DIVERSITY, DISTRIBUTION, AND DYNAMICS OF CORAL REEF BACTERIAL COMMUNITIES ACROSS THE HAWAIIAN ARCHIPELAGO AND WIDER PACIFIC OCEAN

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ZOOLOGY (ECOLOGY, EVOLUTION, AND CONSERVATION BIOLOGY)

DECEMBER 2013

By

Jennifer L. Salerno

Dissertation Committee:

Charles Birkeland, Co-Chairperson
Michael S. Rappé, Co-Chairperson
Brian W. Bowen
Ruth D. Gates
Grieg Steward
DEDICATION

For my parents, Janet and Anthony Salerno. Thank you for instilling a sense of adventure in me, for truly believing in me, and for always encouraging me to chase after my dreams.
ACKNOWLEDGEMENTS

I am indebted to so many who have helped me along on this magnificent, intellectually challenging, physically exhausting, emotionally demanding, at times completely frustrating, but overall absolutely wonderful eye-opening and mind-growing journey. First, I would like to thank Dr. Charles Birkeland for bringing me into his laboratory group in the Zoology department. Of all the prospective advisors I contacted, his work stood out to me because of its implications for marine conservation – something I felt very strongly about incorporating into my doctoral dissertation research. From accompanying me on Molokai field collection trips to simply always having an open door policy for discussing new research project ideas or pressing conservation issues, he has been supportive through and through. I would not have been able to achieve all that I have without his steadfast support and encouragement.

I cannot thank Dr. Michael Rappé enough for “adopting” me as a graduate student in his laboratory even after telling him that the extent of my molecular biology practical laboratory experience was “extracting DNA from an onion once.” I was really a trained microscopist, but he still decided to take a chance on me. He was equally excited about coral microbes from the start and has provided me with logistical and financial support throughout my time at the University of Hawaii and the Hawaii Institute of Marine Biology. I cannot emphasize enough how much I have learned during my tenure in his lab. I think few students come in with an idea of a dream project that they would like to work on as a graduate student and actually get to work on it. So I thank Mike for making that a reality for me.
Dr. Brian Bowen has provided unwavering support from start to finish and I cannot thank him enough. His constant encouragement has kept me going during some very challenging times. I especially enjoyed getting to share so many Northwestern Hawaiian Island cruise adventures with Dr. Bowen. His enthusiasm for all things fish and science are truly inspiring.

I must thank Dr. Ruth Gates for always taking time out to discuss research ideas and caveats with me, as there are often so many when working with corals. Ruth has cheered me on since the very beginning and her energy and excitement for coral research is contagious. Whenever I started to feel mentally saturated about my research topic, a chat with Dr. Gates got me buzzing again. I cannot stress how important this was for boosting my endurance to tackle such a challenging project over the long term.

I will forever remember Dr. Grieg Steward transforming into a motile bacterium in our Marine Microplankton Ecology class to demonstrate movement and scale in the microbial world. I found his teaching to be both enriching and entertaining and knew that he would be invaluable as a committee member. He happily accepted and has not disappointed. I especially appreciated the time and effort he spent reading and critiquing my dissertation proposal.

I cannot thank Dr. Rob Toonen enough. He has been a pillar of support for me throughout my time at HIMB. Dr. Toonen has spent hours going over presentations to help me prepare for meetings and went out of his way to ensure that I had a teaching position during my last semester at HIMB. This is especially commendable as I was not a member of his lab group and he was not on my committee. His unwavering support kept
me going and I am forever grateful for the time he dedicated to help ensure my success as a scientist and student.

I thank Dr. Jo-Ann Leong for supporting me as part of the NOAA-HIMB NWHI research team and for the many times she simply took a minute out of her busy schedule to check in with me to see how I was doing. This meant so much to me as a graduate student.

I had the pleasure of working for Dr. Malia Rivera as an education and outreach graduate assistant during my last semester at HIMB. I found teaching students and the public about marine science to be both professionally rewarding and personally fulfilling and thank her for that opportunity. I greatly admire Dr. Rivera’s passion and dedication to marine education and personally look up to her as a role model. She offered invaluable advice and her full support when I applied for and received a NOAA Knauss Legislative Marine Policy Fellowship.

I would like to thank Representative Lois Capps, who I had the honor to work for during my fellowship year in Washington, D.C., and the rest of the “Capps Family” for not only teaching me everything I know about the legislative process, but for being so open, accepting, trusting, supportive, and fun to work with. Being on the Hill was a truly amazing and eye-opening learning experience.

I cannot thank Dave Pence and Kevin Flanagan from the UH Scientific Diving Program enough. They ensured that I was prepared for every research cruise and field trip and always made time for me if I needed to fit in a last minute dive before a cruise. I learned so much from them and became a better diver because of their teachings.
I must thank the Captain and crew of the Hi‘ialakai for ensuring our safety during every research cruise and dive operation. Our work would literally be impossible without their support. I formed many lasting friendships during my time spent out at sea and I will forever cherish the adventures we shared out on the water. I also extend my most sincere thanks to the wonderful staff at the Papahānaumokuākea Marine National Monument (especially Randy Kosaki, Kelly Gleason, and Elizabeth Keenan Kehn) for their assistance with permitting and cruise preparations.

I also cannot forget to thank the administrative and maintenance staff in the Zoology Department and at HIMB – especially Jan Tatsuguchi, Lynn Ogata, Kathy Nishimura, Steffiny Nelson, Lormona Meredith, Bobby Tangaro, and Wayne Nakamoto who were always ready and willing to help with a smile.

I thank all of the members of the Rappé laboratory (a.k.a. L.A.M.É.) for their support and friendship over the years. Jamie Becker took me through my very first Polymerase Chain Reaction, helped with the many that followed, and enthusiastically accompanied me on a field trip to Molokai despite getting scratched by coral, stung by a jellyfish, and bitten by an unidentified marine creature. Darin Hayakawa has been like a big brother to me and, although he is quite humble about it, harbors an amazing amount of microbial and scientific knowledge. His unwavering support helped me through some of the toughest times. Amy Apprill and I celebrated and commiserated over our triumphs and failures in the lab when it came to working with notoriously difficult coral samples. I appreciated her companionship in the lab and field and thank her for allowing me to draw upon her molecular wisdom. Tracy Campbell and Sara Yeo initially joined the lab as undergraduates and left as Masters students along with Marina Halverson. It was so
wonderful to watch them grow as people and scientists and I learned so much from all of them. Tracy’s quick wit, Sara’s astounding (and aesthetically pleasing) organizational skills, and Marina’s fashion and pop culture knowledge always kept the mood in the lab light and upbeat. It was always a pleasure to come into work to see the ever-positive, smiling faces of Misty Miller and Naomi Wagoner. They simultaneously preserved lab morale while keeping things running smoothly. I must also thank Hollie Putnam and Liz Hambleton, undergraduates who helped me immeasurably in the field and lab and who have both gone on to pursue Ph.D.s of their own. I wholeheartedly thank Ale‘alani Dudoit for her hard work and friendship. She accompanied me on numerous fieldwork excursions, processed hundreds of coral samples with enthusiasm, and was invaluable in helping me to complete several research projects. I also have to thank relative newcomer (now senior lab member) Sean Jungbluth whose mild Midwestern demeanor and great sense of humor have been a wonderful addition to our lab group. Having postdoctoral researchers Megan Huggett, Alexander Eiler, and Jana Grote in the laboratory was a lifesaver for all of us. Their specialized expertise was especially welcomed when it came to analyzing intimidating datasets and preparing for presentations. I would especially like to thank Megan Huggett, who volunteered her time and energy to help me collect coral samples on two exhausting research cruises to the Northwestern Hawaiian Islands and for providing both professional and personal advice.

I also thank members of the Birkeland laboratory – Lance Smith, Jan Dierking, Dan Barshis, and Danielle Jayewardene for helping me to get settled in Hawaii, sharing office space, discussing research, and offering their support and friendship. I thank Dan Reineman of the Gates laboratory for his hard work during our research collaboration and
for all of our discussions about graduate school and marine conservation. I met and became fast friends with Anderson Mayfield, Kevin Hall, Nadiera Sukhraj, and Kenzie Manning during my first year of graduate school and I cannot thank them enough for all of their love and support. Kenzie has become a sister to me and I would not have survived graduate school without her. We have all shared countless adventures, sorrows, and happy times together and I will always cherish our memories in Hawaii and beyond.

Finally, I want to thank my family for their unending love and support. I thank my parents for encouraging me to pursue my dreams and passions, even though they often took me far away from them for extended periods of time. I thank my brothers Tony, Nick, and James and my sister-in-law Beth for cheering me on and encouraging me to stay the course even when things seemed impossible at times. I thank my nieces and nephews Nigel, Maya, Hannah, Lakota, and Lyra for being so understanding when I couldn’t make it home for special birthdays or Christmas. I thank my husband’s family, Pamela, Bob, Nick, and Rikki Schmidle, for offering so much love and support while I was working in Washington D.C. and my husband was deployed. Last but not least, I thank my wonderfully supportive and understanding husband, Christian, whose love and faith in me keep me going every day. We are expecting the arrival of our son in just a few days – our best science experiment to date – and hope to instill in him the same wonder, curiosity, and respect for the natural world that was instilled in us as children.

Of the many positive things I took away from graduate school, expanding my scientific knowledge and forming lasting friendships were, by far, the two most important to me. I thank anyone and everyone who contributed to these wonderful gifts.
ABSTRACT

A comprehensive investigation characterizing the diversity, distribution, and dynamics of coral-associated bacterial communities was carried out through a series of targeted observational and experimental studies. A focus was placed on four overarching research themes deemed fundamental to understanding these complex communities: method optimization, biogeography, natural variability, and environmental effects on bacterial community structure. Protocols for genomic DNA extraction and Polymerase Chain Reaction (PCR) amplification of bacterial 16S rRNA genes were optimized for high-throughput analyses of coral-associated bacterial communities associated with common Hawaiian corals. Successful gene amplification and community profiles varied with sample pre-treatment/nucleic acid extraction method combination and differed significantly between Porites lobata and Porites compressa corals. A baseline assessment of coral-associated bacteria associated with Porites lobata throughout the Hawaiian Archipelago and Indo-Pacific Ocean revealed a weak, but significant isolation by distance pattern, indicating that geographic isolation is partially responsible for driving observed divergences at the archipelagic scale. However, some geographic groupings were not distance-based, indicating that additional environmental factors may also affect the distributions of these communities. The natural variability of bacterial communities associated with colonies of Porites compressa was investigated over a five-month period at two sites around Molokaʻi, Hawaiʻi. Terminal-restriction fragment length polymorphism analysis of PCR-amplified 16S ribosomal RNA genes revealed that bacterial communities did not differ between colonies within a site but differed between sites and fluctuated from month-to-month. Analysis of environmental data revealed site
differences in environmental parameters (seawater salinity, temperature, turbidity, fluorescence, chlorophyll $a$, and nutrients), but for the most part, did not significantly correlate with bacterial community structure (with the exception of silicate at one site). To further investigate the effects of environmental parameters on associated bacteria, an array of experimental aquaria were used to test the effects of increased temperature on bacterial community structure and photophysiology of $Porites compressa$ corals. While the temperature treatment rapidly impacted the photophysiology of the coral host, it did not elicit a statistically significant shift in bacterial community structure. Information gleaned from this research has expanded our limited knowledge of coral-associated bacterial communities and has brought us closer to understanding the roles of bacteria in coral health.
LIST OF TABLES

CHAPTER 2

Table 2.1 Amplification of 16S rRNA genes via PCR as a function of coral species and DNA extraction method\textsuperscript{a} ........................................................................................................ 49

Table 2.2 T-RFLP profiles obtained from PCR amplification of 16S rRNA genes\textsuperscript{a} ....... 50

Table 2.3 Taxonomic Identification of Microorganisms Associated with \textit{Porites compressa} .................................................................................................................................................. 51

Table 2.4 Taxonomic Identification of Microorganisms Associated with \textit{Porites lobata} ...................................................................................................................................................... 53

Table 2.5 Bray-Curtis dissimilarity matrices for \textit{Porites compressa} and \textit{Porites lobata} clone libraries ........................................................................................................................................ 55

CHAPTER 3

Table 3.1 ANOSIM pairwise test values for seawater-associated bacterial communities sampled in 2005 and 2006 and coral-associated bacterial communities sampled in 2005 and 2006 .................................................................................................................. 96

Table 3.2 SIMPER average similarity between and within island/atolls for seawater-associated bacterial communities sampled in 2005 and 2006 and coral-associated bacterial communities sampled in 2005 and 2006 ...................................................................................... 97

CHAPTER 5

Table 5.1 PAM-derived Maximum Quantum Yield regression values during acclimation and manipulation phases of \textit{P. compressa} .................................................................................................................. 166
LIST OF FIGURES

CHAPTER 1

Figure 2.1 Total yield of genomic DNA from airbrushed or crushed starting material using different extraction methods ................................................................. 59
Figure 2.2 NMDS ordination of coral associated bacteria from Porites compressa and Porites lobata corals ................................................................. 59
Figure 2.3 NMDS ordination of coral associated bacteria from Porites compressa corals .......... 60
Figure 2.4 NMDS ordination of coral associated bacteria from Porites lobata corals .......... 61

CHAPTER 2

Figure 3.1 NMDS ordination of seawater and coral-associated bacterial communities sampled in 2005 and 2006 ................................................................. 101
Figure 3.2 NMDS ordination of seawater-associated bacteria sampled in 2005 ............ 102
Figure 3.3 NMDS ordination of seawater-associated bacteria sampled in 2006 .......... 103
Figure 3.4 NMDS ordination of coral-associated bacteria sampled in 2005 .......... 104
Figure 3.5 NMDS ordination of coral-associated bacteria sampled in 2006 .......... 105

CHAPTER 4

Figure 4.1 Map of field sites on the Hawaiian island of Moloka‘i ......................... 136
Figure 4.2 NMDS ordination of coral and seawater bacterial communities ............... 137
Figure 4.3 NMDS ordination of seawater-associated bacteria sampled at high and low tides ................................................................................................. 138
Figure 4.4 NMDS ordination of coral-associated bacterial communities ............... 139
Figure 4.5 PCA ordination of environmental data sampled at high and low tides .... 140
Figure 4.6 NMDS bubble plot showing the relationship between coral-associated bacterial community structure and silicate concentrations of corals at Kamalō .......... 141

CHAPTER 5

Figure 5.1 Porites compressa mounted in a vinyl sheet in holding tank ................. 169
Figure 5.2 Maximum quantum yield of Porites compressa during acclimation to experimental tanks and after a temperature increase of 1°C at Day 10 .......... 170
Figure 5.3 NMDS ordination of all coral and seawater bacterial communities .......... 171
Figure 5.4 NMDS ordination of seawater bacterial communities ....................... 172
Figure 5.5 NMDS ordination of coral-associated bacterial communities .............. 173
TABLE OF CONTENTS

DEDICATION ........................................................................................................ iii
ACKNOWLEDGEMENTS ...................................................................................... iv
ABSTRACT ........................................................................................................... x
LIST OF TABLES ................................................................................................. xii
LIST OF FIGURES ............................................................................................... xiii
CHAPTER 1 ......................................................................................................... 1
GENERAL INTRODUCTION .............................................................................. 1
  Microorganisms and coral disease ................................................................. 2
  Microorganisms and coral health ................................................................. 6
  Outstanding questions: Host specificity, spatial and temporal variability, and response to environmental variables ................................................................. 11
  Aims of the dissertation ............................................................................... 14
  References ..................................................................................................... 18
CHAPTER 2 ......................................................................................................... 25
OPTIMIZATION OF NUCLEIC ACID EXTRACTION AND PCR METHODS FOR CORAL-ASSOCIATED MICROORGANISMS ............................................ 25
  Abstract ...................................................................................................... 26
  Introduction .................................................................................................. 27
  Materials and Methods .............................................................................. 29
    Coral sample collection and processing .................................................... 29
    Nucleic Acid Extraction ......................................................................... 30
    PCR amplification of bacterial SSU rRNA genes ...................................... 31
    T-RFLP analysis ...................................................................................... 33
    Bacterial SSU rDNA clone libraries ......................................................... 34
  Results .......................................................................................................... 36
    Nucleic acid extraction ............................................................................ 36
    PCR amplification of bacterial SSU rRNA genes ...................................... 36
    T-RFLP analysis ...................................................................................... 37
    Bacterial SSU rDNA clone libraries ......................................................... 39
  Discussion ...................................................................................................... 40
  Conclusion .................................................................................................... 44
Acknowledgements ........................................................................................................... 45
References .......................................................................................................................... 46
Figure Legends .................................................................................................................. 56
Figures .................................................................................................................................. 58
Appendix .................................................................................................................................. 63
CHAPTER 3 .......................................................................................................................... 66
Biodiversity and biogeography of microorganisms associated with seawater and reef-building coral across the Hawaiian Archipelago and wider Pacific Ocean ................................................................. 66
Abstract ............................................................................................................................. 67
Introduction .......................................................................................................................... 68
Materials and Methods ........................................................................................................ 71
  Sample collection .............................................................................................................. 71
  DNA extraction and T-RFLP of bacterial SSU rRNA ...................................................... 73
  Statistical analyses ......................................................................................................... 75
Results .................................................................................................................................... 77
  Planktonic and coral-associated bacterial community structure ................................... 77
  Correlations with temperature and depth ...................................................................... 81
  Correlation with geographic distance ........................................................................... 81
Discussion ............................................................................................................................. 82
  Differences between planktonic and coral-associated bacterial communities ............ 82
  Molecular biogeography of planktonic and coral-associated bacteria .......................... 83
  Relationship with Johnston Atoll .................................................................................... 86
  Additional need for environmental and temporal data ................................................... 88
  Summary and Broader Impacts ...................................................................................... 89
Acknowledgements ............................................................................................................. 90
References .............................................................................................................................. 90
Tables .................................................................................................................................... 96
Figure Legends .................................................................................................................... 98
Figures ................................................................................................................................... 101
Appendix ................................................................................................................................ 106
CHAPTER 4 .......................................................................................................................... 109
Dynamics of coral-associated bacterial communities on the Southern Reefs of Moloka‘i, Hawai‘i 109

xv
Abstract ........................................................................................................... 110
Introduction ..................................................................................................... 110
Methods .......................................................................................................... 112
  Site description .......................................................................................... 112
  Sample collection ...................................................................................... 113
  T-RFLP of bacterial SSU rRNA genes ..................................................... 114
  Statistical analysis .................................................................................... 116
Results ............................................................................................................ 119
  Coral vs. seawater-derived bacterial community structure ................. 119
  Planktonic bacterial communities .......................................................... 119
  Coral-associated bacterial communities ............................................. 120
  Correlations with environmental data .................................................. 123
Discussion ..................................................................................................... 124
Conclusion ..................................................................................................... 129
Acknowledgements ..................................................................................... 129
References .................................................................................................... 130
Figure Legend ............................................................................................... 134
CHAPTER 5 .................................................................................................... 141
THE EFFECT OF A SUB-LETHAL TEMPERATURE ELEVATION ON THE STRUCTURE
OF BACTERIAL COMMUNITIES ASSOCIATED WITH THE CORAL PORITES
COMPRESSA .................................................................................................... 142
Abstract ........................................................................................................ 143
Introduction ................................................................................................... 143
Materials and Methods .............................................................................. 146
  Sample collection and experimental design ....................................... 146
  Pulse amplitude modulated (PAM) fluorometry ............................... 148
  T-RFLP of bacterial SSU rRNA genes ............................................... 149
  Statistical analysis .................................................................................. 151
Results ........................................................................................................... 153
  Photophysiology of P. compressa ......................................................... 153
  Planktonic bacterial community analysis ....................................... 154
  Coral-associated bacterial community analysis .............................. 155
Discussion ..................................................................................................... 157
Conclusions .................................................................................................. 161
CHAPTER 1
GENERAL INTRODUCTION

Coral reefs are one of the most productive and diverse ecosystems on the planet. Although they occupy less than 0.1% of the world’s ocean surface, they provide a habitat for 25% of all marine species (Spalding and Grenfell 1997, Spalding et al. 2001, Mulhall 2007). Shallow-water corals are able to build extensive calcium-carbonate reef structures due to the unique symbiosis they maintain with intracellular dinoflagellates (Muscatine et al. 1981). Their dinoflagellate symbionts are of the genus *Symbiodinium*, comprising the largest and most ubiquitous group of endosymbiotic dinoflagellates known. These unicellular algae, colloquially known as “zooxanthellae,” inhabit the endoderm of cnidarians where they translocate photosynthate to the host organism and receive inorganic nutrients in return (e.g., CO2, NH4+). The influx of coral-derived nutrients is what enables zooxanthellae to flourish and subsequently, coral organisms to thrive and grow in warm, otherwise oligotrophic marine environments around the world.

Unfortunately, coral reefs are facing unprecedented threats on a global scale, leading to significant declines in coral health and widespread degradation of reef ecosystems. The most recent edition of the Current Status of Coral Reefs of the World reported that 19% of the existing area of coral reefs has been lost (Wilkinson 2008). A predicted 15% of coral reefs are seriously threatened with loss within the next 10-20 years and 20% are under a longer term threat of collapse in 20-40 years. Coral bleaching and ocean acidification due to global climate change, diseases, plagues, invasive species, sedimentation, eutrophication, chemical pollution, over-fishing and destructive fishing, and coastal development are listed among the top 10 threats and stressors to coral reefs.
Microorganisms and coral disease

The topic of coral disease has received increased attention due to the rise in the prevalence of novel pathogenic diseases resulting in world-wide degradation of coral reefs over the past three decades (Lafferty et al. 2004, Weil et al. 2006). There are currently 18 diseases known to affect more than 845 species of coral. Etiologies for only 6 of them have been determined through the fulfillment of Koch’s postulates (Sutherland et al. 2004, Bourne et al. 2009). Known diseases are associated with pathogens including bacteria, viruses, fungi, and protists and with abiotic stressors including elevated seawater temperature, eutrophication, pollution, and sedimentation (Sutherland et al. 2004).

At the global scale, coral bleaching is the most serious disease symptom currently threatening coral reefs (Rosenberg et al. 2007). Coral bleaching results when the association between the coral host organism and its endosymbiotic zooxanthellae is significantly disrupted. “Bleaching” refers to the whitening of coral tissue due to the loss of symbiotic algae and/or their associated photosynthetic pigments. Coral bleaching most commonly occurs when surface seawater temperatures are anomalously warm (~1 to 2°C above normal for a 4 to 6 week period) and irradiance levels are high (Jokiel and Coles 1990, Lesser 1996, Hoegh-Guldberg 1999, Fitt et al. 2001). However, stress-induced bleaching events can also result from extreme low temperatures (LaJeunesse et al. 2010), low salinity (Goreau 1964), and pollution (Brown 2000). Bleaching negatively affects the coral host by reducing calcification rates and increasing susceptibility to disease (Brandt & McManus 2009, Colombo-Pallotta et al. 2010). If coral hosts do not reacquire a healthy population of zooxanthellae, partial or total mortality of the colony will result (reviewed in Baker et al. 2008).
In two species of coral – *Oculina patagonica* in the Mediterranean Sea and *Pocillopora damicornis* in the Indian Ocean and Red Sea – bleaching has been attributed to bacterial infection (Kushmaro et al. 1996, Kushmaro et al. 1997, Ben-Haim and Rosenberg 2002, Ben-Haim et al. 2003). In *Oculina patagonica*, infection by *Vibrio shiloi* was found to be triggered by an increase in seawater temperature (Kushmaro et al. 1998). Increased temperatures were found to induce bacterial virulence factors, including a cell-surface adhesin that binds to a β-galactose-containing receptor on the coral surface (Toren et al. 1998), superoxide dismutase (Banin et al. 2001), and toxins that inhibit photosynthesis and bleach and lyse the symbiotic zooxanthellae (Banin et al. 2001, Ben-Haim et al. 1999, Rosenberg et al. 2007). Ben-Haim et al. (2003) identified *Vibrio coralliilyticus* as an etiological agent of bleaching in *Pocillopora damicornis*, again with increased bacterial virulence via synthesis of an extracellular proteinase at higher temperatures. These two examples of bacterial-induced coral bleaching initially led to the formation of the “bacterial bleaching hypothesis;” however, follow up studies have shown that *Vibrio shiloi*, or bacterial penetration of tissue in general, are no longer associated with the bleaching of *O. patagonica* and suggest that bacterial involvement in coral bleaching is instead opportunistic colonization (Ainsworth et al. 2008, Bourne et al. 2008).

Many other non-bleaching disease lesions observed on corals have also been attributed to bacterial pathogens; however, determining whether or not bacteria are the causative agents of these lesions has also proven to be quite difficult (Ben Haim and Rosenberg 2002, Cervino et al. 2004, Friaz-Lopez et al. 2004, Pantos and Bythell 2006). Black band disease (BBD), one of the oldest known and most widely studied coral
diseases, exemplifies the difficulty in determining the direct role of bacteria in disease causation (Bourne et al. 2009). BBD is characterized by a dark, dense microbial mat that moves across a coral colony (up to 2 cm/day), killing all living tissue in its path (Kuta and Richardson 2002). Using molecular techniques, a broad consortium of microorganisms have been identified in the mat, including cyanobacteria, sulfate-reducing Desulfovibrio spp., sulfide-oxidizing Beggiatoa spp., and a diversity of heterotrophic microbes (Cooney et al. 2002, Frias-Lopez et al. 2003, Barneah et al. 2007, Viehman et al. 2006, Sekar et al. 2008). The polymicrobial nature of the mat has made it especially difficult to identify a primary causative disease agent; however, recent studies indicate that the microbes might be working synergistically – with the combination of an anoxic microenvironment, sulfide, and cyanobacterial toxins contributing to coral tissue degradation (Carlton et al. 1995, Richardson et al. 2007). However, after nearly four decades of research, scientists have yet to determine the specific conditions which lead to the onset and cause of BBD disease.

Other non-bleaching, bacteria-associated disease lesions have proven equally difficult to elucidate. Bacteria that have been identified as pathogenic agents include newly identified species as well as known pathogens, including many Vibrio spp. White plague affects more than 40 coral species in the Caribbean and is characterized by a distinct line between healthy tissue and freshly exposed coral skeleton. There is no obvious microbial band present as found in BBD; however, white plague has been attributed to Aurantimonas coralicida, the first representative of a new genus (Denner et al. 2003, Weil et al. 2006). The reservoir for this bacterium has not been identified and Sunagawa et al. (2009) did not detect the putative pathogen in a study of healthy and
diseased *Montastraea faveolata*. Another white plague-like disease affecting corals in the Red Sea was confirmed to be caused by the novel species *Thalassomonas loyana* using phage therapy (Barash et al. 2005, Thompson et al. 2006). *Serratia marcescens* is a common human fecal enterobacterium, a known pathogen of humans and domestic animals, and has been shown to cause white pox in *Acropora palmata* in the Caribbean (Patterson et al. 2002). The rate of tissue loss for white pox averages 2.5 cm² per day and is greatest during periods of seasonally elevated temperatures. *Vibrio alginolyticus* and three additional *Vibrio* spp. have been identified as the causative agents of yellow blotch in *Montastraea* spp. in the Caribbean (Cervino et al. 2004). The temperature sensitivity of this disease is similar to that of coral bleaching and evidence indicates that yellow blotch disease primarily affects the symbiotic algae rather than the coral tissue. Finally, *Vibrio carchariae* is presumed to be the primary pathogen in *Acropora* spp. affected by white band; however Koch’s postulates have not been demonstrated for this disease (Ritchie and Smith 1995). Determining coral disease etiologies is problematic because 1) we have a very limited understanding of basic coral physiology and immunology; 2) comprehensive case definitions that systematically characterize diseases at the gross, microscopic, immunologic, and microbial level are lacking (Rosenberg et al. 2007); and 3) very little is known about the types of bacteria associated with corals during disease-free periods and the functional role that these bacteria may play in maintaining host health.
Microorganisms and coral health


Rohwer et al. (2001) first utilized culture-independent, nucleic acid-based techniques to study coral-associated bacteria, which has since led to an abundance of data on bacterial communities associated with diverse coral species (Bourne and Munn 2005, Koren and Rosenberg 2006, Pantos et al. 2003, Rohwer et al. 2002, Frias-Lopez et al. 2002, Cooney et al. 2002, Sekar et al. 2006, Casas et al. 2004). Through these initial studies, several general patterns have emerged: 1) corals harbor a high diversity of bacterial species, most of which are novel to science; 2) coral-associated bacteria are distinctly different from their surrounding seawater environment, indicating specific associations; 3) the species composition of uncultured vs. cultured coral-associated bacteria is significantly different; 4) similar bacteria are found on geographically separated conspecifics, while different coral species in the same location harbor dissimilar bacterial communities; and 5) bacteria found in the mucus are different than those found in the tissue (Rosenberg et al. 2007).
Scientists are now beginning to unravel the complex relationships between corals and their bacterial associates. The bacteria have been proposed to serve a variety of functions including, protecting corals from other harmful bacteria through antibiotic production (Ritchie 2006), providing corals with nutrients (e.g., through nitrogen fixation) (Lesser et al. 2004), and providing an additional food source (Rohwer et al. 2002). Several studies have indicated that beneficial symbioses exist between corals and specific types of bacteria (Rohwer et al. 2002, Lesser et al. 2004).

Bacterial-invertebrate symbioses are common and widespread, with many well-characterized examples found in marine ecosystems. In these associations, bacterial symbionts affect host development, nutrition, reproduction and speciation, defense against predators, and immunity (reviewed in Dale and Moran 2006). The bacterial associates form persistent infections with the host organism and are often beneficial or even obligatory for host survival.

At deep sea hydrothermal vents and cold seeps, bathymodiolin mussels, vesicomyid clams, and vestimentiferan tubeworms host intracellular chemoautotrophic and/or methanotrophic bacterial symbionts and are amongst the most prevalent megafaunal invertebrates at these ecosystems (Nelson and Fisher, 1995). In these associations, the invertebrate host functions as a spatial/temporal bridge to an oxic-anoxic interface where the substrates needed for chemosynthesis are available for use by the symbiotic bacteria (Cavanaugh, 1994). The bacteria use energy yielded from the oxidation of reduced compounds (e.g., hydrogen sulfide, thiosulfate, and/or methane) to fix organic carbon and provide the primary source of nutrition to the host organism (Cavanaugh, 1985; Nelson and Fisher, 1995). As adults, Riftia pachyptila tubeworms
lack functional digestive systems (mouth, gut, and anus) and depend entirely on their bacterial symbionts for nutrition (Hilário et al. 2011). In conjunction with physiological adaptations, these symbioses enable the host organisms to subsist on energy sources that are otherwise unavailable, and in some cases toxic, to eukaryotic cells. Although these symbiotic associations are best described for deep-sea hydrothermal vents and cold seeps, they are ubiquitous in shallow water reducing environments (Stewart and Cavanaugh 2006).

Bacterial-invertebrate symbioses are also prevalent in shallow-water reef ecosystems. The Hawaiian bobtailed squid, *Euprymna scolopes*, engages in a symbiosis with bioluminescent bacteria in order to protect itself from predators (McFall-Ngai and Ruby 1992). Upon hatching from the egg, the juvenile squid is aposymbiotic but soon acquires *Vibrio fischeri* symbionts from the surrounding seawater environment (Nyholm et al. 2000). The bacteria are housed in a specialized light organ on the ventral side of the squid and influence the development of this organ (Foster and McFall-Ngai 1998). As adults, the squid remain buried in the sand of shallow reef flats during the day. At night, they emerge and search for food. The bioluminescent bacteria act to camouflage the squid by counter-illuminating the underside of the animal. Specialized tissues, a shutter mechanism, lens, and filter are used to adjust the color and amount of light emitted from the organ (Tong et al. 2009). The counter-illumination offsets the downwelling moonlight striking and reflecting off the squid, making it practically invisible to predators from below.

Some of the best known and most studied examples of reef invertebrate-bacterial symbioses are found in sponges. Like corals, sponges are found in both shallow
deep water environments, are capable of building-reef structures, are sessile as adults, and have mobile larvae. Sponges also host a diversity of symbiotic microorganisms including archaea, heterotrophic bacteria, cyanobacteria, green algae, red algae, cryptophytes, dinoflagellates, and diatoms (Lee et al. 2001). All sponges are filter feeders, circulating water through a series of pores and canals and filtering out microorganisms and organic particles for consumption. Microorganisms that evade digestion and host immunity can successfully inhabit the sponge (Wilkinson 1987). These microbial symbionts associate with hosts intracellularly (inside host cells or nuclei) and/or extracellularly (attached to outer surface or present in mesohyl). The number of symbiotic microorganisms found in sponge hosts varies, with bacteria making up to 60% of total sponge biomass in some species and only a small percentage in others (Wilkinson 1978a, 1978b, 1978c). The sponge symbiotic bacterial community is highly novel, exhibits temporal and geographic variation, and is species specific (Webster and Hill 2001). Symbiotic bacteria are thought to serve a number of potential beneficial functions for the host, including providing nutrients through nitrogen fixation (Wilkinson 1979); however, the roles of the majority of associated bacteria are largely unknown (Mohamed et al. 2008).

Several studies have indicated that bacteria and corals may also engage in a mutualistic symbiosis. Rohwer et al. (2002) found that the microbial community associated with *Porites astreoides* always contained a specific γ-proteobacterium ribotype (AF365457, PA1) and that *Porites furcata* was always associated with another closely related γ-proteobacterium ribotype (PF1). Other groups of bacteria were also observed to preferentially associate with certain coral species. For example, a group of
closely related α-proteobacteria (Silicibacter spp.) associated with Montastraea franksi and Cytophaga-Flavobacter/Flexibacter-Bacteriodes were more commonly associated with Diploria strigosa over other corals. The specificity of these associations were maintained even when coral colonies of different species were located adjacent to one another; however the specific function of associated microbes is not known.

In addition to specific associations, bacterial activities that are of potential benefit to the coral host have also been identified. Lesser et al. (2004) found unicellular, nonheterocystis cyanobacteria within host cells of Montastraea cavernosa corals. The cyanobacteria coexist with symbiotic zooxanthellae and were found to express the nitrogen-fixing enzyme nitrogenase. The authors proposed that the cyanobacteria contribute this limiting element to the symbiotic association. Ritchie (2006) found that 20% of bacteria cultured from the elkhorn coral Acropora palmata displayed antibiotic activity against one or more tester strains, including the pathogen implicated in white pox disease. A novel coral mucus-mediated selection method was used to culture and identify bacteria that benefitted from nutrients available on the coral surface while providing a benefit to the coral. This mucus-based selection method enriched for a range of bacteria that produced antibiotics. The authors suggest that coral mucus plays a role in the structuring of beneficial coral-associated microbial communities and that associated microbes contribute to the antibacterial activity described for coral mucus.

As more cases of bacterial symbiosis are characterized, the distinction between pathogenesis and mutualism has become increasingly difficult to discern. Infection by a particular bacterium may be beneficial to a host under certain circumstances, but harmful in other parts of the organism, other hosts, or under other environmental conditions (Dale
and Moran 2006). Furthermore, pathogenic and mutualistic bacteria often use the same core molecular mechanisms to maintain their associations with hosts. As we continue to study these associations, we continue to grow our knowledge of the role that bacteria play in host evolution and shaping ecosystems at the smallest scales.

Outstanding questions: Host specificity, spatial and temporal variability, and response to environmental variables

An increasing number of studies regarding coral-bacterial associations have shed light on a range of pertinent topics; however, many fundamental questions surrounding the basic nature of these associations still remain unanswered. For example, we know relatively little about host specificity of coral-associated bacterial communities, their spatial and temporal variability, or how they respond to environmental changes.

Rohwer et al. (2002) found that different coral species, even when physically adjacent, harbored distinct bacterial communities, while corals of the same species, separated by time (~1 year) and space (3,000 km), harbored the same types of bacteria. However, Littman et al. (2009) found that bacterial communities associated with three species of Acropora (Acropora millepora, Acropora tenuis, and Acropora valida) differed by geographic location (Magnetic and Orpheus Islands in Australia), but not between species within each location. The researchers also investigated the temporal stability of bacteria associated with A. millepora over the course of a year at Orpheus Island and found that the dominant members of the bacterial community were relatively stable throughout the seasons despite temperature fluctuations of > 10 °C. On a much smaller scale, Rohwer et al. (2002) observed that bacterial ribotypes were spatially structured within colonies of
the branching coral *Porites furcata* – with tips of branches containing a ribotype that was absent from middle sections. Continuing to investigate patterns of coral-associated bacterial communities on a range of spatial and temporal scales is essential to understanding the influences that microbes have on coral health at both the individual and population levels.

Our understanding of how coral-associated bacterial communities respond to environmental change is even more limited. Although documentation of species-specific associations between corals and bacteria may be indicative a beneficial symbiotic relationship between the two types of organisms (Rohwer et al. 2002), other studies have shown that bacterial communities may shift in response to certain environmental conditions, causing harm to the host coral in some cases. Anthropogenic inputs, including organic carbon and nutrient loading, are increasingly changing the water quality on reefs and have been implicated in the long-term global decline in coral health. However, few studies have been able to directly link poor water quality with coral mortality. Using controlled aquarium experiments, Kline et al. (2006) found that routinely measured parameters of water quality (e.g., nitrate, phosphate, ammonia) did not cause substantial coral mortality in the Caribbean coral *Montastraea annularis*. However, elevated levels of dissolved organic carbon (DOC) increased coral mucus-associated bacterial growth rates by an order of magnitude, resulting in significantly increased coral mortality compared to controls. The researchers propose that the mucus layer bacteria are carbon-limited and that the increase in DOC disrupts the balance between corals and associated microbes by enabling the bacteria to break down more complex and previously unavailable carbon sources. In turn, the elevated microbial growth causes coral death via
oxygen depletion, accumulation of poisons such as hydrogen sulfide and secondary metabolites, and/or microbial predation of the coral host. Kuntz et al. (2005) found similar results in three species of Caribbean corals (*Montastraea annularis, Agaricia tenuifolia, and Porites furcata*) with the probability of coral mortality increasing significantly over time with increased carbon load. However, it is important to note that these experiments were conducted an aquarium setting, which may not be representative of the natural environment. Additionally, a study by Klaus et al. (2005), revealed significant shifts in bacterial community structure along a gradient of human-impacted reef sites in the Caribbean for one of two coral species examined (the most abundant bacterial taxa associated with coral *Diploria strigosa* displayed increased dominance at impacted localities). However, these changes were not documented over time or in response to a specific human-induced environmental disturbance. Ritchie (2006) found that bacteria isolated from healthy *Acropora palmata* tissue during a summer bleaching event did not contain antibiotic-producing bacteria and were dominated by *Vibrio* spp., including species implicated in temperature-dependent coral bleaching. The author suggests that an environmental shift from beneficial bacteria to an overgrowth of opportunistic microbes occurred in response to increased temperatures. Environmental factors such as light, nutrients, and temperature have been correlated with prevalence and progression of BBD disease; however, their exact mechanisms are difficult to elucidate (Boyett et al. 2007, Voss and Richardson 2006). The challenges faced with the study of black band disease are consistent across all studies of corals and their lesions (Bourne et al. 2009).
**Aims of the dissertation**

The aforementioned observations and research studies indicate that bacterial associates play important roles in maintaining and/or destabilizing coral health. If coral-associated bacterial communities are indicative of a coral’s current health state or susceptibility to disease, monitoring these communities could potentially be used to detect physiological stress in a coral before the onset of visible disease occurs. Additionally, this information could be used to predict disease susceptibility in diverse coral species under different environmental conditions and to identify potential pathogens. At this stage in the burgeoning subfield of coral microbial ecology, scientists are challenged with more questions than answers. This dissertation aims to address some of the most fundamental questions in order to help advance the state of research of coral-bacterial associations. For my doctoral research, I used high-throughput, culture-independent molecular methods to investigate the diversity and abundance of bacteria associated with healthy and health-compromised (e.g., temperature-stressed) corals across a gradient of anthropogenic disturbance within the Hawaiian archipelago (main Hawaiian Islands and Northwestern Hawaiian Islands) and Indo-Pacific (Johnston Atoll and American Samoa). Questions regarding coral-associated bacterial host specificity, spatial and temporal variability, and response to environmental change were addressed in four independent research projects – summarized in Chapters 2, 3, 4, and 5 of the dissertation. The underlying goals of this dissertation were to increase our basic knowledge of coral-associated bacterial communities and to investigate their potential roles in influencing coral health.
Throughout this investigation, the following hypotheses were tested:

$H_01$: Assessments of coral-associated bacterial (CAB) community structure (species diversity and relative abundance) do not differ based on the combination of treatment, microbial nucleic acid extraction method, and polymerase chain reaction (PCR)-based technique used (Chapter 2).

$H_02$: CAB community structure does not differ between different colonies of the same coral species (Chapters 2, 3, 4, 5).

$H_03$: CAB community structure does not differ between different coral species (Chapter 2).

$H_04$: CAB community structure does not differ based on distance/geographic location (Chapters 3 and 4).

$H_05$: CAB community structure does not fluctuate over time (Chapter 4).

$H_06$: CAB community structure does not fluctuate in response to changes in environmental variables (e.g. ambient seawater temperature, salinity, turbidity, and nutrients) (Chapters 4 and 5).
The overall objectives of each dissertation chapter are briefly summarized below:

Chapter 2

Since Rohwer et al. (2001) first applied modern molecular techniques to investigating coral-associated bacterial communities, numerous researchers have followed suit. However, subsequent studies have varied widely in their choice of research methods, making it difficult to adequately compare observational and experimental data. Chapter 2, in part, addresses this issue by determining whether or not different nucleic acid extraction methods and polymerase chain reaction (PCR)-based techniques result in significantly different patterns of bacterial community structure for an individual sample. Additional limitations to interpreting data from previous studies include the problem of small sample sizes and difficulty working with certain coral species. This study explores a high throughput method for investigating bacterial communities associated with diverse coral species.

Chapter 3

Despite a significant increase in coral-microbial studies, we have very limited knowledge of how coral-associated bacterial communities vary over a range of spatial scales or of the underlying factors affecting their geographic distributions. Identifying distribution patterns in these communities is fundamental to understanding the potential roles that bacteria play in coral health and disease. Chapter 3 addresses these questions by exploring the biodiversity and biogeography of bacteria associated with *Porites lobata* corals and, for comparison, seawater across the Hawaiian Archipelago, Johnston Atoll, and American Samoa. Chapter 3 also examines the relationship between biogeographic
patterns of *Porites lobata* corals and their bacterial associates to highlight the impact that host dispersal may have on bacterial community structure. Potential influences of environmental factors are also discussed.

**Chapter 4**

As researchers continue to study coral-associated bacterial communities, the need to assess the natural variability of said communities over time remains. Establishing a baseline of microbes associated with non-diseased corals is critical to identifying the bacteria that may contribute to or destabilize the health of the coral host. Of equal importance, is identifying the environmental factors that may influence the composition of associated bacteria, potentially altering the health state of the host coral. These questions are addressed in Chapter 4 by examining the temporal and spatial variability of bacterial communities associated with colonies of the coral *Porites compressa* over a five month period at two sites along the south shore of Moloka‘i, Hawai‘i. Monthly measurements of seawater salinity, temperature, turbidity, fluorescence, chlorophyll a concentration, and nutrients (total nitrogen, phosphorous, and silicate) were collected at each site and used to explore relationships between environmental parameters and bacterial assemblages found in seawater and on corals.

**Chapter 5**

Evidence points to a link between environmental stressors, coral-associated bacteria, and coral disease; however, few studies have examined the details of this relationship under tightly controlled experimental conditions. Chapter 5 addresses this gap using an array of closed-system, precision-controlled experimental aquaria to
investigate the effects of an abrupt temperature increase on the bacterial community structure and photophysiology of *Porites compressa* corals. The experimental design employed in this study was tested for use as a robust, reproducible system for investigating coral microbiology in an aquarium setting.

References


CHAPTER 2

OPTIMIZATION OF NUCLEIC ACID EXTRACTION AND PCR METHODS FOR CORAL-ASSOCIATED MICROORGANISMS

Jennifer L. Salerno¹,², Elizabeth A. Hambleton¹†, and Michael S. Rappé¹*

¹Hawaii Institute of Marine Biology, SOEST, University of Hawaii at Manoa, Kaneohe, HI, 96744
²Department of Zoology, University of Hawaii at Manoa, Honolulu, HI, 96822

*Corresponding author. Mailing address: Hawaii Institute of Marine Biology, SOEST, University of Hawaii at Manoa, P.O. Box 1346, Kaneohe, HI, 96744. Phone: (808) 236-7465, Fax: (808) 236-7443. E-mail: rapp@hawaii.edu

†Present address: Department of Cellular and Molecular Biology, Stanford University, Stanford, CA, 94305

One Sentence Summary:
Running Title: Molecular methods for coral-associated microorganisms
KEY WORDS: corals, disease, molecular methods, microorganisms
Abstract

A variety of nucleic acid extraction methods and polymerase chain reaction (PCR)-based techniques were used to characterize bacterial communities associated with common Hawaiian corals. The objectives of this study were 1) to optimize protocols for genomic DNA extraction and PCR-amplification for high-throughput analyses of coral-associated bacterial communities and 2) to determine if different nucleic acid extraction techniques resulted in significantly different patterns of bacterial community structure. Total genomic DNA, extracted using two processing treatments paired with five DNA extraction methods, varied significantly amongst *Montipora capitata*, *Porites compressa* and *Porites lobata* corals. Amplification of the 16S rRNA gene varied with treatment/extraction combination and appeared to be species-specific. Non-metric multidimensional scaling ordinations of terminal-restriction fragment length polymorphism (T-RFLP) data revealed a significant species-specific difference in bacterial community structure between *Porites compressa* and *Porites lobata* regardless of treatment, colony, or extraction method (MRPP, $A = 0.37$, $p = 0.00$). Overall, each colony/treatment/extraction combination for *Porites compressa* and *Porites lobata* yielded a statistically different T-RFLP profile (MRPP, $p = 0.00$, 0.00) and replicates within a combination were not significantly different (MRPP, $A = 0.76$, 0.86). Inter-colony differences contributed to most of the variation observed in *Porites compressa* T-RFLP profiles, whereas extraction method had a greater affect on *Porites lobata* T-RFLP profiles.
Introduction

In addition to providing a critical habitat for numerous reef-dwelling marine vertebrates and invertebrates, corals support unique and diverse assemblages of microorganisms (Knowlton and Rohwer 2003, Rosenberg et al. 2007). Eukaryotic algae, Bacteria, Archaea, Fungi, and viruses have all been identified as inhabitants of corals during non-diseased states. These microorganisms live in the surface mucus layer, gastro vascular cavity, tissue, and/or skeleton of corals and may benefit them through a variety of functions (Ducklow and Mitchell 1979, Wafer et al. 1990, Rohwer et al. 2001, 2002, Kellogg 2004, Lesser et al. 2004, Wegley et al. 2004). These functions include, but are not limited to, protection from harmful pathogens through antibiotic production, nutrient enrichment (e.g., through nitrogen fixation), and providing a source of nutrition (e.g., through photosynthesis and other nutritional pathways) (Lesser et al. 2004, Lesser et al. 2007, Muscatine and Porter 1977, Ritchie 2006, Rohwer et al. 2002, Shashar et al. 1994). Certain groups of microorganisms, most notably Bacteria, have also been identified as the causative agents of numerous coral diseases and have been linked with bleaching in some coral species (Ben-Haim et al. 1999, Ben Haim and Rosenberg 2002, Cervino et al. 2004, Friaz-Lopez et al. 2004, Pantos and Bythell 2006). Identifying the specific types of Bacteria associated with corals is essential in order to determine the potential roles that they play in maintaining and/or destabilizing coral health.

The relatively recent application of culture-independent, molecular-based methods to study microorganisms in the environment has enabled researchers to investigate the diverse communities of Bacteria associated with various organisms, including numerous coral species (Rohwer et al. 2001, 2002, Knowlton and Rohwer
2003, Lesser et al. 2004, Klaus et al. 2005, Penn et al. 2006). By cloning and sequencing bacterial-specific genes directly from the environment, scientists are currently able to detect a level of bacterial diversity that was significantly underestimated using traditional plate culturing techniques (Olsen et al. 1986, Pace et al. 1986). The availability of nucleic acid extraction kits, along with advances in sequencing technology, have allowed researchers to conduct high-throughput analyses of environmental samples. Furthermore, community fingerprinting techniques, such as terminal-restriction fragment length polymorphism (T-RFLP) analysis, enable scientists to easily compare the structure of complex bacterial communities across environmental gradients. Although, these technologies have resulted in significant progress in the field of microbial ecology, they are accompanied by their respective caveats. Rohwer et al. (2001) found that using different nucleic acid extraction techniques resulted in differing polymerase chain reaction (PCR) amplification efficiencies and subsequently, differing numbers of bacterial species detected in environmental samples through cloning and sequencing. Additionally, Ramette (2007) showed that using different restriction enzymes on an individual sample generated different bacterial community patterns within the sample. As the incorporation of molecular techniques in coral-microbe research increases, so does the need to obtain data that can be compared across diverse coral species and geographic boundaries. This is of considerable importance for techniques that have the potential to be used in monitoring coral health on a global scale.

We set out to develop a minimally-invasive technique to survey the diversity of Bacteria associated with different species of Hawaiian scleractinian corals. Studies by Bourne and Munn (2005) found that the bacterial communities from coral mucus
collected with a syringe were more similar to the surrounding seawater than crushed coral samples. Also, when sampling for mucus only, bacteria that may reside in the gastrovascular cavity, coral tissue, and/or skeleton (Ducklow and Mitchell 1979, Wafar et al. 1990, Rohwer et al. 2001, 2002, Knowlton and Rohwer 2003, Kellogg 2004, Lesser et al. 2004, Wegley et al. 2004) are excluded. Because these bacteria may play important functional roles, we chose a sampling method that included these communities.

Here we use a variety of nucleic acid extraction methods and polymerase chain reaction (PCR)-based techniques to document bacterial communities associated with non-diseased corals from Kaneohe Bay located off the island of Oahu, Hawaii. The objectives of this study are 1) to optimize protocols for bacterial genomic DNA extraction and PCR-amplification for high-throughput analyses of coral-associated bacterial communities and 2) to determine if different nucleic acid extraction techniques result in significantly different patterns of bacterial community structure.

Materials and Methods

Coral sample collection and processing

Non-diseased colonies (per visual assessment) of *Porites lobata*, *Porites compressa*, and *Montipora capitata* were sampled from Kaneohe Bay, Hawaii in January of 2006. A hammer and chisel were used to collect 3 coral fragments, including the overlying mucus layer, tissue, and underlying skeleton, from each colony. Samples were placed in sterile whirl-pak bags and frozen at -80°C until processed. Coral fragments were thawed on ice and a core borer was used to obtain 20 cores (6 mm diameter, 6 mm
height) from each fragment. The resultant core samples were divided in half and subjected to either a crushing or airbrushing treatment.

Half \((n = 10)\) of the coral core samples were crushed using a sterile mortar and pestle (placed in furnace at 450°C for 1 hour). The crushed material was aliquoted into sterile cryovials (300 milligrams of crushate per tube). Vials were kept on ice throughout processing, and then frozen at -80°C. The remaining half of the coral core samples \((n = 10)\) were placed in a sterile whirl-pak bag and airbrushed with 50 ml of filter-sterilized 10X TE buffer (100 mM Tris-HCl, 10 mM EDTA). Care was taken to ensure all tissue was removed from the core samples. The coral tissue/TE buffer slurry was removed from the bag with a sterile pipette and deposited into a conical tube on ice. The slurry was homogenized by vortexing, and then 1.5 ml was added to sterile cryovials on ice. Cryovials were centrifuged at 4°C for 30 minutes at 19,900 X g in a Beckman Coulter Allegra™ 25R centrifuge with a type S5700 rotor. Supernatant was decanted and pipetted, and the remaining tissue pellets were frozen at -80°C until genomic DNA extraction.

**Nucleic Acid Extraction**

Prior to genomic DNA extraction, crushed coral material and airbrushed coral tissue pellets were removed from the -80°C freezer and thawed on ice. For each of the three coral species and two sample processing treatments, five DNA extraction methods were employed. The DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) involved chemical lysis of bacteria contained in coral tissue, as well as selective binding and elution of DNA using a silica-gel column. The DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) also
utilized chemical lysis, as well as mechanical lysis with a QIAshredder© column. A combination of mechanical and chemical lysis was used in the UltraClean Soil DNA Kit and the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA), which both began with bead beating of tissue using a vortex. Both kits contained Inhibitor Removal Solution© to eliminate humic acids and other PCR inhibitors; the PowerSoil DNA Isolation Kit contained extra steps to further eliminate inhibitors. Finally, the CTAB extraction method, which was not a pre-packaged kit, was used for extraction comparisons. The CTAB method involved chemical lysis with a CTAB buffer (2% Cetyl Trimethyl Ammonium Bromide, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% 2-mercaptoethanol, 1% PVP), and multiple phenol/chloroform steps (Dempster et al. 1999). In all methods, DNA was eluted in sterile water and stored frozen at -20°C. Final DNA yield was assessed using two methods: 1) gel electrophoresis (DNA was mixed with loading dye and separated on a 1% agarose/TAE gel run for 30 minutes at 100 volts) and 2) a fluorescent dye assay (Quant-iT PicoGreen dsDNA Assay Kit prepared according to manufacturer’s protocol (Invitrogen)).

**PCR amplification of bacterial SSU rRNA genes**

Bacterial SSU rRNA genes (16S rDNA) from each combination of coral host species, sample processing treatment, and DNA extraction method were amplified via Polymerase Chain Reaction (PCR). The 16S rRNA gene was targeted for PCR amplification using bacterial-specific and universal primers. During initial PCR tests, BIO-X-ACT Short DNA polymerase (Bioline), iProof High-Fidelity DNA polymerase (Bio-Rad Laboratories), the PicoMaxx High Fidelity PCR System (Stratagene), and the
MasterTaq System (5 PRIME) were compared. The MasterTaq System (Eppendorf AG, Hamburg, Germany) was shown to be most effective at amplifying the 16S rRNA gene in our samples and was the primary system used for subsequent PCR reactions. PCR reactions were composed of the following: 5% (vol/vol) genomic DNA template (ranging from 0.1 to 128 µg), 0.2 mM final concentration of each dNTP (Promega, Madison, WI), 10X Taq Buffer with 15mM Mg²⁺ and 25 mM Magnesium Solution to yield a final magnesium concentration of 2.25 mM, 0.5X final concentration of TaqMaster reaction enhancer, 0.2 mM each of forward and reverse primers, 0.5 units of Taq DNA Polymerase, and sterile water for final volumes of either 20 or 50 µl. For PCR reactions testing template amplification ability, 20 µl reaction volumes with oligonucleotide primers 27F-B (5’-AGRGTTYGATYMTGGCTCAG-3’) and 1492R (5’-GGYTACCTTGTTACGACTT-3’) were used. Initial PCR tests revealed that this primer set was superior to 27F-B (5’-AGRGTTYGATYMTGGCTCAG-3’) paired with 519R (5’-GWATTACCGCGGCKCTG-3’) in successfully amplifying bacterial DNA (data not shown). Subsequent PCR reactions to be used for terminal-restriction fragment length polymorphism (T-RFLP) analysis were carried out in volumes of 50 µl with the labeled forward primer 27F-B-6FAM (5’-[6-FAM]AGRGTTYGATYMTGGCTCAG-3’) and 1492R (5’-GGYTACCTTGTTACGACTT-3’). A ‘touchdown’ protocol was programmed into a BIO RAD MyCycler™ thermal cycler (30 cycles of: 3 min at 95°C, 1 min 65°C – 0.5°C per cycle, 2 min at 72°C; 10 cycles of: 30 sec at 95°C, 1 min at 50°C, 2 min at 72°C; 1 cycle of: 30 sec at 95°C, 1 min at 50°C, 20 min at 72°C; 4°C ∞).
**T-RFLP analysis**

All samples that resulted in positive PCR amplification were processed for further analysis. Unincorporated primers and reagents were removed from PCR products using the QIAquick PCR Purification Kit (Qiagen), concentrated, and then subjected to a 6-hour restriction digest using *HAE III* enzyme. Digested PCR products were cleaned using the QIAquick Nucleotide Removal Kit (Qiagen) and then analyzed on an ABI 3100 DNA sequencer at the Evolutionary Genetics Core Sequencing Facility at the Hawaii Institute of Marine Biology.

Nonmetric multidimensional scaling (NMDS), based on Bray-Curtis distance, was used to provide a graphical representation of differences in T-RFLP peak patterns (representing bacterial community structure) among samples. This method was chosen because it makes no assumptions about the underlying distribution of data and is therefore, appropriate for analyzing complex ecological communities. NMDS is a nonparametric ordination method that conducts an iterative search for the position of *n* entities on *k* dimensions that minimizes the stress of the *k*-dimensional configuration (McCune and Grace, 2002). Original T-RFLP data were compiled into a matrix, relativized by total area, and standardized using the variable percentage threshold method described in Osborne et al. (2006). The “slow-and-thorough” autopilot mode of NMDS in PC-ORD (Kruskal 1964, Mather 1976) was used for ordinations. For each ordination, 40 runs of real data, using random starting configurations, were compared to 50 runs of randomized data. The best ordination for the data set was selected by comparing the final stress values for each dimensionality and performing a Monte Carlo test of significance. Final stability was evaluated for each run by examining plots of stress (a measure of the
dissimilarity between ordinations in the original $p$-dimensional space and in the reduced dimensional space) versus the number of iterations.

The Bray-Curtis dissimilarity matrix was subjected to the Multi-response Permutation Procedure (MRPP) in PC-ORD to test the significance of T-RFLP peak differences (i.e. differences in bacterial community structure) due to host species, treatment, and/or extraction method (Mielke and Berry 2001). This procedure provides a non-parametric multivariate test of differences between two or more groups based on the analysis of the ranked distance matrix. If samples within a group are identical, $A = 1$. If heterogeneity within groups equals expectation by chance, $A = 0$, and if there is less agreement than by chance, $A < 0$. In community ecology, values for $A$ are commonly below 0.1 and a value above 0.3 is considered to be fairly high (McCune and Grace 2002). The test statistic describes the separation between groups and is determined by comparing the observed delta (the average within-group distance) to an expected delta to obtain a probability value, $p$. The probability value indicates the likelihood of obtaining a delta as extreme or more extreme than the observed delta.

**Bacterial SSU rDNA clone libraries**

SSU rRNA genes were cloned and sequenced from airbrushed/PowerSoil-extracted samples from each of the three *Porites compressa* colonies and from one airbrushed/PowerSoil-extracted sample and one crushed/PowerSoil-extracted sample from a single *Porites lobata* colony. Clone library sample selections were based on statistical analyses of T-RFLP data that identified variables contributing significantly to differences in bacterial communities between samples.
Bacterial SSU rRNA genes (SSU rDNA) were PCR-amplified for each sample using the same protocol that was employed to generate T-RFLP community profiles, with the exception of using unlabeled primers. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and resultant DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Amplified products were cloned using the pGem-T Easy system (Promega), and sequenced on an ABI 3730XL capillary-based DNA sequencer (Applied Biosystems). Sequences were screened for quality and vector sequences were removed using Sequencher 4.7 software (Gene Codes Corporation). Chimeric sequences were identified using the Chimera Detection program (Ribosomal Database Project II, Michigan State University). Clone sequences were aligned using the SINA Webaligner (Pruesse et al. 2007) and identified to the family level using the Visualization and Analysis of Microbial Populations Structures (VAMPS) online database (http://vamps.mbl.edu).

A Bray-Curtis dissimilarity matrix (Bray and Curtis 1957) was used to compare clone libraries between samples and was constructed using the VAMPS online database. Bray-Curtis dissimilarity is bound between 0 and 1, with 0 indicating that samples have the same composition, and 1 indicating that the two samples do not share any common constituents.
Results

Nucleic acid extraction

A fully nested analysis of variance (alpha level = 0.05) revealed that the total amount of genomic DNA extracted from each coral species differed significantly ($F = 13.10, p = 0.006$) (Figure 2.1; Appendix I: Figures 1-3), with *Porites compressa* yielding the highest amount and *Montipora capitata* yielding the lowest. Within a species, extraction method and treatment contributed significantly to variability in genomic DNA extracted ($F = 2.11, p = 0.009$; $F = 8.06, p = 0.000$; respectively), whereas inter-colony differences did not ($F = 0.76, p = 0.600$).

Overall, the highest yields of genomic DNA extracted from each coral species using different treatment/extraction combinations were; airbrushed/Plant Kit for *Porites compressa* (12,797.1 ± 1,209.1 µg ± s.d.), airbrushed and crushed/Plant Kit for *Porites lobata* (4,883.7 ± 3,299.3 and 4,167.4 ± 4,628.3 µg ± s.d., respectively), and airbrushed/Tissue Kit for *Montipora capitata* (886.5 ± 317.6 µg ± s.d.). Airbrushing and crushing treatments paired with the Soil Kit extraction method resulted in the least amount of total genomic DNA extracted for all three species. We did not observe any significant trends in total genomic DNA extracted with airbrushing and crushing treatments across all extraction methods within or between coral species.

PCR amplification of bacterial SSU rRNA genes

Initial polymerase-chain-reaction (PCR) assays were carried out to determine if bacterial DNA could be amplified using the original concentrations of extracted genomic DNA. In certain cases, dilutions or concentrations of total genomic DNA were needed to
obtain PCR product; and in other instances, adjusting the gDNA template concentrations did not result in successful amplification of the desired PCR product (Table 2.1; Appendix 1: Figures 1-3). We were able to amplify 16S rDNA from all *Porites compressa* and *Porites lobata* extractions, with the exception of the crushed, Plant Kit-extracted samples. The majority of treatment/extraction combinations resulted in 100% amplification for all samples; however, some combinations resulted in patchy, inconsistent amplification (e.g., *Porites compressa* crushed/Tissue Kit). Crushed/Tissue Kit and airbrushed/Soil Kit extractions resulted in 100% amplification for *Montipora capitata*; however, we were unable to amplify 16S rDNA from *Montipora capitata* Plant Kit- and CTAB-extracted samples, regardless of treatment. The remaining treatment/extraction combinations resulted in patchy, inconsistent amplification for this coral species.

*T-RFLP analysis*

Each T-RFLP profile was examined individually and only those considered to be of high quality were included in downstream analyses. Profiles were classified as high quality if peaks were well-defined (e.g., symmetrical) and at least one peak was greater than or equal to 1,000 relative fluorescent units (RFUs). On average, a final concentration of 20 ng/µl of cleaned and restricted PCR product (40 ng total), was needed to obtain a useable T-RFLP profile. In some cases, this target value could not be reached due to a low starting concentration of PCR product and subsequent loss of product during clean-up steps. Overall, the airbrushed/Plant Kit and airbrushed and crushed/PowerSoil Kit extractions were the most consistent in yielding high quality T-RFLP profiles for both
Porites compressa and Porites lobata (Table 2.2). A considerable amount of Porites lobata airbrushed and crushed/CTAB extractions also resulted in high quality T-RFLPs; however the remaining treatment/extraction combinations for all three species resulted in inconsistent or very poor quality T-RFLP profiles. We were not able to obtain any high quality profiles for Montipora capitata extractions.

We observed a significant species-specific difference in bacterial community structure between Porites compressa and Porites lobata regardless of treatment, colony, or extraction method (MRPP, A = 0.37, p = 0.00) (Figure 2.2). Using non-metric multi-dimensional scaling ordination, a three-dimensional solution with a final stress value of 12.63 was selected for this analysis. When data from the two species were analyzed separately, a two-dimensional solution with a final stress value of 7.25 was selected for Porites compressa (Figure 2.3) and a three-dimensional solution with a final stress value of 13.3 was selected for Porites lobata (Figure 2.4). Overall, each colony/treatment/extraction combination for Porites compressa yielded a statistically different T-RFLP profile and replicates within a combination were not significantly different (MRPP, p = 0.00, A = 0.76). For this particular test, only samples with an n >1 were included in the analysis. When all extractions for each colony were grouped together, but separated out based on treatment, there was a significant difference between airbrushed and crushed treatments, but no significant within group difference (MRPP, p = 0.00, A = 0.70). When all treatment/extraction combinations were grouped together, but separated out based on colony, there was a significant difference amongst colonies, but within a colony, profiles were still fairly similar (MRPP, p = 0.00, A = 0.39). When groups were based solely on treatment or extraction, there were significant differences
amongst T-RFLP profiles, but also marked within group variability (MRPP, \( p = 0.00, A = 0.14 \) and \( p = 0.02, A = 0.07 \), respectively). Because \( n=1 \) samples were excluded from the extraction-based analysis, only Plant Kit and PowerSoil-extractions were compared for that particular analysis.

As observed in *Porites compressa*, each colony/treatment/extraction combination for *Porites lobata* yielded a statistically different T-RFLP profile, and replicates within a combination were not significantly different (MRPP, \( p = 0.00, A = 0.86 \)). Again, only samples with an \( n >1 \), were included in the analysis. When samples were partitioned out based on extraction method only, there was a significant difference between extraction methods and a relatively high within group similarity (MRPP, \( p = 0.00, A = 0.37 \)).

Similarly, when all extractions for each colony were grouped together, but separated out based on treatment, there was a significant difference between airbrushed and crushed treatments, and T-RFLP profiles within a colony were fairly similar \( (MRPP, p = 0.00, A = 0.36) \). When all treatment/extraction combinations were grouped together, but separated out based on colony, there was a significant difference amongst colonies, and also significant within colony differences \( (MRPP, p = 0.00, A = 0.19) \). Similar results were obtained when groups were based solely on treatment \( (MRPP, p = 0.00, A = 0.13) \).

*Bacterial SSU rDNA clone libraries*

All processing and extraction combinations examined resulted in successful cloning and sequencing of bacterial 16S rRNA genes from select coral samples (Tables 2.3 and 2.4). Dominant bacterial members of airbrushed, PowerSoil-extracted *Porites compressa* clone libraries varied between colonies. Bacteria of the Gammaproteobacteria
class dominated all three colonies; however, bacteria of the Pseudomonadales order were most abundant in colony 1, while Oceanospirillales were most abundant in colonies 2 and 3. Bray-Curtis coefficients calculated between samples revealed that, in agreement with statistical analysis of T-RFLP data, clone libraries from colonies 2 and 3 were more similar to one another than to colony 1 (Table 2.5A).

Dominant bacterial members also varied in *Porites lobata* clones libraries depending on treatment. The top two most abundant members of the airbrushed, PowerSoil-extracted clone library were from unidentified Bacteria and the Pseudomonadales order, whereas the crushed, PowerSoil-extracted clone library was dominated by the Planctomycetales order and unidentified Bacteria. Bray-Curtis coefficients calculated between samples revealed that, in congruence with statistical analysis of T-RFLP data, clone libraries from the airbrushed and crushed samples exhibited a relatively high degree of dissimilarity (56.1%) (Table 2.5B).

**Discussion**

Characterizing the vast diversity of microbes associated with reef-building corals has become increasingly obtainable with the application of molecular tools to ecological studies. Current research has yielded a wealth of information regarding the specific types of Bacteria associated with coral species from various geographic regions; however, assimilating this knowledge on a global scale is difficult due to the disparity of methodological approaches currently being used. Determining the ultimate effects of upstream processes on subsequent genetic analyses of microbial communities will enable researchers to minimize variability between future assessments of these populations.
This is essential to understanding the role of microbes in coral health and to addressing the global threat of microbial pathogens to coral reefs.

Overall, we observed significant differences in the quality and quantity of total genomic DNA extracted within the three coral species examined depending on the suite of treatment/extraction combinations used. Observed differences may be attributed to interactions between the initial physical and chemical processes of each extraction method with the biochemical makeup, tissue density, skeletal structure, abundance of associated microbes (e.g., zooxanthellae, fungi, bacteria, etc.) and/or mucus concentration of each coral species.

We also observed marked differences within each coral species in terms of positive amplification of 16S rDNA using PCR. Differences in PCR amplification may have also been determined by initial processing and extraction treatments, which subsequently affected the ratio of mixed genomic DNA template from different organisms present in the samples. Additional physical and chemical reactions that occurred during the extraction process may have also affected PCR amplification. For example, both the airbrushed and crushed/Plant Kit extractions yielded similar amounts of genomic DNA for *Porites lobata*; however, only airbrushed treatments PCR amplified. This could possibly be due to a unique biochemical reaction between the skeletal material and Plant extraction kit constituents, shearing of high molecular weight DNA by skeletal fragments, or PCR inhibition due to the presence of calcium ions in the extracted DNA (Bickley et al. 1996). Finally, it is important to note that certain treatment/extraction combinations resulted in inconsistent amplification of the 16S rRNA gene for replicate
samples, whereas others resulted in 100% amplification; thus, indicating that those particular combinations were not as reliable and repeatable as others.

The treatment/extraction combinations that resulted in the highest quality terminal-restriction fragment length polymorphism (T-RFLP) electropherograms were those that were able to provide high concentrations of specific PCR product. High starting concentrations were needed in order to retain enough PCR product for the generation of T-RFLP profiles due to a significant loss of product during clean-up and restriction procedures. Because we were interested in developing a high-throughput method of analyzing coral-associated microbial communities, we avoided pooling samples to compensate for this deficiency. Replication and consistency in generating high quality T-RFLP electropherograms were also of major importance in evaluating treatment/extraction combinations; however, all samples for which high quality T-RFLP electropherograms were obtained were included in the analysis for comparison purposes.

Statistical tests performed individually for both *Porites compressa* and *Porites lobata* revealed that each set of three replicates for individual colony/treatment/extraction combinations were not significantly different, demonstrating that the methods employed in this study were robust. Replicate sets, representing different combinations, were significantly different from one another, indicating that the combination of factors chosen (i.e. colony, treatment, and extraction) had a considerable effect on the resultant bacterial community profile obtained using T-RFLP analysis.

We compared results from running multiple Multi-response Permutation Procedure (MRPP) statistical tests on all T-RFLP data in order to “scale down” the effect that different treatment/extraction combinations had on the resultant bacterial community
structure. Through this analysis, we found that T-RFLP-based bacterial communities were significantly different from one another regardless of the chosen grouping scheme for each species. However, within group variation, represented by the $A$ value, varied with each grouping. For *Porites compressa*, the $A$ value decreased markedly as statistical groupings were changed from colony to treatment to extraction (i.e. groups became less and less similar). For this species, it appeared that the variation in bacterial community structure amongst the three colonies contributed more to observed differences than either treatment or extraction method alone with all three colonies grouped together. The $A$ value was still considerably high when all extractions for each colony were grouped together, but separated out based on treatment. In other words, the treatment had a larger effect than the extraction method for an individual colony.

For *Porites lobata*, the $A$ value was relatively high when sample groups were based solely on extraction method. The $A$ value continued to decrease as statistical groupings were changed from extraction to colony to treatment, indicating that the extraction method had a greater affect than either colony or treatment alone on observed differences for this species. When all extractions for each colony were grouped together, but separated out based on treatment, the resultant $A$ value was similar to the value obtained when sample groups were based on extraction method alone. This suggests that inter-colony variation also had a significant effect on the resultant T-RFLP profiles for this species, but that extraction method contributed most to the observed variation in bacterial community structure.

Overall, it appeared as though inter-colony differences in *Porites compressa* colonies were greater than those found amongst *Porites lobata* colonies. Inter-colony
differences may be species-specific (i.e. larger variability in microbial community structure in one species versus the other) and possibly determined by colony morphology (e.g., finger versus lobe) and the resulting biophysical complexity of the microenvironment surrounding the colony. This could also explain why different extraction methods had a greater affect on *Porites lobata*. Colonies had more homogenous landscapes, resulting in more homogenous bacterial communities amongst colonies.

Cloning and sequencing of select samples confirmed that the gene fragments obtained from T-RFLP analysis were indeed those belonging to Bacteria, with a few possible exceptions. Sequence analysis of bacterial clones for *Porites compressa* revealed inter-colony variation in bacterial communities that agreed with the T-RFLP analysis of these samples. Additionally, sequence analysis of bacterial clones for *Porites lobata* indicated that different treatments (airbrushing vs. crushing) resulted in clone libraries with different bacterial compositions. These findings were also congruent with the T-RFLP analysis of *Porites lobata* samples. Congruence between T-RFLP and clone library data indicates that T-RFLP is a good candidate for high-throughput comparative analyses of coral-associated bacterial communities; however, additional fragment analysis of clones may be needed to ensure that the same phylogenetic groups are equally represented by each analysis.

**Conclusion**

Through the examination of various sample processing and nucleic acid extraction combinations, we were successful in developing and optimizing species-specific
protocols for coral-associated bacterial genomic DNA extraction and PCR-amplification for use in high-throughput terminal-restriction fragment length polymorphism (T-RFLP) analyses. We determined that different treatment/extraction method combinations applied to replicate samples resulted in significantly different T-RFLP community profiles and that the scale on which these factors affected the end product varied within each coral species examined. Furthermore, we observed inter-colony variation in microbial community structure and stress the importance of adjusting protocols to sample this variation. Several of the chosen treatment/extraction method combinations resulted in high quality, replicable T-RFLP profiles for both Porites species; however, these combinations resulted in significantly different T-RFLP profiles. These findings further emphasize the current problems faced by molecular biologists in terms of standardizing and comparing environmental data sets. As a guiding principle in science, the best practice is to be consistent in the chosen methodology. Although we cannot recommend the “best” method, we have demonstrated the importance of carefully choosing your methodology and being aware of its advantages and limitations.

Acknowledgements

Funding for this project was provided by a research partnership between the Northwestern Hawaiian Island Coral Reef Ecosystem Reserve and the Hawaii Institute of Marine Biology (NMSP MOA 2005-008/66882). The assistance of Jamie Becker, Amy Aprill, and Darin Hayakawa is gratefully acknowledged.
References


Pruesse, E., C. Quast, K. Knittl, B. Fuchs, W. Ludwig, J. Peplies, and F. O. Glöckner. 2007. SILVA: a comprehensive online resource for quality checked and aligned
ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* 35: 7188-7196.


Tables

Table 2.1 Amplification of 16S rRNA genes via PCR as a function of coral species and DNA extraction method

<table>
<thead>
<tr>
<th></th>
<th>Plant</th>
<th>PowerSoil</th>
<th>Tissue</th>
<th>CTAB</th>
<th>Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>P. compressa</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>9‡</td>
</tr>
<tr>
<td></td>
<td>9†</td>
<td>5†</td>
<td>8‡</td>
<td>9‡</td>
<td>9‡</td>
</tr>
<tr>
<td>P. lobata</td>
<td>9‡</td>
<td>0</td>
<td>7‡</td>
<td>7‡</td>
<td>8‡</td>
</tr>
<tr>
<td></td>
<td>8‡</td>
<td>8‡</td>
<td>8‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. capitata</td>
<td>0</td>
<td>0</td>
<td>4‡</td>
<td>3‡</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9*</td>
<td>4*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values listed are out of 9 total samples (3 colonies per species with 3 replicates per colony); Zero values indicate that neither dilution nor concentration of gDNA led to successful amplification of 16S rRNA genes.

† 1:10 dilution of gDNA template with sterile water; ‡1:100 dilution of gDNA template with sterile water; ¥ gDNA template dehydrated and re-suspended/concentrated in 25% of the original volume of sterile water; * gDNA template dehydrated and re-suspended/concentrated in 5% of the original volume of sterile water.
Table 2.2 T-RFLP profiles obtained from PCR amplification of 16S rRNA genes\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Plant</th>
<th>PowerSoil</th>
<th>Tissue</th>
<th>CTAB</th>
<th>Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>(P.\ compressa)</td>
<td>9</td>
<td>0</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>(P.\ lobata)</td>
<td>9</td>
<td>0</td>
<td>8</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>(M.\ capitata)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Values listed are out of 9 total samples (3 colonies per species with 3 replicates per colony)
Table 2.3

<table>
<thead>
<tr>
<th>Taxonomic Identification of Microorganisms Associated with <em>Porites compressa</em></th>
<th>Colony 1</th>
<th>Colony 2</th>
<th>Colony 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Actinobacteria; Actinobacteria; Actinomycetales; NA</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Actinobacteria; Actinobacteria; NA; NA</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; NA</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; NA; NA; NA</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; Flammeovirgaceae</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; NA</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; Saprospiraceae</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; NA; NA; NA</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; True Cyanobacteria; Unassigned; Family I</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; True Cyanobacteria; Unassigned; Family IV</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; True Cyanobacteria; Unassigned; Family VIII</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Bacilli; Bacillales; NA</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; NA</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; NA; NA; NA</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae</td>
<td>12</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; NA; NA</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; NA</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Taxonomy</td>
<td>Total Clones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>--------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodobacteraceae</td>
<td>6 4 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae</td>
<td>2 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Burkholderiaceae</td>
<td>1 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae</td>
<td>0 0 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;NA</td>
<td>2 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Oxalobacteraceae</td>
<td>0 0 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Unassigned</td>
<td>2 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Deltaproteobacteria;Desulfbacterales;Desulfbacteraceae</td>
<td>0 1 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Helicobacteraceae</td>
<td>1 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;NA</td>
<td>1 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;NA;NA</td>
<td>3 16 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;NA</td>
<td>5 22 39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae</td>
<td>18 5 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;NA;NA;NA</td>
<td>1 2 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;NA</td>
<td>1 0 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Rubritaleaceae</td>
<td>1 0 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae</td>
<td>0 1 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organelle;Cyanobacteria;Chloroplast;NA;NA</td>
<td>3 1 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organelle;Cyanobacteria;Chloroplast;Rhodophyta;Bangiophyceae</td>
<td>0 2 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organelle;Cyanobacteria;Chloroplast;Stramenopile;Bacillariophyta</td>
<td>0 0 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown;NA;NA;NA;NA</td>
<td>4 0 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Total number of clones per library | 92 95 94 |

*aClones are from airbrushed/PowerSoil extracted samples from each of 3 coral colonies; Values listed are absolute number of clones per library; NA, indicates that the taxonomy was not available for that clone at that taxonomic unit.*
Table 2.4

<table>
<thead>
<tr>
<th>Taxonomic Identification of Microorganisms Associated with <em>Porites lobata</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Colony 1A</th>
<th>Colony 1C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales; Acidobacteriaceae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; NA</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; NA; NA; NA</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; Flammeovirgaceae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; NA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales; Saprospiraceae</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; NA; NA; NA</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; True Cyanobacteria; Unassigned; Family I</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria; Cyanobacteria; True Cyanobacteria; Unassigned; Family VIII</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; NA</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; Clostridia; NA; NA</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria; Firmicutes; NA; NA; NA</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Bacteria; NA; NA; NA; NA</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; NA; NA</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; NA</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Taxonomy</td>
<td>Total Clones</td>
<td>Samples</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Deltaproteobacteria;NA;NA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Coxiellaceae</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;NA</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;NA;NA</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;NA</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria;Proteobacteria;NA;NA;NA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Organelle;Cyanobacteria;Chloroplast;NA;NA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Unknown;NA;NA;NA;NA</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

**Total number of clones per library**

93 94

*aClones are from airbrushed (A) or crushed (C) PowerSoil extracted samples from a single coral colony; Values listed are absolute number of clones per library; NA, indicates that the taxonomy was not available for that clone at that taxonomic unit*
Table 2.5

A.

<table>
<thead>
<tr>
<th></th>
<th>Colony 1</th>
<th>Colony 2</th>
<th>Colony 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony 1</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Colony 2</td>
<td>0.531</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>Colony 3</td>
<td>0.624</td>
<td>0.429</td>
<td>0</td>
</tr>
</tbody>
</table>

Colony 1  Colony 2  Colony 3

\(^a\)Samples from each colony were airbrushed and extracted using the PowerSoil DNA Kit

B.

<table>
<thead>
<tr>
<th></th>
<th>Airbrushed</th>
<th>Crushed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airbrushed</td>
<td>0</td>
<td>---</td>
</tr>
<tr>
<td>Crushed</td>
<td>0.561</td>
<td>0</td>
</tr>
</tbody>
</table>

Airbrushed  Crushed

\(^a\)Samples from colony 1 were extracted using the PowerSoil DNA Kit
Figure Legends

**Figure 2.1:** Total yield of genomic DNA from airbrushed or crushed starting material using different extraction methods. Values expressed as colony averages ± standard deviation. N = 3 colonies per species, with 3 sample replicates per colony (with the exception of crushed, Powersoil extracted *Porites compressa*; n = 2 colonies). PC, *Porites compressa*; PL, *Porites lobata*; MC, *Montipora capitata*.

**Figure 2.2:** Non-metric multi-dimensional scaling ordination plot of coral associated bacterial communities (represented by Terminal-restriction Fragment Length Polymorphism profiles) from *Porites compressa* (gray) and *Porites lobata* (black) corals. Each point represents the bacterial community from a single sample. The superscript adjacent to each point refers to the colony number (e.g., 1, 2, or 3) and extraction method used (CT = CTAB, P = Plant, PS = PowerSoil, and S = Soil). Airbrushed or crushed treatments are not indicated in this plot. The stress value is listed in the bottom right corner.

**Figure 2.3:** Non-metric multi-dimensional scaling ordination plot of coral associated bacterial communities (represented by Terminal-restriction Fragment Length Polymorphism profiles) from *Porites compressa* corals. Each point represents the bacterial community from a single sample. The shape of each point represents the colony number (e.g., circles = samples from colony 1, squares = samples from colony 2, and triangles = samples from colony 3). Gray points were airbrushed prior to extraction and black points were crushed prior to extraction. The superscript adjacent to each point refers to the extraction method used (CT = CTAB, P = Plant, PS = PowerSoil, and S = Soil). The stress value is listed in the bottom right corner.
Figure 2.4: Non-metric multi-dimensional scaling ordination plot of coral associated bacterial communities (represented by Terminal-restriction Fragment Length Polymorphism profiles) from *Porites lobata* corals. Each point represents the bacterial community from a single sample. The shape of each point represents the colony number (e.g., circles = samples from colony 1, squares = samples from colony 2, and triangles = samples from colony 3). Gray points were airbrushed prior to extraction and black points were crushed prior to extraction. The superscript adjacent to each point refers to the extraction method used (CT = CTAB, P = Plant, PS = PowerSoil, and S = Soil). The stress value is listed in the bottom right corner.

Appendix I

Figure 1. A-E; Extracted genomic DNA from *Porites compressa* corals (first column) and subsequent amplification of 16S rRNA genes (second column) from genomic DNA template run on 1% agarose gels; Bioline quantitative DNA marker HyperLadder I (200 to 10,000bp) was run with genomic DNA samples and HyperLadder II (50 to 2,000bp) was run with PCR products; Number/letter headings indicate colony number and airbrushing (A) or crushing (C) treatment; Extraction methods listed in bottom left corner of gels; Numbers on bottom right indicate positive amplification out of 9 samples from airbrushed and crushed treatments (3 colonies with 3 replicates per colony); Plus and minus symbols clarify positive or negative amplification, respectively.

Figure 2. A-E; Extracted genomic DNA from *Porites lobata* corals (first column) and subsequent amplification of 16S rRNA genes (second column) from genomic DNA template run on 1% agarose gels; Bioline quantitative DNA marker HyperLadder I (200 to 10,000bp) was run with genomic DNA samples and HyperLadder II (50 to 2,000bp) was run with PCR products; Number/letter headings indicate colony number and airbrushing (A) or crushing (C) treatment; Extraction methods listed in bottom left corner of gels; Numbers on bottom right indicate positive amplification out of 9 samples from airbrushed and crushed treatments (3 colonies with 3 replicates per colony); Plus and minus symbols clarify positive or negative amplification, respectively.
positive amplification out of 9 samples from airbrushed and crushed treatments (3 colonies with 3 replicates per colony); Plus and minus symbols clarify positive or negative amplification, respectively.

Figure 3. A-E; Extracted genomic DNA from *Montipora capitata* corals (first column) and subsequent amplification of 16S rRNA genes (second column) from genomic DNA template run on 1% agarose gels; Bioline quantitative DNA marker HyperLadder I (200 to 10,000bp) was run with genomic DNA samples and HyperLadder II (50 to 2,000bp) was run with PCR products; Number/letter headings indicate colony number and airbrushing (A) or crushing (C) treatment; Extraction methods listed in bottom left corner of gels; Numbers on bottom right indicate positive amplification out of 9 samples from airbrushed and crushed treatments (3 colonies with 3 replicates per colony); Plus and minus symbols clarify positive or negative amplification, respectively.
Figure 2.1

Figure 2.2
Figure 2.3
Figure 2.4
Appendix

Appendix I
Figure 1.

*Porites compressa*

<table>
<thead>
<tr>
<th>Genomic DNA</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
</tr>
<tr>
<td>Plant</td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
<td></td>
</tr>
<tr>
<td>PowerSoil</td>
<td></td>
</tr>
<tr>
<td><strong>E</strong></td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.

Porites lobata

A  Genomic DNA
   Soil

B  Tissue

C  Plant

D  PowerSoil

E  CTAB

PCR product
A8+ C9+
A7+ C7+
A9+ C9+
Figure 3.

*Montipora capitata*

<table>
<thead>
<tr>
<th>Genomic DNA</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Tissue</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Plant</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
</tr>
<tr>
<td>PowerSoil</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3

BIODIVERSITY AND BIOGEOGRAPHY OF MICROORGANISMS ASSOCIATED WITH SEAWATER AND REEF-BUILDING CORAL ACROSS THE HAWAIIAN ARCHIPELAGO AND WIDER PACIFIC OCEAN

Jennifer L. Salerno\textsuperscript{1,2} and Michael S. Rappé\textsuperscript{1*}

\textsuperscript{1}Hawaii Institute of Marine Biology, School of Ocean and Earth Science and Technology, University of Hawaii at Manoa, Kaneohe, HI, 96744

\textsuperscript{2}Department of Zoology, University of Hawaii at Manoa, Honolulu, HI, 96822

*Corresponding author. Mailing address:
Hawaii Institute of Marine Biology, University of Hawaii, P.O. Box 1346, Kaneohe, HI 96744. Phone: (808) 236-7464. Fax: (808) 236-7443. Email: rappe@hawaii.edu
Abstract

Microorganisms inhabit nearly every ecosystem on the planet; however, the major principles governing their ecological distributions are widely debated and poorly understood. The biogeography of microbes associated with host organisms are of particular interest due to their contributions to organismal health and microbial diversity. Here, we explore the biodiversity and biogeography of bacteria associated with *Porites lobata* corals and seawater across the Hawaiian Archipelago, Johnston Atoll, and American Samoa. Bacterial community composition was characterized using terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial small-subunit (SSU) ribosomal RNA genes. A total of 355 unique terminal restriction fragments (T-RFs) were detected amongst planktonic and coral-associated bacterial community samples collected in 2005 and 2006 (n = 158), with an average of 14 ±5 and 19 ±14 T-RFs per seawater and coral sample, respectively. CLUSTER and ANOSIM analyses indicated that the structure of seawater and coral-associated bacterial communities sampled over the two years was significantly different (*Global R* = 0.425, *P* = 0.001). Overall, we detected relatively weak, but significant isolation by distance patterns for planktonic and coral-associated bacterial communities, indicating that geographic isolation is partially responsible for driving observed divergences at this archipelagic scale. Parallels in biogeographic patterns of *Porites lobata* and bacterial associates highlight the impact that host dispersal may have on bacterial community structure. Both seawater and coral-associated bacterial communities exhibited a biogeographic connection with Johnston Atoll, as observed in other fish and invertebrate species. We did not detect significant correlations with habitat type, temperature, or depth. However,
we detected several geographic groupings that were not distance-based, indicating that additional factors are affecting the distributions of bacterial communities associated with *Porites lobata* and seawater. Future studies incorporating comprehensive measurements of local oceanographic and environmental conditions over appropriate time scales would help elucidate the environmental variables that shape coral-associated bacterial communities. Developing tools to identify specific types of bacteria associated with corals during non-diseased and diseased states, as well as those linked with specific environmental stressors, may enable scientists and resource managers to monitor coral health and mitigate local threats more effectively.

**Introduction**

Microorganisms play a crucial role in sustaining life on earth. Their importance in shaping ecosystems is underscored by the scale upon which they perform essential functions – from forming highly specific symbiotic associations at the cellular level to driving global biogeochemical cycles (Azam 2007, Falkowski et al. 2008, Sachs et al. 2011). Microorganisms inhabit nearly every ecosystem on the planet; however, the major principles governing their ecological distributions are widely debated and poorly understood (Martiny et. al 2006, Hanson et al. 2012). Uncovering the processes that shape microbial biogeography is crucial to distinguishing the fundamental differences between life forms on earth and how microorganisms make the planet habitable.

For the past century, the Baas-Becking hypothesis that “everything is everywhere; but the environment selects,” predominated amongst microbiologists (Baas-Becking 1934). Under this assumption, microorganisms disperse freely, unhindered by geographic
boundaries, settling and thriving only after encountering an optimal environment (de Wit and Bouvier 2006). However, relatively recent advances in molecular technology have given microbiologists a more detailed picture of microbial diversity, indicating that microbes are not as infinitely mobile as previously thought (Head et al. 1998). A growing body of evidence has shown that a wide range of microorganisms display biogeographic patterns, some of which are similar to those of macroorganisms (Green and Bohannan 2006, Martiny et al. 2006, Pommier et al. 2007, Ramette and Tiedje 2007).

In addition to demonstrating biogeographic variation in microbial assemblages, microbial ecologists face the challenge of identifying the underlying processes driving said variation in these communities. Prevailing environmental factors can contribute to observed variation; however, historical events may also affect present-day patterns of microbial biodiversity. For example, dispersal barriers and past climatic conditions can lead to genetic divergence and subsequently, variations in biogeographic distributions (Cox and Moore 2010). Distinct biogeographic provinces, or areas where biotic assemblages reflect historic events, have been identified for macroorganisms (Udvary 1975). However, the existence of such provinces for microorganisms has only recently been addressed, with microbiologists observing biogeographic patterns amongst a wide range of microbes (Fierer and Jackson 2006, Martiny et al. 2006).

Current debate regarding microbial biogeography has focused mainly on free-living microorganisms, but microbes associated with host organisms are also of great interest from a biogeographical perspective due to their important contributions to microbial diversity (Taylor et al. 2004). Host organisms provide unique environmental conditions for microbial colonization compared to that of the surrounding environment.
As a result, eukaryotic hosts may function as habitat ‘islands’ (MacArthur and Wilson 1967), allowing allopatric speciation to occur in microbial populations living on physically separated hosts (Taylor et al 2005). Previous studies have shown that many symbiotic microorganisms display patterns of genetic and morphological diversity paralleling that of their host organism (Distel et al. 1994, Funk et al. 2000).

Here, we explore the biodiversity and biogeography of bacteria associated with reef-building corals and adjacent seawater across the Hawaiian Archipelago. It is well established that corals and seawater harbor distinct bacterial assemblages (Rohwer et al. 2001, 2002, Rosenberg et al. 2007); however, relatively little is known about the fidelity of these associations over a range of spatial scales (Rohwer 2002, Littman 2009, Morrow et al. 2012). Corals, in particular, are known to host a diversity of bacteria in their surface mucous layer, epithelial tissue, and skeleton which are proposed to serve a variety of key functions (Ducklow and Mitchell 1979, Wafar et al. 1990, Rohwer et al. 2001, Lesser et al. 2004, Ritchie 2006). Much like the photosynthetic zooxanthellae that reef-building corals depend on for survival, some of these microbes may be performing life-sustaining processes for the coral host (Knowlton and Rohwer 2003, Rosenberg et al. 2007), and yet others may become pathogenic disease agents under the right conditions (Patterson et al. 2002, Frias-Lopez et al. 2006, Lesser et al. 2007, Rosenberg et al. 2007, Sussman et al. 2008, Richardson et al. 2009). To identify which bacteria are consistently associated with a particular species of coral versus those that are simply transient, it is essential to first understand the temporal and spatial variability of bacterial consortia. It is known that *Porites lobata*, the coral species chosen for this study, conforms to a pattern of isolation by distance across the Hawaiian Archipelago (Polato et al. 2010). If bacteria form
specific associations with *Porites lobata*, we might expect their population genetic structures to correspond with that of their coral host.

Recent studies have revealed that free-living bacteria found in seawater, or bacterioplankton, exhibit biogeographic patterns over various taxonomic and spatial scales (Garcia-Martinez and Rodriguez-Valera 2000, Riemann et al. 2002, Pinhassi et al. 2003, Schwalbach and Fuhrman 2005). Bacterioplankton play an important role in oligotrophic reef ecosystems by recycling limited nutrients (Charpy et al. 2012). In this study, bacterioplankton distributions are compared with those of coral-associated bacteria to examine biogeographic differences between free-living and potentially symbiotic bacterial assemblages. We also set out to identify, in part, the factors that contribute to underlying biogeographic distributions. Understanding the relationship between coral-associated and bacterioplankton communities is valuable because bacteria can directly impact the survival of the coral host and subsequently, the extensive ecosystems that they support.

**Materials and Methods**

**Sample collection**

The hermatypic lobe coral *Porites lobata* (Dana 1846) was targeted for bacterial community assessment due to its widespread Indo-Pacific distribution. The *Porites* genus is ubiquitous throughout the circumtropical region and is amongst the most widely distributed coral genera in terms of both geography and habitats (Veron 2000). *Porites* corals are of major ecological significance as they provide most of the framework for coral reefs in Hawaii (Maragos 1977) and many reefs worldwide (Veron 2000). *Porites*
_lobata_ corals are yellow to greenish in color, have a lobular morphology, and wide distribution throughout the Indo-Pacific. These corals can be found as encrusting colonies in high wave action areas and as large mounds in wave-protected areas.

Coral and seawater samples for bacterial community analysis were collected from sites across the Hawaiian Archipelago, Johnston Atoll (~1,327 km southwest of Oahu), and American Samoa (~4,195 km southwest of Oahu) (Appendix I). The majority of samples collected in Hawaii were taken within the Papahānaumokuākea Marine National Monument (PMNM) in the northwestern portion of the Hawaiian island chain. The NOAA R/V Hi‘ialakai was the main research platform used for sample collection operations during two research cruises in September/October 2005 (22 days) and May/June 2006 (24 days). During the September/October 2005 cruise, samples were collected from multiple sites within French Frigate Shoals, Maro Reef, Pearl and Hermes Atoll, and Kure Atoll (Appendix 1A). During the May/June 2006 samples were collected from Nihoa, French Frigate Shoals, Gardner Pinnacles, and Johnston Atoll (Appendix 1B). Distances between samples ranged from approximately 1 meter to 1,500 kilometers. Samples were collected from several different habitat types including, lagoon, back reef, and fore reef areas.

Coral colonies judged as non-diseased by gross visual assessment were targeted for sample collection. A stainless steel chisel was used to remove a coin-sized piece of coral that consisted of coral tissue, overlying mucous layer, and underlying skeleton. Sub-samples from each colony were put into sterile whirl-pak® bags (Nasco, Fort Atkinson, WI), placed on ice, and transported back to the ship where they were stored frozen. Temperature and depth were recorded at each site with a dive computer.
Seawater samples were also collected to characterize planktonic bacterial communities in the surrounding environment. One liter of seawater was collected adjacent to coral heads, placed on ice, and filtered on board the research vessel through a series of 25 mm diameter, 1.6 µm nominal pore-sized GF/A glass microfiber filters (Whatman International Ltd., Piscataway, NJ) and 13 mm diameter, 0.2 µm pore-sized polyethersulfone membrane filters (Supor-200; Pall Corp., East Hills, NY). Filters were then stored frozen in 250 µL of DNA lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 1.2% v/v Triton X100) (Suzuki et al. 2001). All samples were transported to the Hawaii Institute of Marine Biology where they were further processed for DNA analysis.

**DNA extraction and T-RFLP of bacterial SSU rRNA**

Coral samples were thawed on ice and a flame-sterilized core borer was used to remove a 6-mm diameter, 6-mm deep core from each fragment (3 per colony). Cores from each colony were placed into a sterile whirl-pak® bag containing 2 mL of 0.2 µm-filtered 10X Tris EDTA (100 mM Tris, 10 mM EDTA) buffer solution (pH 7.4) and an air gun outfitted with a sterile pipette tip was used to remove coral tissue from the skeleton. The coral tissue slurry was centrifuged at 19,900 RCF for 30 min at 4°C. The supernatant was removed and the remaining sample pellet was frozen at -80°C until further processed.

Genomic DNA was extracted from coral and seawater samples using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer’s protocol. Total genomic DNA yield was quantified using the Quant-iT
PicoGreen dsDNA Assay Kit (Invitrogen Corp., Carlsbad, CA, USA) and SpectraMax M2 plate reader (Molecular Device Corp., Sunnyvale, CA, USA).

Approximately 69 ±34 ng of genomic DNA was used as template for polymerase chain reaction (PCR) amplification of bacterial small subunit (SSU) ribosomal RNA (16S rRNA) genes in preparation for terminal-restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997).

In addition to genomic DNA template, 50 µl PCR reactions were composed of (final concentrations): 1X MasterTaq reaction buffer (Eppendorf AG, Hamburg, Germany), 2.25 mM Mg\textsuperscript{2+}, 0.5X TaqMaster reaction enhancer (Eppendorf), 0.2 mM each of the fluorescently labeled general bacterial SSU rRNA oligonucleotide primer 27F-B-FAM (5’-FAM-AGRGTTYGATYMTGGCTCAG-3’) and universal SSU rRNA oligonucleotide primer 1492R (5’-GGYTACCTTGTTACGACTT-3’; Lane, 1991), 0.2 mM of each deoxynucleotide (Promega, Madison, WI), 2.5 units of MasterTaq DNA polymerase (Eppendorf), and sterile water. PCR reactions were carried out in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with an initial incubation of 3 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 1 min at 65°C (decreasing by 0.5°C per cycle), and 2 min at 72°C. Reactions were concluded with 10 cycles of 30 sec at 95°C, 1 min at 50°C, and 2 min at 72°C, and 1 cycle of 30 sec at 95°C, 1 min at 50°C, and 20 min at 72°C. Fluorescently labeled PCR amplicons were purified using the QIAquick Multiwell PCR Purification System (Qiagen Inc., Valencia, CA) and approximately 100 ng of each purified amplicon was digested in a 10 µL reaction containing 5 units of HaeIII restriction endonuclease (Promega, Madison, WI). After a 6-h incubation at 37°C, digests were purified via gel filtration chromatography using the
Millipore MultiScreen Assay System (Millipore Corp., Billerica, MA) paired with Sephadex G-50 Superfine (GE Healthcare, Piscataway, NJ). Purified products were adjusted to a final concentration of 30 ng µL⁻¹ and electrophoresed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Size and relative abundance of resulting terminal restriction fragments (T-RFs) was determined using GeneMapper software (Applied Biosystems) and operational taxonomic units (OTUs) were defined as fragments between 50 and 600 base pairs (bp) in length. Fragment lengths were rounded to the nearest integer value, aligned, and manually checked for possible errors in peak determination due to instrument variability. A variable percentage threshold method (Osborne et al. 2006) was used to normalize samples. Finally, peaks were transformed into relative abundance units by dividing integrated peak areas by the total peak area for an individual sample.

Statistical analyses

Statistical analyses were performed using PRIMER 6 Version 6.1.13 and PERMANOVA+ Version 1.0.3 (PRIMER-E Ltd., Plymouth, UK) (Clarke and Warwick 2001, Clarke and Gorley 2006, Anderson and Gorley 2008). A resemblance matrix of Bray-Curtis dissimilarities was calculated from square root-transformed T-RFLP data. Hierarchical clustering analysis (CLUSTER) was used to obtain similarity dendrograms and the similarity profile permutation test (SIMPROF) was used to identify significant “natural groupings” of samples that were not defined a priori. CLUSTER analyses were based on the group average linking option and SIMPROF analyses were performed at a 5% significance level. The analysis of similarity (ANOSIM) routine, which is analogous
to a standard univariate 1-way analysis of variance (ANOVA), was used to test for differences in bacterial community structure (based on T-RF peak abundances) between pre-defined sample groups, based on prior SIMPROF analyses. Nonmetric multidimensional scaling (NMDS) ordination provided a “best fit” 2-dimensional graphical representation of the data (Shepard 1962, Kruskal 1964), illustrating similarities and differences in bacterial community structure amongst samples. The permutational multiple analysis of variance (PERMANOVA) routine was used to test for differences in bacterial community structure between different sites and islands/atolls. PERMANOVA tests the simultaneous response of one or more variables to one or more factors in an analysis of variance (ANOVA) experimental design on the basis of any distance measure and using permutation methods (Anderson 2001). PERMANOVA tests were run with the following specifications: 999 permutations, permutation of residuals under a reduced model, Type III (partial) sum of squares, Monte Carlo tests, and fixed effects sum to zero.

The Bio-Env + Stepwise (BEST) (Clarke and Warwick 2001, Clarke and Gorley 2006) analysis was used to test for relationships between bacterial community structure and environmental variables (temperature and depth). A matrix consisting of temperature and depth data was constructed, values were normalized, and the Bio-Env option, spearman rank correlation method, and permutation test (999 permutations) for significance were chosen for the analysis.

To test for isolation by distance, a matrix based on GPS coordinates for all sample locations was obtained using the Geographic Distance Matrix Generator v.1.2.3 (Ersts, American Museum of Natural History). The RELATE routine, a non-parametric form of the Mantel test, was used to determine if dissimilarity in coral-associated bacterial
species composition amongst samples was significantly correlated with the geographical layout of the samples. Both square root-transformed and presence/absence-transformed T-RFLP data were used to construct resemblance matrices for comparisons.

Results

Planktonic and coral-associated bacterial community structure

Overall, 355 unique T-RFs were detected amongst planktonic and coral-associated bacterial community samples collected in 2005 and 2006 (n = 158), with an average of 14 ±5 and 19 ±14 T-RFs per seawater and coral sample, respectively. CLUSTER and ANOSIM analyses indicated that the structure of seawater and coral-associated bacterial communities sampled over the two years was significantly different (Global $R = 0.425$, $P = 0.001$). SIMPER analysis showed that the average similarity within seawater samples was 35.1%, while average similarity within coral samples was 13.4%. Average dissimilarity between seawater and coral samples was 92.6%, with TRFs 384 (5.9%), 112 (4.8%), 226 (3.5%), 190 (3.3%), and 289 (3.2%) being the major contributors to observed differences. An NMDS ordination provided a “best-fit” 2-dimensional graphical representation of the abundance data which illustrated observed differences between the two sample types (Figure 3.1).

Planktonic samples collected in 2005 and 2006 were analyzed separately since replicate data were not obtained from each island/atoll for both years and therefore, the ability to detect differences based on year of collection were limited. CLUSTER and ANOSIM analyses of plankton-associated bacterial community samples from 2005 (n = 20) indicated that bacterial communities differed significantly between islands/atolls.
PERMANOVA analysis confirmed a significant difference between island/atolls (\textit{Pseudo-F} = 10.637, \textit{P} = 0.001) and revealed that plankton-associated bacterial communities did not significantly differ between sites within an island/atoll (\textit{Pseudo-F} = 3.924, \textit{P} = 0.074). ANOSIM pairwise test values (Table 1A) revealed significant differences between Kure and Maro, Pearl and Hermes and Maro, and Pearl and Hermes and French Frigate Shoals atolls and SIMPER analysis revealed the average similarity between and within island/atolls (Table 2A). T-RFs that contributed most to observed differences between Kure and Maro, Pearl and Hermes and Maro, and Pearl and Hermes and French Frigate Shoals atolls were 290 (6.8%), 384 (8.6%), and 384 (7.8%), respectively. A primary NMDS ordination of all seawater samples from 2005 graphically illustrated differences between individual samples (Figure 3.2A), while a secondary NMDS ordination of ANOSIM pairwise test values summarized overall differences found between islands/atolls (Figure 3.2B).

CLUSTER and ANOSIM analyses of 2006 data (\textit{n} = 26) showed that plankton-associated bacterial communities differed significantly between islands/atolls (\textit{Global R} = 0.219, \textit{P} = 0.007). PERMANOVA analysis also revealed significant island/atoll differences (\textit{Pseudo-F} = 2.677, \textit{P} = 0.031) and indicated that bacterial communities did not significantly differ between sites within an island/atoll (\textit{Pseudo-F} = 1.071, \textit{P} = 0.452). ANOSIM pairwise test values (Table 1B) revealed that American Samoa was significantly different from French Frigate Shoals, Kaneohe Bay, and Johnston Atoll and that Kaneohe Bay was significantly different from French Frigate Shoals and Johnston Atoll. SIMPER analysis revealed the average similarity between and within island/atolls (Table 2B) and that the T-RFs that contributed most to observed differences between
American Samoa and French Frigate Shoals, Kaneohe Bay, and Johnston Atoll were 384 (13.5%), 289 (12.9%), and 384 (7.6%), respectively. T-RFs that contributed most to observed differences between Kaneohe Bay and French Frigate Shoals and Johnston Atoll were 384 (17%) and 289 (16.6%), respectively. An initial NMDS ordination of all seawater samples from 2006 graphically illustrated differences between individual samples (Figure 3.3A), while a subsequent NMDS ordination of ANOSIM pairwise test values (Table 1B) summarized overall differences found between islands/atolls (Figure 3.3B).

As with planktonic samples, coral samples collected in 2005 and 2006 were analyzed separately since replicate data were not obtained from each island/atoll for both years. CLUSTER and ANOSIM analyses of coral-associated bacterial community samples collected in 2005 (n = 76) revealed that bacterial communities differed significantly between islands/atolls (Global R = 0.317, P = 0.001). PERMANOVA analysis confirmed island/atoll differences in bacterial community structure (Pseudo-F = 2.605, P = 0.001) and showed that bacterial communities differed significantly between sites within an island/atoll (Pseudo-F = 1.261, P = 0.002); however, post-hoc pairwise comparisons did not yield significant differences between sites. ANOSIM pairwise test values between islands/atolls (Table 1C) revealed that Maro and Necker were significantly different from each other and all other island/atolls sampled in 2005 and that French Frigate Shoals was also significantly different from Midway Atoll. SIMPER analysis revealed the average similarity between and within island/atolls (Table 2C). T-RFs that contributed most to observed differences between Maro and the other islands/atolls were: 291 (6.9%) for Kure, 191 (6.8%) for Midway, 263 (6%) for Pearl and
Hermes, 315 (6.9%) for French Frigate Shoals, and 57 (7.6%) for Necker. T-RF 57 also contributed most to observed differences between Necker and remaining islands/atolls: 6.9% for Kure, Midway, and Pearl and Hermes, and 7.2% for French Frigate Shoals. T-RF 191 contributed most to observed differences between French Frigate Shoals and Midway (5.6%). A primary NMDS ordination of all coral samples from 2005 graphically illustrated differences between individual samples (Figure 3.4A), while a secondary NMDS ordination of ANOSIM pairwise test values (Table 1C) summarizes overall differences found between islands/atolls (Figure 3.4B).

In 2005, there was an opportunity to obtain replicate samples from four different habitats within French Frigate Shoals atoll (coastal fringing, patch, back, and fore reef). CLUSTER and ANOSIM analyses of coral-associated bacterial samples (n = 17) from French Frigate Shoals showed that bacterial communities did not differ significantly based on habitat type (Global R = 0.06, P = 0.254).

CLUSTER and ANOSIM analyses of coral-associated bacterial community samples (n = 36) collected in 2006 revealed that bacterial communities differed significantly between islands/atolls (Global R = 0.273, P = 0.001). PERMANOVA tests also indicated that coral-associated bacterial communities differed significantly between islands/atolls (Pseudo-F = 1.943, P = 0.001) and between sites within islands/atolls (Pseudo-F = 1.342, P = 0.031); however, post-hoc pairwise comparisons did not yield significant differences between sites. ANOSIM pairwise test values between island/atolls (Table 1D) indicated that French Frigate Shoals significantly differed from Gardner, Kaneohe Bay, and Johnston Atoll. In addition to French Frigate Shoals, Kaneohe Bay also differed significantly from Nihoa and Johnston Atoll. SIMPER analysis revealed the
average similarity between and within island/atolls (Table 2D). T-RFs that contributed most to observed differences between French Frigate Shoals and the islands/atolls listed above were 324 (10.4%), 251 (11.8%), and 57 (11%), respectively. T-RF 251 also contributed most to observed differences between Kaneohe Bay and Nihoa and Johnston Atoll at 12.1% and 12.2%, respectively. An initial NMDS ordination of all coral samples from 2006 graphically illustrates differences between individual samples (Figure 3.5A), while a subsequent NMDS ordination of ANOSIM pairwise test values (Table 1D) summarizes overall differences found between islands/atolls (Figure 3.5B).

**Correlations with temperature and depth**

BEST analysis results did not reveal significant correlations between temperature or depth with bacterioplankton community structure for 2005 or 2006. Similarly, the analysis did not indicate a significant correlation between temperature or depth with coral-associated bacterial community structure for 2005, but did show a significant correlation for both variables with community structure in 2006 ($\rho = 0.129; P = 0.04$).

**Correlation with geographic distance**

RELATE analyses did not reveal a significant relationship between Bray-Curtis dissimilarities, based on square root-transformed T-RFLP data, and geographic distances for planktonic bacterial communities sampled in 2005 ($\rho = 0.106, P = 0.161$). However, when T-RFLP data were transformed to presence/absence only, a weak, but significant relationship was revealed ($\rho = 0.190, P = 0.031$). For planktonic communities sampled in 2006, RELATE analyses indicated a weak, but significant relationship between Bray-
Curtis dissimilarities and geographic distances for square root-transformed ($\rho = 0.133$, $P = 0.028$) and presence/absence-transformed data ($\rho = 0.127$, $P = 0.037$). Similarly, RELATE analyses revealed a weak, yet significant relationship between Bray-Curtis dissimilarities and geographic distances for coral-associated bacterial communities sampled in 2005 (square root-transformed: $\rho = 0.16$, $P = 0.001$; presence/absence: $\rho = 0.14$, $P = 0.002$) and 2006 (square root-transformed: $\rho = 0.12$, $P = 0.015$; presence/absence: $\rho = 0.112$, $P = 0.019$).

**Discussion**

*Differences between planktonic and coral-associated bacterial communities*

Our findings indicate that corals harbor distinctly different bacterial communities from their surrounding seawater environment, concordant with previous observations (Rohwer et al. 2001, 2002, Rosenberg et al. 2007). Although we detected the same order of magnitude of T-RFs amongst sample types, differences in community composition were evident and coral-associated bacteria samples were more variable than seawater samples. The lack of significant overlap between the two sample types reinforces the idea that corals provide a unique habitat for microbial assemblages and that microbes form unique associations with the coral host organism (Rohwer and Knowlton 2002).

For the most part, T-RFs contributing most to observed differences between islands/atolls were different for each sample type and year. However, a few T-RFs were common amongst several pairs of islands/atolls within a sample type for a particular year and others, such as T-RF 57, were common across both sample types and years.
Sequence analysis would resolve the identity of these individual bacterial ribotypes and perhaps shed some light on their ecological functions within seawater and coral habitats.

*Molecular biogeography of planktonic and coral-associated bacteria*

Overall, we detected relatively weak, but significant distance-based relationships for planktonic and coral-associated bacterial communities sampled within the Hawaiian Archipelago and wider Pacific Ocean. Thus, geographic isolation is, in part, responsible for driving observed divergences (Martiny et al. 2006). As a logical first assumption, high bacterial dispersal rates might explain these relatively weak correlations (Peterson and Denno 1998, Hanson et al. 2012). However, we also detected several geographic groupings amongst island/atolls that were not distance-based, indicating that additional factors are affecting the distributions of bacterial communities associated with *Porites lobata* corals and seawater.

Differences in dispersal modes may, in part, explain our observation that planktonic bacterial communities exhibited higher connectivity than coral-associated bacterial communities at both the inter- and intra-island/atoll scale – with coral-associated communities differing between and within islands/atolls and planktonic communities differing between islands/atolls to a lesser degree, but not within island/atolls. Long-range dispersal in both types of bacterial communities may be highly dependent on ocean currents; however, association with an invertebrate host may markedly affect or even limit the dispersal capabilities of coral-associated bacterial communities (Taylor et al. 2005). Additionally, differences in homogeneity between the two substrate types may also explain observed differences. In the long-term, planktonic
communities are continuously suspended in the plankton and subjected to a relatively mixed seawater environment, whereas coral-associated communities are exposed to numerous microniches within a benthic, immobile substrate (Gates and Ainsworth 2011).

It is important to note that while the PERMANOVA global test revealed significant pairwise differences between sites within an island/atoll for coral-associated communities, post-hoc pseudo-t tests did not. It is known that a global test can detect a difference of means even in cases where no individual post-hoc tests of the pairs of means will yield a significant result due to differences in sensitivity of the two types of tests. The post-hoc tests are regarded as being more conservative than global tests and in some cases, the data are sufficient to reveal that true means differ but not which pairs of means differ. Our sample sizes at the site level may have not enabled us to detect pairwise differences and increased replicates could help resolve this issue.

Determining the role of dispersal in structuring coral-associated bacterial communities is further complicated by the gradient of host-bacteria specificity possible within these associations. Researchers have only recently begun to investigate and identify potential bacterial symbioses in corals (Apprill et al. 2009, Sharp et al. 2010). The underlying nature of these symbioses (e.g., mutualistic, commensal, parasitic), which for the most part remain unexplored, undoubtedly impact the distribution of coral-associated bacterial communities.

Identifying the mode of transmission in these symbioses (e.g., vertical or horizontal) remains an unresolved priority (Dale and Moran 2006). If corals take up bacteria from the environment as free-swimming larvae in the plankton, we might expect a more cosmopolitan or homogenous bacterial community (Laue and Nelson 1997,
Nelson and Fisher 2000, Harmer et al. 2008). However, if larvae are brooded or if offspring initiate associations with locally adapted bacteria after settlement and metamorphosis, we might expect more heterogeneous and geographically structured communities. Similarly, vertical, or maternal transmission, would result in relatively structured populations that reflect the phylogeography of the host coral (Peek et al. 1998, Hosokawa et al. 2006).

Our study species, *Porites lobata*, is a gonochoric broadcast spawner which obtains its symbiotic zooxanthellae through vertical transmission (Fadlallah 1983). Due to this built-in nutritional source, the species is thought to have a relatively long planktonic larval duration and therefore, high dispersal potential (Glynn et al. 1994, Polato et al. 2010). Polato et al. (2010) found that *Porites lobata* corals, collected from the same islands/atolls investigated here, conformed to a pattern of isolation by distance within the Hawaiian Archipelago and were strongly isolated from Johnston Atoll corals. They concluded that while the majority of gene flow was occurring at adjacent reefs, inter-island distances were insufficient to generate strong population structure across the archipelago. Similarly, we detected, weak, but significant isolation by distance patterns in coral-associated bacterial communities and found that Johnston Atoll differed significantly from some, but not all islands/atolls within the Hawaiian Archipelago. The parallels in these findings support the existence of a close relationship between *Porites lobata* corals and their bacterial associates and also highlight the impact that host dispersal may have on bacterial community structure. However, the ratio of truly symbiotic resident bacteria versus transient “visitor” bacteria is essentially unknown for these corals (Ritchie 2006), and this is likely to skew correlations between bacterial
community structure and geographic distance. Identifying and developing specific
genetic markers for symbionts, prevalent bacteria, and bacteria driving observed
differences between locales, may help resolve the biogeography of these communities on
a finer scale.

*Relationship with Johnston Atoll*

The biogeographic connection between Johnston Atoll and the Hawaiian
Archipelago is of further interest when interpreting the results of this study. Johnston
Atoll is the only island outside of the Hawaiian Archipelago that is considered to be part
of the Hawaiian biogeographic province based on faunal similarities and there is
compelling evidence that it serves as a stepping stone to species colonization in the
simulations and high-resolution ocean current data to identify two potential larval
transport corridors – one connecting Johnston with the middle portion of the Hawaiian
Archipelago in the vicinity of French Frigate Shoals and another connecting Johnston
with lower islands in the vicinity of Kauai. Recent genetic analyses and larval dispersal
simulations of several Indo-Pacific fish species have also provided evidence of a
dispersal corridor between Johnston Atoll and the middle of the Hawaiian Archipelago
(Gaither et al. 2011, Rivera et al. 2011, Andrews et al. *in prep*). Finally, some
invertebrate species, such as *Acropora cytherea* table coral, exhibit geographic
distributions concurrent with the presence of a dispersal corridor between the two locales
(Kenyon et al. 2007).
Our observations for seawater and coral-associated bacterial communities revealed similar connections to Johnston Atoll. Seawater bacterial communities from Johnston Atoll were significantly different from communities at the lower end of the archipelago (Oahu), but not from the middle portion (French Frigate Shoals and Nihoa). Coral-associated bacterial communities from Johnston Atoll were also significantly different from those at the lower end of the archipelago (Oahu), but not from the middle portion (Gardner Pinnacles and Nihoa), with the exception of French Frigate Shoals. Dispersal strategies in seawater versus coral-associated bacterial communities might explain differences observed with French Frigate Shoals. Population genetic analysis of *Porites lobata* by Polato et al. (2010) revealed little gene flow between Johnston Atoll and the Hawaiian Islands; however, evidence of rare dispersal events between the two populations was observed. Specifically, genotypes with migrant ancestry were found in the Hawaiian Archipelago (French Frigate Shoals, Gardner Pinnacles, and Pearl & Hermes Atoll) and Johnston Atoll, indicating that larval exchange occurs in both directions and that connectivity between these populations is maintained over evolutionary, if not ecological, time scales. Seawater bacterial communities, on the other hand, may not be as dispersal limited. The observed biogeographic patterns of seawater and coral-associated bacterial communities in this study provide evidence that both free-living and host-associated microbes are subjected to similar processes that influence the biogeography of marine macrofauna. Further study is needed to elucidate the mechanisms governing the microbial structuring of marine ecosystems.
Additional need for environmental and temporal data

The fact that some geographic groupings amongst island/atolls were not distance-based, further supports the presence of additional environmental factors affecting the distributions of these bacterial communities (Martiny et al. 2006). We examined a limited range of environmental variables and our findings are discussed below.

Our observation that coral-associated bacterial communities did not differ amongst habitats at French Frigate Shoals is notable, but warrants further study. Results should be interpreted with caution because data were only obtained for a single year at one atoll due to logistic constraints. It is more likely that a suite of environmental conditions coinciding with habitat type shape coral-associated bacterial communities and those variables were not measured in this study. This is emphasized by the observation that similar habitats within other islands/atolls differed significantly.

For the most part, we did not observe correlations between temperature or depth with bacterioplankton or coral-associated bacterial community structure – with the exception of coral samples from 2006. However, temperature data were collected at a single point in time and it is possible that temperature variation is more important than any single value. Relationships with temperature and *Porites lobata* bacterial communities could be explored further by assessing temporal fluctuations in temperature and examining potential shifts in bacterial community structure (Littman et al. 2010). Future studies incorporating comprehensive measurements of local oceanographic and environmental conditions over appropriate time scales would help elucidate the affects that specific environmental variables have on shaping coral-associated bacterial communities.
Summary and Broader Impacts

We chose to examine coral-associated bacterial communities due to the critical role that corals play in maintaining reef ecosystems and because they are critically threatened by bleaching and disease – both of which have been associated with bacterial pathogens – on a global scale. By exploring the biodiversity and biogeography of bacteria associated with *Porites lobata* corals and seawater across the Hawaiian Archipelago and wider Pacific Ocean, we demonstrated that seawater bacterial communities are more dispersive than coral-associated bacterial communities. We also found that in addition to dispersal, other unidentified factors – environmental conditions or stochastic variation – have a significant influence on the distribution and structure of these bacterial communities. Understanding the processes that affect coral-associated bacterial communities, in particular, has significant implications for coral health and the future of coral reef ecosystems. This study serves as a first baseline assessment of coral-associated bacterial community variability in an environment that is relatively removed from direct human impacts. However, corals from locations around the globe, including these, remain threatened by global scale disturbances (e.g., global climate change, plastic pollutants and other marine debris). Addressing threats to coral health on such a large scale is difficult and impractical. However, focusing on the maintenance of coral health at the local level may be the best possible approach for ensuring the conservation of coral reef ecosystems. Developing tools to identify specific types of bacteria associated with corals during non-diseased and diseased states, as well as those linked with specific environmental stressors, may enable scientists and resource managers to monitor coral health and mitigate local threats more effectively.
Acknowledgements

This research was funded by a research partnership between the Northwestern Hawaiian Island Coral Reef Ecosystem Reserve and the Hawaii Institute of Marine Biology (NMSP MOA 2005-008/66882). We gratefully acknowledge the assistance of Jamie Becker, Anderson Mayfield, Megan Huggett, Elizabeth Hambleton, and Ale’alani Dudoit for their assistance in the field and laboratory. We thank the Captain and crew of the Hi’ialakai for ensuring our safety during research cruises and dive operations. We also extend our gratitude to the staff at the Papahānaumokuākea Marine National Monument for their assistance with permitting and cruise preparations.

References


de Wit, R. and Bouvier, T. 2006. ‘Everything is everywhere, but, the environment selects’; what did Baas Becking and Beijerinck really say? *Environmental Microbiology* 8: 755-758.
Tables

Table 3.1

<table>
<thead>
<tr>
<th></th>
<th>KU</th>
<th>MI</th>
<th>PE</th>
<th>MA</th>
<th>FFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MI</td>
<td>0.607</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PE</td>
<td>0.300</td>
<td>0.055</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MA</td>
<td>0.981*</td>
<td>1.000</td>
<td>0.559*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>0.729</td>
<td>0.429</td>
<td>0.388*</td>
<td>0.278</td>
<td>---</td>
</tr>
<tr>
<td>NE</td>
<td>0.750</td>
<td>1.000</td>
<td>0.164</td>
<td>0.583</td>
<td>0.321</td>
</tr>
</tbody>
</table>

* p < 0.05

<table>
<thead>
<tr>
<th></th>
<th>AS</th>
<th>FFS</th>
<th>NI</th>
<th>KB</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>0.243*</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NI</td>
<td>-0.241</td>
<td>0.778</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>KB</td>
<td>0.610*</td>
<td>1.000*</td>
<td>1.000</td>
<td>---</td>
</tr>
<tr>
<td>JA</td>
<td>0.203*</td>
<td>0.037</td>
<td>-0.036</td>
<td>0.591*</td>
</tr>
</tbody>
</table>

* p < 0.05

<table>
<thead>
<tr>
<th></th>
<th>KU</th>
<th>MI</th>
<th>PE</th>
<th>MA</th>
<th>FFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MI</td>
<td>0.071</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PE</td>
<td>-0.058</td>
<td>-0.074</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MA</td>
<td>0.347*</td>
<td>0.442*</td>
<td>0.496**</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>0.186</td>
<td>0.298*</td>
<td>0.260</td>
<td>0.354**</td>
<td>---</td>
</tr>
<tr>
<td>NE</td>
<td>0.439**</td>
<td>0.452**</td>
<td>0.434**</td>
<td>0.173**</td>
<td>0.348**</td>
</tr>
</tbody>
</table>

* p < 0.05
** p < 0.01

<table>
<thead>
<tr>
<th></th>
<th>GA</th>
<th>FFS</th>
<th>NI</th>
<th>KB</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>0.294*</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NI</td>
<td>0.209</td>
<td>0.059</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>KB</td>
<td>0.118</td>
<td>0.605*</td>
<td>0.508*</td>
<td>---</td>
</tr>
<tr>
<td>JA</td>
<td>0.213</td>
<td>0.289*</td>
<td>0.108</td>
<td>0.668*</td>
</tr>
</tbody>
</table>

* p < 0.05
Table 3.2

<table>
<thead>
<tr>
<th></th>
<th>KU</th>
<th>MI</th>
<th>PE</th>
<th>MA</th>
<th>FFS</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU</td>
<td>56.647</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MI</td>
<td>49.887</td>
<td>82.623</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PE</td>
<td>43.147</td>
<td>51.071</td>
<td>45.766</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MA</td>
<td>35.313</td>
<td>37.908</td>
<td>36.114</td>
<td>56.422</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>45.443</td>
<td>57.894</td>
<td>42.354</td>
<td>59.006</td>
<td>68.484</td>
<td>---</td>
</tr>
<tr>
<td>NE</td>
<td>43.413</td>
<td>57.267</td>
<td>44.895</td>
<td>51.390</td>
<td>62.847</td>
<td>76.018</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>AS</th>
<th>FFS</th>
<th>NI</th>
<th>KB</th>
<th>JA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS</td>
<td>29.696</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>29.249</td>
<td>69.741</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NI</td>
<td>32.796</td>
<td>47.681</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>KB</td>
<td>15.486</td>
<td>21.976</td>
<td>20.697</td>
<td>66.240</td>
<td>---</td>
</tr>
<tr>
<td>JA</td>
<td>22.845</td>
<td>40.944</td>
<td>26.471</td>
<td>10.137</td>
<td>27.747</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>KU</th>
<th>MI</th>
<th>PE</th>
<th>MA</th>
<th>FFS</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td>KU</td>
<td>10.749</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MI</td>
<td>10.230</td>
<td>9.3709</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NE</td>
<td>7.307</td>
<td>7.6645</td>
<td>6.6822</td>
<td>12.235</td>
<td>8.817</td>
<td>15.117</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GA</th>
<th>FFS</th>
<th>NI</th>
<th>KB</th>
<th>JA</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>17.242</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>FFS</td>
<td>8.822</td>
<td>28.564</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NI</td>
<td>10.771</td>
<td>17.511</td>
<td>14.846</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>KB</td>
<td>12.492</td>
<td>3.2948</td>
<td>5.254</td>
<td>22.627</td>
<td>---</td>
</tr>
</tbody>
</table>

97
Figure Legends

**Figure 3.1:** NMDS ordination of seawater (n = 46) and coral-associated (n = 112) bacterial communities sampled in 2005 and 2006, based on Bray-Curtis dissimilarities calculated from the square root transformation of T-RFLP relative abundance data. Seawater samples are represented by open circles and coral samples are represented by squares.

**Figure 3.2:** (A) NMDS ordination of seawater-associated bacterial communities sampled in 2005 (n = 20), based on Bray-Curtis dissimilarities calculated from the square root transformation of T-RFLP relative abundance data. Sample collection location is indicated by closed circles (Kure), closed squares (Midway), closed triangles (Pearl and Hermes), open circles (Maro), open squares (French Frigate Shoals), and open triangles (Necker). (B) Secondary NMDS ordination of ANOSIM pairwise test values (Table 1A) to summarize similarities between islands/atolls.

**Figure 3.3:** (A) NMDS ordination of seawater-associated bacterial communities sampled in 2006 (n = 26), based on Bray-Curtis dissimilarities calculated from the square root transformation of T-RFLP relative abundance data. Sample collection location is indicated by closed circles (American Samoa), closed squares (French Frigate Shoals), closed triangles (Nihoa), open circles (Kaneohe Bay), and open squares (Johnston Atoll). (B) Secondary NMDS ordination of ANOSIM pairwise test values (Table 1B) to summarize similarities between islands/atolls.
**Figure 3.4:** (A) NMDS ordination of coral-associated bacterial communities sampled in 2005 (n = 76), based on Bray-Curtis dissimilarities calculated from the square root transformation of T-RFLP relative abundance data. Sample collection location is indicated by closed circles (Kure), closed squares (Midway), closed triangles (Pearl and Hermes), open circles (Maro), open squares (French Frigate Shoals), and open triangles (Necker). (B) Secondary NMDS ordination of ANOSIM pairwise test values (Table 1C) to summarize similarities between islands/atolls.

**Figure 3.5:** (A) NMDS ordination of coral-associated bacterial communities sampled in 2006 (n = 36), based on Bray-Curtis dissimilarities calculated from the square root transformation of T-RFLP relative abundance data. Sample collection location is indicated by closed circles (Gardner Pinnacles), closed squares (French Frigate Shoals), closed triangles (Nihoa), open circles (Kaneohe Bay), and open squares (Johnston Atoll). (B) Secondary NMDS ordination of ANOSIM pairwise test values (Table 1D) to summarize similarities between islands/atolls.

**Table 3.1:** ANOSIM pairwise test values for seawater-associated bacterial communities sampled in 2005 (A) and 2006 (B) and for coral-associated bacterial communities sampled in 2005 (C) and 2006 (D). A single asterisk indicates significance at the $\alpha = 0.05$ level and a double asterisk indicates significance at the 0.01 level.

**Table 3.2:** SIMPER average similarity between and within island/atolls for seawater-associated bacterial communities sampled in 2005 (A) and 2006 (B) and for coral-
associated bacterial communities sampled in 2005 (C) and 2006 (D). Values expressed as percentages.
Figures

Figure 3.1

2D Stress: 0.237

- coral
- seawater
Figure 3.2

A

B

2D Stress: 0.145

- KU
- MI
- PE
- MA
- FFS
- NE

2D Stress: 0.055

MI

PE

FFS

MA

KU

NE
Figure 3.3

A

B

2D Stress: 0.19

• AS
■ FFS
▲ NI
○ KB
□ JA

2D Stress: 0

KB

AS
NI

JA

FFS
Figure 3.4

A

2D Stress: 0.251

- KU
- MI
- PE
- MA
- FFS
- NE

B

2D Stress: 0
Figure 3.5

A

B

2D Stress: 0.23

GA

FFS

NI

KB

JA

2D Stress: 0
## Appendix

### Appendix IA

<table>
<thead>
<tr>
<th>sample ID</th>
<th>year</th>
<th>Island/atoll</th>
<th>site</th>
<th>latitude</th>
<th>longitude</th>
<th>habitat</th>
<th>depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>905 2</td>
<td>2005</td>
<td>FFS</td>
<td>23</td>
<td>23.8657</td>
<td>-166.23965</td>
<td>back reef</td>
<td>4</td>
</tr>
<tr>
<td>905 4</td>
<td>2005</td>
<td>FFS</td>
<td>33</td>
<td>27.785633</td>
<td>-175.8232167</td>
<td>patch reef</td>
<td>11</td>
</tr>
<tr>
<td>905 6</td>
<td>2005</td>
<td>FFS</td>
<td>H6</td>
<td>23.880583</td>
<td>-166.27675</td>
<td>fore reef</td>
<td>15</td>
</tr>
<tr>
<td>905 8</td>
<td>2005</td>
<td>FFS</td>
<td>R46</td>
<td>23.7694687</td>
<td>-166.261333</td>
<td>fringing reef</td>
<td>12</td>
</tr>
<tr>
<td>905 38</td>
<td>2005</td>
<td>KU</td>
<td>R33</td>
<td>28.41832</td>
<td>-178.371167</td>
<td>fore reef</td>
<td>20</td>
</tr>
<tr>
<td>905 40</td>
<td>2005</td>
<td>KU</td>
<td>R36</td>
<td>28.42052</td>
<td>-178.37151</td>
<td>back reef</td>
<td>3</td>
</tr>
<tr>
<td>905 42</td>
<td>2005</td>
<td>KU</td>
<td>17</td>
<td>28.4316</td>
<td>-178.365933</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>905 44</td>
<td>2005</td>
<td>KU</td>
<td>17</td>
<td>28.4316</td>
<td>-178.365933</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>905 10</td>
<td>2005</td>
<td>MA</td>
<td>R12</td>
<td>25.471107</td>
<td>-170.632167</td>
<td>patch reef</td>
<td>18</td>
</tr>
<tr>
<td>905 12</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>25.3786667</td>
<td>-170.564667</td>
<td>patch reef</td>
<td>9</td>
</tr>
<tr>
<td>905 14</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>25.3982</td>
<td>-170.578833</td>
<td>patch reef</td>
<td>10</td>
</tr>
<tr>
<td>905 28</td>
<td>2005</td>
<td>MI</td>
<td>R3</td>
<td>28.1903333</td>
<td>-177.399533</td>
<td>fore reef</td>
<td>18</td>
</tr>
<tr>
<td>905 30</td>
<td>2005</td>
<td>MI</td>
<td>R7</td>
<td>28.1903333</td>
<td>-177.399533</td>
<td>fore reef</td>
<td>11</td>
</tr>
<tr>
<td>905 46</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57355</td>
<td>-154.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>905 48</td>
<td>2005</td>
<td>NE</td>
<td>2</td>
<td>23.5782187</td>
<td>-154.7064</td>
<td>fringing reef</td>
<td>19</td>
</tr>
<tr>
<td>905 18</td>
<td>2005</td>
<td>PE</td>
<td>R32</td>
<td>27.8391667</td>
<td>-175.753033</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>905 20</td>
<td>2005</td>
<td>PE</td>
<td>33</td>
<td>27.785633</td>
<td>-175.8232167</td>
<td>fore reef</td>
<td>6</td>
</tr>
<tr>
<td>905 22</td>
<td>2005</td>
<td>PE</td>
<td>R26</td>
<td>27.7859</td>
<td>-175.7803167</td>
<td>fore reef</td>
<td>12</td>
</tr>
<tr>
<td>905 24</td>
<td>2005</td>
<td>PE</td>
<td>26</td>
<td>27.8579</td>
<td>-175.8022</td>
<td>back reef</td>
<td>3</td>
</tr>
<tr>
<td>905 26</td>
<td>2005</td>
<td>PE</td>
<td>R39</td>
<td>27.9406167</td>
<td>-175.861733</td>
<td>back reef</td>
<td>19</td>
</tr>
<tr>
<td>AS 23</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 300</td>
<td>-14.18355</td>
<td>-159.66022</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 9</td>
<td>2006</td>
<td>AS</td>
<td>Tutuila, Fagaitua Bay</td>
<td>-14.27032</td>
<td>-161.64834</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 11</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 400</td>
<td>-14.178312</td>
<td>-169.65338</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 21</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 300</td>
<td>-14.18355</td>
<td>-169.60622</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 15</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 400</td>
<td>-14.17812</td>
<td>-169.65338</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 17</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 300</td>
<td>-14.18355</td>
<td>-169.66022</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 27</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 300</td>
<td>-14.18355</td>
<td>-169.66022</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>AS 28</td>
<td>2006</td>
<td>AS</td>
<td>Cfu. pool 300</td>
<td>-14.18355</td>
<td>-169.66022</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>506 002</td>
<td>2006</td>
<td>FFS</td>
<td>R18</td>
<td>23.8509833</td>
<td>-166.3296</td>
<td>patch reef</td>
<td>4</td>
</tr>
<tr>
<td>506 003</td>
<td>2006</td>
<td>FFS</td>
<td>H6</td>
<td>23.880583</td>
<td>-166.27375</td>
<td>fore reef</td>
<td>6</td>
</tr>
<tr>
<td>506 004</td>
<td>2006</td>
<td>FFS</td>
<td>R29</td>
<td>23.87885</td>
<td>-166.145883</td>
<td>patch reef</td>
<td>2</td>
</tr>
<tr>
<td>506 005</td>
<td>2006</td>
<td>FFS</td>
<td>37</td>
<td>23.6743</td>
<td>-166.168</td>
<td>patch reef</td>
<td>2</td>
</tr>
<tr>
<td>506 008</td>
<td>2006</td>
<td>FFS</td>
<td>48</td>
<td>23.8735833</td>
<td>-166.257833</td>
<td>patch reef</td>
<td>3</td>
</tr>
<tr>
<td>506 009</td>
<td>2006</td>
<td>FFS</td>
<td>23</td>
<td>23.8657</td>
<td>-166.23655</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>506 14</td>
<td>2006</td>
<td>JA</td>
<td>JOH-5P</td>
<td>16.77415</td>
<td>-169.4970167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>506 18</td>
<td>2006</td>
<td>JA</td>
<td>25</td>
<td>16.7768167</td>
<td>-169.4900333</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>506 010</td>
<td>2006</td>
<td>JA</td>
<td>JOH-20</td>
<td>16.7611667</td>
<td>-169.5266667</td>
<td>fringing reef</td>
<td>2</td>
</tr>
<tr>
<td>506 011</td>
<td>2006</td>
<td>JA</td>
<td>JA-HIMB3</td>
<td>16.7429333</td>
<td>-169.5378167</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>506 012</td>
<td>2006</td>
<td>JA</td>
<td>JOH-22</td>
<td>16.7643</td>
<td>-169.526833</td>
<td>fore reef</td>
<td>4</td>
</tr>
<tr>
<td>506 013</td>
<td>2006</td>
<td>JA</td>
<td>JOH-23</td>
<td>16.7473</td>
<td>-169.509167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>506 015</td>
<td>2006</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.780333</td>
<td>-169.461833</td>
<td>fore reef</td>
<td>5</td>
</tr>
<tr>
<td>506 016</td>
<td>2006</td>
<td>JA</td>
<td>JOH-5P</td>
<td>16.7185167</td>
<td>-169.551333</td>
<td>back reef</td>
<td>2</td>
</tr>
<tr>
<td>SBE 6.28.06</td>
<td>2006</td>
<td>KB</td>
<td>SBE</td>
<td>21.4190167</td>
<td>-157.7807833</td>
<td>patch reef</td>
<td>1</td>
</tr>
<tr>
<td>CB 6.28.06</td>
<td>2006</td>
<td>KB</td>
<td>CB</td>
<td>21.45705</td>
<td>-157.81135</td>
<td>patch reef</td>
<td>1</td>
</tr>
<tr>
<td>506 001</td>
<td>2006</td>
<td>NI</td>
<td>North side</td>
<td>23.0582667</td>
<td>-161.92855</td>
<td>fringing reef</td>
<td>4</td>
</tr>
<tr>
<td>Sample ID</td>
<td>Year</td>
<td>Island/Aтолл</td>
<td>Site</td>
<td>Longitude</td>
<td>Depth (m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>------</td>
<td>--------------</td>
<td>------</td>
<td>-----------</td>
<td>-----------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1309</td>
<td>2005</td>
<td>PE</td>
<td>R44</td>
<td>175.9080333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1310</td>
<td>2005</td>
<td>MI</td>
<td>R3</td>
<td>177.3998333</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1326</td>
<td>2005</td>
<td>MI</td>
<td>R5</td>
<td>177.3998333</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1333</td>
<td>2005</td>
<td>MI</td>
<td>2</td>
<td>177.3998333</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1334</td>
<td>2005</td>
<td>MI</td>
<td>R3</td>
<td>177.3998333</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1252</td>
<td>2005</td>
<td>PE</td>
<td>33</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1343</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1612</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1611</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1608</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1614</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1613</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1609</td>
<td>2005</td>
<td>KU</td>
<td>KU 17</td>
<td>178.3620333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1140</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>170.5675867</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1141</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>170.5666867</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1258</td>
<td>2005</td>
<td>PE</td>
<td>R26</td>
<td>175.78050167</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1254</td>
<td>2005</td>
<td>PE</td>
<td>33</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1256</td>
<td>2005</td>
<td>PE</td>
<td>R26</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1257</td>
<td>2005</td>
<td>PE</td>
<td>33</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1291</td>
<td>2005</td>
<td>PE</td>
<td>33</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1293</td>
<td>2005</td>
<td>PE</td>
<td>R39</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 1305</td>
<td>2005</td>
<td>PE</td>
<td>R44</td>
<td>175.8320167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3PS</td>
<td>2006</td>
<td>KB</td>
<td>KB</td>
<td>35.5073833</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44PS</td>
<td>2006</td>
<td>KB</td>
<td>KB</td>
<td>35.5073833</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1026</td>
<td>2005</td>
<td>FFS</td>
<td>H6</td>
<td>166.27350167</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1001</td>
<td>2005</td>
<td>FFS</td>
<td>33</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1002</td>
<td>2005</td>
<td>FFS</td>
<td>33</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1004</td>
<td>2005</td>
<td>FFS</td>
<td>33</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1005</td>
<td>2005</td>
<td>FFS</td>
<td>33</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1101</td>
<td>2005</td>
<td>FFS</td>
<td>23</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1012</td>
<td>2005</td>
<td>FFS</td>
<td>23</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1013</td>
<td>2005</td>
<td>FFS</td>
<td>23</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1014</td>
<td>2005</td>
<td>FFS</td>
<td>23</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1016</td>
<td>2005</td>
<td>FFS</td>
<td>23</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1025</td>
<td>2005</td>
<td>FFS</td>
<td>H6</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1027</td>
<td>2005</td>
<td>FFS</td>
<td>H6</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1030</td>
<td>2005</td>
<td>FFS</td>
<td>R46</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1032</td>
<td>2005</td>
<td>FFS</td>
<td>R46</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1033</td>
<td>2005</td>
<td>FFS</td>
<td>R46</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FFS1036</td>
<td>2005</td>
<td>FFS</td>
<td>R46</td>
<td>166.27350167</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4PS</td>
<td>2005</td>
<td>KB</td>
<td>KB</td>
<td>35.5073833</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 MA 6 1174</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>170.5675867</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 MA 12 1103</td>
<td>2005</td>
<td>MA</td>
<td>R12</td>
<td>170.5675867</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 MA 6 1171</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>170.5675867</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1005 NE 4 1641</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>164.70350167</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>506 GA 7 141</td>
<td>2006</td>
<td>GA</td>
<td>7</td>
<td>164.70350167</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>606 JOH-1AP 346</td>
<td>2006</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7860333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>606 JOH-1AP 346</td>
<td>2006</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7860333</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>506 FR 30 201</td>
<td>2006</td>
<td>FFS</td>
<td>30</td>
<td>164.9675833</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>506 NI NS-1 092</td>
<td>2006</td>
<td>NI</td>
<td>NS-1</td>
<td>161.92850167</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>506 NI NS-2 054</td>
<td>2006</td>
<td>NI</td>
<td>NS-2</td>
<td>161.92850167</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 MA 6 1175</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>170.5675867</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 MA 12 1104</td>
<td>2005</td>
<td>MA</td>
<td>R12</td>
<td>170.5675867</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>905 MA 6 1173</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>170.5675867</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1005 NE 4 1642</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>164.70350167</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>506 GA 7 142</td>
<td>2006</td>
<td>GA</td>
<td>7</td>
<td>164.70350167</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>606 JOH-1AP 366</td>
<td>2006</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7860333</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample ID</td>
<td>year</td>
<td>island/atoll</td>
<td>site</td>
<td>latitude</td>
<td>longitude</td>
<td>habitat</td>
<td>depth (m)</td>
</tr>
<tr>
<td>---------------</td>
<td>------</td>
<td>--------------</td>
<td>--------</td>
<td>----------</td>
<td>------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>006 JOH-5P 388</td>
<td>2005</td>
<td>JA</td>
<td>JOH-5P</td>
<td>16.77415</td>
<td>-169.497167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>006 FR 30 202</td>
<td>2005</td>
<td>FFS</td>
<td>30</td>
<td>23.849833</td>
<td>-166.297433</td>
<td>patch reef</td>
<td>2</td>
</tr>
<tr>
<td>006 NI NS-2 003</td>
<td>2005</td>
<td>NI</td>
<td>NS-2</td>
<td>23.002183</td>
<td>-161.920333</td>
<td>fringing reef</td>
<td>4</td>
</tr>
<tr>
<td>1005 NE 4 1648</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>905 MA 6 1176</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>25.3982</td>
<td>-170.579833</td>
<td>patch reef</td>
<td>10</td>
</tr>
<tr>
<td>905 MA R 12 1105</td>
<td>2005</td>
<td>MA</td>
<td>R12</td>
<td>25.471117</td>
<td>-170.642167</td>
<td>patch reef</td>
<td>18</td>
</tr>
<tr>
<td>1005 NE 2 1650</td>
<td>2005</td>
<td>NE</td>
<td>2</td>
<td>23.5782167</td>
<td>-164.7014</td>
<td>fringing reef</td>
<td>19</td>
</tr>
<tr>
<td>1005 NE 4 1643</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>506 GA 7 143</td>
<td>2006</td>
<td>GA</td>
<td>7</td>
<td>25.0000383</td>
<td>-167.99855</td>
<td>fringing reef</td>
<td>6</td>
</tr>
<tr>
<td>606 JOH-1AP 367</td>
<td>2006</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7660333</td>
<td>-169.481333</td>
<td>fore reef</td>
<td>5</td>
</tr>
<tr>
<td>606 JOH-5P 389</td>
<td>2006</td>
<td>JA</td>
<td>JOH-5P</td>
<td>16.77415</td>
<td>-169.497167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>506 FR 33 205</td>
<td>2005</td>
<td>FFS</td>
<td>33</td>
<td>23.8357</td>
<td>-166.2658667</td>
<td>patch reef</td>
<td>1</td>
</tr>
<tr>
<td>1005 NE 4 1649</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>905 MA 6 1177</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>25.3982</td>
<td>-170.579833</td>
<td>patch reef</td>
<td>10</td>
</tr>
<tr>
<td>905 MA 22 1142</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>25.3785667</td>
<td>-170.567467</td>
<td>patch reef</td>
<td>9</td>
</tr>
<tr>
<td>1005 NE 2 1652</td>
<td>2005</td>
<td>NE</td>
<td>2</td>
<td>23.5782167</td>
<td>-164.7054</td>
<td>fringing reef</td>
<td>19</td>
</tr>
<tr>
<td>1005 NE 4 1644</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>506 GA 7 144</td>
<td>2006</td>
<td>GA</td>
<td>7</td>
<td>25.0000383</td>
<td>-167.99855</td>
<td>fringing reef</td>
<td>6</td>
</tr>
<tr>
<td>606 JOH-5P 390</td>
<td>2006</td>
<td>JA</td>
<td>JOH-5P</td>
<td>16.77415</td>
<td>-169.497167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>506 NI NS-2 005</td>
<td>2006</td>
<td>NI</td>
<td>NS-2</td>
<td>23.0621833</td>
<td>-161.920333</td>
<td>fringing reef</td>
<td>4</td>
</tr>
<tr>
<td>1005 NE Shark's Bay 1710</td>
<td>2005</td>
<td>NE</td>
<td>Shark's Bay</td>
<td>23.5782167</td>
<td>-164.7014</td>
<td>fringing reef</td>
<td>7</td>
</tr>
<tr>
<td>905 MA 6 1178</td>
<td>2005</td>
<td>MA</td>
<td>6</td>
<td>25.3982</td>
<td>-170.579833</td>
<td>patch reef</td>
<td>10</td>
</tr>
<tr>
<td>905 MA 22 1143</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>25.3785667</td>
<td>-170.567467</td>
<td>patch reef</td>
<td>9</td>
</tr>
<tr>
<td>1005 NE 2 1652</td>
<td>2005</td>
<td>NE</td>
<td>2</td>
<td>23.5782167</td>
<td>-164.7054</td>
<td>fringing reef</td>
<td>19</td>
</tr>
<tr>
<td>1005 NE 4 1645</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>506 GA 7 145</td>
<td>2006</td>
<td>GA</td>
<td>7</td>
<td>25.0000383</td>
<td>-167.99855</td>
<td>fringing reef</td>
<td>6</td>
</tr>
<tr>
<td>606 JOH-1AP 377</td>
<td>2006</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7860333</td>
<td>-169.481333</td>
<td>fore reef</td>
<td>5</td>
</tr>
<tr>
<td>506 FR 23 252</td>
<td>2006</td>
<td>FFS</td>
<td>23</td>
<td>23.8057</td>
<td>-166.23965</td>
<td>patch reef</td>
<td>2</td>
</tr>
<tr>
<td>506 NI NS-2 006</td>
<td>2006</td>
<td>NI</td>
<td>NS-2</td>
<td>23.0621833</td>
<td>-161.920333</td>
<td>fringing reef</td>
<td>4</td>
</tr>
<tr>
<td>1005 NE Shark's Bay 1711</td>
<td>2005</td>
<td>NE</td>
<td>Shark's Bay</td>
<td>23.5782167</td>
<td>-164.7014</td>
<td>fringing reef</td>
<td>7</td>
</tr>
<tr>
<td>905 MA R 12 1100</td>
<td>2005</td>
<td>MA</td>
<td>R12</td>
<td>25.471117</td>
<td>-170.642167</td>
<td>patch reef</td>
<td>18</td>
</tr>
<tr>
<td>905 MA 22 1144</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>25.3785667</td>
<td>-170.567467</td>
<td>patch reef</td>
<td>9</td>
</tr>
<tr>
<td>1005 NE 2 1653</td>
<td>2005</td>
<td>NE</td>
<td>2</td>
<td>23.5782167</td>
<td>-164.7054</td>
<td>fringing reef</td>
<td>19</td>
</tr>
<tr>
<td>1005 NE 4 1647</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>606 JOH-1AP 308</td>
<td>2005</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7860333</td>
<td>-169.481333</td>
<td>fore reef</td>
<td>5</td>
</tr>
<tr>
<td>606 JOH-1AP 379</td>
<td>2005</td>
<td>JA</td>
<td>JOH-1AP</td>
<td>16.7860333</td>
<td>-169.481333</td>
<td>fore reef</td>
<td>5</td>
</tr>
<tr>
<td>506 FR 23 254</td>
<td>2006</td>
<td>FFS</td>
<td>23</td>
<td>23.8057</td>
<td>-166.23965</td>
<td>patch reef</td>
<td>2</td>
</tr>
<tr>
<td>506 NI NS-2 051</td>
<td>2006</td>
<td>NI</td>
<td>NS-2</td>
<td>23.0621833</td>
<td>-161.920333</td>
<td>fringing reef</td>
<td>4</td>
</tr>
<tr>
<td>905 MA R 12 1101</td>
<td>2005</td>
<td>MA</td>
<td>R12</td>
<td>25.471117</td>
<td>-170.642167</td>
<td>patch reef</td>
<td>18</td>
</tr>
<tr>
<td>905 MA 22 1145</td>
<td>2005</td>
<td>MA</td>
<td>22</td>
<td>25.3785667</td>
<td>-170.567467</td>
<td>patch reef</td>
<td>9</td>
</tr>
<tr>
<td>1005 NE 2 1654</td>
<td>2005</td>
<td>NE</td>
<td>2</td>
<td>23.5782167</td>
<td>-164.7054</td>
<td>fringing reef</td>
<td>19</td>
</tr>
<tr>
<td>1005 NE 4 1647</td>
<td>2005</td>
<td>NE</td>
<td>4</td>
<td>23.57395</td>
<td>-164.7038</td>
<td>fringing reef</td>
<td>11</td>
</tr>
<tr>
<td>606 JOH-23 313</td>
<td>2006</td>
<td>JA</td>
<td>JOH-23</td>
<td>16.7473</td>
<td>-169.593167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>606 JOH-5P 386</td>
<td>2006</td>
<td>JA</td>
<td>JOH-5P</td>
<td>16.77415</td>
<td>-169.497167</td>
<td>back reef</td>
<td>1</td>
</tr>
<tr>
<td>506 FR 33 157</td>
<td>2006</td>
<td>FFS</td>
<td>33</td>
<td>23.8357</td>
<td>-166.2658667</td>
<td>patch reef</td>
<td>1</td>
</tr>
<tr>
<td>506 NI NS 001</td>
<td>2006</td>
<td>NI</td>
<td>NS-1</td>
<td>23.0982667</td>
<td>-161.92855</td>
<td>fringing reef</td>
<td>4</td>
</tr>
<tr>
<td>1005 NE Shark's Bay 1714</td>
<td>2005</td>
<td>NE</td>
<td>Shark's Bay</td>
<td>23.5782167</td>
<td>-164.7054</td>
<td>fringing reef</td>
<td>7</td>
</tr>
</tbody>
</table>
CHAPTER 4

DYNAMICS OF CORAL-ASSOCIATED BACTERIAL COMMUNITIES ON THE
SOUTHERN REEFS OF MOLOKA‘I, HAWAI‘I

Jennifer L. Salerno\textsuperscript{1,2}, Charles Birkeland\textsuperscript{2}, and Michael S. Rappé\textsuperscript{1*}

\textsuperscript{1}Hawaii Institute of Marine Biology, School of Ocean and Earth Science and
Technology, University of Hawaii at Manoa, Kaneohe, HI, 96744

\textsuperscript{2}Department of Zoology, University of Hawaii at Manoa, Honolulu, HI, 96822

*Corresponding author. Mailing address:
Hawaii Institute of Marine Biology, University of Hawaii, P.O. Box 1346, Kaneohe, HI
96744. Phone: (808) 236-7464. Fax: (808) 236-7443. Email: rappe@hawaii.edu
Abstract

The temporal and spatial variability of bacterial communities associated with colonies of the coral *Porites compressa* were examined at two sites along the south shore of Moloka‘i, Hawai‘i, monthly for a five-month period. Terminal-restriction fragment length polymorphism (T-RFLP) analysis of polymerase chain reaction-amplified 16S ribosomal RNA genes was used to assess the structure of seawater and coral-associated bacterial communities. Seawater and coral-associated bacterial communities were unique (ANOSIM Global R = 0.859, p = 0.001) and site differences were observed for both types of communities (ANOSIM Global R = 0.859, p = 0.001 and Global R = 0.435, p = 0.001, respectively). Analysis of environmental data mirrored this result, revealing significant differences in the environmental parameters measured at each site. Environmental parameters did not significantly correlate with planktonic communities (BEST ρ = 0.423, p = 0.052); however, silicate concentrations significantly correlated with coral-associated bacterial community structure at the Kamalō site (BEST ρ = 0.569, p = 0.01). Bacterial communities did not differ amongst colonies within a site (PERMANOVA Pseudo-F = 1.177, p = 0.074), but fluctuated from month-to-month (PERMANOVA Pseudo-F = 1.589, p = 0.001).

Introduction

Our current understanding of the coral holobiont system is that bacterial associates may function as both beneficial and harmful agents to the host coral organism (Rosenberg et al. 2007). Healthy corals harbor diverse and unique bacterial assemblages that may benefit the coral organism through antibiotic production (Ritchie 2006), nutrient
acquisition (Wafar et al. 1990, Lesser et al. 2004), and carbon transformations (Ducklow and Mitchell 1979, Rohwer et al. 2001, 2002). In contrast, bacteria have also been identified as the causative agents for a number of lethal coral diseases (e.g. Patterson et al. 2002, Rosenberg et al. 2007, Sussman et al. 2008, Richardson et al. 2009). However, relatively little is known about the diversity and dynamics of coral-associated bacterial communities during transition from healthy to diseased states. Current research indicates that specific environmental stressors may cause detrimental shifts in the resident microbiota, ultimately leading to disease (Lesser et al. 2007), or, in some cases, that harmful foreign pathogens may be introduced to corals through human activities (Lipp et al. 2002). Further complicating our understanding of coral disease processes is the lack of information regarding the temporal and spatial variability of coral-bacterial associations during non-diseased states.

Sedimentation is particularly prevalent within the back reef areas of southern Moloka‘i, with corals being exposed to suspended sediment concentrations that have been shown to adversely affect coral health (Dodge et al. 1974, Dodge and Vaisnys 1977). Exposure of this magnitude has been implicated in substantial reef degradation along this coast (Jokiel et al. 2004). Sedimentation is known to negatively affect corals by inhibiting larval recruitment (Wittenberg and Hunte 1992), reducing fecundity, decreasing calcification, shielding light needed by zooxanthellae for photosynthesis (Bak 1978, Hayward 1982), and smothering the cnidarian host (Rogers 1990, Tomascik and Sander 1985, 1987, Riegl and Branch 1995, Nugues and Roberts 2003). Although some coral species are able to tolerate significant sedimentation and may even use sediment-bound organic matter as a food source (Te 1992, Woolfe and Larcombe 1998, Rosenfeld
et al. 1999, Anthony 2000), most are impaired by moderate levels of sedimentation (Rogers 1983, 1990; Szmant et al. 2000). In addition to directly affecting the coral organism, increased sediment loading may indirectly impact coral health by causing changes in the composition of coral-associated bacterial communities through nutrient enrichment and/or by introducing foreign bacteria from terrestrial sources (Frias-Lopez et al. 2002, Patterson et al. 2002, Smith et al. 1996).

Here, we set out to answer some fundamental questions pertaining to coral-bacterial associations by tracking changes in the bacterial community composition of *Porites compressa* coral colonies along the south shore of Moloka‘i over a 5-month period. In addition to addressing the overall temporal and spatial variability of these associations, we sought to examine how environmental variables may influence the structure and stability of coral-associated bacterial communities. To address this particular objective, physical and chemical properties of seawater were measured including temperature, salinity, pH, chlorophyll *a*, and nutrient concentrations. In addition, sediment resuspension was approximated with a turbidity measurement due to its potential role in affecting coral-associated bacterial communities and possibly, coral health.

**Methods**

**Site description**

Two back reef sites on the island of Moloka‘i, Hawaii were selected because of existing coral cover and sedimentation data at these locations (Ogston et al. 2004, Storlazzi et al. 2004, Presto et al. 2006). One is located near the town of Kamalō, downstream of the Kamalō gulch, and the other is located near the town of Kamiloloa,
offshore of the Kamiloloa condominium complex and downstream of the Kaloko‘eli fishpond (Figure 1). Both sites were located approximately 750 m from the shoreline at a high tide depth of approximately 3 m.

Sample collection

Seawater and corals were sampled monthly from April to August 2007, 1 to 3 days following the new moon. *Porites compressa* (Dana 1846), a common and ecologically important reef-building coral endemic to Hawaii, was chosen as the target species for this study. Samples were collected from four randomly chosen colonies within a reef at each site, with 2 to 4 meters separating colonies. In order to address intra-colony variability, three random sub-samples were obtained from each colony via a stainless steel core borer and combined for subsequent analyses. Each sample consisted of a 6-mm diameter, 6-mm deep core that included the coral tissue, overlying mucus layer, and underlying skeleton. Coral samples were immediately placed on ice and transported to the laboratory, where they were stored frozen at -80 °C until further processing. Due to poor visibility during high tides, coral samples were only collected during low tides.

Seawater samples were collected at both low and high tide except for the month of April, where only low tide samples were obtained. One liter of seawater was collected adjacent to the sampled coral heads and filtered through a 25 mm diameter, 1.6 μm pore-sized GF/A glass microfiber filter (Whatman International Ltd., Piscataway, NJ) followed by a 13 mm diameter, 0.2 μm pore-sized polyethersulfone membrane filter (Supor-200; Pall Corp., East Hills, NY). Filters were stored in 250 μL of DNA lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA
pH 8.0, 1.2% v/v Triton X100) (Suzuki et al. 2001) and frozen at -80 °C until further analysis.

An instrumented probe (YSI, Yellow Springs, Ohio) was used to measure salinity, temperature, turbidity, and fluorescence in the water column at each site during high and low tides. An additional seawater sample (150 ml) was collected at each site for spectrophotometric analyses of chlorophyll $a$ in the laboratory. Finally, a 250-ml seawater sample was collected and frozen (-20°C) for subsequent nutrient analysis (total nitrogen, phosphorous, and silicate).

**T-RFLP of bacterial SSU rRNA genes**

After thawing, 2 mL of 0.2 µm-filtered 10X Tris EDTA (100 mM Tris, 10 mM EDTA) buffer solution (pH 7.4) was added to each sample. Coral tissue was removed from the skeleton using an air gun with an attached sterile pipette tip, and the resulting slurry was centrifuged at 19,900 RCF for 30 min at 4°C. The supernatant was subsequently removed from the sample and the remaining sample pellet was frozen at -80°C until processed further.

After thawing, genomic DNA was extracted from the coral tissue pellets and seawater filters using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer’s protocol. Genomic DNA was eluted in sterile, 0.1 µm-filtered water and stored frozen at -20°C. Total genomic DNA yield was assessed on a SpectraMax M2 plate reader (Molecular Device Corp., Sunnyvale, CA, USA) using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Corp., Carlsbad, CA, USA), prepared according to manufacturer’s protocol.
For terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997), the general bacterial primers 27F-B-FAM (5’-FAM-AGRGTTYGATYMTGGCTCAG-3’) and 1492R (5’-GGYTACCTGGTTACGACTT-3’) were used for the amplification of small subunit ribosomal RNA (16S rRNA) genes from each sample via the polymerase chain reaction (PCR). The MasterTaq System (Eppendorf AG, Hamburg, Germany) was used for all PCR reactions, which were composed of the following (final concentrations): 1X MasterTaq reaction buffer, 2.25 mM Mg^{2+}, 0.5X TaqMaster reaction enhancer, 0.2 mM each of the forward and reverse primers, 0.2 mM of each dNTP (Promega, Madison, WI), approximately 138 ±58 ng of genomic DNA template, 2.5 units of MasterTaq DNA polymerase, and sterile water to a final reaction volume of 50 µl. A MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) and touchdown protocol were used, which, after a 3 min incubation at 95°C, consisted of 30 cycles of 30 sec at 95°C, 1 min at 65°C (decreasing by 0.5°C per cycle), and 2 min at 72°C. This was followed by 10 cycles of 30 sec at 95°C, 1 min at 50°C, and 2 min at 72°C, and 1 cycle of 30 sec at 95°C, 1 min at 50°C, and 20 min at 72°C.

The fluorescently labeled amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc, Valencia, CA) following the manufacturer’s instructions. Approximately 100 ng of each purified amplicon was subsequently digested in a 10 µL reaction containing 5 units of HaeIII restriction endonuclease (Promega, Madison, WI) at 37°C for 6 hours. After purification via gel filtration chromatography with Sephadex G-50 (Amersham Biosciences, Sweden), the restricted samples were adjusted to a final concentration of 40 ng µL^{-1} and separated via capillary electrophoresis on an automated
ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). GeneMapper Software version 3.0 (Applied Biosystems, Foster City, CA) was used to estimate the size and relative abundance of the resulting terminal restriction fragments (T-RFs), which were defined as fragments between 50 and 600 base pairs (bp) in length. Fragment lengths were rounded to the nearest integer value, aligned, and manually checked for possible errors in peak determination due to such factors as instrument variability. The threshold below which peaks were excluded was determined via the variable percentage threshold method as described in Osborne et al. (2006).

Statistical analysis

After square root transformation of the T-RFLP data matrix, all subsequent statistical analyses were carried out in PRIMER 6 Version 6.1.13 and PERMANOVA + Version 1.0.3 (PRIMER-E Ltd., Plymouth, UK) (Clarke and Gorley 2006, Anderson and Gorely 2008, Clarke and Warwick 2001).

A resemblance matrix was constructed based on Bray-Curtis similarity (REFERENCE). Hierarchical clustering analysis (CLUSTER) of the resemblance matrix was used to construct similarity dendrograms and a similarity profile permutation test (SIMPROF) was used to identify significant “natural groupings” of samples that were not structured a priori. CLUSTER analyses were based on the group average linking option and SIMPROF analyses were performed at a 5% significance level.

Nonmetric multidimensional scaling (NMDS) ordination of the resemblance matrix was used to provide a 2-dimensional graphical representation of similarities in bacterial community structure (based on T-RFLP peak abundances) amongst samples.
(Shepard 1962, Kruskal 1964). NMDS was chosen because it makes no assumptions about the underlying distribution of data and is therefore appropriate for analyzing complex ecological communities. In some cases, data from CLUSTER analyses were used to overlay Bray-Curtis similarity boundaries onto NMDS plots to display percent similarity amongst samples.

An analysis of similarity (ANOSIM), performed on the resemblance matrix, was used to test for differences in bacterial community structure between pre-defined sample groups. The ANOSIM test is analogous to a standard univariate 1-way analysis of variance (ANOVA) and group specifications were made a priori. A ‘similarity percentages’ routine (SIMPER) was used to identify the role of individual species in contributing to group differences as well as within group closeness. SIMPER dissects average Bray-Curtis dissimilarities between all pairs of samples into percentage contributions from each species and lists them in decreasing order of contribution (Clarke and Gorley 2006). A 90% cut-off was employed to focus on higher-contributing variables.

A permutational multiple analysis of variance (PERMANOVA) of the resemblance matrix was used to test for differences in bacterial community structure between sites, colonies, months, and tides. PERMANOVA tests the simultaneous response of one or more variables to one or more factors in an analysis of variance (ANOVA) experimental design on the basis of a resemblance measure, using permutation methods. The routine calculates an appropriate distance-based pseudo-F statistic for each term in the model based on the expectations of mean squares, and p-values are obtained using an appropriate permutation procedure for each term (Anderson et al. 2008).

117
PERMANOVA tests were run with the following specifications: 999 permutations, permutation of residuals under a reduced model, Type III (partial) sum of squares, Monte Carlo tests, and fixed effects sum to zero.

A data matrix containing all measurements of environmental variables was constructed for use in Principle Components Analysis (PCA). Prior to analysis, environmental data (with the exception of temperature, salinity and pH) were log-transformed and values for all parameters were normalized. PCA is a non-parametric mathematical procedure that is used to identify patterns in complex, multivariate data sets. Here, we used PCA to examine differences in physical and chemical parameters measured in the surrounding seawater environment. Using orthogonal transformation, samples, represented as points distributed in high-dimensional variable space (with as many dimensions as there are variables), are projected onto a low-dimensional plane that is the “best fit” of the data. The new axes, or principal components, capture as much of the variability in the original space as possible and a percent variance is assigned to each axis, along with linear coefficients defining each principle component (Clarke and Gorely 2006). A minimum of 10 principal components was selected for the analysis.

The Bio-Env + Stepwise (BEST) analysis was used to examine the relationship between bacterial community structure amongst samples and the environmental variables associated with those samples (i.e. to determine the degree to which the environmental data explain the observed biotic pattern). For this analysis, environmental data (with the exception of temperature, salinity, and pH) were log-transformed and values for all parameters were normalized. The Bio-Env option, spearman rank correlation method, and permutation test (99 permutations) for significance were chosen for the analysis.
Results

Coral vs. seawater-derived bacterial community structure

Both CLUSTER and ANOSIM analyses revealed that the structure of coral-associated (n=33) and planktonic (n=18) bacterial communities sampled over the course of the study were significantly different (Global $R = 0.859$, $p = 0.001$). An NMDS ordination derived from all 51 coral and seawater samples for which T-RFLP-based bacterial community structure data was obtained also visually resolved two distinct clusters (Figure 2). Overall, there were 184 different ribotypes found in seawater samples and 387 found in coral samples, with 172 ribotypes shared between the two sample types. Only 12 ribotypes were unique to seawater samples, while coral samples contained 215 unique ribotypes.

Planktonic bacterial communities

CLUSTER and ANOSIM analyses revealed a significant difference between the planktonic bacterial communities sampled from the Kamiloloa and Kamalō sites (Global $R = 0.25$, $p = 0.002$). Additionally, a PERMANOVA test revealed significant differences between the two sites (Pseudo-$F = 2.243$, $p = 0.038$). SIMPER analysis indicated an average dissimilarity of 50.6% between Kamiloloa and Kamalō planktonic bacterial communities. Terminal restriction fragments (T-RFs) with lengths of 66, 225, 385, 384, and 209 base pairs (bp) were the top contributors to observed differences, accounting for 2.8%, 2.7%, 2.5%, 2.0%, and 1.8% of the average dissimilarity between the two sites, respectively. All five T-RFs were present at both sites, but varied in abundance. For example, Kamalō exhibited 55.3% average within-group similarity with T-RFs of 66,
385, 221, 225, and 189 bp being ranked as the top contributors and Kamiloloa exhibited 50.3% average within-group similarity with T-RFs of 385, 221, 66, 189, and 181 bp being ranked as the top contributors.

Using PERMANOVA tests on samples separated by site, it was determined that monthly changes in planktonic bacterial community structure were non-significant (Pseudo-\(F = 1.646, p = 0.053\)), and no significant differences were observed between high and low tides for each month (Pseudo-\(F = 1.590, p = 0.152\)). An NMDS ordination of planktonic bacterial community structure data underscored the separation between samples derived from the Kamiloloa and Kamalō field sites, and also highlighted variability observed within each site (Figure 3).

*Coral-associated bacterial communities*

CLUSTER and ANOSIM analyses of coral-associated bacterial community structure revealed a distinct difference between coral samples derived from the Kamiloloa and Kamalō study sites (Global \(R = 0.435, p = 0.001\)). A PERMANOVA test confirmed this observation (Pseudo-\(F = 2.785, p = 0.025\)). SIMPER analysis revealed an average dissimilarity of 67.3% between Kamiloloa and Kamalō coral-associated bacterial communities. T-RF with lengths of 264, 308, 191, 203, and 226 bp were the top contributors to observed differences, accounting for 2.3%, 1.6%, 1.5%, 1.5% and, 1.4% of the average dissimilarity between the two sites, respectively. All five T-RFs were present at both sites, but varied in abundance. For example, Kamalō exhibited 35.2% average within-group similarity with T-RFs of 166, 225, 203, 264, and 226 bp being ranked as the top contributors, and Kamiloloa exhibited 39.3% average within-group
similarity with T-RFs of 203, 191, 225, 286, and 166 bp being ranked as the top contributors.

A PERMANOVA test showed that coral-associated bacterial communities did not differ between colonies within a site for a particular month (Pseudo-F = 1.177, p = 0.074), but revealed significant temporal variability within a site from month to month (Pseudo-F = 1.589, p = 0.001). An NMDS ordination of T-RFLP derived coral-associated bacterial community structure data illustrates the separation between the Kamiloloa and Kamalō field sites, as well as the monthly variability of these communities at each site (Figure 4).

An ANOSIM analysis of coral-associated bacterial communities from Kamiloloa supported the observation that communities varied from month to month (Global R = 0.206, p = 0.011). Samples collected during the month of April contributed most to observed differences, and were significantly different from those collected in May (R = 0.479), June (R = 0.537), and August (R = 0.406). The remaining months were not significantly different from one another.

SIMPER analysis indicated an average within-group similarity of 43.1% for the month of April. T-RFs of 203, 246, and 166 bp were the top contributors to observed within-group similarity, accounting for 6%, 4.4%, and 4.2%, respectively. For the month of May, the average within-group similarity was 36.5%, with T-RFs 203, 191, and 225 contributing to 12.2%, 10.8%, and 7%, respectively. Average within-group similarity for the month of June was 37.3%, with T-RFs 203, 191, and 166 contributing to 15.6%, 7.5%, and 7%, respectively. Average within-group similarity for the month of July was 43.6%, with T-RFs 203, 225, and 406 contributing to 6.7%, 5%, and 4.7%, respectively.
Finally, SIMPER analysis showed that within-group similarity for the month of August was 41.6%, with T-RFs 203, 286, 225 contributing to 7.1%, 4.4%, and 4.3%, respectively.

An ANOSIM analysis of coral-associated bacterial communities collected from the Kamalō site also indicated that community structure varied temporally (Global R = 0.499, p = 0.002). Samples taken in April were significantly different than those taken during May (R = 1), June (R = 0.917), July (R = 1), and August (R = 0.833), with limited overlap in bacterial community structure. Samples collected in May were also different from those collected in June (R = 0.444), July (R = 0.63), and August (R = 0.481), but still overlapped somewhat. Samples from June greatly overlapped with those from July (R = -0.296) and August (R = 0). July and August also significantly overlapped (R = 0.148).

SIMPER analysis indicated an average within-group similarity of 51.1% for the month of April. T-RFs of 191, 225, and 400 bp were the top contributors to observed within-group similarity, accounting for 7.7%, 7.4%, and 5.7%, respectively. Average within-group similarity for May was 45.7%, with T-RFs 166, 225, and 191 contributing to 6.6%, 6.6%, and 5.9%, respectively. The month of June had an average within-group similarity of 41.2%, with T-RFs 264, 166, and 225 contributing to 9.6%, 8.6%, and 6.2%, respectively. Average within-group similarity for July was 37.8%, with T-RFs 264, 166, and 225 contributing to 11.3%, 8.8%, and 8.4%, respectively. Finally, SIMPER analysis revealed that August had a within-group similarity of 35.2%, with T-RFs 226, 166, and 203 contributing to 14.4%, 13.2%, and 9.4%, respectively.
Correlations with environmental data

A PCA ordination of environmental variables revealed distinct differences in the physical and chemical parameters measured at the two sites (Figure 5). PCA eigenvalues indicated that axes PC1 and PC2 accounted for 41% and 20% of variability amongst samples, respectively. Turbidity and phosphate concentrations contributed primarily to the PC2 axis, along with nitrite, silicate, ammonia, temperature and chlorophyll \(a\) all increasing in the negative PC2 direction. Chlorophyll \(a\) did not account for a significant portion of the variability observed amongst samples. Salinity, total nitrogen, and pH contributed mainly to the PC1 axis, with salinity and total nitrogen increasing and pH decreasing in the positive PC1 direction.

Overall, seawater from the Kamiloloa site was characterized as being slightly warmer, more turbid, more basic, less saline and having higher concentrations of phosphate and silicate relative to Kamalō seawater. Samples from both sites had increased concentrations of nitrite, ammonia, and total nitrogen towards the positive PC2 and negative PC1 directions.

Results from the BEST analysis for Kamiloloa indicated that the combination of silicate, pH, and turbidity ranked highest in correlating with patterns of planktonic bacterial community structure at that site. However, the rank correlation of \(\rho = 0.406\) was not statistically significant (\(p = 0.54\)). Results from the BEST analysis for Kamalō indicated that phosphate, total nitrogen, silicate, nitrite, and ammonium ranked highest in correlating with patterns of bacterioplankton community structure at that site. However, the rank correlation of \(\rho = 0.542\) was also not statistically significant (\(p = 0.33\)).
The BEST analysis was also used to explore possible correlations between environmental data and coral-associated bacterial community structure. Results from the BEST analysis for Kamiloloa indicated that the combination of phosphate, total nitrogen, ammonia, salinity, and turbidity during low tides ranked highest in correlating with patterns of coral-associated bacterial community structure at that site. However, the rank correlation of $\rho = 0.289$ was relatively low and not statistically significant ($p = 0.05$). During high tides, the combination of phosphate, temperature, and pH ranked highest in correlating with patterns of coral-associated bacterial community structure at that site. However, the rank correlation of $\rho = 0.307$ was relatively low and not statistically significant ($p = 0.29$). Results from the BEST analysis for Kamalō data indicated that silicate concentrations during low tides ranked highest in correlating with patterns of coral-associated bacterial community structure with a statistically significant rank correlation ($\rho = 0.569$, $p = 0.01$). During high tides, total nitrogen and ammonia ranked highest in correlating with patterns of coral-associated bacterial community structure with a statistically significant rank correlation ($\rho = 0.569$, $p = 0.01$). However, the rank correlation of $\rho = 0.238$ was relatively low and not statistically significant ($p = 0.27$).

**Discussion**

We observed that bacterioplankton community composition at the two study sites was distinctly different from the consortium of bacteria detected on corals. Although corals and seawater shared an abundance of ribotypes, corals harbored an order of magnitude larger number of unique ribotypes, further supporting previous observations that corals provide a distinctive niche for unique types of microorganisms (Rohwer et al.
2002). We also found that the temporal stability of bacterial community structure differed between seawater and corals, with bacterioplankton communities remaining relatively stable between high and low tides and sampling months and coral-associated communities fluctuating between sampling months. It is plausible that the benthic nature of the coral colonies may have enabled us to detect temporal variability on a finer scale than possible with continually mixing reef water. Furthermore, it is important to consider that coral-associated bacteria may be exposed to localized conditions for a longer period of time than bacterioplankton, and therefore, these communities may be more reflective of local exposure to environmental stressors (Klaus et al. 2007).

Although differences between bacterial community type (e.g., bacterioplankton vs. coral-associated) prevailed over differences between geographic location (i.e., coral-associated bacterial communities from the two sites were more similar to one another than to bacterioplankton communities from either site), we also observed significant site differences in bacterial community structure within each community type (e.g., coral-associated bacterial communities differed significantly between sites). Site differences for both corals and seawater may indicate that localized environmental conditions play a significant role in shaping bacterial communities over time (Fuhrman et al. 2006). Similar site-specific patterns have been observed in other studies, challenging the previously held idea that bacterial associations are geographically preserved amongst coral species (Rowher et al. 2002). Littman et al. (2009) found that coral-associated bacterial diversity differed with location, but not species, for three acroporid corals at two sites on the Great Barrier Reef. While certain bacterial groups were associated with corals at both sites, the dominant members of the bacterial community differed between sites. In this study,
corals at both sites harbored similar terminal-restriction fragment lengths (T-RFs), indicative of specific bacterial groups, but in significantly different abundances. In both cases, long-term disparities in environmental conditions at geographically separated coral reefs may be responsible for observed differences.

The observation that coral colonies within a site did not harbor significantly different bacterial communities on any given sampling day, but fluctuated between sampling days, also indicates that the environment may play a marked role in shaping these communities on shorter time scales. Although within-site inter-colony homogeneity could possibly be attributed to vertical transmission of microbial consortia from parent colonies, recent studies have indicated that most mass-spawning corals acquire their bacterial associates from the environment after larval settlement (Sharp et al. 2010). Sunagawa et al. (2010) suggested that if corals need to continuously replenish their microbial population via horizontal transmission throughout their lifespan, adult corals might be expected to harbor a mixed consortium of bacteria. In this case, the types of bacteria growing in the surrounding habitat, and subsequently selected by host corals, may be highly dependent on, and fluctuate with, local environmental conditions. However, we also know that specificity exists between some coral species and certain types of bacteria (Rohwer et al. 2002). In this instance, host corals might be seeded with a particular bacterial community from the environment at a specific developmental stage which subsequently responds to surrounding environmental conditions over time. Most likely, a combination of these two scenarios exists.

Although temporal fluctuations in coral-associated bacterial community composition differed between the two study sites, the month of April stood out as being
particularly unique from remaining months at both sites. Samples taken during April had a relatively high within-group similarity that was clearly distinguishable from other months; however, the particular T-RFs contributing to this similarity differed at the two sites. Although it is difficult to draw a solid conclusion from this observation, it is possible that bacterial communities at each site were experiencing and responding to similar changes in environmental conditions, but historical differences in environmental conditions, and resident bacteria between sites, resulted in different bacteria driving observed changes. In support of this explanation, the Principle Components Analysis of environmental data (low tide only – no high tide data for April) revealed a general trajectory of change from April through August at both sites, with April falling out at one end of the spectrum and remaining months overlapping. Most notably, April samples from both sites had lower concentrations of nitrite, ammonia, and total nitrogen relative to remaining months. A well-documented temporal shift in the amount of resuspended land-derived sediment along Moloka‘i’s southern fringing reef (Ogston et al. 2004, Storlazzi et al. 2004, Presto et al. 2006) shows that the greatest amount of resuspension occurs when trade winds are seasonally strong, from April to November, and at high tide (Ogsten et al. 2004). It is possible that our April sampling period took place just before this seasonal shift occurred.

We further explored linkages between environmental variables and bacterial community structure at both sites to determine the degree to which the environment may be shaping bacterial assemblages on Moloka‘i’s coral reefs. When we examined correlations between environmental variables and bacterioplankton, we identified a suite of properties that ranked highest in correlating with community structure; however, the
correlation was non-significant. We obtained similar results when we examined correlations between environmental variables and coral-associated bacterial community structure, with the exception of a correlation with silicate concentrations at the Kamalō site. Bacteria do not generally require silica for growth and therefore, are not thought to play a significant role in the silica cycle (Bidle and Azam 1999). However, certain types of bacteria are known to enhance the dissolution of silicate minerals in freshwater aquifers (Vandevivere et al. 1994; Bennett et al. 1996) and marine diatom frustules (Patrick and Holding 1985; Bidle and Azam 1999; Azam and Malfatti 2007). It remains unclear whether or not bacterial communities were directly responding to varying silicate concentrations in seawater or if bacteria were actively altering concentrations of silicate in seawater. Further studies are needed to elucidate the intricacies of this interesting correlation.

The fact that we did not observe significant correlations between environmental factors and bacterial community structure for all sample types and sites may partially be attributed to our limited sampling scope and effort. It is likely that a combination of environmental variables influence bacterial communities on multiple scales, but that our methods were not designed to sample the variability in a way that elucidated meaningful patterns and relationships. Despite these limitations, our findings underscored the importance of sampling bacterial communities on a range of spatial and temporal scales. Resident bacterial communities at geographically separated reefs may respond differently to similar environmental changes over time, which has important implications for the susceptibility of local coral populations to specific environmental stressors. We are currently in the exploratory stage of determining how environmental variables may be
working in concert to shape bacterial communities, and this study was intended to broadly address some of the first order questions that are needed to understand these processes.

Conclusion

Coral reef ecosystems are under immense pressure from human impacts. Threats ranging from local overfishing and pollution to global climate change are compromising coral reefs at unprecedented rates. Although large scale issues such as climate change are difficult to address directly, mitigating stressors on a local scale may increase the resiliency of coral reefs, enabling them to better adapt to changing global conditions. Methods for monitoring coral health, such as those proposed in this study, are needed to better understand how corals respond and adapt to an ever-changing world and what might possibly be done to successfully conserve these unique ecosystems.

Acknowledgements

We thank Mike Fields for logistical support in completing this study, and Hollie Putnam, Elizabeth Hambleton, Nadiera Suhkraj, Jamie Becker and Alealani Dudoit for assistance in the field. This research was funded by a USGS Ridge to Reef Program grant, a research partnership between the Northwestern Hawaiian Island Coral Reef Ecosystem Reserve and the Hawaii Institute of Marine Biology (NMSP MOA 2005-008/66882), and a National Science Foundation (grant no. OCE-0928806).
References


Figure Legends

**Figure 4.1:** Map of the Hawaiian island of Molokaʻi. Kamiloloa (A) and Kamalō (B) sites. Yellow stars mark the specific reef locations where coral colonies were sampled.

**Figure 4.2:** Non-metric multidimensional scaling analysis of coral (n=33) and seawater (n=18) bacterial community structure over the five-month survey, based on T-RFLP. The dashed lines indicate a 30% Bray-Curtis similarity threshold.

**Figure 4.3:** Non-metric multidimensional scaling analysis of seawater bacterial community structure sampled at high and low tides once a month over the five-month survey, based on T-RFLP. Individual samples are identified by the month and tide (H, high; L, low) during which the sample was collected.

**Figure 4.4:** Non-metric multidimensional scaling analysis of coral-associated bacterial community structure sampled over the five-month survey, based on T-RFLP. Individual samples are identified by colony number and month of sample collection.

**Figure 4.5:** PCA ordination of environmental data sampled at high and low tides over a 5-month period (n=18). Individual samples are identified by the month and tide (H, high; L, low) during which the sample was collected. Environmental variables are noted above vectors.
**Figure 4.6:** An NMDS bubble plot showing the relationship between coral-associated bacterial community structure and silicate concentrations of colonies at the Kamalō site (n = 14). An NMDS ordination of a Bray-Curtis similarity matrix was calculated from a square root transformation of the T-FLP relative abundance data matrix and silicate concentrations were overlaid onto the NMDS plot points. The diameter of the bubbles are directly proportional to the concentration of silicate in the surrounding seawater at the time of sampling (µmol/L). The colony identification and month of sample collection is listed above each data point.
Figures

Figure 4.1
Figure 4.2
Figure 4.3
Figure 4.4
Figure 4.5

The figure shows a principal component analysis (PCA) plot with site indicators for Kamiloia and Kamaloh. The plot includes measurements such as nitrate-nitrite (N-N), nitrogen (NH4), silicate, dissolved oxygen (DO), chlorophyll-a (CHL A), and temperature (Temp C) plotted against PC1 and PC2 axes.
Figure 4.6
CHAPTER 5

THE EFFECT OF A SUB-LETHAL TEMPERATURE ELEVATION ON THE STRUCTURE OF BACTERIAL COMMUNITIES ASSOCIATED WITH THE CORAL *PORITES COMPRESSA*

Jennifer L. Salerno\(^1,2\), Dan R. Reineman\(^1,3\), Ruth D. Gates\(^1\), and Michael S. Rappé\(^1*\)

\(^1\)Hawaii Institute of Marine Biology, School of Ocean and Earth Science and Technology, University of Hawaii at Manoa, Kaneohe, HI, 96744

\(^2\)Department of Zoology, University of Hawaii at Manoa, Honolulu, HI, 96822

\(^3\)Current address: Emmett Interdisciplinary Program in Environment and Resources, Stanford University, Stanford, CA, 94305

*Corresponding author. Mailing address:
Hawaii Institute of Marine Biology, University of Hawaii, P.O. Box 1346, Kaneohe, HI 96744. Phone: (808) 236-7464. Fax: (808) 236-7443. Email: rappe@hawaii.edu

Published by the Hindawi Publishing Corporation
Journal of Marine Biology
Volume 2011, Article ID 969173, 9 pages
doi: 10.1155/2011/969173
Reprinted with permission
Abstract

Evidence points to a link between environmental stressors, coral-associated bacteria, and coral disease; however, few studies have examined the details of this relationship under tightly controlled experimental conditions. To address this gap, an array of closed-system, precision-controlled experimental aquaria were used to investigate the effects of an abrupt 1°C above summer ambient temperature increase on the bacterial community structure and photophysiology of *Porites compressa* corals. While the temperature treatment rapidly impacted the photophysiology of the coral host, it did not elicit a statistically significant shift in bacterial community structure from control, untreated corals as determined by terminal restriction fragment length polymorphism analysis of 16S rRNA genes. Two of three coral colonies were determined to harbor more closely related bacterial communities at the time of collection and, despite statistically significant shifts in bacterial community structure for both control and treatment corals during the 10-day acclimation period, maintained this level of relatedness over the course of the experiment. The experimental design used in this study proved to be a robust, reproducible system for investigating coral microbiology in an aquarium setting.

Introduction

The worldwide degradation of coral reef ecosystems is due, in part, to the emergence of novel pathogenic diseases affecting scleractinian corals (Lafferty et al. 2004, Harvell et al. 2007) and it has been speculated that the widespread proliferation of coral diseases is linked to increasing sea surface temperatures (SSTs)
Many disease outbreaks correlate with temperature anomalies and seasonal warming (Harvell et al. 2002, Kuta and Richardson 2002, Jones et al. 2004), and increased SSTs have also been shown to affect the virulence of coral disease pathogens (Cervino et al. 2004). For example, infection of *Pocillopora damicornis* by the bacterial pathogen *Vibrio coralyticus* increases rapidly with increased temperatures (Ben-Haim and Rosenberg 2002), and disease outbreaks often follow or co-occur with temperature-induced coral bleaching (Harvell et al. 2002, Jones et al. 2004, Brandt and Mcmanus 2009). Discriminating between bacteria acting as causative agents of coral bleaching (Ben-Haim et al. 1999, Banin et al. 2001) versus post-bleaching opportunists has been ambiguous (Lesser et al. 2007, Ainsworth et al. 2008). However, it remains undisputed that bacteria play important roles in both maintaining and destabilizing the health of the coral holobiont, which is composed of coral host polyps, symbiotic dinoflagellates known as zooxanthellae, and a diverse assemblage of associated algae, fungi, Bacteria, Archaea, and viruses associated with the skeleton, tissues, and mucus layer of adult coral colonies (Knowlton and Rohwer 2003, Rohwer et al. 2002).

Our limited understanding of the resident bacterial biota associated with corals during non-diseased states hinders our ability to understand many of the processes involved in coral disease. Corals harbor diverse bacterial communities that facilitate the transformation of organic carbon (Rohwer et al. 2002, Ducklow and Mitchell 1979), acquisition and transfer of macronutrients (Wafar et al. 1990, Rohwer et al. 2001), and resistance to pathogen invasion (Lesser et al. 2004, Ritchie 2006). The characterization of the diversity, function, and stability of these
communities suggests a link between environmental stressors, coral-associated bacteria and coral disease. For example, increased temperatures, dissolved organic carbon loading, elevated nutrient concentrations, reduced pH, and point source coastal pollution have all been shown to drive shifts in the composition of bacterial community in corals (Kline et al. 2006, Klause et al. 2007, Thurber et al. 2009). A shift towards “disease associated” bacteria has been observed in some cases (Thurber et al. 2009), and coral mortality in others (Kline et al. 2006). Shifts in coral associated bacteria have also been shown to occur in corals infected with a known coral pathogen (Pantos et al. 2003) and changes in bacterial biota are often detectable prior to disease symptoms becoming visible. Coral associated bacterial communities are responsive to infection by disease agents and environmental stressors. As such, the structure of the coral-associated bacterial community could potentially serve as a biological indicator of coral health.

The goals of this study were to (1) examine the feasibility and potential effects of using a closed-system, precision-controlled experimental aquarium system to study the structure and dynamics of microbial communities associated with corals, and (2) assess the effect of a sub-lethal, abrupt change in temperature on coral-associated microbial community structure in the context of the photo-physiological health of the coral. Shifts in the bacterial communities associated with the Hawaiian reef building coral *Porites compressa* were assessed in coral fragments exposed to an abrupt 1°C increase in seawater temperature above ambient summer levels following a 10 d acclimation at ambient temperature. Terminal-restriction fragment length polymorphism (T-RFLP) analysis, a bacterial community fingerprinting technique
based on the 16S rRNA gene, allowed for high-throughput examinations of seawater and coral-associated bacterial community structure throughout the course of the experiment. The finger coral *Porites compressa* was targeted because it is endemic to Hawaii and ecologically important in the region. The photo-physiological response of *P. compressa* to the temperature increase was assessed using Pulse Amplitude Modulated (PAM) fluorometry (Jones and Hoegh-Guldberg 1999, Maxwell and Johnson 2000, Warner et al. 1999) and experimental aquaria allowed for the precise control of environmental variables (such as temperature) and replication of treatments. The results show that the aquaria provided a robust platform for controlled and replicated alterations of environmental conditions that are of a relevant scale to fluctuations either experienced or anticipated to be experienced by corals in the near future, and that the thermal stress applied in this study rapidly impacted the photophysiology of the coral host. In contrast, no change in the structure of coral-associated bacterial communities was detected with the combination of temperature and duration of treatment used in this study.

**Materials and Methods**

*Sample collection and experimental design*

On August 4, 2007, approximately 45 branch tips of *Porites compressa* (Dana 1846) 3 to 5 cm in length and 1 to 1.5 cm diameter were removed from each of three coral colonies judged as non-diseased by gross visual assessment in central Kaneohe Bay off of the island of Oahu, Hawaii, in the Pacific Ocean. Source colonies were sampled at a depth of approximately 4 m, and were located within 10 m of each
other. Three fragments from each colony were immediately frozen in liquid nitrogen for analysis of bacterial community structure associated with the source colonies. The remaining fragments were immediately placed in containers with aerated seawater and transported back to the Hawaii Institute of Marine Biology (HIMB). In addition to the coral sampling, water samples were collected to characterize the bacterial communities in the planktonic environment surrounding the corals. Approximately 1 L of seawater was collected ~1.5 m above the reef, filtered through 25 mm diameter, 0.2 µm pore-sized polyethersulfone membrane filters (Supor-200; Pall Corp., East Hills, NY) and frozen for DNA analysis in 250 µL of DNA lysis buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 1.2% v/v Triton X100) (Suzuki et al. 2001).

Upon return to the laboratory at the HIMB, coral fragments were rinsed with 1 µm-filtered seawater and placed in a holding tank of 1 µm-filtered seawater maintained at the ambient temperature of 27°C. Four fragments from each colony were mounted randomly in each of eight 0.76 mm thick clear vinyl sheets (Figure 5.1). A sheet was placed in each of eight independently controlled 60 L experimental aquaria. An additional 2 - 4 extra fragments per colony were also mounted in sheets as backups for fragment mortality. To minimize exposure to disturbance and handling upon removal from the reef, coral samples were collected between 0900 and 0945 and mounted in their final position in the aquaria by 1400 the same day.

All aquaria were filled with 1 µm-filtered, UV-treated seawater, which recirculated through the individual tanks for the duration of the experiment. Each tank was equipped with a full spectrum light (white and UV) on a 12:12 hr light:dark cycle, a
protein skimmer, and a submersible pump flowing at 20 gal min\(^{-1}\). The volume of seawater in each tank was monitored daily and salinities kept constant by the addition of sterile, milli-Q freshwater. Temperature and light levels in the tanks were monitored with Onset Computer HOBO temperature and light pendant loggers (UA-002-64 Onset Computer Corporation, Bourne, MA). The loggers were fixed horizontally (light meter facing up) to the vinyl sheets containing coral fragments with cable ties, and the sheets supporting the mounted corals were fixed to the bottom of the tank with suction cups. The seawater temperature was maintained at the ambient temperature of 27˚C for 10 d to allow corals to acclimate.

After the acclimation period, four tanks were randomly assigned as controls (tanks 1, 8, 9, and 13) and four as treatment (tanks 3, 4, 15, and 16). At 1300 on that day, seawater and coral bacterial communities were sampled and the seawater temperature in the treatment tanks abruptly increased to 28°C (+1°C over) in treatment tanks. Corals were subsequently sampled from the aquaria at the same time of day (i.e. initiated at 1300 and completed by 1430) after 2, 4, and 6 d of incubation, and aquarium seawater after 2 and 7 d. Coral sampling consisted of removing one randomly selected coral fragment per colony per tank, and freezing it in liquid nitrogen. Seawater (250 mL) was removed from each tank using acid-washed polycarbonate bottles, and subsequently filtered through 25 mm diameter Supor-200 membranes (Pall Corp.). Filters were placed in 200 µL of DNA lysis buffer and stored at -80˚C until further processed.

_Pulse amplitude modulated (PAM) fluorometry_

Pulse amplitude modulated fluorometry was performed by taking one measurement per fragment on the fragment tip with a Walz Diving-B PAM
Fluorometer (Heinz Walz GmbH, Germany) with an 8-mm fiber optic cable. The measurement tip of the PAM cable was sheathed in opaque tubing to prevent light pollution of the other fragments during measurements, and to create a standard distance between the sample and the measuring tip. Sampling was performed in the evening after approximately 1 h of dark adaptation. The resulting Maximum Quantum Yield (MQY) data was analyzed with Minitab 15.0 statistical analysis software (Minitab Inc, State College, PA).

**T-RFLP of bacterial SSU rRNA genes**

Individual coral fragments were thawed, and a flame-sterilized stainless steel core borer was used to remove three random sub-samples. Each sub-sample consisted of a 6-mm diameter, 6-mm deep core that included the coral tissue, overlying mucus layer, and underlying skeleton. The three sub-samples were placed into one sterile bag containing 2 mL of 0.2 µm-filtered 10X Tris EDTA (100 mM Tris, 10 mM EDTA) buffer solution (pH 7.4), and airbrushed with an air gun and sterile pipette tip. The resultant slurry was centrifuged at 19,900 RCF for 30 min at 4°C. The supernatant was subsequently removed from the sample and the remaining sample pellet was frozen at -80°C until processed further.

After thawing, genomic DNA was extracted from the coral tissue pellets and seawater filters using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer’s protocol. Genomic DNA was eluted in sterile, 0.1 µm-filtered water and stored frozen at -20°C. Total genomic DNA yield was assessed on a SpectraMax M2 plate reader (Molecular Device Corp., Sunnyvale, CA,
USA) using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Corp., Carlsbad, CA, USA), prepared according to manufacturer’s protocol.

For terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997), the general bacterial primers 27F-B-FAM (5’-FAM-AGRGTTYGATYMTGGCTCAG-3’) and 1492R (5’-GGYTACCTTGTACGACTT-3’) were used for the amplification of small subunit ribosomal RNA (16S rRNA) genes from each sample via the polymerase chain reaction (PCR). The MasterTaq System (Eppendorf AG, Hamburg, Germany) was used for all PCR reactions, which were composed of the following (final concentrations): 1X MasterTaq reaction buffer, 2.25 mM Mg²⁺, 0.5X TaqMaster reaction enhancer, 0.2 mM each of the forward and reverse primers, 0.2 mM of each dNTP (Promega, Madison, WI), approximately 160 to 280 ng of genomic DNA template, 2.5 units of MasterTaq DNA polymerase, and sterile water to a final reaction volume of 50 μl. A MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) and touchdown protocol were used, which, after a 3 min incubation at 95°C, consisted of 30 cycles of 30 sec at 95°C, 1 min at 65°C (decreasing by 0.5°C per cycle), and 2 min at 72°C. This was followed by 10 cycles of 30 sec at 95°C, 1 min at 50°C, and 2 min at 72°C, and 1 cycle of 30 sec at 95°C, 1 min at 50°C, and 20 min at 72°C.

The fluorescently labeled amplicons were purified using the QIAquick PCR purification kit (Qiagen Inc.) following the manufacturer’s instructions. Approximately 100 ng of each purified amplicon was subsequently digested in a 10 μL reaction containing 5 units of HaeIII restriction endonuclease (Promega, Madison, WI) at 37°C for 6 hours. After purification via gel filtration chromatography with Sephadex G-50
(Amersham Biosciences, Sweden), the restricted samples were adjusted to a final concentration of 35 ng µL⁻¹ and separated via capillary electrophoresis on an automated ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). GeneMapper software (Applied Biosystems) was used to estimate the size and relative abundance of the resulting terminal restriction fragments (T-RFs), which were defined as fragments between 42 and 613 base pairs (bp) in length. Fragment lengths were rounded to the nearest integer value, aligned, and manually checked for possible errors in peak determination due to such factors as instrument variability, etc. The threshold below which peaks were excluded was determined via the variable percentage threshold method as described in Osborne et al. (2006).

Statistical analysis

After square root transformation of the T-RFLP data matrix, all subsequent statistical analyses were carried out in PRIMER 6 Version 6.1.13 and PERMANOVA + Version 1.0.3 (PRIMER-E Ltd., Plymouth, UK) (Clarke and Gorley 2008, Anderson et al. 2008, Clarke and Warwick 2001).

A resemblance matrix was constructed based on Bray-Curtis similarity. Hierarchical clustering analysis (CLUSTER) of the resemblance matrix was used to construct similarity dendrograms and a similarity profile permutation test (SIMPROF) was used to identify significant “natural groupings” of samples that were not structured a priori. CLUSTER analyses were based on the group average linking option and SIMPROF analyses were performed at a 5% significance level.
Nonmetric multidimensional scaling (NMDS) ordination of the resemblance matrix was used to provide a 2-dimensional graphical representation of similarities in bacterial community structure (based on T-RFLP peak abundances) amongst samples (Shepard 1962, Kruskal 1964). NMDS plots can be generally interpreted as follows: points that are closer together are very similar in community composition and points that are further apart correspond to very different communities. NMDS was chosen because it makes no assumptions about the underlying distribution of data and is therefore appropriate for analyzing complex ecological communities. In some cases, data from CLUSTER analyses were used to overlay Bray-Curtis similarity boundaries onto NMDS plots to display percent similarity amongst samples.

An analysis of similarity (ANOSIM), performed on the resemblance matrix, was used to test for differences in bacterial community structure between pre-defined sample groups. The ANOSIM test is analogous to a standard univariate 1-way analysis of variance (ANOVA) and group specifications were made a priori. A ‘similarity percentages’ routine (SIMPER) was used to identify the role of individual species in contributing to group differences as well as within group closeness. SIMPER dissects average Bray-Curtis dissimilarities between all pairs of samples into percentage contributions from each species and lists them in decreasing order of contribution (Clarke and Gorley 2006). An 80% cut-off was employed to focus on higher-contributing variables.

A permutational multiple analysis of variance (PERMANOVA) of the resemblance matrix was used to test for differences in bacterial community structure between temperature treatments, tanks, coral colonies, and time points. PERMANOVA
tests the simultaneous response of one or more variables to one or more factors in an analysis of variance (ANOVA) experimental design on the basis of a resemblance measure, using permutation methods. The routine calculates an appropriate distance-based pseudo-F statistic for each term in the model based on the expectations of mean squares, and p-values are obtained using an appropriate permutation procedure for each term (Anderson et al. 2008). PERMANOVA tests were run with the following specifications: 999 permutations, permutation of residuals under a reduced model, Type III (partial) sum of squares, Monte Carlo tests, and fixed effects sum to zero.

Results

Photophysiology of P. compressa

Initially, both control and treatment fragments of P. compressa exhibited very high variability in MQY, presumably due to shock associated with their collection from the field, loading into sheets, and positioning within the aquaria (data not shown). By day 3, however, the MQY had stabilized, and remained nearly constant throughout the acclimation period (Figure 5.2). Statistical analyses revealed that the MQY of the control and treatment groups did not differ significantly during this acclimation phase (two sample t-test: t = 0.74, DF = 481, P = 0.459), indicating there were no tank effects. In addition, the MQY of all colonies on the first and last days of the acclimation period were also not significantly different (two sample t-test: t = 0.17, DF = 239, P = 0.867), indicating that the MQY stabilized during acclimation. After the initiation of the temperature manipulation on Day 10 (+1°C), the MQY of both treatment and control samples decreased. However, linear regression of the control and treatment samples
indicated a greater decline in the treatment samples over time (Table 5.1; Figure 5.2) with the slope of the control sample linear regression decreasing by a factor of 2.33 and the slope of the treatment by a factor of 5.50.

**Planktonic bacterial community analysis**

CLUSTER and ANOSIM analyses of all coral-associated (n=99) and seawater bacterial community (n=26) samples collected over the course of this study showed that the bacterial communities were significantly different between the two sample types (Global R = 0.864, p = 0.001). An initial NMDS ordination of all coral and seawater samples overlaid with a 20% Bray-Curtis similarity boundary resolved two distinct clusters clearly demonstrating this difference (Figure 5.3).

The microbial community in seawater from Kaneohe Bay taken near the corals sampled for this experiment (n=1), from the source water produced by the HIMB seawater system (n=1), and from the aquaria throughout the course of the experiment (0, 2, and 7 days; n=24) were compared using CLUSTER and ANOSIM. These tests revealed a tight group of microbial communities from the seawater sample from Kaneohe Bay and from the HIMB seawater system, which was significantly different from the aquaria planktonic microbial communities regardless of whether they originated from control or treatment tanks (Figure 5.4) (Global R = 0.981, p = 0.003). Thus, the planktonic bacterial community structure within the aquaria shifted over the 10 days between the time of collection and start of the experiment. A PERMANOVA test was used to investigate the differences in seawater bacterial communities amongst different aquaria throughout the experiment. This test revealed no difference between control or
treatment tanks (Pseudo-\(F = 0.619, p = 0.824\)). However, the test did reveal differences in the bacterial communities in aquarium seawater over time (Pseudo-\(F = 2.173, p = 0.003\)), and between replicate tanks within control and treatment (Pseudo-\(F = 2.119, p = 0.001\)). An ANOSIM test confirmed differences in aquarium seawater bacterial communities over time (Global R = 0.131, p = 0.019), but only identified time points 0 d and 7 d as being significantly different from one another (R = 0.33, p = 0.003). Differences between tanks within control or treatments were also confirmed via ANOSIM (Global R = 0.372, p = 0.001), and pair-wise comparisons revealed a range of similarities between tanks from both the control and treatment sets. For example, control tank 8 was highly distinct from the other three control tanks, with R values approaching 1 (8/9 R = 0.704; 8/1 R = 0.852; 8/13 R = 0.889), whereas control tank 4 and treatment tank 13 were highly similar (R = 0).

*Coral-associated bacterial community analysis*

Coral samples collected from source colonies in the field (n = 3) were compared to all experimental and control coral samples (0, 2, 4, and 6 d time points; n = 96) using CLUSTER and ANOSIM tests, which revealed a significant difference between source and aquaria maintained colonies (Global R = 0.688, p = 0.001). Thus, coral associated bacterial community structure appeared to shift between the time of collection and the start of the experiment 10 d later. A PERMANOVA test including all time points indicated that coral-associated bacterial communities did not differ between the control or treatment sets (Pseudo-\(F = 0.791, p = 0.807\)), or over time (Pseudo-\(F = 1.257, p = 0.145\)). Despite significant differences between tanks within control or experimental tank sets
(Pseudo-F = 1.532, p = 0.003), significant inter-colony differences in bacterial community structure were maintained over the course of the experiment across all tanks (Pseudo-F = 8.338, p = 0.001).

To further investigate intra-colony differences, separate PERMANOVA tests were performed for each colony. This confirmed that bacteria associated with colonies 2 and 3 were not significantly affected by the treatment (Pseudo-F = 0.900, p = 0.502; Pseudo-F = 1.068, p = 0.377), time (Pseudo-F = 1.433, p = 0.062; Pseudo-F = 0.842, p = 0.754), or tank (Pseudo-F = 1.232, p = 0.110; Pseudo-F = 1.009, p = 0.487). While test results for colony 1 revealed that bacterial community structure was not significantly affected by treatment (Pseudo-F = 0.846, p = 0.793), it was significantly affected by tank (Pseudo-F = 1.305, p = 0.036) and time (Pseudo-F = 1.524, p = 0.017). An ANOSIM test of colony 1 data supported these observations (Global R = 0.097, p = 0.028), with significant differences between time points 0 and 4 d (R = 0.218, p = 0.015), and between 2 and 6 d (R = 0.209, p = 0.043), but not between 0 and 2 d (R = 0.111, p = 0.136), 2 and 4 d (R = -0.049, p = 0.716), 4 and 6 d (R = 0.027, p = 0.352), or 0 and 6 d (R = 0.106, p = 0.095). SIMPER analysis indicated that the terminal restriction fragment of 303 base pairs in length (T-RF 303) was the major contributor to observed differences between times, accounting for 4.85% of the average dissimilarity between time 0 and 4 d groups, and 5.37% of average dissimilarity between 2 and 6 d groups. The relative abundance of T-RF 303 bp increased during the intermediate time points in control tanks 1, 8, and 13, and treatment tank 16, thus contributing to observed differences amongst tanks.

An NMDS ordination of the data set illustrated the shift in bacterial community structure between colonies sampled in the field and those sampled during the
experimental time points (Figure 5.5a). The NMDS ordination also revealed that bacterial communities associated with source colonies 2 and 3 were more similar to one another than to colony 1. In spite of the overall shift in community structure between source and experimental coral-associated microbial communities observed for all three colonies, inter-colony differences were preserved over the course of the experiment: colonies 2 and 3 remained similar, while colony 1 remained distinct (Figure 5.5a). An ANOSIM test confirmed that the three colonies were significantly different from one another during the experiment (Global R = 0.33, p = 0.001), with pair-wise comparisons highlighting that colonies 2 and 3 were more similar to one another than to colony 1 (2/3 R = 0.164; 2/1 R = 0.427; 3/1 R = 0.437; p = 0.001 for all tests).

SIMPER was used to identify individual T-RFs that contributed most greatly to group differences, and defined within group similarities. Overall, T-RF 303 and T-RF 264 played large roles in discerning inter-colony differences (Figures 5.5b and c). T-RF 303 contributed to 6.61% of average dissimilarity between colonies 1 and 2, and 6.48% between colonies 1 and 3. T-RF 264 contributed to 4.78% of average dissimilarity between colonies 1 and 2, and 5.78% between colonies 1 and 3.

**Discussion**

As the integrity of coral reefs decline on a global scale, the development of effective means to monitor coral health has become critical. In particular, methods that detect the onset of physiological stress in corals prior to disease or death are essential for effective management. In addition to their utility in other areas of coral health and disease, we hypothesized that fluctuations in bacterial community structure associated
with corals could be used as a sensitive indicator of physiological stress by the coral host. However, most studies to date that have related coral-associated bacterial community structure with coral health have focused on distant end-members (e.g., “healthy” vs. “non-healthy” or diseased) (Pantos et al. 2003, Frias-Lopez et al. 2004, Pantos and Bythell 2006), and have not been able to tease apart the large gradient of health states in between these widely separated extremes. Experimental aquarium systems provide an opportunity to tightly control the environmental conditions experienced by the corals (and thus their associated microbiota), and consequently allow for the investigation of small, systematic, and ecologically relevant environmental disturbances.

In general, corals live within a relatively narrow temperature margin and will bleach in response to both high and low sea surface temperature anomalies (Coles and Fadlallah 1991, Jokiel and Coles 1990). Bleaching due to elevated seawater temperatures occurs more frequently, and even a small increase (e.g., 1-2˚C) for several weeks during the summer season can induce bleaching (Jokiel and Coles 1990, Brown 1997). Using these observations as rationale, we exposed fragments of *P. compressa* to a one degree, sub-lethal temperature elevation over the ambient summer temperature at the time of sampling. This temperature increase elicited a rapid response by the coral host, resulting in a progressive decrease in MQY over the course of the six-day treatment, which we interpret to indicate a decrease in the photosynthetic efficiency of the coral. The control corals that were not subjected to the temperature increase also showed a progressive but far less dramatic decrease in MQY over time. Thus, while unidentified components of the experimental aquarium set up appeared to have a negative impact on the photosynthetic efficiency of the coral with time, the additive adverse affect of the minor increase in
temperature was of significant enough magnitude to overshadow the tank affect. By contrast, after 6 d of experimental manipulation, no clear systematic segregation was detected between bacterial communities associated with control and treatment corals.

Our results do not imply that coral-associated bacterial communities were static: these communities changed significantly during the 10-day acclimation period when no treatment was applied, resulting in a distinct difference between the parent “source” colonies and the experimental coral fragments. Despite this divergence from source colony community structure during the acclimation period, for the most part the coral-associated bacterial communities did not continue to systematically change during the treatment period. We interpret this to indicate that the coral-associated microbial community structure changed in response to acclimation to the experimental aquarium system, stabilized over the course of the initial 10-day period, and subsequently did not systematically respond to the temperature elevation treatment or experimental aquarium environment. The most plausible explanation for this observation is that the temperature increase was not significant enough in magnitude and/or duration to either directly impact the growth of the coral-associated bacterial biota, or indirectly affect their growth by impacting the physiology of the coral to a degree that would impact the microbial community. In essence, PAM fluorometry appeared to be detecting changes in the photophysiology of the coral host that had no impact on the community structure of associated bacteria for the duration of our experiment. In future experiments, it will be valuable to extend the duration of the temperature treatment significantly (i.e. from days to weeks) to correlate bacterial community structure to a broader gradient of impact to the
coral host, including the ultimate fate of the coral fragments subjected to sub-lethal increases in temperature.

It is interesting to note that the three *P. compressa* source colonies sampled for this experiment all possessed statistically different microbial communities. The structure of the bacterial communities of two of these colonies were much more closely related to each other than to the third, and, despite changes during the 10-day acclimation phase, this relationship was maintained throughout the duration of the experiment. The maintenance of this relationship throughout the acclimation and experimental periods suggests that the microbial communities either did not randomly diverge upon containment, but rather changed systematically, or that they diverged but the differences did not reach a significant enough magnitude for our methodology to detect. Extension of the duration of the experimental treatment would help to tease apart the interplay between these non-exclusive options.

Throughout the course of this study, several aspects of the experimental aquarium system were identified that had the potential to negatively impact this research by forcing environmental conditions to diverge from the natural environment, and thus would require additional attention in the future. In particular, technical limitations required that the seawater used to fill the experimental aquaria be re-circulated within each individual tank. Thus, to limit microbial growth, the seawater initially supplied to the tanks was filtered to remove eukaryotic phytoplankton, and treated with UV radiation to decrease the load of viable bacteria. As might be expected, the structure of planktonic bacterial communities within the experimental aquaria appeared to change in an unpredictable fashion over the course of this study, resulting in significant between tank differences.
irrespective of whether they originated from treatment or control groups. While cellular abundance was not monitored, it is probable that the filtration and UV treatments initially decreased the cellular load, but the tank environment provided a hospitable environment for subsequent microbial growth. Ultimately, all tanks exhibited significant changes in planktonic bacterial community structure by the end of the experiment. The source coral colonies sampled in this study were subject to significant, variable flow, and thus experience a constantly refreshed microbial community. While the impact that aquarium-contained planktonic seawater bacterial communities may have on the corals in these aquaria (or their closely associated microbiota) cannot be predicted at the present time, it is a potentially confounding factor that can be eliminated by employing a flow-through seawater system with natural seawater as source.

**Conclusions**

The primary goal of this study was to begin to explore the relationship between coral-associated bacterial community structure and coral health during an abrupt but sub-lethal temperature anomaly. More broadly, we sought to relate successional changes in bacterial community structure to gradients of physiological stress in the coral host, before the symptoms of the stress became visually apparent (e.g., bleaching, disease symptoms). While the thermal stress we applied rapidly impacted the photophysiology of the coral host, no changes in the bacterial community structure were detectable with the combination of temperature and duration of treatment used. Data from this study provide new information on the variability of coral associated bacterial communities amongst colonies of the same species, and serve as a guide to improve the design of future
experiments aimed at elucidating the role that bacterial communities play in the responses of corals to climate change stressors such as elevated sea surface temperatures and ocean acidification.

Acknowledgements

The authors would like to thank Chelsea Ale‘alani Dudoit for her assistance with field collection and sample processing, and Andy Taylor for his assistance with statistical data analysis. This research was funded by a grant/cooperative agreement from the National Oceanic and Atmospheric Administration, Project number R/CR-12, which is sponsored by the University of Hawaii Sea Grant College Program under Institutional Grant No. NA05OAR4171048 (UNIHI-SEAGRANT-XM-06-03), a research partnership between the Northwestern Hawaiian Island Coral Reef Ecosystem Reserve and the Hawaii Institute of Marine Biology (NMSP MOA 2005-008/66882), and the National Science Foundation (grant no. OCE-0928806). This is SOEST contribution 8028 and HIMB contribution 1415.

References


Tables

**Table 5.1** PAM-derived Maximum Quantum Yield regression values during acclimation and manipulation phases of *P. compressa*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>Slope</th>
<th>$R^2$</th>
<th>T statistic</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Acclimation</td>
<td>-0.00168</td>
<td>0.052</td>
<td>-3.61</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Manipulation</td>
<td>-0.00392</td>
<td>0.070</td>
<td>-3.26</td>
<td>0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td>Acclimation</td>
<td>-0.00159</td>
<td>0.049</td>
<td>-3.54</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Manipulation</td>
<td>-0.00875</td>
<td>0.273</td>
<td>-7.43</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 5.1: Photograph of *P. compressa* mounted in a vinyl sheet and held in a seawater holding tank prior to being placed into the experimental aquaria.

Figure 5.2: Maximum quantum yield of *P. compressa* during acclimation to experimental tanks (acclimation) and after a temperature increase of 1°C at Day 10 (treatment group tanks only; manipulation). Each point represents a single PAM measurement (points are shifted slightly in the ±x direction in order to reduce overlap). Lines are least-squares linear regressions. Control colonies: closed circles, solid regression; treatment colonies: x’s, dashed regression. Note: the treatment and control regression lines overlap during the “acclimation” phase.

Figure 5.3: NMDS ordination of all coral (n = 99) and seawater (n = 26) bacterial communities sampled over the course of this study, based on a Bray-Curtis similarity matrix calculated from a square root transformation of the T-RFLP relative abundance data matrix. The dashed line indicates the 20% Bray-Curtis similarity threshold. Seawater samples are represented by squares and coral samples are represented by circles. Control and treatment samples are represented by open and closed symbols, respectively.

Figure 5.4: NMDS ordination of seawater bacterial communities sampled over the course of this study (n = 26), based on a NMDS ordination of a Bray-Curtis similarity matrix calculated from a square root transformation of the T-RFLP relative abundance data matrix. The dashed line indicates the 20% Bray-Curtis similarity threshold. “Field”
indicates the seawater bacterial community sampled from the field site in Kaneohe Bay, while “source” indicates the seawater system at HIMB used as a source for the aquaria. Control aquaria are represented by open symbols and are individually numbered 1, 8, 9, and 13, while treatment aquaria are represented by closed symbols and are individually numbered 3, 4, 15, and 16. Sampling times are indicated by circles (t = 0), squares (t = 2 d), and triangles (t = 7d).

**Figure 5.5:** NMDS ordination of coral-associated bacterial communities based on Bray-Curtis similarity. Points represent coral-associated bacterial communities sampled directly from the three source colonies in Kaneohe Bay (PC1, PC2, and PC3) and from experimental tanks. (a) Sampling times are listed adjacent to points, and colonies are represented by circles (colony 1), squares (colony 2), and triangles (colony 3). Control and treatment samples are represented by open and closed symbols, respectively. In (b) and (c), colony numbers are superimposed on the relative abundance bubbles, and the diameter of each bubble is correlated to the square root transformed relative abundance of (b) T-RF 303 or (c) T-RF 264 for each sample. The solid (a) and dashed (a)–(c) lines demarcate 40% and 25% Bray-Curtis similarity thresholds, respectively.
Figures

Figure 5.1
Figure 5.2

![Graph showing maximum quantum yield over time during acclimation and manipulation phases. The graph compares treatment corals (X) and control corals (●) with their respective regression lines.](image)
Figure 5.3

2D stress: 0.207

- Treatment seawater
- Control seawater
- Treatment corals
- Control corals
Figure 5.5

(a) Source: Colony 1, Control: Colony 1, Treatment: Colony 1

(b) Source: Colony 2, Control: Colony 2, Treatment: Colony 2

(c) Source: Colony 3, Control: Colony 3, Treatment: Colony 3

2D stress: 0.27
CHAPTER 6
SUMMARY AND CONCLUSION

Since the initiation of this dissertation research and within the time covered by the 2004 and 2008 “Status of coral reefs of the world” reports, a number of coral reefs have made promising recoveries while others have faced serious declines (Wilkinson 2004, 2008). What has remained relatively constant is the large percentage of coral reef ecosystems still at risk of being completely lost (15% in the next 10-20 years and 20% in the next 20-40 years) if current anthropogenic impacts are not lessened and the many known and preventable risks to coral health are not addressed. Although the 2008 report states that 46% of the world’s reefs are regarded as healthy and not under immediate threat of destruction, it also notes that this estimate does not take the threat of unpredictable global climate change into account.

Despite these grave estimates, some major advances have been made including: the recovery of corals in the Indian Ocean and Western Pacific from a devastating 1998 bleaching event, the increased use of socioeconomic assessments in coral reef management decisions, the establishment of several large scale marine protected areas (e.g., the Papahānaumokuākea Marine National Monument, the Phoenix Islands Protected Area, the Chagos Marine Protected Area, and the Cook Islands Marine Park), and a surge in global initiatives dedicated to allocating resources to conserve coral reef resources (e.g., the Coral Triangle Initiative, the Caribbean Challenge, the Coral Reef Targeted Research and Capacity Building for Management Program, the Coral Reefs, Initiatives for the Pacific, and the work for the Pew Environment Group). Together, these failures and successes further emphasize the need for continued research pertaining to coral reef health and most importantly, the need for improved communication between scientists, resource managers, and policy makers to ensure that scientific information is implemented into current policy and effective change.
The major objectives of the dissertation were to increase our basic knowledge of coral-associated bacterial communities and to investigate their potential roles in influencing coral health. These objectives were pursued by addressing some of the most fundamental research questions regarding coral-associated bacterial host specificity, spatial and temporal variability, and response to environmental change. Below is a summary of specific findings from each research chapter, an overall assessment of hypotheses tested through the dissertation research, followed by a discussion of future research needed to further our understanding of coral-associated bacterial communities and how such small-scale interactions may be affecting large-scale ecosystem health.

Summary of findings

Chapter 2

This chapter addressed the lack of standardization in current methods used to study coral-associated bacterial communities. Combinations of nucleic acid extraction methods and polymerase chain reaction (PCR)-based techniques for three species of corals were examined to develop a high-throughput method that could be used to for a wide variety of coral species. Findings revealed that: 1) the total amount of genomic DNA extracted varied significantly amongst different coral species; 2) the treatment/extraction method used affected the success of 16S rRNA gene amplification for all samples; 3) Species-specific differences in coral-associated bacterial community structure were evident regardless of treatment/extraction method; 4) Each coral colony/treatment/extraction combination yielded a statistically different bacterial community profile; 5) Inter-colony differences contributed to most of the variation observed in *Porites compressa* corals, whereas extraction method had a greater affect on
*Porites lobata* bacterial community profiles. Ultimately, through exploring a combination of methods, I was able to identify the benefits and caveats of using different treatments and nucleic acid extraction techniques, optimize species-specific protocols for genomic DNA extraction and PCR-amplification for high-throughput analyses of associated bacteria for several coral species and, emphasize the importance of using standardized methods to obtain comparative data for coral-associated bacteria monitoring purposes.

**Chapter 3**

Fundamental questions regarding coral-associated bacterial biodiversity and biogeography were addressed in this chapter. An assessment of bacterial communities associated with *Porites lobata* corals and seawater across the Hawaiian Archipelago and greater Indo-Pacific Ocean showed that 1) planktonic and coral-associated bacterial communities consisted of a large diversity of unique bacterial ribotypes; 2) the structure of seawater and coral-associated bacterial communities was significantly different; 3) Weak, but significant isolation by distance patterns were detected for planktonic and coral-associated bacterial communities, indicating that geographic isolation is partially responsible for driving observed divergences at the archipelagic scale; 4) parallels in the biogeographic patterns of *Porites lobata* corals and their bacterial associates indicate that host dispersal may have an effect on bacterial community structure; 5) seawater and coral-associated bacterial communities exhibited a biogeographic connection with Johnston Atoll, as observed in other fish and invertebrate species; and 6) additional environmental factors (not identified in this study) affect the distributions of bacterial communities associated with *Porites lobata* and seawater (based on the observation that
several geographic groupings were not distance-based). Through this research, the first baseline assessment of coral-associated bacterial communities associated with *Porites lobata* corals throughout the Hawaiian Archipelago and greater Indo-Pacific Ocean was conducted. Further development of tools used to identify specific types of bacteria associated with corals during non-diseased and diseased states, as well as those linked with specific environmental stressors, may enable scientists and resource managers to monitor coral health and mitigate local threats more effectively.

Chapter 4

In this chapter, the temporal and spatial variability of coral-associated bacterial communities was addressed. Bacteria associated with colonies of the coral *Porites compressa* were examined at monthly intervals, for a five-month period at two sites along the south shore of Moloka‘i, Hawai‘i. Major findings included: 1) seawater and coral-associated bacterial communities were distinct and unique; 2) site differences were observed for both types of communities; 3) environmental parameters measured at each site were significantly different; 4) environmental parameters did not significantly correlate with planktonic community structure, but silicate concentrations significantly correlated with coral-associated bacterial community structure at the Kamalō site; 5) bacterial communities did not differ amongst colonies within a site; 6) seawater and coral-associated bacterial communities fluctuated from month-to-month. Understanding the natural variability of coral-associated bacterial communities over time is an essential step to establishing a baseline from which we can begin to more closely examine the interplay between bacteria, the environment, and coral health.
Chapter 5

In this chapter, the relationship between environmental conditions (specifically, increased temperature) and coral-associated bacterial community structure was addressed. An array of closed-system, precision-controlled experimental aquaria were used to investigate the effects of an abrupt 1°C above summer ambient temperature increase on the bacterial community structure and photophysiology of *Porites compressa* corals. Major findings were: 1) the temperature treatment rapidly impacted the photophysiology of the coral host; 2) the temperature treatment did not elicit a statistically significant shift in bacterial community structure from control, untreated corals; 3) two of three coral colonies harbored more closely related bacterial communities at the time of collection; 4) despite statistically significant shifts in bacterial community structure for both control and treatment corals during the 10-day acclimation period, coral colonies maintained the same pattern of relatedness over the course of the experiment; and 5) a robust, reproducible system for investigating coral microbiology in an aquarium setting was successfully designed and tested.

Hypotheses addressed by dissertation research:

\( H_01: \) Assessments of coral-associated bacterial (CAB) community structure (species diversity and relative abundance) do not differ based on the combination of treatment, microbial nucleic acid extraction method, and polymerase chain reaction (PCR)-based technique used.

**Based on Chapter 2 findings, we reject the null hypothesis.**
H_02: CAB community structure does not differ between different colonies of the same coral species (Chapters 2, 3, 4, 5).

Based on findings from Chapters 2, 3, and 5, we reject the null hypothesis. However, based on findings from Chapter 4, we fail to reject the null hypothesis. The discrepancy in these findings highlights the need for continued research on how environmental stressors may influence the composition of coral-associated bacterial communities over space and time.

H_03: CAB community structure does not differ between different coral species (Chapter 2).

Based on Chapter 2 findings, we reject the null hypothesis.

H_04: CAB community structure does not differ based on distance/geographic location (Chapters 3 and 4).

Based on findings from Chapters 3 and 4, we reject the null hypothesis.

H_05: CAB community structure does not fluctuate over time (Chapter 4).

Based on findings from Chapter 4, we reject the null hypothesis.

H_06: CAB community structure does not fluctuate in response to changes in environmental variables (e.g. ambient seawater temperature, salinity, turbidity, and nutrients) (Chapters 4 and 5).
Results varied according to location and environmental parameter being investigated. In Chapter 4, environmental parameters did not significantly correlate with planktonic community structure at either site over time or with coral-associated bacterial community structure at the Kamiloloa site. Therefore, we fail to reject the null hypothesis for these three cases. However, silicate concentrations were significantly correlated with coral-associated bacterial community structure at the Kamalō site, leading us to reject the null hypothesis for this particular case. Based on findings from Chapter 5, which showed that the temperature treatment did not elicit a statistically significant shift in bacterial community structure from control, untreated corals, we fail to reject the null hypothesis.

Future research

Information gleaned from this body of work builds upon the currently limited pool of knowledge regarding the roles that coral-associated bacteria play in coral health. However, as with any extensive scientific investigation, it produced nearly as many questions as answers in regard to the topic. Many of these questions are within the scope of the dissertation, but require additional research. Being that coral microbial ecology is a relatively nascent subfield, it would greatly benefit from research that continues to focus on providing answers to fundamental ecological questions – especially those pertaining to biogeography (MacArthur and Wilson 1967). Beginning with the basics, we continue to seek the answers to the fundamental questions of who is where and how many with regard to coral-associated bacterial communities. With recent technological advances, it
is becoming more feasible to address said questions by studying these unique ecosystems on a range of spatial and temporal scales. Equipped with this essential knowledge, scientists will be better prepared to further explore interactions between coral-associated microbial communities and the surrounding environment and the important roles that bacteria play in coral health. The following paragraphs discuss suggested focus areas for future research.

Method standardization

Method standardization continues to be an important issue in coral-associated bacterial studies. Since the vast majority (>99%) of marine microorganisms do not grow on enriched media (Azam 1998), researchers have employed a host of different culture-independent, molecular-based methods to assess the diversity of coral-associated bacterial communities. Sequence-based assessments of bacterial ribosomal RNA (rRNA) genes amplified from nucleic acids (Olsen et al. 1986) provide a high taxonomic resolution across large sets of environmental samples based on nucleotide heterogeneity. Cloning and sequencing of bacterial 16S rRNA genes (Rohwer et al. 2001, 2002; Frias-Lopez et al. 2002; Bourne and Munn 2005; Koren and Rosenberg 2006) and community fingerprinting techniques such as automated ribosomal intergenic spacer analysis (ARISA) (Daniels et al. 2011), Denaturing Gradient Gel Electrophoresis (DGGE) (Littman et al. 2009), Terminal-Restriction Fragment Length Polymorphism (T-RFLP) analysis (Klaus et al. 2005), and tag sequencing (Sunagawa et al. 2009) are a few examples of techniques used in recent studies. Although these methods are all based on Polymerase Chain Reaction (PCR) amplification of bacterial 16S ribosomal DNA
(rDNA), they differ in their detection limits, making it difficult to obtain standardized comparisons. Fingerprinting techniques have lower taxonomic resolution than clone sequence libraries; however, trends of phylogenetic diversity observed using both techniques are generally consistent and fingerprinting techniques provide greater qualitative assessments of large numbers of samples or assemblages (Mouchka et al. 2010).

In addition to disparities in the types of methods used, standardization is also challenged by how individual researchers choose to determine “cutoffs” for DNA sequence identity defining operational taxonomic units and allowing for comparisons between bacterial communities (Rohwer et al. 2001, 2002; Klaus et al. 2005; Guppy and Bythell 2006; Littman et al. 2009). Operator-defined taxonomic resolution can significantly influence the outcomes of similarity analyses between bacterial communities and it is currently unclear which nucleotide identity cutoffs are appropriate for defining ecologically meaningful taxonomic levels (Mouchka et al. 2010).

Finally, as revealed by Chapter 2 of the dissertation and other studies (Klaus et al. 2005), the tissue type targeted for nucleic acid extraction, as well as the type of nucleic acid extraction technique utilized varies from study to study and has a significant impact on similarities/differences detected between samples. It is known that healthy corals harbor diverse and unique bacterial communities in their surface mucus layer, gastrovascular cavity, tissue, and skeleton (Ducklow and Mitchell 1979, Wafar et al. 1990, Rohwer et al. 2001, 2002, Kellogg 2004, Lesser et al. 2004, Wegley et al. 2004) and that the types of bacteria found may differ by location within the coral host (Rosenberg et al. 2007). Klaus et al. (2005) compared bacterial diversity between a
syringe extracted coral mucus sample and an airbrushed coral tissue slurry sample and found that clone diversity retrieved from a coral mucus library more closely reflected the diversity of surrounding seawater than a corresponding coral tissue clone library. This study highlights the difficulty of being able to effectively sample one particular tissue type without contamination from another type (e.g. mucus vs. seawater or coral tissue) and stresses the importance of developing a standardized sample collection method for comparisons across studies.

Ultimately, differences between research findings highlight the multifaceted and dynamic nature of coral-associated bacterial communities, and scientists should be careful to not over-simplify or over-generalize the nature of these associations (Mouchka et al. 2010). With additional studies increasing our general knowledge of coral associated bacterial communities, along with decreased costs and increased use of advanced technologies such as next-generation sequencing (Wegley 2007), we are beginning to close the gap between studies by making data more comparable. A long-term future goal may be to create an open-access global database of coral-associated bacterial DNA sequences from locations around the world.

Diversity and distribution

Extensive surveys of bacterial 16S rRNA genes have uncovered an enormous diversity of bacterial ribotypes, the majority of which are not closely related to previously identified, cultivated or uncultivated, microorganisms (Mouchka et al. 2010). In one of the first comprehensive studies of coral-associated bacterial diversity, Rohwer et al. (2002) sequenced over 1,000 bacterial 16S rDNAs from 3 species of Caribbean corals in
two locations and estimated that additional sequencing would yield another 6000 bacterial ribotypes. Subsequent studies have uncovered a similar magnitude of bacterial diversity from a variety of coral species around the world (Bourne and Munn 2005, Klaus et al. 2005, Koren and Rosenberg 2006, Sekar et al. 2006, Wegley et al. 2007, Koren and Rosenberg 2008, Littman et al. 2009). Researchers are finding that coral-associated bacterial communities are like plankton communities in that they are dominated by a few different taxonomic units with many rare types found in the long tail of the species-distribution curve (Rohwer et al. 2002), known as the “rare” biosphere (Sogin et al. 2006, Mouchka et al. 2010). Despite the volume of data that has been collected regarding coral-associated bacteria, we are still far from understanding the true diversity of these communities and how they are distributed across species, space, and time.

We must continue to investigate the diversity and distribution of coral-associated bacterial communities on a range of spatial scales relevant to coral health. For example, researchers may choose to focus on bacteria that are consistently associated with a particular host coral species in greater detail and at smaller scales to examine the bacteria’s role in health at the individual colony level. On the other hand, researchers may choose to examine coral-associated bacteria that are ubiquitous on a global scale to examine the effects of climate change and/or disease on coral populations. As mentioned previously, improvement and increased access to advanced technologies such as Fluorescent in Situ Hybridization (FISH) and next generation sequencing are making these goals more achievable.
Relationship with natural environment

Identifying specific environmental variables that affect coral-associated bacterial communities is essential to understanding the intricate balance between coral hosts and associated microbes and how large scale disturbances such as climate change and ocean acidification may affect coral health and subsequently, coral reef ecosystems. Although we have begun to identify particular factors that alter bacterial communities (e.g., temperature, carbon loading), they are often detected using short-term, small-scale aquarium experiments involving a single coral species. Larger-scale, long-term monitoring of coral-associated bacterial assemblages and environmental parameters in the field and in the laboratory is needed to fully assess how the coral holobiont will respond to localized and widespread global environmental change. After identifying specific types of bacteria that respond to particular environmental stressors, we may begin to target microbes for culture and continued study in the laboratory and field.

Identification of bacterial symbionts and functional roles

Recent studies have started to uncover a diversity of potential bacterial symbionts in many different coral species (Lesser et al. 2004, Apprill et al. 2009, Sharp et al. 2010, Sharp et al. 2012); however, many questions remain as to what beneficial functions these prospective symbionts perform for the coral host and how they are transmitted from generation to generation. Advances in gene sequencing, Fluorescent in Situ Hybridization, and microscopy techniques (Ainsworth et al. 2006, Neulinger et al. 2009) have improved our ability to specifically localize bacteria within host tissue and to
screen for functional genes, but continued study is needed to truly understand the
dynamics of these unique associations and their roles within the coral holobiont system.

Conclusion

Continued research on coral ecosystem health is important to preserving the
future of coral reefs; however, science alone cannot save them from widespread
devastation. Of equal importance is ensuring that information gleaned from scientific
studies extends far beyond the reaches of academia. Increased communication between
scientists, resource managers, and policy makers is vital to developing effective
conservation tools and for improving and implementing legislation aimed at managing
and protecting marine ecosystems.

This dissertation research was part of an ongoing collaboration between
scientists at the Hawaii Institute of Marine Biology, University of Hawaii and resource
managers from the Papahānaumokuākea Marine National Monument (National Oceanic
and Atmospheric Administration, the U.S. Fish and Wildlife Service, and the Hawaii
State Department of Land and Natural Resources). As a group, we were able to increase
communication and understanding amongst stakeholders, identify the scientific research
needs of the Monument, streamline permitting processes between agencies, expand
education and outreach, and address concerns of the Native Hawaiian community. From a
scientist’s perspective, this arrangement provided a unique opportunity to develop
innovative ways to address basic science questions while simultaneously incorporating
applications for management could serve as a model for future endeavors pertaining to
coral reef ecosystem based management and research.
References


