INVESTIGATING COASTAL MICROBIAL COMMUNITIES: DETECTING INTRODUCTIONS OF ENTERIC BACTERIA AND CHANGES IN THE CORAL MICROBIOME

By

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(Under the Direction of Erin Lipp and William Fitt)

ABSTRACT

Coastal development and maintaining the integrity of coastal ecosystems is a global issue, especially with the global population growing. In the Florida Keys, over 60,000 people live on a small archipelago (356 km^2). This dense population in close proximity to the only U.S. barrier reef has attracted much attention in research for the impacts on the health of coastal ecosystems. This dissertation investigates the level of water contamination and the coral health along the entire reef tract. In the remote Dry Tortugas National Park there is limited access and during peak activity, visitors swimming or wading introduced detectable levels of human enteric bacteria. This study confirms the introduction of enteric bacteria in coastal environment throughout the Florida Keys, which threatens the coral reef ecosystem. *Serratia marcescens* is one enteric bacterium that causes disease, white pox disease acroporid serratiosis, in the threatened coral species, *Acropora palmata*. The bacterial community of *A. palmata* was investigated to describe their healthy and disease states communities. These communities were highly diverse and responded to large environmental events, such as a Saharan dust storm. Overall, the bacteria Order Rhodobacterales increased in diseased coral lesions but neither *S.*

marcescens nor its parent Order Enterobacteriales were specifically associated with disease lesions. An advanced molecular assay was developed to specifically detect this bacterium throughout the Florida Keys. When three years of *A. palmata* samples and reef water were screened for *S. marcescens* there was only a 9% detection rate. However, it was more likely to detect the bacterium in the coral mucus than the water. Coral mucus is capable of concentrating and supporting the growth of introduced bacteria, like *S. marcescens*. The issue of the Florida Keys high population density and its influence on the coral reef resource is improving because local government agencies are upgrading the wastewater management. This improvement has dramatically improved the coastal water quality and minimized sewage contamination on the coral reef. However, complete etiologies of white pox disease along with other coral diseases are still illusive. More detailed monitoring and efforts are needed to identify pathogens.

INDEX WORDS: Enteric Bacteria, Coral Disease, White Pox Disease, *Serratia marcescens,* Coral Microbiome

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BS, Florida State University, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2014

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May 2014

DEDICATION

I dedicate this to Russell and Tamra Joyner, who instilled my early love for science and nature. I could not have come this far without their support and encouragement.

ACKNOWLEDGEMENTS

This dissertation research would not have been possible with out the support of my advisors and committee. Friends and undergraduate students made all the field, lab and analysis work possible and enjoyable. I would especially like to thank Jason Westrich, Carrie Futch, MaryKatherine Crews, Beth Mote, Jeff Turner, Leena Mayalil, Dusty Kemp, Keri Goodman, Beck Frydenborg, Tina Okereke, Jamilla Pham, Kelsey Montgomery, Virginia Schutte, Tom Barnum, Bill McDowell, Sara Budischak, Adam Rivers, Ashton Griffin, and Brett Berry. During the Dry Tortugas project I am particularly indebted to the help and support of Doug Morrison (USNPS, Retired) and the staff of the Dry Tortugas National Park for logistical support. Erich Bartels at MOTE Tropical Research Laboratory was an amazing captain and support for the lower FL Keys coral disease work. The wastewater treatment plant operators in Key Largo, Marathon, and Key West greatly facilitated collecting sewage samples.

Funding for the various projects and personal stipend was provided by the National Oceanic Atmospheric Association through an Oceans and Human Health Traineeship with the Georgia Oceans and Health Initiative and the Nancy Foster Scholarship. A National Science Foundation Ecology of Infectious Disease grant provided funding for the research on white pox coral disease. The United States Geological Service, US National Park Service, International Women's Fishing Society, UGA School of Ecology, and UGA Graduate School provided research and travel support through the years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Coral reefs are important ecologically and economically, contributing about $375 billion a year through ecosystem services around the world (Costanza, d'Arge et al. 1997). However, their existence is threatened because of a narrow habitat range and unclear resistance and resilience to impacts from climate change and coastal development, among other things. Twenty-percent of the world's coral reef ecosystems are degraded with little chance of recovery and 26% of the remaining reefs face impending collapse (Wilkinson, Science et al. 2004). The loss of coral reefs will translate to a global loss in biodiversity, diminishing many of the coastal ecosystem functions that benefit human health (Wilkinson 1996; Costanza, d'Arge et al. 1997; Hughes, Bellwood et al. 2002; Roberts, McClean et al. 2002).

Coastal water quality and ecosystem health

The poor disposal and containment of human wastewater has vast negative effects on the coastal environment and human populations. Impacts to the coastal environment grow in proportion to the growth of the coastal populations globally (Vitousek, Mooney et al. 1997). In less developed regions of the world, human wastewater is minimally treated before being released into coastal waters (Islam and Tanaka 2004). Along developed coastlines, including the Florida Keys, the traditional disposal methods for human wastewater are cesspits and aging septic systems, which are known to leak or directly release into coastal waters worldwide (Paul, Rose et al. 1997; Keller and Causey 2005). These discharges result in habitat degradation and pathogen introduction. Many popular Florida beaches are actively monitored and from 2006 to

2009 fecal contamination advisories were posted for 24 of 56 beaches (Natural Resources Defense Council 2009). The contaminated beaches and subsequent closures have a heavy impact on coastal economies through decreased tourism (Henrickson, Wong et al. 2001; Dorfman, Stoner et al. 2007). The BEACH (Beaches Environmental Assessment and Coastal Health) Act requires governments of coastal states in the U.S. to adopt consistent systems and procedures to regulate and monitor coastal water quality for sewage contamination (BEACH Act 2000). While this critical legislation protects recreational water quality and public health, water quality management still has its weaknesses. There is limited legislation that addresses water quality for non-beach recreational waters, such as coral reefs. Furthermore, existing legislation does not specifically address non-human health impacts, such as degradation of benthic communities (Kuersteiner and Herbach 1978; Sleasman 2009; Mallin, Haltom et al. 2010).

Coastal water quality has been a critical issue in the Florida Keys where many of the islands are densely populated. Most of the coastal ecosystems of the Florida Keys are state and federally protected; notable areas are the Florida Everglades, Florida Keys National Marine Sanctuary (FKNMS), The Dry Tortugas National Park (DTNP), and a few national wildlife refuges. These areas provide for the preservation of the coastal ecosystems in the face of growing coastal development and tourism (Keller, Gleason et al. 2009). Buildings in the Florida Keys primarily used septic and cesspit systems to dispose of waste. Despite ongoing upgrades to centralized wastewater treatment, these initial wastewater containment systems remain a source of sewage contamination to the surrounding coastal environment via groundwater discharge (Paul, Rose et al. 1995; Lipp, Futch et al. 2007; Futch, Griffin et al. 2010). These systems in the Florida Keys are minimally effective as wastewater disposal and containment solutions (Paul, Rose et al. 1997; Paul, McLaughlin et al. 2000; Sleasman 2009; Sleasman 2010). The primary

reason the traditional wastewater systems are ineffective is because the Florida Keys is an archipelago and a former coral reef. This means that most of the substrate for coastal development is porous limestone, fossilized coral, which has channels for wastewater to travel through and contaminate ground water, followed by the coastal environment (Shinn, Reese et al. 1994; Paul, Rose et al. 1997). Public and ecosystem health are at risk with the concurrent release of nutrients, chemicals, and enteric microbes (Pastorok and Bilyard 1985; Chabanet, Adjeroud et al. 2005).

Nutrification (the increase of nutrients in the coastal zone) has myriad negative effects, particularly in coral reef ecosystems, which rely on low nutrient conditions for high-light penetration to function efficiently. The higher level of nutrients promote algal growth, increase prevalence of marine diseases, and increase the abundance of bioeroders such as some worms and sponges on the coral reef (Lapointe and Clark 1992; Green and Bruckner 2000; Lapointe, Barile et al. 2004; Fabricius 2005; Ward-Paige, Risk et al. 2005). Alongside an increase in nutrients to the coral reef ecosystem, groundwater discharge also results in elevated levels of bacteria and viruses associated with sewage (Lipp, Jarrell et al. 2002; Lipp and Griffin 2004). These microbes, even at low levels in water, can accumulate in marine organisms such as corals and sponges (Lipp and Griffin 2004; Futch, Griffin et al. 2011). In some cases marine animals are able to survive with no long-term effects of exposure to such contamination (Kefalas, Castritsi-Catharios et al. 2003). Furthermore, some marine sponges can actually prosper when exposed to some level of sewage contamination and their abundance and location can be an indicator for contamination (Perez 2000; Ward-Paige, Risk et al. 2005). The accumulation of enteric bacteria and viruses in corals and sponges also allow these animals to be potential

biological sentinels for contamination; however, exposure to potential pathogens is also possible (Patterson, Porter et al. 2002; Lipp and Griffin 2004).

Coastal water quality monitoring

The history of sewage contamination along the coastal areas of the Florida Keys has generated concern for human and ecosystem health. Therefore, local and state agencies regularly monitor recreational areas for sewage contamination, where there is a greater potential for human exposure. However, in the remote the Dry Tortugas National Park (DTNP; about 112 km northwest of Key West), enteric microbes can still be detected in the coastal environment, which suggests that other sources of contamination, not related to waste disposal from a large population, may be important (Donaldson, Griffin et al. 2002; Santavy, Summers et al. 2005; Lipp, Futch et al. 2007). Access to the DTNP is limited and the onsite wastewater treatment and containment system was recently upgraded to limit possible contamination. Besides sewage potential contamination sources of enteric bacterial contamination in coastal waters are: terrestrial run-off, wildlife excrement, and human activities like boating and swimming (Fabricius 2005; Loehr, Beegle-Krause et al. 2006; Graczyk, Majewska et al. 2008; Wang, Solo-Gabriele et al. 2010). Identifying the sources of enteric microbes is essential for effective remediation and prevention of future coastal water contamination, a process referred to as source tracking (Field and Samadpour 2007; Plummer and Long 2007). Wildlife and domestic animal excrement also cause water quality problems along beaches and make identifying human sources of contamination more difficult (Shibata, Solo-Gabriele et al. 2010; Wang, Solo-Gabriele et al. 2010). Direct sewage contamination in the Florida Keys likely overwhelms the contamination caused by other potential sources, however they all contribute to decreased coastal water quality. Compared to the main islands of the Florida Keys, there are fewer marine vessels around DNTP,

but they are still a potential source of sewage contamination, mostly because of leaking holding tanks and lack of compliance with regulations to reduce dumping in nearshore waters (National Park Service Public Use Statistics ; Shafer and Yoon 1998; Mallin, Haltom et al. 2010).

To facilitate coastal water quality monitoring for sewage contamination, indicators for human health risk have been identified from among common enteric bacteria, which are now as a group referred to as fecal indicator bacteria (FIB). These FIB are the traditional system for assessing water quality in terms of human health risk (USEPA 2002; EPA 2004). Common FIB are *Escherichia coli*, enterococci, *Clostridium perfringens*, and the broader group of fecal coliform bacteria; culturing these bacteria from the environment is the primary and official detection methodology used by the federal and state governments (Cabelli, Dufour et al. 1983; Fujioka and Shizumura 1985; Dufour, Ericksen et al. 1986; EPA 2004). While useful as an indicator for health risk (e.g., gastrointestinal illness), FIB often cannot be used to identify contamination sources, nor do they represent the survival of human pathogens in the environment (Griffin, Lipp et al. 2001). The time and materials required for culture-based assays to identify and enumerate FIB also limits the scale of water quality monitoring along a coastline by the number of samples that can be processed. The accuracy of culturing results is also low because not all bacteria can be cultured and the presence of environmental inhibitors in the sample.

Molecular techniques (*e.g.*, quantitative polymerase chain reaction, qPCR) are being developed to increase the accuracy of detection and quantification of contamination of key microbial contaminants in the coastal environment (Siefring, Varma et al. 2008; Haugland, Varma et al. 2010; Sinigalliano, Fleisher et al. 2010). Molecular markers can also be used to improve the identification of contamination sources through the ability to directly detect human

pathogens instead of using other bacteria as indirect indicators (Lipp, Rivera et al. 2003; Fong and Lipp 2005; Shanks, Atikovic et al. 2008; Sauer, VandeWalle et al. 2011). The development of new indicators and methods strengthens water quality monitoring, which improves near-shore environments for both human health and the health of the coral reef ecosystem.

Coral holobiont a part of coral reef resilience

A key revelation for research and efforts to preserve corals and the coral reef ecosystem was the notion that the coral colonies function as holobionts (Fautin and Buddemeier 2004; Reshef, Koren et al. 2006; Rosenberg, Koren et al. 2007; Krediet, Ritchie et al. 2013). Coral holobionts are the conglomeration of the coral animal and micro-symbionts, such as dinoflagellates, bacteria, viruses, archaea, and fungi. These symbioses are extremely interdependent and the holobiont is part of the evolutionary process. According to the Hologenome Theory of Evolution, the holobiont acts as a unit for natural selection (Zilber-Rosenberg and Rosenberg 2008; Rosenberg, Sharon et al. 2009).

To understand the complexity of the coral holobiont, advanced molecular techniques have been used, most recently sequencing of the microbial community with next generation sequencing platforms. Sequencing studies of the coral microbiota with these advanced molecular techniques have been used to describe the composition of bacterial communities and are beginning to be used to investigate viral, archaeal, and fungal communities. These methods have helped to establish that the microbiota of the coral holobiont are made up of unique communities in the coral skeleton, tissue, and surface mucus layer (Sunagawa, Woodley et al. 2010; Sweet, Croquer et al. 2011). Proteobacteria, particularly the alpha- and gamma-Proteobacteria, comprise the majority of the coral bacterial communities in the mucus and tissue (Rohwer, Seguritan et al. 2002; Wegley, Edwards et al. 2007; Littman, Willis et al. 2009; Ceh,

Van Keulen et al. 2011; Morrow, Moss et al. 2012; Wilson, Aeby et al. 2012). There are also nitrogen-fixing bacteria and endolithic fungi specific to the coral skeleton (Shashar, Cohen et al. 1994; Golubic, Radtke et al. 2005). However, these communities are dynamic and the relative abundance of specific taxa change when the coral holobiont is bleached or diseased (Bourne, Iida et al. 2008; Thurber, Willner-Hall et al. 2009; Littman, Willis et al. 2011; Kelly 2013). The mechanisms that drive the changes in the microbial communities and the recovery of the communities to the their previous state are still being explored (Thurber, Willner-Hall et al. 2009; Littman, Willis et al. 2011; Wilson, Aeby et al. 2012). Overall, a simplistic view of the coral microbial community is that it is highly diverse and dynamic.

Sequencing of entire communities has expanded the field of coral reef ecology with identifications of new microbial taxa and functional roles within the coral holobiont symbiosis. The results of defining the coral microbial communities are generating efforts to understand the most common (e.g., core taxa) to the most unique taxa. In addition to identifying the taxa of coral symbionts, molecular techniques are also able to reveal functional roles of the microbiota within the holobiont (Garcia, Croquer et al. 2004; Beman, Roberts et al. 2007; Wegley, Edwards et al. 2007; Burke, Steinberg et al. 2011).

Functional gene sequencing and analyses of the coral holobiont have revealed that the symbiotic microbiota are essential contributors to the innate immune system of the coral holobiont (Burke, Steinberg et al. 2011; Kvennefors, Sampayo et al. 2012; Bay, Guérécheau et al. 2013; Kelly 2013). As part of the innate immune system, symbiotic microbes prevent infection through competitive exclusion, inhibiting enzymes produced by pathogens, interfering with pathogen cell-to-cell signaling, and producing antimicrobial compounds (Shnit−Orland and Kushmaro 2009; Teplitski and Ritchie 2009; Krediet, Ritchie et al. 2013). Specific studies of the

coral holobiont have identified and subsequently isolated antibacterial compounds, produced by symbiotic bacteria that contribute to the defense of coral colonies from pathogens (Ritchie 2006; Teplitski and Ritchie 2009; Shnit-Orland, Sivan et al. 2012). Similar to how symbiotic microbiota confer resistance to pathogen invasion of the coral holobiont, these microbiota may assist in the coral holobiont recovery following changes instigated by extreme environmental stress (Reshef, Koren et al. 2006; Rosenberg, Koren et al. 2007).

Common environmental stressors for the coral holobiont are associated with climate change and human activities (Wilkinson 1996; Obura 2005; Hoegh-Guldberg, Mumby et al. 2007). As the impacts from climate change and coastal development grow, the threats to coral reefs amplify and environmental conditions (e.g., temperature, nutrients, pH, physical damage) become more favorable for diseases (Harvell, Mitchell et al. 2002; Bruno, Petes et al. 2003; Sokolow 2009; Ateweberhan, Feary et al. 2013). If coral holobionts are stressed beyond a tolerance threshold such as during extreme thermal anomalies or nutrification, the susceptibility to infection increases and higher disease incidences are observed (Jackson 2008; Mydlarz, McGinty et al. 2009). When environmental conditions return to their previous state, the common microbiota of the holobiont may aid in recovery and adapt to respond to future infection by the same pathogen (Reshef, Koren et al. 2006; Rosenberg, Koren et al. 2007). The supportive roles of the symbiotic microbiota may facilitate the persistence of the coral holobiont and subsequently increase the resilience of the coral reef.

Coral diseases

There are currently about 20 described coral diseases with only 6 potential pathogens identified; the wide range is indicative of the difficulty in identifying and describing unique coral diseases (Sutherland, Porter et al. 2004; Rosenberg, Kellogg et al. 2007; Weil and Rogers 2011).

Microbes that generally cause diseases are categorized as primary or opportunistic pathogens. Where a primary pathogen of the coral would cause disease by invading and colonizing the coral holobiont, an opportunistic coral pathogen does not usually cause disease and may already be a part of the coral holobiont, but under certain environmental conditions it can colonize the coral holobiont or rapidly proliferate to cause disease (Ritchie 2006; Lesser, Bythell et al. 2007). The distinction between a primary pathogen and opportunistic pathogen for coral is currently vague because the coral holobiont is still not completely defined and it is unclear what conditions, environmental or physiological, cause a shift in the symbioses for diseases to occur (Casadevall and Pirofski 2003; Work, Richardson et al. 2008).

Koch's postulates are the primary method to identify or confirm potential pathogens. Koch's postulates state that the pathogenic microorganism occurs in every case of the disease being studied, it does not occur in another disease, it can be repeatedly isolated and grown in pure culture, can induce the disease in healthy individuals, and can be recovered from the newly diseased individuals (Evans 1976; Grimes 2006). With the growing abilities offered by molecular techniques, Koch's postulates as a system to confirm a pathogen have been re-evaluated. Depending on the study system there have been a few proposed techniques suggested to modernize Koch's postulates (Firth and Lipkin 2013). One is a series of four assertions: congruence, consistency, cumulative dissonance, and curtailment (Inglis 2007). In short, these assertions state that the potential pathogen always causes disease when encountering its host, there is no external cause before encounter to encourage the disease, the disease progresses as it was described initially after each encounter and when the pathogen is removed (i.e., antibiotic treatment) from the host the condition of the host improves (Inglis 2007). Another novel process to determine the cause of disease, but in the context of modern advanced sequencing methods,

are the revised Metagenomic Koch's Postulates. The revised postulates are: 1) the diseased metagenome must be significantly different from the healthy control metagenome and the suspected traits must be more abundant in disease metagenomes; 2) a healthy individual shows signs of disease when exposed to a sample from the diseased individual; and 3) the traits in the metagenome identified in the first step are again more abundant in the newly diseased individual's metagenome (Mokili, Rohwer et al. 2012).

Key challenges that remain in confirming coral pathogens include the ability to grow putative pathogens in pure culture and confirming the pathogen by inducing the disease (Ritchie, Polson et al. 2001). In an aqueous environment, it is difficult to contain potential pathogens during direct exposure to just a few colonies of a coral species, without re-introducing the pathogen into the environment, and ensure adequate exposure to the colony. Aquaria experiments can remedy these issues but do not replicate the highly dynamic environmental conditions found in a coral reef ecosystem (Ritchie, Polson et al. 2001; Sutherland, Shaban et al. 2011). Additionally, there are regulations that restrict the collection of coral for experiments in US territories, especially corals that are listed under the Endangered Species Act (ESA 1973; Hogarth 2006). The struggle to identify pathogens is neither new nor unique to coral diseases. Pathogens for many known human diseases have not fulfilled Koch's postulates, such as typhoid fever and leprosy, and the same may be true for coral diseases (Evans 1976; Grimes 2006; Firth and Lipkin 2013). In the case of opportunistic coral pathogens, virulence may depend on certain environmental conditions, which means that even if a microorganism can be isolated it may not always cause disease when tested because of differences in those key conditions (Ritchie, Polson et al. 2001; Muller and Woesik 2012). Regardless of the process to describe the cause of a coral disease, clear identification of coral pathogens requires a large number of samples, records of

environmental conditions, and utilizing modern molecular methods (Richardson, Smith et al. 2001; Pollock, Morris et al. 2011).

For the few identified or presumptive coral pathogens, there are a variety of disease etiologies. *Vibrio* bacteria have been associated with many coral diseases and these bacteria are both common in the marine environment and a part of the normal coral holobiont (Ben-Haim, Zicherman-Keren et al. 2003; Talledo, Rivera et al. 2003; Ritchie 2006; Cervino, Thompson et al. 2008; de Castro, Araujo et al. 2010; Sharon and Rosenberg 2010; Apprill, Hughen et al. 2013). *Vibrio coralliilyticus* was isolated from *Pocillopora damicornis* and *V. shiloi* was isolated from *Oculina patagonica* when there were signs of coral bleaching (Ben-Haim and Rosenberg 2002). Further testing illustrated how at temperatures greater than 25°C, *V. coralliilyticus* infected the symbiotic algae of *P. damicornis* to cause the observed bleaching now referred to as *Vibrio*-induced bleaching (Ben−Haim, Banim et al. 1999; Ben-Haim, Zicherman-Keren et al. 2003). Some of the mechanisms for how *Vibrio* spp. cause bleaching, such as inhibiting photosynthesis of the symbiotic zooxanthellae, have been elucidated using the *V. shiloi* and *O. patagonica* system (Ben−Haim, Banim et al. 1999; Banin, Israely et al. 2000). However since 2003, exposing *O. patagonica* to *V. shiloi* no longer results in coral bleaching; this is likely because the healthy corals are no longer susceptible (Reshef, Koren et al. 2006; Rosenberg, Koren et al. 2007). This is an example of a recent observation that some coral diseases are changing, which makes identifying disease etiologies and potential pathogens even more of a challenge (Bourne, Garren et al. 2009). The probiotic hypothesis explains that this change is due to the microbiota, as part of the coral holobiont, which have adapted and are able to prevent future infection and colonization of pathogens (Reshef, Koren et al. 2006). Some coral diseases have had an extreme effect on a coral population, such that the remaining population may

contain colonies that are more resistant to disease through their innate immune system (Bosch 2008; Vollmer and Kline 2008; Reed, Muller et al. 2010).

There are few but important coral diseases with identified pathogens that have been introduced into the environment (unlike the coral diseases caused by the marine bacteria, *Vibrio*). The introduced pathogens are from non-marine sources to coral reefs – the bacterium *Serratia marcescens* and the fungus *Aspergillus sydowii*. The fungus *A. sydowii* appears to have been introduced multiple times and Koch's postulates have been fulfilled to support that it is an opportunistic pathogen of gorgonian sea fans in the Caribbean (Smith, Ives et al. 1996; Rypien, Andras et al. 2008; Hallegraeff, Coman et al. 2014). This fungus is thought to have multiple methods of introduction from the terrestrial environments, including water run-off and dust storms (Weir-Brush, Garrison et al. 2004; Rypien, Andras et al. 2008). *S. marcescens* is a bacterium also commonly found in the terrestrial environment (e.g., plants, insects, soil) (Hejazi and Falkiner 1997; Azambuja, Garcia et al. 2005). Rather recently this bacterium has been found in coastal waters through sewage pollution (Patterson, Porter et al. 2002). Identical strains of this bacterium were also found in white pox coral disease lesions during the same time frame (Sutherland, Porter et al. 2010).

White pox disease as a specific threat to coral reefs

In 2002, Patterson and colleagues first reported that *S. marcescens* was an etiological agent of lesions described morphologically as white pox disease in *Acropora palmata* (elkhorn coral). The agent was confirmed via fulfillment of Koch's postulates, and following detection of *S. marcescens* white pox disease signs are referred to as acroporid serratiosis (Patterson, Porter et al. 2002). Between 1996 and 1999, 85% of *A. palmata* coral cover was lost in the Florida Keys, with much of this loss attributable to widespread disease prevalence (Patterson, Porter et al.

2002). This severe impact from disease on *A. palmata* resulted in their listing as threatened species according to the Endangered Species Act (ESA 1973; Precht, Bruckner et al. 2002; Hogarth 2006). When corals with signs of white pox disease were further investigated during outbreaks in 2002 and 2003, *S. marcescens* was found in disease lesions, uninfected corals, and *Coralophilia abbreviata* (corallivorous snail) (Sutherland, Porter et al. 2010). The marine isolated strains of *S. marcescens* matched strains found in human sewage using pulsed field gel electrophoresis (PFGE) (Sutherland, Porter et al. 2010; Sutherland, Shaban et al. 2011). It is unclear what the current prevalence of *S. marcescens* is on the coral reef, or how many morphologically described white pox disease cases are acroporid serratiosis. A molecular assay similar to the new water quality techniques would provide a method to accurately identify white pox disease lesions in *A. palmata* and if they are acroporid serratiosis when confirmed with *S. marcescens* infections.

Dissertation project goals

The goals of this dissertation were to: 1) Evaluate the introduction of enteric bacteria in a remote location with limited human and coastal development to introduce contamination. 2) Describe the microbial community of a critical coral species, *Acropora palmata*, threatened by the introduction of a human enteric bacterium. 3) Develop a specific molecular assay to detect the enteric bacterium and coral pathogen, *Serratia marcescens*, in near shore and reef environments. 4) Survey the microbial communities of *A. palmata* in the FL Keys for *S. marcescens* using this molecular assay. This dissertation will address the impacts of coastal water contamination and contribute more information about the microbial communities of this coral holobiont.

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CHAPTER 2: POTENTIAL FOR ANTHROPOGENIC INTRODUCTION OF ENTERIC BACTERIA IN A REMOTE MARINE CORAL REEF SYSTEM[1]

[1] Joyner, Jessica L.; Futch, J. Carrie; Griffin, Dale; Lipp, Erin; Human activity as a direct source of enteric bacteria in a remote coastline. To be submitted to Marine Pollution Bulletin.

Abstract

The Dry Tortugas National Park (DTNP) is a remote marine park in the southern Gulf of Mexico, which provides critical habitat for coral reefs. In the DNTP, and elsewhere, these remote and protected areas are also a draw for tourists. Access to the DTNP is regulated but concerns about water quality remain. This study examines the DTNP coastal environment for evidence of fecal contamination associated with human activities. Abundance, distribution and prevalence of fecal indicator bacteria (FIB; enterococci, fecal coliform bacteria and *Clostridium perfringens*) and *Serratia marcescens* (a pathogen of elkhorn coral) were evaluated quarterly for one year. Bacterial levels were low throughout the study (enterococci concentrations averaged 3.7 CFU 100 ml^{-1}, fecal coliform bacteria 1.9 CFU 100 ml^{-1}, and *C. perfringens* and *S. marcescens* <1 CFU 100 ml^{-1}), but fecal coliform bacteria and enterococci were always detected at the docks and beach. During the quarterly sampling the FIB most often detected were fecal coliform bacteria (20/52 samples) and enterococci (17/52 samples), whereas *C. perfringens* (7/52 samples) and *S. marcescens* (0/52 samples) were rarely detected. During a 42-h beach-bather time series study, both fecal coliform bacteria and *S. marcescens* levels in nearshore waters were significantly correlated with bather density. Although levels were low, indicating minimal risk to public health, results demonstrate that human activities are a source of enteric bacteria introduction, even in a remote locale.

Introduction

While many water quality studies are conducted in highly populated areas given the potential for high impact and exposure risk, very little is known for remote marine locations, which are often assumed to be relatively un-impacted. However, with growing interest in eco-

tourism, many remote areas are becoming a draw for tourists and efforts to understand potential impacts and risks to both humans and the environment are needed. In the case of tropical systems, recent work suggests that even regulated human access to offshore reefs can increase risks to coral health (Lamb and Willis 2011); however, few studies have assessed the impact of regulated human access and activity on water quality in such remote areas. To begin to explore this issue, we examined the water quality of the Dry Tortugas National Park (DTNP). Located 110 km west of Key West in the Florida Keys, tourist accessibility to the DTNP is highly limited. The national park was initially established in 1992 to preserve the Civil War era Fort Jefferson on Garden Key and later was extended to include the surrounding ecosystems. DTNP is considered to be unspoiled, relative to other heavily populated coastal regions, including the Florida Keys. Even with limited access and the few resident park rangers, fecal indicator bacteria and evidence of human enteric viruses have been reported anecdotally in studies conducted over the past 15 years (Paul, Rose et al. 1997; Donaldson, Griffin et al. 2002; Griffin, Donaldson et al. 2003; Griffin, Shinn et al. 2006). Fecal contamination was often attributed to two traditional in-ground waste disposal systems (*i.e.*, septic systems). Prompted in part by concerns for potential contamination, in 2004, DTNP converted to an aerobic digestion system within the Fort and a composting system for overnight visitors at the campground (Kimball 2005). New policies also required visitors, who arrive daily by ferryboats from Key West, to only use the toilets provided on the ferryboat; the island's few composting toilets were reserved for park staff (Dry Tortugas National Park 2007).

In contrast to DTNP, the greater Florida Keys archipelago is densely populated with in-ground disposal of untreated waste *via* septic systems as the primary disposal method, which is a primary source of sewage contamination to the surrounding marine environment (Paul, Rose et

al. 1995; Lipp, Futch et al. 2007; Futch, Griffin et al. 2010). Tracer studies in the 1990s demonstrated that viruses migrated quickly from septic systems and shallow injection wells into canals and continued offshore within hours of being introduced (Paul, Rose et al. 1995; Paul, Rose et al. 1997; Donaldson, Griffin et al. 2002). More recent work demonstrated that human enteric viruses could be found as far as the coral reef (Lipp, Futch et al. 2007; Futch, Griffin et al. 2010). For highly populated coastal areas, like the FL Keys, enteric bacteria can have other sources of introduction, though minor in relation to sewage contamination. Marine vessels can introduce human enteric bacteria through faulty waste containment or poor treatment from onboard facilities (Shafer and Yoon 1998; Loehr, Beegle-Krause et al. 2006). A non-human source of enteric bacteria is wildlife, terrestrial run-off will carry excrement from terrestrial animals and bird excrement can directly contaminate the water (Kleinheinz, McDermott et al. 2006; Graczyk, Majewska et al. 2008; Wang, Solo-Gabriele et al. 2010). The presence of enteric microbes within the DTNP, a remote location with limited access and improvements in local wastewater systems, suggest contamination from other sources or confounding stressors in this system. The contamination of enteric microbes in DTNP could be direct introduction from bather shedding as well as the highly mobile birds and boats (Gerba 2000; Kleinheinz, McDermott et al. 2006; Elmir, Wright et al. 2007; Loge, Lambertini et al. 2009).

Given the association between ecosystem health and anthropogenic stressors (including the introduction of enteric microbes), we examined the coastal marine environment of the Dry Tortugas National Park for evidence of sewage contamination and potential sources of enteric bacteria to the nearshore and offshore environment, including direct introduction by the limited number of swimmers that visit the DTNP.

Methods

Location

Samples were collected from four sites within the DNTP; locations were chosen along a range of human influence (Figure 2.1). The South Swim Beach and Ferry Dock, located on Garden Key, were considered to be the most likely impacted by human activities, as this is the focal point of all tourist activities within the park. Two reef stations were located about 1 km (Long Key Reef) and 5 km (Perfection Reef) offshore of Garden Key; both reefs have restricted human access. Visitor records during the sampling periods were obtained from the public database within the National Park Service (National Park Service Public Use Statistics). DTNP park staff record numbers of visitors that arrive by recreational vessels, seaplane, ferryboat, and others not included in those categories. Non-recreational visitors including commercial vessels (including fishing vessels) and overnight governmental vessels are also recorded.

Seasonal Study

Samples were collected at each of the four sites in December 2009, April 2010, June 2010 and September 2010. Because of inclement weather, Perfection Reef was not sampled in September 2010. At the beach and dock, surface water (1 L), sediment interstitial water (also referred to as pore water) (up to 1 L) and sediment (50 g) were collected. Surface water samples (grab samples) were collected ~0.5 m below the surface at each site using sterile 1 L polypropylene collection bottles. A modified *in situ* suction system was used to collect pore water. Briefly, sterile tygon tubing attached to a screened PVC pipe was inserted >0.5 m under the sediment and water collected into sterile containers via a gentle suction generated with a peristaltic pump. Sediment samples were collected from the first few centimeters using sterile 50 ml tubes to scoop and store the sample. Sponge tissue (5 g per sample) was collected from two

individuals selected haphazardly along the moat wall, adjacent to the beach site; no sponges were collected near the dock. At each of the two reefs, surface water (1 L) and sediment (50 g) were collected as described above. Additionally, three individual coral and sponges were sampled at Perfection Reef; six of each were collected from Long Key Reef (a larger system than Perfection Reef). Coral samples were comprised of 100 ml of the coral surface microlayer (mucus) collected from three species, *Acropora palmata*, *A. cervicornis* or *A. prolifera*, using a sterile needleless syringe. Five grams of sponge tissue was collected from four species: *Desmapsama anchorata*, *Iotrochota birotulata*, *Amphimedon compressa* and *Speciospongia vesparians*. Sponge tissue was only excised from the most distal branch or portion of individuals.

Water temperature, salinity, and pH were measured with a YSI 556 multi parameter sonde at each sampling point. Data were also obtained from the nearest NOAA weather buoy in Key West (#8724580) to corroborate *in situ* environmental measurements and determine approximate conditions for the December sampling event, when direct measurements could not be made.

Beach Study

In June 2010, a 2-day intensive sampling effort was completed at the South swimming beach on Garden Key (Figure 2.1), the site most popular for bathers. Surface water samples (2 L) were collected for 42 h every three hours, beginning at 06:00 day one until midnight day two. At each collection time, temperature, pH and weather conditions were noted. Additionally, people on the beach and in the water were counted (observations over several days confirmed that most beach goers were also bathers in the summer months).

Analysis of Enteric Bacteria

All surface water and pore water samples were filtered onto 47 mm, 0.45µm pore-size sterile mixed cellulose ester membranes, in duplicate, and placed onto appropriate selective agar plates (described below). Sediment samples (5 g) were agitated by vortexing for 2 min in sterile DI water (50 ml) and particles were briefly allowed to settle; 5 ml of the supernatant fluid was filtered in duplicate onto membranes as described above and placed onto appropriate selective media. Sponge samples (1 g) were macerated with a sterile razor blade and agitated in sterile DI water (50 ml) by vortexing; the resulting slurry was filtered in duplicate (5 ml per membrane) and incubated on appropriate selective media. Up to 10 ml of coral mucus was filtered in duplicate before placing membrane onto appropriate selective agar media.

Enterococci was grown on mEI agar and incubated at 41°C for 18 ± 4 hours; all colonies with a blue halo were considered to be positive (USEPA 2002). Fecal coliform bacteria were grown on mFC agar at 44.5°C for 18 ± 4 hours in a water bath; all blue colonies were counted (APHA 1995). *Clostridium perfringens* was grown on mCP agar and incubated anaerobically at 44.5°C for 18 ± 4 hours; all yellow colonies that turned pink upon brief exposure (~30 s) to ammonium hydroxide fumes were considered to be positive (Bisson and Cabelli 1979). *S. marcescens* was enumerated using the two-step culture method described by Sutherland et al. (2010). Briefly, pink colonies cultured on McConkey Sorbital agar (MCSA) with colistin and grown at 37ºC for 18 ± 4 hours were picked and transferred to DNase agar with Toluidine Blue supplemented with cephalothin (DTC) and grown at 41ºC for up to 48 hours (Farmer III, Silva et al. 1973; Grasso, d'Errico et al. 1988). Colonies that produced a red halo on DTC were considered as presumptive *S. marcescens* and were subsequently confirmed using PCR, as

described by Sutherland and colleagues (2010). Sterile phosphate buffered saline (PBS) was used for negative controls with all assays.

Statistical Analysis

The distributions of all enteric bacteria (FIB and *S. marcescens*) concentrations were tested using the D'Agostino and Pearson omnibus test for normality (including both non-transformed and log transformed data). Any counts below the limit of detection were considered to be zero for statistical analyses. Mean concentrations of bacteria were compared between sample dates, station, and sample type using the Kruskall-Wallis test for non-normally distributed data followed by the Dunn's Multiple Comparison test to determine pair-wise differences. Data from the beach study were analyzed separately from the data collected seasonally. A cross-correlative time series analysis with time lag was used to evaluate the relationship between numbers of beachgoers and swimmers and the bacterial levels. In all cases, significance was declared at $P < 0.05$. Statistical tests were carried out in RGUI (v and Graph Pad Prism (v 5.04).

Results

For the duration of the study, pH averaged 8.17, water temperature averaged 29.5°C, and salinity averaged 34.2 (Table 2.1). During the months sampled, the number of visitors ranged from 2,526 in September to 7,073 in July with a mean of 4,557 tourists per month (Table 2.1). There were no significant correlations noted between bacterial concentrations and measured environmental parameters. Additionally, bacterial concentrations were not correlated with total monthly visitor levels.

Concentrations of all FIB and *S. marcescens* were relatively low throughout the year-long study period. For all samples combined (N=120), fecal coliform bacteria were detected at a mean of 1.9 CFU 100 ml^{-1}, enterococci at 3.7 CFU 100 ml^{-1}, and *C. perfringens* and *S. marcescens* at <1 CFU 100 ml^{-1}. Fecal coliform concentrations were significantly greater than those of *C. perfringens* and *S. marcescens* ($P < 0.05$). Similarly, enterococci concentrations were significantly greater than *S. marcescens* ($P < 0.05$). When compared only by frequency of detection (i.e., presence/absence), fecal coliform bacteria were also found more often than other enteric bacteria (21.7% of all samples were positive compared to 14.2%, 6.7% and 0.8% positive for enterococci, *C. perfringens* and *S. marcescens*, respectively).

By station, fecal coliform bacteria and enterococci levels were both significantly greater at the dock (13.4 CFU 100 ml^{-1} and 31.9 CFU 100 ml^{-1}) than at reef stations (concentrations <1 CFU 100 ml^{-1}; $P < 0.05$). Enterococci levels at the dock were also significantly greater than concentrations at the beach ($P < 0.05$; Figure 2.2). All of the FIB were most frequently detected at the dock (75%, 66.7% and 41.7% of all samples were positive for fecal coliform bacteria, enterococci and *C. perfringens*, respectively; N = 12) and the beach (40%, 35% and 10% of all samples were positive, respectively; N = 20). However, *S. marcescens* was only detected at the beach (5% positive; one sample).

Compared by sample type, fecal coliform bacteria and enterococci were both found at significantly greater concentrations in pore water (means of 10.5 CFU 100 ml^{-1} and 15.6 CFU 100 ml^{-1}, respectively; N = 8) than in surface water (N = 19), sediment (N = 19), sponges (N = 41) or corals (N = 33; $P < 0.0001$; Figure 2.2). Likewise, FIB were most often detected in samples from pore water (87.5% of samples positive for fecal coliform bacteria and enterococci and 37.5% of samples positive for *C. perfringens*; N = 32). Among surface water samples, both

fecal coliform bacteria and enterococci were detected at significantly greater concentrations than in sponges (which were always negative) ($P < 0.05$) (Figure 2.2). While there were no significant differences between any of the bacterial concentrations between pore water sources (i.e., beach *versus* dock) or sediment sources (i.e., beach, dock, and both reefs), fecal coliform bacteria and enterococci were concentrations were significantly greater in dock surface water than other stations ($P < 0.05$). No differences were noted for either *C. perfringens* or *S. marcescens*, which remained at very low levels (not detected) for most samples (Figure 2.2).

Beach Study

During the course of the 42-h beach study, fecal coliform bacteria, enterococci and *S. marcescens* levels averaged 2.0, 0.3 and 2.6 CFU 100 ml^{-1}, respectively ($N = 13$). The highest daily concentrations for fecal coliform bacteria were 19 CFU 100 ml^{-1} at 18:00 on day one and 2 CFU 100 ml^{-1} at 15:00 on day two. Similarly, the highest concentrations of *S. marcescens* were noted at 18:00 at 20 CFU 100 ml^{-1} on day one and at 15:00 at 7.5 CFU 100 ml^{-1} on day two. For enterococci, levels peaked at 1.5 CFU 100 ml^{-1} on day two at 12:00. Beach-goers and bathers were confined to hours between 10:00 and 17:00 each day based on the daily ferry service schedule. On day one, 44 total beach-goers were counted with the greatest number of people (37) noted at 12:00. On day two, 33 total beach-goers were counted with the greatest number of people (23) again noted at 12:00.

The noted daily peaks in FIB and *S. marcescens* were significantly correlated with the number of people near or in the water over the same time period (Figure 2.3). Cross correlation analysis indicated a 6-hour lag between the peaks in beachgoer numbers and the subsequent peaks in the levels of fecal coliform bacteria ($P = 0.001$) and *S. marcescens* ($P < 0.001$).

Concentrations reached their highest levels on both days 6 hours after the peak number of people on the beach and in the water, and returned to baseline levels within the following 6 hours.

Discussion

The Dry Tortugas National Park (DTNP) has successfully established restrictions for visitor use that limit the overall direct human impact to the island and surrounding waters. The low levels of bacteria indicate that the upgraded treatment system, installed in 2004 at Fort Jefferson, has reduced bacterial levels in the coastal waters. It also suggests that enteric bacteria in surface water around the Fort are currently not likely to originate from the wastewater systems at the Fort. Fecal indicators were detected consistently at the swimming beach and boat docks, but at relatively low levels. Based on the time and location of detection, the introduction of these bacteria could be associated with bathers, presence of boats, and birds (Elmir, Wright et al. 2007; Graczyk, Majewska et al. 2008). Additionally, given the relatively high levels in sediment and pore water, sand in these areas could also represent a source of these FIB (Kleinheinz, McDermott et al. 2006; Stewart, Gast et al. 2008; Mallin, Haltom et al. 2010; Phillips, Solo-Gabriele et al. 2011). The higher abundance and frequency of fecal indicators specifically detected at the dock may be attributed to bird populations that frequent this area or a higher density of boats, including occasional live-aboard vessels. The DTNP is known for large populations of migrating terns and other shore birds (Colchero, Bass Jr et al. 2010) and the boat dock serves as typical congregation place. Future work should specifically investigate the influence of the density of birds around Garden Key using recently developed molecular markers for sources of avian fecal contamination (Ryu, Griffith et al. 2012).

Bathers appear to introduce a significant amount of enteric bacteria to the surrounding water, although concentrations declined to background levels within 6 hours. Other studies have shown similar trends of fecal indicator bacteria and potential pathogens in greater abundance when there was greater human use of the environment for swimming or recreating (Gerba 2000; Fleisher, Fleming et al. 2010). In addition to bathers directly shedding bacteria, their activity disturbs and resuspends sediment that may contain FIB, which together can contribute to a detectable rise of FIB in the water (Winslow 1976; Le Fevre and Lewis 2003; Phillips, Solo-Gabriele et al. 2011). However, FIB levels in the sediment throughout this study were low, supporting that the main source of FIB introduction during the beach study was bathers shedding bacteria.

Improved water quality monitoring as well updated sewage treatment and containment, as seen in the DTNP, can limit the introduction and impact from potential pathogens in coastal environments. With the exception of enteric bacteria introduced during swimming or beach activities, results suggest that water quality is typically good surrounding the remote islands within the DTNP. Small-scale introductions of human enteric bacteria are unlikely to pose a significant human health risk and enteric bacteria were found infrequently among coral and sponge samples. However, the presence of human enteric bacteria may be indicative of potential exposure risk and should be taken into consideration regarding conservation measures within this and similar remote marine parks.

Acknowledgements

This work was supported by a USGS – US National Park Service (Park Oriented Biological Support Program) Grant to DWG and EKL (GR10MN00BG54900). An NSF EEID award also

provided additional funding to EKL (OCE1015342) and a NOAA Oceans and Human Health Graduate Training Initiative (S0867882), which provided funding for JLJ and JCF. Lodging and field support were graciously provided by the Park staff at the Dry Tortugas National Park. We are particularly indebted to the help and support of Dr. Douglas Morrison (USNPS, Retired) for logistical support.

Disclaimer - The use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government

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Tables

Table 2.1. Monthly environmental characteristics of Garden Key.

Sampling Month	Number of Visits[a]	Water Temp. (C°)	Salinity	pH
December	3847	27.7	***	***
April	4785	29.1	36.5	8.3
July	7073	31.5	32.8	8.0
September	2526	29.8	33.5	8.2

[a] Recreational visitors during sampling month

Figures

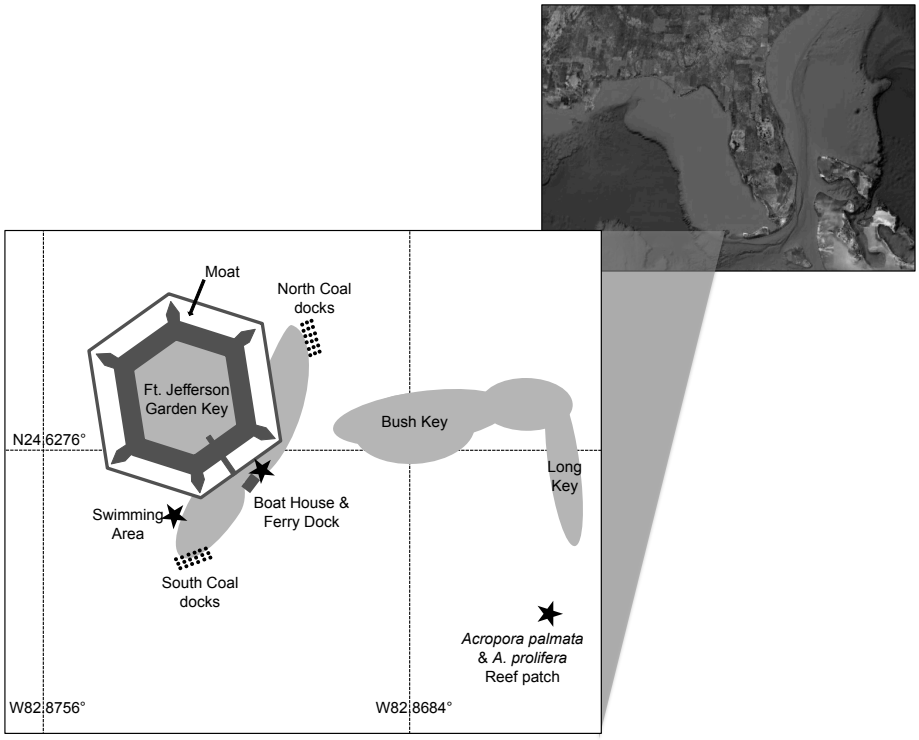

Figure 2.1. Map of the Dry Tortugas National Park.

Samples were collected at the locations marked with stars: the ferry dock, the south swimming beach area, the *Acropora palmata* and *A. prolifera* reef patch (also referred to as Long Key Reef), and a distant reef patch (Perfection Reef) located ~ 5 km northwest from Garden Key; not pictured).

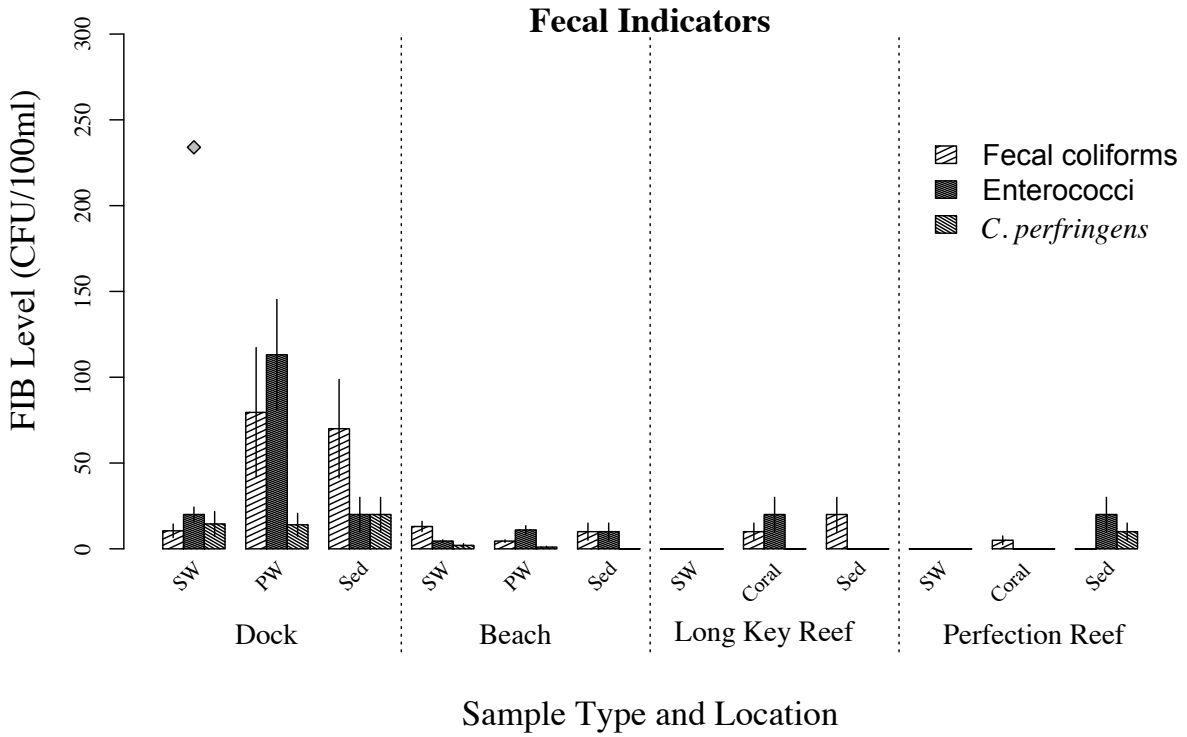

Figure 2.2. Fecal indicator bacteria (fecal coliform bacteria, enterococci and Clostridium perfringens) levels for each sample type and location.

Sample type: PW = pore water, Sed = sediment, SW = surface water, and Coral at the reef sites. Sponges were excluded from the figure because all samples were negative for FIB.

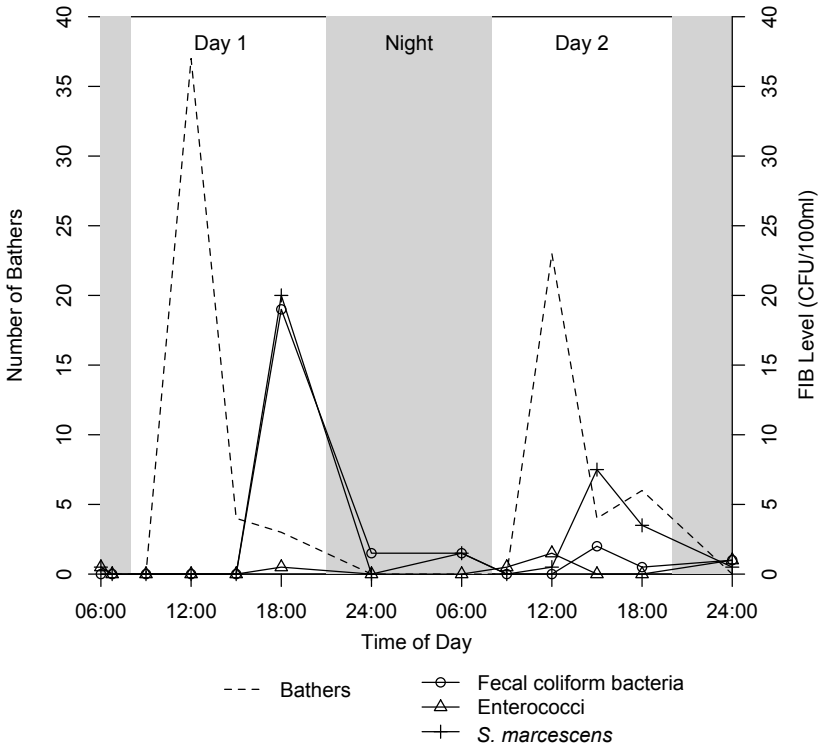

Figure 2.3. The number of people (dashed line) and enteric bacteria concentrations (solid line) during the bather study in July 2010.

This study lasted for two days, the white background indicates daytime sampling and the grey background indicates evening sampling.

CHAPTER 3: BACTERIAL COMMUNITY OF THE MUCU-POLYSACCHARIDE LAYER OF *ACROPORA PALMATA* IN THE FLORIDA KEYS[1]

[1] Joyner, Jessica L.; Kemp, Dustin; Porter, James; Lipp, Erin. To be submitted to the International Society for Microbial Ecology journal.

Abstract

The microhabitat of the surface layer of coral colonies or 'coral mucus' has a unique and diverse microbial community important for coral survival. One contributing cause for the decline of coral reefs is disease, which is linked to both a change in the symbiotic microbial communities and environmental stressors. This study compared bacterial communities of coral mucus from apparently healthy and white pox diseased *A. palmata* colonies. Samples were collected from Molasses (4 colonies and 16 samples from 2011 to 2012) and Looe Key (11 colonies and 46 samples from 2011 to 2013) reefs in the FL Keys; the 16S rRNA V4 region of bacteria was sequenced using Illumina MiSeq PE250. At Molasses, the average Shannon Diversity index (H') was 7.81 and 7.98 for the samples from healthy (N=16) and abnormal areas (N=4) of colonies, respectively. At Looe Key, the average H' was 7.02 for healthy (N=35) and 8.06 for abnormal (N=3) and 5.90 for diseased (N=8) colonies. The Permanova of the weighted UniFrac distance matrix for Looe Key indicated that the bacterial communities from diseased samples that occurred during the summer were significantly different than healthy corals ($P<0.05$). While there was no overall difference in the identified taxonomic community composition by season, changes in relative abundance of certain taxa were associated with disease status. In healthy coral mucus samples (N=51), Rhodobacterales made up an average of 5% of the bacterial community while in the disease samples (N=8) they contributed an average of 27%. This change in Rhodobacterales supports previous studies that have shown an increase in Alphaproteobacteria (as well as Rhodobacterales) in diseased corals. Results from these coral mucus bacteria communities also indicated a significant change in communities from episodic events in addition to other, more typical seasonal environmental differences.

Introduction

Microbial communities of plants and animals are critical for the survival of the host organism through nutrient exchange and protection from pathogens or predators (Azambuja et al., 2005; Ingham et al., 1985; Reshef et al., 2006; Rosenberg et al., 2007). Not surprisingly, research on coral microbial communities has revealed the revealed the importance and diversity of symbiotic microbes (Ainsworth et al., 2010; Johnston and Rohwer, 2007; Littman et al., 2009; Ritchie and Smith, 1997; Ritchie, 2006; Rohwer et al., 2002). According to the holobiont theory, the symbiosis between coral (animal) host, dinoflagellate symbionts (zooxanthellae) and microbes is interwoven to the extent that evolution acts on them as a unit (Zilber-Rosenberg and Rosenberg 2008). However, the short-term variation and response of the microbial communities to environmental changes and exposure to potential disease agents is unclear (Ainsworth et al., 2010; Bourne et al., 2008; Ceh et al., 2011; Guppy and Bythell, 2006; Koren and Rosenberg, 2006; Littman et al., 2009). Seasonal changes in the environment often correspond with significant changes in the coral symbionts and microbial community because of stress from increased water temperatures and higher irradiance, observed as coral bleaching or disease (Fitt et al., 2001; Hoegh-Guldberg et al., 2007). The probiotic hypothesis is an extension of the holobiont theory and proposes that microbial community shifts in response to environmental stress can contribute to the adaptation and survival of both the microbiota and the symbiotic host (Reshef et al. 2006). Additionally, the microbial communities provide services to coral hosts by producing antibiotic metabolites that prevent the invasion of foreign bacteria (Nissimov et al., 2009; Ritchie, 2006; Shnit-Orland and Kushmaro, 2009) and assist in making or acquiring necessary nutrients for corals (Shashar et al. 1994).

Despite the potential positive outcome predicted by the probiotic hypothesis, changes to the coral microbial community in response to stress may also negatively impact coral health by affecting disease susceptibility. Coral diseases are increasing in abundance throughout the world and research efforts are rapidly growing to address potential causes (Lesser et al., 2007; Pollock et al., 2011). There are up to 30 described coral diseases and only six diseases with an isolated and described pathogen (Sutherland et al. 2004, Weil 2004, Rosenberg et al. 2007). In the Caribbean, the iconic elkhorn coral (*Acropora palmata*) has been impacted by a number of observed diseases (e.g., white plague, white band, and white pox) (Peters et al. 1983, Ritchie and Smith 1998, Porter et al. 2001, Patterson et al. 2002). Since 1999, because of disease, hurricanes and human activity, living *A. palmata* coral tissue only covers about 2% of its potential habitat (Patterson et al. 2002). The dramatic decline observed for *A. palmata* earned the species formal recognition as a threatened species according to the Endangered Species Act (ESA 1973, Precht et al. 2004, Hogarth 2006). One of the few coral diseases with an identified pathogen is acroporid serratiosis, caused by a bacterium *Serratia marcescens*, which is initially is described through field surveys as white pox disease (Patterson et al., 2002; Sutherland et al., 2011). However, increased monitoring and tracking of disease occurrences suggest that *A. palmata* diseases, including signs of white pox disease, may be associated with additional pathogens or underlying causes (Ritchie 2006, Lesser et al. 2007, May et al. 2010). In other words, acroporid serratiosis may be just a single variant of white pox disease signs and additional work to understand the microbial community, as a whole is needed.

The goal of this study was to systematically examine the surface microbial community of the threatened coral *A. palmata* over multiple years and colonies. The study spanned three years, three seasons, two reefs and 16 coral colonies. We hypothesized that coral mucus from colonies

with and without observed signs of white pox disease would have distinctly different microbial communities and that communities would vary between studied seasons. These results contribute to broader efforts to describe coral microbial communities and the etiologies of coral diseases.

Methods

Sample Collection and DNA Extraction

Acropora palmata mucus and water were collected from physically distinct colonies at Molasses (MR) and Looe Key Reef (LKR) from 2011 to 2013. Presence of white pox disease on the coral reef was determined by conducting monitoring surveys at each sampling date. *A. palmata* colonies were tagged and the same colonies photographed at every sampling to document white pox disease lesions and general health status of the colony. During field surveys, divers considered colonies to have white pox disease if colonies were observed with irregularly shaped distinct patches (1-15cm^2) of tissue loss where the skeleton was bright white, indicating rapid tissue loss (Patterson et al. 2002), and no evidence of other causes (e.g., predation) were noted. When disease was present, samples were collected along the lesion margin and paired with a healthy region of the same coral colony, at least 10 cm away from the lesion (generally on a separate branch).

At each sampling time, coral mucus was carefully aspirated from at least 3 coral colonies with sterile 20 ml or 12 ml needless syringes. Water was collected with a sterile syringe (up to 60 ml) approximately one meter above the sampled colonies. The same colonies were re-sampled, whenever possible, throughout the study period. Samples were placed on ice within 30 minutes of collection and processed within 3 hours. Duplicate 2 ml aliquots of each sample were centrifuged at ~13,000 x *g* for 20 min and the supernatant fluid discarded. The remaining

bacteria-containing pellet was stored at -20°C until DNA could be extracted. The protocol of Boström and colleagues (Boström et al. 2004) was used to extract environmental DNA from frozen pellets, with slight modifications. Lysis buffer [175µl of 400mM NaCl, 750mM sucrose, 20mM EDTA (ethylenediaminetetraacetic acid), 50mM Tris-HCL (pH 9.0)] and lysozyme (1µl of 10 mg ml^{-1}) were added to the pelleted sample. Following incubation at 37°C for 30 min, proteinase K (100 µg ml^{-1} final concentration) and SDS (1% w/v final concentration) were added and tubes incubated at 55° C for 16-18 h. To aid in the precipitation of DNA, tRNA (50 µg) was used as a carrier molecule. Precipitation of DNA was initiated by adding 20 µl of 3M NaAc and 120 µl isopropyl alcohol then incubating for an hour at -20°C. Samples were centrifuged (~13,000 x *g* for 20 min) and the supernatant fluid decanted, retaining the pelleted DNA in the original tube. Samples were then washed with 500 µl EtOH (70%), centrifuged (~13,000 x *g* for 20 min) and supernatant fluid discarded. A SpeedVac (Eppendorf Concentrator 5301) was used to dry the DNA pellet, which was then resuspended in 50 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Next-Generation Sequencing

The hypervariable V4 region of the 16S rRNA gene was amplified using tagged primers containing Illumina adaptors and sequencing primer as described in Caporaso and colleagues (Table 3.1) (Caporaso et al. 2010). Each PCR included 12.5 µl Qiagen Taq PCR Master Mix, 0.5 µl forward and reverse primers (2 µM final concentration), 2 µl extracted DNA, and commercial sterile nuclease free water added to a final volume of 25 µl. Reactions were run in triplicate and amplified product (253 bp) confirmed by gel electrophoresis. Each band was excised and purified (MoBIO's UltraClean® GelSpin® DNA Extraction Kit, Carlsbad, CA) products from the triplicate reactions were combined to reduce the effects of PCR bias during sequencing.

Pooled samples were normalized to the sample with the lowest concentration, mixed with 10% PhiX (to reduce over clustering because of low phylogenetic diversity typical for 16S sequencing), and run on an Illumina MiSeq platform using a PE250 run (UGA Georgia Genomics Facility, Athens, GA). MR and LKR samples were sequenced on separate sequencing runs.

Sequencing Analysis

Sequences were demultiplexed and barcodes removed with the Illumina software (MiSeq Control Software 2.2.0.2 & 2.3.0.8). Illumina adaptors were trimmed using Trimmomatic in addition to trimming bases with poor quality scores (Lohse et al. 2012). A sliding window consisting of 4 basepairs was used and sequences trimmed whenever the average quality score of the window dropped below 15. The ends of the sequences were trimmed if the quality score was below 30. The minimum length, or number of nucleotide bases for a sequence to be retained was 100 nucleotide bases. The FastX toolkit (Gregory Hannon; http://hannonlab.cshl.edu/fastx_toolkit/index.html) was used to filter sequences that had minimum of 95% of bases with a quality score of 20 and to convert sequence files from fastq format to fasta. The QIIME pipeline was used for all further sequence processing and data analysis, selecting default options unless stated otherwise (Caporaso et al. 2010). The fasta files were modified for the desired QIIME input format by adding labels and concatenating the sequence files. Before picking OTUs, mitochondrial sequences were removed. This was accomplished by searching GenBank for 16S rRNA sequences within the mitochondrial database then downloading the results as a fasta file. The sample sequences were blasted against this database and sequences that matched the mitochondrial database with an e-value of at least 1e-10 and 97% alignment were considered mitochondria sequences and removed. The resulting

sequences were used to pick OTUs. OTUs were picked using an open reference frame and taxonomy assigned with the green genes database (gg_13_5.fasta) (DeSantis et al., 2006; Edgar, 2010; Wang et al., 2007). Chimera Slayer was used to detect and remove chimeric sequences (Haas et al. 2011). Sequences that were not taxonomically classified within the Bacterial kingdom were removed; these were Archaea, chloroplasts, and unclassified sequences. The most abundant sequence for each OTU, excluding singletons, was selected to create a representative set of sequences for all of the bacterial OTUs. The representative set of OTUs was aligned with PyNast and a phylogenetic tree was generated with FastTree using the 'fastest' method (Caporaso et al., 2009; DeSantis et al., 2006; Price et al., 2010).

Standard alpha diversity assessments were completed, calculating Chao1 and within sample diversity of the bacterial communities. Paired water samples were used to generate an OTU dataset to identify and remove water-specific OTUs from coral mucus samples. For beta diversity assessments common water OTUs were removed; these were OTUs present in at least 50% of the water samples. This filtering allowed the study to focus on coral mucus specific bacterial communities while retaining some water OTU sequences to still be able to observe potential changes in the water between sampling dates. Proportions of the remaining OTUs for replicate samples were averaged to create bacterial community profiles for each date, sample type, and diseased corals.

Samples were analyzed with QIIME and RGui for alpha and beta diversity to assess potential difference in the bacterial communities among the categories of reef, sampling date, sample type, and disease presence. The weighted UniFrac distance matrix was calculated to complete principle components analysis (PCoA). Differences in community composition between samples were determined with the distance matrix using permutation-based multivariate

analysis of variance (PERMANOVA) (Lozupone and Knight 2005, Lozupone et al. 2007). The PERMANOVA (adonis function in vegan package for RGui; (Oksanen 2005, Oksanen et al. 2007) of the weighted UniFrac metric distance matrix was stratified by reef and interactions between the sampling type, date, and disease status. To analyze changes in the abundance of bacteria, proportions of each Order were square root arcsine transformed to approximate a normal distribution and transformed data were used in ANOVAs for each reef with date, sample type (coral mucus or water), and disease used as interacting factors (Sokal and Rohlf 1995).

Results

Definitive white pox disease signs were recorded in summers of 2012 and 2013. At LKR in August 2011 abnormal tissue areas were observed in 9.6% of colonies (3 of 31 colonies surveyed) but white pox disease was not confirmed (Table 3.2). At MR in June 2011 abnormal tissue areas were recorded in 33% of corals (4 out of 12 surveyed corals) but disease was not confirmed. In September 2011, 80% (8/10) of MR reef colonies had signs of white pox disease. For LKR, definitive white pox signs were noted on 32% of colonies (10 of 31 colonies surveyed) in August 2012 and on 22% of colonies (6 of 27 colonies surveyed) in July 2013. Disease signs were not noted in samples collected in the winter (December – February).

Compared to previous coral microbiome studies, the bacterial communities (regardless of disease state or collection date) had high numbers of OTUs and diversity (Cárdenas et al. 2012, Morrow et al. 2012, Kimes et al. 2013). Despite normalization of amplified DNA between samples before sequencing there was a wide range of OTUs, (131 to 3180) with an average of 1348 OTUs. There was also a high diversity within each sample, an average estimation from Shannon index (H') of 7.34 and phylogenetic diversity of 49.8 (Table 3.2). When comparing the

phylogenetic similarity (weighted UniFrac distance matrix) of the bacterial communities, the sampling date was significant and explained more than 50% of the differences between samples (P=0.001; Table 3.4). The interactions between sample type and sample date was significant (P=0.001; Table 3.4) and interaction between the sample date and disease presence was significant (P=0.03; Table 3.4).

A large portion of all the bacterial communities was composed of unknown bacteria. When the core water OTUs were removed, 139 bacteria OTUs were unknown (from a total of 965 OTUs when OTUs were filtered for at least 100 counts/OTU; Figure 3.1) for an average of 22.3% of the bacterial communities. According to the PCoA, bacterial communities between all the samples did change according to sample type, sample collection date between winter (December – February) and summer (July – September), and if disease was present. The PCoA generally showed clusters for each sampling event and disease lesion samples were separate from healthy coral samples (Figure 3.2).

To isolate the potential contributions of specific bacterial groups on the composition of coral microbial communities, the relative abundance of bacterial Orders (representing at least one percent of the overall coral microbial community at each reef and assigned a definitive Order taxonomic classification) were compared for differences according to disease state and date (30 Orders tested of the 111 total Orders; Figure 3.2). At MR and LKR a significant difference in the relative abundance of Orders was noted between healthy and abnormal (no disease signs collected in August 2011) sample communities for only Burkholderiales (fewer in MR abnormal and rare at LKR; P<0.001; Figure 3.3). Additional orders were significantly different for abnormal coral samples at LKR: Enterobacteriales (fewer in disease samples, P<0.001), Lactobacillales (fewer in disease samples, P<0.001), Planctomycetales (more in disease samples,

P<0.001), Rhizobiales (fewer in disease samples, P=0.0105), Thiotrichales (fewer in disease samples, P<0.001), Xanthomonadales (fewer in disease samples, P<0.001). Healthy and diseased coral communities were compared for samples from LKR (as no disease was noted at MR). Significant differences in the relative abundance of Orders were recorded (Figure 3.3): Rhodobacterales (P<0.001), Pseudomonadales (P<0.001), Thiotrichales (P<0.001), Alteromonadales (P<0.001). At LKR, Rhodobacterales and Thiotrichales significantly increased in disease samples. The Rhodobacterales relative abundance within the MR coral mucus also increased in abnormal samples in June 2011 from 3% in healthy samples to 13% in abnormal samples; however, the difference between sample types was not significant. At LKR in August 2011 the relative abundance increased from 5% in healthy samples to 6% in abnormal samples. In August 2012, Rhodobacterales expanded from 11% in healthy samples to 36.6% in definitively diseased samples; and, in July 2013 from 0.6% in healthy samples to 17.6% in diseased samples. The increase in Rhodobacterales contributed to an overall higher percentage of the class Alphaproteobacteria in mucus from diseased coral lesions. At one sampling point, July 2013 (LKR) all samples (i.e., water, healthy coral, and diseased coral colonies) showed a significant increase in Pseudomonadales from an average of 4% in all previous samples to an average of 32% in the July 2013 samples (P=0.0031) and Vibrionales from about 3% in all previous samples to 30% in the July 2013 samples (P=0.00123). Other bacterial Orders also varied between samples and through the year to contribute to the overall described bacterial community variation (Appendix 1).

Discussion

Coral microbial communities are highly diverse and the communities in this study had greater diversity than many previously reported (Barott et al. 2012, Cárdenas et al. 2012, McKew et al. 2012, Morrow et al. 2012, Lesser and Jarett 2014). Early research on the *A. palmata* bacterial communities relied on degradient gel electrophoresis (DGGE) band patterns (based on the 16S rRNA gene) and 16S rDNA clone libraries to estimate bacterial community diversity and composition (Pantos and Bythell 2006, Ritchie 2006). These studies provided the foundation for current efforts to identify coral microbiomes with next generation sequencing, which now have a much greater sampling depth and refined taxonomic assignment. Previous studies that used next generation sequencing to describe the coral bacterial community among other corals had lower levels of diversity than the healthy *A. palmata* (H'=6.53 – 8.22) in this study: *A. palmata* (H'= 3.11 – 5.56; (Lesser and Jarett 2014)), *Orbicella faveolata* (H'=4.2 – 6.1; (Morrow et al. 2012)), *O. annularis* (H'=2.8 – 5.0; (Barott et al. 2012)), *Porities asteroides* (H'=0.8 – 4.3; (Morrow et al. 2012)), *Siderastrea siderea* (H'=5.4 – 5.5; (Cárdenas et al. 2012)), and *Diploria strigosa* (H'=4.4 – 4.6; (Cárdenas et al. 2012)). However, even within different next generation sequencing studies the variation in described communities can be attributed to different sampling techniques and the community differences between the microhabitats of the coral holobiont (i.e., the skeleton, tissue, or surface mucus layer) (Ainsworth et al. 2010, Sweet et al. 2011).

The taxonomic composition of the bacterial community is dynamic within the surface mucus layer of *A. palmata* colonies of in the Florida Keys. The composition of the bacterial community varied by season with differences noted between summer and winter without a single taxa driving the variation (except for the unique changes observed in July 2013). Furthermore, corals from each reef, LKR and MR, had distinct microbial communities (PCoA). This

difference may be because of geographic distance as has been previously reported (Littman et al. 2009, Morrow et al. 2012). However, the bacterial community data was collected on two separate sequencing runs and this may have also generated enough variation to confound real differences in the communities.

Coral mucus from disease lesions had a different community of bacteria than healthy coral mucus. Previous work has shown that the microbial communities from different regions within an individual *A. palmata* colony are homogeneous (Kemp et al., in revision); therefore, observed differences between healthy and diseased regions within a single colony can be clearly linked to disease state rather than underlying heterogeneity in these corals. Coral mucus samples collected from a healthy area on a diseased colony did not differ, in regards to bacterial community composition or diversity, from samples collected from apparently healthy colonies. Conversely, the composition of mucus bacterial communities was significantly different between healthy tissue and disease lesions mucus bacterial communities, in contrast to a recent study of white pox disease in *A. palmata* (Lesser and Jarett 2014) but similar to what other coral disease studies (in *A. palmata* and other coral species) have reported (Pantos et al. 2003, Sunagawa et al. 2009).

Despite overall differences in communities between the two reefs studied, white pox disease samples had a greater relative abundance of Rhodobacterales (Class Alphaproteobacteria). Previous descriptions of bacterial communities in healthy and diseased corals (i.e., black band disease, white plague) have also reported a general increase in Alphaproteobacteria in the diseased corals and some studies specifically recorded an increase in Rhodobacterales in coral disease lesions (e.g., plague-like disease, atramentous necrosis, black band disease) (Pantos et al. 2003, Jones et al. 2004, Sekar et al. 2006, Mouchka et al. 2010,

Cárdenas et al. 2012). The association of Rhodobacterales with disease lesions may indicate either that the bacteria are part of the initial infection of coral or a response to newly available substrate in response to disease conditions. The generality of the Rhodobacterales detected with most coral diseases suggests that the reported increase from this study is neither specific to white pox disease nor another coral disease and is potentially a response to any disease that causes tissue loss (Sekar et al. 2006, Mouchka et al. 2010, Cárdenas et al. 2012). Rhodobacterales are abundant in marine waters and maintain a variety of roles within microbial communities such as colonizing a marine surface early in the establishment of marine microbial communities, which alone may explain the abundance associated with disease lesions because they are initiating a new microbial community in the exposed skeleton (Buchan et al. 2005, Dang et al. 2008, Witt et al. 2011). Our work corroborates these findings and provides additional support that the general increase of Alphaproteobacteria in diseased corals recorded in early studies with poor taxonomic resolution may have been observed because of an increase in Rhodobacterales as is recorded now with improved sequencing capabilities and taxonomic identification.

To date, only one causative agent for white pox disease signs has been confirmed. When the bacterium *Serratia marcescens* is confirmed in white pox disease lesions, the disease is referred to as acroporid serratiosis. *S. marcescens* is a gram-negative bacterium in the Order Enterobacteriales, Class Gammaproteobacteria (Patterson et al., 2002). Given limitations in identification using short regions of the 16S rRNA gene, bacteria could not be resolved to the species level in this study and identification to the genus level were not considered to be robust, especially for Enterobacteriales (Naum et al. 2008). Enterobacteriales were found in bacterial communities from all samples; however, there was no increase in abundance in disease samples or in samples collected during the summer when disease was present on the reef. Therefore, any

white pox disease signs, such as those observed during this study period, are likely not acroporid serratiosis (Lesser et al. 2007, Sutherland et al. 2010, Lesser and Jarett 2014)). Although analysis at the Order level might mask specific and important compositional shifts at the genus or species level, the data from this study suggest that other etiological agents should be considered for signs associated with white pox disease.

The coral holobiont can rapidly change, responding to perturbation, which is one of the challenges in describing coral diseases (Reshef et al. 2006, Thurber et al. 2009). Previous coral disease studies have proposed that the microbial communities associated with disease lesions are opportunistic bacterial growth (Reis et al. 2009). Synthesizing our results of greater levels of Rhodobacterales and no change in the abundance of Enterobacteriales may indicate that the mucus samples from these white pox disease lesions do not represent the initial infection of healthy tissue or disease margin. The bacterial communities described from disease samples may instead represent a transitional community, one that follows the disease front and is composed of bacteria opportunistically colonizing newly available space (Krediet et al. 2013). Many coral disease studies are limited by the ability to capture the initiation of disease and it is often not clear in the field at what stage of disease a coral may be experiencing.

An episodic environmental event during the 2013 summer illustrated the interconnectedness of the marine microbial communities with strong and rapid influence from environmental changes. In the day before samples were collected in July 2013 at Looe Key, aerosolized Saharan dust passed over the Florida Keys. Dust deposition events are known to occur in the Caribbean and subsidize ecosystems with micronutrients and trace metals (including iron) eliciting a biological response (Shinn et al. 2000, Garrison et al. 2003, Pulido-Villena et al. 2008, Hill et al. 2012). Vibrionales are especially thought to proliferate under conditions with a

higher level of available iron (Westrich et al. in prep); dust transported from Africa and deposited in the North Atlantic and Caribbean is known to provide a source of bioavailable iron (Sigel and Payne 1982, Garrison et al. 2003, Maranón et al. 2010). In this case, the overlap of this dust deposition event and sampling of the bacterial communities in July 2013 may explain the very large increase in the relative abundance of the Vibrionales and Pseudomonadales. Westrich and colleagues (personal communication) also noted a significant increase in culturable *Vibrio* concentrations from the reef water column during this event. This was a serendipitous sampling event, where the deposition of Saharan dust coincided with sampling of the *A. palmata* bacterial community. The results provide preliminary support that desert dust deposition can have a significant effect on the coral holobiont. In addition to this increase in the abundance of these taxa in all July 2013 samples, the abundance of Rhodobacterales was still elevated in samples from disease lesions relative to the water and healthy coral samples.

Recent studies focusing on the function and composition of the microbial community in coral holobionts suggest that the resident microbiota might be specific to individual species (Ritchie and Smith, 1997; Ritchie, 2006; Rohwer et al., 2001). Characterizing this microbial community structure over time and space is a critical step in understanding the role of this community in the dynamics of coral disease, such as white pox disease (Ainsworth and Hoegh-Guldberg, 2009; Guppy and Bythell, 2006). Future changes or adaptations in microbial communities and the implication for the health of the holobiont can only be detected with a well-established baseline of natural variability. Regional to global climate change and local anthropogenic disturbances are the two main sources of stress on coral reef communities and recent trends forecast increasing impacts (Hoegh-Guldberg et al., 2007). Therefore, there is great

potential for selective pressure on the microbial symbionts of corals and a need for regular, long-term surveys of both coral colonies and associated microbiomes.

This study has described the baseline bacterial community of *A. palmata* in the Florida Keys and begins to explain the etiology and progression of white pox disease. The bacterial communities were described for three years including diseased corals and some bacteria are predominate because of strong influences from the environment and others during disease progression, but it was unclear as to stage of the disease these communities represented. Future studies about white pox disease in *A. palmata* need to be refined assessments of the microbial communities from the disease margin. Additional research about the *A. palmata* microbiota will assist in identifying the cause of white pox disease when *S. marcescens* is absent from samples. It is also possible that some microbes respond in similar ways to the coral disease progression regardless of the coral species and potential pathogen. Further research about the coral microbiome can clarify the coral holobiont response to infection and the disease process; separating out the pathogenic microbes from the holobiont microbial response and the microbes capitalizing on the holobiont's plight and colonizing new territory.

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Tables

Table 3.1. Primers used for Illumina PE250 sequencing (Caporaso et al. 2011).

Sequences are listed in the 5’ to 3’ direction.

Primer	16S V4 PCR primer	Barcode	Adaptor	Linker	Illumina Sequencing Primer
Forward	AATGATACGGCGACCACCGAGATCTACAC	N/A	TATGGTAATT	GT	GTGCCAGCMGCCGCGGTAA
Reverse	CAAGCAGAAGACGGCATACGAGAT	TCCCTTGTCTCC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT

Table 3.2. Individual samples collected with total sequences after quality control filtering. White pox disease (WPD) presence on the reef is also given for context. When a bacterial community was not sequenced it is indicated by (-).

Molasses Reef					
Sample Date	**WPD**	**Coral Colony**	**Healthy (# Seqs)**	**Abnormal (# Seqs)**	**Paired Water (# Seqs)**
June 2011	0%	1	111010	69103	158458
		7	78218	71604	-
		9	41409	25995	-
		17	-	39838	23812
		22	92201	-	84692
Sept. 2011	80%	1	45250	-	53455
		7	46486	-	49950
		9	38918	-	43989
		17	122248	-	26014
Feb. 2012	0%	1	51442	-	74470
		7	43384	-	-
		9	61805	-	63935
		17	54471	-	24357
May 2012	0%	1	90599	-	36775
		6	-	-	91023
		7	85168	-	-
		9	70772	-	-
		17	57496	-	68326
Looe Key Reef					
Sample Date	**WPD**	**Coral Colony**	**Healthy (# Seqs)**	**Diseased (# Seqs)**	**Paired Water (# Seqs)**
August 2011	0%	21	12814	6413 (abnormal patch)	-
		24	16208	-	15841
		30	8418	-	16813
		57	22146	-	16233
		60	20228	-	-
		62	29301	-	21925
		64	20581	-	14331
		66	15274	-	-
		68	18772	-	12615
		69	9877	11744 (abnormal patch)	-
		71	11762	6495 (abnormal patch)	2413
Feb. 2012	0%	24	19385	-	15561
		30	18720	-	18880
		57	18243	-	-
		62	13321	-	-
		64	9699	-	-
		68	21352	-	-
		71	17265	-	-

August 2012	32.4%	24	9773	15451	-
		62	6876	13379	-
		68	4823	14697	-
Feb. 2013	0%	24	18533	-	-
		29	11946	-	12099
		30	26037	-	-
		57	20095	-	-
		60	14185	-	-
		62	16632	-	-
		64	13839	-	-
		68	13447	-	8788
		71	14662	-	16991
July 2013	22.2%	30	14133	14822	11451
		57	16362	15631	1036
		60	26216	18691	14938
		64	17422	7205	20848
		71	16349	20769	20345

Table 3.3. The initial alpha diversity summary with no core water OTUs removed. Individual sample metrics were averaged and reported below for each sample type. MR = Molasses Reef, LKR = Looe Key Reef.

Reef	Date	Sample Type	Sample Size	Filtered Seqs (μ #)	OTUs (μ #)	Chao1 (μ #)	Shannon (μ H')	Phylogenetic Diversity
MR	June 2011	Water	3	65660	2389.33	3061.85	8.02	82.91
MR	June 2011	Coral	4	57410	2628	3327.30	8.17	95.92
MR	June 2011	Abnormal	4	36655	2082.75	2745.5	7.98	83.55
MR	Sept. 2011	Water	4	36155	1806.5	2386.02	7.75	71.34
MR	Sept. 2011	Coral	4	53595	2047	2669.64	6.95	78.84
MR	Feb. 2012	Water	3	45148	2280.33	2893.76	8.06	90.04
MR	Feb. 2012	Coral	4	43996	2364.75	2933.89	8.23	90.42
MR	May 2012	Water	3	55129	2000	2617.12	7.61	61.83
MR	May 2012	Coral	4	60041	2342.5	2995.32	7.91	73.77
LKR	Aug. 2011	Water	7	98723	863.43	1283.50	7.69	33.39
LKR	Aug. 2011	Coral	11	11538	1020.55	1497.94	7.99	39.69
LKR	Aug. 2011	Abnormal	3	5697	766.67	1183.06	8.06	31.62
LKR	Feb. 2012	Water	2	12451	995.5	1386.04	7.87	37.48
LKR	Feb. 2012	Coral	7	11586	792.57	1082.50	6.95	30.30
LKR	Aug. 2012	Coral	3	5558	588	890.74	7.08	26.85
LKR	Aug. 2012	Disease	3	13395	686	926.31	5.76	29.59
LKR	Feb. 2013	Water	3	8231	844.67	1227.99	8.19	33.27
LKR	Feb. 2013	Coral	9	12432	941.44	1369.75	6.53	36.83
LKR	July 2013	Water	5	10031	666	1038.54	6.12	24.98
LKR	July 2013	Coral	5	15407	1064.20	1604.07	6.54	33.35
LKR	July 2013	Disease	5	12564	904.8	1386.36	6.04	32.14

Table 3.4. Permanova table for the analysis of the weighted UniFrac distance matrix for to test for the significant factors influencing the bacterial communities.

adonis(formula = DistanceMatrix ~ SampleType*Date*Disease, data = MappingFile, permutations = 999, strata = MappingFile$Reef)						
	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
SampleType	1	0.06961	0.069609	12.3844	0.04708	0.001 ***
Date	7	0.83722	0.119603	21.2789	0.56627	0.001 ***
Disease	1	0.00392	0.003924	0.6981	0.00265	0.489
SampleType:Date	6	0.09524	0.015873	2.8240	0.06442	0.001 ***
Date:Disease	3	0.03970	0.013234	2.3544	0.02685	0.035 *
Residuals	77	0.43280	0.005621		0.29273	
Total	95	1.47849			1.00000	
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

Figures

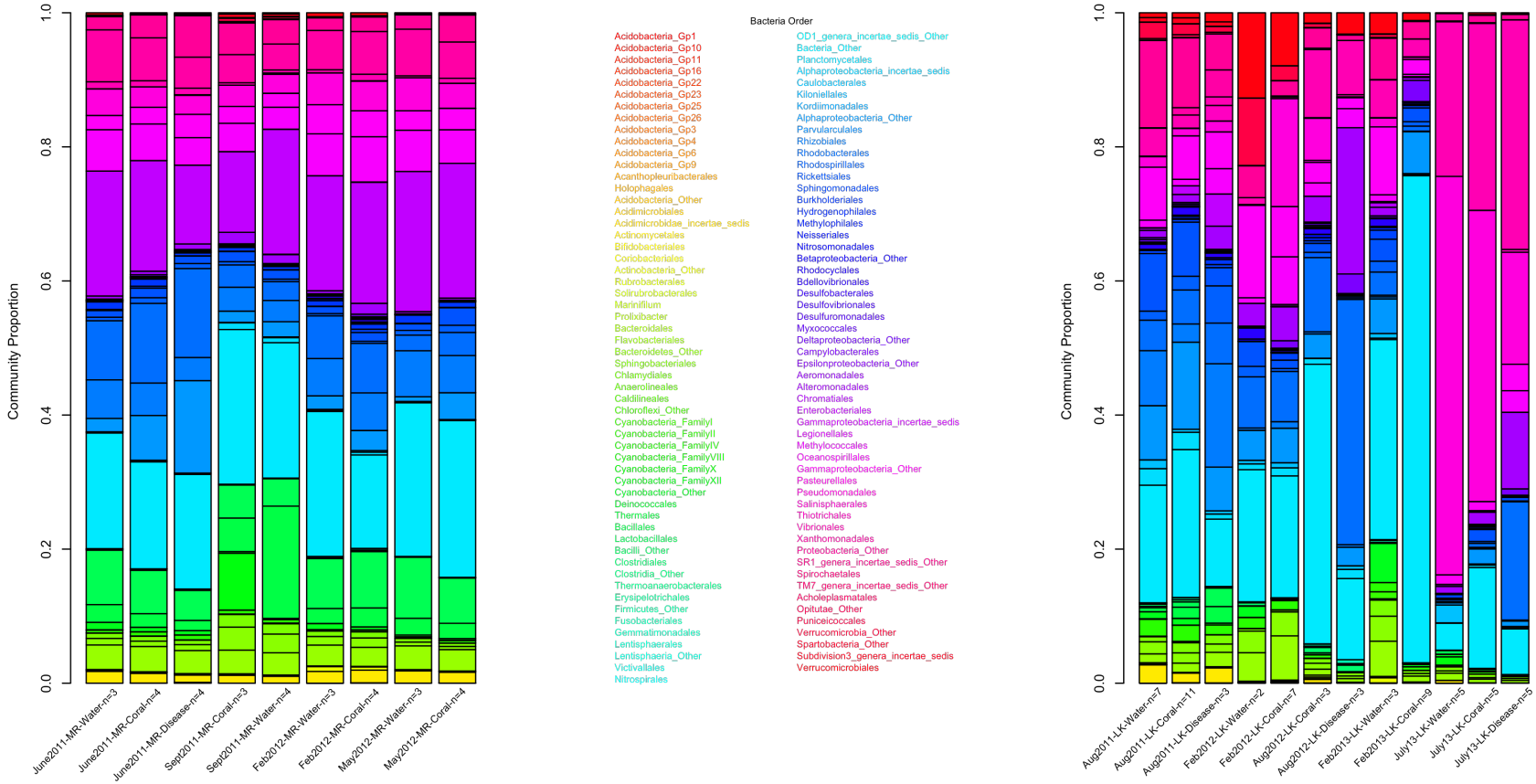

Figure 3.1. Complete bacterial community profiles.

Individual samples were averaged excluding a coral sample from MR in Sept 2011 that had an abnormal abundance of the Order Bacillales.

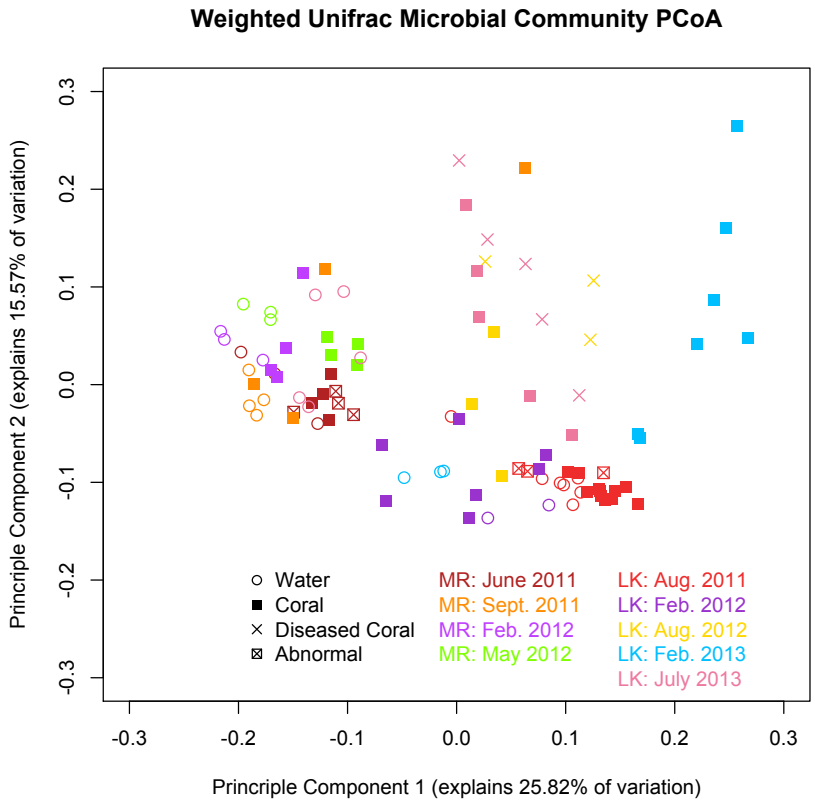

Figure 3.2. The principle components analysis graph for all reef samples.

Samples from each reef form separate clusters with each sampling date as a secondary cluster. Greater variation between replicates is observed as the distance between points and similarity (or difference) between sample types and dates is observed by the overlap (or the lack of overlap) of clusters.

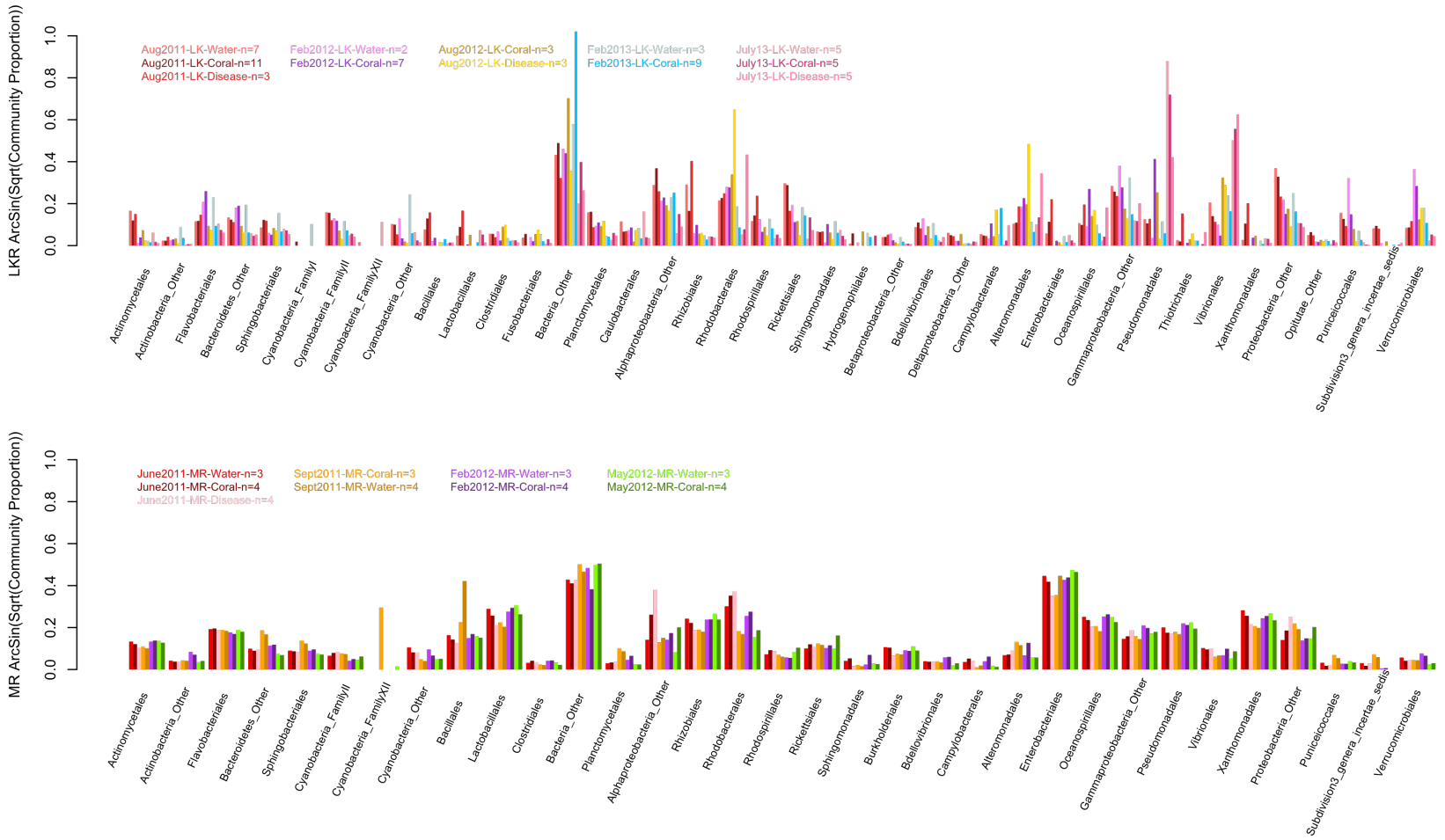

Figure 3.3. Top bacterial Orders for MR and LKR.

Each Order represented at least 1% of the relative abundance for the whole reef microbial community. Data presented is the relative abundance proportions that were arcsine transformed for statistical analysis.

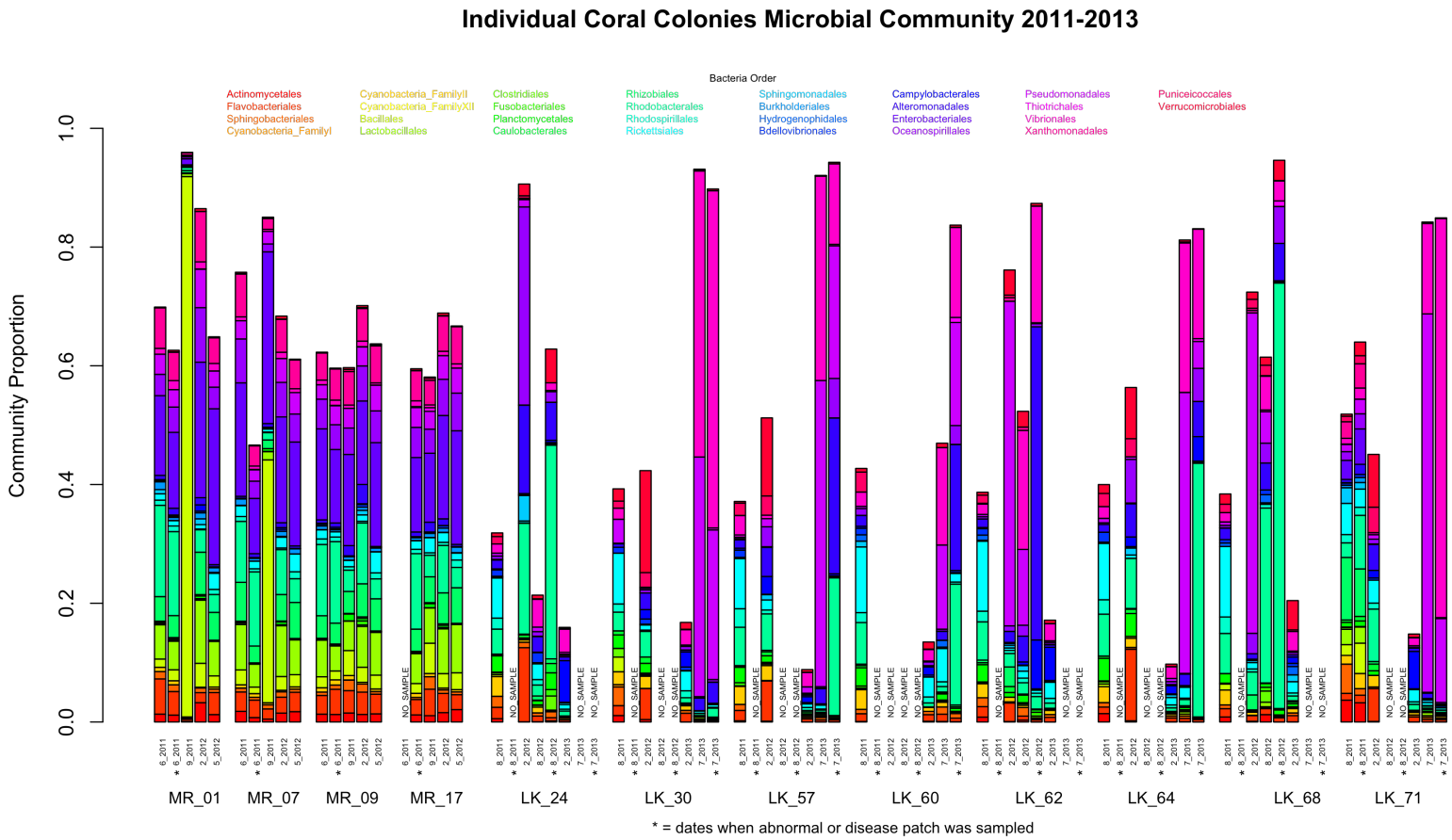

Figure 3.4. The relative abundance of 30 bacterial Orders within individual coral colony samples through time.

Each Order represented at least 1% of the relative abundance for the whole reef microbial community but not all bacteria are represented. When a coral was not sampled it is indicated. When a sampled colony had signs of abnormal tissue or WPD the column of that sample is indicated with an asterisks.

CHAPTER 4: DIRECT DETECTION OF *SERRATIA MARCESCENS* IN MARINE AND OTHER AQUATIC ENVIRONMENTS USING QUANTITATIVE REAL TIME PCR[1]

[1] Joyner, J., D. Wanless, C. D. Sinigalliano, and E. K. Lipp. 2014. Applied and Environmental Microbiology 80:1679-1683.

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Abstract

Serratia marcescens is the etiological agent of acroporid serratiosis, a distinct form of white pox disease in the threatened coral *Acropora palmata*. The pathogen is commonly found in untreated human waste in the Florida Keys, which may contaminate both nearshore and offshore waters. Currently there is no direct method for detection of this bacterium in the aquatic or reef environment and culture-based techniques may underestimate the abundance of this microbe in marine waters. A quantitative real-time PCR assay was developed to detect *S. marcescens* directly from environmental samples, including marine water, coral mucus, sponge tissue and wastewater. The assay targeted the LuxS gene and was able to distinguish *S. marcescens* from other *Serratia* species with a reliable quantitative limit of detection of 10 cell equivalents (CE) per reaction. The method could routinely discern the presence of *S. marcescens* for as few as 3 CE per reaction, but could not be reliably quantified at this level. The assay detected environmental *S. marcescens* in complex sewage influent samples at up to 761 CE ml^{-1} and in septic system impacted residential canals in the Florida Keys at up to 4.1 CE ml^{-1}. This detection assay provided rapid quantitative abilities and good sensitivity and specificity, which should offer an important tool for monitoring this ubiquitous pathogen that can potentially impact both human health and coral health.

Introduction

Serratia marcescens is a ubiquitous bacterium in the environment, naturally found in water and soil and in association with plants and animals, often as a pathogen (Hejazi and Falkiner 1997, Azambuja et al. 2005, Tu et al. 2010). *S. marcescens* is also an opportunistic pathogen for humans, commonly associated with hospital-acquired infections (Maltezou et al.

2012, Musham et al. 2012). In 1999, it was found within the mucus layer of elkhorn coral (*Acropora palmata*) and later identified as an etiological agent of white pox disease (designated as acroporid serratiosis when *S. marcescens* is present) (Patterson et al. 2002, Maragakis et al. 2008). In the Florida Keys and broader Caribbean, multiple white pox disease outbreaks have contributed to the decline of elkhorn coral since the late 1990s (Patterson et al. 2002). During the 2002-2003 Florida Keys' outbreak, where acroporid serratiosis was confirmed, the dominant strain of *S. marcescens* circulating among diseased corals and reef water was concurrently detected in human sewage (strain type PDR60), but in no other potential sources; this lead to the hypothesis that wastewater treatment practices may have a direct impact on coral health (Sutherland et al. 2010).

Current methodology to detect *S. marcescens* from aquatic samples requires a multistep process for culture, detection and then identification of the bacterium (Farmer III et al. 1973, Grasso et al. 1988, Sutherland et al. 2010). The protocol used for detection in marine waters and coral includes an initial culture on a selective medium (McConkey Sorbital Agar amended with colistin; MCSA), verification on a second selective medium (DNAse agar amended with toluidine blue and cephalothin; DTC), followed by PCR for a *Serratia* specific region of the 16S rRNA gene (Sutherland et al. 2010). This process likely underestimates the total concentration and without all three steps, lacks the specificity for *S. marcescens* to determine the true abundance of the bacterium in the environment; it can also take days to confirm results. The time and materials required for the culture-based assays effectively limits the number of samples that can be screened. Some assays using PCR or quantitative real-time PCR (qPCR) are available for *S. marcescens*; however, their applications were designed for specific settings (*i.e.*, clinical, cultured cells, building debris) and may not be effective for environmental samples of diverse

microbial communities (Saikaly et al. 2007, Zhu et al. 2008, Dalvi and Worobec 2012). A rapid, culture-independent and quantitative method is needed to screen large numbers of environmental samples, which is critical for determining the prevalence of this organism among diseased, apparently healthy corals, other organisms and surrounding water. Efficient detection at high resolution (*i.e.*, large numbers of samples collected over space and time) is also required to better inform models of disease dynamics and transmission. In addition to a fast screening assay for environmental samples, any direct detection technique should also be applicable as a diagnostic tool. A rapid diagnostic assay would provide a method to accurately identify diseased lesions in corals as acroporid serratiosis *versus* another (as yet unknown) potential agent of white pox disease (Bruckner 2002, Pollock et al. 2011). Therefore, our overall objective in this study was to develop an efficient and quantitative real-time PCR (qPCR) assay to detect *S. marcescens* directly from environmental samples.

Materials and Methods

Selection of Amplification Target

Multiple common genetic regions were explored *in silico* as suitable gene targets for a *S. marcescens* specific assay, including *gyr*B, 16S rRNA, 23S rRNA and *luxS* (Saikaly et al. 2007, Zhu et al. 2008). The LuxS gene, associated with quorum sensing, was selected for additional consideration given its potential for higher specificity for *S. marcescens*, compared to other possible targets, according to submitted gene sequences within National Center for Biotechnology's (NCBI; www.ncbi.nlm.nih.gov) GenBank. The LuxS gene in *S. marcescens* diverged from other *luxS* containing bacteria but was highly conserved among *S. marcescens* strains (Figure 4.1). A previous study by Zhu and colleagues also identified *lux*S as suitable to

detect *Serratia* spp. in environmental samples using traditional PCR (Zhu et al. 2008). Finally, *lux*S has the additional benefit of having only a single copy within the *S. marcescens* genome, making specific quantification through qPCR simpler.

Primer and Probe Design

NCBI's Primer BLAST (Ye et al. 2012) was used to create forward and reverse primers for a region within the LuxS gene that was highly specific to *S. marcescens* (about 516 base pairs [bp] in *S. marcescens* [GenBank accession # EF164926.1 and AJ628150.1]). In developing the candidate primer pair, the amplicon size was restricted to $\leq$ 300 bp in length with primer lengths between 18 and 22 bp. Corresponding candidate sequences for a 5'-exonuclease-hydrolysis probe (*i.e.*, TaqMan® probe) were designed by aligning *S. marcescens* sequences with other *Serratia* species and closely related bacteria using the MAFFT multiple sequence alignment program (Katoh et al. 2009). The probe was also chosen to be between 20 and 30 bp in length with a melting temperature greater than the melting temperature of the associated primers.

Three sets of primers and two hydrolysis probes for *lux*S were evaluated. Probes were designed to increase the assay specificity by exclusively aligning *lux*S with a variety of *Serratia* species and other closely related bacteria (Figure 4.2). The final primers and probe combination (Table 4.1) had only minor secondary structures as confirmed using Primer Express (Applied Biosystems, Foster City, CA).

Controls

Pure cultures of known strains of *S. marcescens* (ATCC 13880 and Db11) were grown overnight in LB broth (Fisher BP1426) at 37°C to an estimated cell density of 10^8 cells ml^{-1}. The DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) was used to extract DNA according to the manufacturer's protocol for Gram-negative bacteria. DNA quantity and quality were checked

with a Nanodrop1000 (ThermoScientific, Wilmington, DE). DNA with an $A_{260/280}$ purity ratio of 1.8 to 2.0 and $\geq$ 20 ng μl^{-1} was used. Invitrogen TOPO TA PCR Cloning Kit (Life Technologies, Grand Isle, NY) was used to clone the *lux*S amplicon (PCR assay described below) of *S. marcescens* Db11 into a plasmid. The Invitrogen Plasmid Miniprep kit was used to extract plasmid DNA, which was used as a positive control and in the development of standard curves. Plasmid DNA was checked for purity, quantified, divided into aliquots and stored at -80^{o}C.

Sensitivity and Specificity

To optimize the qPCR protocol, *S. marcescens* Db11 *lux*S-containing plasmid was serially diluted in 10-fold increments over a 9-log scale. This serial dilution (10 points) was used to create the standard curve, in triplicate, for quantification of environmental samples. In addition to the sequence alignments completed when designing the primers, the designed assay (developed primers, probe and reaction conditions) was applied to four other *Serratia* species for verifying specificity and non-cross reactivity of the primers: *S. plymuthica* (ATCC 27593), *S. liquefaciens* (ATCC 27592), *S. rubidaea* (ATCC 33670), and *S. odorifera* (ATCC 33077). Additionally, other bacteria (non-*Serratia* spp.) were screened for primer cross-reaction, *Enterococci faecalis* (ATCC 19433), *Escherichia coli* (ATCC 15597), *Vibrio cholerae* (O1 strain; ATCC 14035), and *V. parahaemolyticus* (ATCC 17803). These species were chosen because they represent other genera that carry the LuxS gene and are found in the environment naturally or through wastewater contamination.

Primers and reaction conditions were initially screened using SYBR Green based qPCR (BioRad, Hercules, CA) on a StepOne Plus platform (Applied Biosystems, Life Technologies, Grand Isle, NY). All qPCR reactions were completed with duplicate technical replicates and duplicate no-template negative controls. Following successful reactions for duplicate qPCR runs

with no evidence of non-specific primer binding, reaction conditions were optimized for TaqMan based qPCR (QuantiTect Probe Kit, Qiagen, Valencia, CA). A successful preliminary standard curve was created and used to further test the sensitivity and specificity for *S. marcescens* in environmental samples. Final reactions included 0.9 µM of forward and reverse primers, 0.06 µM of TaqMan Black Hole Quencher probe, 1 X of Taq master mix (as provided in the QuantiTect Probe Kit), 1 µl of sample DNA and PCR grade water for a total reaction volume of 25 µl. Using this complete reaction master mix formula, a temperature gradient was run on a StepOne Real-Time PCR System (Life Technologies, Grand Isle, NY) from 60 °C to 67 °C to determine the best primer annealing temperature of 62 °C, which was also effective for extension. The completed run program was 95 °C for 15 min then 45 cycles of 95 °C for 5 s and 62 °C for 40 s.

Evaluation of Inhibition in Environmental Samples

The sample matrix from environmental sources was tested for inhibition of the qPCR reaction. Extracted DNA from duplicate environmental samples (coral mucus, sponge pore-water, sediment, canal water, wastewater, and 1:10 diluted wastewater), see below for extraction method, was mixed 1:1 with 10^4 CE (from plasmids) for a total of 2 µl and added to 48 µl of reaction master mix, in the concentrations described earlier. The complete reaction volume was divided and used as duplicate technical replicates in 25 µl qPCR assays. An equivalent concentration of plasmid CE, achieved by using PCR grade water instead of sample DNA, was used in qPCR assays as a standard to evaluate the environmental extracts for inhibitory characteristics.

Application to Environmental Samples

Coastal canal water, sediment, sponge tissue, coral mucus, and sewage influent from the Florida Keys were collected to evaluate the performance of this qPCR for environments previously known to harbor culturable *S. marcescens* (Sutherland et al. 2010). Water samples were collected in 1 L sterile polypropylene bottles from just below the surface in residential canals of the Florida Keys (September 2011 and August 2012). Sediment (N = 3) and marine sponge species (N = 3) were also collected (about 5 g each from near-shore Key Largo, FL in August 2012) and after vigorous vortexing and settling of the sample, the supernatant fluid (2 ml) was saved for DNA extraction. Mucus was collected from the surface of the coral *Siderastrea radians* (N = 3) from near-shore Key Largo, FL in August 2012 by aspirating the mucus with needless syringes. Sewage influent (post-bar screen) was collected in 1L sterile polypropylene bottles with the assistance of the treatment plant staff using their established protocol for plant monitoring. Sewage samples were collected from Key West, FL and Marathon, FL plants in September 2011 and August 2012. After collection, all samples were placed on ice and processed within 3 hours.

In the field laboratory, water, mucus and sewage samples were split to compare culture and qPCR based detection. For molecular detection, replicate 2 ml aliquots of each sample (biological replicates) were centrifuged at ~13,000 x *g* for 20 min and the supernatant fluid decanted. The bacteria-containing pellet was stored at -20°C until DNA could be extracted (described below). The remaining sample was used immediately for the detection of *S. marcescens* by culture. Up to 25 ml of water and 10 ml of coral mucus were filtered onto 47 mm diameter 0.45 µm pore sized mixed cellulose ester membranes (Millepore, Billerica, MA). Filters were placed onto selective agar for *S. marcescens* (MCSA). Up to 100 µl of sewage

influent were spread directly onto MCSA agar plates. Sponge and sediment samples were not cultured. MCSA plates were incubated for 19 – 24 h at 37^{o}C and presumptive *Serratia* colonies (pink colonies indicative of sorbitol fermentation) were transferred to DTC agar for phenotypic confirmation (indicated by red halos around colonies), as described by Sutherland and colleagues (Farmer III et al. 1973, Sutherland et al. 2010). Isolated colonies of presumptive *S. marcescens* were saved in deep agar stabs (LB agar), following two rounds of isolation, until further genotypic confirmation to species level by PCR (or qPCR).

DNA was extracted from saved isolates by growing a sub-culture in 5 ml LB broth (Fisher BP1426) for 12 – 16 h at 37 °C. Cells were centrifuged (4,000 x *g* at 24 °C for 5-10 min) and the pellet washed three times with 1 X phosphate buffered saline (PBS). The final pellet was resuspended in 1 ml of 1 X PBS and brought to a temperature of 100°C for 10 min. The lysed cell suspensions were centrifuged for 10 min at ~13,000 x *g* and the supernatant fluid (containing DNA) was stored at -80°C or diluted and used immediately for qPCR.

The ethanol precipitation protocol of Boström and colleagues (Boström et al. 2004) was used to extract environmental DNA from frozen pellets, with slight modifications. A sterile 2 mL centrifuge tube was used as an extraction negative control during each extraction process. Lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA (ethylenediaminetetraacetic acid), 50mM Tris-HCL (pH 9.0), and lysozyme (1 mg ml^{-1}) was added to the pelleted sample. Following incubation at 37°C for 30 min, proteinase K (100 µg ml^{-1} final concentration) and SDS (1% w/v final concentration) were added and tubes incubated at 55° C for 16-18 h. To aid in the precipitation of DNA, tRNA (50 µg; to act as a DNA carrier molecule), 0.1 volume NaAc, and 2.5 volume EtOH (99%) were added and incubated for an hour at -20°C. Samples were centrifuged (~13,000 x *g* for 20 min) and the supernatant fluid decanted, retaining pelleted DNA

in the original tube. DNA pellets were then washed with 500 µl EtOH (70%), centrifuged (~13,000 x *g* for 20 min) and supernatant fluid decanted. A SpeedVac (Eppendorf Concentrator 5301) was used to dry the DNA pellet, which was then resuspended in 100 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Final DNA suspension was stored at -80°C or used immediately for qPCR. All samples were subjected to qPCR as two technical replicates. Additionally, runs included two no-template negative controls, extraction negative controls, and a 3-point standard curve, run in duplicate, with *luxS* plasmid standards.

Amplicons of the *luxS* qPCR from sewage (N = 2) and presumptive isolates of *S. marcescens* (from canal water and sewage in the Florida Keys; N = 10) were submitted for sequencing by primer extension (Macrogen, Rockville, MD). The sequences were screened through the NCBI BLAST search engine and aligned to the *S. marcescens* Db11 LuxS gene sequence using the MAFFT alignment tool (using the Q-INS-i strategy, scoring matrix of 1PAM/K=2, and the default gap opening penalty of 1.53).

Results

Sensitivity and Specificity

A final standard curve for the qPCR assay was established from 10 dilutions and 3 reaction replicates. The final curve showed a y-intercept of 38.842, slope of -2.883, and a mean efficiency of 122% (Figure 4.3). Since there is only one *lux*S copy in the *S. marcescens* genome, cell equivalents (CE) and genome equivalents are the same and CE is used as the quantification unit. The assay was able to quantify *S. marcescens* to an optimized limit of detection of 10 CE per reaction and could regularly detect as few as 3 CE per reaction, but without reliable quantification. Thus, values in the range below 10 CE per reaction should be considered as

"detected but not quantified" (DNQ). Subsequent qPCR runs confirmed the standard curve. The assay was also highly specific; there was no cross-reaction detected with any of the *Serratia* or non-*Serratia* strains tested (*i.e.*, no amplification detected) (Table 4.2).

Inhibition Analysis

Positive control plasmid DNA (10^4 CE μl^{-1}) was seeded into all extracts of sample matrices and Cq values were compared to template DNA in PCR-grade water. No inhibition (i.e., no change in Cq values) was noted for water, coral mucus, sponge and sediment. Undiluted sewage caused significant inhibition, noted by a delayed Cq (p-value=0.0124); a ten-fold dilution removed the inhibitory effect (Figure 4.4).

Environmental Application

Sewage influent and canal water were the only samples in which *S. marcescens* was detected using both culture and qPCR methods. *S. marcescens* was not detected in the coral, sponge, and sediment samples (screened using culture and qPCR). All DNA extraction controls were screened and all were below the assay's detection limits.

Among the canal samples (N = 3), *S. marcescens* was detected at a mean of 3.63 CE ml^{-1}; ranging from 2.8 to 4.1 CE ml^{-1}. Concentrations of *S. marcescens* using culture averaged 0.5 CFU ml^{-1} (ranging from 0.2 to 1 CFU ml^{-1}). Among sewage samples (N = 3), *S. marcescens* was detected at a mean of 277.3 CE ml^{-1} ranging from 9 to 761 CE ml^{-1}. Concentrations of *S. marcescens* based on culture resulted in a mean level of 40 CFU ml^{-1} (20 – 50 CFU ml^{-1}) (Table 4.3).

The amplicon sequences of environmental isolates and sewage samples from September 2011 (presumptive *S. marcescens* based on conventional culture method) were confirmed as *S. marcescens* and aligned with the LuxS gene of *S. marcescens* strain Db11 (Figure 4.5; Appendix

2). All 9 sequences from environmental isolates matched *S. marcescens lux*S sequences (NCBI accession # EF164926.1 and AJ628150.1). With the exception of one sequence, all showed identity with *S. marcescens luxS* at greater than 91%. Environmental isolate SC42 showed 88% sequence homology to *S. marcescens luxS* and a 96% sequence homology to a region of *S. liquefaciens* ATCC 27592 (NCBI accession # CP006253.1, submitted June 2013). The amplicon sequences from qPCR-positive sewage samples were identified using BLAST nucleotide searching as *S. marcescens lux*S (NCBI accession # EF164926.1 and AJ628150.1) with 98% (Key Largo) and 99% (Marathon) max identity.

Discussion

This assay was able to specifically detect *Serratia marcescens* in marine environmental and sewage samples using qPCR directed at the single-copy LuxS gene. The assay was not cross-reactive with known *Serratia* strains or other *luxS* containing bacteria tested in the laboratory. Of the 12 qPCR amplicons submitted for sequencing 11 were confirmed for *S. marcescens* (the final one was poor sequence quality and not included in the analyses). While one amplicon (environmental isolate SC42) also showed homology with both *S. liquefaciens* (an intergenic, unannotated region) and *S. marcescens* (*lux*S), the *S. liquefaciens* control strain (ATCC 27592) was never amplified in this assay nor was it identified in sewage samples These results suggest that this assay is specific for *S. marcescens lux*S over other related sequences and is highly sensitive, with a detection limit as low as 3 CE per reaction.

Comparison of this qPCR method to culture based detection in environmental samples demonstrated similar results for surface water samples, with qPCR (CE) concentrations slightly greater than concentration determine by culture. The literature also suggests that qPCR typically

results in a higher concentration compared to culture due to detection of both dead and viable but non-culturable cells (Josephson et al. 1993, Gedalanga and Olson 2009). In this case, qPCR concentrations were within one order of magnitude of those from culture and results were obtained with a smaller sample volume (2 ml for qPCR *versus* 5 or 15 ml for culture). Among raw sewage samples, concentrations determined by qPCR were similar to those determined by culture for two of the wastewater treatment plants but were significantly greater than culture in one plant (Key West). This large difference may be due to the high level of non-specific growth on the MCSA spread plates from sewage, which may have reduced detection of presumptive *S. marcescens* colonies. While *S. marcescens* were not detected in the few sponges and corals collected for this study, data from efficiency, specificity, sensitivity and inhibition assays suggest that the bacteria were absent from these samples, rather than simply not detected.

No inhibition was noted for the tested canal surface waters but sewage was likely to contain significant inhibitors; this could generally be alleviated with a 1:10 dilution of sample extract before qPCR. Inhibitors in environmental or other complex samples can increase the likelihood of false negatives by PCR and reduce the concentration estimates in qPCR. This is a common issue for PCR and qPCR detection assays and can be addressed by the development of a specific internal control to calculate the inhibition within each qPCR reaction (Sen et al. 2007, Cao et al. 2012, Haugland et al. 2012). In the absence of a unique internal control sequence, template can be spiked into sample extracts to estimate inhibition effects, as was done here (Haugland et al. 2012).

In addition to increased sensitivity and specificity for *S. marcescens,* the qPCR detection assay significantly reduces time to obtain results compared to culture based techniques (Haugland et al. 2005, Clark et al. 2011). Furthermore, qPCR provides a platform for high

throughput detection and analysis. To date, *S. marcescens* is the only confirmed etiological agent of white pox disease (termed acroporid serratiosis when this etiological agent is confirmed in disease lesions) in the threatened elkhorn coral (Patterson et al. 2002, Sutherland et al. 2010). Outbreaks of disease consistent with signs of white pox continue to occur in the Florida Keys and elsewhere in the Caribbean; however, in many cases efforts to assign these outbreaks as acroporid serratiosis have not been carried out due to the lack of a simple diagnostic tool. In order to better describe patterns of disease associated with occurrence and distribution of *S. marcescens versus* general white pox symptoms, rapid and high through put tools are needed to screen large numbers of samples from a variety of environments (*e.g.*, corals, water, etc.). Such detailed observations are also needed to track potential pathogen sources or reservoirs (Bruckner 2002, Pollock et al. 2011). Using this qPCR assay to detect *S. marcescens* within a white pox disease lesion and confirm acroporid serratiosis is a key advance to the study and management of the coral disease.

Acknowledgments

The bacterial strain *S. marcescens* Db11 used in this work was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). Assay development began while JLJ interned with CS at NOAA AOML, completing a NOAA Oceans and Human Health (OHH) Graduate Training fellowship (S0867882) under EKL. An NSF Ecology and Evolution of Infectious Disease award provided additional funding to EKL (OCE1015342). Additional support for work conducted at NOAA AOML was funded in part by Oceans and Human Health Center grants from NSF and NIEHS (NSF 0CE0432368/0911373 and NIEHS P50ES12736, respectively).

Disclaimer – The use of trade names is for descriptive purposes only and does not imply endorsement by the U.S. Government

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Tables

Table 4.1. *Serratia marcescens* real time quantitative primers and probe sequences as well as the nucleotide position and melting temperature.

LuxS qPCR Primers and Probes	Nucleotide Position	Melting Temp (^{o}C)
Forward: 5'-TGCCTGGAAAGCGGCGATGG-3'	306 – 325	66.6
Reverse: 5'-CGCCAGCTCGTCGTTGTGGT-3'	480 – 461	66.6
Probe: 5'-6FAM-GTGGTACCTACCACATGC ACTCGCTGGAA-BHQ1a	384 – 413	70.3

Table 4.2. Specificity of the LuxS qPCR assay.

Serratia marcescens as well as other bacteria known to be similar to *S. marcescens* or contain the LuxS gene were screened. Only the *S. marcescens* strains tested were positive with this assay.

Species	Strain #	*lux*S qPCR reaction
Serratia marcescens	Db11	+
S. marcescens	ATCC 13880	+
S. plymuthica	ATCC 27593	-
S. liquefaciens	ATCC 27592	-
S. rubidaea	ATCC 33670	-
S. odorifera	ATCC 33077	-
Enterococcus faecalis	ATCC 19433	-
Escherichia coli	ATCC 15597	-
Vibrio cholerae O1	ATCC 14035	-
V. parahaemolyticus	ATCC 17803	-

Table 4.3. *Serratia marcescens* found in Florida Key's environmental samples (collected August 2012) using culture and qPCR methods.

Culture data were recorded as colony forming units (CFU) and qPCR data were recorded as cell equivalents (CE), as calculated by the standard curve.

Sample Description	Location (Lat. / Long.)	Culture (CFU ml^{-1})	qPCR (CE ml^{-1})
Canal water – Eden Pines	24° 41.463'N, 81° 22.674'W	1	4
Canal water – Doctor's Arm	24° 42.092'N, 81° 21.124'W	0.4	2.8
Freshwater Lens – Blue Hole	24° 42.368'N, 81° 22.837'W	0.2	4.1
Sewage influent – Key Largo	25° 6.041'N, 80° 25.930'W	20	8.9
Sewage influent – Marathon	24° 43.855'N, 81° 0.241'W	50	62
Sewage influent – Key West	24° 34.115'N, 81° 47.818'W	50	76

Figures

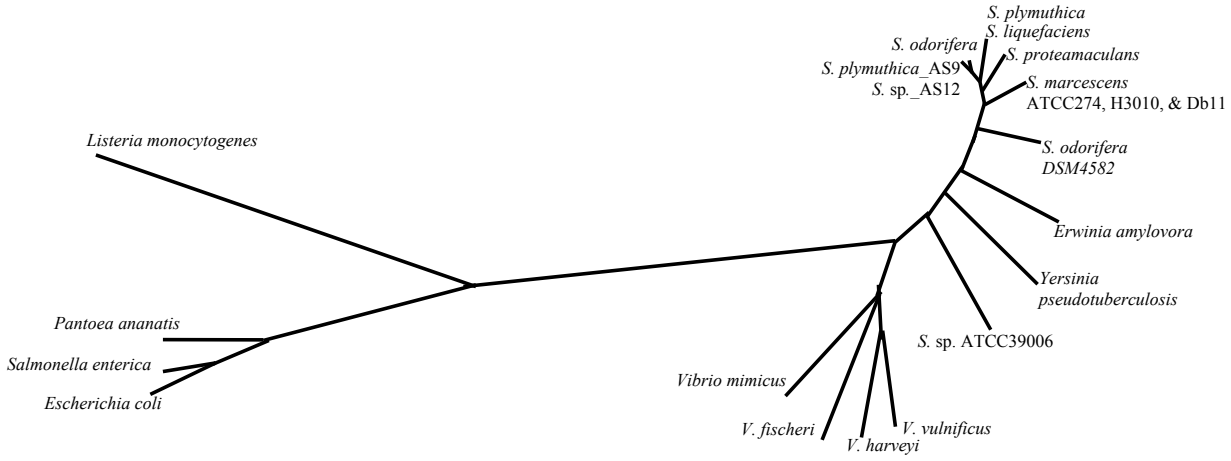

Figure 4.1. Unrooted phylogenetic tree for the LuxS gene.

*Serratia marcescens lux*S QPCR – gene alignment with primer/probe location

1- *S. marcescens*_DB11
2- *S. marcescens*_H3010
3- *S. marcescens*_ATCC274
4- *S. liquefaciens*_ATCC27592
5- *S. proteamaculans*_568
6- *S. odorifera*
7- *S. plymuthica*
8- *S. plymuthica*_AS9
9- S. sp._AS12
10- S. sp._ATCC39006
11- *Yersinia pestis* biovar Microtus_91001
12- *Erwinia amylovora*_611

```
1-        TGCCGGAAGA GCAGCGCGTT GCCGATGCCT GGAAAGCGGC GATGGCCGAC
2-        ********** ********** ********** ********** **********
3-        ********** ********** ********** ********** **********
4-        ********** ******T*** ********G* ********** **********
5-        ********** ******T*** ********** ********** A***AGT***
6-        ********** ********** ********G* ********** ****T*****
7-        ********** ********** **T*****G* ********** ****T*****
8-        ********** ********** ********G* ********** ****T*****
9-        ********** ********** ********G* ********** ****T*****
10-       C*****C*** A**A****** **T**G**T* *******A** A*****G**T
11-       CA**T**C** ***A**G*** **T******* ********** A*********
12-       *******C** ******T**G ***C*G**G* ****G***** *****GT**T

1-        GTGCTGAAAG TGACCGACCA GCGCAAGATC CCTGAGCTGA ACGAGTACCA
2-        ********** ********** ********** ********** **********
3-        ********** ********** ********** ********** **********
4-        ********** ****T***** ***T**A**T ******T*** ****A*T***
5-        ********** *******T** ******A**T ********** *T****T***
6-        ********** ****T***** ******A**T ********** *T****T***
7-        ********** ****T***** ******A**T ********** *T****T***
8-        ********** ****T***** ******A**T ********** *T****T***
9-        ********** ****T***** ******A**T ********** *T****T***
10-       **A******* *C**T**T** ***T**A*** **G*****C* *T*T***T**
11-       ********G* ****T***** ***G****** ********** *T**A**T**
12-       ***T****** *TG******* ***TC***** **C******* *T*****T**

1-        GTGTGGTACC TACCACATGC ACTCGCTGGA AGAAGCGCAG GAAATCGCCA
2-        ********** ********** ********** ********** **********
3-        ********** ********** ********** ********** **********
4-        ***C**G**T *****T**** ********** ***G****** ********G*
5-        ***C*****T ********** ********** ******A*** **********
6-        ***C**G**T **T******* ****TT**** ********** **********
7-        ***C**G**T **T******* ****TT**** ********** **********
8-        ***C**A**T **T**T**** ****TT**** ********** **********
9-        ***C**A**T **T**T**** ****TT**** ********** **********
10-       ***C**A*GT **TACA**** *T**TT**** G********* **T**T**TC
11-       ***C**G**T **T**T**** **T******* ******T*** AGT**T**T*
12-       *********T **TG*A**** ********** T********* **T******C

1-        AGCACATTCT GGATAACGGC GTGGTGGTGA ACCACAACGA CGAGCTGGCG
2-        ********** ********** ********** ********** **********
3-        ********** ********** ********** ********** **********
4-        ********** T**CC*T*AT **T**A**** ********** A*********
5-        *******C** T***C***A* *****A**** *T******** A*********
6-        ********** C***C***A* *****A**** ********** A*********
7-        ********** C***C***A* *****A**** ********** A*********
8-        ********** T***C***A* *****A**** ********** A*********
9-        ********** T***C***A* *****A**** ********** A*********
10-       GT**T**C** G**A**G*AT ***T*A**A* *TAGA**T** ***TT****A
11-       *AG*T***** T**CCGT*AT ***CGTA*C* ********** A***T****A
12-       GT**T**CA* C**A**G*AT A*TCGC**T* ***G****** T********A
```

Figure 4.2. Abbreviated luxS sequence alignment for *Serratia marcescens* and closely related bacterial species.

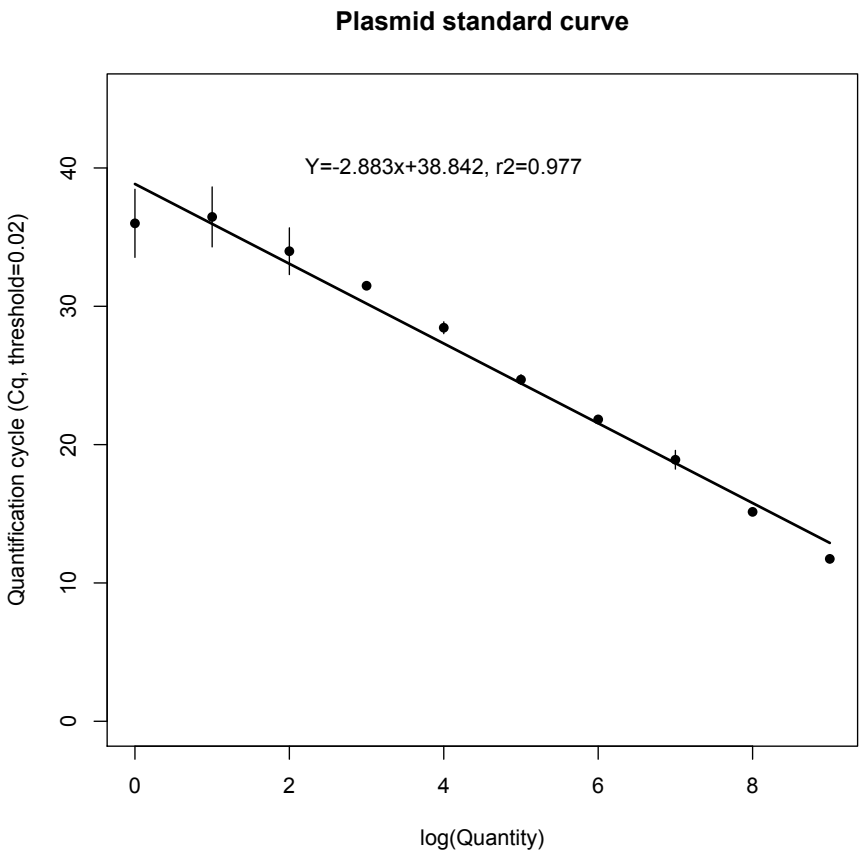

Figure 4.3. *Serratia marcescens* real-time quantitative PCR standard curve using plasmids of LuxS gene extracted from the Db11 strain.

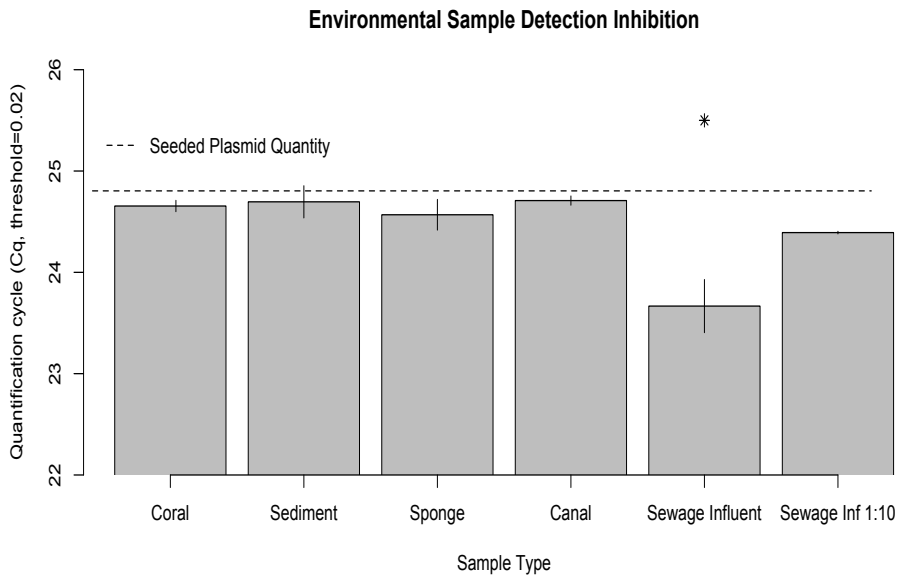

Figure 4.4. Inhibition effects of environmental matrix on the detection of *S. marcescens*.

*Serratia marcescens lux*S QPCR – gene alignment with environmental isolate sequences

1- *lux*S	7- KWWWI_24
2- TL47	8- KWWWI_22
3- TL35	9- KLWWI_7
4- SC59	10- KLWWI_6
5- SC42	11- Sewage-KL
6- BH28	12- Sewage-MA

```
1-   TGCCTGGAAA GCGGCGATGG CCGACGTGCT GAAAGTGACC GACCAGCGCA
2-   ---------- ---------- ---------- ---------- -GA*****C-
3-   ---------- ---------- ---------- ---------- ------***-
4-   ---------- ---------- ---------- ---------- CGA*N****-
5-   ---------- ---------- ---------- ---------- ----------
6-   ---------- ---------- ---------- ---------- -*T***N**-
7-   ---------- ---------- ---------- ---------- -GA******-
8-   ---------- ---------- ---------- ---------- -----****-
9-   ---------- ---------- ---------- ---------- -----****-
10-  ---------- ---------- ---------- ---------- ------***-
11-  ---------- ---------- ---------- ---------- ----------
12-  ---------- ---------- ---------- ---------- ----------

1-   AGATCCCTGA GCT-----GA ACGAGTACCA GTGTGGTACC TACCACAT--
2-   ********** ***-----** ********** ***C**C*** *****T**--
3-   **N******* ***-----** ********** ********** ********--
4-   ********** ***-----** ********** ********** ********--
5-   ---------- -T*-----*- **NNA*T*N* ***C**G**T *****T**--
6-   N********* ***-----*- *******T** N**C**C**T *****T**--
7-   ********** ***-----** ********** ***C**C**T *****T**--
8-   ****-N**** ***-----** ********N* ********** ********--
9-   ********** ***-----** ********** ********** ********--
10-  ********** ***-----** ********** ********** ********--
11-  -AT*T****C ***TCTTCC* G*****G*-- A*******GG *******CTG
12-  -AT*T****C ***TCTTCC* G*****G*-- A*******GG *******CTG

1-   GCACTCGCTG GAAGAAGCGC AGGAAATC-- GCCAAGCACA TTCTGGATAA
2-   ********** ********** ********-- ********** ********C*
3-   ********** ********** ********-- ********** **********
4-   ********** ********** ********-- ********** **********
5-   ********** ********** ********-- **G******* ****T**CC*
6-   ********** ********** ****G***-- *****A**** **********
7-   ********** ********** ********-- ********** ********C*
8-   ********** ********** ********-- ********** **********
9-   ********** ********** ********-- ********** **********
10-  ********** ********** ********-- ********** **********
11-  *T*****T*C -----***T* ***-G***TT **G------- ----------
12-  *T*****T*C -----***T* ***-G***TT **G------- ----------

 1-   CGGCGTGGTG GTGAAC---C ACAACGACGA GC-TGGC--- -------G
 2-   ********** ******---* ********** **-****--- -------*
 3-   ********** ******---* ********** **-****--- -------*
 4-   ********** ******---* ********** **-****--- -------*
 5-   T*A***T**A ******---* ********** **-****--- -------*
 6-   **AT****** ******---* ********** **-****--- -------*
 7-   ********** ******---* ******N*** **-****--- -------*
 8-   ********** ******---* *****CC*** **-**C*--- -------*
 9-   ********** ******---* ********** **-****--- -------*
 10-  ********** ******---* ********** **-****--- -------*
 11-  ----C****C *GTC**TTT* *GC***T**G C*T*C**CCC TTTCCAG*
 12-  ----C****C *GTC**TTT* *GC***T**G C*T*C**CGC TTTCCAG*
```

Figure 4.5. Sequence alignment for environmental isolates collected in September 2011.

CHAPTER 5: WHITE POX DISEASE AND THE ENVIRONMENTAL PREVALENCE OF *SERRATIA MARCESCENS* IN THE FLORIDA KEYS[1]

[1] Joyner, Jessica L.; Sutherland, Kathryn P.; Kemp, Dustin; Porter, James; Lipp, Erin. To be submitted to Environmental Microbiology.

Abstract

Throughout the Florida Keys, disease has contributed to the significant decline of the branching elkhorn coral *Acropora palmata*. White pox disease was first described in 1999 and *Serratia marcescens* was confirmed as one etiological agent (referred to as acroporid serratiosis when *S. marcescens* are found in association with white pox lesions). Owing in part to a lack of a rapid and simple diagnostic test, there have been few systematic assessments of the prevalence of acroporid serratiosis (versus general white pox signs). This study surveys six reefs in the Florida Keys for three years to determine disease status by both visual assessment and prevalence of *S. marcescens* using a species-specific qPCR assay in conjunction with traditional culture based assays to obtain isolates. Four of the 6 reefs had evidence of white pox disease in last two years year of the study; in all cases, disease was only recorded in the summer months (July – September). Disease prevalence (based on visual assessment) ranged from absent on Western Sambo reef throughout the study to 32% (10/31) colonies with signs of disease at Looe Key reef August 2012. While disease prevalence was high on some reefs (i.e., Looe Key), little disease-associated mortality was observed and coral disease was not recorded by the following winter sampling, suggesting colony recovery. The bacterium was never cultured from *A. palmata* and qPCR only detected the bacterium in 10.8% of *A. palmata* samples (n=240). There was no statistical relationship between the detection of *S. marcescens* on either a reef with disease signs or colonies with a WPD lesion. However, *S. marcescens* was regularly detected, by both culture and molecular methods, in non-*A. palmata* corals. These results indicate that *S. marcescens* is still present on the coral reef. While WPD lesions may still be observed on *A. palmata* it is not likely that the disease is acroporid serratiosis. This study further illustrates the challenges in identifying coral disease etiologies and pathogens.

Introduction

In the Caribbean, the combination of physical stress and disease has been responsible for the decline of many corals (Miller et al. 2002, Gardner et al. 2003). White pox disease (WPD) of *Acropora palmata* has contributed to coral's population decline, especially in the Florida Keys (Patterson et al. 2002, Miller et al. 2008). Following outbreaks of white pox in the late 1990s and early 2000s, the bacterium *Serratia marcescens* was identified as an etiological agent by fulfillment of Koch's postulates (Patterson et al. 2002, Sutherland et al. 2010). To distinguish the disease caused by this bacterium from broader signs of WPD, it is referred to as acroporid serratiosis when *S. marcescens* is confirmed from a lesion on an *A. palmata* colony (Sutherland et al. 2010, Sutherland et al. 2011).

Between 1999 and 2006, two strains of *S. marcescens* were associated with large outbreaks of WPD (acroporid serratiosis) in the Florida Keys. The strain found in association with outbreaks in 2002 and 2003 was identical to a strain concurrently found in human sewage from the nearby islands that compose the Florida Keys archipelago (Sutherland et al. 2010, Sutherland et al. 2011). In 2000, the local governments of the Florida Keys began the process to build infrastructure for centralized wastewater treatment facilities in order to improve nearshore water quality by decommissioning the old methods in in-ground disposal (Sleasman 2009). Historically, wastewater disposal was managed by septic systems and cesspits in the Florida Keys, with only Key West having a full scale advanced treatment plant (Paul et al. 1995, Paul et al. 1997, Keller and Causey 2005). The conversion from traditional septic tanks and cesspits for wastewater disposal to treatment facilities is expected to decrease local sewage pollution and limit the threat to the nearby coral reef (Lipp et al. 2002, Masago et al. 2007, Futch et al. 2011).

In more recent studies, researchers have reported that *S. marcescens* could not always be isolated from colonies displaying signs of white pox disease (Polson et al. 2008, Sutherland et al. 2010, Lesser and Jarett 2014). These observations suggest that other disease agents may cause signs similar to white pox disease and highlights the importance of differentiating acroporid serratiosis or identifying it as a specific type of white pox disease. Given that there are only a limited number of outward manifestations that a coral may display in response to a disease, diagnosis and disease identification has been an on-going problem in the coral disease ecology field (Work and Aeby 2006, Pollock et al. 2011). Additionally, coral disease samples can be collected at any stage of disease progression, which can generate varying results for potential pathogen occurrences (Work and Aeby 2006).

In effort to document the prevalence of white pox signs and the contribution of *S. marcescens* to disease patterns, this study used a high-through put qPCR assay to assess presence of *S.* marcescens in conjunction with detailed visual assessments spanning multiple seasons, years and reefs in the Florida Keys.

Methods

Sample Sites and Collection Strategy

Samples were collected at six reefs spanning the length of the Florida Keys National Marine Sanctuary (FKNMS). Stations (listed from Upper Keys, near Key Largo to Lower Keys, near Key West) included Carysfort, Sombrero, Molasses (only for culture work), Looe Key, Rock Key, and Western Sambo (Figure 1). At each station and sampling point at least two sample types were collected: water (collected as a grab sample 1 m above the reef) and coral surface mucus samples. SCUBA divers using 12 ml or 20 ml sterile needleless syringes collected

mucus from the surface of the coral colonies. *A. palmata* colonies at each station were mapped and labeled with a unique identifier to allow for tracking individual colonies between sampling times. At each sampling, each colony was examined for visual signs of WPD (patchy necrosis with a distinct border between healthy and disease areas; per Patterson and colleagues (2002) and overall health status (i.e., other disease, bleaching, or predation scares) was recorded. When apparent disease lesions were present, an additional sample was collected at the active lesion border. For corals with disease lesions (and a few with abnormal patches), healthy tissue was from a branch of the same colony for which no lesions were present. On three occasions (August 2012, February 2012 and August 2013) mucus samples were collected from other ('non-host') corals, which are not associated with signs of WPD, including *Orbicella faveolata*, *O. annularis*, *Siderastrea siderea*, *Porities porities*, and *P. asteroides*. In addition to coral mucus samples, sediment and *Coralophilia abbreviata* snails, which prey on *A. palmata*, were collected at Molasses and Looe Key reef sites (primarily for culture work). Unless otherwise noted, samples were collected three times per year between 2011 and 2013 ('winter' [December – February], 'spring' [May and June] and 'summer' [July - September]).

In addition to the reef survey, samples were also collected from residential canals in the Florida Keys that have a history of contamination from septic systems (Griffin et al. 2001, Sutherland et al. 2010). Tropical Lane and Sexton Cove are residential canals on Key Largo in the Upper Keys, an area under going conversion from septic systems to centralized wastewater treatment during the period of this study. Eden Pines and Doctor's Arm are residential canals on Big Pine Key, an area that was not yet converted to centralized wastewater treatment at the time of this study. Blue Hole is a freshwater lens also located on Big Pine Key, in the Lower Keys. Surface water from each site was collected as grab samples from about 0.5 m below the surface

of the water using sterile 1 L polypropylene bottles.

Finally, as a control for detection of *S. marcescens*, sewage influent was collected from three wastewater treatment plants in Key Largo (25°6.041'N, 80°25.930'W), Marathon (24°43.855'N, 81°0.241'W), and Key West (24°34.115'N, 81°47.818'W) (Figure 5.1). Final effluent was also collected to evaluate potential discharge of *S. marcescens* from these plants. Wastewater influent and effluent samples were collected according to the wastewater plant's operator protocol; typically for influent this was post bar-screen grab sample but occasionally a composite sample was used for influent and the effluent collection method was consistently a grab sample from the last effluent holding tank.

Samples were immediately placed on ice and transported to the field laboratory and processed within 2 hours of collection.

White Pox Disease Survey

A. palmata colonies at each site were photographed with standard scale bar, with extra care devoted to documenting disease lesions. The number of *A. palmata* colonies surveyed varied by reef because of a wide-range of abundance throughout the FL Keys reef track. Coral colonies within the study were tagged for tracking throughout the survey period (Table 5.1). The prevalence of disease from 2011 to 2013 for each reef was recorded as a percent of the monitored colonies that had white pox lesions. Lesions were defined as irregularly shaped white patches (1-15cm^2) of tissue loss where the remaining skeleton was bright white, indicating rapid tissue loss (Patterson et al. 2002). Lesions were occasionally multifocal and coalesced when individual lesions grew.

Molecular Detection of S. marcescens

Samples were split into 2 ml aliquots, excluding snails and sediment, and were centrifuged at ~13,000 x *g* for 20 min. The supernatant fluid was discarded and the bacteria-containing pellet was stored at -20°C pending DNA extraction. A modified ethanol precipitation protocol was used to extract environmental DNA from one of the frozen pellets, maintaining a back-up sample for future analysis (Boström et al. 2004). Briefly, lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM EDTA (ethylenediaminetetraacetic acid), 50mM Tris-HCL (pH 9.0), and lysozyme (1 mg ml^{-1}) was added to the pelleted sample. Following incubation at 37°C for 30 min, proteinase K (100 µg ml^{-1} final concentration) and SDS (1% w/v final concentration) were added and tubes incubated at 55° C for 16-18 hrs. To increase the precipitation of DNA, tRNA (a DNA carrier molecule; 50 µg), 0.1x volume NaAc, and 2.5x volume EtOH (99%) were added and incubated for an hour at -20°C. Samples were centrifuged (~13,000 x *g* for 20 min) and the supernatant fluid decanted, retaining pelleted DNA in the original tube. DNA pellets were then washed with 500 µl EtOH (70%), centrifuged (~13,000 x *g* for 20 min) and supernatant fluid decanted. A SpeedVac (Eppendorf Concentrator 5301) was used to dry the DNA pellet, which was then resuspended in 100 µl of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The final DNA suspension was stored at -80°C.

Samples were screened with quantitative real-time PCR (qPCR) as technical replicates (Joyner et al. 2014). Extraction controls were completed and inhibition tests were completed similar to the original assay design (Joyner et al. 2014). The standard curve for the qPCR assay has a detection limit of at least 3 genome equivalents /µl (a maximum Cq threshold value of 38.84) and any reaction result greater than this limit were considered positive.

Isolation of Culturable S. marcescens

In order to obtain isolates of *S. marcescens* for genetic fingerprinting, surface water (up to 50 ml) and coral mucus (up to10 ml) were filtered onto 47 mm diameter 0.45 μm pore sized mixed cellulose ester membranes (Millipore, Billerica, MA). Sediment samples (up to 5 g) were resuspended in sterile 1% phosphate buffered saline (PBS) and 5 ml of the supernatant fluid was filtered through 0.45 μm pore sized membranes. Membrane filters were placed onto selective agar for *S. marcescens* (MacConkey Sorbitol Agar amended with colistin, (Sutherland et al. 2010)). Snail tissue was macerated and streaked onto MCSA agar with a sterile cotton swab. Sewage influent samples (100 μl) were spread directly onto MCSA agar plates. Sterile 1% PBS was used for negative controls. MCSA plates were incubated for at 37^{o}C and presumptive *Serratia* colonies (pink colonies indicative of sorbitol fermentation) were transferred to DNAse agar amended with toluidine blue and cephalothin (DTC) for phenotypic confirmation (indicated by red halos around colonies) (Farmer III et al. 1973, Grasso et al. 1988, Sutherland et al. 2010). DTC plates were incubated at 30^{o} and 41^{o}C for 18 ± 4 hrs. Isolated colonies of presumptive *S. marcescens* from DTC were saved in deep-agar stabs (LB agar); after two rounds of isolation, genotypic confirmation to species level was completed with PCR (Sutherland et al. 2010). After PCR confirmation of a colony as *S. marcescens*, pulsed field gel electrophoresis (PFGE) was completed (as described by Sutherland and colleagues) for strain identification (Tenover et al. 1995, Sutherland et al. 2010)

Statistical Analysis

A generalized linear model with mixed effects using extensions for correlation analysis was applied to the data using the PROC GLIMMIX procedure in SAS (version 9.3; Cary, NC). Preliminary analysis indicated that samples from healthy coral, abnormal coral (generally

discolored but not diseased) and coral white pox disease lesions were not significantly different; therefore, they were grouped together as coral samples for final analysis. The statistical model was used to determine if correlations existed between qPCR detection of *S. marcescens* and the sample type (coral or water) or reef disease status. The collection year, season, and reef were linked together as the random effect in the model. Significance was determined at $P < 0.05$.

Results

Survey for White Pox Disease

WPD was recorded during the study in 2012 and 2013 (Table 5.1). Lesions often appeared as small clusters that sometimes merged, most colonies recovered and lesions were no longer active by the winter sampling effort (Figure 5.2). Some reefs did not have signs of WPD – Western Sambo and Rock Key; both reefs are located in the lower Florida Keys and only have a few *A. palmata* colonies within the survey area. Looe Key reef has a history of a high frequency for WPD (Porter et al. 2001) and this study period was no exception, 32.26% (10 of 30) colonies had signs of WPD in 2012 and 22.22% (6 of 27) colonies in 2013. Sombrero and Carysfort reefs also had diseased colonies present. Sombrero reef had WPD lesions on 25% (2 of 8) colonies in June 2011, 14.29% (1 of 7) colonies in August 2012, and 25% (2 in 8) colonies in August 2013. Carysfort reef had abnormal patches on 25% (2 of 8) colonies in June 2011 and 16.67% (1 of 6) colonies in August 2013. Molasses reef had abnormal patches on 80% (8 of 10) colonies in September 2011, WPD on 33% (3 of 9) colonies in August 2012, and WPD on 16.67% (1 of 6) colonies in July 2013. No reef or colony had white pox disease present through the entire study period; furthermore, colony mortality associated with disease was never observed.

Nearshore S. marcescens

S. marcescens was regularly cultured from the canals and sewage influent samples. In 2011, 10 canal samples were collected with two positive for *S. marcescens* cultures in the spring (winter was not sampled) and four samples positive for *S. marcescens* cultures in the summer and qPCR was positive in one spring sample and one summer sample. In 2012, 15 canal samples were collected and only five Lower Keys samples were positive for *S. marcescens* cultures and qPCR was only positive in the three summer samples (spring 2012 was not tested with qPCR). Compared to previous years, the most samples were tested positive in 2013 using culture and molecular methods. Canal samples (N=15) had positive *S. marcescens* cultures in four winter samples, all eight spring samples, and three summer samples. *S. marcescens* was detected with qPCR in 12 of the 15 samples, with only random negative samples.

Wastewater treatment plant sewage influent were always positive for *S. marcescens* using both cultures and qPCR methods. Effluent samples were never positive for *S. marcescens* cultures and only seven were qPCR positive, which was likely residual DNA retained after the treatment process.

All confirmed *S. marcescens* isolates were processed with PFGE to determine the strain. PDL100 and PDR60 PFGE strains were recovered from the coral reef during previous WPD outbreaks and confirmed as a coral pathogen during challenge experiments (Patterson et al. 2002, Sutherland et al. 2010, Sutherland et al. 2011). Few isolates were identified as the PDR60 strain, one isolate from Key Largo Wastewater influent in August 2011 and two isolates from Key Largo Wastewater influent in August 2012. No PDL100 strains were found.

Coral Reef S. marcescens

Few samples collected from the reef were positive for *S. marcescens*. No *A. palmata* samples were positive for *S. marcescens* by culture. qPCR screening was completed for 240 *A. palmata* samples and 26 samples (10.8%) were positive for *S. marcescens* (Table 5.3). Of the 240 coral samples screened with qPCR, 26 were from the disease margin of colonies with WPD lesions and 7 samples (26.9%) were positive for *S. marcescens*. During the summer, when WPD was most often recorded, the total *A. palmata* colonies that were qPCR positive was none (0/25) for August 2011, 22% (9/41) for August 2012, and 17.5% (7/40) for July 2013. Reef water samples that were qPCR positive was none (0/17) for August 2011, 17.4% (4/23) for August 2012, and 6.7% (1/15) for July 2013. There was no significant trend of detecting *S. marcescens* in samples collected from disease lesions or reefs that had WPD recorded. The *A. palmata* coral samples from combined healthy, abnormal, and disease lesions, did have significantly more samples qPCR positive than overlying water samples ($P<0.05$, Table 5.4).

Coralophilia abbreviata snails and sediment culturing efforts did not detect *S. marcescens* and they were not screened with qPCR. Coral mucus collected from non-host corals, *Porities porities*, *P. asteroides*, *Orbicella annularis*, *O. faveolata*, and *Siderastrea siderea* were positive by culture 2.6 % of the time (7 of 274). *P. porities* had the most positive culture samples (4/17) with *P. asteroides* (2/10) and *Orbicella faveolata* (1/10) also having positive culture samples. Of non-host corals tested with qPCR, *Porities porities*, *P. asteroides*, *Orbicella* spp., 21 of 26 samples (11%) were positive (Table 5.3). When WPD was observed on a reef, *S. marcescens* was detected in non-host corals by qPCR in 81% of the samples.

All confirmed *S. marcescens* isolates were processed with PFGE to determine the strain. In August 2012, isolates from *P. asteroides* and *P. porities* (a total of 11 isolates) were *S. marcescens* PDR60; none matched PDL100.

Discussion

White pox disease (WPD) was present throughout the Florida Keys but was most commonly observed at Looe Key reef. Through 2011 to 2013, WPD was not as prevalent as recorded during previous disease outbreaks (Porter et al. 2001, Patterson et al. 2002). This extensive survey also indicates a potential shift in the severity of the disease; colonies that died during the three years were never because of WPD. The WPD lesions did not progress to overtake the entire coral colony and result in complete colony mortality. Strong storms impacted the study sites and caused typical physical damage (including colony loss) on the reefs; the tracks of two tropical storms and a tropical depression brought them near the Florida Keys 2011 to 2013 (NOAA National Hurricane Center).

This is the first widespread survey for *S. marcescens* in the Florida Keys' and it confirms the bacterium's presence in near shore as well as coral reef environments. Sporadic detection of the bacterium in the coral reef environment contrasts with the regular detection in the canals, which suggests the canals are still an introduction source of *S. marcescens* to the coastal environment (Griffin et al. 1999, Sutherland et al. 2010). However, detection of *S. marcescens* at the reef tract was not correlated with the presence of *A. palmata* colonies with WPD. When *S. marcescens* was cultured from the coral reef, in this study and in previous studies, a small percentage of the isolates were identified as the tested coral pathogenic strains PDR60 and PDL100 (Sutherland et al. 2010). *S. marcescens* PDR60 was found in the coral reef environment

the summer of 2012, which confirms that the pathogenic strain is still capable of reaching or circulating in the reef tract but it was not causing disease. Therefore, these white pox observations are not considered to be acroporid serratiosis, further highlighting the need for better disease diagnostics.

Potential pathogens, in addition to *S. marcescens*, may be associated with WPD signs. Additional surveys applying advanced molecular techniques may help to identify causative agents for what may be multiple manifestations of WPD. Acroporid serratiosis could be considered a known type of WPD, which differs from a distinct WPD type that dominated during this study and possibly other recent studies of WPD (May et al. 2010, Lesser and Jarett 2014). Only WPD acroporid serratiosis would have the specific association with the presence of *S. marcescens* within disease lesions. At this time, the differences between acroporid serratiosis and WPD observed in 2012 and 2013 in the Florida Keys are noted by a decreased mortality (whole colony mortality) and a lack of *S. marcescens* detection.

The ambiguity in descriptions for coral disease etiologies stems in part from lack of knowledge about the coral holobiont, pathogen ecology and a low number of potential disease signs among corals (Pollock et al. 2011, Weil and Rogers 2011). Traditional methods to isolate coral pathogens assumes that the pathogen is culturable; however, many marine bacteria are not culturable and some bacteria become viable but not culturable under non-ideal conditions or when in association with corals (Sharon and Rosenberg 2010). Novel molecular methods are improving what is known about the coral holobiont and may facilitate pathogen detection (Cook et al. 2013, Krediet et al. 2013, Lesser and Jarett 2014). The application of this qPCR method to detect *S. marcescens* is an example of an improved method for coral disease studies specifically about acroporid serratiosis to ascertain the prevalence of the bacterium in the coastal

environment. *S. marcescens* is uncommon in the marine environment and any positive detection using this qPCR suggests introduction to the coral reef environment and potential for acroporid serratiosis.

Of the known etiologies for coral diseases some are associated with a single pathogen others with a consortium of bacteria. Current research supports the notion that many diseases are not identified as having a single pathogen but a consortium of microbes responsible for the disease signs (i.e., red band and black band diseases) (Pantos et al. 2003, Sekar et al. 2006, Klaus et al. 2011). Where a single coral pathogen has been identified, some of those pathogens have ceased to cause or be associated with the signs of the disease in subsequent studies, which is similar to what is now observed with WPD and *S. marcescens* (Banin et al. 2000, Ben-Haim et al. 2003, Reshef et al. 2006). Other pathogens may have always existed that produce similar disease signs of identified coral diseases, such as white pox disease. This study is an example of the complexity in defining a coral disease through the absence of confirmed acroporid serratiosis when coral disease surveys identified white pox disease lesions.

Similar to the records of other coral diseases, the nature of WPD may be changing in response to selective pressures caused by disease itself, resulting from either the microbial community controlling levels of pathogenic bacteria or changes in coral host resistance (Reshef et al. 2006, Reed et al. 2010). The probiotic hypothesis states that such a change in an apparent pathogen's virulence is possibly due to the coral holobiont's adaptation; the microbiota of the holobiont can respond to the selective pressure from the pathogen to resist future infections (Reshef et al. 2006). In addition to the immune benefits from the holobiont's microbiota, the corals are also have an innate immune system and may have resistance to diseases following non-fatal infections (Loker et al. 2004, Mydlarz et al. 2009, Reed et al. 2010). There are only a

few *A. palmata* genotypes remaining in the Caribbean, which may increase the probability that these elements of the holobiont's immune system, microbiome adaptation and coral innate immunity, all confer a greater resistance to the pathogens that caused the massive decline in *A. palmata* population (Baums et al. 2005, Vollmer and Kline 2008).

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Tables

Table 5.1. *A. palmata* colonies that were tagged and photographed as part of the WPD survey with percent diseased colonies in parentheses.

Site	Location	Number Coral Colonies in WPD Survey							
		2011		2012			2013		
		Spring	Summer	Winter	Spring	Summer	Winter	Spring	Summer
Carysfort	25°13.248'N, 80°12.594'W	8 (25%)	6 (0%)	7 (0%)	7 (0%)	6 (0%)	5 (0%)	6 (0%)	6 (16.67%)
Sombrero	24°37.518'N, 81° 6.696'W	8 (25%)	7 (0%)	9 (0%)	8 (0%)	7 (14.29%)	8 (0%)	8 (0%)	8 (25%)
Molasses	25° 0.528'N, 80° 22.590'W	12 (0%)	10 (80%)	10 (0%)	10 (0%)	9 (33.33%)	7 (0%)	6 (0%)	6 (16.67%)
Looe Key	24°32.7'N, 81°24.4'W	33 (0%)	32 (0%)	28 (0%)	30 (0%)	31 (32.26%)	31 (0%)	27 (0%)	27 (22.22%)
Rock Key	24°27.270'N, 81°51.534'W	6 (0%)	6 (0%)	6 (0%)	6 (0%)	6 (0%)	6 (0%)	6 (0%)	6 (0%)
Western Sambo	24°28.680'N, 81°43.026'W	11 (0%)	9 (0%)	9 (0%)	8 (0%)	7 (0%)	6 (0%)	7 (0%)	7 (0%)

Table 5.2. *A. palmata* colonies that were tested for *S. marcescens* using qPCR.

NA indicates when a sample was unable to be collected or tested.

Year	Season	Reef	Sample Type	WPD on the reef	Total Samples	qPCR Positive
2011	Spring	Carysfort	Coral	+	4	0
2011	Spring	Carysfort	Pox	+	2	0
2011	Spring	Carysfort	Water	+	4	0
2011	Spring	LooeKey	Coral	-	9	0
2011	Spring	LooeKey	Abnormal	-	1	1
2011	Spring	LooeKey	Water	-	NA	NA
2011	Spring	RockKey	Coral	-	5	0
2011	Spring	RockKey	Abnormal	-	3	0
2011	Spring	RockKey	Water	-	1	0
2011	Spring	Sombrero	Coral	+	6	0
2011	Spring	Sombrero	Pox	+	2	0
2011	Spring	Sombrero	Water	+	4	0
2011	Spring	WesternSambo	Coral	-	6	0
2011	Spring	WesternSambo	Water	-	3	0
2011	Summer	Carysfort	Coral	-	4	0
2011	Summer	Carysfort	Water	-	4	0
2011	Summer	LooeKey	Coral	-	NA	NA
2011	Summer	LooeKey	Water	-	NA	NA
2011	Summer	RockKey	Coral	-	6	0
2011	Summer	RockKey	Water	-	5	0
2011	Summer	Sombrero	Coral	-	7	0
2011	Summer	Sombrero	Abnormal	-	2	0
2011	Summer	Sombrero	Water	-	3	0
2011	Summer	WesternSambo	Coral	-	6	0
2011	Summer	WesternSambo	Water	-	5	0
2012	Winter	Carysfort	Coral	-	6	0
2012	Winter	Carysfort	Water	-	5	0
2012	Winter	LooeKey	Coral	-	8	6
2012	Winter	LooeKey	Abnormal	-	1	0
2012	Winter	LooeKey	Water	-	4	1
2012	Winter	RockKey	Coral	-	7	0
2012	Winter	RockKey	Water	-	4	0
2012	Winter	Sombrero	Coral	-	4	0
2012	Winter	Sombrero	Water	-	2	0
2012	Winter	WesternSambo	Coral	-	4	0
2012	Winter	WesternSambo	Water	-	4	0
2012	Spring	Carysfort	Coral	-	NA	NA
2012	Spring	Carysfort	Water	-	NA	NA
2012	Spring	LooeKey	Coral	-	NA	NA
2012	Spring	LooeKey	Water	-	NA	NA
2012	Spring	RockKey	Coral	-	6	0
2012	Spring	RockKey	Abnormal	-	1	0
2012	Spring	RockKey	Water	-	4	0
2012	Spring	Sombrero	Coral	-	NA	NA
2012	Spring	Sombrero	Water	-	NA	NA
2012	Spring	WesternSambo	Coral	-	8	0
2012	Spring	WesternSambo	Water	-	3	0
2012	Summer	Carysfort	Coral	-	6	0
2012	Summer	Carysfort	Water	-	4	0

2012	Summer	LooeKey	Coral	+	8	4
2012	Summer	LooeKey	Pox	+	9	4
2012	Summer	LooeKey	Water	+	6	1
2012	Summer	RockKey	Coral	-	6	0
2012	Summer	RockKey	Water	-	5	1
2012	Summer	Sombrero	Coral	+	3	0
2012	Summer	Sombrero	Pox	+	4	1
2012	Summer	Sombrero	Water	+	4	2
2012	Summer	WesternSambo	Coral	-	5	0
2012	Summer	WesternSambo	Water	-	4	0
2013	Winter	Carysfort	Coral	-	5	0
2013	Winter	Carysfort	Water	-	3	0
2013	Winter	LooeKey	Coral	-	9	3
2013	Winter	LooeKey	Water	-	3	0
2013	Winter	RockKey	Coral	-	6	0
2013	Winter	RockKey	Water	-	5	0
2013	Winter	Sombrero	Coral	-	8	0
2013	Winter	Sombrero	Water	-	4	0
2013	Winter	WesternSambo	Coral	-	6	0
2013	Winter	WesternSambo	Water	-	4	0
2013	Spring	Carysfort	Coral	-	NA	NA
2013	Spring	Carysfort	Water	-	NA	NA
2013	Spring	LooeKey	Coral	-	NA	NA
2013	Spring	LooeKey	Water	-	NA	NA
2013	Spring	RockKey	Coral	-	4	0
2013	Spring	RockKey	Water	-	3	0
2013	Spring	Sombrero	Coral	-	6	0
2013	Spring	Sombrero	Abnormal	-	2	0
2013	Spring	Sombrero	Water	-	4	0
2013	Spring	WesternSambo	Coral	-	5	0
2013	Spring	WesternSambo	Water	-	3	0
2013	Summer	Carysfort	Coral	+	6	0
2013	Summer	Carysfort	Pox	+	1	0
2013	Summer	Carysfort	Water	+	3	0
2013	Summer	LooeKey	Coral	+	5	5
2013	Summer	LooeKey	Pox	+	5	2
2013	Summer	LooeKey	Water	+	5	1
2013	Summer	RockKey	Coral	-	6	0
2013	Summer	RockKey	Abnormal	-	3	0
2013	Summer	RockKey	Water	-	2	0
2013	Summer	Sombrero	Coral	+	6	0
2013	Summer	Sombrero	Pox	+	3	0
2013	Summer	Sombrero	Water	+	2	0
2013	Summer	WesternSambo	Coral	-	5	0
2013	Summer	WesternSambo	Water	-	3	0

Table 5.2. Samples collected from additional coral species for culture and qPCR detection of *S. marcescens*.

Percent positive samples are in parentheses.

		2012		2013			
		Summer		Winter		Summer	
Site	**Coral species**	Culture	qPCR	Culture	qPCR	Culture	qPCR
Looe Key	*P. asteroides*	10 (20%)	5 (80%)	10 (0%)		10 (0%)	
	P. porities	10 (20%)	5 (100%)	10 (0%)		10 (20%)	3 (33%)
	O. spp.	10 (0%)		10 (0%)		10 (10%)	3 (100%)
	S. siderea	10 (0%)		10 (0%)		10 (0%)	
Molasses	*P. asteroides*	8 (0%)		9 (0%)		10 (0%)	
	P. porities	10 (0%)		11 (0%)		10 (0%)	3 (33%)
	O. spp.	10 (0%)		8 (0%)		8 (0%)	3 (0%)
	S. siderea	10 (0%)		10 (0%)		10 (0%)	
Rock Key	*P. asteroides*				5 (100%)		
	P. porities				5 (60%)		

Table 5.3. The presence of *S. marcescens* in the Florida Key's canal samples.

Includes both culture (C) and molecular (M) methods. Some samples are presumptive positives until further confirmation (+*).

	2011				2012						2013					
	Spring		Summer		Winter		Spring		Summer		Winter		Spring		Summer	
Sites	C	M	C	M	C	M	C	M	C	M	C	M	C	M	C	M
Sexton Cove	-	-	-	-	-	-	-		-		-	-	-	-	-	+
Tropical Lane	-	+	+	-	-	-	-		-		+*	+	+*	+	+	+
Blue Hole	-	-	+	-	-	-	+*		+	+	+*	+	-	-	-	+
Eden Pines	+	-	+	-	+	-	+*		+	+	+*	+	-	+	-	+
Doctors Arm	+	-	+	+	+	-	+*		+	+	+*	+	-	+	-	+

Table 5.4. Generalized linear mixed effects model results.

Fixed effects were the presence of white pox disease on the reef and the sample type (coral or water). id=sample year, sample season, and reef.

Fit Statistics	
-2 Res Log Pseudo-Likelihood	445.73
Generalized Chi-Square	40.05
Gener. Chi-Square / DF	0.51

Covariance Parameter Estimates		
Cov Parm	Estimate	Standard Error
*id*Diseased*	4.5124	1.6710
Residual (VC)	0.5135	0.09234

Solutions for Fixed Effects							
Effect	DiseasedReef	Sample_type	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept			-3.2633	0.9774	27.27	-3.34	0.0024
Diseased	NoDisease		-2.0228	1.1248	27.95	-1.80	0.0829
Diseased	PoxPresent		0	.	.	.	.
Sample_*type*		Coral	0.9572	0.3926	62.16	2.44	0.0177
Sample_*type*		Water	0	.	.	.	.

Odds Ratio Estimates							
DiseasedReef	Sample_type	DiseasedReef	Sample_type	Estimate	DF	95% CI	
NoDisease		PoxPresent		0.132	27.95	0.013	1.325
	Coral		Water	2.604	62.16	1.188	5.709

Type III Tests of Fixed Effects						
Effect	*Num DF*	*Den DF*	*Chi-Square*	*F Value*	*Pr > ChiSq*	*Pr > F*
Diseased	1	27.95	3.23	3.23	0.0721	0.0829
Sample_type	1	62.16	5.94	5.94	0.0148	0.0177

Figures

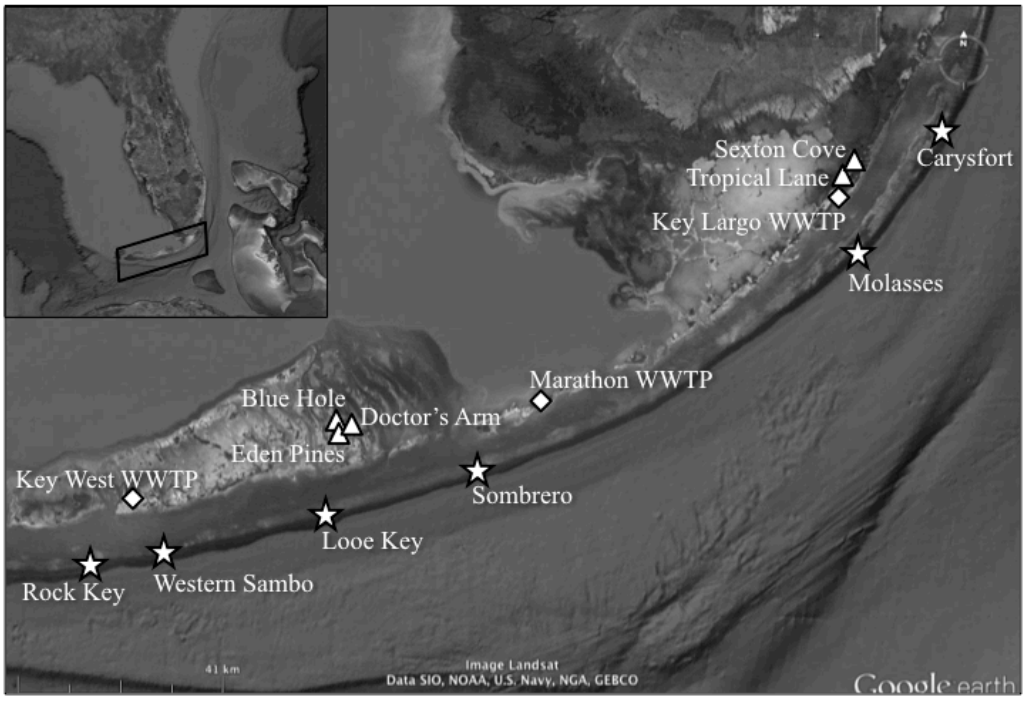


Figure 5.1. Sample locations in the Florida Keys – stars indicate reef sites, triangles indicate canal sites, and diamonds indicate wastewater treatment plants (WWTP).

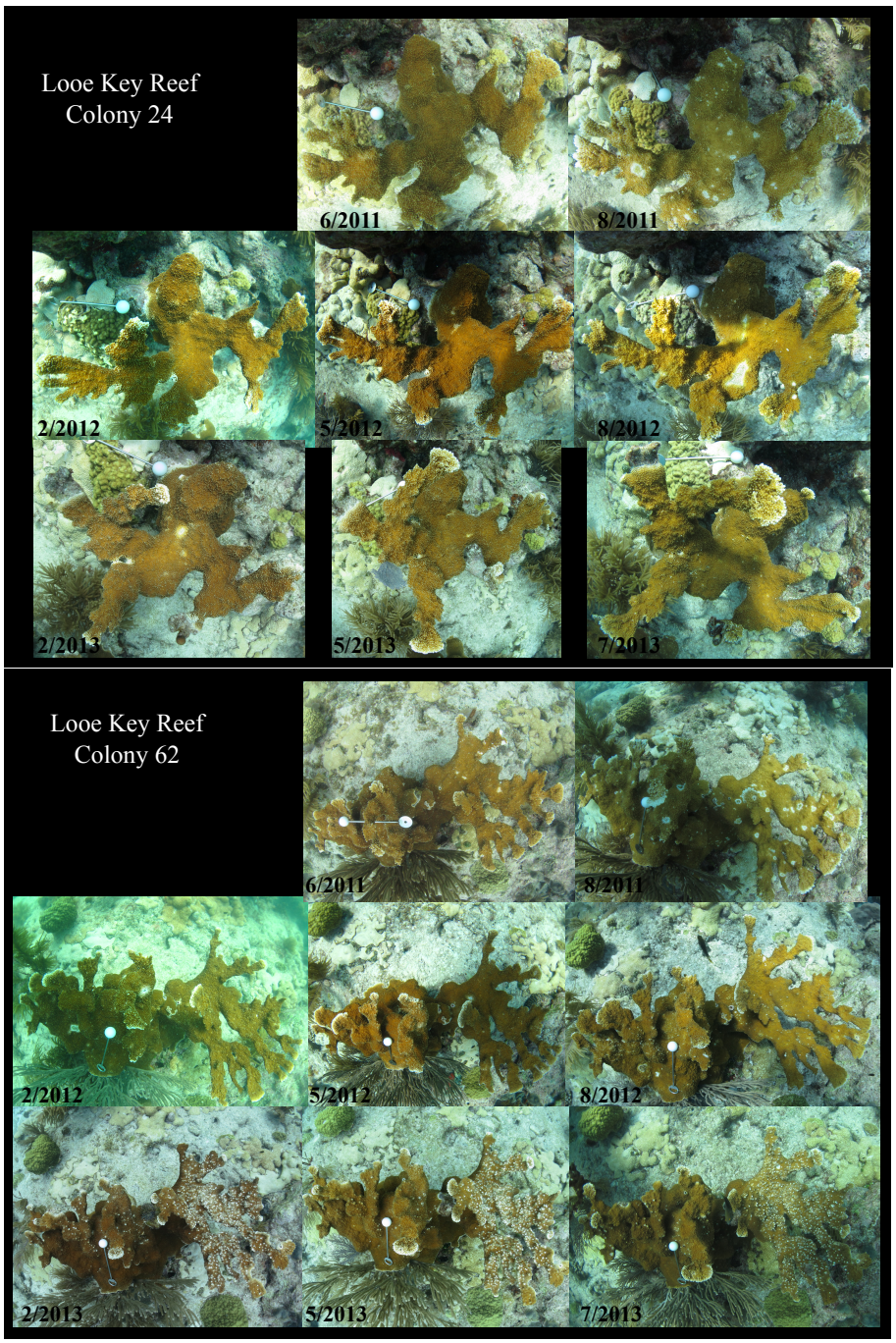


Figure 5.2. Example of WPD photographic survey, two corals are shown from Looe Key reef from August 2011 to July 2013.

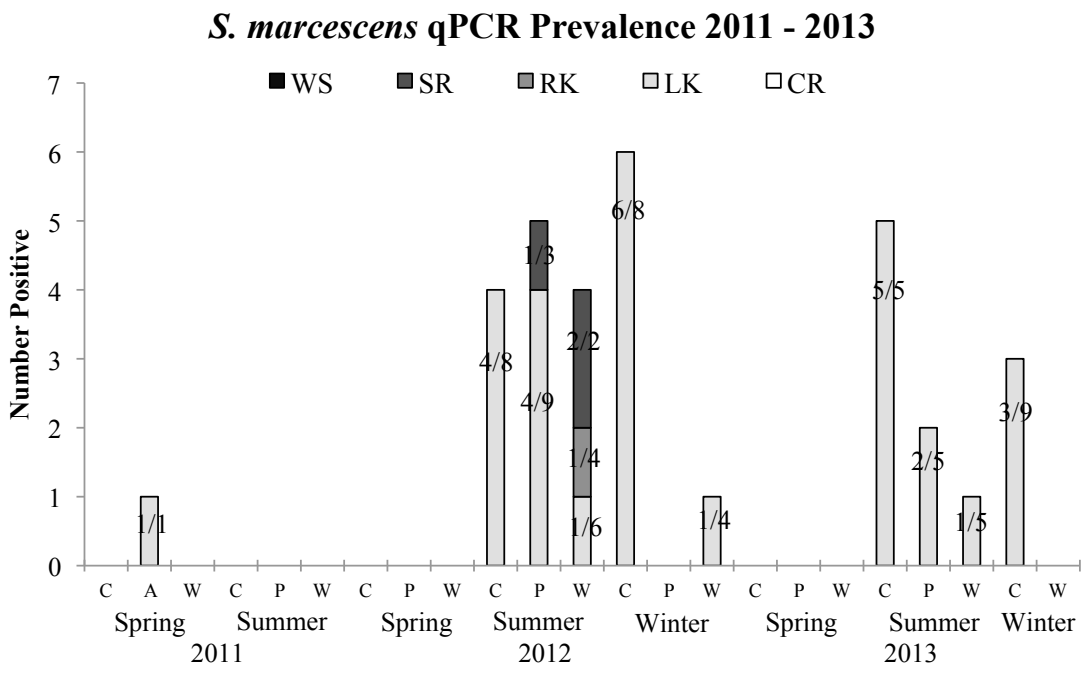


Figure 5.3. *S. marcescens* qPCR positive coral reef samples, *A. palmata* corals and water. Axis labels are coral (C), abnormal (A), white pox disease lesions (P), reef water (W). Bar shading is according to reef CR = Carysfort, SR = Sombrero Reef, LK = Looe Key, WS = Western Sambo, RK = Rock Key. Numbers on the bars represent the number positive of the total number of samples.

CHAPTER 6: CONCLUSION

The global population is growing and the greatest density of people is along the coastlines. Coastal development pressure combined with the changing climate is a considerable threat to the health of the coastal ecosystems. This dissertation investigated two ensuing issues: the contamination of coastal waters with human enteric bacteria and potential pathogen introduction to the coral reef ecosystem. The studies took place along the Florida Keys archipelago where there is an intimate relationship between the coastal communities and ecosystem health. The goals of this dissertation were to evaluate the introduction of human enteric microbiota in a remote location with low risk of introduction, describe the coral microbiota of the threatened coral *A. palmata* that is susceptible for infection by a human enteric bacterium (*S. marcescens*), then design an advanced molecular detection assay to determine the prevalence of *S. marcescens* in the coastal environment.

In the Dry Tortugas National Park there is a decreased risk of coastal contamination with human enteric bacteria. This remote location also has restricted visitor access and protects over 3 km^2 of coral reef habitat. Traditional culture methods to detect human enteric bacteria (enterococci, *S. marcescens*, *Clostridium perfringens,* and a broad group of fecal coliforms) showed that the coastal waters have persistent enteric bacteria but the amount detected were not a human health concern. Since there were very low levels of enteric bacteria, this was an ideal location to study the influence on the coastal microbiota from visitors swimming or wading in the beach areas. Remarkably there was a significant rise in the enteric bacteria in the water

following the peak number of people in or around the water. This study illustrated that coastal contamination of enteric bacteria is a persistent occurrence for tropical areas like the Florida Keys, even without the influence from increasing coastal development and poor infrastructure for wastewater containment. While the levels may not reach the government regulation threshold for a human health risk, coastal marine animals are still being exposed to the enteric bacteria and the effects are only recently being investigated.

The Florida Key's coral reef is a threatened ecosystem and many coral populations have dramatically declined. Therefore of the marine animals, corals are at an exposure risk for coastal contamination in addition to the other growing threats to the ecosystem (e.g., nutrification, ocean acidification, boat groundings). The second study in this dissertation examined the microbiota of the threatened coral, *A. palmata*, to understand the potential impact from human enteric bacteria and environmental changes. A persistent threat for this coral species is white pox disease; this is one of a few *A. palmata* diseases that have caused Caribbean-wide population declines. Early studies of this disease indicated that the human enteric bacterium *Serratia marcescens* was a causal agent for white pox disease acroporid serratiosis. Therefore for three years, *A. palmata* coral colonies were sampled and the bacterial community was sequenced. These bacterial communities helped elucidate about the coral holobiont dynamics as well as the etiology of white pox disease. The microbiota of *A. palmata* was more similar for when and where the samples were collected, such as summer samples generally clustering while water bacterial communities were different from coral bacterial communities. These bacterial communities also revealed the strong influence of episodic environmental events, such as Saharan dust clouds. A dust cloud in July 2013 deposited micro-nutrients and trace metals into the coral reef ecosystem, which correlated with the proliferation of bacteria in the Order Vibrionales. Additionally, a few

bacterial taxa had a higher relative abundance in the disease lesions than in healthy corals, one of which was the Order Rhodobacterales. More studies are needed to understand the role this taxon in the disease process or if it is only responding to newly available space for colonization. However, in all of the bacterial communities, especially the white pox disease lesions, there was little to no *S. marcescens* as part of the community.

To improve the method used to directly detect *S. marcescens* in the coastal environment, I developed a quantitative real-time PCR (qPCR) assay. Detecting and quantifying this bacterium in the environment evaluated a human enteric bacterium in the coastal environment and demonstrated the presence of this coral pathogen in the coral reef ecosystem. The assay I designed uses a *S. marcescens* specific region of the *lux*S gene as the molecular target. This assay increased the sensitivity of detecting the bacterium in the environment, which was needed because traditional culture methods were taking valuable time to process and were not very sensitive. While *A. palmata* continues to exhibit signs of white pox disease *S. marcescens* has not been isolated from a disease lesion for over five years. The *S. marcescens* qPCR assay was used on 240 unique coral samples from the Florida Keys through 2010 to 2013 and only 26 samples were positive for the bacterium. Implementing this qPCR assay improved the detection of the bacterium in the coastal environment, and it confirmed that the bacterium is present at very low abundance. There was also no correlation to when a sample was positive for *S. marcescens* and if it was from a diseased coral. Since the pathophysiology for white pox disease is not completely known, it is unclear if the lack of *S. marcescens* associated with disease is because the samples from diseased corals were before or after when *S. marcescens* was most abundant in the disease progression or if the bacterium is always associated with the disease.

The *S. marcescens* strains that are coral pathogens are linked to the introduction of human enteric bacteria into the coastal environment. However, using traditional and advanced molecular detection techniques, *S. marcescens* is not naturally abundant in the marine microbiota. In the remote location of the Dry Tortugas National Park the bacterium was only detected at very low levels correlated to when people were swimming. The only time that *S. marcescens* was regularly detected in marine samples was from the sewage impacted canals throughout the Florida Keys. *S. marcescens* does not survive long enough in the environment, which explains the low transport to the coral reef ecosystem. The qPCR surveys for *S. marcescens* will elucidate some ecological aspects of the bacterium in the marine environment, however it does not address the disassociation of this bacterium with white pox disease.

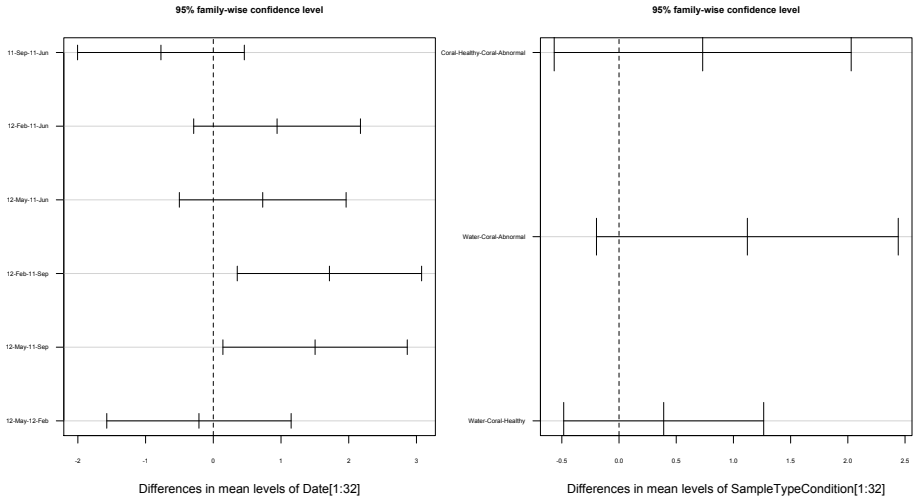
APPENDIX 1

ANOVAs were completed to analyze the top bacterial Orders of MR and LKR coral bacteria communities. These Orders represented at least 1% of the relative abundance within the bacterial communities. The resulting ANOVA tables are reported for the simplest model for each Order (Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1). A post-hoc Tukey's Honestly Significant Difference (HSD) was completed for each ANOVA and the resulting 95% confidence interval (CI) is plotted below the ANOVA tables, significant comparisons are when the CI does not cross zero on the x-axis.

Actinomycetales Results

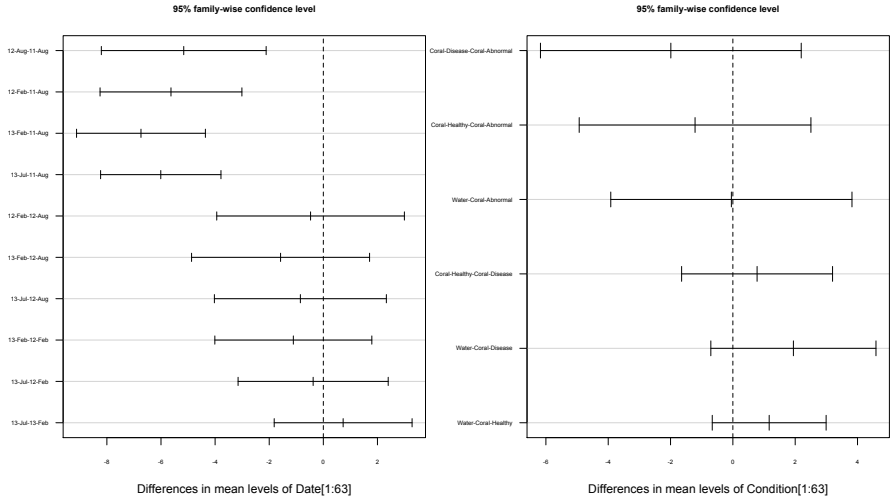
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1:32]	3	12.886	4.295	4.985	0.00728 **
SampleTypeCondition[1:32]	2	5.179	2.589	3.005	0.06697 .
Residuals	26	22.402	0.862		



Looe Key Reef

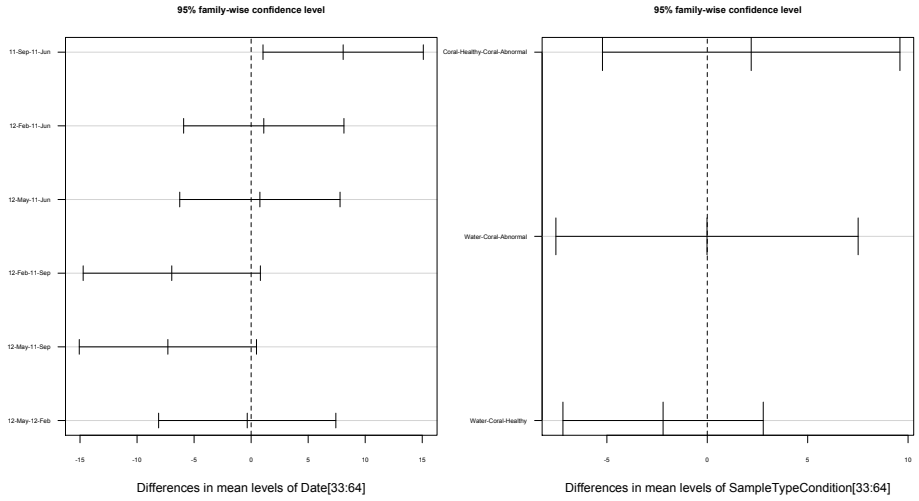
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1:63]	4	518.5	129.63	23.823	1.82e-11 ***
SampleTypeCondition[1:63]	3	34.2	11.39	2.093	0.112
Residuals	55	299.3	5.44		



Bacillales Results

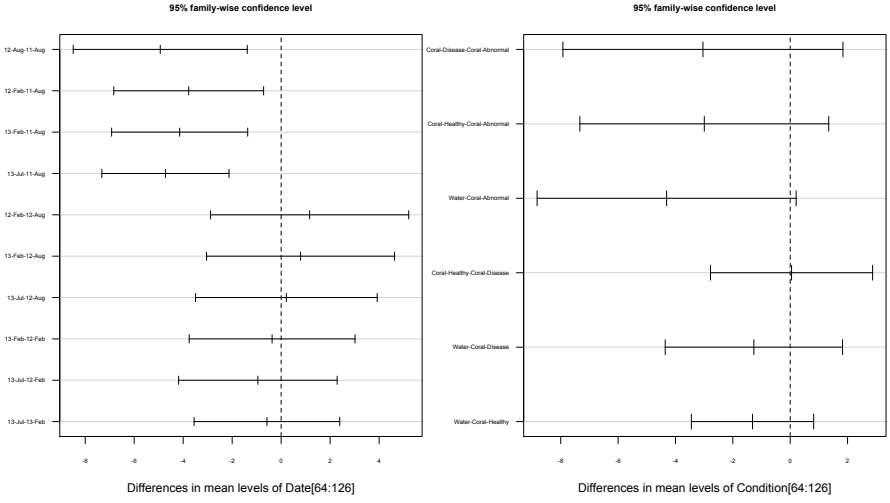
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[33:64]	3	316.8	105.61	3.764	0.0228 *
SampleTypeCondition[33:64]	2	40.2	20.08	0.715	0.4984
Residuals	26	729.6	28.06		



Looe Key Reef

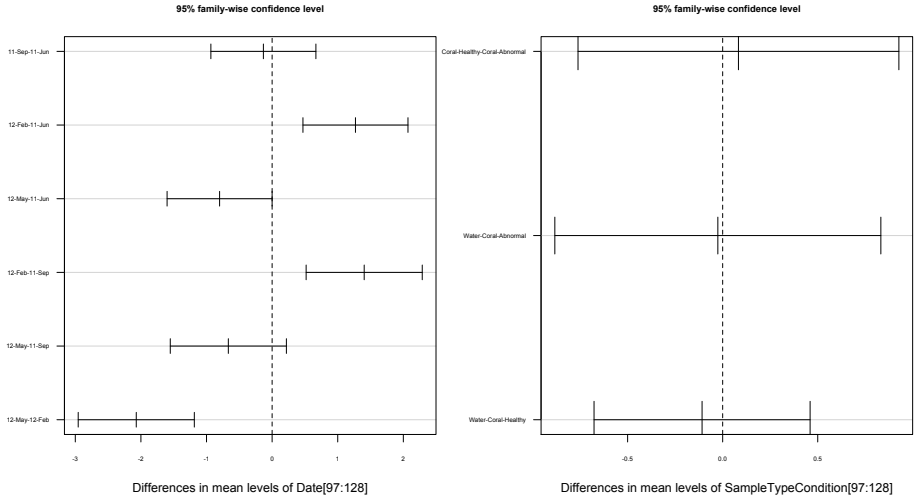
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[64:126]	4	276.8	69.20	9.341	7.81e-06 ***
SampleTypeCondition[64:126]	3	55.7	18.55	2.504	0.0686 .
Residuals	55	407.4	7.41		



Bdellovibrionales Results

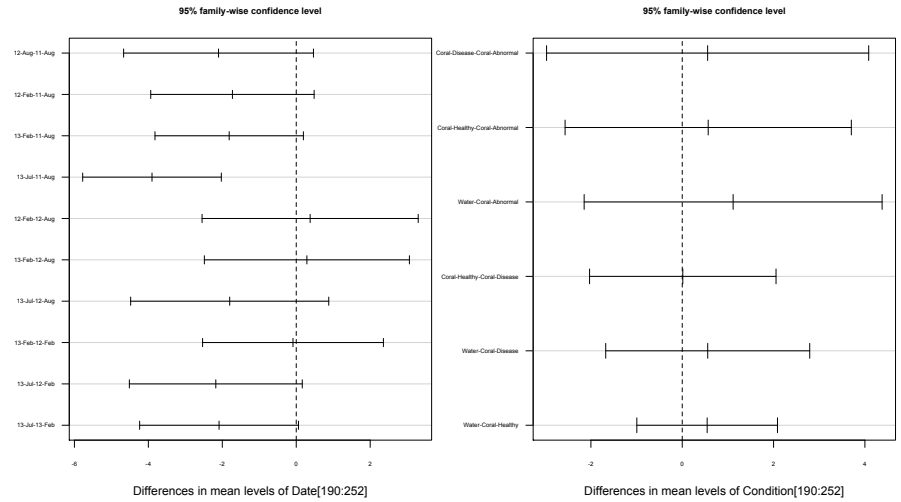
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[97:128]	3	15.732	5.244	14.40	1e-05 ***
SampleTypeCondition[97:128]	2	0.087	0.044	0.12	0.888
Residuals	26	9.469	0.364		



Looe Key Reef

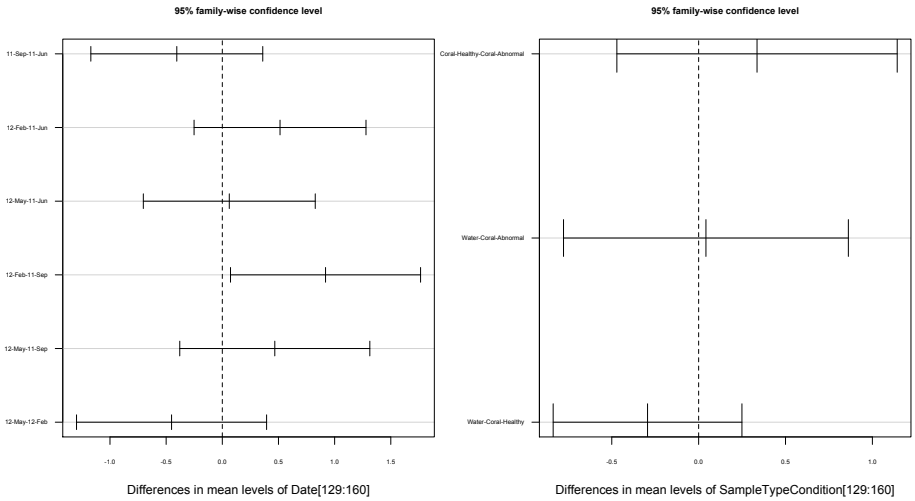
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[190:252]	4	134.47	33.62	8.694	1.64e-05 ***
SampleTypeCondition[190:252]	3	5.31	1.77	0.458	0.713
Residuals	55	212.67	3.87		



Caulobacterales Results

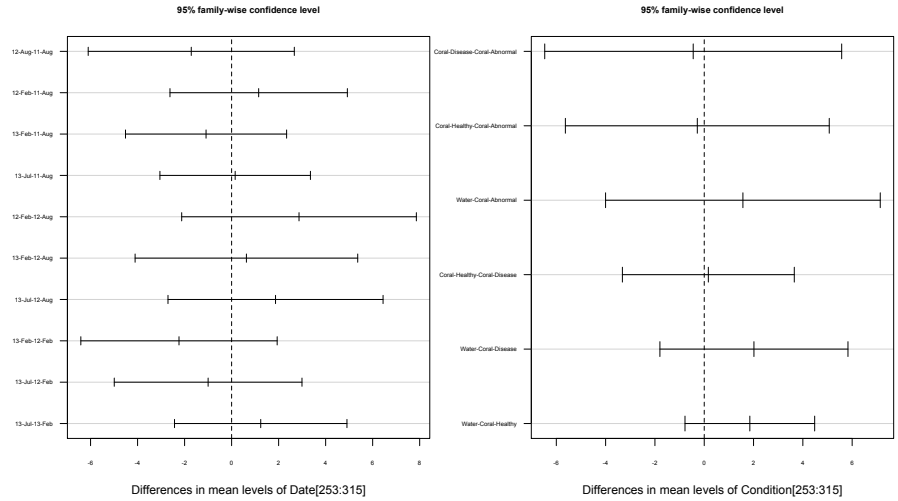
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[129:160]	3	2.980	0.9933	2.984	0.0496 *
SampleTypeCondition[129:160]	2	0.789	0.3943	1.185	0.3219
Residuals	26	8.655	0.3329		



Looe Key Reef

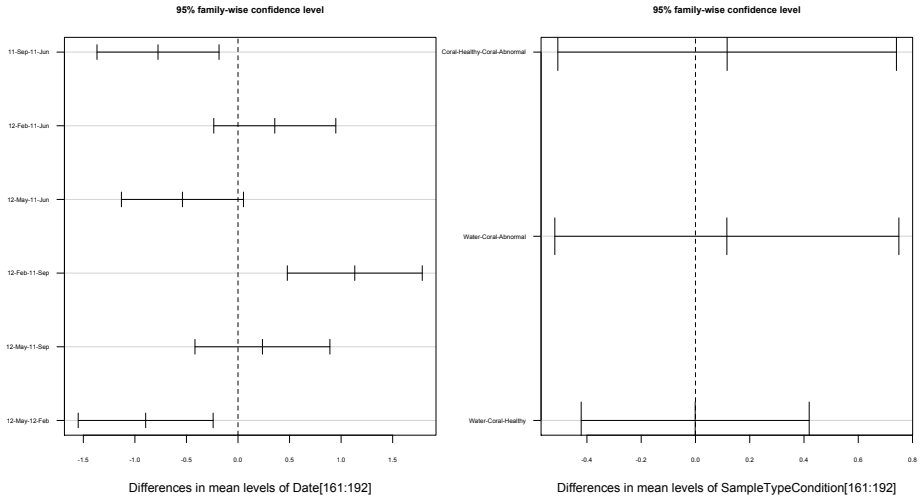
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[253:315]	4	42.3	10.58	0.938	0.449
SampleTypeCondition[253:315]	3	46.7	15.56	1.380	0.258
Residuals	55	620.1	11.27		



Clostridiales Results

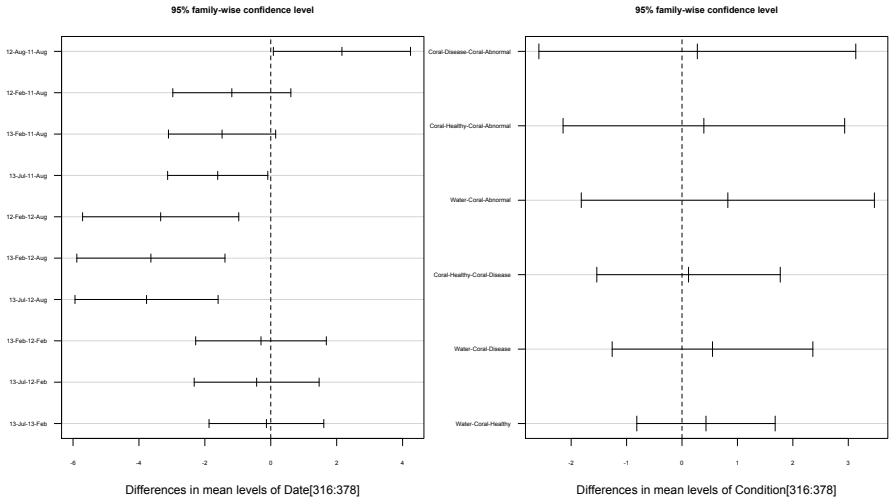
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[161:192]	3	5.732	1.9107	9.596	0.000194 ***
SampleTypeCondition[161:192]	2	0.065	0.0325	0.163	0.850208
Residuals	26	5.177	0.1991		



Looe Key Reef

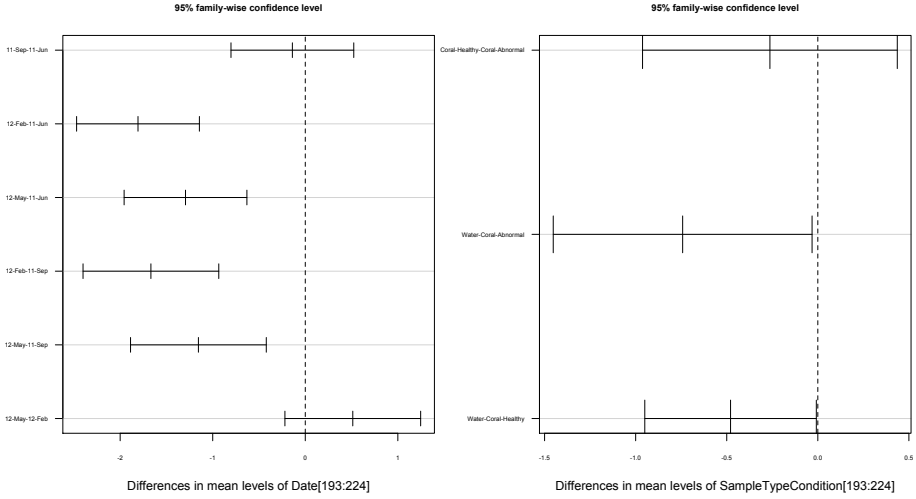
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[316:378]	4	80.79	20.197	7.951	3.93e-05 ***
SampleTypeCondition[316:378]	3	3.49	1.162	0.457	0.713
Residuals	55	139.72	2.540		



Cyanobacteria_Family_II Results

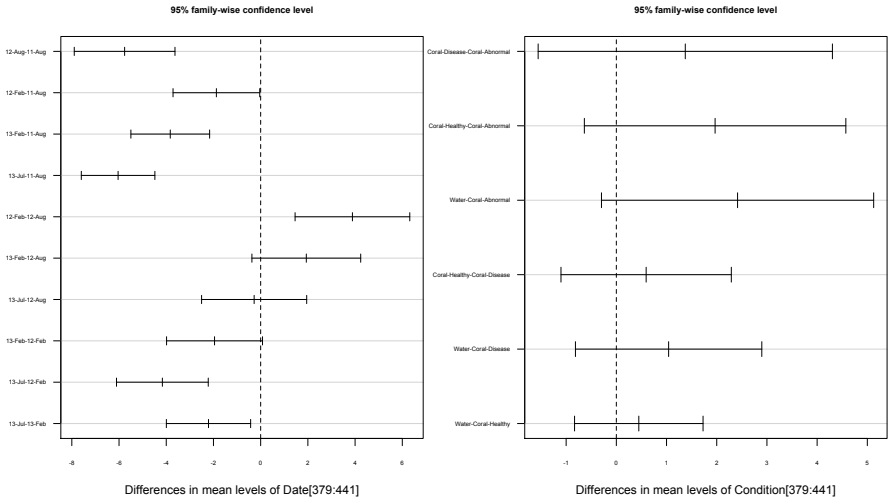
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[193:224]	3	18.640	6.213	24.847	8.44e-08 ***
SampleTypeCondition[193:224]	2	2.817	1.408	5.632	0.00929 **
Residuals	26	6.501	0.250		



Looe Key Reef

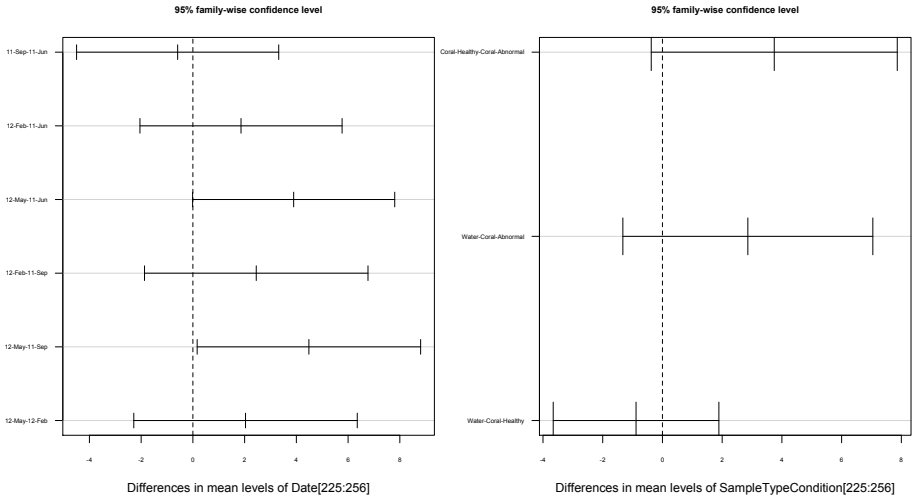
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[379:441]	4	393.1	98.26	36.795	5.95e-15 ***
SampleTypeCondition[379:441]	3	18.9	6.29	2.354	0.082 .
Residuals	55	146.9	2.67		



Enterobacteriales Results

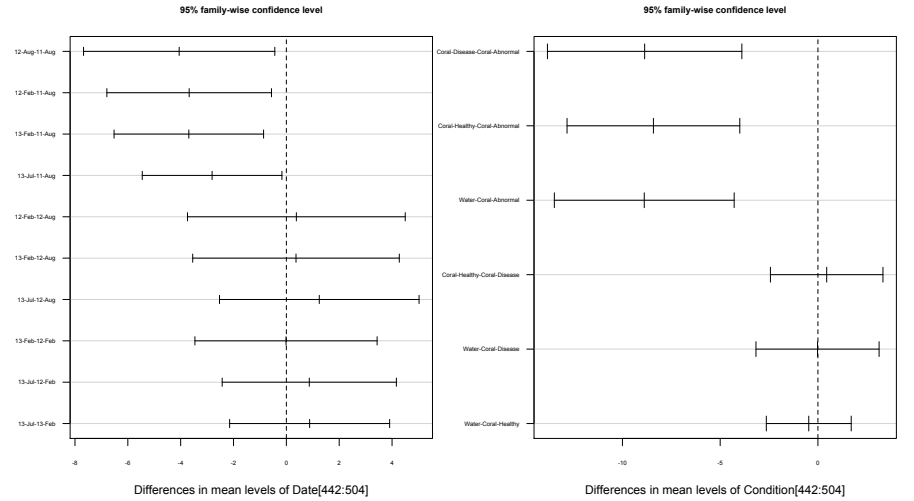
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[225:256]	3	92.01	30.669	3.534	0.0285 *
SampleTypeCondition[225:256]	2	58.28	29.140	3.358	0.0504 .
Residuals	26	225.61	8.677		



Looe Key Reef

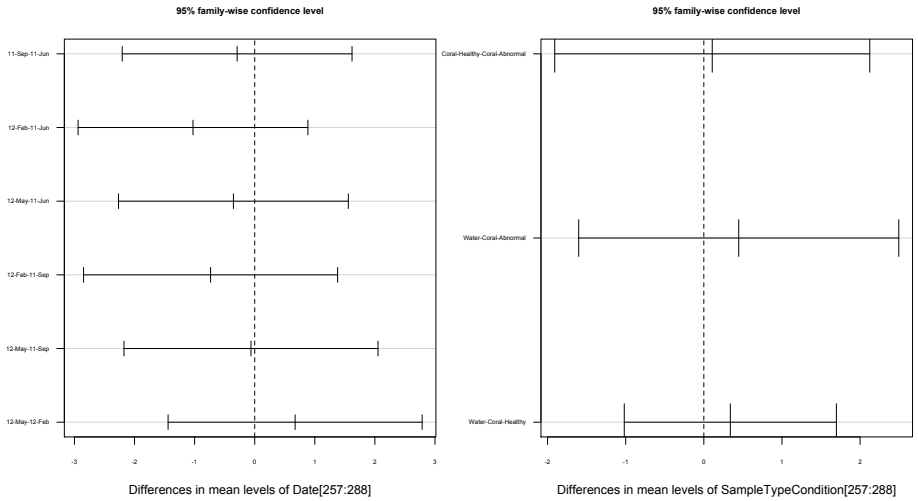
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[442:504]	4	173.8	43.46	5.655	0.000693 ***
SampleTypeCondition[442:504]	3	242.5	80.83	10.518	1.43e-05 ***
Residuals	55	422.7	7.69		



Flavobacteriales Results

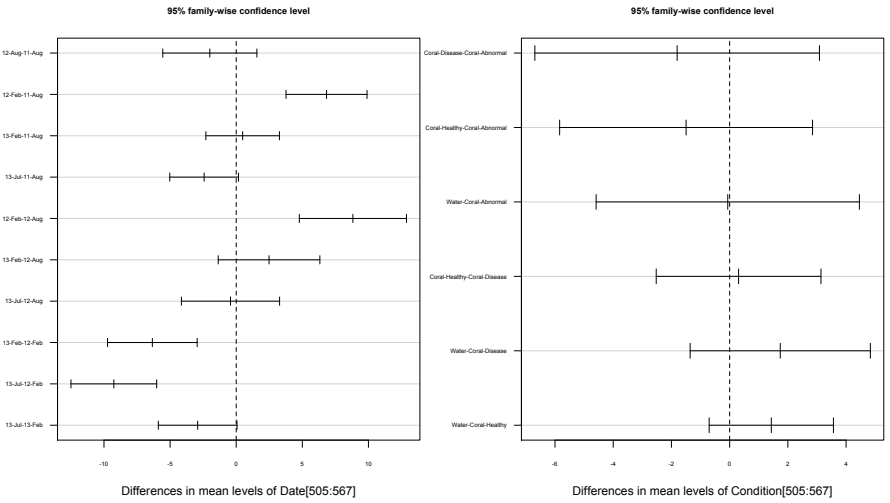
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[257:288]	3	4.57	1.5231	0.733	0.542
SampleTypeCondition[257:288]	2	1.17	0.5873	0.283	0.756
Residuals	26	54.04	2.0784		



Looe Key Reef

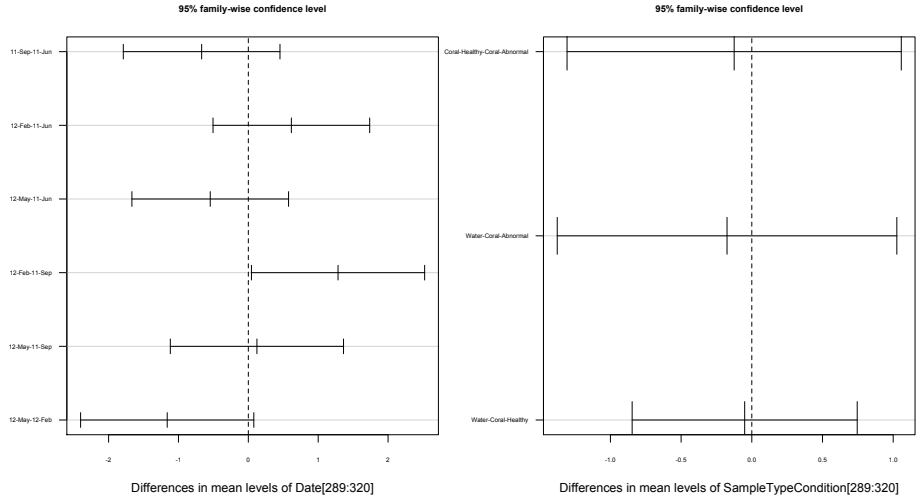
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[505:567]	4	529.2	132.30	17.809	1.97e-09 ***
SampleTypeCondition[505:567]	3	36.1	12.02	1.618	0.196
Residuals	55	408.6	7.43		



Fusobacteriales Results

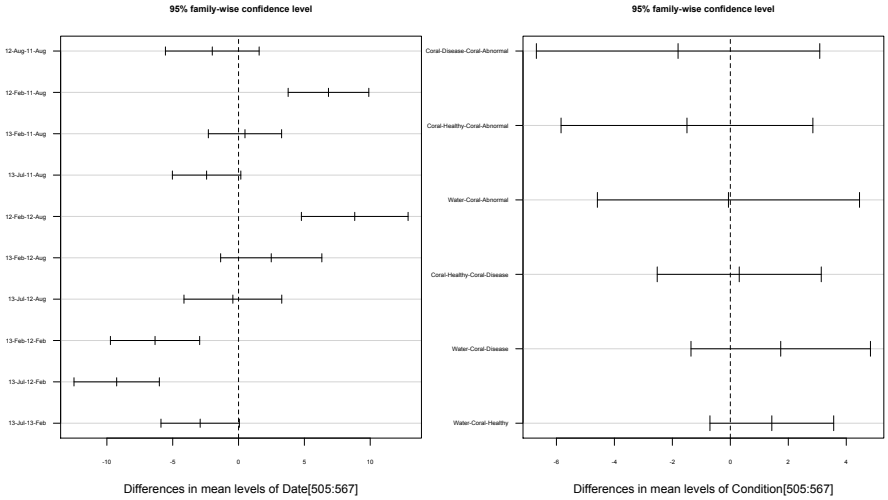
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[289:320]	3	7.315	2.4383	3.413	0.0322 *
SampleTypeCondition[289:320]	2	0.126	0.0628	0.088	0.9161
Residuals	26	18.577	0.7145		



Looe Key Reef

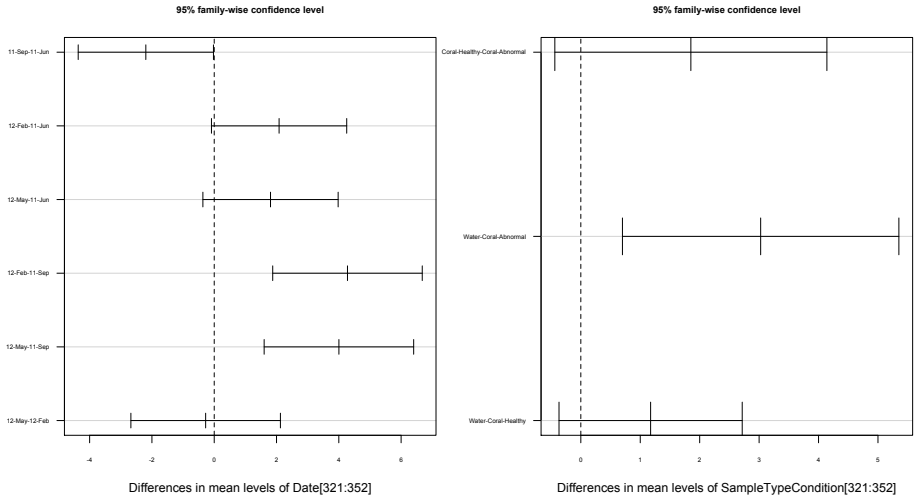
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[568:603]	2	2.75	1.3754	1.070	0.355
SampleTypeCondition[568:603]	2	1.74	0.8681	0.675	0.516
Residuals	31	39.85	1.2855		



Lactobacillales Results

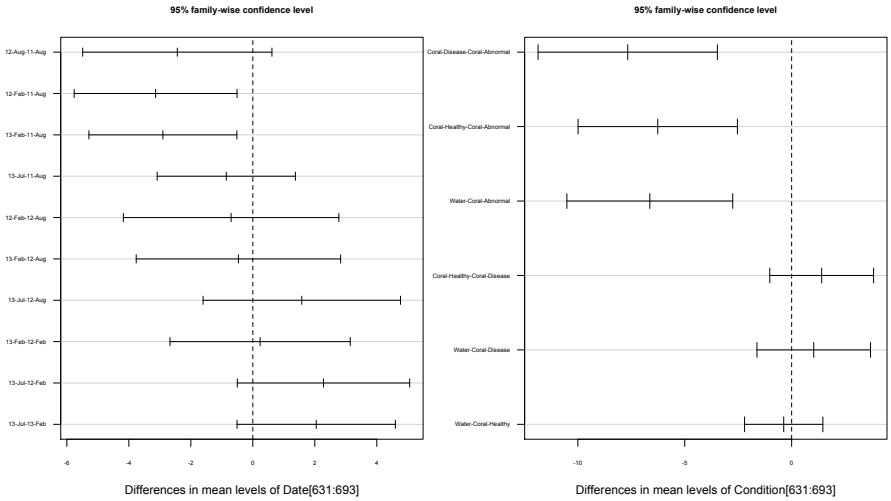
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[321:352]	3	82.58	27.526	10.273	0.000122 ***
SampleTypeCondition[321:352]	2	38.13	19.065	7.116	0.003431 **
Residuals	26	69.66	2.679		



Looe Key Reef

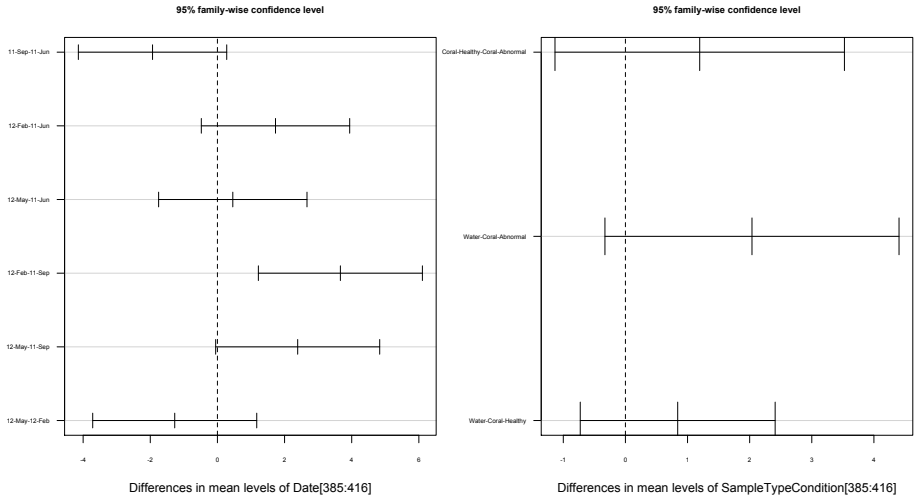
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[631:693]	4	106.1	26.53	4.853	0.00201 **
SampleTypeCondition[631:693]	3	164.3	54.77	10.018	2.29e-05 ***
Residuals	55	300.7	5.47		



Oceanospirillales Results

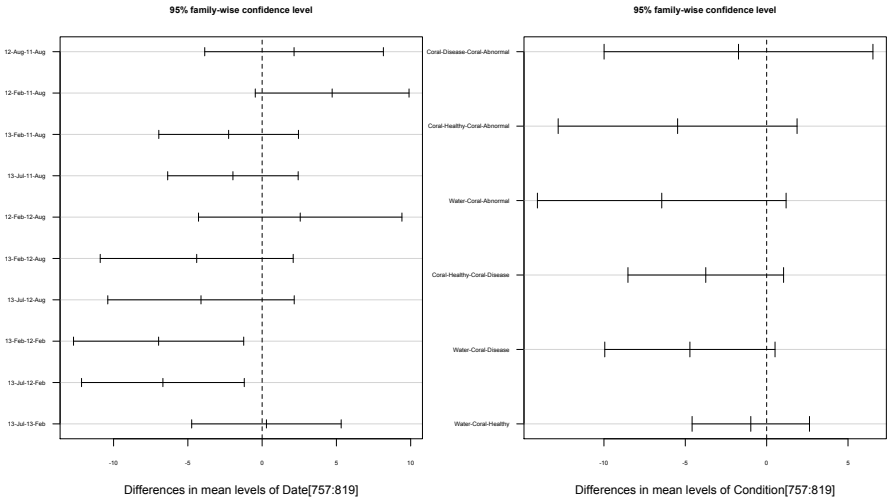
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[385:416]	3	48.46	16.155	5.820	0.0035 **
SampleTypeCondition[385:416]	2	17.47	8.733	3.147	0.0597 .
Residuals	26	72.16	2.775		



Looe Key Reef

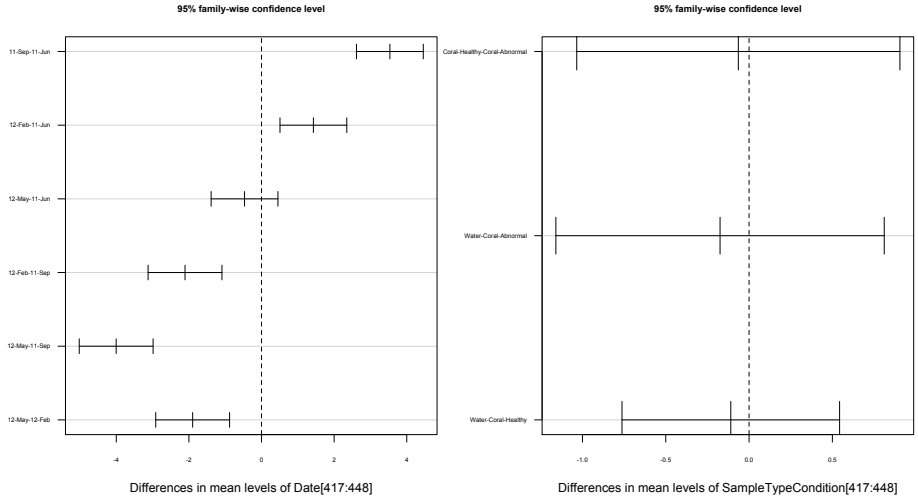
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[757:819]	4	346.7	86.67	4.085	0.00571 **
SampleTypeCondition[757:819]	3	237.8	79.27	3.737	0.01623 *
Residuals	55	1166.8	21.21		



Planctomycetales Results

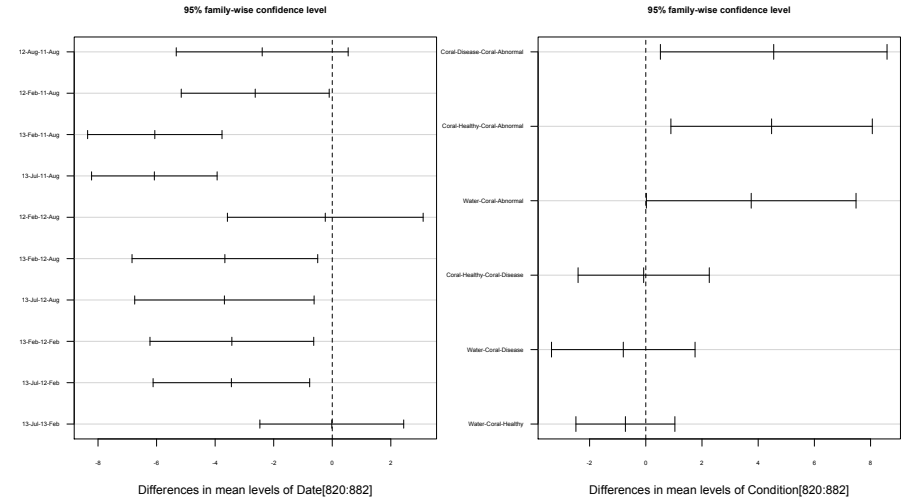
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[417:448]	3	72.36	24.120	50.143	6.01e-11 ***
SampleTypeCondition[417:448]	2	0.15	0.076	0.158	0.854
Residuals	26	12.51	0.481		



Looe Key Reef

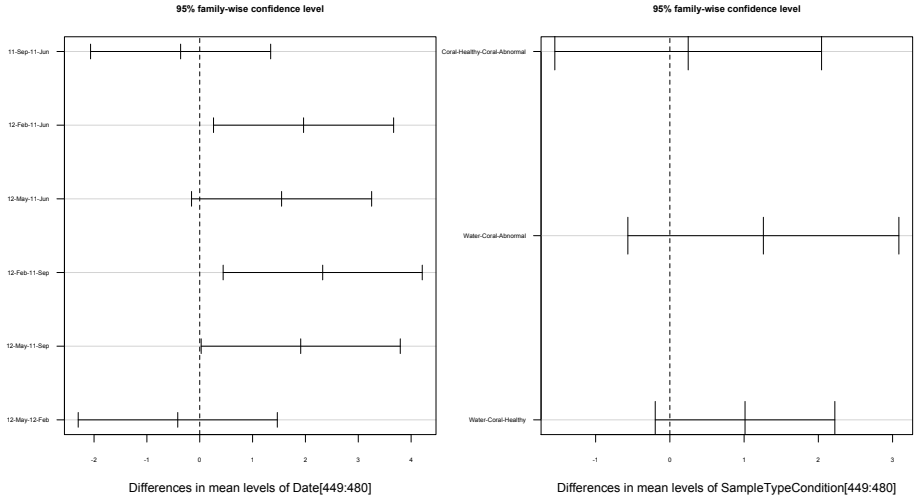
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[820:882]	4	444.1	111.03	21.960	7.16e-11 ***
SampleTypeCondition[820:882]	3	68.6	22.88	4.526	0.0066 **
Residuals	55	278.1	5.06		



Pseudomonadales Results

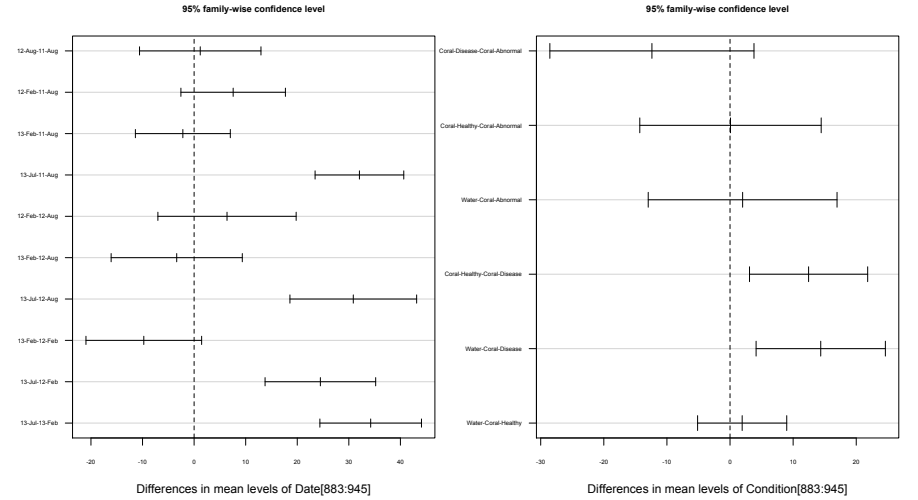
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[449:480]	3	29.53	9.842	5.963	0.0031 **
SampleTypeCondition[449:480]	2	9.91	4.956	3.003	0.0671 .
Residuals	26	42.91	1.650		



Looe Key Reef

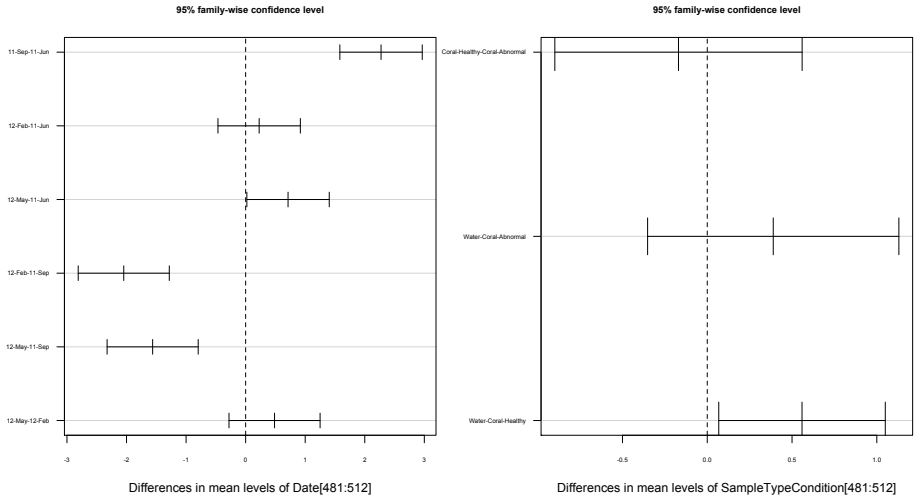
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[883:945]	4	11537	2884.1	35.471	1.22e-14 ***
SampleTypeCondition[883:945]	3	1759	586.2	7.209	0.000364 ***
Residuals	55	4472	81.3		



Puniceicoccales Results

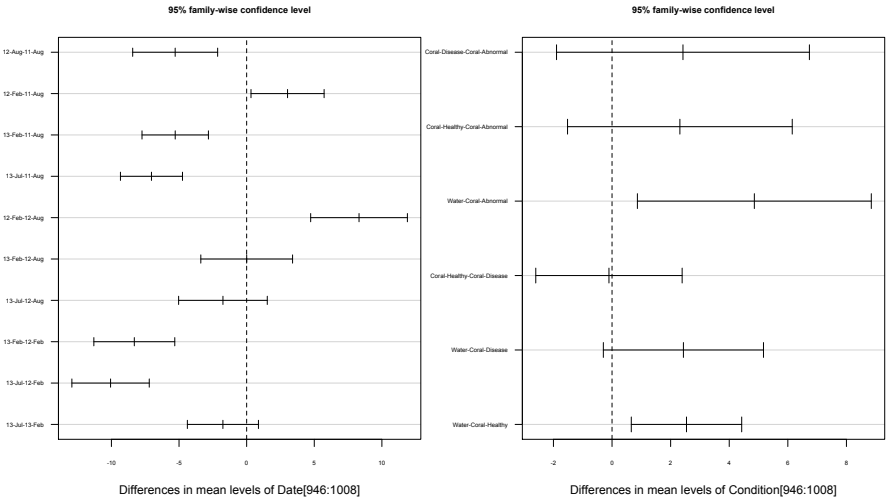
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[449:480]	3	29.53	9.842	5.963	0.0031 **
SampleTypeCondition[449:480]	2	9.91	4.956	3.003	0.0671 .
Residuals	26	42.91	1.650		



Looe Key Reef

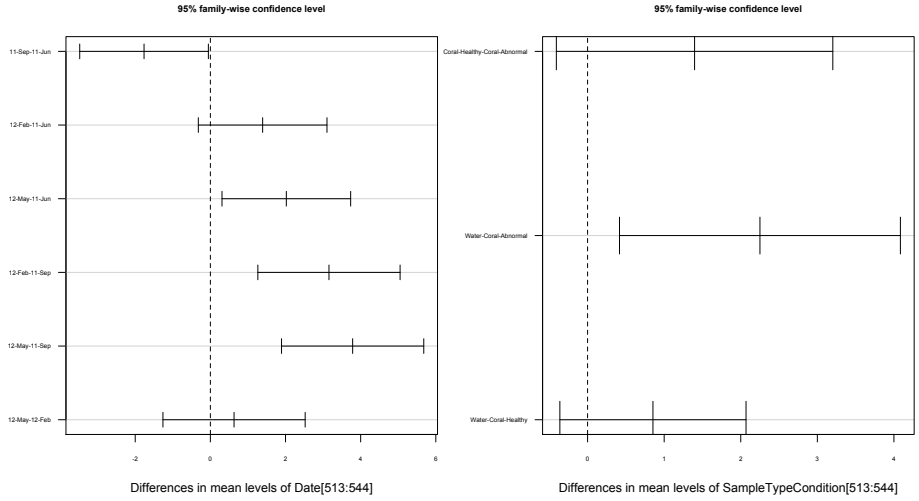
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[946:1008]	4	850.4	212.59	36.731	6.16e-15 ***
SampleTypeCondition[946:1008]	3	107.4	35.79	6.184	0.00107 **
Residuals	55	318.3	5.79		



Rhizobiales Results

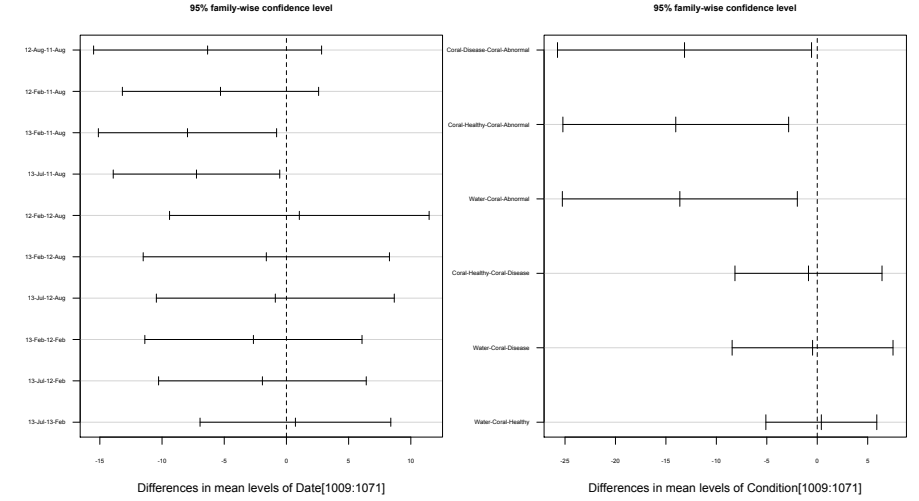
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[513:544]	3	59.81	19.937	11.97	4.14e-05 ***
SampleTypeCondition[513:544]	2	21.03	10.513	6.31	0.00584 **
Residuals	26	43.32	1.666		



Looe Key Reef

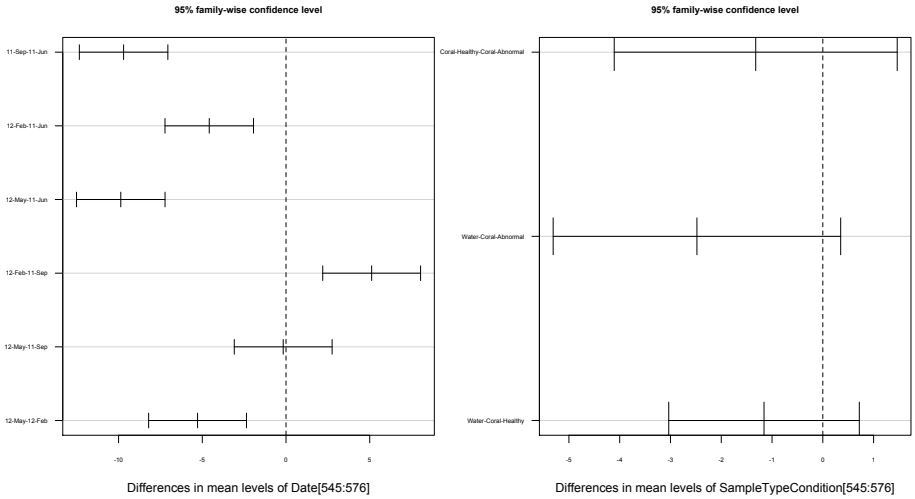
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1009:1071]	4	705.8	176.44	3.581	0.0115 *
SampleTypeCondition[1009:1071]	3	608.6	202.87	4.118	0.0105 *
Residuals	55	2709.8	49.27		



Rhodobacterales Results

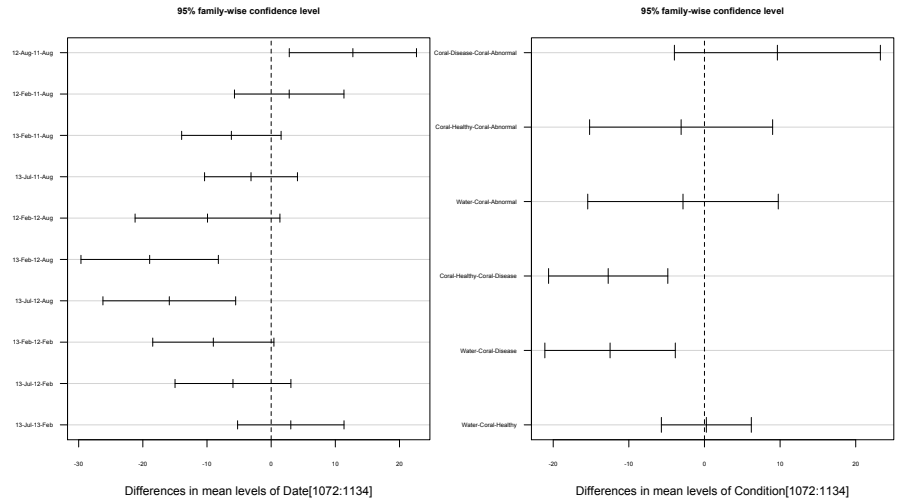
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[545:576]	3	594.2	198.05	49.882	6.37e-11 ***
SampleTypeCondition[545:576]	2	26.7	13.33	3.358	0.0504 .
Residuals	26	103.2	3.97		



Looe Key Reef

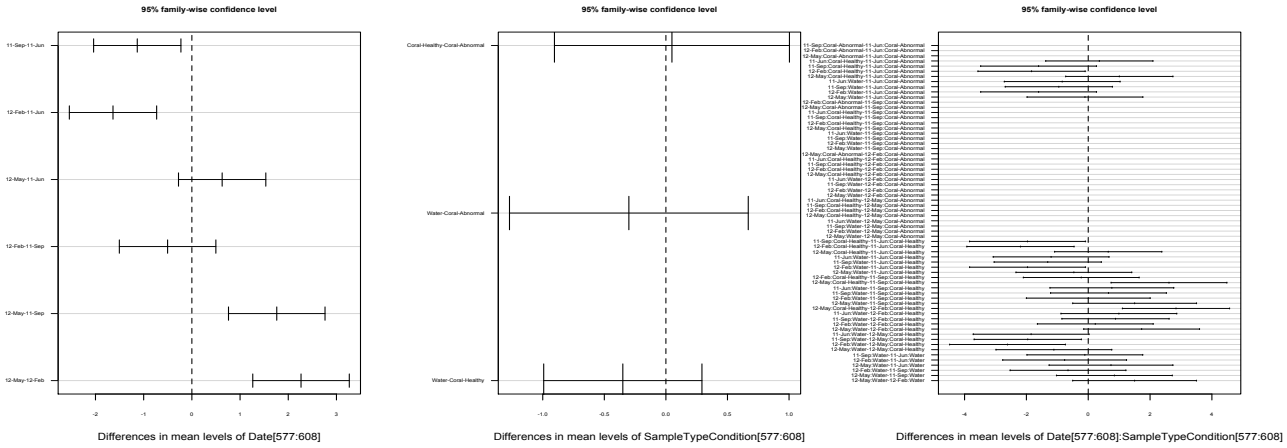
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1072:1134]	4	1647	411.9	7.145	0.000105 ***
SampleTypeCondition[1072:1134]	3	1580	526.8	9.139	5.29e-05 ***
Residuals	55	3170	57.6		



Rhodospirillales Results

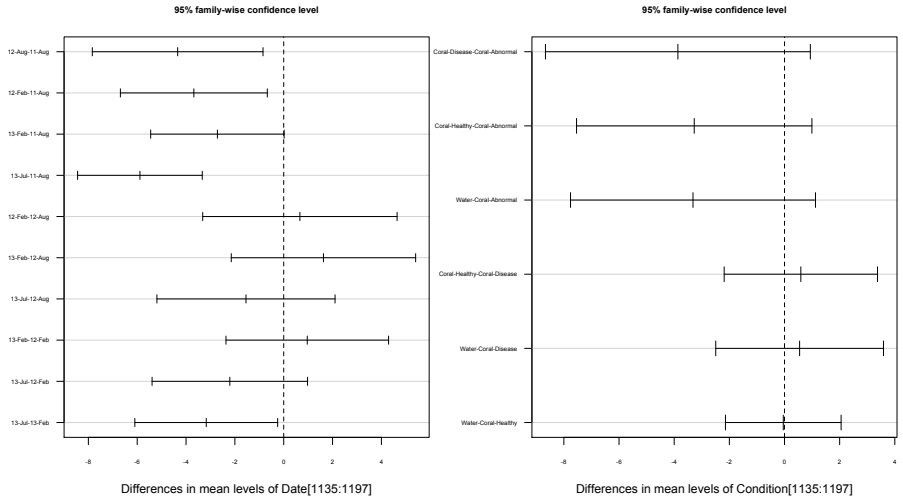
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[577:608]	3	23.479	7.826	17.077	4.7e-06 ***
SampleTypeCondition[577:608]	2	0.937	0.469	1.022	0.3756
Date[577:608]:SampleTypeCondition[577:608]	3	4.539	1.513	3.302	0.0383 *
Residuals	23	10.541	0.458		



Looe Key Reef

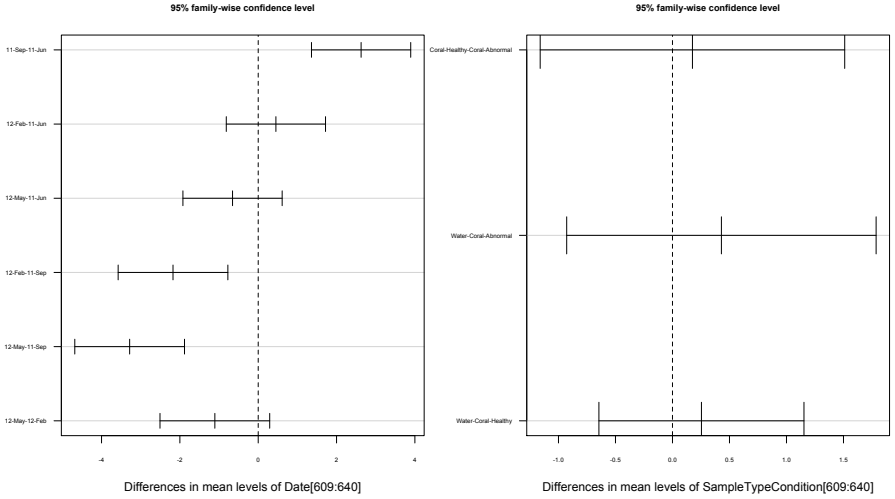
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1135:1197]	4	328.8	82.21	11.469	7.7e-07 ***
SampleTypeCondition[1135:1197]	3	41.7	13.91	1.941	0.134
Residuals	55	394.2	7.17		



Sphingomonadales Results

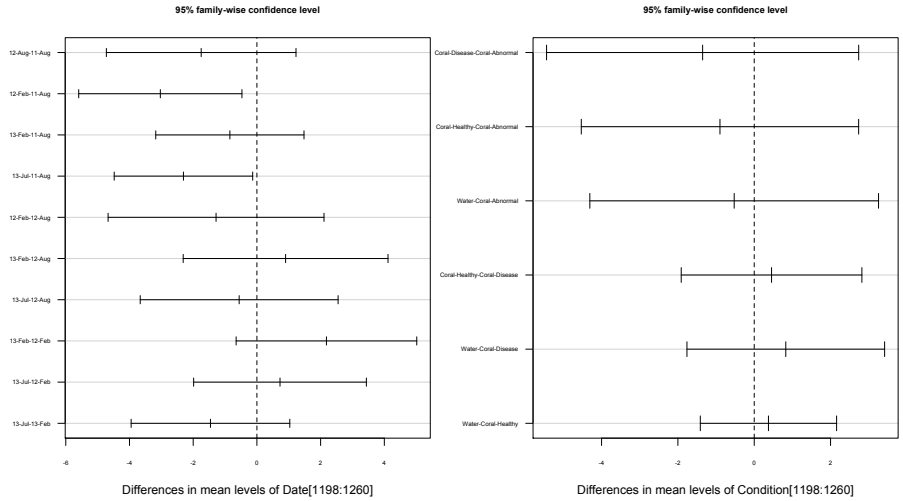
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[609:640]	3	43.75	14.583	15.986	4.3e-06 ***
SampleTypeCondition[609:640]	2	0.89	0.444	0.487	0.62
Residuals	26	23.72	0.912		



Looe Key Reef

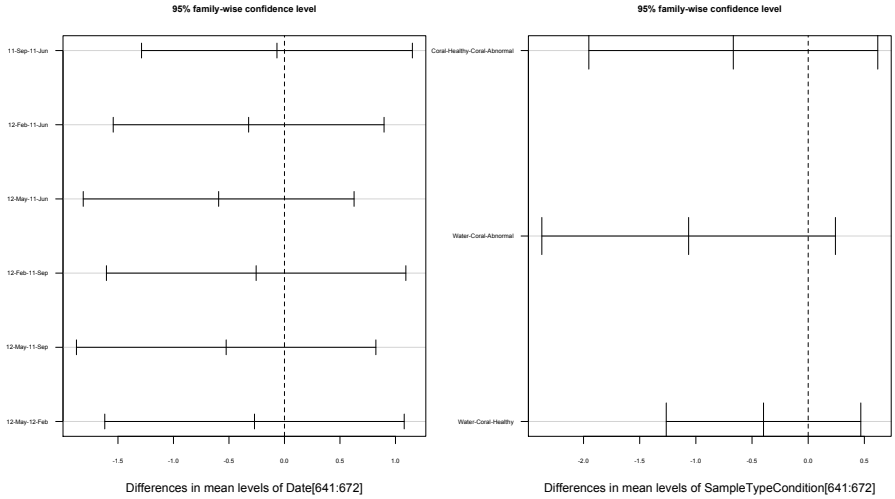
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1198:1260]	4	81.32	20.330	3.911	0.00726 **
SampleTypeCondition[1198:1260]	3	7.90	2.634	0.507	0.67922
Residuals	55	285.89	5.198		



Thiotrichales Results

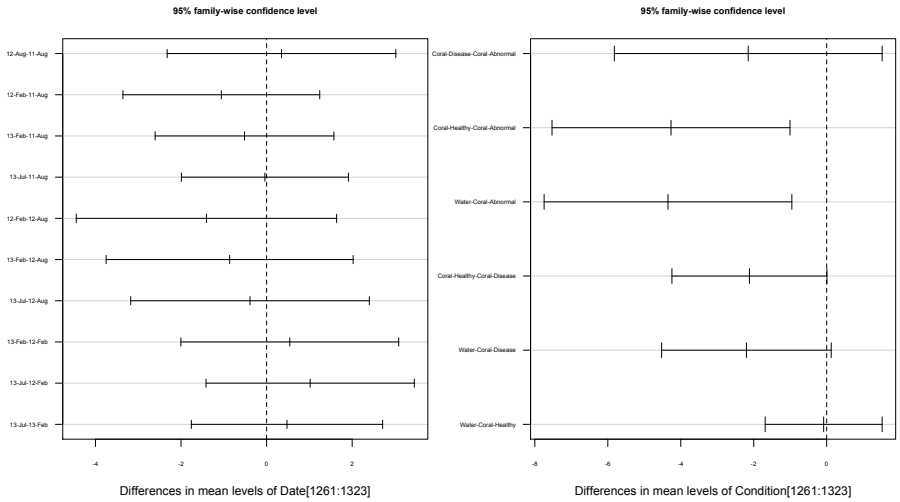
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[641:672]	3	1.742	0.5806	0.686	0.5685
SampleTypeCondition[641:672]	2	4.695	2.3473	2.775	0.0809 .
Residuals	26	21.996	0.8460		



Looe Key Reef

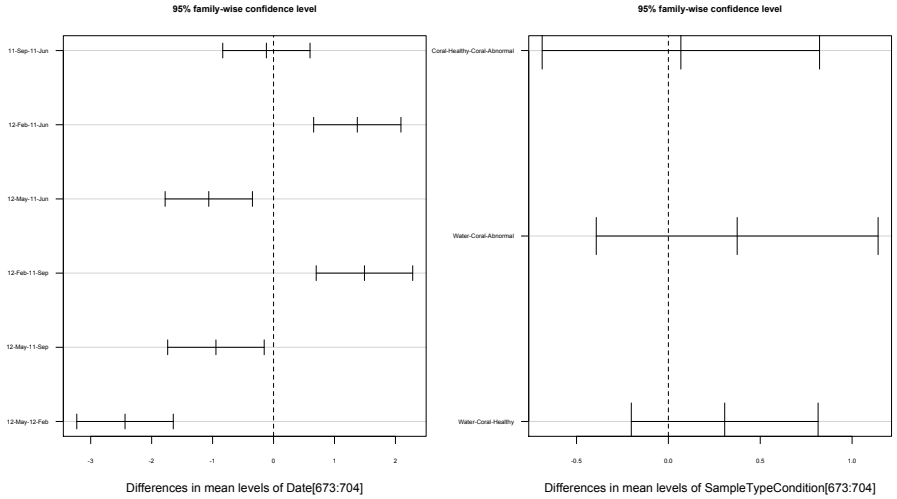
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1261:1323]	4	10.81	2.702	0.645	0.632724
SampleTypeCondition[1261:1323]	3	87.84	29.280	6.990	0.000456 ***
Residuals	55	230.37	4.189		



Verrucomicrobiales Results

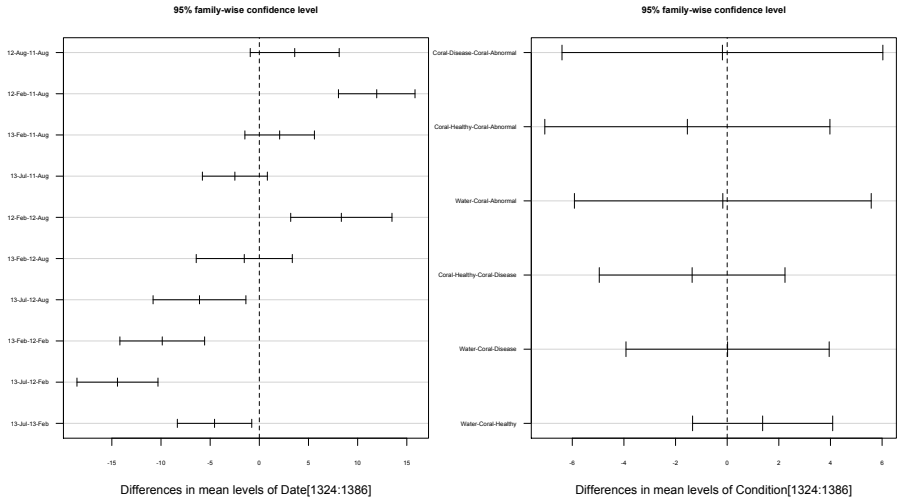
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[673:704]	3	21.162	7.054	24.199	1.08e-07 ***
SampleTypeCondition[673:704]	2	0.894	0.447	1.533	0.235
Residuals	26	7.579	0.292		



Looe Key Reef

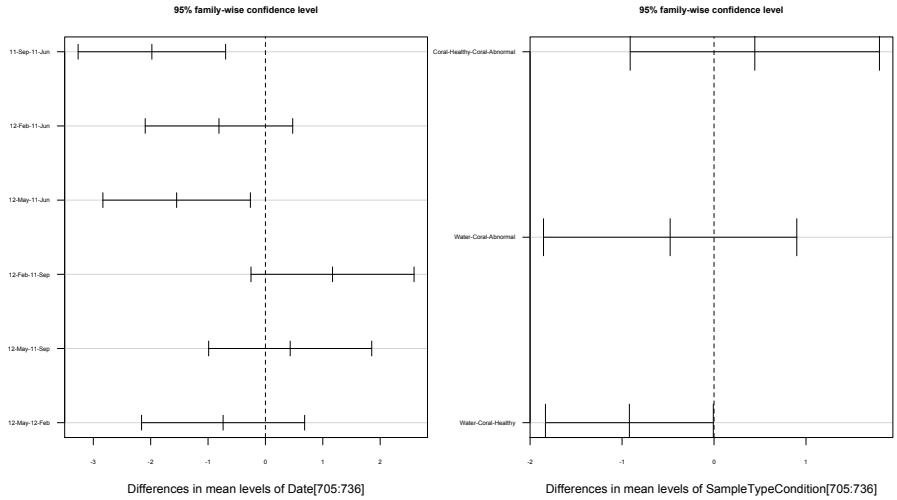
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1324:1386]	4	1289.2	322.3	26.855	2.24e-12 ***
SampleTypeCondition[1324:1386]	3	33.9	11.3	0.941	0.427
Residuals	55	660.1	12.0		



Vibrionales Results

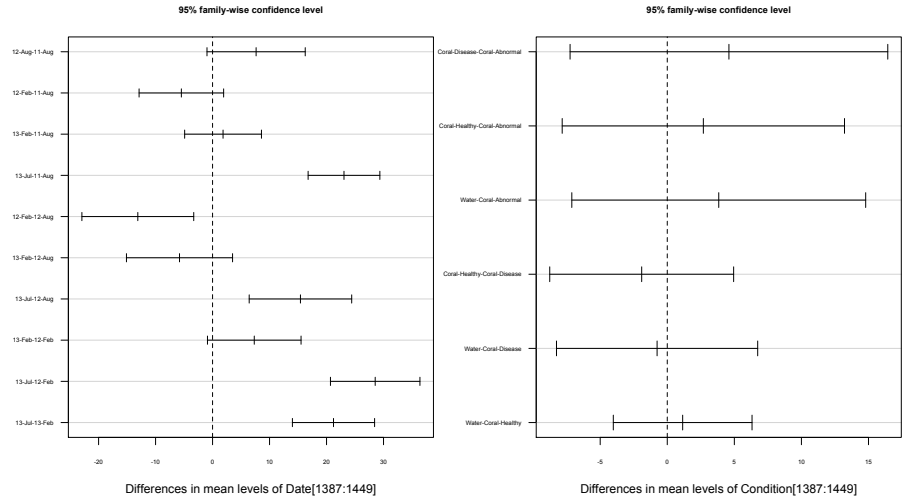
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[705:736]	3	19.98	6.660	7.091	0.00123 **
SampleTypeCondition[705:736]	2	5.99	2.995	3.189	0.05773 .
Residuals	26	24.42	0.939		



Looe Key Reef

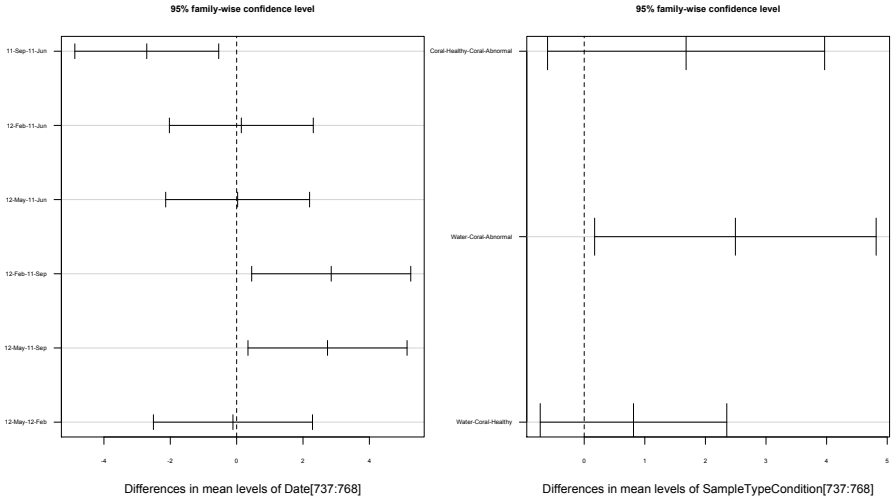
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1387:1449]	4	6529	1632.3	37.449	4.2e-15 ***
SampleTypeCondition[1387:1449]	3	78	26.0	0.597	0.62
Residuals	55	2397	43.6		



Xanthomonadales Results

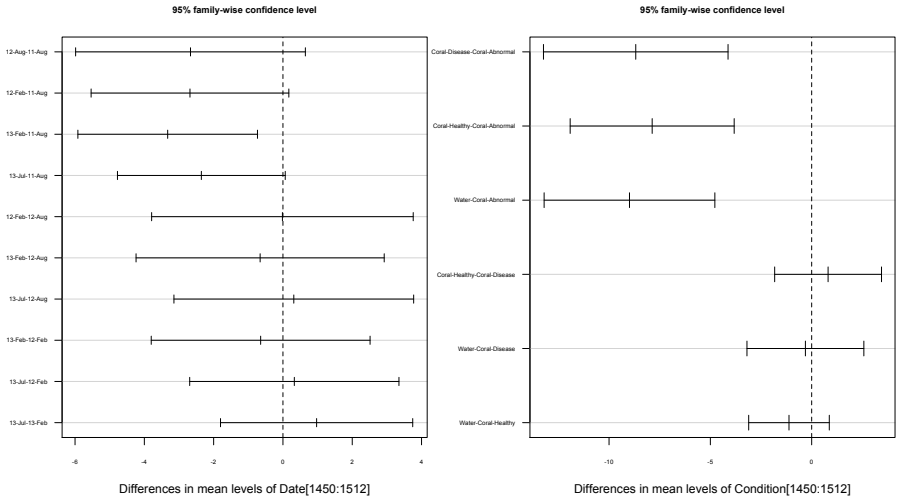
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[737:768]	3	41.65	13.883	5.195	0.00604 **
SampleTypeCondition[737:768]	2	25.51	12.757	4.774	0.01714 *
Residuals	26	69.48	2.672		



Looe Key Reef

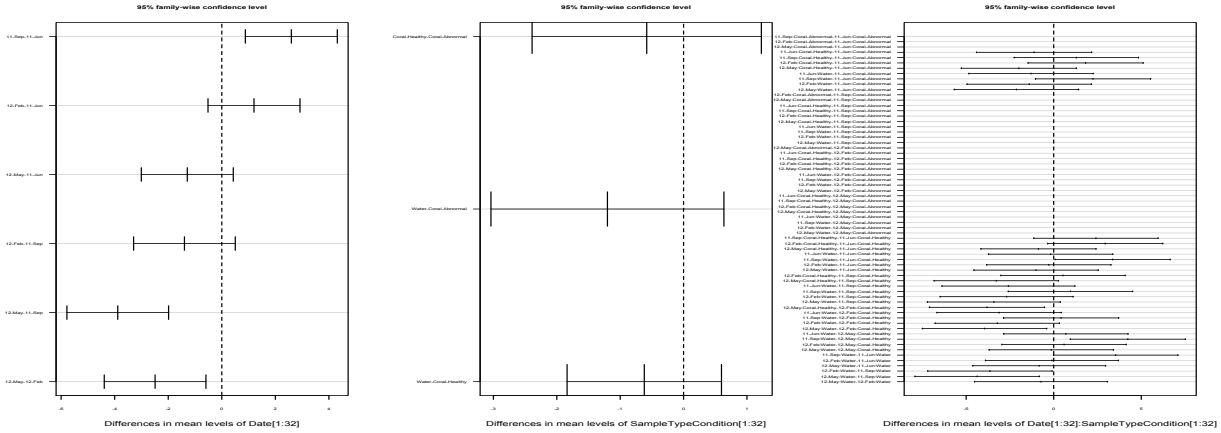
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1450:1512]	4	112.1	28.02	4.346	0.00399 **
SampleTypeCondition[1450:1512]	3	238.7	79.56	12.340	2.78e-06 ***
Residuals	55	354.6	6.45		



Alteromonadales Results

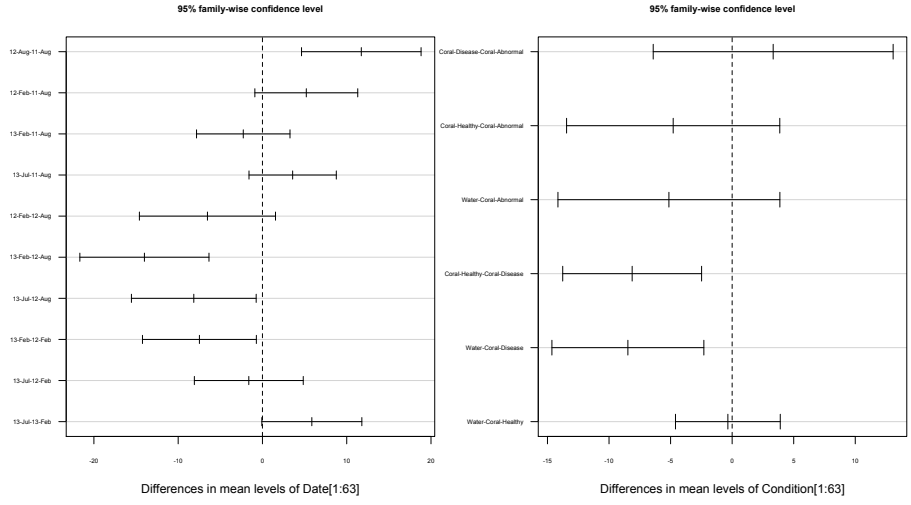
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1:32]	3	59.28	19.761	11.999	6.24e-05 ***
SampleTypeCondition[1:32]	2	6.49	3.246	1.971	0.1621
Date[1:32]:SampleTypeCondition[1:32]	3	16.58	5.528	3.357	0.0363 *
Residuals	23	37.88	1.647		



Looe Key Reef

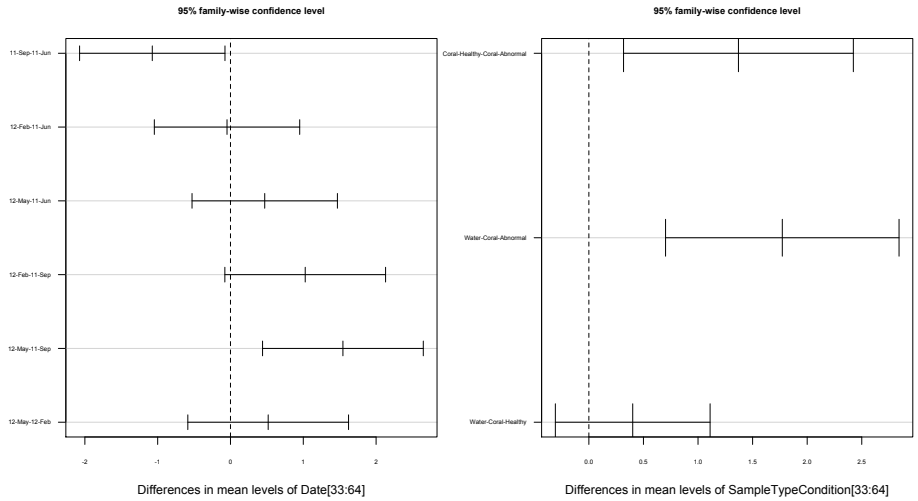
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[1:63]	4	996.4	249.11	8.441	2.2e-05 ***
SampleTypeCondition[1:63]	3	687.1	229.04	7.761	0.000207 ***
Residuals	55	1623.2	29.51		



Burkholderiales Results

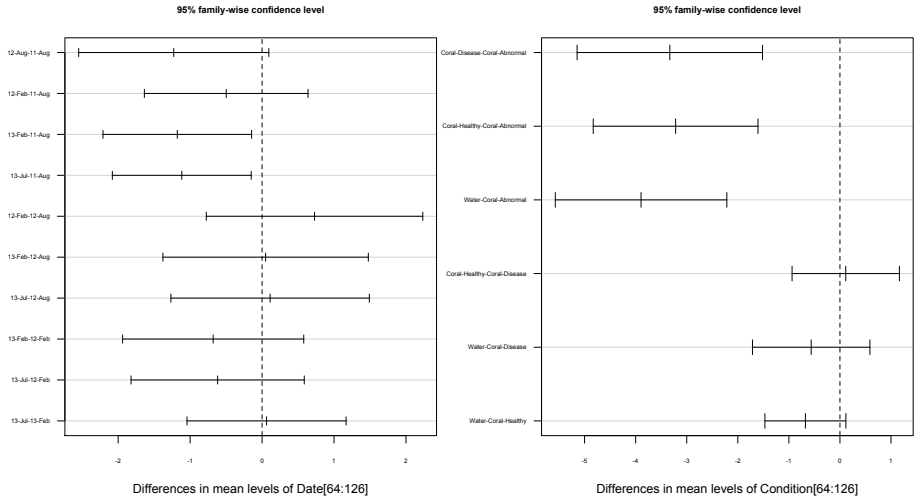
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[33:64]	3	8.99	2.997	5.289	0.005558 **
SampleTypeCondition[33:64]	2	12.97	6.485	11.445	0.000272 ***
Residuals	26	14.73	0.567		



Looe Key Reef

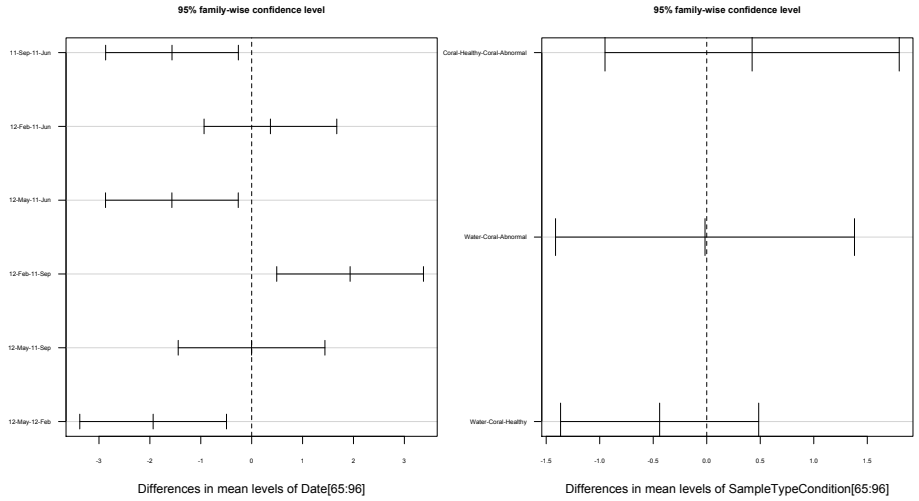
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[64:126]	4	17.63	4.409	4.303	0.00423 **
SampleTypeCondition[64:126]	3	42.00	14.000	13.666	8.93e-07 ***
Residuals	55	56.34	1.024		



Campylobacterales Results

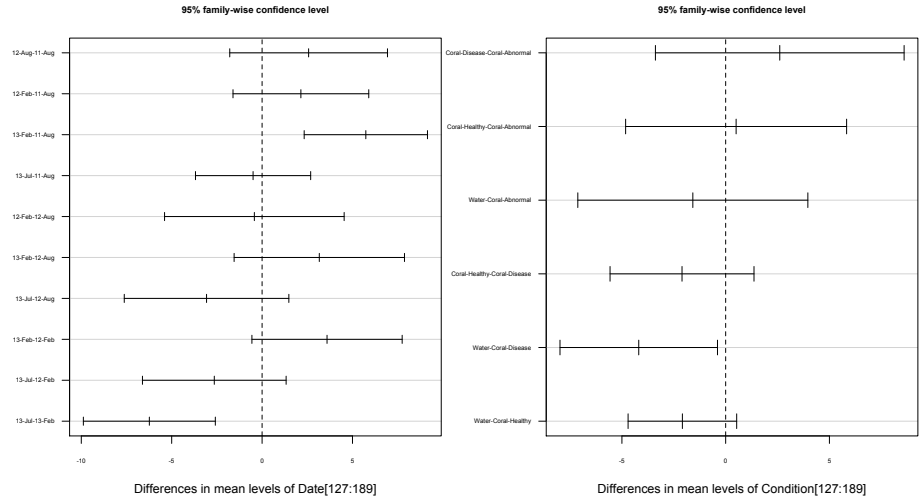
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[65:96]	3	23.603	7.868	8.143	0.000549 ***
SampleTypeCondition[65:96]	2	1.578	0.789	0.816	0.453002
Residuals	26	25.120	0.966		



Looe Key Reef

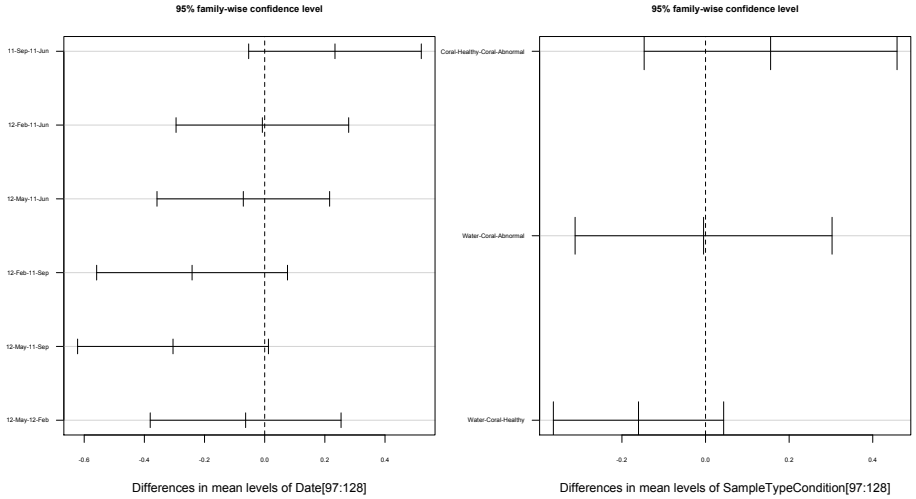
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[127:189]	4	333.3	83.32	7.464	7.08e-05 ***
SampleTypeCondition[127:189]	3	129.0	43.01	3.853	0.0142 *
Residuals	55	613.9	11.16		



Cyanobacteria_Family_I Results

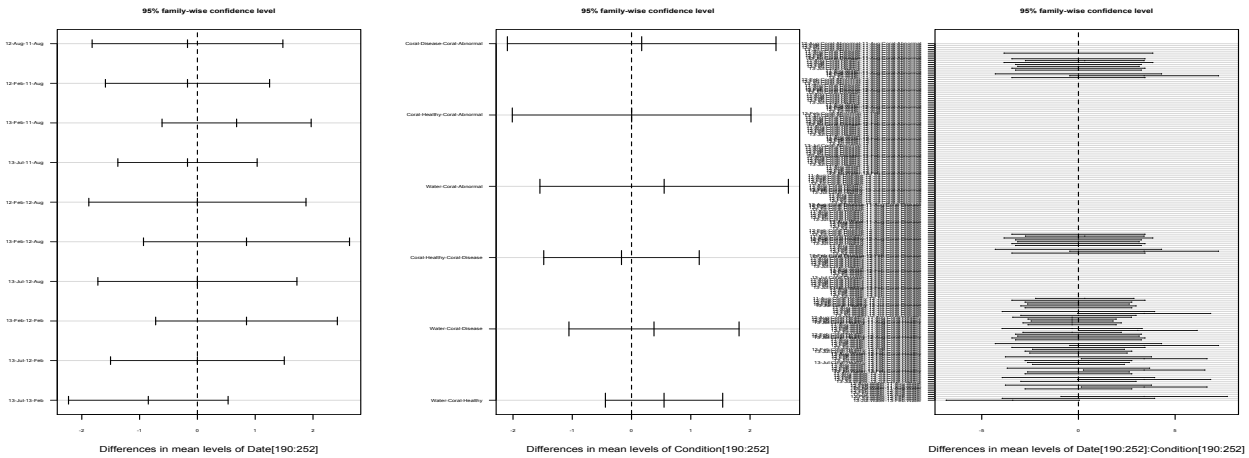
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[97:128]	3	0.3813	0.1271	2.716	0.0653 .
SampleTypeCondition[97:128]	2	0.2103	0.1051	2.247	0.1259
Residuals	26	1.2167	0.0468		



Looe Key Reef

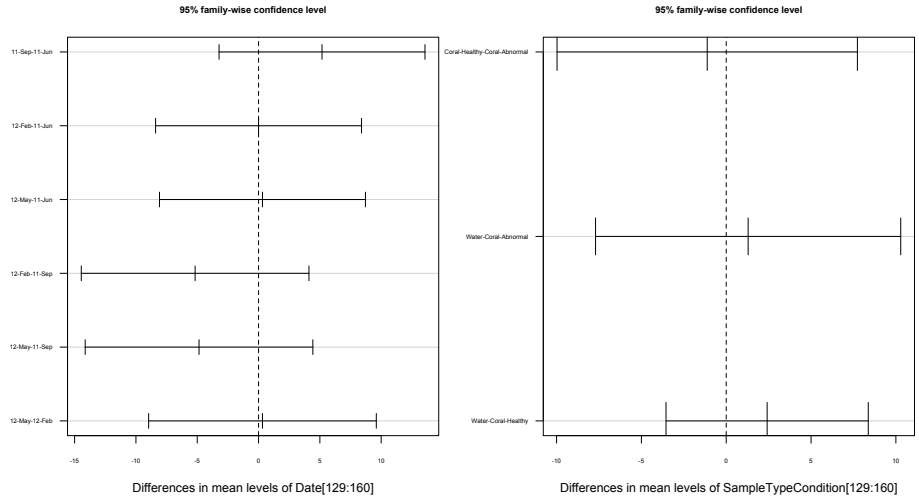
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[190:252]	4	6.26	1.564	0.984	0.4244
SampleTypeCondition[190:252]	3	3.70	1.234	0.776	0.5126
Date[190:252]:SampleTypeCondition[190:252]	4	22.84	5.709	3.593	0.0118 *
Residuals	51	81.03	1.589		



Cyanobacteria_Family_XII Results

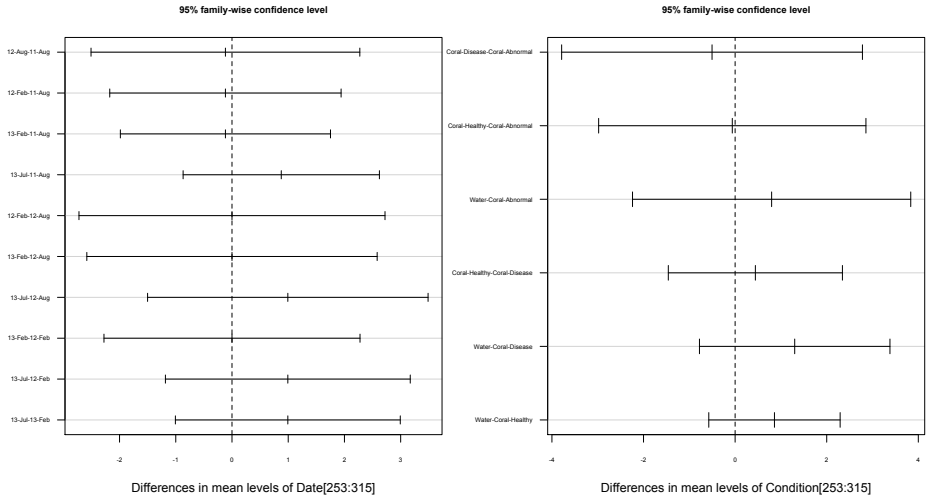
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[129:160]	3	141.9	47.30	1.180	0.336
SampleTypeCondition[129:160]	2	41.2	20.58	0.514	0.604
Residuals	26	1042.0	40.08		



Looe Key Reef

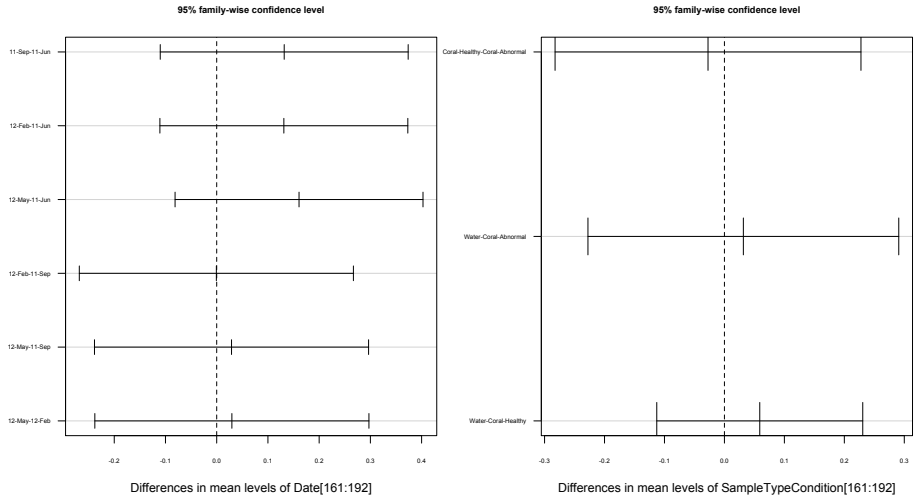
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[253:315]	4	10.30	2.576	0.768	0.550
SampleTypeCondition[253:315]	3	13.86	4.620	1.378	0.259
Residuals	55	184.37	3.352		



Hydrogenophilales Results

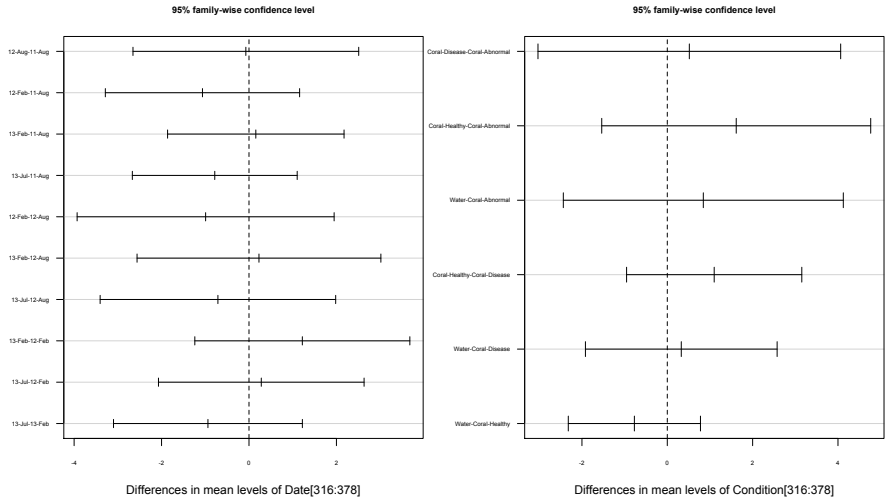
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[161:192]	3	0.1479	0.04931	1.481	0.243
SampleTypeCondition[161:192]	2	0.0246	0.01229	0.369	0.695
Residuals	26	0.8654	0.03329		



Looe Key Reef

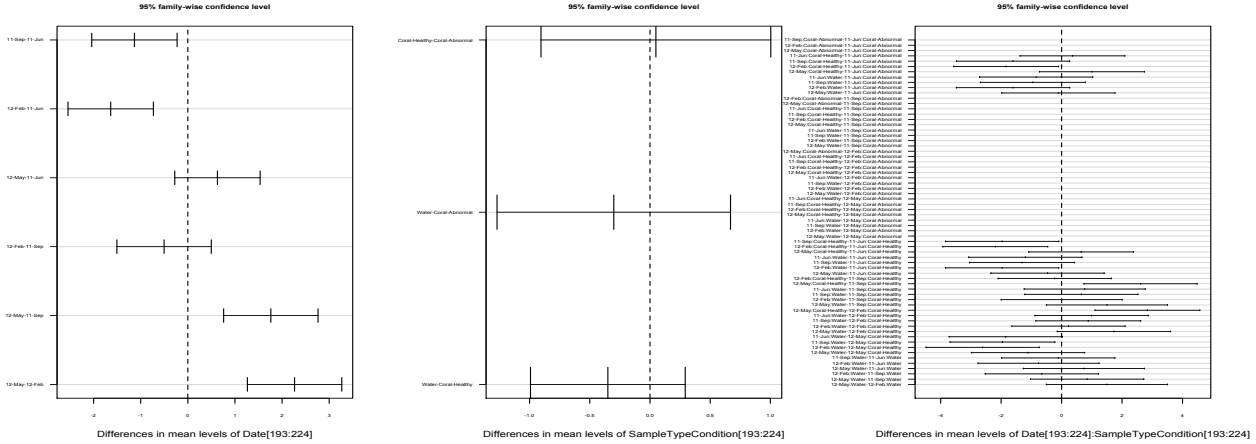
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[316:378]	4	13.44	3.359	0.859	0.494
SampleTypeCondition[316:378]	3	18.70	6.232	1.594	0.201
Residuals	55	215.06	3.910		



Rickettsiales Results

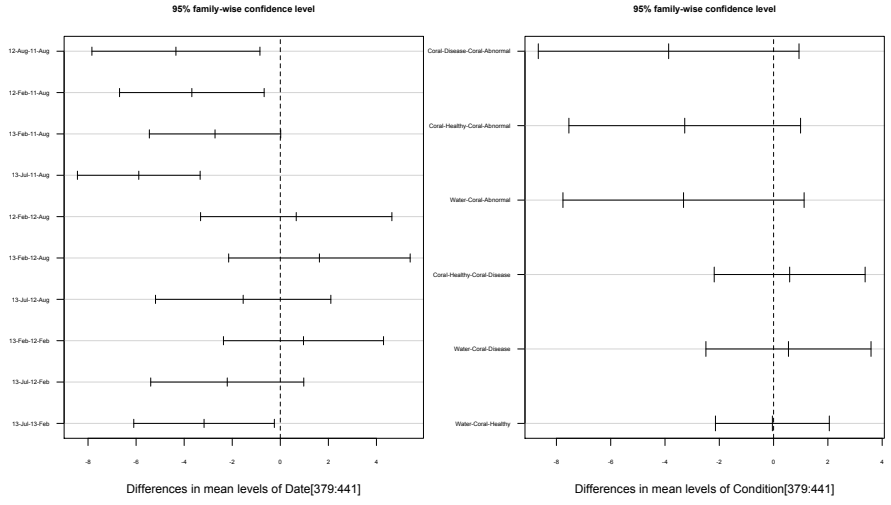
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[193:224]	3	23.479	7.826	17.077	4.7e-06 ***
SampleTypeCondition[193:224]	2	0.937	0.469	1.022	0.3756
Date[193:224]:SampleTypeCondition[193:224]	3	4.539	1.513	3.302	0.0383 *
Residuals	23	10.541	0.458		



Looe Key Reef

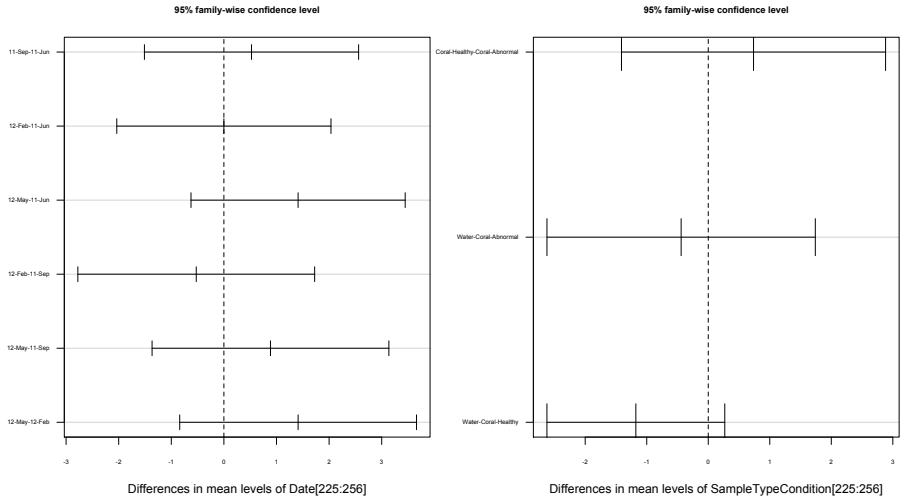
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[379:441]	4	328.8	82.21	11.469	7.7e-07 ***
SampleTypeCondition[379:441]	3	41.7	13.91	1.941	0.134
Residuals	55	394.2	7.17		



Rhodospirillales Results

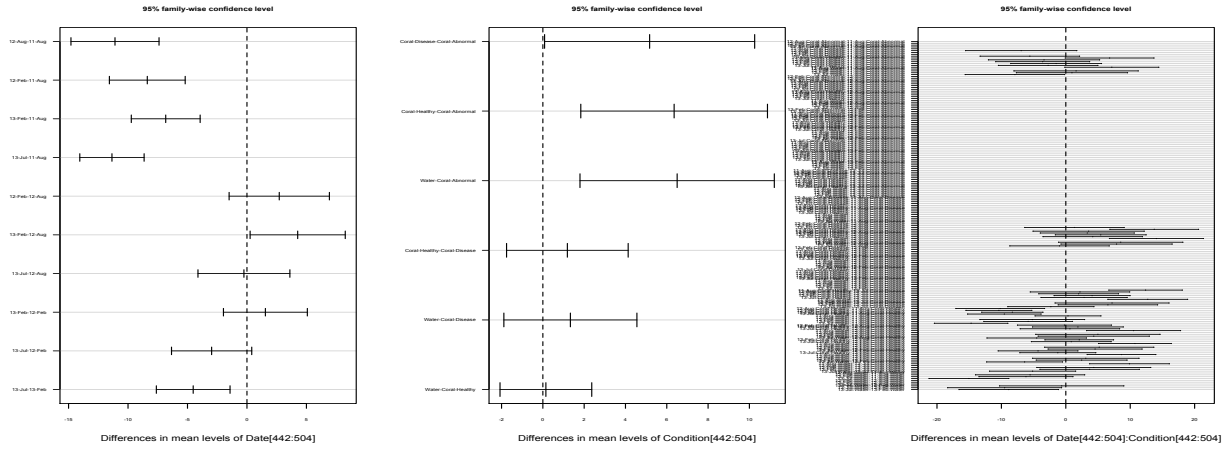
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[225:256]	3	10.12	3.375	1.432	0.256
SampleTypeCondition[225:256]	2	9.91	4.956	2.103	0.142
Residuals	26	61.26	2.356		



Looe Key Reef

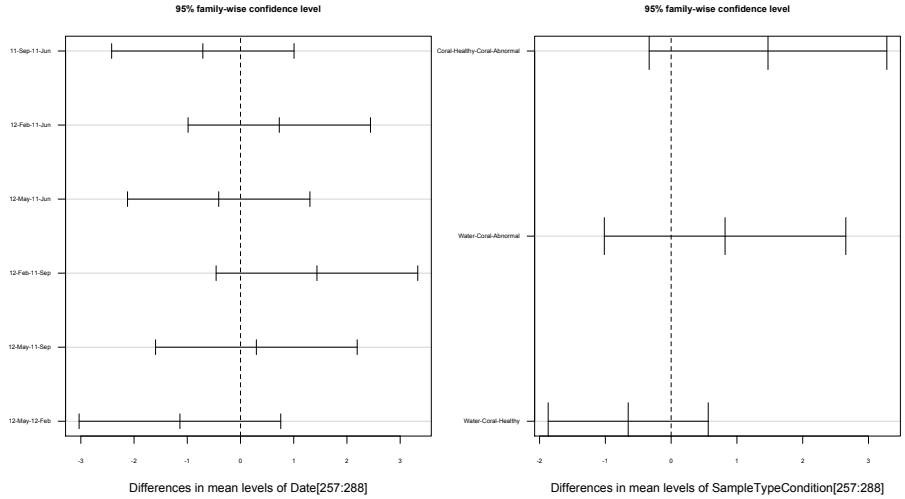
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[442:504]	4	1396.5	349.1	43.630	7.14e-16 ***
SampleTypeCondition[442:504]	3	131.5	43.8	5.478	0.00243 **
Date[442:504]:SampleTypeCondition[442:504]	4	133.6	33.4	4.174	0.00533 **
Residuals	51	408.1	8.0		



Sphingobacteriales Results

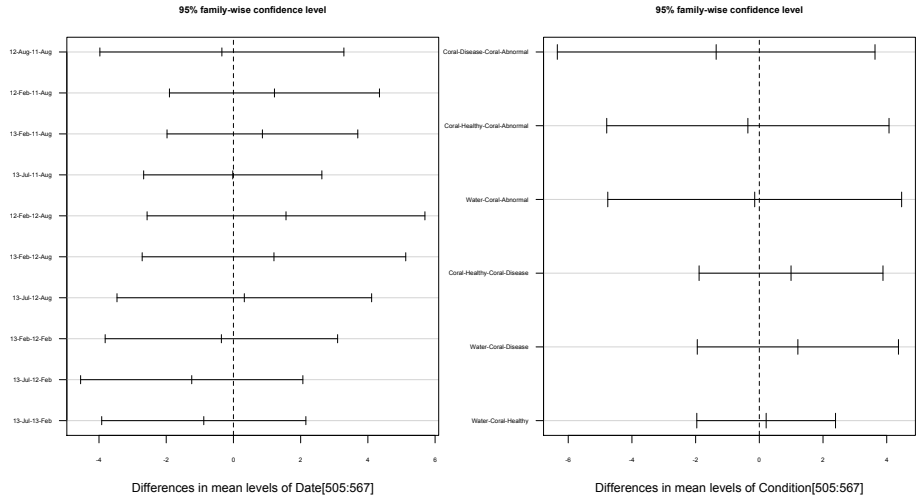
Molasses Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[257:288]	3	8.16	2.721	1.631	0.2065
SampleTypeCondition[257:288]	2	9.40	4.702	2.817	0.0781 .
Residuals	26	43.39	1.669		



Looe Key Reef

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Date[505:567]	4	17.3	4.332	0.560	0.692
SampleTypeCondition[505:567]	3	12.9	4.309	0.557	0.645
Residuals	55	425.2	7.732		



APPENDIX 2

LuxS qPCR amplicon sequence NCBI BLAST search results. Listed is the top tier of the results for each amplicon based on the E-value distribution. SC40 amplicon sequence is excluded because of poor BLAST search results. Amplicon names correspond to site where the environmental isolate was cultured. KLWWI# is the Key Largo Wastewater Influent, KWWWI# is the Key West Wastewater influent, SC# and TL# are from residential canals in Key Largo (coordinates 25° 9.933'N, 80° 23.102'W and 25° 8.047'N, 80° 24.462'W, respectively), BH# is from the Blue Hole (a freshwater lens, coordinates 24° 42.368'N, 81° 22.837'W) on Big Pine Key. Sequences 12 and 13 are confirmed amplicons of the LuxS qPCR assay from sewage influent samples collected at treatment plants in Key Largo and Marathon, respectively.

Amplicon Name	NCBI Sequence Match Description	Max Score	Total Score	Query Coverage	E-value	Max Identity	Accession Number
TL47	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene,complete cds	215	215	97%	7.00E-53	96%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	215	215	97%	7.00E-53	96%	AJ628150.1
	Serratia marcescens WW4, complete genome	209	215	97%	3.00E-51	95%	CP003959.1

TL35	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	228	228	99%	9.00E-57	98%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	228	228	99%	9.00E-57	98%	AJ628150.1
	Serratia marcescens WW4, complete genome	209	215	97%	3.00E-51	98%	CP003959.1
SC59	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	233	233	94%	2.00E-58	99%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	233	233	94%	2.00E-58	99%	AJ628150.1
	Serratia marcescens WW4, complete genome	228	228	94%	1.00E-56	98%	CP003959.1
SC42	*Serratia liquefaciens* ATCC 27592, complete genome	176	176	92%	3.00E-41	96%	CP006252.1
	Serratia marcescens strain H3010 autoinducer-2 synthase (*lux*S) gene,	121	121	88%	1.00E-24	88%	EF164926.1

	complete cds						
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	121	121	88%	1.00E-24	88%	AJ628150.1
	Serratia marcescens WW4, complete genome	115	115	88%	6.00E-23	87%	CP003959.1
BH28	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	169	169	92%	6.00E-39	91%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	169	169	92%	6.00E-39	91%	AJ628150.1
KWWWI24	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	206	206	96%	5.00E-50	95%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	206	206	96%	5.00E-50	95%	AJ628150.1
	Serratia marcescens WW4, complete genome	200	200	96%	2.00E-48	94%	CP003959.1

KWWWI22	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	206	206	14%	4.00E-49	95%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	206	206	14%	4.00E-49	95%	AJ628150.1
	Serratia marcescens WW4, complete genome	200	200	14%	2.00E-47	94%	CP003959.1
KLWWI7	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	233	233	99%	6.00E-61	99%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	233	233	99%	6.00E-61	99%	AJ628150.1
	Serratia marcescens WW4, complete genome	228	228	99%	1.00E-59	98%	CP003959.1
KLWWI6	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	231	231	96%	7.00E-58	99%	EF164926.1
	Serratia marcescens LuxS	231	231	96%	7.00E-58	99%	AJ628150.1

	gene for autoinducer-2 synthase, strain ATCC 274						
	Serratia marcescens WW4, complete genome	226	226	96%	3.00E-56	98%	CP003959.1
Sewage-KL	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	213	213	69%	6.00E-55	99%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	213	213	69%	6.00E-55	99%	AJ628150.1
	Serratia marcescens WW4, complete genome	207	207	69%	3.00E-53	98%	CP003959.1
Sewage-MAR	*Serratia marcescens* strain H3010 autoinducer-2 synthase (*lux*S) gene, complete cds	207	207	51%	3.00E-53	98%	EF164926.1
	Serratia marcescens LuxS gene for autoinducer-2 synthase, strain ATCC 274	207	207	51%	3.00E-53	98%	AJ628150.1
	Serratia marcescens WW4, complete genome	202	202	51%	1.00E-51	97%	CP003959.1