TEMPERATURE EFFECTS ON MICROBIAL COMMUNITIES

OF SEA FAN CORAL AS AN INDICATOR

OF DISEASE SUSCEPTIBILITY

By

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ABSTRACT

Coral reefs are in decline due to increases in coral disease. We suspect that one of the main sources of coral immunity is the strength the residential, native microbiota. Based on vertebrate models I suspect that disturbing the normal microbial community will lead to disease. The goal of this study is to use the coral-microbiota-disease hypothesis as a tool to establish how an increase in sea surface temperature leads to disease. I tested this by first exposing cuttings of sea fan coral (*Gorgonia ventalina*) to average summer sea surface temperature as well as to higher than average temperatures. Then, I analyzed the microbial community structure using denaturing gradient gel electrophoresis (DGGE). Finally, I identified individual bands through sequencing. I found that there were not significant differences in the microbial communities due to changes in temperature. However, the microbial community structures varied greatly when comparing coral on reefs and those in aquaria.

ACKNOWLEDGMENTS

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Of course, I also have to thank my family. I may actually finish before Alden!! Mom and Dad, thank you for talking my off the ledge those many times and for asking "So…when are you going to be done?!"

Thank you.

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INTRODUCTION

Coral reefs are valuable, fragile marine ecosystems, recently threatened by human activity. They have monetary value as biodiversity hotspots and for the services the unique ecosystem provides. In an area less than one-tenth of the total marine environment, coral reefs host 25% of all marine species (Burke et al. 2011). As a unique ecosystem they generate approximately $30 billion per year, for the food they produce, the money they generate from tourism, for their value as a disturbance regulator (wave breakers), and for their role in waste management (Cesar et al. 2003). Despite the monetary value of reefs more than 60% of the global reefs are immediately and directly affected by local sources like overfishing, coastal development, watershed-based pollution, or marine-based pollution and damage (Burke et al. 2011). When thermal stress (such as the rise in the sea surface temperature) is taken into account, about 75% of the world's coral reefs are considered threatened from local pressures (overfishing and pollution) and global pressures (climate change) (Burke et al. 2011).

Climate change has resulted in an increase in sea surface temperature. There is a correlation between the higher sea surface temperatures and an increase in coral diseases and bleaching. However, the mechanism of coral disease is unknown. The goal of this study is to test whether an increase in sea surface temperature causes a change in microbial diversity, an indicator of coral susceptibility to disease.

Therefore, it is imperative to understand these stresses and their causes in order to develop ways to mitigate threats to coral reefs.

What are Coral and Coral Reefs?

A single coral structure is often made up of many individual, genetically identical, coral polyps. The general anatomy of a coral consists of the polyp, calcium carbonate exoskeleton, endosymbiotic algae called zooxanthellae, and various associated viruses, bacteria and fungi.

Coral are in the phylum Cnidaria, and the class Anthozoa, which also includes sea anemones (Nybakken & Bertness 2004). The number of arms on the polyps indicates whether the coral is in the subclass Hexacorallia (multiples of six) or subclass Octocorallia (multiples of eight). Hexacoral include reef building coral that produce an external calcium carbonate skeleton. Octocoral include soft coral, which are flexible. Within Octocoral are sea fans, order Gorgonacae. Sea fans were used in this study and have a gorgonin, or proteinaceous, axis and calcium carbonate spicules (Nybakken & Bertness 2004).

Coral reproduce asexually and sexually. Most coral are colonial, meaning that the coral skeleton is made up of many individual polyps. These polyps are produced asexually through budding, producing genetically identical polyps (NOAA 2011). Therefore, the polyps producing any one coral structure are, theoretically, all genetically identical. Coral can also grow asexually through fragmentation, where a portion of a larger colony is broken off and is able to grow into a new colony (NOAA 2011).

Some coral, like gorgonians also reproduce sexually through either broadcast spawning or brooding (Brazeau & Lasker 1989, Fitzsimmons-Sosa et al. 2004). Most coral are broadcast spawners and produce both male and female gametes, which are released into the water column (NOAA 2011). The release of the gametes is precisely timed to environmental cues so that the sperm and eggs are released at the same time (NOAA 2011). Some coral reproduce through brooding, where only the male gametes are released into the water column (NOAA 2011). They then sink and if they come in contact with a female polyp they are taken in by the female polyp, where fertilization occurs (NOAA 2011). The resulting planula is then later released at a development stage where it is capable of settling onto a substrate (NOAA 2011). When the planula find a location that falls within acceptable parameters the coral grow and eventually reproduce.

Coral reefs grow under specific conditions. Most reefs occur in the tropics, between the tropics of Cancer and Capricorn. This is because the optimum reef development occurs in average sea surface temperatures between 23-25 °C, although some coral can tolerate sea surface temperatures up to 36-40 °C (Nybakken & Bertness 2004). Most coral grow in less than 25 m of water (Nybakken & Bertness 2004). This is due to light as a limiting factor, since the zooxanthellae (the endosymbiotic algae) require sunlight for photosynthesis. Other factors that limit the scope of where coral can grow are salinity (32-35 psu), turbidity, wave action, and exposure to air (Nybakken & Bertness 2004).

Light availability, in particular, is an important factor because coral polyps get their energy from two sources: what they ingest and what the zooxanthellae (*Symbiodinium* spp.) provide them. Zooxanthellae are photosynthetic endosymbiotic

dinoflagellates. The zooxanthellae live within the polyp, and the zooxanthellae provide the coral polyp with simple carbohydrates for energy that the zooxanthellae produce through photosynthesis (Figure 1) In healthy corals, the zooxanthellae provide up to 100% of the host's daily energy requirements (Borell & Bischof 2008).

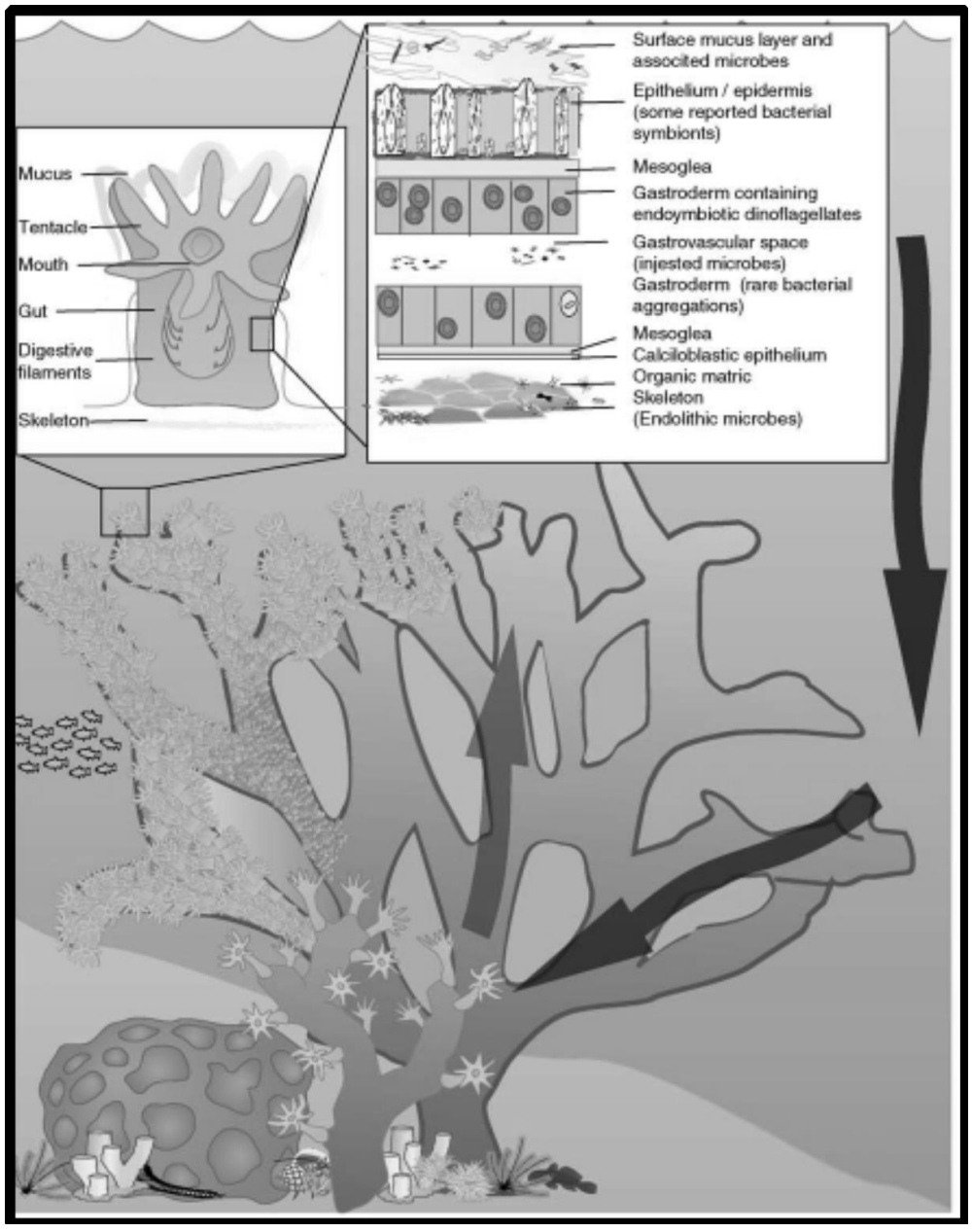

Figure 1. Diagram of Coral and Associated Microbiota (Ainsworth et al. 2007).

The polyp transports inorganic carbon (i.e. CO_2), nitrogen (i.e. NH_3, NO_3^- and N_2), and phosphates (i.e. PO_4^{-3}) to the endosymbiotic algae through the host's tissue (Yellowlees et al. 2008). Normally, the host would expel these metabolic byproducts. Instead they are used by the algae in photosynthesis. In return the zooxanthellae export to the polyp glycerol, glucose, amino acids, and lipids (Yellowlees et al. 2008). The zooxanthellae produce glycerol and glucose which can be up to 100% of the carbon requirements for the coral polyp (Lesser et al. 2004). Research suggests that the coral polyp is able to curb the growth of the zooxanthellae by limiting the amount of inorganic nitrogen available to the algae (Lesser et al. 2004, Yellowlees et al. 2008).

Causes of Coral Reef Decline

Globally, coral reefs are encountering threats from many sources. Some of the causes of coral reef decline are directly due to human activity, such as exploitation, harvesting, and over fishing. Human activity indirectly causes coral bleaching and coral disease. Research estimates that approximately 75% of the coral reefs could be categorized as threatened (Burke et al. 2011).

Direct human activity, such as exploitation and overfishing, cause localized, immediate threats. Exploitation of coral reefs, is not as prevalent as overfishing, but occurs when fishermen harvest coral from the reefs for decoration in aquaria and for use in jewelry (Nybakken & Bertness 2004). Over fishing causes a decline in coral reefs. Researchers estimate over fishing and destructive fishing threaten approximately 55% of the worlds reefs (Burke et al. 2011). The removal of herbivorous fish results in a reef

overrun with algae. The algae overgrowth prevents coral larvae recruitment (Gladfelter 1982).

Thermal stress is a pervasive, global threat. Within the last 30 years, the global surface temperature has increased about 0.2 °C per decade (Hansen et al. 2006, Fang et al. 2008). In addition, it is estimated that the current global temperature is within 1 °C of the maximum temperature of the past million years, with 1998 the warmest year on record (Hansen et al. 2006, Saunders & Lea 2008).

When sea surface temperature increases, the coral become stressed which can cause them to expel the zooxanthellae, also known as bleaching (Brown & Howard 1985, Hoegh-Guldberg 1999). Bleaching occurs when there is a disruption in the symbiosis, due to some stressor (i.e. high temperature), between the coral polyp and endosymbiotic zooxanthellae, resulting in the expulsion of the zooxanthellae (Brown 1997). The term "bleaching" is because the coral appear white after the expulsion of the zooxanthellae, which give the coral their color (Brown 1997, Nybakken & Bertness 2004). If the temperature increase continues for a long enough period of time and coral polyp does not recover its zooxanthellae then coral polyp will die because it is not able to supplement the amount of food the zooxanthellae provides (Brown 1997, Hoegh-Guldberg 1999). For example 1998 was one of the warmest years on record and coincided with the most severe bleaching event recorded (Hoegh-Guldberg 1999).

Diseases in the Ocean

Since the early 1970s researchers have recorded 29 mass mortalities (more than 10% of the population) of marine animals, from a wide array of sea life animals such as

porpoises, urchins, kelp and coral (Harvell et al. 1999). Harvell et al (1999) presented evidence that the frequency of reports of marine diseases has increased. Ward and Lafferty (2004) corrected for increased reporting and still found an increase in coral diseases.

Marine disease can have a significant ecological impact, even if the disease affects only one species. For example, in 1983 there was a disease that wiped out 97-100% of the long-spined black sea urchin, the key herbivore, throughout the Caribbean (Lessios et al. 1984). Three years later, without the main algal herbivore (Tuya et al. 2004), algae had overgrown the reef, with up to 96% cover (Hughes et al. 1987). Algae overgrowth smothers the reef and inhibits the growth of live, existing coral, and prevents the recruitment of coral larvae (Hughes et al. 1987). The algal overgrowth led to a near complete collapse of the reef ecosystem.

In another widespread case Gladfelter (1982) first described the white band disease of the coral *Acropora palmata* and *A. cervicornis*. In some areas the coral cover was down to about 5% from over 50% before white band disease became widespread (Aronson & Precht 2001). A suite of bacteria cause white band disease, but researchers isolated one species *Vibrio carchariae* (Aronson & Precht 2001, Pantos & Bythell 2006, Ritchie 2006, Rosenberg et al. 2007). The loss of reef building *Acropora* spp. resulted in a decrease of reef structural complexity, a decrease in fish community diversity (Gladfelter 1982), and an increase in algae and other herbivorous invertebrates (Aronson & Precht 2001). It is suspected that if the *Acropora* spp. do not recover, algae will continue to dominate Caribbean reefs, along with brooding corals, in particular *Agaricia* spp. and *Porites* spp. (Aronson & Precht 2001).

Smith et al (1996) first described the fungus *Aspergillus sydowii* as the pathogen that had caused mass mortalities in Caribbean sea fans. Sea fans infected with *A. sydowii* exhibit lesions, galling, and purpling of the tissue. Continued infection can lead to the death of the colony (Smith et al. 1998). Aspergillus is a common soil fungus but not a common marine microbe and it does not form spores in the marine environment (Smith et al. 1996, Kim & Harvell 2004, Kim et al. 2006). Therefore scientists consider the outbreak of aspergillosis an emergent disease (Kim & Harvell 2004).

Coral have defenses against various microbial diseases. The defenses can be physical, chemical, and even immunological. As physical defenses sea fans have sclerites and form tumors around algae (Alker et al. 2004). One of the signs of aspergillosis is a purpling of the tissue that is actually an increase in the amount of sclerites in the area. These usually have tissue necrosis (Alker et al. 2004, Kim & Smith 2005).
While primitive, coral do mount an immune response. Coral produce antifungal (Kim et al. 2000) and antibacterial (Ritchie 2006) extracts. *Gorgonia* spp. have granular acidophilic amoebocytes that are involved in wound repair (Mydlarz et al. 2008). Researchers have found that these cells respond to pathogens, such as *A. sydowii*, and react to temperature stress to the coral (Mydlarz et al. 2008).

Another factor contributing to the increase in coral disease is an increase in novel pathogens and an increase in the virulence of known pathogens. As seen in the *Gorgonia-Aspergillus* system, *A. sydowii* is a novel coral pathogen (Smith et al. 1996). Even though higher temperatures result in an increased production of antifungal compounds, at the higher temperatures the pathogen growth rate also increased (Kim et al. 2000, Kim & Smith 2005, Ward et al. 2007). Ben-Haim et al. (2003) determined that while water

temperature above 25°C was necessary for infection of *Pocillopora damicornis* with *Vibrio coralliilyticus*, it was unclear whether the infection was due to increased susceptibility of the coral or due to an increased virulence of *V. corallilyticus*. Another example, *Vibrio* strain AK-1, causes extensive bleaching in the Mediterranean coral, *Oculina patagonica*. The bacterium expresses adhesion genes at temperatures higher than the optimal temperature for coral (Toren et al. 1998); therefore temperatures that stress the coral are optimal for the pathogenicity for the *Vibrio* sp.

Coral as a Holobiont

The coral holobiont is the entire community of living organisms that make up the coral: the coral polyp, zooxanthellae, and associated bacteria, fungi, and viruses (Bourne et al. 2009). The holobiont includes the idea that the coral needs to be considers as a whole: each piece is integral to how the rest interacts.

If any one of these (polyp, zooxanthellae, or bacteria) are disturbed the entire system is affected. The disruption of the holobiont is possibly why bleached coral are more likely to become diseased (Harvell et al. 2001). The loss of the zooxanthellae results in a significant loss of energy and nutrients. The photosynthetic, symbiotic zooxanthellae provide most of the energy to the coral (Lesser et al. 2004), so any disruption to the symbiotic relationship would make the coral more susceptible to diseases.

Not all bacteria found on coral are pathogenic and some are even symbiotic (Knowlton & Rohwer 2003, Ritchie 2006). For example, some of the resident microbiota (bacteria) produce antibiotics against transient, and possibly pathogenic, bacteria (Ritchie

2006). The bacteria may benefit the coral by producing vitamins (Ritchie 2006) or they may become a source of food for the coral polyp (Sorokin 1973).

Microbiota as Defense

Coral microbiota are primarily found in the surface mucus layer, which covers the coral and provides both a physical and chemical barrier. The mucus is composed of lipids, proteins, monosaccharides and polysaccharides (Ducklow & Mitchell 1979, Ritchie 2006). The carbon in the mucus layer is from the zooxanthellae, but the mucus is produced by mucus secreting cells in the polyps (Ritchie & Smith 2004). Environmental stressors, such as temperature increases can change the mucus composition (Glynn et al. 1985), and lead to a change in the microbiota that live off of the nutrients in the mucus (Ducklow & Mitchell 1979, Gil-Aguedlo et al. 2006, Ritchie 2006).

Some would argue that a change in microbiota is caused by a shift in composition of the existing zooxanthellae. Researchers have found evidence, in scleractinian coral, that a single coral colony can have a combination of algal symbionts (Baker & Romanski 2007). Some have suggested that housing a diversity of zooxanthellae has an evolutionary advantage when exposed to higher temperatures because certain zooxanthellae are more heat resistant than others (Baker & Romanski 2007). Therefore, in the presence of higher temperatures natural selection favors the more thermophilic zooxanthellae. Since zooxanthellae contribute to the coral mucus composition, any change in the composition of zooxanthellae would cause a change in the coral microbiota. If the temperature increase leads to a change in zooxanthellae in Gorgonians, then this might explain the changes in microbial composition within the same coral colony. The

changing substrate would lead to different microbes able to survive in the mucus. However, research with *G. ventalina* showed that the types of zooxanthellae remained stable even when exposed to higher temperatures (Kirk et al. 2005). This reinforces the hypothesis that any changes that are seen are due to stresses on the zooxanthellae (Kim & Ward in prep) and not differences in zooxanthellae composition.

The microbiota, or bacteria, as defense model is based on the mammalian intestinal microbial community. Intestinal bacteria perform necessary functions for the health of the host. For example, major functions of intestinal bacteria include the enhanced absorption of nutrients. The indigenous bacteria protect the colonized host against the invasion of foreign microbes (the barrier effect) (Guarner & Malagelada 2003, Dethlefsen & Relman 2010). Researchers view the relationship between the bacterial community and the hosts as a range between symbiosis, commensalism and pathogenicity (Hooper & Gordon 2001). Furthermore, the commensal, indigenous bacteria modulate the expression of the host's genes and affect the physiology of the host (Hooper et al. 2001). When there is a disruption in the community structure, the relationships can shift from commensalism to pathogenicity, as seen in some diseases like inflammatory bowl diseases (Hooper & Gordon 2001, Guarner & Malagelada 2003). Therefore, the composition of the host's intestinal bacteria confers a real and important part of the host's immune system.

A similar system is seen in coral. Bacteria that normally live on the coral may also be considered part of the coral's immune system by preventing the colonization of other, possibly pathogenic, bacteria. Symbiotic bacteria may also produce antibacterial, antifungal, or antiviral compounds for themselves, but the presence benefits the coral

(Koren & Rosenberg 2006, Ritchie 2006).

Opportunistic pathogens are bacteria found on healthy coral but have the potential to become pathogens (Ritchie 2006). For example, if the temperature increases the coral become stressed, and the opportunistic bacteria would dominate and cause disease. Often opportunistic bacteria are kept in check by antibiotics from other bacteria or from the coral themselves by colonization resistance by other bacteria, or because the environmental conditions are not optimal (i.e. mucus composition and temperature). These bacteria normally don't cause problems but they can if conditions change.

The coral-microbiota-disease hypothesis regards the coral as a holobiont (polyp, zooxanthellae, and microbiota) to determine how environmental perturbations affect the coral. This hypothesis undermines the theory of how corals become diseased. The hypothesis proposes that changes in the environment lead to an unstable microbial community (Kim & Ward in prep). Changes in the microbial community indicate a disruption of the symbiosis between coral and bacteria and consequently there is a loss of coral health. This in turn means that the coral become immunocompromised.

The coral-microbiota-disease hypothesis makes several predictions. One is that environmental perturbation, such as an increase in temperature, will lead to changes in the microbial community. The change in microbial community will in turn affect coral immunity, and the loss of immunity means the coral are more susceptible to disease. The decrease in disease resistance due to changes in the normal microbial community has been documented with other animals, in particular the digestive tract of humans (Hooper & Gordon 2001, Hooper et al. 2001, Guarner & Malagelada 2003).

The normal microbiota are essential to host health. They prevent the colonization by pathogens and have a unique relationship with the host. Normal microbiota create a line of resistance to colonization by foreign microbes, which prevent the invasion of the host by pathogens by preventing the attachment and infection of pathogenic bacteria into the epithelial cells of the intestine (Hooper et al. 1999, Guarner & Malagelada 2003). The colonization resistance also applies to the opportunistic bacteria, which are present but have restricted growth (van der Waaij 1989). Experiments confirm that germ-free animals are much more susceptible to disease (Guarner & Malagelada 2003). However, when antibiotics were used, the normal microbiota were disrupted and this led to the overgrowth of a potential pathogen, toxigenic *Clostridium difficile* (van der Waaij 1989). Therefore, any changes in the residential microbial makeup negatively affect the health of the host.

Goal of This Study

This study uses the aspergillus pathogen system to test the coral-microbiota-disease hypothesis. Others have studied the infection of *Gorgonia ventalina* with *Aspergillus sydowii* (Kim et al. 2000, Alker et al. 2001, Kim et al. 2006, Ward et al. 2007, Mydlarz et al. 2008), and they noted a change in the surface microbiota after infection was detected (Kim & Smith 2005). However, it was not clear whether the change in microbiota occurred before infection, making *G. ventalina* more susceptible to *A. sydowii*, or if the infection caused the change in the microbial community.

The goal of this study is to use the coral-microbiota-disease hypothesis as a tool to establish how an increase in sea surface temperature leads to disease. I am testing the

prediction that an increase in temperature will result in a shift in the coral microbiota. I tested this by first exposing cuttings of sea fan coral (*Gorgonia ventalina*) to average summer sea surface temperature as well as to higher than average temperatures. Then, I analyzed the microbial community structure using denaturing gradient gel electrophoresis (DGGE). Finally, I identified individual bands through sequencing.

MATERIALS AND METHODS

Field Experiment

I collected the sea fan pieces on Ithaca Reef, in the Florida Keys, USA (Figure 2). From 12 apparently healthy coral, I took three cuttings (4cm x 4cm) of each and transported them in seawater back to the lab.

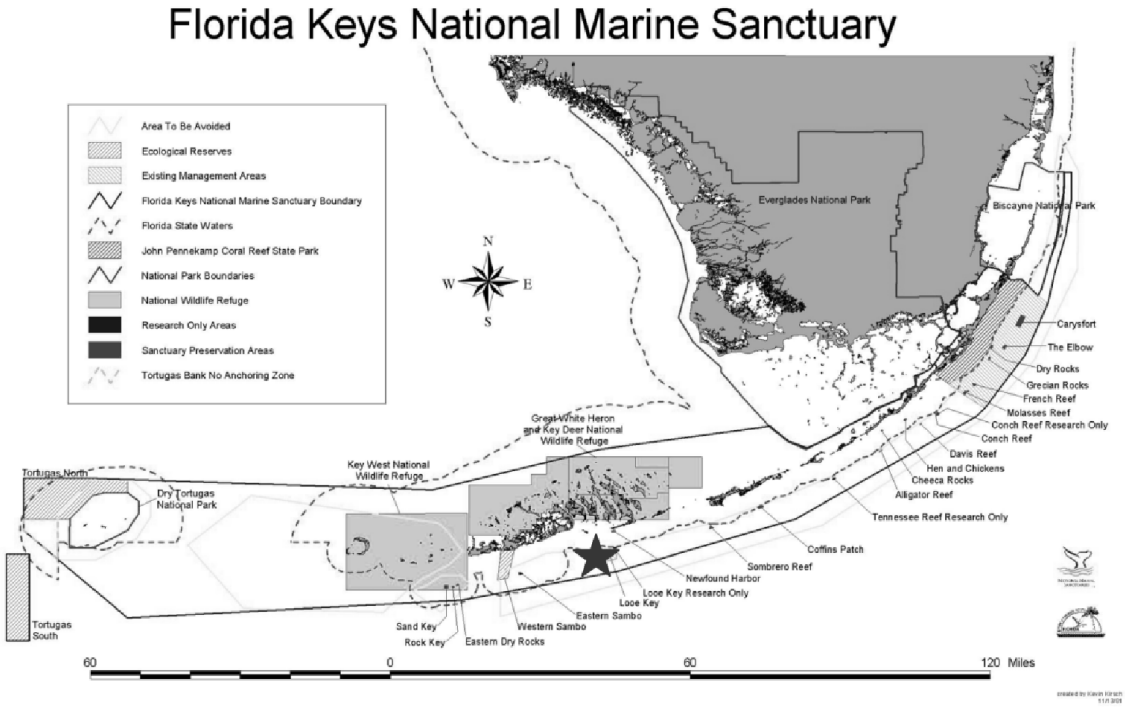

Figure 2. Map of Sampling Location in the Florida Keys.

Note: The star marks the location of Ithaca Reef where the sea fan samples were taken.

I immediately processed the initial pieces at Mote Marine Lab, in Summerland Key, FL. The control pieces were randomly assigned a 114 liter aquarium (4 cuttings per

tank) for 10 days, with the temperature kept at 28-29 °C (Figure 3). This temperature range is similar to summer ocean temperatures in the Florida Keys. In a similar fashion, I randomly assigned the treated coral pieces to a 114 liter aquarium in which the temperature was increased 0.5 °C per day until the temperature reached above seawater temperatures, 31-32 °C (Figure 3). I chose this temperature because it is the temperature at which *G. ventalina* begin to bleach. The experiment lasted for 10 days.

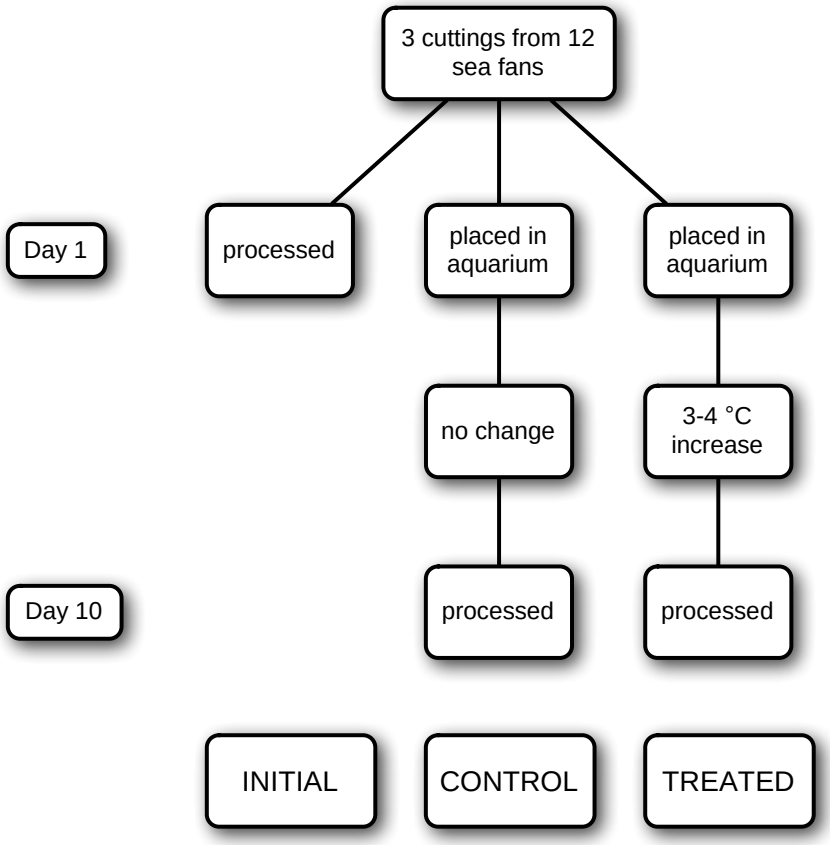

Figure 3. Diagram of Experimental Methods.

At the end of 10 days, I processed the sea fan samples as follows: the coral was cut in a 50 ml centrifuge tube into 3, approximately uniform 4 cm x 4 cm pieces (16 cm^2).

Each piece was vortexed for 1 min in 40 ml of sterile seawater, producing mucus water slurry. A digital photo was taken of each piece in order to estimate tissue area by using ImageJ v.1.41o (Rasband 2008).

Molecular Work

To compare the community structure of the microbiota in each of the different treatments I used denaturing gradient gel electrophoresis (DGGE). For this work, I centrifuged down 3 ml of the slurry, of which 0.5 ml was used for DNA extraction by employing the PowerSoil Kit (MoBio, San Diego).

To amplify a section of the 16S rRNA region I used polymerase chain reaction (PCR). The first set of PCR was STAND15, using 27F (5'AGAGTTTGATCMTGGC-TCAG) as the forward primer and 1492R (5'TACGGYTACCTTGTTACGACTT) as the reverse primer. Each reaction consisted of 45 µl High Fidelity Master Mix (Invitrogen, Carlsbad), 5 µl of 10 µM 27F primer, 5 µl of 10 µM 1942R primer, and 50-150 ng DNA. I then placed the reaction in a thermocycler set for the following cycles (Table 1).

Table 1

First PCR Cycle

Temperature (°C)	Time (min)	
94	5	
94	1	repeat 30 cycles
55	1	
72	2	
72	5	
4	hold	

I used nested PCR (Table 2) to amplify a specific 193 base-pair region and to add a CG clamp for denaturing gradient gel electrophoresis (DGGE). The primers used were 341F with CG clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG-GGGGCCTACGGGAGGCAGCAG) and 534R (5′ATTACCGCGGCTGCTGG). The primers correspond to the highly variable V3 region that is common to all eubacteria (Muyzer et al. 1993). Again, I used 45 µl High Fidelity Master Mix (Invitrogen), 5 µl of 10 µM 341F primer, 5 µl of 10 µM 534R primer, and 50-150 ng DNA. The following cycles were used in amplification.

Table 2

Second (Nested) PCR Cycle

Temperature (°C)	Time (min)	
94	5	
94	1	-1°C every 2 cycles until get to 50°C
65	1	
72	3	
72	5	
4	hold	

I used electrophoresis with a 1.5% agarose gel to determine the presence and size of the PCR product. Then, DGGE was performed to separate the operational taxonomic units (OTUs), or unique sequence, based on sequence. The DGGE gel was a 10% acrylamide gel with a 20%-60% formamide-urea gradient. The gels were run in 1xTAE buffer at 160 volts for 6 hours on the BioRad DGGE system. The DGGE gel separates

the PCR products of the same sized based on sequence by melting point. The DGGE ladder was a compilation of several bacterial species, so that bands could be compared across gels.

I stained the gel using SYBRgold made up with 1μl SYBRgold to 10 ml 1xTAE buffer and photographed to identify bands for excision. The excised bands were completely unique (not seen in other lanes) or were commonly found across three or more lanes. I also identified specific bands based on brightness: bright bands were easy to identify and make a clear excision. Brighter bands were also an indication of more rRNA, indicating the possibility of obtaining sequencing results. I used sterile blades to remove the bands from the gel.

I placed the bands in PCR tubes containing 100 μl sterile water and stored at 4 °C overnight to elute the DNA. The tubes were then shaken for 1-2 hours then spun down at 10,000 rpm for 1 minute. I then prepared the product for sequencing after PCR, using 5 μl of template solution, and the nested PCR primers. The PCR products were then sent out to be sequenced (University of Washington, High-Throughput Genomics Unit) using the nested PCR primers, and prepared per the instructions of the lab.

I performed a preliminary analysis of the banding patterns using an analysis of variance (ANOVA) to see if there were significant differences between the initial, control, and treated. I tallied the total number of bands in each well, and assigned a rank to each. The ranks were assigned 1, 2, and 3, corresponding to the fewest, middle, and most bands. Rank was used to determine the effects of elevated temperature on bacterial

numbers relative to each sea fan. It was also used to normalize the number of bands in the case of outliers that could skew the data.

Phylogenetic Analysis

Sequencing of the bands was then done to identify selected bands. First, all the sequences were input into Geneious (Drummond AJ 2011). Only the trimmed sequences were used for analysis: base pairs that fell under a quality confidence threshold of 20 not used. Then, the forward and reverse sequences from each operational taxonomic unit (OTU) were aligned using ClustalW2 (Larkin MA 2007), and a consensus sequence was determined.

I excised and sent out for sequencing more bands than are highlighted in the gels. The highlighted bands are those that returned with both forward and reverse sequences. Of the 79 forward and 79 reverse sequences sent out for sequencing, resulted in only 34 consensus sequences, with an average length of 162 base pairs. I resolved ambiguities by determining which sequence had a higher quality read at that base.

The consensus sequences were put into BLAST for a comparison to known sequences in the NCBI database. I recorded the top sequence, based on the E-score and percent identity (how similar they were). I did not find any results below 97% identity. I used PAUP to create a distance phylogenetic tree using neighbor-joining from the consensus sequences, using known coral pathogens (*Serratia marcescens* and *Vibrio coralliilyticus*) as outgroups.

RESULTS

DGGE Analysis

The number of bands per well ranged from 26 (sea fan A, Initial) to 2 bands (sea fans K and L) (Table 3). Sea fans A and F, with 18 unique bands, had the most unique bands in each treatment. Sea fan I contained 10 bands found in common (at the same position) across all three treatments, the most for any of the sea fans.

Table 3

Quantitative Analysis of the Banding Patterns for the Treatments of Each Sea Fan

Sea Fan	Treatment	Total Bands	Unique Bands	Bands Common (to at least 1 other treatment/ sea fan)	Bands Common (to all 3 treatments/ sea fan)
Average	Initial	12.417	4.333	8.083	4.417
	Control	10.083	3.500	6.583	
	Treated	9.333	2.083	7.250	

Since the PCR products are all the same length, the sequences separate by melting point (G-C content) and not by size. It is assumed that individual bands (Figure 4) represent unique sequences of the same length, and thus, possibly different strains of bacteria, or operational taxonomic unit (OTU).

From the gels, I identified bands of interest, excising them for sequencing. Direct sequencing of the PCR product was not ideal because the results may underestimate

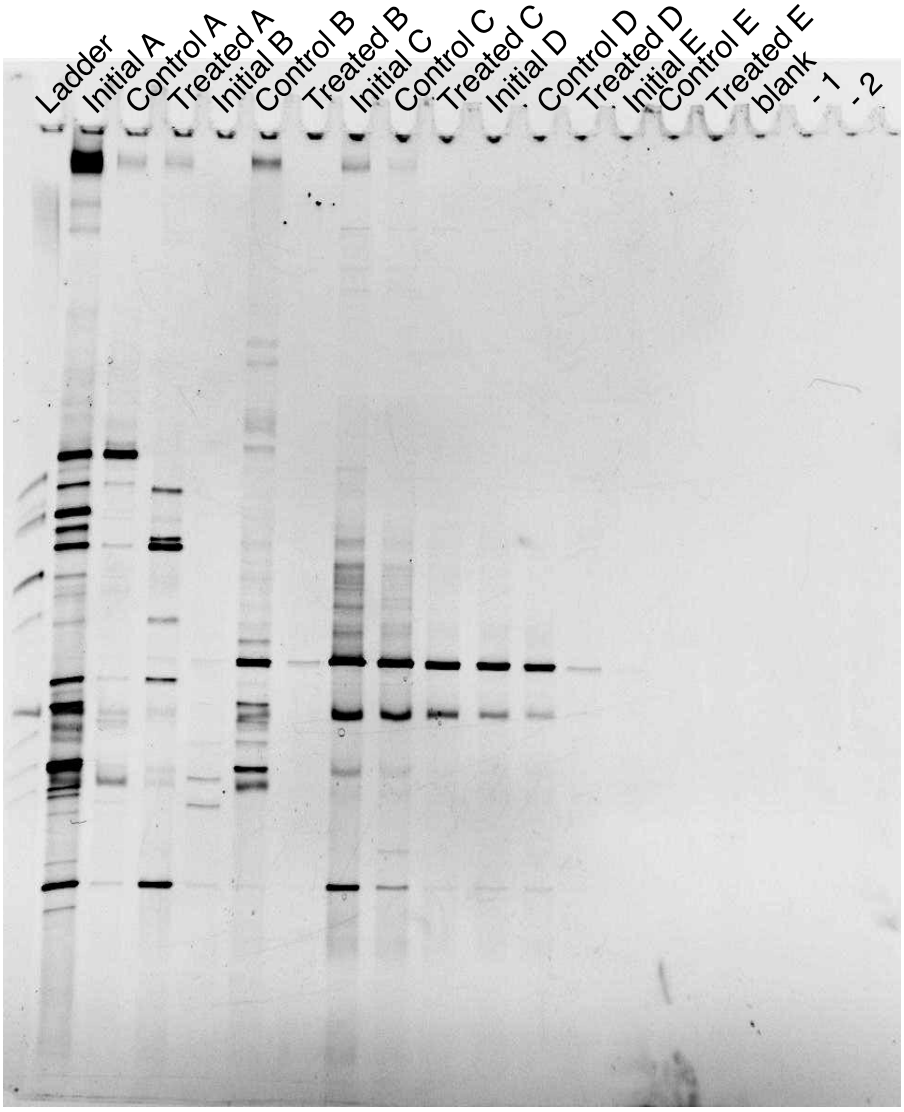

Figure 4. A Photograph of a DGGE Gel for Sea Fans A-E of the Initial, Control, and Treated Sea Fan Coral.

Note. The first lane is a ladder. The ladder is a composite of bacterial isolates that are amplified using the second PCR cycle. The blank is PCR grade water used in DNA extraction and put through both rounds of PCR. (-1) is PCR grade water that went through both cycles of PCR, and (-2) is PCR grade water used in the second PCR cycle.

sequence diversity. Bands of interest include those that are found in most of the lanes as well as those unique to particular lanes. Bands that found in the majority of the lanes may

represent bacteria common to sea fans, and were excised. Bands were also chosen based on the brightness, which indicated a higher concentration of rRNA and it was assumed to result in more PCR product, and therefore more likely to produce a reliable sequence. In all, bands that were chosen and excised best represented the overall banding pattern and

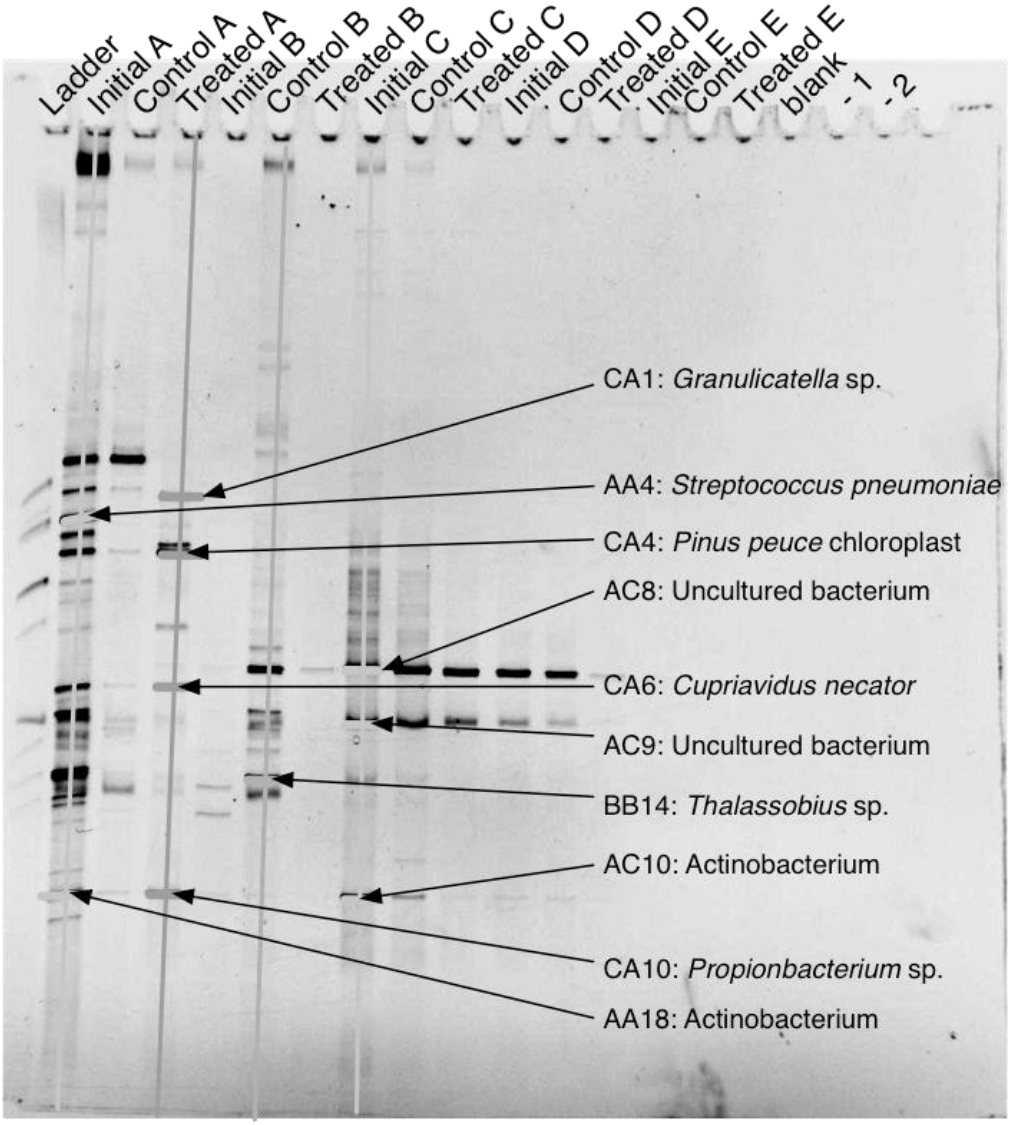

Figure 5. Sequenced DGGE Bands Highlighted and Annotated for Sea Fans A-E.

Note. The letters note the sea fan the band is from and the number references the band number of that sea fan. The highlighted bands were sequenced and identified.

those most likely to yield enough rRNA for sequencing.The ladder on the right most lane in the gels is a product of a several *Pseudoalteromonas* sp. strains. The ladder was used so that, in theory, there would be some ability to compare the banding patterns across gels.

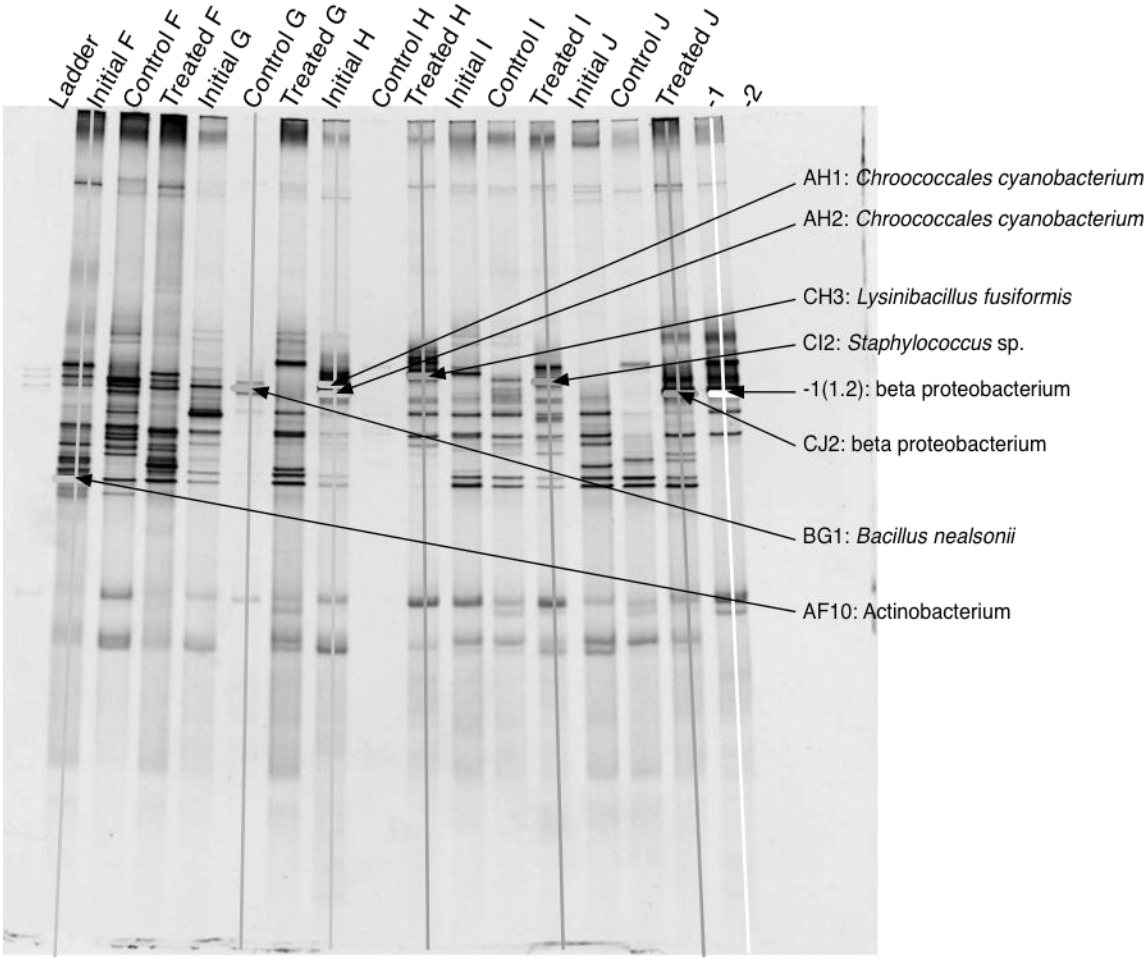

Figure 6. Sequenced DGGE Bands' Location and Identification, Sea Fans F-J.

Note. The highlighted bands are bands that were sequenced and identified.

Overall, the initial sea fans had the most bands, the greatest number of OTUs. Three of the initial sea fan sequences were Actinobacteria (Figures 5 &6). Another three

sequences were *Burkholderia* sp. (Figures 7 & 8), two were identified as cyanobacteria (Figure 6), and one as *Streptococcus pneumoniae* (Figure 5). The initial sea fans were the only samples with bands sequenced as *Burkholderia* sp.

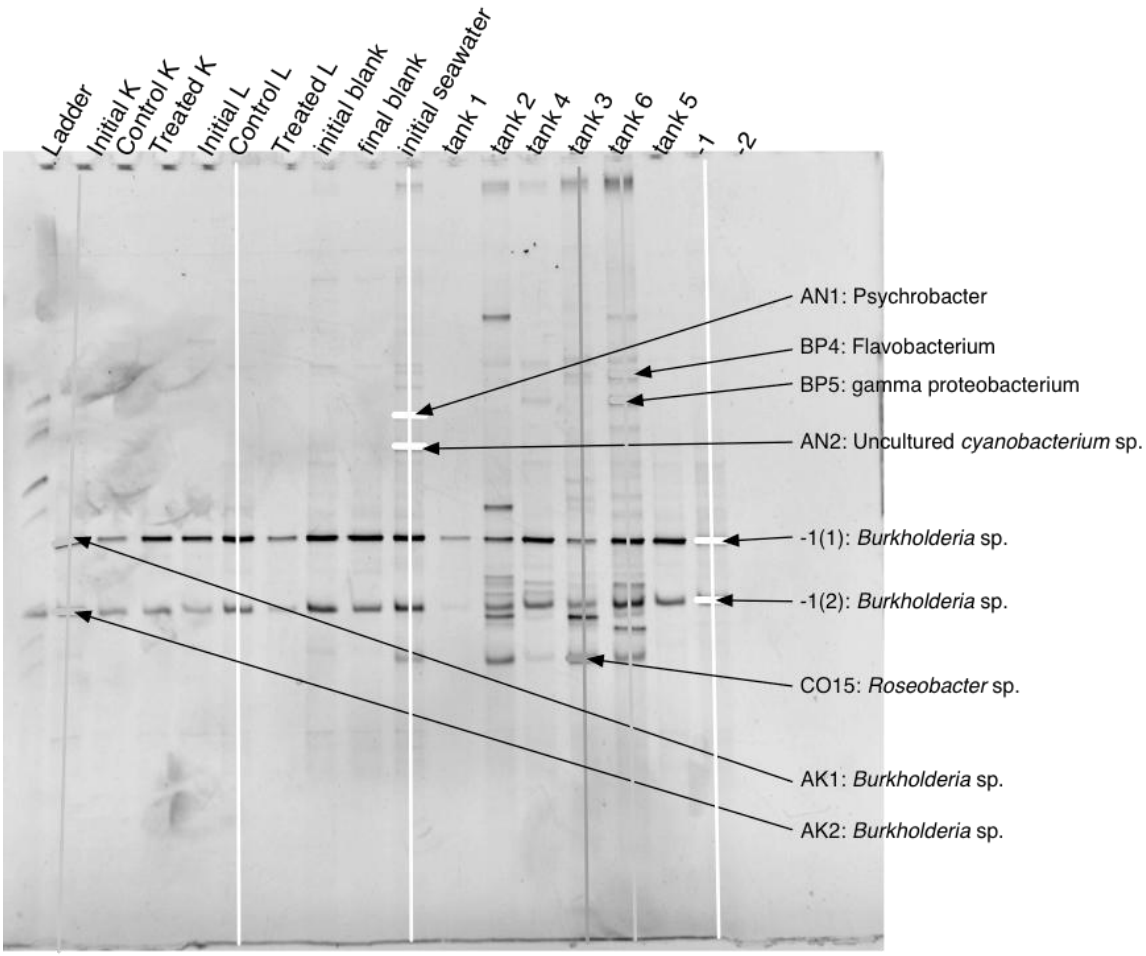

Figure 7. Sequenced DGGE Bands' Location and Identification, Sea Fans K-L, Seawater and Tank Water.

Note. The highlighted bands are bands that were sequenced and identified.

Only two sequences from the control sea fans were identified. The two sequences were identified as *Thalassobius* sp. (Figure 5) and *Bacillus nealsonii* (Figure 6).

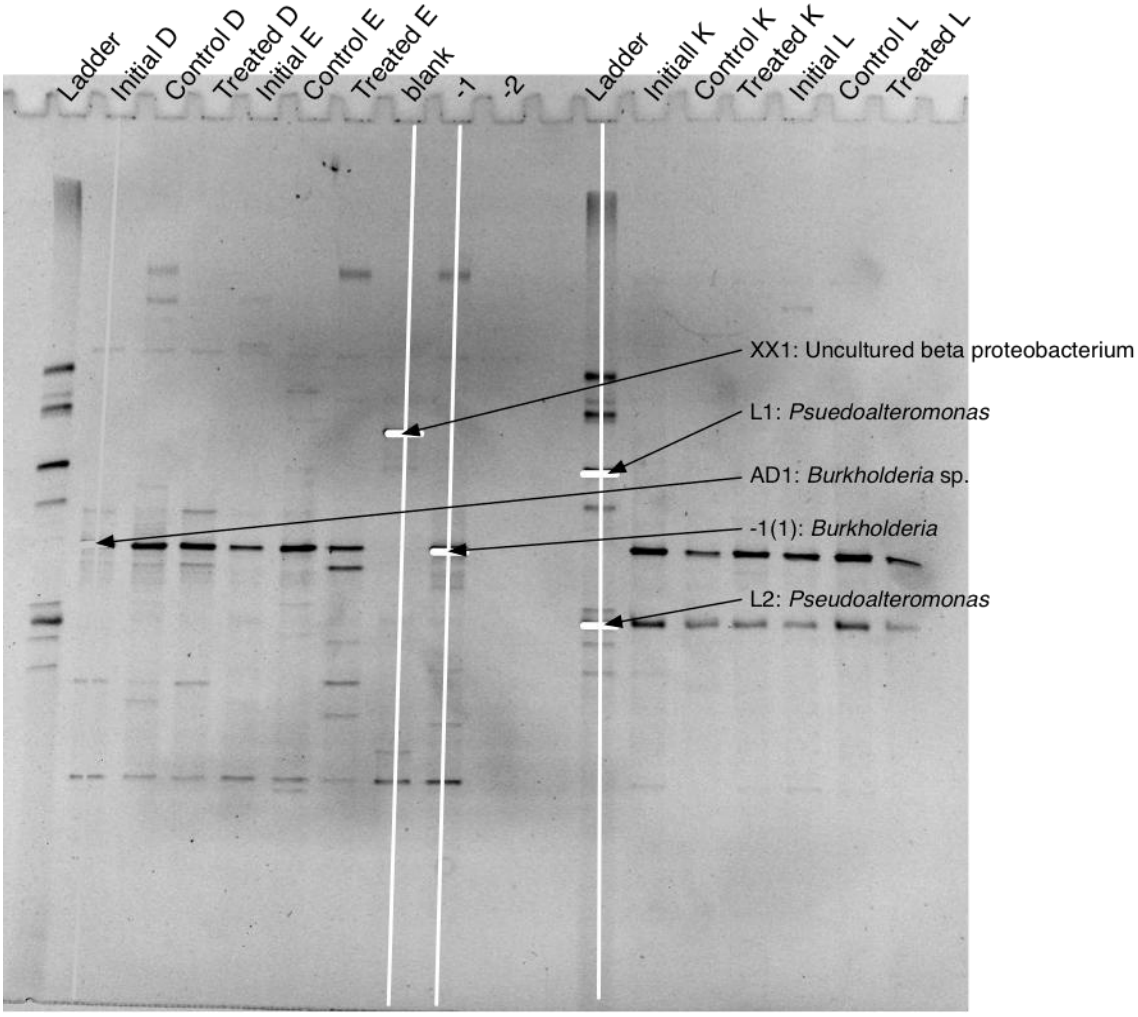

Figure 8. Sequenced DGGE Bands' Location and Identification, Sea Fans D-E, K-L.

Note. The highlighted bands are bands that were sequenced and identified.

Treated sea fans included sequences for *Propionibacterium* sp., *Cupravidas nector*, *Granulicatella* sp., and a chloroplast. Treated sea fans samples were also identified as *Bacillus*, sp., which includes *Lysinibacillus* sp. and *Staphylococcus* sp. (Figure 6).

The banding pattern for the sea fans was different than that for the sea water and the tank water. Sea fans K - L (Figure 7) have a similar banding pattern, while the samples from Tank 2-5 have unique banding patterns. The initial sea fan K and the molecular control (-1) hosted *Burkholderia* sp., while sequencing did not reveal

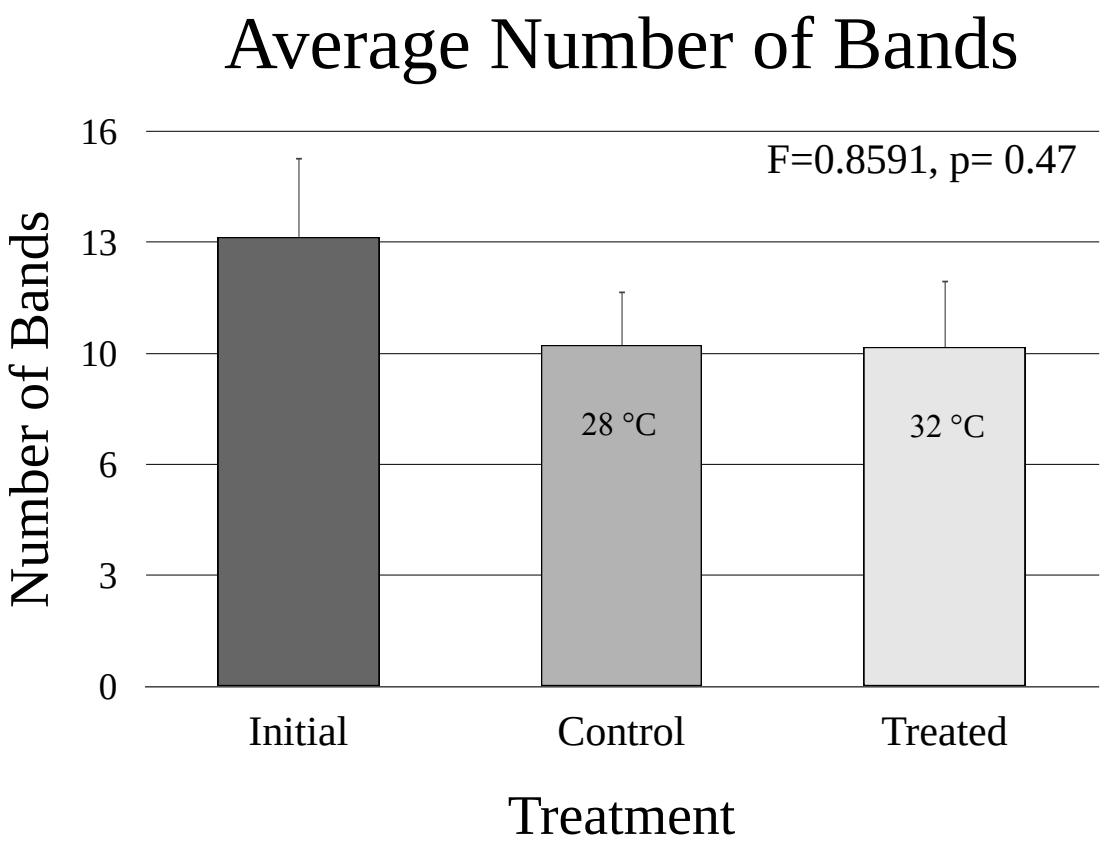

Figure 9. The Average Number of Bands Found for the Initial, Control and Treated Sea Fans from Figures 5, 6, 7 and 8.

Note. Error bars are standard errors.

Burkholderia sp. in any of the tank samples. The *Burkholderia* sp. bands were identified as the same accession number, and the only differences were the length of the sequences.

Burkholderia sp. are present in the (-1), molecular control, as well as in the initial sea fan D (Figure 8). Both ladder bands were sequenced as *Psuedoalteromonas* sp.

I counted the number of bands for each treatment and averaged them for each treatment (Figure 9). An ANOVA showed there was no difference in the number of bands (bacterial strains) between the initial, control, and treated sea fans (F=0.8591, p=0.43).

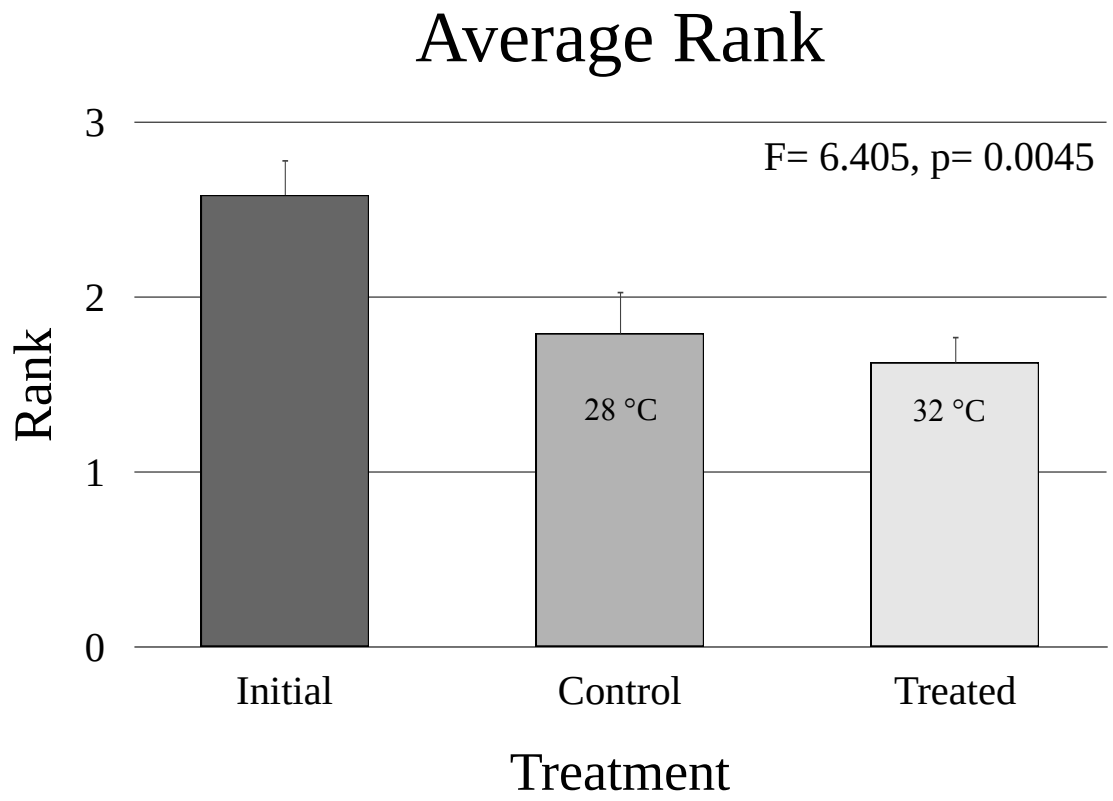

Figure 10. Average Rank of Bands for Each Sea Fan Treatment.

Note. Each treatment per sea fan was assigned a rank based on number of bands (fewest bands = 1, most bands = 3). The ranks were averaged for each treatment. Error bars are standard errors.

However, when I normalized the data to look at how the microbial community changed for each sea fan there was a significant difference. Ranking the treatments for

each sea fan normalized the data because the interest was in the relative change for each sea fan when the temperature changed. I tallied the number of bands for each treatment, and then assigned a rank of 1, 2 or 3 for each treatment per sea fan. A rank of 1 meant the treatment had the fewest bands per sea fan; a rank of 3 meant the treatment had the most bands. Ties were resolved by averaging the rank (i.e. a rank of 1.5 or 2.5). For example, for sea fan A the initial had 26 bands, the control had 4 bands, and the treated had 17 bands. Therefore, the initial was ranked as 3, the control as 1, and treated as 2.

Figure 10 illustrates the average rank for each treatment. The means are significantly different ($F = 6.4049$, $p = 0.0045$). A Tukey-Kramer post-hoc analysis (Table 4) shows that the rank of the initials was significantly higher than the ranks of the controls and treated sea fan pieces.

Table 4

Tukey-Kramer Analysis and Significantly Different Treatments

Level			Mean
Initial	A		2.58333
Control		B	1.79167
Treated		B	1.62500

Note. Levels not connected by same letter are significantly different.

I also did an ANOVA to determine if there was a significant difference in the number of novel bands in each treatment, for each sea fan (bands found in one treatment but not the others). After analyzing the number of unique bands for each treatment, I found no difference ($F= 1.066$, $p = 0.36$). Even when the number of unique

bands was ranked, to normalize for the differences in each sea fan, there was still no difference ($F = 1.983$, $p = 0.064$).

Sequence Analysis

The resulting phylogenetic tree (Figure 11) is based distance and using neighbor-joining of the consensus sequences, shows that there appear to be 3 main subgroups. They appear to generally group into actinobacteria, β-proteobacteria, and firmicutes/cyanobacteria. The most common genus was *Burkholderia* sp. with 6 of the total sequences. The largest group appears to be β-proteobacteria, which includes *Burkholderia* sp. and *Cupriavidus* sp.

Most of the accession numbers are for bacteria found in soil or water, with some that have been found on or around coral reefs (Table 5). Several bands of interest are CA4, AC10, and BG1. CA4 is most similar to a chloroplast sequence; AC10 is most similar to an actinobacterium; and BG1 is most similar to *Bacillus nealsonii*.

Overall, there were relatively more bands found in the initial sea fans and fewer in the control and elevated temperature sea fans. The most common bacterial sequences were *Burkholderia* sp.

Troubleshooting

I ran more than one gel of each set of samples, but those gels that had the best resolution are shown here, however only the data presented was analyzed. This is mentioned because the banding patterns of the lanes in the other gels were similar. For

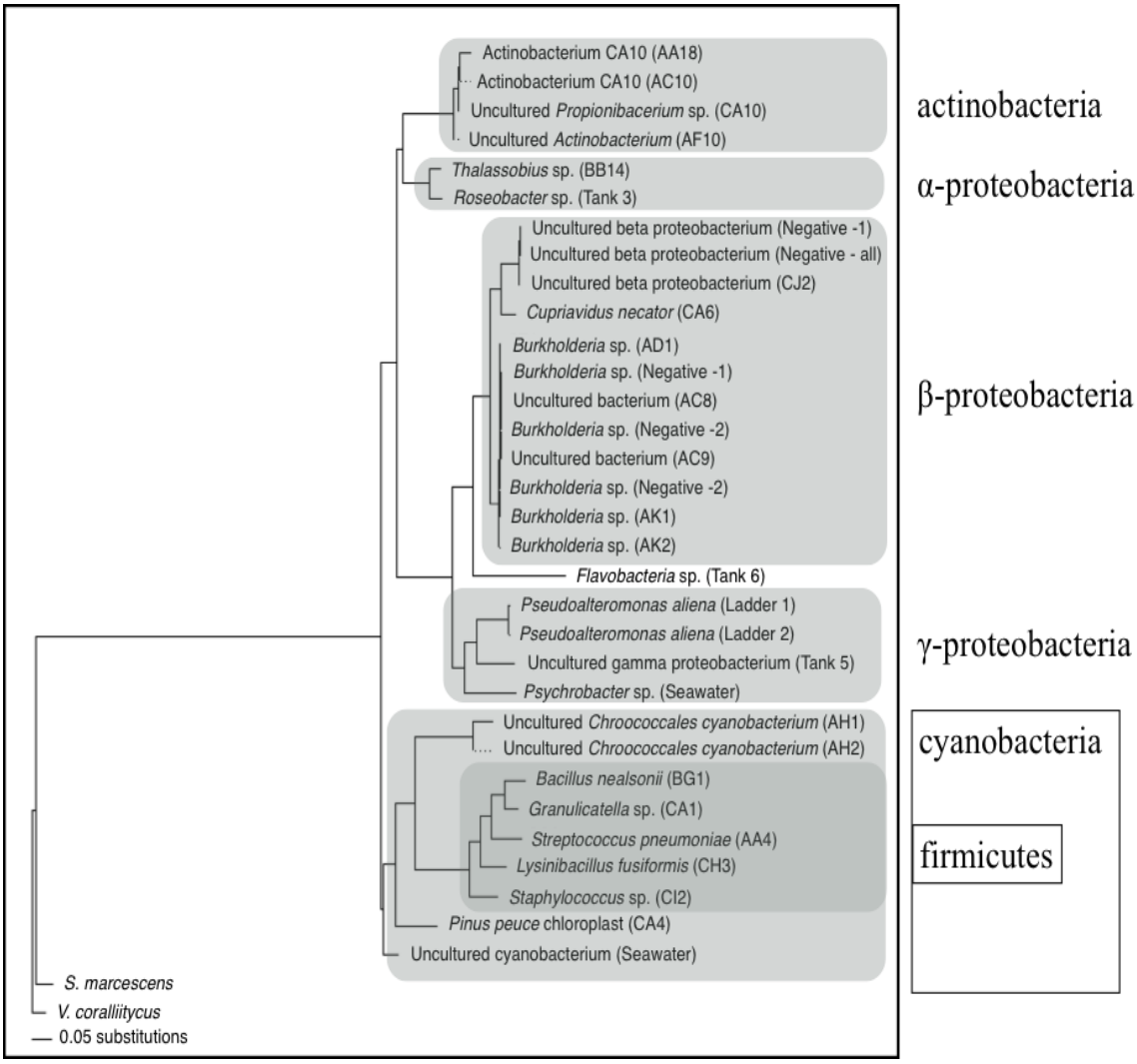

Figure 11. Distance Phylogenetic Tree, Using Neighbor-Joining, of the Consensus Sequences and Illustrating the Major Groupings of Bacteria.

Note. The letters (i.e. AA) refer to the treatment and sea fan the band came from, while the number (i.e. 18) refers to the specific band within the lane. AA-CL are from sea fans, AN is initial seawater, -(1)s and XX are molecular negative controls, CO is from Tank 3, BP is from Tank 6, and Ls are bands from the ladder.

Table 5

BLAST Results of Sequenced Bands

Treatment	Band	Accession	E-score	Length of Alignment	% Identity	Description
Initial	AA4	CP002176.1	9e-63	129	100	*Streptococcus pneumoniae* 670-6B
	AA18	FJ662870.1	1e-57	113	100	Actinobacterium CA10 (inhibition of *Streptococcus pyogenes* biofilm formation by coral-associated actinomycetes on *Acropora digitifera*)
	AC8	FJ156714.1	2e-66	129	100	Uncultured bacterium (marine water from Roi-roi reef)
	AC9	FJ156714.1	5e-67	130	100	Uncultured bacterium (marine water from Roi-roi reef)
	AC10	FJ662870.1	1e-51	102	100	Actinobacterium CA10 (*Acropora digitifera*)
	AD1	HM461232.1	1e-69	136	100	*Burkholderia* sp. (soil bacterial from Colombia)
	AF10	GU015971.1	3e-52	103	100	Uncultured actinobacterium (soils in central Taiwan)
	AH1	EF160008.1	9e-46	99	97	Uncultured *Chroococcales cyanobacterium* (cyanobacterial diversity in a tropical intertidal lagoon)
	AH2	EF160008.1	2e-47	99	98	Uncultured *Chroococcales cyanobacterium* (cyanobacterial diversity in a tropical intertidal lagoon)
	AK1	HM461232.1	5e-61	120	100	*Burkholderia* sp. (soil bacterial from Colombia)
	AK2	HM461232.1	4e-62	122	100	*Burkholderia* sp. (soil bacterial from Colombia)

Treatment	Band	Accession	E-score	Length of Alignment	% Identity	Description
Control	BB14	DQ681190.1	2e-54	107	100	*Thalassobius* sp. (marine bacterioplankton in the NW Mediterranean Sea)
	BG1	HM100206.1	9e-64	125	100	*Bacillus nealsonii* (coral associated bacteria against fish pathogens)
Treated	CA1	GQ900875.1	3e-67	131	100	*Granulicatella* sp. (bacteria associated with cystic fibrosis)
	CA4	FJ899582.1	2e-47	98	100	*Pinus peuce* chloroplast
	CA6	GU168986.1	1e-68	133	100	*Cupriavidus necator* (culturable bacteria in activated sludge in Malaysia)
	CA10	GQ369207.1	1e-58	115	100	Uncultured *Propionibacterium* sp. (bacterial community in two rice fields)
	CH3	HQ003444.1	8e-65	127	100	*Lysinibacillus fusiformis* (psychrophilic bacteria from Gurudongmar Lake, India)
	CI2	HQ141278.1	2e-66	133	99	*Staphylococcus* sp. (soil contaminated with oil)
	CJ2	HM480197.1	1e-62	123	100	Uncultured β-proteobacteria (hypersaline microbial mat)
Seawater	AN1	GU371682.1	3e-61	120	100	*Psychrobacter* sp. (Shenzen coastal waters, Zhujiang river estuary)
	AN2	GU170796.1	3e-46	92	100	Uncultured *Cyanobacterium* sp. (coastline of the northeastern Gulf of Mexico)
	CO15	GQ246649.1	1e-53	106	100	*Roseobacter* sp. (bacterial communities from marine environment in Dokdo)
	BP4	AB496663.1	4e-61	120	100	Flavobacteria bacterium (seawater)
	BP5	FJ745222.1	2e-66	130	100	Uncultured γ proteobacterium (surface water at the UGA Marine Institute)

Treatment	Band	Accession	E-score	Length of Alignment	% Identity	Description
Negative Controls	XX1	HM480197.1	1e-68	134	100	Uncultured β-proteobacteria (hypersaline microbial mat)
	-1(1)	HM461232.1	5e-67	131	100	*Burkholderia* sp. (soil bacterial from Colombia)
	-1(1.2)	HM480197.1	2e-67	132	100	Uncultured β-proteobacteria (microbial mats of hypersaline lakes)
	-1(2)	HM461232.1	2e-67	132	100	*Burkholderia* sp. (soil bacterial from Colombia)
	-1(2.2)	HM461232.1	7e-66	129	100	*Burkholderia* sp. (soil bacterial from Colombia)
Ladder	L1	HQ003447.1	1e-68	134	100	*Pseudoalteromonas aliena* (psychrophilic bacteria from Gurudongmar Lake)
	L2	HQ003447.1	2e-67	132	100	*Pseudoalteromonas aliena* (psychrophilic bacteria from Gurudongmar Lake)

example, when the samples from Figure 6 were run again the bands from control treatments of sea fans G and H were still faint, as were sea fans D and E (Figure 4).Also, to ensure that the pattern was due to the rRNA, and not an artifact of the gel preparation, all treatments of sea fans K-L were run again on a separate gel (see Figure 8).

There is one band that is found in nearly each well, even in the negative control. This band may represent contamination or it may be a result of primer dimerization during PCR. Bands that are found in most wells, but not all, could represent a common strain found on all sea fans irrespective of the temperature or time. It does appear that there was some contamination, since there are bands in the -1 sample (the first PCR negative control), but not the -2 sample (the second PCR negative control). This indicates that the samples were contaminated during the first round of PCR, but not when the second round of nested PCR was done.

DISCUSSION

Researchers have correlated increases in temperature with an increase in coral disease (Hoegh-Guldberg 1999, Ward et al. 2007). They have also shown that the composition of the surface mucus layer changes with changes in the environment, as when the temperature changes (Ritchie 2006). An increase in temperature may decrease the ability of the normal bacteria to maintain normal growth, thereby causing a change in the bacterial community. Resident bacteria can produce antibiotics against transient bacteria (Ritchie & Smith 2004), and this change may decrease the coral's immunity. It is possible then for opportunistic pathogens already on the coral or pathogens that are already present in the water column, to take over. The change in an established bacterial community causing disease is consistent with data on human disease and microbiota (van der Waaij 1989, Hooper & Gordon 2001, Eckburg et al. 2005, Dethlefsen & Relman 2010). For example, when antibiotics were used on humans, the change in the normal microbiota led to the overgrowth of a potential pathogen, toxigenic *Clostridium difficile* (van der Waaij 1989).

My prediction was that an increase in temperature would cause a change in the microbiota diversity. There was a difference among the treatments when the relative numbers of bands were analyzed. According to the ANOVA there was a significant difference between the initial sea fans, and the control and treated sea fans. This indicates that there was a higher diversity of bacteria present on the sea fans before they were

placed into the aquaria. Therefore, while there was a change in the bacterial diversity, temperature was not the determining factor.

Aquarium Effect

The results of this work suggest that there was an aquarium effect (Figures 10 and 11, Table 3). The overall diversity decreased when the sea fan fragments were in aquaria, irrespective of the temperature. Others have observed a decrease in microbial diversity in experiments where the coral were kept in aquaria (Kooperman et al. 2007, Ainsworth & Hoegh-Guldberg 2009). The notable shifts were losses of actinobacteria and cyanobacteria (Kooperman et al. 2007). Although every attempt was made to make the aquaria as similar to conditions on the reef, these data indicate that there is in fact a change in the physiology of the coral when put in an aquarium (Kooperman et al. 2007, Ainsworth & Hoegh-Guldberg 2009).

The change in microbial community may indicate that coral adapt to new environmental conditions, as suggested by Kooperman et al. (2007). However, the decrease in microbial diversity, is similar to the decrease in microbial diversity of a diseased coral (Pantos et al. 2003) as well as after a bleaching event (Ainsworth & Hoegh-Guldberg 2009). This decrease suggests that aquaria stress the coral. If this is the case, then keeping coral in aquaria for physiology or microbial studies may not be appropriate.

Banding Pattern

It is clear that the banding patterns are not due to bacteria found in the water column. The banding patterns of the sea fan samples are distinct from the seawater and

tank samples (Figure 7). This indicates that the water column does not influence the structure of the sea fan microbiota.

While I predicted that the initial and the control treatments would be more similar than the treated sea fans, comparing the number of bands between the treatments shows no significant difference. However, the patterns of the bands do not remain consistent within and between treatments of the sea fans. That is, each sea fan may have unique microbiota. Therefore, it was important to look at the relative change: was there a change for each sea fan? The relative numbers of bands in a treatment for a given sea fan showed that the initial sea fans had relatively more bands than the control and heat-treated sea fans ($p=0.047$). This indicates that there is some change in the microbial community structure. The relative numbers of bands in the control and heated treatments were not significantly different from each other. This suggests that after the initial treatment there was a decrease in the bacterial diversity of the coral. The banding patterns were not apparently similar between the control and heated treatments. It is possible that although the numbers of bands are similar, the types of bacteria present are different.

While there was a shift in the relative number of bands (a quantitative change) it was not clear if the change was due to a bacterium taking over and others being out competed (a qualitative change). If a few species were being selected, then more unique bands would be present in the initial sea fans samples than in the control or heat-treated sea fans. Bands found only in the final treatment may represent opportunistic or pathogenic bacteria that have found a niche on the coral when the temperature was increased. However, the ANOVAs showed no one treatment had significantly more unique bands than another.

No treatment had significantly more bands that were not found in either of the other treatments, or unique bands. The lack of unique bands indicates that while there were some bacteria that were introduced to the control and elevated temperature sea fans, they were not a set of novel bacteria. The lack of unique bands in the initial sea fan samples shows that a few bacteria did not outcompete others. The banding patterns could be due to some bacteria that were lost and that others were emphasized.

Sequences

The changes in the banding patterns do not take into account the functional changes in the microbial community that occurred among the treatments. It is possible that there is a fundamental functional shift in the types of bacteria found in each treatment: there may be a shift in the functions the bacteria play from predominantly bacteria producing antibiotics to mostly denitrifying bacteria. Most of the sequences were identified as bacteria found in either the soil or marine environments (Figure 12 and Table 4). I suspect that one of the reasons many soil bacteria were found may be due to the sampling conditions. On the day I collected the sea fans and filled the aquaria, the ocean was turbid from a tropical storm. It is possible that the bacteria present in the aquaria were able to take hold but which bacteria they were may have depended on the temperature of the aquaria and how well established the indigenous bacteria were.

Some of the more notable sequences were CA4 (a chloroplast sequence) and BG1 (*Bacillus nealsonii*). It is possible that CA4 is actually a section of a cyanobacterium, since chloroplast were once free-living cyanobacteria (Martin et al. 2002) and they have similar rRNA (Bergsland & Haskelkorn 1991). AC10 is most similar to an

actinobacterium, associated with the inhibition of *Streptococcus pyogenes* and isolated from the coral *Acropora digitifera*. BG1 is most similar to *Bacillus nealsonii*, a coral associated bacteria that are antagonistic toward fish pathogens.

Kooperman et al (2007), found that when they placed coral in aquaria there was a loss of actinobacteria and an increase β-proteobacteria. Similarly, although three sequences identified as actinobacteria were found on the initial sea fans, only one was present on the elevated temperature samples (Figure 11). Actinobacteria are known to produce antibiotic compounds, and their loss may have an impact on the corals' disease susceptibility (Kooperman et al. 2007).

One β-proteobacteria group consisted of *Burkholderia* sp. Burkholderiales group includes many pathogenic bacteria, particularly plant pathogens (Burkholder 1950).

Utility of DGGE in Microbial Community Analysis

One of the main assumptions about DGGE is that each band represents a unique sequence and can resolve sequences with even one base pair difference (Muyzer et al. 1993). Therefore, the assumption is that bands that travel the same distance from the well have the same sequence.

However, this assumption may be incorrect. For example, the lower band of the ladder (Figure 8) was sequenced and identified as *Pseudoalteromonas* sp. However, those bands that appear to be at the same position, but on a different gel (Figure 7), were sequenced as *Burkholderia* sp. It is possible that the bands are not at the same position, but very close. It is also possible that the rRNA in the bands was mixed between excision and sequencing, but only one strand was sequenced. Another example can be seen in

Figure 5, where bands AC8 and AC9 are located at different latitudes, but have the same sequence. It is possible that there was some slippage during PCR and one of the sequences had one or 2 more base pairs, resulting in an increased melting point, therefore showing up as a unique band.

Another concern is that DGGE relies on PCR products, causing a possible PCR bias (Acinas et al. 2005). PCR bias results in an overrepresentation of certain strains due to the primers annealing better to certain sequences than to others. Consequently, some strains would replicate more readily than others and this results in some strains appearing more abundant, even if the starting product had equal amounts of both.

Other Sources of Error

It appears that there was some contamination, as there were several bands present in two sets of negative controls, labeled as blank (Figure 8) and (-1) on the DGGE gels (Figures 6, 7, and 8). The blank negative control was the PCR grade water that went through all the same procedures as the samples: DNA extraction and both rounds of PCR. Another negative control, (-1), was PCR grade water that was put through both the first and second rounds of PCR. Interestingly, there was no contamination in the second negative control, (-2). It was not possible to identify contamination after the first round of PCR because of the small amount of product. Therefore, the first time I noticed any contamination was when PCR electrophoresis was done after the second round of PCR. At this time it was thought the bands in the first negative control were due to primer-dimers.

CONCLUSION

Overall, there was a decrease in the microbial diversity of the control and heat-treated sea fans, from the initial sea fans. However, any change caused by the temperature was masked by a strong aquarium effect, where relative number of bands between the control and heat-treated sea fans were insignificant. It is possible that the change in the microbial community is a precursor to disease, especially with the large proportion of *Burkholderia* sp. present. Future studies will have to account for the aquarium affect to determine exactly what influence temperature has on coral microbial diversity.

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