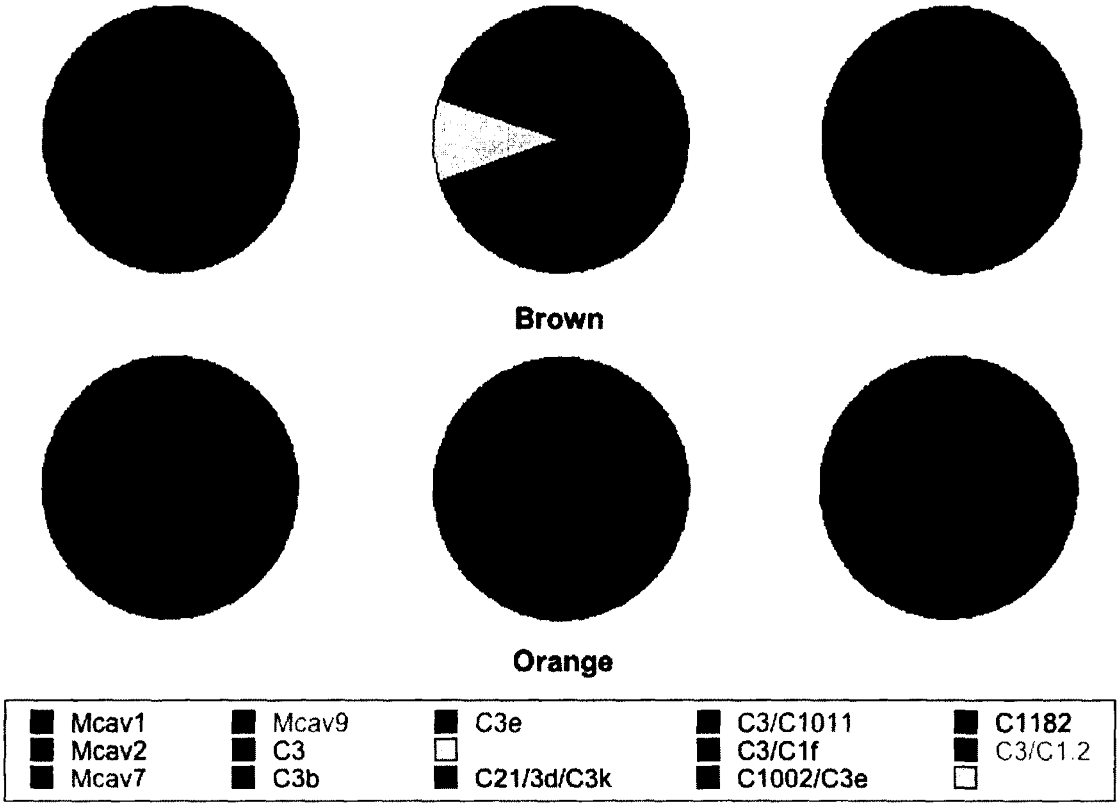
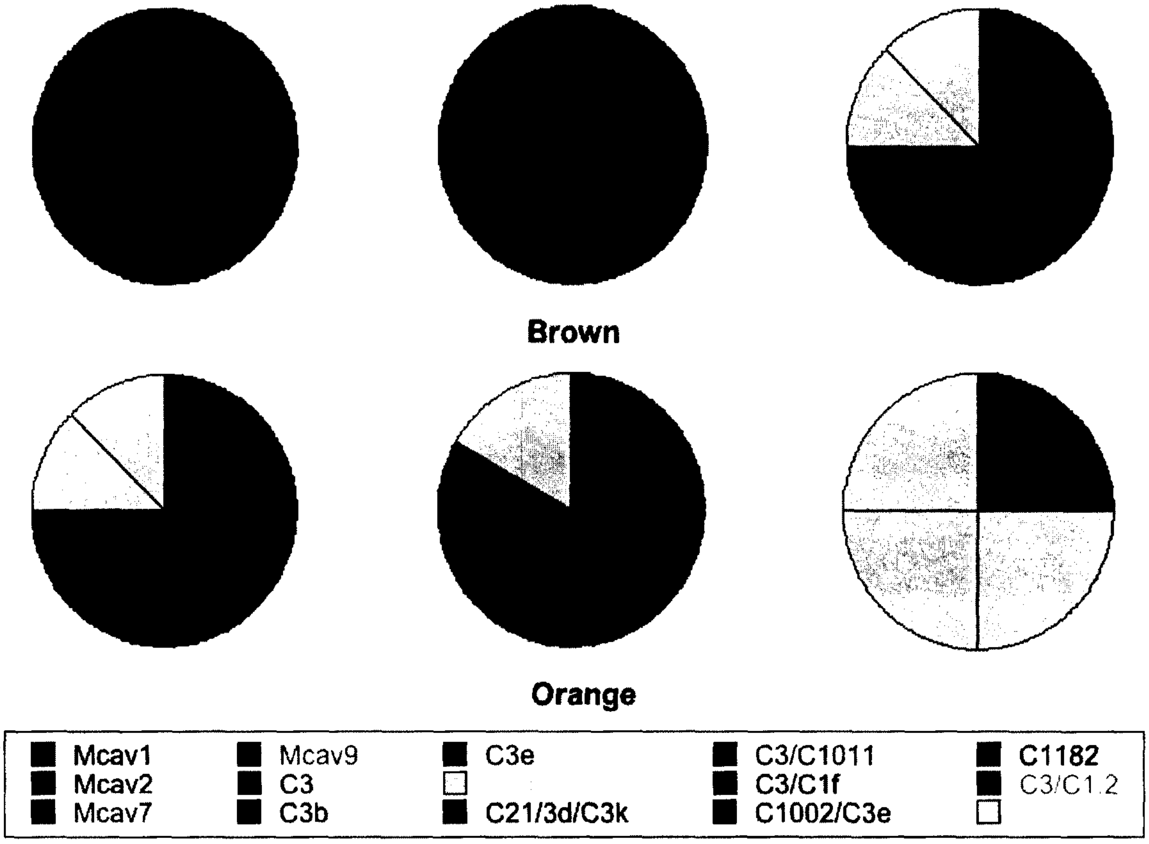


Figure 4.8. Density of *Symbiodinium* cells in tissue of brown and orange *Montastraea cavernosa* in control and treatment tanks during thermal stress experiment. Error bars are standard deviation.

A.


Brown
Orange
Mcav1
Mcav2
Mcav7
Mcav9
C3
C3b
C3e
C21/3d/C3k
C3/C1011
C3/C1f
C1002/C3e
C1182
C3/C1.2

B.


Brown
Orange
Mcav1
Mcav2
Mcav7
Mcav9
C3
C3b
C3e
C21/3d/C3k
C3/C1011
C3/C1f
C1002/C3e
C1182
C3/C1.2

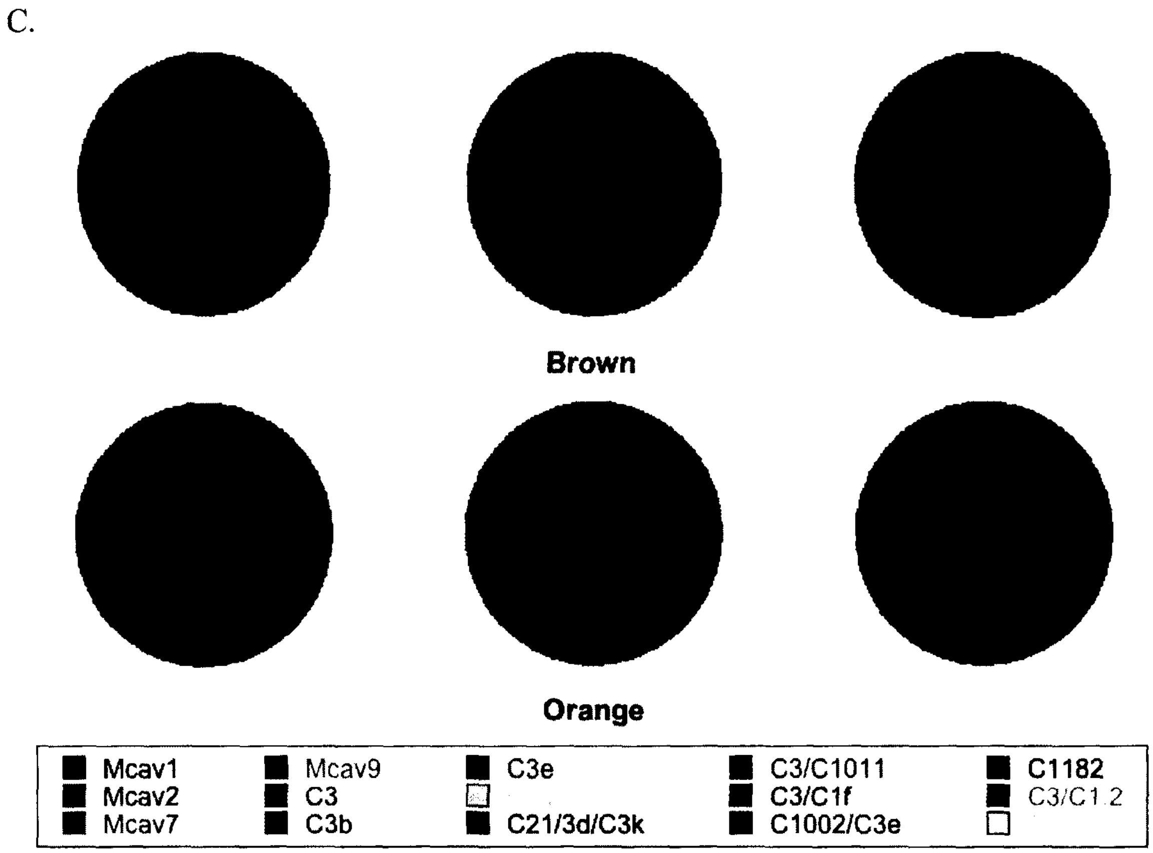


Figure 4.9. *Symbiodinium* rRNA ITS2 types from brown and orange colonies of *Montastraea cavernosa* colonies from (a) the Florida Keys; (b) Little Cayman, Cayman Islands; (c) Lee Stocking Island, Bahamas. Each pie chart represents a single coral colony, and each sector represents a single ITS2 sequence. The number of sequences per colony varies as indicated by the number of sectors.

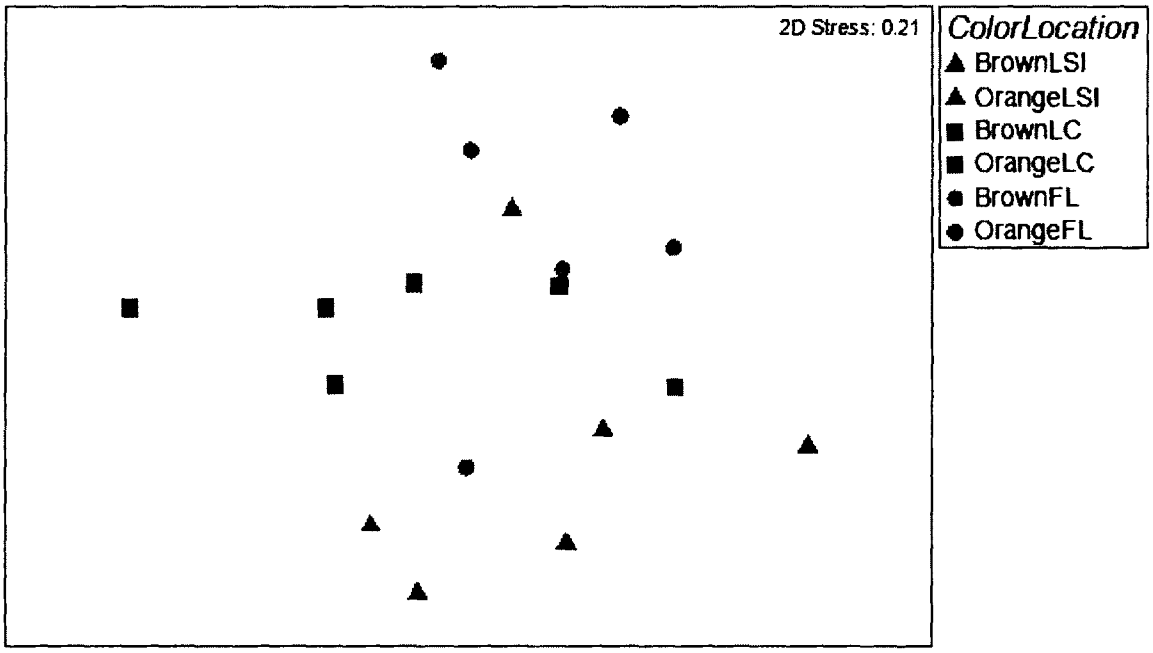


Figure 4.10. Multidimensional scaling plot of differences in *Symbiodinium* rRNA ITS2 types from brown and orange colonies from the Florida Keys (FL), Little Cayman (LC), and Lee Stocking Island, Bahamas (LSI). Plotted from Bray-Curtis similarity measure on square-root transformed abundance data.

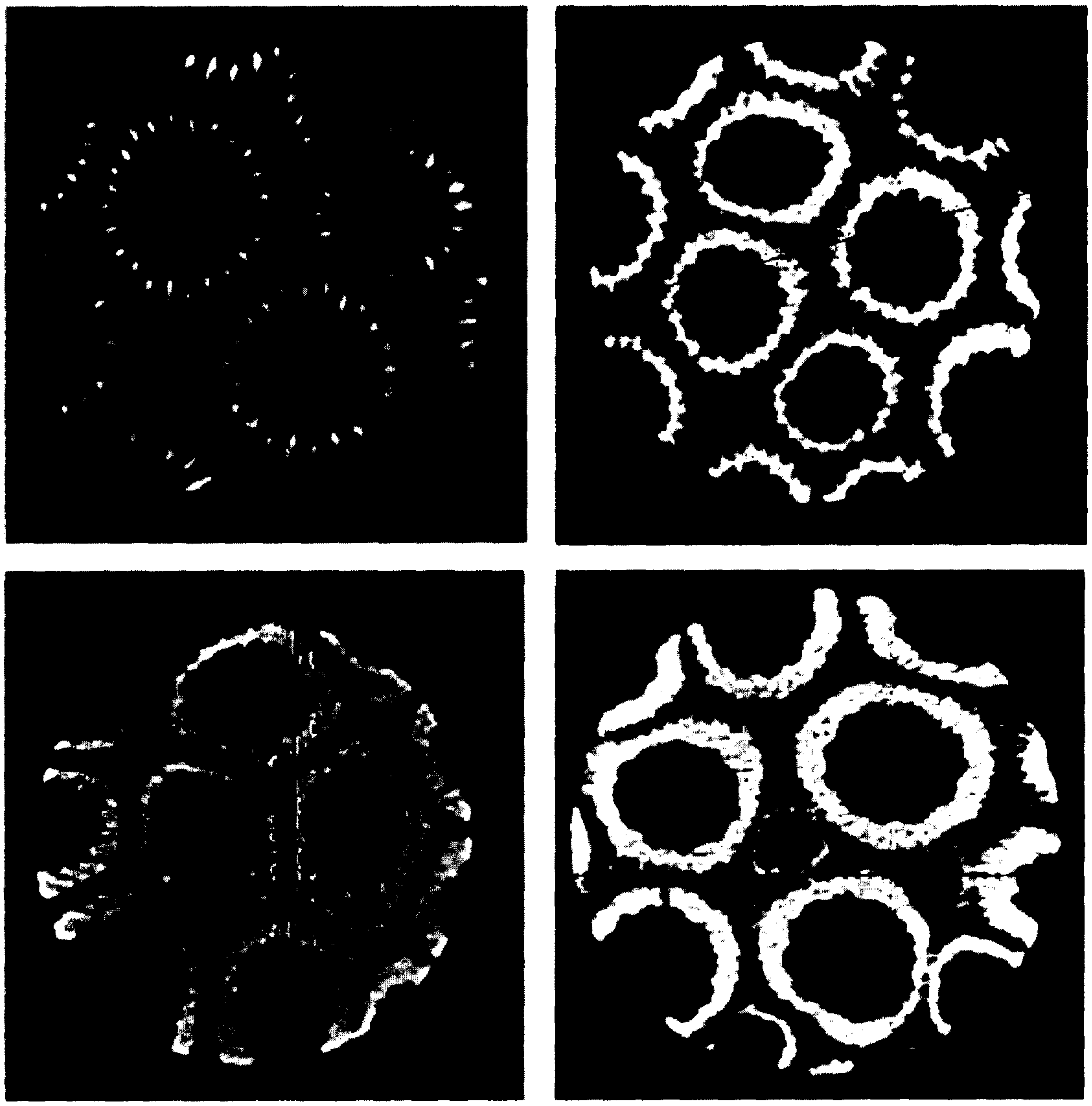


Figure 4.11. Views of representative *Montastraea cavernosa* cores from reconstructed computed tomography 3-D images. Views are perpendicular to the growth axis, equivalent to the visible surface of the colony. These images illustrate the high degree of polymorphism in skeletal morphology within the whole population, not differences between brown and orange colonies specifically.

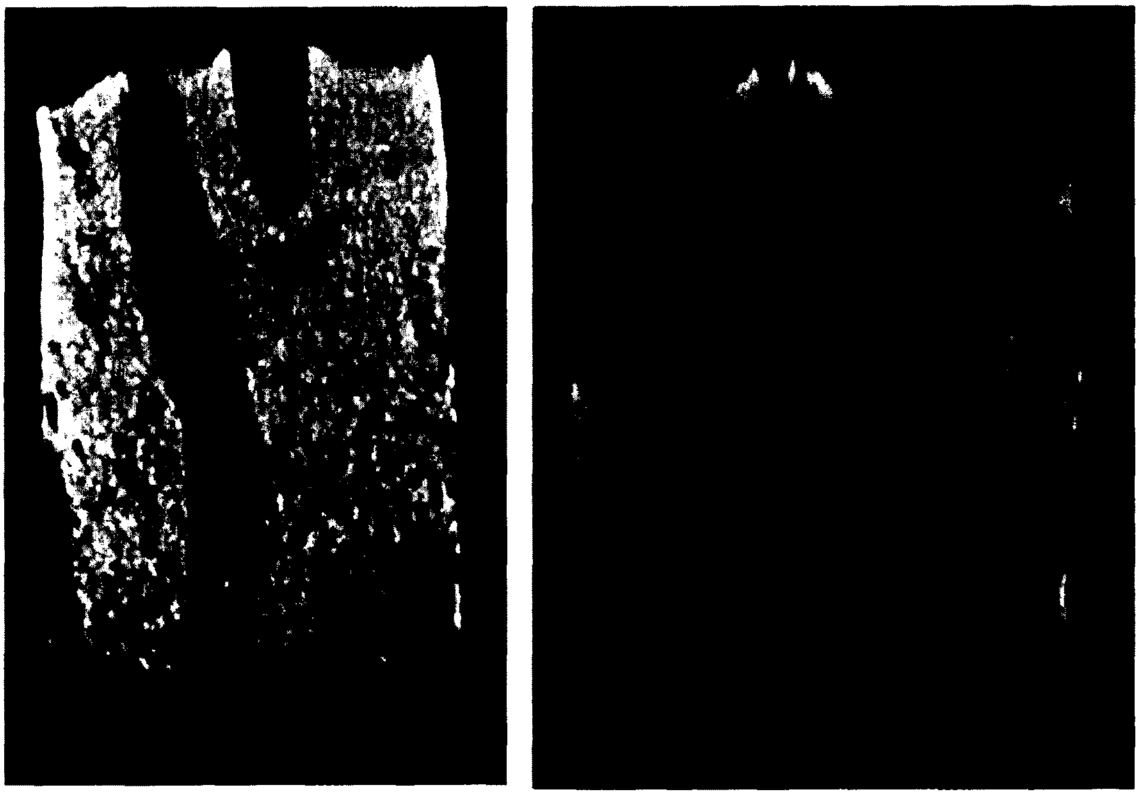


Figure 4.12. Virtual slices from reconstructed computed tomography 3-D images of cores of *Montastraea cavernosa*, showing the high degree of variation in skeletal density within the population. Both cores were sampled from orange colonies from Lee Stocking Island, Bahamas, but variations in density were also present in brown colonies. Slices shown are roughly parallel to the growth axis.

CONCLUSIONS

Symbioses, defined in the broadest sense as the living together of dissimilar organisms (sensu de Bary, 1879), can be classified in a variety of ways. Any given partnership can be placed along a continuum based on its persistence in time and space, the specificity of the relationship, necessity for the survival of both partners, and whether it is beneficial or detrimental to each partner. Symbioses occupying many positions on this continuum are critical to the existence, formation, and ecological function of coral reefs. In particular, the coral animal harbors intracellular, symbiotic, photosynthetic dinoflagellates, *Symbiodinium* (Freudenthal, 1962). In the broadest sense this relationship is facultative for *Symbiodinium* but obligate for the coral because without these symbionts the coral will die. However, most studies have shown that both partners benefit, the coral gaining a source of fixed carbon and the algae gaining a protected habitat where they reach high population densities. The relationship has persisted over evolutionary time, and both partners have developed varying degrees of specificity depending on the host and genetics of the symbiont (Weis, *et al.*, 2001, LaJeunesse, *et al.*, 2004, Mauricio, *et al.*, 2004, Wood-Charlson, *et al.*, 2006).

Corals are also found in symbiosis with a wide variety of other microbes, including Fungi, bacteria, Archaea, and viruses (Rohwer, *et al.*, 2002, Wegley, *et al.*, 2004, Wegley, *et al.*, 2007, Vega Thurber, *et al.*, 2008, Amend, *et al.*, 2011, Littman, *et al.*, 2011) with unknown benefits or consequences . Some bacteria are intracellular (Lesser, *et al.*, 2004, Ainsworth, *et al.*, 2006), whereas others are associated with the skeleton (Lukas, 1974, Sweet, *et al.*, 2010) or mucus rather than the tissue (Reia & John, 2006, Sweet, *et al.*, 2010). Some bacteria are consistently found with the same corals even in locations separated geographically over long distances (Rohwer, *et al.*, 2002) and in different seasons (Littman, *et al.*, 2009), implying a degree of specificity and potentially an obligate symbiosis. Other bacteria proliferate only during specific environmental conditions such as increased temperature, and may become opportunistic pathogens (Ritchie, 2006, Sunagawa, *et al.*, 2009, Vega Thurber, *et al.*, 2009).

A recently discovered symbiosis on coral reefs is that of the coral *Montastraea cavernosa* with nitrogen-fixing cyanobacteria, in addition to the typical dinoflagellate *Symbiodinium* (Lesser, *et al.*, 2004, Lesser, *et al.*, 2007). The cyanobacteria are present in only some colonies, raising important questions about the costs and benefits of this relationship and its prevalence in the population. If the cyanobacteria are beneficial, why are they not more common? If they are detrimental, why do they persist? The goal of this research was to characterize this symbiosis and explore its effects on the coral host and the other functionally important microbial associates of the coral. Here, I review and integrate the results of this study and suggest areas where further research is necessary.

A preliminary identification of the symbiotic cyanobacteria was made based on sequencing of a single orange colony with eubacterial primers. The cyanobacterial population associated with the orange colony appears to include several distinct lineages, including a *Pleurocapsa*-like and a *Gloeocapsopsis*-like group, both of which are related to other known symbionts. In order to conclusively identify the cyanobacteria, it will be necessary to recover similar sequences from multiple orange colonies and use fluorescent in-situ hybridization (FISH) or a similar technique to localize these cells within coral tissue. The presence of a mixed population of cyanobacteria suggests that specificity in this association may be lower than anticipated, despite the intracellular location of symbionts. Sequencing of additional coral colonies will reveal if the same types of cyanobacteria, or closely related types, are consistently present. Relationships of this type are common in sponges; many bacterial symbionts are species-specific but are closely related to the symbionts found in other species of sponges (Schmitt, *et al.*, 2012).

The presence of cyanobacteria does not significantly affect the abundance or diversity of the rest of the coral-associated prokaryotic community. However, ongoing analysis of the metatranscriptome may reveal differences in the gene expression and activity of shared taxa. Low-abundance taxa in particular have been shown to have disproportionately high activity levels in previous studies (Campbell, *et al.*, 2011, Gaidos, *et al.*, 2011). A high number of putative mRNA contigs in the metatranscriptome were assigned to Burkholderiales, despite the fact that this group made up only a small percentage of 16S rRNA amplicon libraries. This suggests that low-abundance prokaryotes may be highly transcriptionally active and functionally important in *M. cavernosa.*

Prokaryotic communities included numerous sequences from disease- and stress-associated taxa such as Vibrionales and Alteromonadales in coral samples. Although the Vibrionales in particular are frequently associated with stressed corals and have been implicated as opportunistic pathogens in coral diseases (Frias-Lopez, *et al.*, 2002, Garren, *et al.*, 2009, Sunagawa, *et al.*, 2009), this group may also be important nitrogen-fixers in the holobiont. Nitrogen-fixing *Vibrio* have been isolated from the mucus of *Mussismilia hispida* in Brazil (Chimetto, *et al.*, 2008), and the abundance of *Vibrio*-like nitrogenase (*nifH*) sequences was positively correlated with *Symbiodinium* density in two species of *Montipora* from Hawaii (Olson, *et al.*, 2009), suggesting a benefit was conferred by the bacteria. It is possible that these taxa or related taxa may contribute to the nitrogen fixation previously observed in orange colonies of *M. cavernosa*. Multi-isotope imaging mass spectrometry (MIMS) has been used successfully in shipworms to definitely identify the site of nitrogen fixation and the fate of the nitrogen products (Lechene, *et al.*, 2007). Applying this approach to the *M. cavernosa* system in conjunction with stable isotopes would definitively demonstrate if the cyanobacteria or some other coral-associated bacteria are responsible for nitrogen fixation.

The composition of prokaryotic communities varied significantly at different locations, with Lee Stocking Island distinguished from Florida and Little Cayman by a lower abundance of some proteobacterial taxa, including Vibrionales and Alteromonadales. The similarity between Little Cayman Island and Florida was unexpected. Little Cayman is the most geographically isolated of the sites studied and has a very small permanent human population, whereas the Florida Keys are densely populated and the water quality is heavily impacted by runoff of nutrients and sediment (Lapointe & Clark, 1992). However, these sites are hydrographically linked by the Caribbean Current, which becomes the Loop Current as it passes through the Gulf of Mexico and eventually the Gulf Stream off the Florida coast (Kameo, *et al.*, 2004). In contrast, the Bahamas are influenced by the Antilles Current, which may account for the differences in bacterial populations at this site.

Samples from Lee Stocking Island were chosen for metatranscriptomic analysis and many contigs were differentially expressed in brown and orange colonies. These significant differences suggest that changes in components of the holobiont may result in important functional differences between the two colony morphs. Further analysis and annotation of the metatranscriptome will reveal if different members of the holobiont such as Fungi or prokaryotes are transcriptionally active in brown and orange colonies, and how the gene expression of the coral host and *Symbiodinium* may differ. The size of the dataset and the replicated samples will enable powerful statistical comparisons of colony types. This in-depth profile of the transcriptional activity of normal adult coral holobionts with fully established symbioses will also serve as an important comparison to existing transcriptomic data sets, which originate primarily from aposymbiotic coral larvae, stressed adult corals, or cultures of *Symbiodinium* (Polato, *et al.*, 2011, Traylor-Knowles, *et al.*, 2011, Bayer, *et al.*, 2012).

The effects of cyanobacteria on fitness appear to be neutral, although a significant effect on the quantum yield of photochemistry was observed in the thermal stress experiment. Growth, the ability to deter predators and produce toxins were unaffected by the presence of cyanobacterial symbionts, but investigation of other aspects of fitness may be more fruitful. *Symbiodinium* have higher growth rates in orange colonies (Lesser, *et al.*, 2007), and may also be able to translocate more carbon to the coral host. Alone or in conjunction with fixed nitrogen products, this could lead to differences in reproductive output that might have a large impact on coral fitness. Intriguingly, brown and orange colonies form two genetically distinct populations at Lee Stocking Island, and possibly at other sites as well, although this was not investigated. The high dispersal potential of this species along with the small spatial scale of sampling implicates local selection as the driver of these genetic differences. This further suggests that fitness differences may exist, and that such traits may be under selection.

With this research we are only beginning to understand the nature of the symbiosis between *M. cavernosa* and cyanobacteria, and many questions remain unanswered. The cyanobacteria do not appear to be parasitic, but their exact placement on the continuum from mutualism to parasitism is unclear. While the symbiosis is clearly not obligate for *M. cavernosa* as a species, the genetic differences in brown and orange colonies leave open the possibility that it may be obligate for the population of orange colonies. Further research will help to clarify unresolved questions. In particular, MIMS or a similar technique is warranted to establish if the intracellular cyanobacteria or other prokaryotes are fixing nitrogen, which would clarify their functional role in the holobiont. Microscopic and molecular investigation of freshly spawned gametes could reveal if cyanobacteria and perhaps other microbes are vertically transmitted, as they are in some sponges (Schmitt, *et al.*, 2008). Vertical transmission tends to occur in symbioses that are more obligate and specific and have a longer evolutionary history (Bright & Bulgheresi, 2010). The cyanobacterial symbionts of *M. cavernosa* are distinguished from many of the other associated prokaryotes by their intracellular location and presumably intimate relationship with the host, but the nature of most of these symbioses and their ecological importance are still obscure.

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APPENDIX

THE UNIVERSITY OF MISSISSIPPI INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

ANIMAL STUDY APPLICATION APPROVAL

Application No.: 09-018 Approval Date: 12-19-08 Project Expiration Date: 12-19-11

Approved Pain Category: A/B Animals to be Used & No. Approved: 180 Pufferfish for 3 year approval period

TO: Dr. Marc Slattery
Department of Pharmacognosy

FROM: Virginia D. Cantú
IACUC Research Compliance Specialist

SUBJECT: Protocol 09-018, Population differences in susceptibility to natural products

Your application was reviewed by the Institutional Animal Care and Use Committee (IACUC) and determined appropriate for designated review. The assigned designated reviewers completed their review and approved protocol 09-018 on December 19, 2008.

They realize you have extensive knowledge and experience in this field. Since the dose of MS 222 was not specified, they asked that this excerpt from the AVMA Guidelines on Euthanasia be sent for the record. A reference to this excerpt was added to #28 C on the attached protocol application.

> "**TRICAINE METHANE SULFONATE (MS 222, TMS)** MS 222 is commercially available as tricaine methane sulfonate (TMS), which can be used for the euthanasia of amphibians and fish. Tricaine is a benzoic acid derivative and, in water of low alkalinity (< 50 mg/L as $CaCO_3$); the solution should be buffered with sodium bicarbonate.104 A 10 g/L stock solution can be made, and sodium bicarbonate added to saturation, resulting in a pH between 7.0 and 7.5 for the solution. The stock solution should be stored in a dark brown bottle, and refrigerated or frozen if possible. The solution should be replaced monthly and any time a brown color is observed.105 For euthanasia, a concentration ≥ 250mg/L is recommended and fish should be left in this solution for at least 10 minutes following cessation of opercular movement.104 In the United States, there is a 21-day withdrawal time for MS 222; therefore, it is not appropriate for euthanasia of animals intended for food."

Regulations require that you:

1. **Post a copy of this approval letter in your animal room for the three-year IACUC approval period**
   (PHS Policy IV, D, a-e; AWAR §2.35, a, 2, and 3)

2. **Submit a renewal application to continue this study beyond the three-year approval period**
   (PHS Policy IV, C, 1 – IV, C, 4) (We will remind you well before the 3-year expiration date.)

In order to maintain your approval status, you must submit an annual report of progress on this study (AWAR §2.31, d, 5 and PHS Policy IV, C, 5) using a Protocol Annual Update form. (We will send this form to you electronically several months before the due date.)

Animal purchase requisitions for labs located in the University Vivarium must be submitted to Ms. Penni Bolton, Animal Facility Supervisor.

Procedure, personnel, and other changes must be first approved by the Committee. Complete one of the following forms:

- Non-Personnel Protocol Amendment
- Protocol Amendment for Personnel Changes Only

Approval for adding personnel requires that the new person(s):

- Complete required Health & Safety and species-specific training BEFORE engaging in any activity involving live vertebrate animals.
- If conducting surgery, complete mandatory training and performance of surgical techniques in the procedure(s) and species indicated. Must receive surgery proficiency certification from the Attending Veterinarian (Dr. Fyke).
- Submit the Occupational Health Evaluation and OHSP Risk Inventory forms to the Attending Veterinarian (Dr. Fyke, B104 NCNPR) for review and approval by the Occupational Health Physician.
- Read the materials on asthma and allergy for animal handlers.
- Check to make sure tetanus vaccination is current. The Centers for Disease Control and Prevention recommends tetanus vaccinations every 10 years, and the IACUC strongly urges personnel to get this free vaccination. (The ORSP Division of Research Integrity and Compliance pays the $45.00 fee.).

Please contact the IACUC office staff at 915-7482 or askiacuc@olemiss.edu if you need assistance or have any questions.

cc: Ms. Penni Bolton, Animal Facility Supervisor
Dr. Deborah Gochfeld, Project Director