

ABUNDANCE AND DIVERSITY OF CULTURABLE BACTERIA FROM HEALTHY  
AND SUSPECT WHITE PLAGUE TYPE II-INFECTED CORALS IN THE FLOWER  
GARDEN BANKS NATIONAL MARINE SANCTUARY

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Abundance and Diversity of Culturable Bacteria from Healthy and Suspect White  
Plague Type II-infected Corals in the Flower Garden Banks National Marine Sanctuary

A thesis submitted in partial fulfillment of the requirements for the degree of Master of  
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## **DEDICATION**

This work is dedicated to my mom, Catherine S. Winters, for her love and support.

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## LIST OF ABBREVIATIONS

16S rRNA	16 small-subunit ribosomal ribonucleic acid
ANOVA	analysis of variance
bp	base pair
C	Celsius
CFU	colony forming unit
cm	centimeter
D	coral tissue from the disease margin
DEPC	diethyl pyrocarbonate
diH <sub>2</sub> O	deionized water
DNA	deoxyribonucleic acid
dDNTP	dideoxyribonucleotide triphosphate
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FEC	FGBNMS East Bank Coral
FWC	FGBNMS West Bank Coral
FGBNMS	Flower Garden Banks National Marine Sanctuary
g	gram
GMU	George Mason University
H	apparently healthy coral tissue from an unaffected colony
HD	apparently healthy coral tissue on a diseased colony
LH-PCR	length heterogeneity polymerase chain reaction
ml	milliliter
mm	millimeter
nmi	nautical miles
OTU	operational taxonomic unit
PCO	principal coordinate analysis
PCR	polymerase chain reaction
rpm	revolutions per minute
SCUBA	self contained underwater breathing apparatus
TE	tris-EDTA buffer
WP	white plague
WPI	white plague type I
WPII	white plague type II
WPIII	white plague type III

$\mu\text{g}$	microgram
$\mu\text{l}$	microliter
$\mu\text{M}$	micromolar

## **ABSTRACT**

### **ABUNDANCE AND DIVERSITY OF CULTURABLE BACTERIA FROM HEALTHY AND SUSPECT WHITE PLAGUE TYPE II-INFECTED CORALS IN THE FLOWER GARDEN BANKS NATIONAL MARINE SANCTUARY**

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George Mason University, 2008

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The increasing number and geographic spread of coral diseases over the past three decades is threatening the diversity and viability of coral reef ecosystems. Little is known about the causes of disease outbreaks in coral; how coral diseases are transmitted; or the relative impact of climate, environmental stressors, and anthropogenic factors on coral disease, making the prevention and/or mitigation of coral disease impacts difficult if not impossible. The coral disease white plague type II (WPII) is a particularly virulent and fast moving disease, affecting 38 documented species of Caribbean scleractinian corals. WPII has the ability to destroy coral tissue at a rate of up to 10 cm/day, with the ability to kill entire colonies within days. Loss of tissue from the margin of several coral species at the Flower Garden Banks National Marine Sanctuary in the Gulf of Mexico in May 2005 suggested that the corals were affected by WPII. The goals of this study were to

enumerate the microbial communities associated with suspect WPPII coral disease, to compare the microbial abundance and community composition (diversity) between the healthy and diseased coral tissue, and to verify whether a previously proposed etiological agent of WPPII, the bacterium *Aurantimonas coralicida*, was present in the diseased coral tissue. Triplicate coral core samples were collected from suspect WPPII diseased-margin tissue, apparently healthy tissue on diseased coral colonies, and healthy tissue from four paired coral colonies from the Flower Garden Banks National Marine Sanctuary in the Gulf of Mexico. The culturable microbial communities were enumerated and then examined by molecular fingerprinting and 16S rRNA gene sequencing. The bacterial abundance data suggest that variations may be due, in part, to coral species-specific and site-specific factors. The length heterogeneity polymerase chain reaction (LH-PCR) fingerprinting and 16S rRNA gene sequencing data suggest that for selected bacterial colonies there was a relatively high degree of similarity among the culturable bacterial populations associated with apparently healthy tissue on diseased coral, healthy coral tissue, and diseased-margin coral tissue. Much of the variability in the presence of these bacterial populations was found in the less abundant taxa. The bacterium *A. coralicida* was not detected using LH-PCR to characterize the culturable bacterial colonies, even though isolates were selected based on morphological characteristics of *A. coralicida* to focus on likely candidates.

## **1. Introduction**

### **The Current State of Coral Reef Communities**

Coral reefs are highly diverse, complex ecosystems (Wilkinson 2002) that serve as important resources for the global community. Coral reef communities represent major reservoirs of biological diversity, serving as home to 32 of the 34 recognized animal phyla (Wilkinson 2002). In addition, they provide for the livelihood, in whole or in part, for an estimated 500 million people worldwide, with the value of goods and services derived from coral reef ecosystems estimated at U.S. \$375 billion annually (Wilkinson 2002). These reef-related goods and services include producing seafood (coral reefs account for 9–12% of the world's fisheries), providing physical protection from coastal erosion, serving as the spawning grounds for numerous organisms, and providing recreation and tourism opportunities that support a number of human communities worldwide (Moberg et al. 1999).

While in their natural state, coral reefs are considered to be highly stable ecosystems that demonstrate a great degree of ecological complexity (Spalding et al. 2001). However, coral reef communities worldwide are being affected by a growing list of threats, including: (1) increasing development and tourism in coastal areas, (2) rising surface sea temperatures, (3) increasing sedimentation and turbidity, (4) increasing carbon dioxide levels and associated decreases in pH, (5) pollution, primarily with

respect to nutrient loading and to a lesser extent toxicants, (6) overfishing and the use of destructive fishing practices, (7) oil spills and discharge of oily ballast water from ships, and (8) pathogenic microorganisms. These threats appear to be affecting coral communities at an increasing rate (Bryant et al. 1998, Spalding et al. 2001, Lesser et al. 2007). While little data are available on the relative impact of such threats to coral reef communities or possible synergistic or additive impacts from combined threats, these threats are rarely found in isolation and are on the rise with increased losses of coral reef communities (Wilkinson 2004).

### **An Overview of Coral Disease**

During the past three decades, the number of coral diseases and syndromes identified (Goreau et al. 1998, Richardson et al. 1998a, Harvell et al. 1999, Porter et al. 2001, Bythell et al. 2004, Voss and Richardson 2006), the number of disease cases reported, and the number of coral species impacted by coral disease all have increased (Porter et al. 1999, Green and Bruckner 2000, Porter et al. 2001, Weil 2004, and Voss and Richardson 2006). This proliferation of coral disease threatens the diversity and viability of coral reef ecosystems. The first coral disease (black band disease) was reported in 1973 (Antonius 1973). Reports of new coral diseases were relatively rare over the next two decades, with plague (later termed white plague type I) first reported in 1977 (Dustan 1977), white band disease type I first reported in 1977 (Gladfelter et al. 1977), and red band disease first reported in 1983 (Goreau et al. 1998). However, beginning in the 1990s, reports of new coral diseases began to increase steadily (Sutherland et al.

2004), with current estimates indicating that at least 102 coral species have been affected on reefs in more than 54 countries worldwide (Green and Bruckner 2000). Worldwide, the majority of disease outbreaks occur in the Caribbean (Spalding et al. 2001, Sutherland et al. 2004), with 66% of all reported cases of coral diseases coming from the Caribbean region, which, notably, constitutes only 8% of the world's coral reef area (Green and Bruckner 2000).

Diseases are broadly defined as “impairment(s) of the normal state of the living animal...or one of its parts that interrupts or modifies the performance of the vital functions, is typically manifested by distinguishing signs and symptoms, and is a response to environmental factors..., to specific infective agents..., to inherent defects of the organism..., or to combinations of these factors,” (Medline Plus® 2008) and, therefore, may result from biotic agents, environmental factors, genetic anomalies, and/or nutritional disorders. Due to a lack of baseline data and epizootiological information, little is known about the causes of disease outbreaks in coral; how coral diseases are transmitted; the impact of these diseases on marine environments; and the relative impact of climate, environmental stressors, and anthropogenic factors on coral disease. From a search of the available literature, eight coral diseases have been identified in the Caribbean, with only half having documented etiological agents (Table 1).

**Table 1: Coral Diseases Affecting the Caribbean Region**

Coral Disease	Etiological Agent/Date Identified	Host Species	Geographic Location	Date Identified
<b>Aspergillosis</b> <sup>1</sup>	<i>Aspergillus sydowii</i> <sup>1</sup>	Gorgonian corals  (Koch's postulates have been fulfilled for <i>Gorgonia ventalina</i> and <i>G. flabellum</i> species, confirming the etiological agent as <i>A. sydowii</i> . <sup>2</sup>	Caribbean <sup>2</sup>	1996 <sup>2</sup> (Geiser et al.)
<b>Black Band Disease</b>	Originally thought to consist of a consortium of cyanobacteria ( <i>Phormidium corallyticum</i> ), sulfide-oxidizing bacteria ( <i>Beggiatoa</i> spp.), sulfate-reducing bacteria ( <i>Desulfovibrio</i> spp.), marine fungi, and other microbes <sup>3</sup> , with <i>P. corallyticum</i> hypothesized to be the primary pathogen. <sup>4</sup>  Subsequent research (using molecular methods rather than previous microscopic studies) has shown that the consortium is more complex and possibly less defined, consisting of a cyanobacterium species (possibly from one of three taxa, depending on the consortium sampled), <i>Desulfovibrio</i> spp., and <i>Cytophaga</i> spp. These recent molecular studies are also showing that <i>Beggiatoa</i> spp. are not present. <sup>5</sup> Other possible bacteria present in the consortium potentially include a proteobacterium similar to the etiological agent for juvenile oyster disease <sup>6</sup> and/or <i>Firmicutes</i> spp. <sup>7</sup>	Numerous species of scleractinian coral <sup>3</sup> and gorgonian coral species <sup>8</sup> .	Caribbean, Indo-Pacific, the Red Sea <sup>9</sup>	1973 (Antonius) <sup>10</sup>
<b>Dark Spots Disease</b> <sup>11</sup>	Unknown	Scleractinian corals, most commonly <i>Siderastrea</i>	Caribbean	1990s

<sup>1</sup> Alker 2001<sup>2</sup> Sutherland et al. 2004<sup>3</sup> Richardson et al. 1997, Viehman and Richardson 2002, Cooney et al. 2002<sup>4</sup> Cooney et al. 2002<sup>5</sup> Cooney et al. 2002 and Frias-Lopez et al. 2004<sup>6</sup> Cooney et al. 2002<sup>7</sup> Frias-Lopez et al. 2004<sup>8</sup> Dinsdale 2000 and Cooney et al. 2002<sup>9</sup> Bruckner and Bruckner 1997b and Dinsdale 2000<sup>10</sup> Antonius 1973 and Cooney et al. 2002

Coral Disease	Etiological Agent/Date Identified	Host Species	Geographic Location	Date Identified
		<i>siderea</i>		
<b>Red Band Disease</b> <sup>12</sup>	Unknown. Sutherland et al. 2004 reported that red band disease in scleractinian corals may be caused by a consortium consisting of cyanobacteria, sulfur oxidizing bacteria, and a nematode. However, because recent discoveries indicate that black band disease is associated with several different cyanobacteria species and because there have been no new reports of red band disease since the early 1990s, there is reason to suspect that red band disease is not distinct from black band disease. Research is also being conducted on red band disease in gorgonian corals. Richardson 1998 reported that photographs of the disease on gorgonians suggest it may actually be a stage of aspergillosis on sea fans.	<i>Gorgonia ventalina</i> , <i>Montastraea annularis</i> , and <i>Montastraea faveolata</i>	Bahamas, Puerto Rico, Florida Keys, Belize, Colombian Caribbean	1983
<b>White Band Disease</b> <sup>13</sup> (Two Types--- White Band I and II)	Unknown for WBI.  Current research indicates that the etiological agent for WBII is a <i>Vibrio</i> species, closely related to <i>V. harveyi</i> . <sup>14</sup>	Acroporid corals, specifically: <i>Acropora palmata</i> and <i>Acropora cervicornis</i>	Caribbean	WBI: 1977 WBII: early 1990s
<b>White plague</b> (Three Types--- White Plague I, II, and III)	<i>Aurantimonas corallicida</i> (WPII); Unknown for WPI and WPIII. <sup>15</sup>	Numerous species of scleractinian corals <sup>16</sup>	Caribbean; Indo-Pacific region <sup>15</sup>	WPI: 1977 WPII: 1995 <sup>17</sup> WPIII: 1999 <sup>14</sup>
<b>White Pox</b> <sup>18</sup>	<i>Serratia marcescens</i>	Exclusively affects <i>Acropora palmata</i>	Caribbean	1996
<b>Yellow Blotch/ Band Disease</b>	Current research presents the proposed Causal agent as four as yet unidentified species of <i>Vibrio</i> , possibly representing a bacterial consortium. <sup>19</sup>	Scleractinian corals, most commonly <i>Montastraea annularis</i> <sup>20</sup>	Caribbean <sup>18</sup>	1994 <sup>18</sup>

<sup>11</sup> Goreau et al. 1998 and Sutherland et al. 2004

<sup>12</sup> Goreau et al. 1998, Richardson 1998, Sutherland et al. 2004

<sup>13</sup> Sutherland et al. 2004

<sup>14</sup> Gil-Agudelo et al. 2006

<sup>15</sup> Richardson et al. 2001

<sup>16</sup> Sutherland et al. 2004

<sup>17</sup> Holden 1996

<sup>18</sup> Patterson et al. 2002

<sup>19</sup> Cervino et al. 2004

<sup>20</sup> Sutherland et al. 2004

While other coral diseases have been identified and/or proposed, including: skeletal eroding band disease, yellow band disease, pink-line syndrome, fungal-protozoan syndrome, and *Vibrio*-induced bleaching, these diseases, thus far, have not been identified in the Caribbean region (Sutherland et al. 2004). It is important to note that of these diseases, only one, *Vibrio*-induced bleaching, has had an etiological agent identified—the bacterium *Vibrio shiloi* in the case of the coral *Oculina patagonica* (Banin et al. 2000 and Sutherland et al. 2004), and the bacterium *Vibrio coralliilyticus* in the case of the coral *Pocillopora damicornis* (Ben-Haim et al. 2003).

### **An Overview of White Plague**

White plague (WP) is a disease that presents a significant threat to coral communities, given its ability to rapidly destroy coral tissue on multiple scleractinian coral species, which serve as important structural components of coral reef ecosystems. While the mechanism by which WP disease affects the soft tissue of coral is not known (Bythell et al. 2004), the disease is characterized in the literature as exhibiting a sharp line between apparently healthy coral tissue and white, exposed coral skeleton (Richardson et al. 1998a, Richardson et al. 2001, Denner et al. 2003). The margin of the affected coral tissue adjacent to the recently denuded skeleton is often straight and smooth—not serrated, undulating, or serpiginous—with clear separation between the remaining apparently healthy coral tissue and polyps and the exposed coral skeleton as coral tissue disappears from the surface of the coral (Work and Aeby 2006).

Corals exhibiting WP-like signs have been found in the Caribbean (Richardson 1998, Green and Bruckner 2000, Sutherland et al. 2004), the Gulf of Mexico (NOAA 2005a, Hickerson et al. 2005), the Indo-Pacific region (Sutherland et al. 2004), and most recently in the Red Sea (Barash et al. 2005, Thompson 2006). To date, 38 Caribbean scleractinian coral species have been affected by WP-like disease (Sutherland et al. 2004).

In 1995, an outbreak of WP-II occurred in the northern Florida Keys, affecting 17 of the 43 scleractinian corals in the coral reef ecosystem and 38% of a single species, *Dichocoenia stokesi*, in as little as 10 weeks (Denner et al. 2003). In 1996, WP-II was found in the lower Florida Keys and the Dry Tortugas, and in 1997, WP-II was identified in the upper Florida Keys (Richardson et al. 2001). In August and December 1996, WP was first reported on reefs near La Parguera, Puerto Rico. Appearing about two weeks after a hurricane hit the area, WP quickly spread among the genera *Colpophyllia*, *Dendrogyra*, *Diploria*, *Montastraea*, and *Siderastrea*. Disease signs were most prevalent in *Diploria labyrinthiformis* on Mario Reef, affecting 47% of the reef population of this species (Bruckner and Bruckner 1997a). In February 2005, the first widespread outbreak of WP-II-like syndrome was reported in the Gulf of Mexico at the Flower Garden Banks National Marine Sanctuary (FGBNMS) when WP-II-like signs were identified on the coral species *Diploria strigosa*, *Porites astreoides*, *Montastraea faveolata*, and *Montastraea franksii* (NOAA 2005a). Whereas WP-II-like signs had been observed in the FGBNMS prior to February 2005, it had been limited to individual, isolated colonies (NOAA 2005a).

WP was first documented in the Florida Keys in 1977 by Phil Dustan (Dustan 1977, Richardson et al. 1998a, Richardson et al. 2001). Dustan characterized plague as potentially a “suite of diseases that result in the death of coral tissue, leaving behind only a stark white skeleton” (Dustan 1977). Originally termed “plague,” the disease was later renamed white plague type I (WPI) to distinguish it from two diseases, white plague type II (WP II) and white plague type III (WP III), which were later shown to exhibit similar disease signs but progressed at a more rapid rate (Sutherland et al. 2004). To date, 13 species of Caribbean coral have reportedly been impacted by WPI (Sutherland et al. 2004). The linear rates of tissue loss from WPI-infected corals are relatively slow, measuring, at most, 3.1 mm/day; killing an entire colony in four months (Dustan 1977).

Experiments to test the transmission of WPI were conducted by Dustan using a sterile syringe to sample along the diseased margin and then emptying the syringe onto the surface of a test coral that had been abraded. Where lesions developed on the test coral, microscopic examination of the lesions revealed rod-shaped bacteria. Microscopic examination of the inoculants revealed small amounts of filamentous blue green algae accompanied by bacteria (Dustan 1977). No attempts were made by Dustan to culture or isolate bacteria from the samples (Dustan 1977, Richardson 1998), and, to date, no etiological agent for WPI has been described.

In 1995, a faster moving, more virulent form of a disease exhibiting the characteristic WP-like disease signs was documented in the Florida Keys (Richardson et al. 1998a, Richardson et al. 2001, Denner et al. 2003). The disease exhibited a similar pattern of tissue loss to WPI (Richardson et al. 1998a); however, the disease progressed at a more

rapid rate (up to 10 cm/day) (Richardson et al. 1998a, Richardson et al. 2001). Given the disease's similarities to the earlier disease outbreak characterized as plague, this epizootic was designated white plague II (Richardson et al. 1998a). To date, 18 species of Caribbean coral have reportedly been impacted by WP II (Sutherland et al. 2004).

Coral tissue and mucus samples collected from the diseased margin of three of the 1995 WP II-infected coral species using a syringe showed a predominance of small, motile, Gram-negative rod-shaped bacteria. The tissue and mucus samples collected with a syringe from the diseased margin, healthy tissue, and exposed tissue of three of these coral species were plated on Marine Agar 2216 and exhibited differences in bacterial populations (Richardson et al. 1998b, Denner 2003). Samples from the diseased margin were uniformly 1.5–2.0 mm in diameter and translucent but would turn yellow after several days in incubation (Richardson et al. 1998b).

Infection experiments demonstrated that bacterial isolates from a sample collected from the diseased margin of a WP II-infected coral could cause WP-like disease signs in the healthy coral. The experiments were conducted using two healthy *D. stokesi* coral colonies placed on marine agar plates inoculated with the bacterial isolate derived from the diseased margin of a WP II-infected *D. stokesi*. The healthy tissue exhibited WP II disease signs, beginning at their bases and losing all tissue within three days (Richardson et al. 1998a).

Carbon utilization testing of a bacterial isolate from a diseased-margin coral tissue sample plated on marine agar indicated that the bacterium was most closely related to the genus *Sphingomonas* (Richardson et al. 1998a, Richardson et al. 2001). Genetic analysis

of a 300-base pair 16S rRNA gene sequence of this isolate indicated that the sample was in the  $\alpha$ -subclass of *Proteobacteria*, matching most closely with *Erythromicrobium*, *Erythrobacter*, and *Sphingomonas*—exhibiting an identity of 89%, 87%, and 86%, respectively (Richardson et al. 1998a, Richardson et al. 2001). It should be noted that the 86–89% matches do not provide genus-level identification for the sample (Mignard and Flandrois 2006). Given that the first two groups, *Erythromicrobium* and *Erythrobacter*, are facultative aerobic photoheterotrophs and cultures of the disease isolate incubated under light yielded no photosynthetic pigment, these groups were ruled out, and it was decided that the bacterium was most closely related to the genus *Sphingomonas* (Richardson et al. 1998a, Richardson et al. 2001).

The strain of this bacterium and presumptive etiological agent of WP11 underwent further analysis in 2003. A replication sequence comparison of the redetermined 16S rRNA gene sequence from this study to the sequence from the original 1995 sample did not show significant degree of similarity (~82%). The researchers gave little explanation for the discrepancy, only that the discrepancy relates to the sequence originally deposited in GenBank (Denner et al. 2003). Phylogenetic analysis of the redetermined 16S rRNA gene sequence showed that it was in the class  $\alpha$ -proteobacteria and order Rhizobiales (Denner et al. 2003). The sequence fell within the group of families that includes Rhizobiaceae, Bartonellaceae, Brucellaceae, and Phyllobacteriaceae, but showed no similarity with any of the established taxa in these families at a rate greater than 92%; it was shown to be closest to *Mesorhizobium mediterraneum* (Denner et al. 2003). As a

result, researchers felt that it belonged in a new genus and species and proposed the new species *Aurantimonas coralicida* (Denner et al. 2003).

In 1999, a disease exhibiting white plague-like signs, which advanced at an even more rapid rate, was documented and named white plague type III (WPIII) (Richardson 2000, Richardson et al. 2001, NOAA 2005b). Linear tissue loss occurred at a rate of up to 10 cm/day and could begin on the sides or top of coral colonies (NOAA 2005b). Two species of Caribbean coral, *Colpophyllia natans* and *Montastraea annularis*, have reportedly been affected by WPIII (Sutherland et al. 2004). An etiological agent for WPIII has not been identified (Richardson 2000, Lesser et al. 2007).

In 2002, a rapid tissue loss, WP-like disease was first observed on the Eilat coral reef in the Gulf of Aqaba in the Red Sea (Barash et al. 2005). Two major reef-building coral genera, *Favia* and *Goniastrea*, were most affected by the disease, with approximately 10% of these coral colonies either dead or showing signs of the disease (Barash et al. 2005). Signs of this form of WP-like disease begin as a small spot of denuded skeleton (Barash et al. 2005). The disease can spread at a rate of up to 11 mm/day (Barash et al. 2005).

Infection experiments in which healthy coral tissue was placed in an aquarium with either diseased coral tissue or samples of water surrounding a diseased coral resulted in infection, and that infection was not coral species dependent (Barash et al. 2005). Further testing identified a culturable bacterial isolate (BA-3) that, in combination with a filtrate created from a 0.2  $\mu\text{m}$ -filtered-sample of water taken surrounding an infected coral colony, caused disease signs in healthy coral tissue when placed into an aquarium with

the healthy tissue (Barash et al. 2005). Researchers hypothesized that this isolate is an extracellular virulence factor required for infection and that it could act either by causing the coral host to become more susceptible to disease or by causing the bacterium to become more virulent (Barash et al. 2005).

Phenotypic characterization of the BA-3 bacterial strain indicated that it was a Gram-negative, motile, rod-shaped bacterium with a single polar flagellum (Barash et al. 2005). Initial phylogenetic analysis of the 16S rRNA gene sequence showed that the BA-3 strain (1356 base pairs) was most closely related to *Eubostrichus dianaepibacterium* (94%) (Barash et al. 2005). In 2006, the BA-3 bacterial strain was further characterized. Cells grown on Marine Agar 2216 were 0.5–0.8 µm in width and 1.0–2.0 µm in length (Thompson 2006). Cells formed translucent, convex, smooth-rounded colonies with entire margins. After three days in incubation at 25 °C, the colonies became cream colored and grew to 3 mm in size (Thompson 2006). Phylogenetic analysis of the 16S rRNA gene sequence showed it to be a member of the *Colwelliaceae* family most closely related to *Thalassomonas ganhawensis* and *T. viridians* (95% and 94%, respectively) (Thompson 2006). Based on the fact that researchers found that several key phenotypic and chemotaxonomic characteristics of the genus *Thalassomonas*, such as oxidase activity, pigment, and growth exhibited at 15 or 37°C, were not exhibited by the bacterial strain, the researchers proposed that the bacterium represented a novel species and identified that species as *T. loyana*, sp. nov. (Thompson 2006).

## 2. Hypotheses

Bacteria are a normal part of the coral-surface microlayer, which extends a few millimeters above the surface of the coral, and are thought to play a role in nutrient cycling within the reef community (Lyons et al. 1998). However, when coral communities are affected by coral diseases, the normal coral-surface microbial community can be disrupted, potentially resulting in damage to coral colonies, their microflora, and ultimately the entire reef structure.

Research into the causes of coral diseases and how these diseases affect healthy coral communities is difficult, given that currently little is known about the abundance and diversity of bacterial populations found in healthy coral communities, the role they play in coral biology, or how these populations become altered with the introduction of etiological agents into coral communities. While limited, current research examining the diversity and structure of bacterial populations associated with healthy coral communities indicates that the bacterial populations associated with individual coral species are not random associations (Rohwer et al. 2002). Different coral species, even those found adjacent to one another, possess distinct bacterial communities (Rohwer et al. 2002), and individual coral species, even those separated by large distances, are associated with

distinct bacterial populations (Rohwer et al. 2001, Frias-Lopez et al. 2004, Rohwer et al. 2002).

When coral communities are affected by coral diseases, are such changes caused by etiological agents introduced from outside of the coral reef system (e.g., through sewage or terrestrial runoff)? Is the etiological agent part of the “normal” environment of the coral community, but potentially being affected by environmental factors (e.g., increased ultraviolet radiation, warmer water temperatures, increased nutrients)? If so, are environmental factors either (1) creating a favorable environment, that allows the etiological agent(s) to reach large enough population numbers to become harmful to coral communities or (2) altering these etiological agent(s), causing them to become more virulent to the coral reef community? Is the same etiological agent always associated with disease (e.g., white plague-like signs) or could there be more than one etiological agent producing the same disease signs in different geographic locations?

This investigation focused on the culturable bacterial community, using 16S rDNA fingerprinting and sequencing techniques to study diseased tissue samples from coral communities from the FGBNMS that showed signs similar to WP11 (1) to compare the microbial abundance and diversity between the healthy and diseased coral tissue, and (2) to determine whether the proposed etiological agent of WP11, the bacterium *A. coralicida*, was present in the diseased coral tissue. The null hypotheses tested for this research were:

- H<sub>1</sub>: The abundance of culturable bacterial communities does not differ among tissue from healthy coral colonies, apparently healthy tissue from diseased coral colonies, and tissue from the diseased margin.
- H<sub>2</sub>: The species diversity of culturable bacterial communities does not differ among healthy coral tissue, healthy tissue on diseased coral, and diseased-margin coral tissue samples.
- H<sub>3</sub>: *Aurantimonas coralicida*, the proposed etiological agent for WPPII, is found only in diseased-margin coral tissue samples.

### 3. Methods

#### Sample Location

All samples were collected from the FGBNMS, which is located in the Gulf of Mexico, approximately 110 nautical miles (nmi) southeast of Galveston, Texas (DOI 1999) (Figure 1). The sanctuary, designated in 1992, is the northernmost coral reef on the continental shelf of North America (DOI 1999). Geographically, the FGBNMS' coral reef ecosystem sits atop two salt domes that have been overlain by calcium carbonate deposited by reef-building corals and calcareous algae (DOI 1999). The sanctuary totals approximately 42 nmi<sup>2</sup> (FGBNMS 2005) and includes three separate geographic areas—East Flower Garden Bank, covering 19.2 nmi<sup>2</sup>, located at 27°54'35.9"N, 93°35'49.7"W; West Flower Garden Bank, covering 22.5 nmi<sup>2</sup>, located at 27°52'35.1"N, 93°48'54.1"W; and Stetson Bank, covering less than 1 nmi<sup>2</sup>, located at 28°09.956'N, 94°17.859'W (FGBNMS 2005).



**Figure 1:** Map of the Flower Garden Banks National Marine Sanctuary (Image courtesy of [http://soundwaves.usgs.gov/2005/07/.](http://soundwaves.usgs.gov/2005/07/))

Diverse in marine community structure, the sanctuary supports 20 species of corals, more than 120 species of fishes, and over 253 invertebrate species (DOI 1999). The sanctuary consists mostly of large brain and star corals and lacks gorgonian soft corals and branching corals (e.g., staghorn corals) (Gittings 1994). Primary threats to the FGBNMS reef ecosystem are thought to include potential impacts from hydrocarbon drilling in the region and the increase in recreational use following the creation of the FGBNMS (Gittings et al. 1992). Studies of the reef ecosystem dating from 1992 indicate that the coral populations on the FGBNMS have remained relatively unchanged with respect to population species dominance, diversity, and evenness and that coral growth rates have remained steady since the late 1970s, when studies of the reef ecosystem were first undertaken (Gittings et al. 1992). These studies also demonstrated a relatively low rate of coral disease in the reef ecosystem, with mortality rates of less than 1.6%.

## Sample Collection

Samples were collected during a research cruise sponsored by the FGBNMS on board the *M/V Fling* on May 22-26, 2005. All samples were collected by Dr. Robert B. Jonas and Geoffrey M. Cook using standard SCUBA techniques. As outlined below in Table 2, coral tissue samples were collected at four sites in the FGBNMS, two on the West Bank—Buoy #1 and Buoy #2—and two on the East Bank—Buoy #1 and Buoy #2. Tissue and skeleton cores were collected from healthy corals and corals showing signs of active white plague-like disease. When the samples were collected, it was noted that the algal turf associated with the coral was often in close proximity to the disease-healthy tissue interface, suggesting that the disease progression was relatively slow. In total, three different species/morphologies of coral were sampled: *C. natans* (at the West Bank sampling site at Buoy #1), *M. franksii* (at the West Bank sampling site at Buoy #2), and *M. faveolata* (at the East Bank sampling sites at Buoys #1 and #2).

It should be noted that *M. franksii*, *M. faveolata*, and a third coral “species,” *M. annularis*, have very similar morphologies, are known to share intermediate morphologies, and have been shown to have a very close genetic relationship (Manica and Carter 2000). Because of these similarities, these three coral “species” are often treated as a single taxon (Manica and Carter 2000). For the purposes of this study, *M. franksii* and *M. faveolata* are identified as separate species. However, the implications of these species’ close morphological and genetic relationship are considered.

**Table 2:** Sampling Scheme for FGBNMS Study

<b>Location</b>	<b>Buoy #</b>	<b>Latitude/ Longitude</b>	<b>Date Sampled</b>	<b>Coral Species Sampled</b>
West Bank	1	27°52'35.1"N, 93°48'54.1"W	May 25, 2005	<i>C. natans</i>
West Bank	2	27°52'31.3"N, 93°48'51.3"W	May 25, 2005	<i>M. franksii</i>
East Bank	1	27°54'35.9"N, 93°35'49.7"W	May 23-24, 2005	<i>M. faveolata</i>
East Bank	2	27°52'31.3"N, 93°48'51.3"W	May 24, 2005	<i>M. faveolata</i>

For each of the four sampling sites chosen, a total of nine cores were collected. Three samples were taken from the diseased margin of a coral colony, three samples were taken from the apparently healthy tissue on the diseased colony, and three samples were taken from a separate, but nearby, control colony of the same species having no evidence of disease.

Each coral sample was labeled to designate whether it was taken from the West Bank (FWC) or East Bank (FEC) of the FGBNMS. Each pair of healthy and diseased corals from a sampling site was labeled with a paired alphanumeric system—101D/101HD/106H, 201D/201HD/206H, 301D/301HD/306H, and 401D/401HD/406H—with the diseased-margin coral tissue/apparently healthy tissue on diseased coral/healthy coral tissue samples labeled respectively, as shown in Table 3.

**Table 3:** Labeling Scheme for FGBNMS Samples

<b>Pair #1</b> <i>(C. natans)</i>	<b>Pair #2</b> <i>(M. franksii)</i>	<b>Pair #3</b> <i>(M. faveolata)</i>	<b>Pair #4</b> <i>(M. faveolata)</i>
FWC101D1	FWC201D1	FEC301D1	FEC401D1
FWC101D2	FWC201D2	FEC301D2	FEC401D2
FWC101D3	FWC201D3	FEC301D3	FEC401D3
FWC101HD1	FWC201HD1	FEC301HD1	FEC401HD1
FWC101HD2	FWC201HD2	FEC301HD2	FEC401HD2
FWC101HD3	FWC201HD3	FEC301HD3	FEC401HD3
FWC106H1	FWC206H1	FEC306H1	FEC406H1
FWC106H2	FWC206H2	FEC306H2	FEC406H2
FWC106H3	FWC206H3	FEC306H3	FEC406H3

Key:

- FW or FE = FGBNMS West Bank or FGBNMS East Bank
- C = Coral
- 101/106, 201/206/301/306/401/406 = Coral Pair Numbering System (101, 201, 301, 401 correspond to diseased tissue; 106, 206, 306, 406 correspond to apparently healthy tissue from a non-diseased colony)
- D/HD/H = Diseased-Margin Coral Tissue/Healthy Tissue from a Diseased Coral Colony/Apparently Healthy Coral Tissue from a Non-Diseased Colony
- 1, 2, 3 = Identification of triplicate sample

Samples were collected by using a two-pound sledge to hammer a 1.6-cm stainless steel core tube, with a sharpened edge, into the coral tissue to a depth of about 0.5–1.0 cm (Cook 2006). The carbonate disks were broken free from the colony by gently tapping the side of the core tube with the sledge. In an effort to minimize potential contamination of healthy samples with material from diseased coral samples were taken in the following order: (1) control colony tissue, (2) apparently healthy tissue on the diseased colony, and (3) tissue from the diseased margin (Cook 2006). Once freed from the coral colony, disk samples contained in the core tubes were immediately transferred to 50-ml centrifuge tubes and capped. The holes in the coral colony left as a result of sampling were patched using underwater epoxy (JB Epoxy Waterweld, JB Weld

Company, Sulphur Springs, TX), with care taken to ensure that the patching materials were smooth and flush with the level of tissue of the coral colony (Cook 2006). A photograph of the colony was then taken, with a written record of the appropriate sample label visible in the picture.

Immediately upon returning to the boat, each carbonate disk sample was removed from the stainless steel core tube using a steel rod, forceps, and/or locking pliers that were sterilized and depurinated in a 10% bleach solution (10 ml bleach:90 ml seawater) and then rinsed in filtered (0.2  $\mu\text{m}$  porosity), boiled seawater (Cook 2006). Care was taken to ensure that disks were handled in such a manner that only the sample disks' edges were touched with the forceps and locking pliers. Once removed from the core tube, the carbonate disks were trimmed to approximately 3 mm in thickness using a diamond-edged wheel on a Dremel® tool, which was sterilized, depurinated, and rinsed, as described above. The disks were then cut in half, with one-half of each disk used for molecular analysis (not addressed in this thesis research) and the other half used for culturable bacterial community analysis. As soon as possible, but always within 4 hours of sample collection, samples were crushed, diluted in sterile seawater, and spread onto plates of half-strength Marine Agar 2216 (Difco, Becton, Dickinson, and Company, Sparks, MD), as detailed below. The inoculated plates were incubated in the dark at approximately 23 °C while on board the research vessel and, on return to port, shipped to the George Mason University (GMU) campus in Prince William County, Virginia. Samples for molecular community analysis were placed into cryovials, frozen at an approximate temperature of -196 °C, and also shipped to GMU.

### **Culturable Microbial Community Analysis:**

The culturable microbial community for each sample was analyzed using serial dilution and spread plating of the samples on half-strength Marine Agar 2216. To prepare the dilution series, one-half of each of the core samples [measuring 0.8 cm in diameter and 1.0 mm in (coral tissue) thickness<sup>21</sup>, for a total volume of 0.10 ml of coral tissue; as  $1/2 (\Pi r^2 \times h = 1/2 (3.14 \times 0.8 \text{ cm} \times 0.8 \text{ cm} \times 0.1 \text{ cm}) = 0.10 \text{ cm}^3 = 0.10 \text{ ml})$ ] was crushed using stainless steel needle-nose pliers that had been sterilized, depurinated, and rinsed with seawater from the sample site. The crushed material was further ground directly in the bottom of the conical, polypropylene tube using a bleached and rinsed steel rod with a rounded end. Nine milliliters of filtered (0.2  $\mu\text{m}$  porosity), sterilized seawater were added directly to the pulverized coral sample in the conical tubes. This produced a nominal initial dilution of  $10^{-1}$ . The coral suspension was then vortexed at high speed for 2 minutes before serial dilution. Serial dilutions were then carried out as needed (between  $10^{-2}$  and  $10^{-6}$ ), using 1:10 dilution ratios of filtered, sterilized seawater.

Sample dilutions were plated onto half-strength Marine Agar 2216. The agar was prepared using Difco™ Marine Broth 2216, at one-half the concentration recommended, with additional agar added to bring the final concentration to 2%. The increased agar content helps keep motile bacteria from covering the surface of the medium. Half-strength artificial seawater (Seven Seas Marine Mix, Utility Chemical Co., Paterson, NJ)

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<sup>21</sup> While the coral samples (composed of both coral skeleton and tissue) were trimmed to a thickness of approximately 3.0 mm, the bacterial abundance measurements were based on a 1.0 mm thickness, which represents the presumed thickness of the coral's surface microlayer of tissue (minus the coral skeleton) (Ritchie and Smith 2004). While some bacterial colonies are associated with coral skeleton, the majority are associated with this coral surface microlayer.

adjusted to pH 8.2 was used to prepare the medium to maintain full-strength salt concentration in the final products. When preparing the petri dishes, all of the medium was dispensed in a laminar flow hood to reduce the possibility of contamination of the plates. Once the plates were poured, they were allowed to sit covered in the hood for three to four days without forced air flow (24 hours with forced air flow is equally efficient) to allow the medium to dry slightly to reduce excess moisture on the surface of the medium. This procedure was necessary to control bacterial migration on the surface of the medium in high-humidity environments.

Triplicate plates were inoculated with 0.1 ml aliquots of each sample dilution ( $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ ). The aliquots were then distributed over the surface with sterile, polypropylene spreaders. Total incubation time ranged from 7 to 10 days at approximately 23 °C.

After incubation, high-resolution digital photographs of all of the plates were obtained with a Nikon D80 SLR camera mounted on a tripod. Lighting was provided by two fluorescent light banks, one on either side of the plate. Where possible, these digital images of plates with 20–200 individual colony forming units (CFUs) per plate were analyzed to enumerate the total culturable microbial abundance for each sample using the BioQuant Nova Prime (version 6.90.10) image analysis software. This range of CFUs per plate is desirable for estimating bacterial abundance using BioQuant imaging analysis software. In some cases, the abundance of CFUs per plate exceeded this value even at the highest dilution. In those cases, the highest dilution plates were used for analysis. After

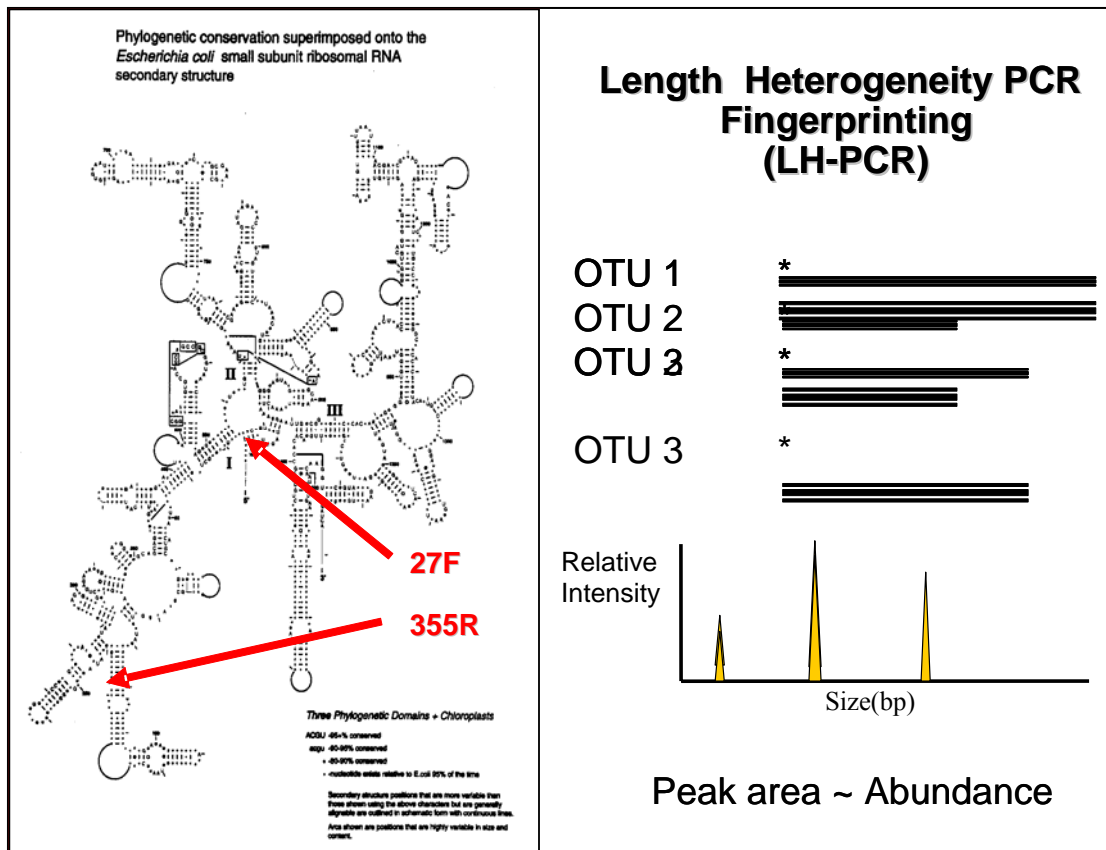
the photographs were taken, the plates were packaged in plastic bags and refrigerated at 4 °C.

Depending upon the size, uniformity, and density of bacterial colonies on the spread plates in the photos, the following three types of BioQuant analyses and/or a combination of these analyses were used to enumerate the bacterial colonies in each plate photograph: (1) trace-based array counts, (2) pixel-based array counts, and/or (3) manual counts. (For a more detailed explanation of the BioQuant analysis, see Appendix 1.)

### **Molecular Analysis**

Fingerprinting and sequencing of 16S rDNA of selected bacteria colonies from apparently healthy coral tissue, diseased-margin tissue, and apparently healthy tissue on diseased coral plated on half-strength Marine Agar 2216 were used to analyze the community composition. DNA fingerprinting relies on highly conserved regions of the 16S rRNA gene (Figure 2) and uses naturally occurring differences in the length of the gene that occur among different species to develop a genetic profile, or fingerprint, of a microbial community (Mills 2007). In LH-PCR, the fluorescently labeled products are sequenced using an automated DNA sequencer that converts the fluorescence data into electropherograms, the peaks of which are used to represent different OTUs, and the height of each peak is used to represent the relative abundance for that bacterial segment found in a bacterial sample (Mills 2007). Differences in the lengths of amplicons correspond to different OTUs, and the OTUs can be used to represent individual organisms and to describe bacterial diversity and, potentially, relative abundance within a

microbial community. Because it is possible for different taxa to have identical amplicon lengths and for a single taxon to have multiple operons, positive taxonomic identification requires DNA sequencing.



**Figure 2:** Diagram of the 16S rRNA Gene. Use of the 27F and 355R universal bacterial primers exploits variability in the length and genetic sequence in the 16S rRNA gene of all prokaryotic organisms. The attachment of a fluorescent probe to the 27F primer enables quantification of the relative abundance of different OTUs. (Image courtesy of Dr. Patrick Gillevet.)

## **Selection of Culturable Colonies**

The high-resolution digital photographs were used to differentiate and select examples of morphologically distinct types of bacterial colonies cultured from each sample site. For each sample (i.e., triplicate dilution samples of H, D, HD coral tissue samples taken from each of four sampling sites, for a total of 36 individual samples), a total of eight distinct colonies were selected for analysis, for a total of 288 bacterial colonies sampled. Each set of dilution plate photographs from a sample (including the entire dilution series) was examined to identify eight morphologically distinct colonies using visual characteristics—including colony size, shape, color, elevation, and edge type. Care was taken to select eight of the morphologically most distinct colonies within a given sample and also across a given set of triplicate samples from a sampling site. Particular care was taken to select for translucent, yellow bacterial colonies (Richardson 1998b) and opaque, circular, entire, convex, smooth and golden-orange bacterial colonies (Denner 2003) characteristic of *A. coralicida*. The graphical software program Microsoft Paint was used to mark the selected colonies on the digital photos, and the selected photos and a description of the selected colonies were recorded in a lab notebook.

## **Sampling, Cell Lysis, and Extraction of DNA**

Using the digital photos to identify the selected colonies on the corresponding spread plates, the subsamples of the selected colonies on the stored plates were collected using autoclaved, sterilized toothpicks. These subsamples were placed into the individual wells of a 96-well plate and mixed with 50.0  $\mu$ l Tris-EDTA (TE) (pH 8.0) (Cook 2006).

The plate was then placed into a centrifuge (Jouan CR412 Benchtop Centrifuge) and spun momentarily until the centrifuge reached 2000 rpm to ensure that the buffer and sample were thoroughly mixed. The cells were then lysed by placing the 96-well plate into the 96-Well GeneAmp® PCR System 9700 (Applied Biosystems; Foster City, CA) and heating the samples at 96 °C for 15 minutes. The plates were then returned to the centrifuge and spun at 2000 rpm for 3 minutes to separate out the lysed cell fragments into pellet form and leave the DNA in the supernatant (Cook 2006). The samples were then either refrigerated (if held overnight) or frozen (if kept longer than overnight) until amplification using the Polymerase Chain Reaction (PCR).

### **PCR Amplification and DNA Fingerprinting**

PCR amplification was conducted using universal bacterial primers (including a fluorescently labeled forward and a reverse primer) to amplify the 328-base pair region between the 27 forward primer (27 F; 5'-AGA GTT TGA TCM TGG CTC AG-9') and 355 reverse primer (355R; 5'-GCT GCC TCC CGT AGG AGT-9') regions of the samples. The 27F primer was labeled using a fluorescent phosphoramidite dye, 6-FAM, which allowed the relative abundance of Operational Taxonomic Units (OTUs) to be estimated (Suzuki et al. 1998). For each PCR, a master mix was created containing DEPC water, 10X reaction buffer, 25 mM magnesium mixture, dNTPs (2.5 mM each of each of the four dNTPs), 0.1% BSA, 5 units/μl of Taq polymerase, 10 μM of a fluorescently labeled universal 27F bacterial primer, and 10 μM of a universal 355R bacterial primer. The master mix was then aliquoted into PCR tubes, and negative controls (no DNA

added), positive controls (*E. coli* DNA added), and DNA extracts from the sample colonies were added (Mills 2003).

To amplify the DNA segments, the prepared samples were loaded into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA), which was set to perform 35 cycles using the following temperature-time profile: (1) 95 °C for 11 seconds to denature the DNA strands, (2) cycling between 95 °C for 30 seconds (to denature the DNA strands), 48 °C for 30 seconds (to allow the primers to anneal), and 72 °C for 2 minutes for a total of 34 cycles, with 5 seconds added to each step during each successive cycle (to allow the polymerase to elongate the new DNA strands), and finishing the cycles with (3) 72 °C for 30 minutes. At completion, the samples were cooled to 4 °C and held at 4 °C until the products were analyzed (Mills 2003).

PCR reaction products were analyzed using electrophoresis with a 1% agarose gel containing ethidium bromide in 1X TAE buffer solution to confirm the purity and size of the PCR product. The Lambda DNA Hind III size marker was run on the agarose gel with the PCR products to approximate their molecular weights.

Length heterogeneity (LH)-PCR was used to characterize the diversity and relative abundance of the selected culturable bacterial colonies associated with coral tissue samples. The fluorescently (6-FAM) labeled PCR products were separated and (relatively) quantified using the SpectuMedix SCE9610 capillary electrophoresis genetic analysis system (Mills 2003). The sequencer converted the fluorescence data into electropherograms, the peaks of which represent different sizes of bacterial fragments and the height of each peak represents the relative abundance for that bacterial segment.

Bacterial lengths were calculated using a size standard. The amplicon length heterogeneity profiles were used to determine the degree of bacterial diversity in the samples, with the number of peaks in the profile corresponding to the differing amplicon lengths detected—operational taxonomic units—and the relative quantities of the amplicons represented by the areas under the peaks in the profile (Mills 2007).

### **PCR Amplification and DNA Sequence Analysis**

As with the DNA fingerprinting, PCR amplification for DNA sequence analysis was conducted using universal bacterial primers to amplify the 328-base pair region between the 27F primer and 355R primer regions of the samples. For each PCR, the master mix was created identically to the master mix for DNA fingerprinting, with the exception that 10  $\mu$ M of un-labeled universal 27F bacterial primer was used. To amplify the DNA segments for sequencing, the prepared samples were loaded into the GeneAmp® PCR System 9700, which was set to perform 35 cycles using the same temperature-time profile as for the DNA fingerprinting (Mills 2003). As above, the reaction products were cooled to 4 °C and held at 4 °C until the products were analyzed. The PCR products were analyzed using gel electrophoresis as described above.

To purify the PCR products for sequencing, 10  $\mu$ l of product was pipetted into a clean 96-well plate (for those PCR products totaling less than 10  $\mu$ l, deionized water (diH<sub>2</sub>O) water was added, in quantities no more than 3  $\mu$ l in volume, to bring the total volume to 10  $\mu$ l) and mixed with 18  $\mu$ l of AMPure PCR Bind buffer (Agencourt® Bioscience Corporation, Beverly, MA)—the buffer selectively binds PCR amplicons 100

base pairs and larger to magnetic beads. After being thoroughly mixed, the purification plate was placed onto a magnetic SPRIplate (a 96-well magnet plate) to separate out the magnetic beads from solution. The liquid in each of the 96 wells was then aspirated, and the wells were rinsed with 70% ethanol and then aspirated twice to remove any contaminants (e.g., excess nucleotides, salts, and enzymes) from the beads. The 96-well plate was then air dried. Once dried, 40  $\mu$ l of TE buffer was added to each well to extract the PCR amplicons back into solution. The plate was then spun in the centrifuge for 30 seconds and remixed by pipetting the solution up and down in the plate. The magnetic beads were then separated from the elution mixture by placing the 96-well plate on the magnetic SPRIplate. A small amount of cleaned PCR product (4.0  $\mu$ l) was removed for quantification, the remaining quantity of cleaned PCR product was covered with Nashua® 322 foil tape (Covalence Adhesives, Franklin, MA) and frozen until the sequencing reaction was performed. To ensure that sufficient product remained after cleaning, 4.0  $\mu$ l of the cleaned PCR product was analyzed using 1% agarose gel electrophoresis as above.

The sequencing reaction was conducted using the cleaned PCR products. Into a new 96-well plate, the following were added to each well: 4  $\mu$ l of Big Dye Terminator™ (Ready Reaction Mix) (Applied Biosystems, Foster City, CA), 2  $\mu$ l of 5X reaction buffer, 2  $\mu$ l diH<sub>2</sub>O, 1.5  $\mu$ l primer (at a final concentration of 3.2 pmol), and 9.0  $\mu$ l cleaned PCR product plus 1.5  $\mu$ l diH<sub>2</sub>O. PGem® (Promega, Madison, WI) was used as a positive control, and M13 was used as a negative control. The plate was then covered with Nashua® 322 foil tape, placed in the centrifuge, and spun to 2000 rpm to thoroughly mix

the reaction mixture. To conduct the dye terminator sequencing reaction, the prepared 96-well plate was loaded into the GeneAmp® PCR System 9700, which was set to perform one cycle at 96 °C for 1 minute followed by 50 cycles of the following: 96 °C for 30 minutes, followed by 50 °C for 15 seconds, followed 60 °C for 4 minutes. Reaction products were cooled to 4 °C and held at 4 °C until clean up (Mills 2003).

The sequencing reaction mixture was then cleaned up using Sephadex G-50® powder (Sigma Chemical Company, St. Louis, MO) to remove excess, unincorporated dye terminator. To prepare the Sephadex columns for filtration, dry Sephadex G-50® was loaded into the wells of a Multiscreen® PCR<sub>96</sub> Millipore 96-well filter plate, and a multiscreen filtration plate was attached. To hydrate the plate, 300 µl of diH<sub>2</sub>O was added to each lane of the plate, and the plate was capped and allowed to sit for 3 hours. After 3 hours, an additional 50 µl of diH<sub>2</sub>O was added to each well of the plate. The plate was then placed into the centrifuge and spun at 2000 rpm for 3 minutes to spin excess water out of the plate. To load the sequencing reaction products, the Sephadex columns (i.e., each well of the 96-well Millipore filter plate) were pre-wetted with 25 µl diH<sub>2</sub>O and then 20 µl of the sequencing reaction were loaded into the wells of the plate. The plate was then loaded into the centrifuge and spun at 2000 rpm for 8 minutes to filter the sequencing reaction products. The DNA, which collects in the 96-well reaction plate, was then dried in the Speed Vac for 110 minutes. Once dried, the sample plate was covered with Nashua® 322 foil tape and frozen at -20 °F until sequencing analysis. Sequencing was conducted using the SpectruMedix® SCE9610 capillary electrophoresis genetic analysis system (Mills 2003).

Sequence data were analyzed and manually corrected using Sequencer 4.1 software (Gene Codes Corporation, Ann Arbor, MI). Using the Sequencer 4.1 software, the chromatograms were aligned, using a minimum match percentage of 85% and minimum overlap of 20 base pairs. The chromatograms were compared and analyzed, and individual base pair edits were made manually, as necessary. DNA sequences corresponding to chromatograms that did not comply with the 85% match criteria and, upon manual inspection, appeared faulty, were deleted. The corrected sequence data were then analyzed using the Ribosomal Database Project II, Release 8.1, which compares the sequence data against a database of known DNA sequences (Ribosomal Database Project 2007, Cole 2005) in GenBank, the European Molecular Biology Laboratory, and the DNA Data Bank of Japan (Maidak et al. 2001).

## RESULTS

### **Culturable Bacterial Count Data**

#### *Mean Abundance Data -- All Samples*

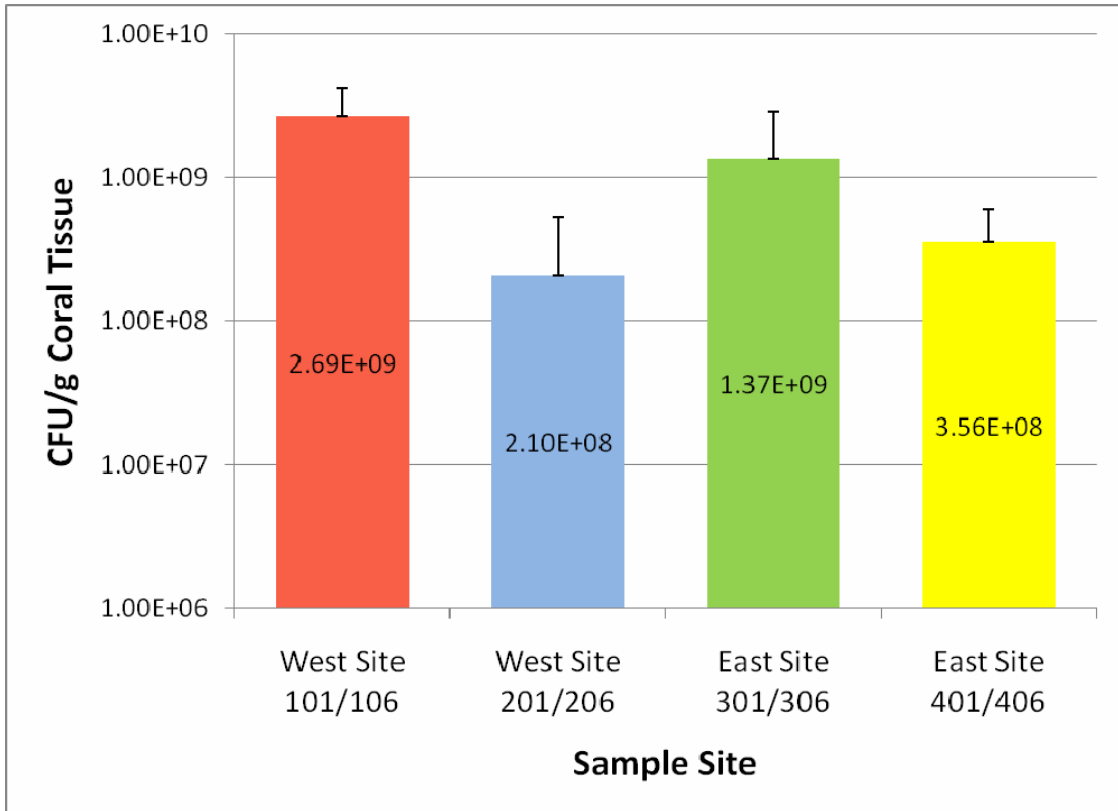
Overall, mean bacterial abundance was large at  $1.16 \times 10^9$  CFU/g of coral tissue (Table 4), and there was substantial variability in bacterial abundance among all the coral samples [standard deviation (SD) =  $1.46 \times 10^9$  CFU/g coral tissue]. The highest average tissue type-specific bacterial abundances occurred in the diseased coral tissue while the lowest was in the apparently healthy tissue of diseased corals (Table 4). There was substantial variability in bacterial abundance among sample types, as indicated by the large standard deviation. However, a one-way analysis of variance (ANOVA) of means of bacterial abundance among all three tissue types ( $P = 0.153$ ) (Appendix 2, Table A-1) did not support a hypothesis of significant differences among these coral sample types.

**Table 4:** Mean and Standard Deviation of Culturable Bacterial Abundance for All Coral Tissue Samples

<b>Coral Tissue Sample Type</b>	<b>N</b>	<b>Minimum (CFU/gram coral tissue)</b>	<b>Maximum (CFU/gram coral tissue)</b>	<b>Mean (CFU/gram coral tissue)</b>	<b>Standard Deviation</b>
All Coral Tissue Samples	102	9.10E+06	6.53E+09	1.16E+09	1.46E+09
D	35	8.19E+07	3.89E+09	1.50E+09	1.25E+09
HD	33	1.82E+07	4.21E+09	8.12E+08	1.09E+09
H	34	9.10E+06	6.53E+09	1.13E+09	1.87E+09

***Mean Abundance Data -- By Sample Site***

The differences in mean bacterial abundance among all four sample sites were significant ( $P = .000$ ) (Appendix 2, Table A-2). The West Bank 101/106 site had the greatest mean culturable bacterial abundance, followed by East Bank 301/306, East Bank 401/406, and West Bank 201/206 (Figure 3). Bacterial abundance at site 101/106 was significantly greater than in sites 201/206, 301/306, and 401/406 ( $P = .000$ ,  $.012$ , and  $.000$ , respectively) (Table 5). Bacterial abundance at sites 301/306 and 401/406 were significantly greater than site 201/206 ( $P = .012$  and  $.021$ , respectively) (Table 5). However, differences in bacterial abundance among sites 301/306 and 401/406 (both *M. faveolata* coral colonies) were not statistically significant ( $P = .101$ ) (Table 5).



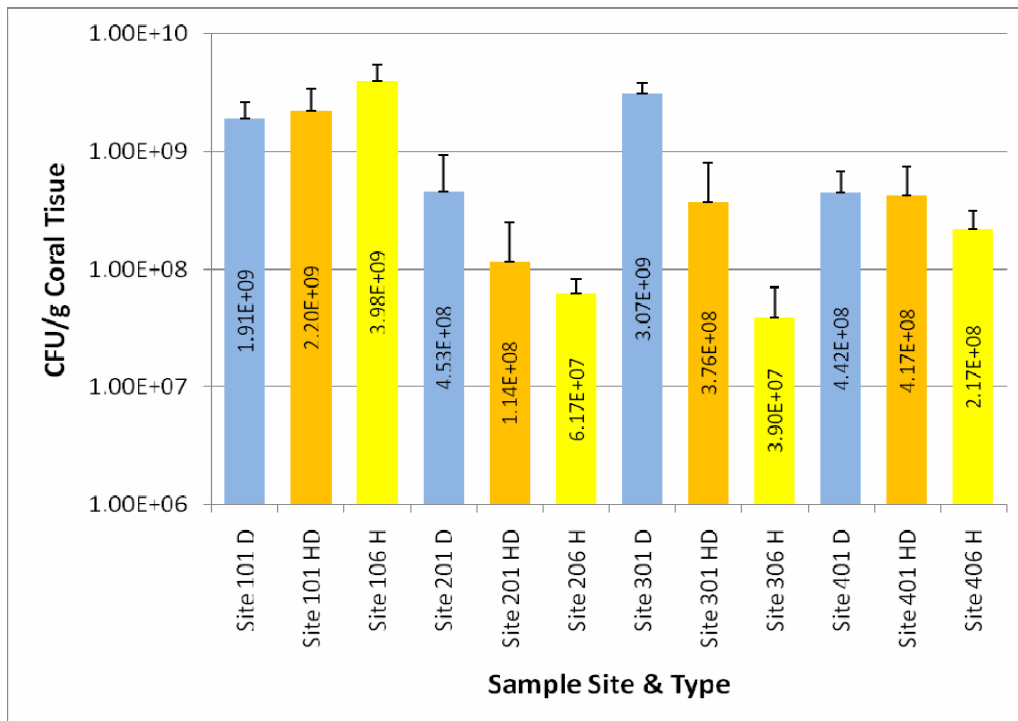
**Figure 3:** Mean Culturable Bacterial Abundance by Sample Site

**Table 5:** Pair Relationships for Site-Specific Bacterial Abundance Data Analyzed Using Wilcoxon Matched Pair Signed-Rank Test

Site 101/106	Tissue Type Pairing	Site 101/106> Site 201/206	Site 101/106> Site 301/306	Site 101/106> Site 401/406
	P	.000	.012	.000
Site 201/206	Tissue Type Pairing	Site 201/206< Site 101/106	Site 201/206< Site 301/306	Site 201/206< Site 401/406
	P	.000	.012	.021
Site 301/306	Tissue Type Pairing	Site 301/306< Site 101/106	Site 301/306> Site 201/206	Site 301/306> Site 401/406
	P	.012	.012	.101
Site 401/406	Tissue Type Pairing	Site 401/406< Site 101/106	Site 401/406> Site 201/206	Site 401/406< Site 301/306
	P	.000	.021	.101

***Mean Abundance Data -- By Sample Site and Tissue Type***

At all four sample sites mean bacterial abundance varied among tissue types. For three sites—West Bank 201/206, East Bank 301/306, and East Bank 401/406—the average bacterial abundance was greatest in the diseased-margin coral tissue, intermediate in the apparently healthy tissue on diseased coral, and least in the tissue of apparently healthy coral (Figure 4). However, at the West Bank 101/106 site, this trend in average bacterial abundance was reversed (Figure 4). At each sample site, there was substantial variation in bacterial abundance among all sample types, as indicated by the large standard deviation values associated with the sample means (Table 4).



**Figure 4:** Mean Culturable Bacterial Abundance by Sample Site and Coral Tissue Type

There are significant differences in bacterial abundance among the tissue types at sites 101/106, 201/206, and 301/306 ( $P = .002, .015, \text{ and } .000$  respectively) (Appendix 2, Tables A-4 to A-6). For sites 201/206, 301/306, and 401/406, bacterial abundance was significantly greater in the diseased-margin tissue than in the healthy coral tissue ( $P = .007, .018, \text{ and } .024$ , respectively) (Table 6). For site 301/306, bacterial abundance in the diseased-margin tissue also exceeded that in the apparently healthy tissue on the diseased coral colony ( $P = .027$ ) (Table 6). In contrast, for site 101/106, bacterial abundance in the healthy coral tissue was greater than in both the diseased-margin coral tissue ( $P = .011$ ) and the apparently healthy tissue on diseased coral ( $P = .051$ ) (Table 6). In contrast, no significant differences in bacterial abundance was found among the three tissue types from East Bank 401/406 ( $P = .131$ ) (Appendix 2, Table A-7).

**Table 6:** Tissue Type Pair Relationships for Site-Specific Bacterial Abundance Data Analyzed Using Wilcoxon Matched Pair Signed-Rank Test

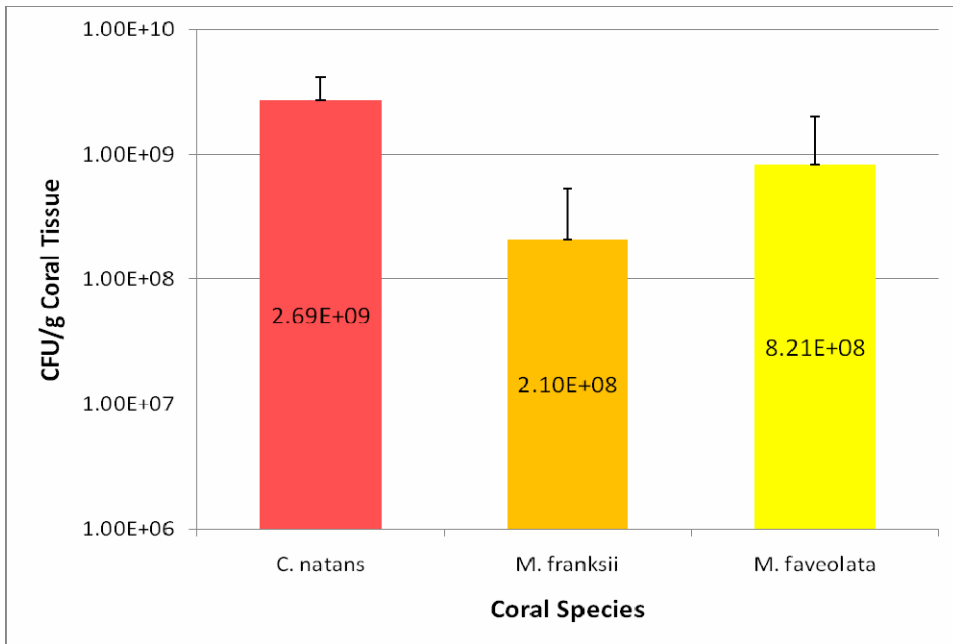
Site	Tissue Type Pairing	H>D	HD>D	H>HD
101/106	P	.011	.767	.051
Site	Tissue Type Pairing	D>H	D>HD	HD>H
201/206	P	.007	.139	.498
Site	Tissue Type Pairing	D>H	D>HD	HD>H
301/306	P	.018	.027	.249
Site	Tissue Type Pairing	D>H	D>HD	HD>H
401/406	P	.024	.089	.260

**Table 7:** Mean and Standard Deviation of Bacterial Abundance by Sample Site and Coral Tissue Type

<b>Coral Tissue Sample Type</b>	<b>N</b>	<b>Minimum</b>	<b>Maximum</b>	<b>Mean (CFU/g coral tissue)</b>	<b>Standard Deviation</b>
<i><b>Sample Site 101/106 (FGB West Bank)</b></i>					
All Site 101/106 Coral Tissue Samples	27	7.19E+08	6.53E+09	2.69E+09	1.46E+09
D	9	7.19E+08	3.29E+09	1.91E+09	7.14E+08
HD	9	7.19E+08	4.21E+09	2.20E+09	1.22E+09
H	9	2.78E+09	6.53E+09	3.98E+09	1.45E+09
<i><b>Sample Site 201/206 (FGB West Bank)</b></i>					
All Site 201/206 Coral Tissue Samples	27	1.82E+07	1.17E+09	2.10E+08	3.24E+08
D	9	8.19E+07	1.17E+09	4.53E+08	4.70E+08
HD	9	1.82E+07	4.10E+08	1.14E+08	1.37E+08
H	9	3.64E+07	9.10E+07	6.17E+07	2.07E+07
<i><b>Sample Site 301/306 (FGB East Bank)</b></i>					
All Site 301/306 Coral Tissue Samples	22	9.10E+06	3.89E+09	1.37E+09	1.54E+09
D	9	1.80E+09	3.89E+09	3.07E+09	7.50E+08
HD	6	1.82E+07	1.06E+09	3.76E+08	4.25E+08
H	7	9.10E+06	9.10E+07	3.90E+07	3.10E+07
<i><b>Sample Site 401/406 (FGB East Bank)</b></i>					
All Site 401/406 Coral Tissue Samples	26	1.27E+08	1.03E+09	3.56E+08	2.56E+08
D	8	1.55E+08	6.92E+08	4.42E+08	2.41E+08
HD	9	1.37E+08	1.03E+09	4.17E+08	3.35E+08
H	9	1.27E+08	3.64E+08	2.17E+08	9.42E+07

### ***Mean Abundance Data – By Coral Species***

The differences in mean bacterial abundance among the three different coral species were significant ( $P = .000$ ) (Appendix 2, Table A-3). *C. natans* had the greatest mean abundance at  $2.69E+09$  CFU/g, which is an order of magnitude larger than the mean bacterial abundance for both *M. franksii* and about three times that in *M. faveolata* (Figure 5). Bacterial abundance in *C. natans* was significantly greater than that in either *M. franksii* or *M. faveolata* ( $P = .000$  and  $.005$ , respectively) (Table 8), and bacterial abundance in *M. faveolata* was significantly larger than in *M. franksii* ( $P = .001$ ) (Table 8).



**Figure 5:** Mean Culturable Bacterial Abundance by Coral Species

**Table 8:** Pair Relationships for Species-Specific Bacterial Abundance Data Analyzed Using Wilcoxon Matched Pair Signed-Rank Test

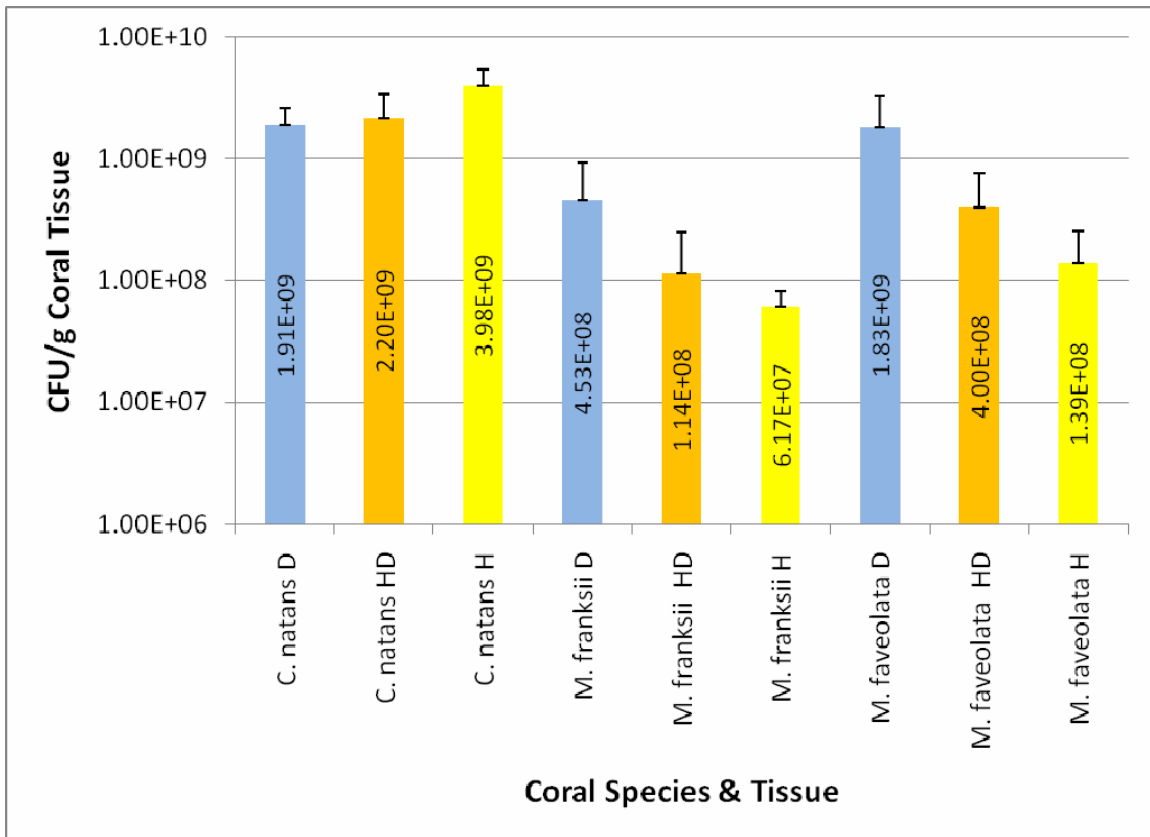
<i>C. natans</i>	<b>Tissue Type Pairing</b>	<i>C. natans</i> > <i>M. franksii</i>	<i>C. Natans</i> > <i>M. faveolata</i>
	P	.000	.005
<i>M. franksii</i>	<b>Tissue Type Pairing</b>	<i>M. franksii</i> < <i>C. natans</i>	<i>M. franksii</i> < <i>M. faveolata</i>
	P	.000	.001
<i>M. faveolata</i>	<b>Tissue Type Pairing</b>	<i>M. faveolata</i> < <i>C. natans</i>	<i>M. faveolata</i> > <i>M. franksii</i>
	P	.005	.001

***Mean Abundance Data – By Coral Species and Tissue Type***

Bacterial abundance varied by tissue type among the coral species/morphotypes. For two coral species—*M. franksii* and *M. faveolata*—average bacterial abundance was greatest in the diseased-margin coral tissue, intermediate in the apparently healthy tissue on diseased coral, and least in the apparently healthy coral tissue (Figure 6). For *C. natans*, this trend in average abundance was reversed. For each coral species/morphotype, there was substantial variation in bacterial abundance among the sample types, as indicated by the large standard deviation values associated with the sample means (Table 9).

**Table 9:** Mean and Standard Deviation of Bacterial Abundance by Coral Species and Tissue Type

<b>Coral Tissue Sample Type</b>	<b>N</b>	<b>Minimum</b>	<b>Maximum</b>	<b>Mean (CFU/g coral tissue)</b>	<b>Standard Deviation</b>
<b><i>Colpophyllia natans</i> (FGB West Bank 101/106)</b>					
All <i>C. natans</i> Coral Tissue Samples	27	7.19E+08	6.53E+09	2.69E+09	1.46E+09
D	9	7.19E+08	3.29E+09	1.91E+09	7.14E+08
HD	9	7.19E+08	4.21E+09	2.20E+09	1.22E+09
H	9	2.78E+09	6.53E+09	3.98E+09	1.45E+09
<b><i>Montastraea franksii</i> (FGB West Bank 201/206)</b>					
All <i>M. franksii</i> Coral Tissue Samples	27	1.82E+07	1.17E+09	2.10E+08	3.24E+08
D	9	8.19E+07	1.17E+09	4.53E+08	4.70E+08
HD	9	1.82E+07	4.10E+08	1.14E+08	1.37E+08
H	9	3.64E+07	9.10E+07	6.17E+07	2.07E+07
<b><i>Montastraea faveolata</i> (FGB East Bank 301/306 &amp; 401/406)</b>					
All <i>M. faveolata</i> Coral Tissue Samples	48	9.10E+06	3.89E+09	8.21E+08	1.16E+09
D	17	1.55E+08	3.89E+09	1.83E+09	1.46E+09
HD	15	1.82E+07	1.06E+09	4.00E+08	3.59E+08
H	16	9.10E+06	3.64E+08	1.39E+08	1.16E+08



**Figure 6:** Mean Culturable Bacterial Abundance by Coral Species and Tissue Type

The differences in bacterial abundance among the tissue types for *C. natans*, *M. franksii*, and *M. faveolata* were significant ( $P = .002$ ,  $.015$ , and  $.000$ , respectively) (Appendix 2, Tables A-7 to A-10). For *M. franksii* and *M. faveolata*, bacterial abundance was significantly greater in the diseased-margin tissue than in healthy coral tissue ( $P = .007$  and  $.001$ , respectively) (Table 10). In *M. faveolata*, abundance in the diseased-margin tissue was also greater than that in the apparently healthy tissue on the diseased coral colony ( $P = .006$ ) (Table 10). By contrast, in *C. natans* abundance in healthy coral tissue was greater than in diseased-margin samples ( $P = .011$ ) and in healthy tissue on the

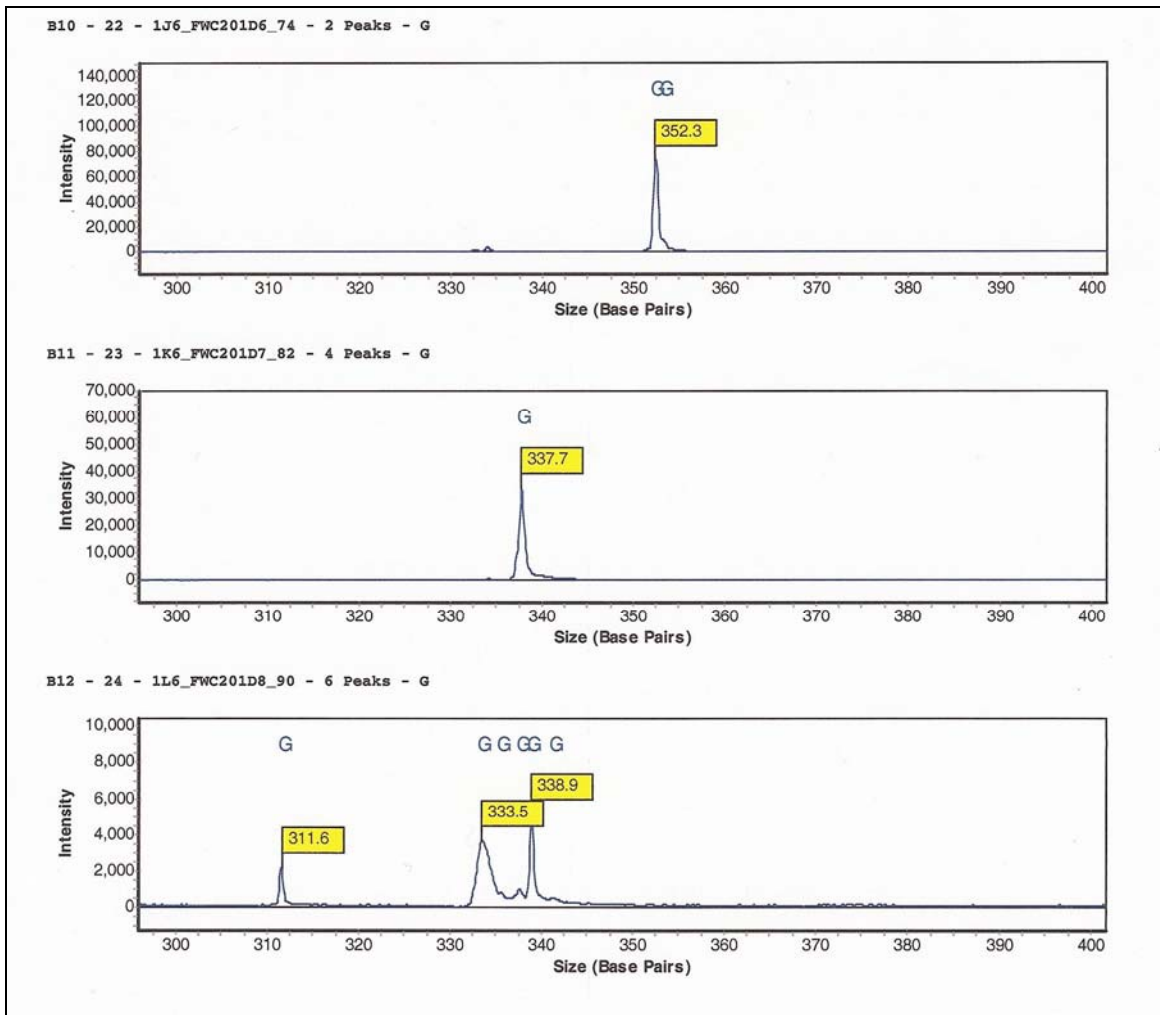
diseased coral colony (P = .051) (Table 10). For both *Montastraea* species (i.e., the *M. annularis* complex), there was no significant difference between the healthy tissue samples and the samples of apparently healthy tissue on those diseased colonies (P = .498 and .061) (Table 10).

**Table 10:** Tissue Type Pair Relationships for Species-Specific Bacterial Abundance Data Analyzed Using Wilcoxon Matched Pair Signed-Rank Test

<i>C. natans</i>	<b>Tissue Type Pairing</b>	<b>H&gt;D</b>	<b>HD&gt;D</b>	<b>H&gt;HD</b>
	P	.011	.767	.051
<i>M. franksii</i>	<b>Tissue Type Pairing</b>	<b>D&gt;H</b>	<b>D&gt;HD</b>	<b>HD&gt;H</b>
	P	.007	.139	.498
<i>M. faveolata</i>	<b>Tissue Type Pairing</b>	<b>D&gt;H</b>	<b>D&gt;HD</b>	<b>HD&gt;H</b>
	P	.001	.006	.061

### Genetic Fingerprint Data

LH-PCR was used to characterize the diversity and relative abundance of the 288 bacterial colonies that were selected. For a number of the FGBNMS bacterial colonies selected (139 out of the 288 samples), the LH-PCR generated multiple OTUs. Bacteria frequently possess multiple operons, between 1 and 15, and 16S rRNA sequences can differ up to several percent between operons (Acinas 2004, Mills 2007). To account for the possibility that the bacterial colonies selected for this study contain multiple operons, when multiple peaks occurred in the electropherogram data, all peaks representing at least 25% of the total were considered to be discrete OTUs (Figure 7).



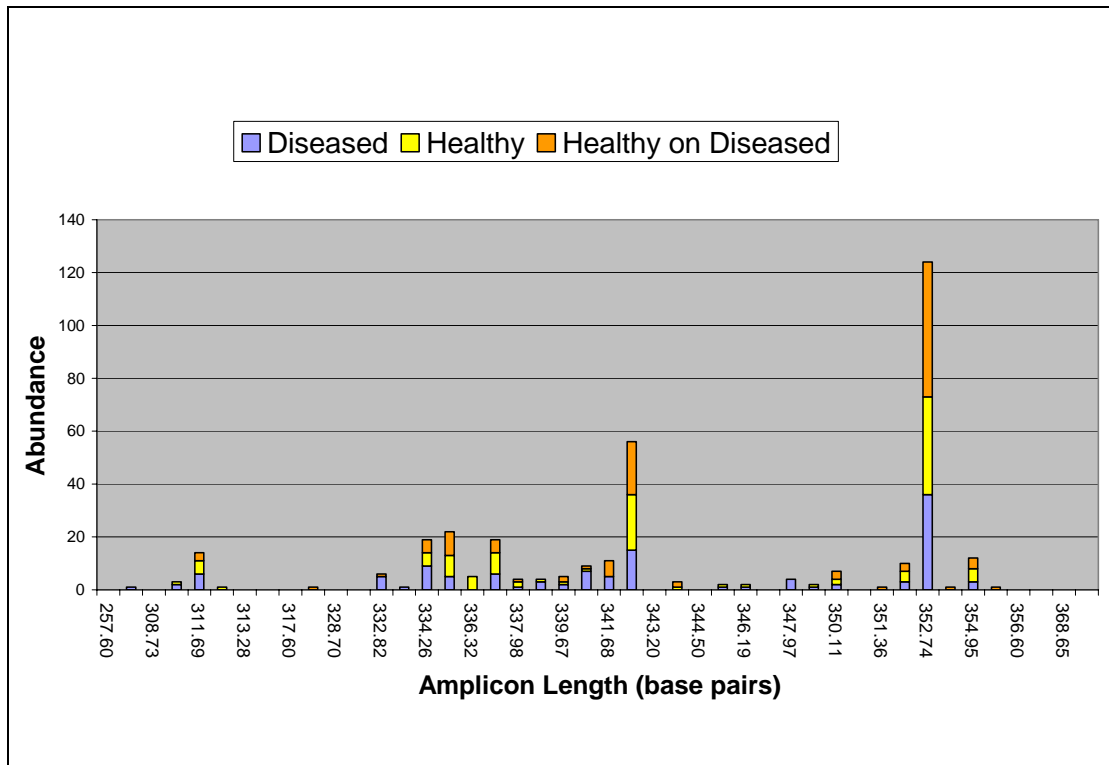
**Figure 7:** SpectruMedix Printout of Electropherograms of OTUs from Selected Bacterial Colonies from the FGBNMS (FWC201D6, FWC201D7, and FWC201D8). Each peak represents an amplicon of a different base pair length. Multiple OTUs are seen in FWC201D8, and single OTUs are seen in FWC201D6 and FWC201D7. In this study, all peaks representing at least 25% of the total were considered to be discrete OTUs. For example, for bacterial colony FWC201D8, three peaks were identified on the electropherogram at 311.6, 333.5, and 338.9 base pairs. Because the 311.6 OTU made up less than 25% of the total electropherogram peak area, it was not considered to be a discrete OTU, and, therefore, only the 333.5 and 338.9 OTUs were considered to be discrete OTUs for this sample.

***Amplicon Abundance and Diversity – Among All Bacterial Colonies***

Among the bacterial colonies selected, there was a fairly high degree of diversity, with a total of 29 different OTUs identified (Table 11). Amplicons ranged from 270.20 to 356.03 base pairs in length (Figure 8). However, most of the bacterial colonies selected (25%) belonged to only one of two OTUs. The most common OTU for all samples was 352.74, representing 17% of all colonies fingerprinted. The second most common OTU was 342.42, representing 8% of all colonies fingerprinted. The remaining 27 different OTUs were much less common, all representing less than 3% of the total number of OTUs found, with many representing less than 1% of the total.

**Table 11:** Number of Different OTUs from Selected Bacterial Colonies by Tissue Type from the FGBNMS

<b>Sample Type</b>	<b>Number of Unique OTUs</b>	<b>Number of D OTUs</b>	<b>Number of HD OTUs</b>	<b>Number of H OTUs</b>
All FGB Samples	29	22	19	21



**Figure 8:** Amplicon Abundance for Selected Bacterial Colonies Sampled from the FGBNMS. Results from each tissue type are stacked above the appropriate OTU. The height of each bar segment represents the relative number of selected bacterial colonies within each tissue type that shared a particular OTU.

### *Amplicon Abundance and Diversity – Among All Bacterial Colonies and Tissue Types*

There was a great deal of similarity in selected bacterial colonies among the three tissue types. Each tissue type possessed roughly the same number of different OTUs—22 among the diseased-margin tissue samples, 19 among the apparently healthy tissue on diseased coral samples, and 21 among the healthy coral tissue samples (Table 11). As in the combined bacterial colony data, the majority of bacterial colonies selected, regardless of tissue type, belonged to only one of two OTUs, the same two described above. The most common amplicon length among all tissue types was 352.74, representing 30%,

43%, and 33% of the total bacterial colonies selected from the D, HD, and H tissue samples, respectively (Table 12). The second most common amplicon length among all sample types was 342.42, representing 13%, 17%, and 19% of the total bacterial colonies selected from the D, HD, and H tissue samples, respectively (Table 12). The remaining 27 different OTUs were much less common among all three tissue types (Table 12).

**Table 12:** Amplicon Totals for All Selected FGBNMS Bacterial Colonies. Table shows the total number of selected bacterial colonies within each tissue type that shared a particular OTU as well as the percentage of the total OTUs for a given tissue type that this OTU represents.

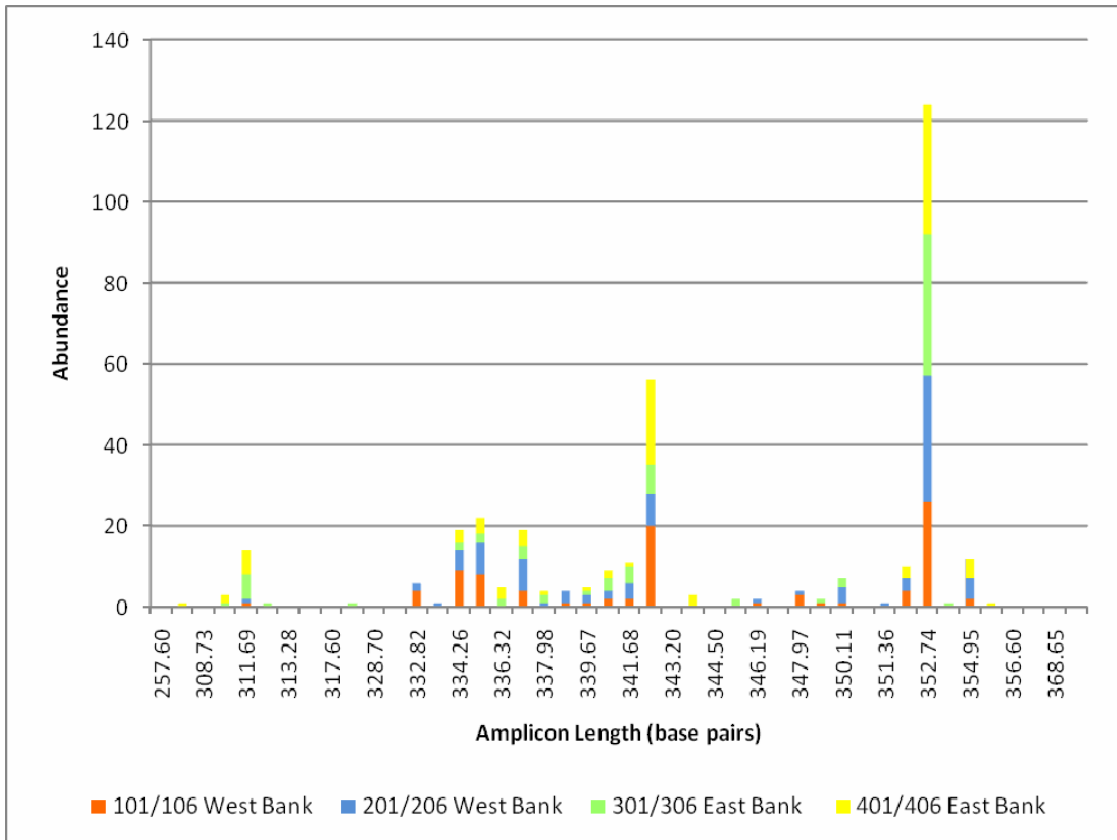
Tissue Type	OTU (Base Pair)										
	257.60	270.20	308.73	310.43	311.69	312.40	313.28	314.60	317.60	327.00	328.70
<b>D</b>	0	1	0	2	6	0	0	0	0	0	0
<b>H</b>	0	0	0	1	5	1	0	0	0	0	0
<b>HD</b>	0	0	0	0	3	0	0	0	0	1	0
% of Total OTUs for D Tissue Samples	0%	1%	0%	2%	5%	0%	0%	0%	0%	0%	0%
% of Total OTUs for HD Tissue Samples	0%	0%	0%	1%	5%	1%	0%	0%	0%	0%	0%
% of Total OTUs for H Tissue Samples	0%	0%	0%	0%	3%	0%	0%	0%	0%	1%	0%
	<b>330.70</b>	<b>332.82</b>	<b>333.45</b>	<b>334.26</b>	<b>335.59</b>	<b>336.32</b>	<b>337.40</b>	<b>337.98</b>	<b>338.73</b>	<b>339.67</b>	<b>340.79</b>
<b>D</b>	0	5	1	9	5	0	6	1	3	2	7
<b>H</b>	0	0	0	5	8	5	8	2	1	1	1
<b>HD</b>	0	1	0	5	9	0	5	1	0	2	1
% of Total OTUs for D Tissue Samples	0%	4%	1%	8%	4%	0%	5%	1%	3%	2%	6%
% of Total OTUs for HD Tissue Samples	0%	0%	0%	5%	7%	5%	7%	2%	1%	1%	1%
% of Total OTUs for H Tissue Samples	0%	1%	0%	4%	8%	0%	4%	1%	0%	2%	1%

Tissue Type	OTU (Base Pair)										
	341.68	342.42	343.20	343.71	344.50	345.24	346.19	347.05	347.97	348.88	350.11
<b>D</b>	5	15	0	0	0	1	1	0	4	1	2
<b>H</b>	0	21	0	1	0	1	1	0	0	1	2
<b>HD</b>	6	20	0	2	0	0	0	0	0	0	3
% of Total OTUs for D Tissue Samples	4%	13%	0%	0%	0%	1%	1%	0%	3%	1%	2%
% of Total OTUs for HD Tissue Samples	0%	19%	0%	1%	0%	1%	1%	0%	0%	1%	2%
% of Total OTUs for H Tissue Samples	5%	17%	0%	2%	0%	0%	0%	0%	0%	0%	3%
	<b>350.90</b>	<b>351.36</b>	<b>352.17</b>	<b>352.74</b>	<b>354.06</b>	<b>354.95</b>	<b>356.03</b>	<b>356.60</b>	<b>358.33</b>	<b>368.65</b>	<b>375.60</b>
<b>D</b>	0	0	3	36	0	3	0	0	0	0	0
<b>H</b>	0	0	4	37	0	5	0	0	0	0	0
<b>HD</b>	0	1	3	51	1	4	1	0	0	0	0
% of Total OTUs for D Tissue Samples	0%	0%	3%	30%	0%	3%	0%	0%	0%	0%	0%
% of Total OTUs for HD Tissue Samples	0%	0%	4%	33%	0%	5%	0%	0%	0%	0%	0%
% of Total OTUs for H Tissue Samples	0%	1%	3%	43%	1%	3%	1%	0%	0%	0%	0%

### ***Amplicon Abundance and Diversity – By Sample Site***

Roughly the same number of different OTUs was found at each site—17 OTUs at West Bank 101/106, 19 at West Bank 201/206, 18 at East Bank 301/306, and 17 at East Bank 301/306 (Table 14). In terms of the total number of different OTUs, 31% of all selected bacterial colonies (comprising 9 different OTUs) were common to all four sampling sites. In terms of the total number of OTUs fingerprinted, the vast majority—278 OTUs, or 79% of all OTUs fingerprinted—were found at all four sites.

The majority of bacterial colonies selected belonged to one of two OTUs (Figure 9). For all the samples sites, the most common OTU was 352.74, representing 29%, 34%, 46%, and 34% of the bacterial colonies selected from sites 101/106, 201/206, 301/306, and 401/406, respectively (Table 13). The second most common OTU for all sample sites was 342.43, representing 22%, 9%, 9%, and 23% of the bacterial colonies selected from sites 101/106, 201/206, 301/306, and 401/406, respectively (Table 13). The remaining 27 OTUs were much less common among all tissue types at all four sites (Table 13), suggesting that most of the variability among the sample sites and tissue types is found in the less common taxa in the selected bacterial population. For example, OTUs 312.40 and 327.00 were found only at East Bank 301/306 (Table 13).



**Figure 9:** Amplicon Abundance for Bacterial Colonies Selected from West Bank 101/106 and 201/206 and East Bank 301/306 and 401/406. Results from each sample site are stacked above the appropriate OTU. The height of each bar segment represents the relative number of selected bacterial colonies within each sample site that shared a particular OTU.

**Table 13:** Amplicon Totals for All Selected FGBNMS Bacterial Colonies by Sample Site. Table shows the total number of selected bacterial colonies for each sample site that shared a particular OTU as well as the percentage of the total OTUs at a given sample site that this OTU represents.

Tissue Type	OTU (Base Pair)										
	257.60	270.20	308.73	310.43	311.69	312.40	313.28	314.60	317.60	327.00	328.70
<b>101/106</b>											
West Bank	0	0	0	0	1	0	0	0	0	0	0
<b>201/206</b>											
West Bank	0	0	0	0	1	0	0	0	0	0	0
<b>301/306</b>											
East Bank	0	0	0	1	6	1	0	0	0	1	0
<b>401/406</b>											
East Bank	0	1	0	2	6	0	0	0	0	0	0
<b>% of Total OTUs at 101/106</b>											
West Bank	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%
<b>% of Total OTUs at 201/206</b>											
West Bank	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%
<b>% of Total OTUs at 301/306</b>											
East Bank	0%	0%	0%	1%	8%	1%	0%	0%	0%	1%	0%
<b>% of Total OTUs at 401/406</b>											
East Bank	0%	1%	0%	2%	6%	0%	0%	0%	0%	0%	0%
	<b>330.70</b>	<b>332.82</b>	<b>333.45</b>	<b>334.26</b>	<b>335.59</b>	<b>336.32</b>	<b>337.40</b>	<b>337.98</b>	<b>338.73</b>	<b>339.67</b>	<b>340.79</b>
<b>101/106</b>											
West Bank	0	4	0	9	8	0	4	0	1	1	2
<b>201/206</b>											
West Bank	0	2	1	5	8	0	8	1	3	2	2
<b>301/306</b>											
East Bank	0	0	0	2	2	2	3	2	0	1	3
<b>401/406</b>											
East Bank	0	0	0	3	4	3	4	1	0	1	2
<b>% of Total OTUs at 101/106</b>											
West Bank	0%	4%	0%	10%	9%	0%	4%	0%	1%	1%	2%
<b>% of Total OTUs at 201/206</b>											
West Bank	0%	2%	1%	5%	9%	0%	9%	1%	3%	2%	2%
<b>% of Total OTUs at 301/306</b>											
East Bank	0%	0%	0%	3%	3%	3%	4%	3%	0%	1%	4%
<b>% of Total OTUs at 401/406</b>											
East Bank	0%	0%	0%	3%	4%	3%	4%	1%	0%	1%	2%

Tissue Type	OTU (Base Pair)										
	341.68	342.42	343.20	343.71	344.50	345.24	346.19	347.05	347.97	348.88	350.11
101/106 West Bank	2	20	0	0	0	0	1	0	3	1	1
201/206 West Bank	4	8	0	0	0	0	1	0	1	0	4
301/306 East Bank	4	7	0	0	0	2	0	0	0	1	2
401/406 East Bank	1	21	0	3	0	0	0	0	0	0	0
% of Total OTUs at 101/106 West Bank	2%	22%	0%	0%	0%	0%	1%	0%	3%	1%	1%
% of Total OTUs at 201/206 West Bank	4%	9%	0%	0%	0%	0%	1%	0%	1%	0%	4%
% of Total OTUs at 301/306 East Bank	5%	9%	0%	0%	0%	3%	0%	0%	0%	1%	3%
% of Total OTUs at 401/406 East Bank	1%	23%	0%	3%	0%	0%	0%	0%	0%	0%	0%
	350.90	351.36	352.17	352.74	354.06	354.95	356.03	356.60	358.33	368.65	375.60
101/106 West Bank	0	0	4	26	0	2	0	0	0	0	0
201/206 West Bank	0	1	3	31	0	5	0	0	0	0	0
301/306 East Bank	0	0	0	35	1	0	0	0	0	0	0
401/406 East Bank	0	0	3	32	0	5	1	0	0	0	0
% of Total OTUs at 101/106 West Bank	0%	0%	4%	29%	0%	2%	0%	0%	0%	0%	0%
% of Total OTUs at 201/206 West Bank	0%	1%	3%	34%	0%	5%	0%	0%	0%	0%	0%
% of Total OTUs at 301/306 East Bank	0%	0%	0%	46%	1%	0%	0%	0%	0%	0%	0%
% of Total OTUs at 401/406 East Bank	0%	0%	3%	34%	0%	5%	1%	0%	0%	0%	0%

### ***Amplicon Abundance and Diversity – By Sample Site and Tissue Type***

Among the three tissue types, small differences were seen in the number of different OTUs, ranging from only 8 to 14, when all sites were compared (Table 14). At both West Bank sites, the greatest number of unique OTUs occurred in the D tissue and least in the H tissue (Table 14). However, this pattern was reversed for both East Bank sites. There, the D tissue contained the least OTUs (or D=HD at East Bank 301/306), and the greatest was found in the H tissue (or H=HD at East Bank 401/406) (Table 14).

**Table 14:** Total OTUs Observed in Selected Bacterial Colonies from the FGBNMS - by Sample Site

<b>Sample Type</b>	<b>Number of Unique OTUs</b>	<b>Number of D OTUs</b>	<b>Number of HD OTUs</b>	<b>Number of H OTUs</b>
101/106 FGB West Samples	17	14	9	8
201/206 FGB West Samples	19	14	10	9
301/306 FGB East Samples	18	10	10	12
401/406 FGB East Samples	17	10	9	10

### ***Amplicon Abundance and Diversity – By Coral Species***

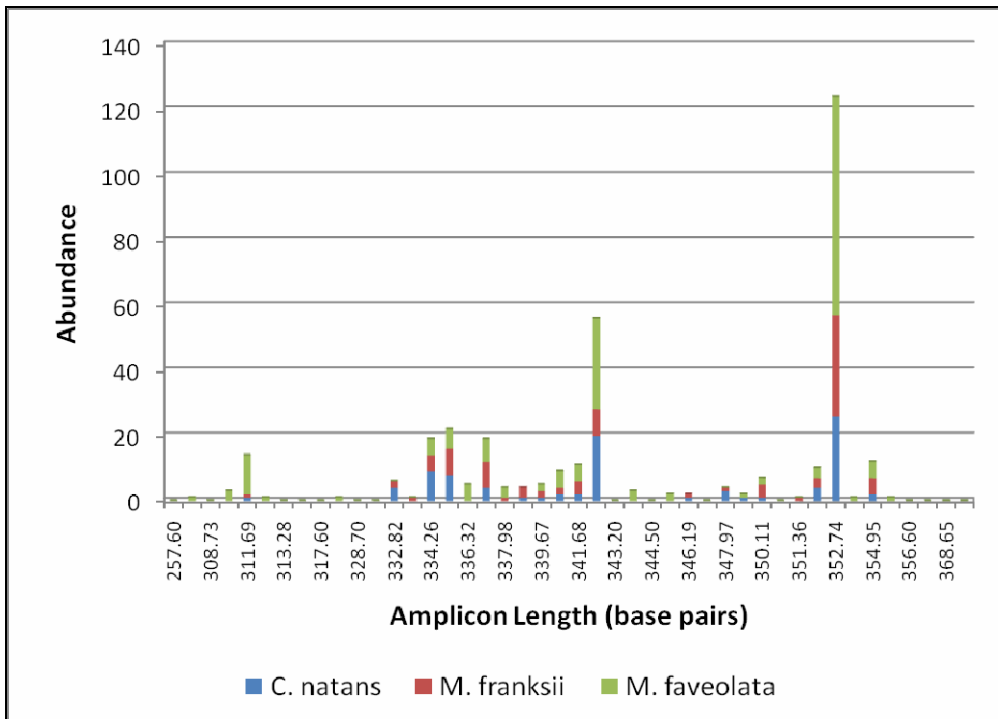
There was a great degree of similarity in bacterial diversity among the coral species. The *M. faveolata* bacterial community was slightly more diverse (23 OTUs) than the *M. franksii* (19 OTUs) and *C. natans* (17 OTUs) communities (Table 15). However, at least some of this difference may be related to the greater bacterial sampling (2X) from *M. faveolata* tissue. There was a great deal of similarity in the organisms found among the three species. Of the 29 different OTUs that were identified among all the bacterial colonies sampled, 41% (12 different OTUs), were found in all three species. This

represents 88% of the 308 total OTUs that were found among all the bacterial colonies sampled.

**Table 15:** Total OTUs Observed in Selected Bacterial Colonies from the FGBNMS – by Coral Species

Sample Type	Number of Unique OTUs	Number of D OTUs	Number of HD OTUs	Number of H OTUs
<i>Colpophyllia natans</i> (FGB West Bank 101/106)	17	14	9	8
<i>Montastraea franksii</i> (FGB West Bank 201/206)	19	14	10	9
<i>Montastraea faveolata</i> (FGB East Bank 301/306 & 401/406)	23	13	16	16

Again, the majority of bacterial colonies selected belonged to two different OTUs (Figure 10). For the three species sampled, *C. natans*, *M. franksii*, and *M. faveolata*, the most common amplicon length was again 352.74 (29%, 34%, and 34% of the bacterial colonies sampled, respectively) (Table 16). As in the site-specific data, among all the coral species/morphotypes, the second most common amplicon length was 342.43, representing 22%, 9%, and 9% of the bacterial colonies selected, respectively (Table 16). Again, the remaining 27 OTUs were generally found in much lesser quantities among the three coral species/morphotypes. This suggests that most of the variability in the selected bacterial populations among the coral species is found in the less common taxa.



**Figure 10:** Amplicon Abundance for Bacterial Colonies Selected from *C. natans*, *M. franksii*, and *M. faveolata*. Results from each corals species are stacked above the appropriate OTU. The height of each bar segment represents the relative number of selected bacterial colonies from each coral species that shared a particular OTU.

**Table 16:** Amplicon Totals for All Selected FGBNMS Bacterial Colonies by Coral Species. Table shows the total number of selected bacterial colonies for each coral species that shared a particular OTU as well as the percentage of the total OTUs for a given coral species that this OTU represents.

Tissue Type	OTU (Base Pair)										
	257.60	270.20	308.73	310.43	311.69	312.40	313.28	314.60	317.60	327.00	328.70
<i>C. natans</i>	0	0	0	0	1	0	0	0	0	0	0
<i>M. franksii</i>	0	0	0	0	1	0	0	0	0	0	0
<i>M. faveolata</i>	0	1	0	3	12	1	0	0	0	1	0
% of Total OTUs for <i>C. natans</i>	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%
% of Total OTUs for <i>M. franksii</i>	0%	0%	0%	0%	1%	0%	0%	0%	0%	0%	0%
% of Total OTUs for <i>M. faveolata</i>	0%	1%	0%	2%	7%	1%	0%	0%	0%	1%	0%
	<b>330.70</b>	<b>332.82</b>	<b>333.45</b>	<b>334.26</b>	<b>335.59</b>	<b>336.32</b>	<b>337.40</b>	<b>337.98</b>	<b>338.73</b>	<b>339.67</b>	<b>340.79</b>
<i>C. natans</i>	0	4	0	9	8	0	4	0	1	1	2
<i>M. franksii</i>	0	2	1	5	8	0	8	1	3	2	2
<i>M. faveolata</i>	0	0	0	5	6	5	7	3	0	2	5
% of Total OTUs for <i>C. natans</i>	0%	4%	0%	10%	9%	0%	4%	0%	1%	1%	2%
% of Total OTUs for <i>M. franksii</i>	0%	2%	1%	5%	9%	0%	9%	1%	3%	2%	2%
% of Total OTUs for <i>M. faveolata</i>	0%	0%	0%	3%	4%	3%	4%	2%	0%	1%	3%
	<b>341.68</b>	<b>342.42</b>	<b>343.20</b>	<b>343.71</b>	<b>344.50</b>	<b>345.24</b>	<b>346.19</b>	<b>347.05</b>	<b>347.97</b>	<b>348.88</b>	<b>350.11</b>
<i>C. natans</i>	2	20	0	0	0	0	1	0	3	1	1
<i>M. franksii</i>	4	8	0	0	0	0	1	0	1	0	4
<i>M. faveolata</i>	5	28	0	3	0	2	0	0	0	1	2
% of Total OTUs for <i>C. natans</i>	2%	22%	0%	0%	0%	0%	1%	0%	3%	1%	1%
% of Total OTUs for <i>M. franksii</i>	4%	9%	0%	0%	0%	0%	1%	0%	1%	0%	4%
% of Total OTUs for <i>M. faveolata</i>	3%	9%	0%	2%	0%	1%	0%	0%	0%	1%	1%

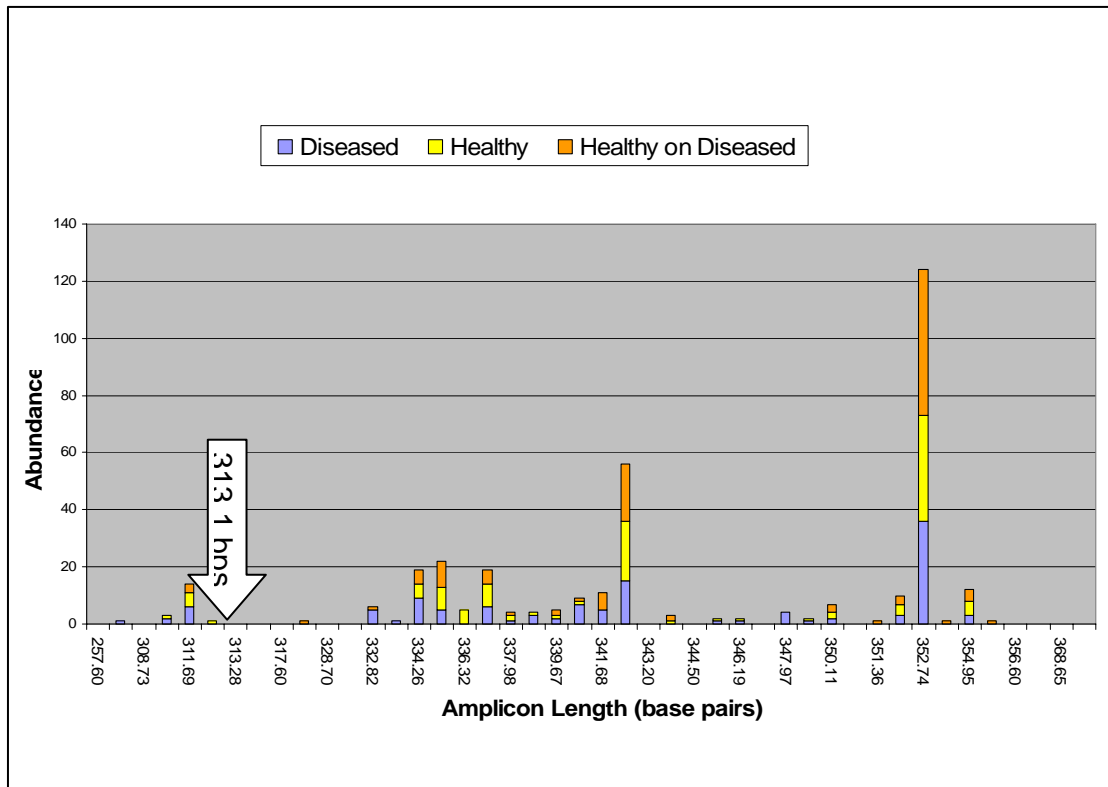
Tissue Type	OTU (Base Pair)										
	350.90	351.36	352.17	352.74	354.06	354.95	356.03	356.60	358.33	368.65	375.60
<i>C. natans</i>	0	0	4	26	0	2	0	0	0	0	0
<i>M. franksii</i>	0	1	3	31	0	5	0	0	0	0	0
<i>M. faveolata</i>	0	0	3	67	1	5	1	0	0	0	0
% of Total OTUs for <i>C. natans</i>	0%	0%	4%	29%	0%	2%	0%	0%	0%	0%	0%
% of Total OTUs for <i>M. franksii</i>	0%	1%	3%	34%	0%	5%	0%	0%	0%	0%	0%
% of Total OTUs for <i>M. faveolata</i>	0%	0%	2%	34%	1%	3%	1%	0%	0%	0%	0%

### ***Amplicon Abundance and Diversity – By Coral Species and Tissue Type***

Few differences in bacterial diversity were found among the three tissue types of the species/morphotypes sampled, where the total number of unique OTUs ranged from 8 to 16 overall (Table 15). There were no clear trends in OTU diversity among tissue types for the coral species/morphotypes sampled. A similar pattern of OTU distribution among tissue types was observed for *C. natans* and *M. franksii*, with the greatest number of different OTUs found in the D tissue (14 OTUs in each), followed by the HD tissue (9 and 10 OTUs, respectively), and the H tissue (8 and 9 OTUs, respectively) (Table 15). *M. faveolata* represented a distinct pattern with regard to the total number of different OTUs identified, with the greatest number of unique OTUs found in the HD (16 OTUs) and H (16 OTUs) tissue samples and the least number of unique OTUs found in the D tissue (13 OTUs) (Table 15).

### *Presence of Aurantimonas coralicida*

Genomic DNA from a pure culture of *Aurantimonas coralicida* supplied by Dr. Laurie Richardson of Florida International University was extracted and amplified for LH-PCR by Geoffrey M. Cook. The OTU identified for the *A. coralicida* sample was 313.1 base pairs (Cook 2006). This 313.1 base pair length did not match any of the bacterial colonies selected from any of the four FGBNMS sampling sites (Figure 11), despite efforts to select colonies that matched the description of *A. coralicida*.



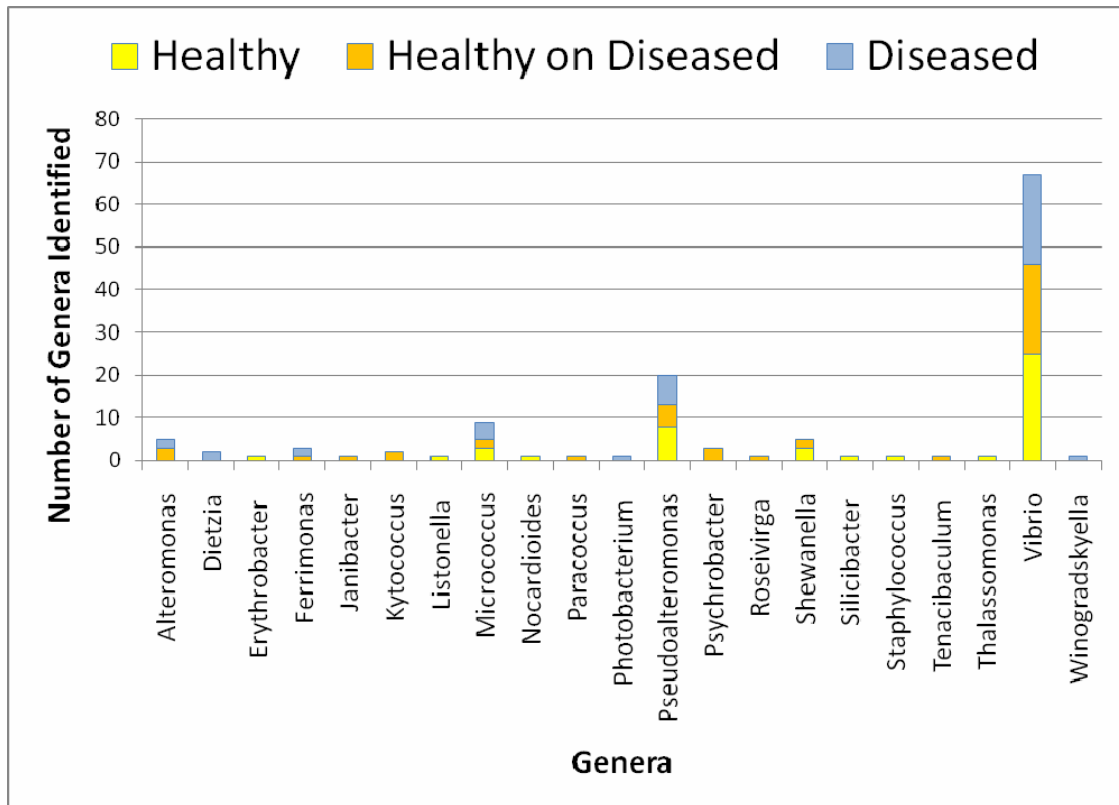
**Figure 11:** Amplicon Abundances for All Bacterial Colonies Selected, with the Amplicon Length for *A. coralicida* (313.1 base pairs) Identified. This 313.1 base pair length for *A. coralicida* does not match any of the OTUs for the bacterial colonies selected from any of the FGBNMS sampling sites.

## **16S rRNA Gene Sequencing**

Because it is possible for different taxa to have the same amplicon length and for a taxon to have multiple operons, dye terminator DNA sequencing was used to sequence the region of the 16S rRNA gene that had been fingerprinted. Only sequences with at least an 80% match to known DNA sequences from previously identified organisms were considered for analysis. A total of 284 culturable bacterial samples from the four FGB sample sites were amplified and prepared for sequencing. Of these, 127 yielded a greater than 80% match to known DNA sequences from previously identified organisms in GenBank, the European Molecular Biology Laboratory, and the DNA Data Bank of Japan (Maidak et al. 2001).

### ***Gene Sequence Abundance and Diversity – All Samples***

A total of 21 different genera were represented among the 127 bacterial colonies with a database match. *Vibrio* (52%), *Pseudoalteromonas* (16%), and *Micrococcus* (7%) were the most abundant genera. The remaining 18 genera were found in less than 4% of the samples, and more than half were found in less than 1% (Figure 12). These data support the previous findings from the fingerprint data that, among the bacterial colonies selected, there is a high degree of bacterial diversity, with respect to the total number of different taxa identified, but the taxa are unevenly distributed, with only a few genera representing a relatively large percentage of all the selected colonies.

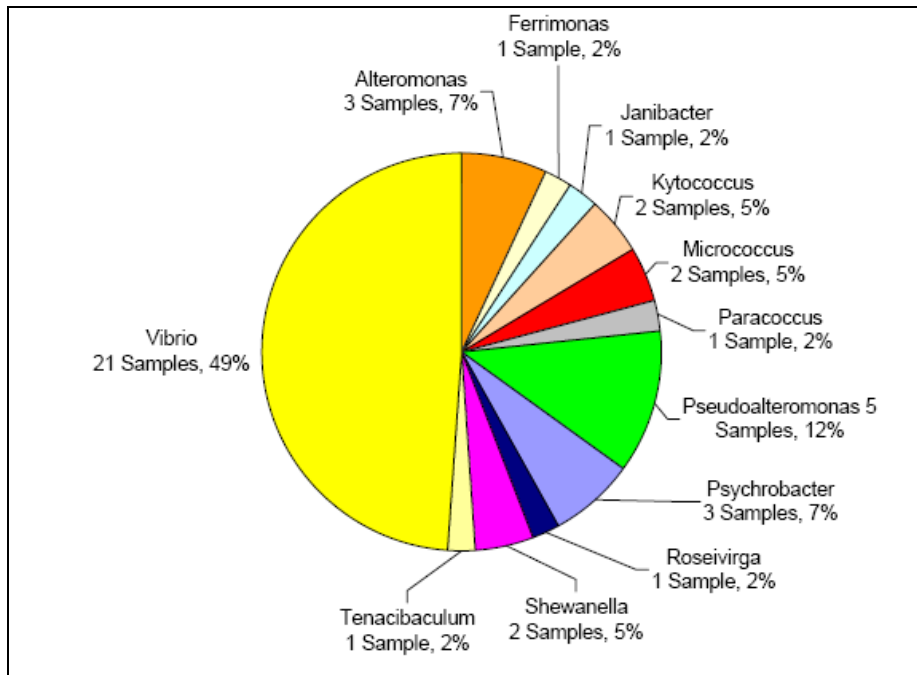


**Figure 12:** Genera Identified for All Bacterial Colonies Selected by Tissue Type. The bars represent the number of selected bacterial colonies that belong to a given genera. The most abundant genera among all selected bacterial colonies and tissue types are *Vibrio*, followed by *Pseudoalteromonas* and *Micrococcus*.

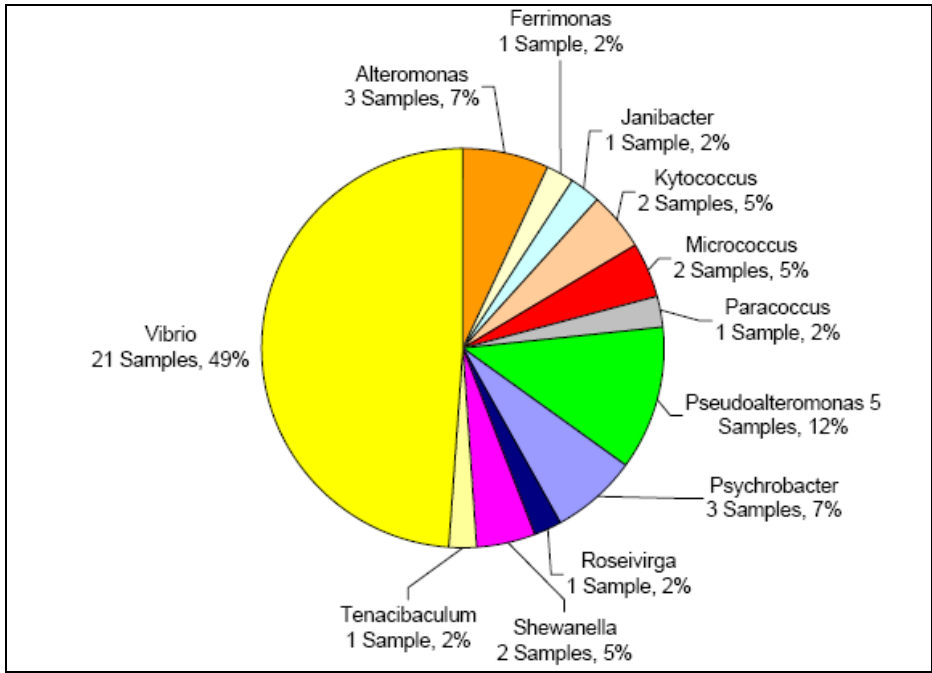
### ***Gene Sequence Abundance and Diversity – All Samples by Tissue Type***

The genera varied by tissue type. For all three types, *Vibrio* was the most abundant genus found in the culturable bacterial samples, representing 53% of all D samples, 49% of all HD samples, and 57% of all H samples (Figures 13–15). Two other

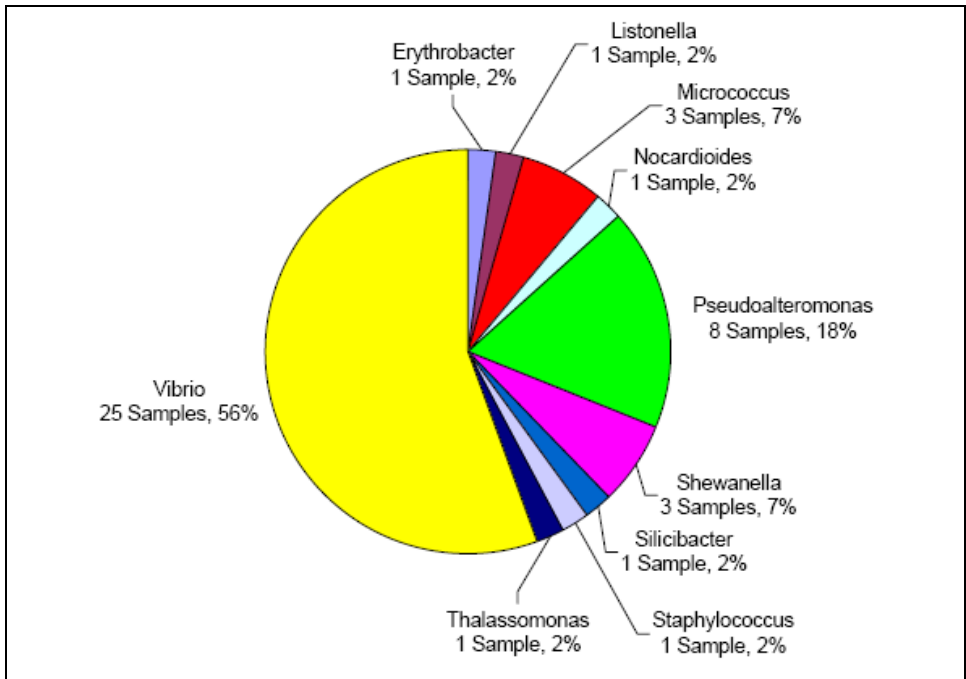
genera, *Pseudoalteromonas* and *Micrococcus*, were identified in all three tissue types, but they represented a lower proportion of the colonies selected (Figures 13–15).



**Figure 13:** Number and Percentage of Genera from Diseased-Margin Tissue Samples



**Figure 14:** Number and Percentage of Genera from Apparently Healthy Tissue on Diseased Coral



**Figure 15:** Number and Percentage of Genera from Apparently Healthy Coral Tissue

Two genera, *Alteromonas* and *Ferrimonas*, were found only in the D and HD samples. *Alteromonas* represented 5% and 7% of the bacterial colonies selected from the D and HD tissue types, respectively. *Ferrimonas* represented 5% and 2% of the bacterial colonies selected from the D and HD tissue types, respectively (Figures 13–15). One genus, *Shewanella*, was identified only in the HD and H tissue types. *Shewanella* represented 5% and 7% of the bacterial colonies selected from the HD and H tissue types, respectively (Figures 13–15). The remaining genera identified, *Dietzia*, *Erythrobacter*, *Janibacter*, *Kytococcus*, *Listonella*, *Nocarioides*, *Paracoccus*, *Photobacterium*, *Psychrobacter*, *Roseivirga*, *Silibacter*, *Staphylococcus*, and *Winogradskyella*, were found in only one of the three tissue types and ranged between 2% and 7% of the total bacteria selected for a given tissue type (Figures 13–15).

## DISCUSSION

### **FGBNMS Total Culturable Bacterial Abundance**

The mean total culturable abundance for all bacterial colonies grown on half-strength Marine Agar 2216 was large ( $1.16 \times 10^9$  CFU/g of coral tissue) (Table 4), a value similar to many organic-rich sediments (Atlas and Bartha 1981, Ford 1993, Schmidt et al. 1998, Austin 1998, Cook 2006). Bacterial abundances varied among the three tissue types, with the highest abundance on the diseased-margin tissue and the lowest on the apparently healthy tissue on diseased coral (Table 4), although the differences were not statistically significant ( $P = 0.153$ ) (Appendix 2, Table A-1).

Data from previous studies of coral colonies affected by WPPII and WPPII-like disease demonstrate some trending toward greater culturable bacterial abundance in diseased-margin compared to healthy coral tissue samples and some indication that bacterial abundance is higher in apparently healthy tissue found on diseased coral compared to diseased-margin tissue. Because these studies used different collection methods (i.e., syringe and core samples), direct comparison of bacterial abundance among studies is not possible. However, comparisons of the general trends in bacterial abundance among the tissue types sampled are useful. The studies include:

- A 1998 study by Richardson et al., in which coral tissue and mucus samples were taken using a syringe from diseased-margin tissue, apparently healthy tissue, and denuded skeleton from three coral species affected by WP2—*Dichocoenia stokesi*, *Dendrogyra cylindrus*, and *Diploria labyrinthiformis*—and plated on Marine Agar 2216. Bacterial abundance was highest on disease-margin tissue ( $10^5$  cells/ml), intermediate on samples from the denuded skeleton ( $10^4$  cells/ml), and lowest on the healthy tissue ( $10^3$  cells/ml) (Richardson et al. 1998a and 1998b, Denner 2003).
- A 2005 study by Barash et al., in which both mucus samples, taken via a syringe, and core samples were taken from a *Favia fava* colony affected by WP2-like disease. For both the mucus and core samples, bacterial abundance was higher on the diseased compared to the healthy tissue. In the mucus samples, 10,000 times more culturable bacteria were found in the diseased tissue when compared to the healthy tissue. In the coral core samples, 80-100 times more culturable bacteria were found on the diseased tissue compared to the healthy tissue (Barash et al. 2005). The actual bacterial abundance totals were not provided by Barash et al. 2005.
- A 2006 study by Cook, in which core coral samples were taken from *Montastraea annularis* affected by WP2. Higher bacterial abundance was found on diseased-margin tissue ( $3.09 \times 10^8$  CFU/g) compared to healthy tissue ( $2.83 \times 10^8$  CFU/g). However, the highest average bacterial abundance was found on the apparently

healthy tissue on diseased coral ( $4.18 \times 10^8$  CFU/g). Statistical analysis of the bacterial abundance data was not provided in the Cook study (Cook 2006).

- A 2004 study by Rothenberger, in which core tissue samples were taken from *M. annularis* affected by WPII. In this study, the bacterial abundance was significantly higher on the apparently healthy tissue on diseased coral ( $6.30 \times 10^7$  CFU/cm<sup>3</sup>) than on diseased-margin tissue ( $1.87 \times 10^7$  CFU/cm<sup>3</sup>) ( $P = <0.02$ ).

One important result from the current work is that culturable bacterial abundances at the FGBNMS were about  $10^9$ /g of coral tissue, at least an order of magnitude higher than the bacterial abundance values reported in other comparable studies (Rothenberger 2004, Cook 2006). While the results for mean bacterial abundances among all tissue types from this study are not statistically different, the data suggest the possibility of slightly higher bacterial abundance in diseased-margin versus apparently healthy coral tissue. This is similar to the trend seen in the Richardson, Barash, Cook, and Rothenberger studies, which show some trending toward greater culturable bacterial abundance in diseased-margin compared to healthy coral tissue samples. Empirically, this makes sense given that, when stressed by disease or bleaching events, coral communities have been shown to increase mucus production and, as a result, culturable bacterial colonies associated with the mucus could increase in number and become more active (Ritchie and Smith 1995, 2004). However, unlike in both the Cook and Rothenberger studies, which found the highest average bacterial abundance on the HD tissue, this study found the average bacterial abundance to be lowest in the HD tissue, compared to both the D and H tissues. This variation among studies may be due, at least in part, to site-specific (e.g.,

temperature, nutrients, turbidity) and species-specific differences among the coral colonies sampled. Clearly, to elucidate trends in bacterial abundance among tissue types in corals affected by WP-II and suspect WP-II disease many more coral colony samples from different coral species and geographic locations will need to be tested.

### **FGBNMS Culturable Bacterial Abundance: Coral Species and Site-Specific Comparisons**

In this study, significant variability in bacterial abundance among the three tissue types occurred in both the three coral species, or species-complex members, and the four sampling sites. This suggests that both coral species- and site-specific factors play a role in determining the abundance, and probably the structure, of bacterial communities found on the surface of coral colonies.

Among the three coral “species” sampled, a similar pattern in average bacterial abundance occurred in both *M. franksii* and *M. faveolata*, which are members of the *M. annularis* complex. The highest bacterial abundance for both species was on the D tissue, intermediate on the HD tissue, and lowest on the H tissue samples (Figure 6, Table 9). The pattern was reversed in *C. natans*, with the average bacterial abundance highest on the H tissue, intermediate on the HD tissue, and lowest on the D tissue (Figure 6, Table 9). Wilcoxon matched pair-signed ranking tests showed that for *C. natans*, H>D; for *M. franksii*, D>H (P = .007); and for *M. faveolata*, D>H and D>HD (P = .001 and .006, respectively) (Table 10). These results indicate that species-specific factors likely play a role in determining the abundance of bacterial colonies associated with the surface mucus layer of coral species.

The FGBNMS study results also may suggest that site-specific factors (e.g., temperature, nutrients, and turbidity) play a role in determining the structure and abundance of bacteria on coral. At three sampling sites, three coral pairs—West Bank 201/206 (*M. franksii*), East Bank 301/306 (*M. faveolata*), and East Bank 401/406 (*M. faveolata*)—demonstrated a similar pattern in bacterial abundance among tissue types, with the highest mean bacterial abundance at each site found on the D tissue, intermediate on the HD tissue, and lowest on the H tissue samples (Figure 4, Table 6). This relationship was statistically significant for the West Bank 201/206 (*M. franksii*) ( $P = .015$ ) and East Bank 301/306 (*M. faveolata*) ( $P = .000$ ) coral pairs (Appendix 2, Tables A-5 and A-6), but was not for the East Bank 401/406 (*M. faveolata*) coral pair ( $P = .131$ ) (Appendix 2, Table A-7). The pattern in bacterial abundance was entirely reversed in *C. natans* from West Bank 101/106, with the highest bacterial abundance was on the H tissue, intermediate on the HD tissue, and lowest on the D tissue. These results are statistically significant ( $P = .002$ ) (Appendix 2, Table A-4).

For all four sampling sites, the relationship between the healthy coral tissue and diseased-margin coral tissue was significant. For West Bank 101/106,  $H > D$  ( $P = .011$ ); for West Bank 201/206 and East Bank 301/306,  $D > H$  ( $P = .007$  and  $.018$ , respectively); and for East Bank 401/406,  $D > H$  and  $D > HD$  ( $P = .024$  and  $.089$ , respectively) (Table 6). These results indicate that site-specific factors likely play a role in determining the abundance, and potentially the structure, of bacterial colonies associated with the surface layer of coral species.

A similar pattern in bacterial abundance among the three tissue types was seen in both the *Montastraea* species/morphotypes sampled, which included *M. franksii* (West Bank 201/206) and *M. faveolata* (East Bank 301/306 and 401/406). Such results are not surprising, given that bacterial populations have been shown to be more similar within coral of a single genus (Ritchie and Smith 2004). However, differences in relative abundance among tissue types between *M. faveolata* species from East Bank 301/306 and 401/406 (Figure 4) suggest that differences in bacterial abundance within a coral species may be a result of small-scale geographical differences that may play a role in shaping the bacterial flora associated with the mucus-rich surface layer of coral species.

#### **FGBNMS Bacterial Diversity: LH-PCR Data**

While a relatively diverse set of bacteria was found among the 288 bacterial colonies selected for LH-PCR analysis, with 29 different OTUs identified, there was little variation in selected colony abundance and diversity among the three tissue types. The number of different OTUs among the tissue types was similar, with 22 different OTUs among D tissue samples, 19 among the HD tissue samples, and 21 among the H tissue samples (Table 11). Among all three tissue types, two OTUs, 352.74 and 342.42, were the most prominent among those selected from the culturable community. Both of these OTUs are comprised of a number of different bacterial genera, with 352.74 corresponding to *Alteromonas*, *Janibacter*, *Micrococcus*, *Pseudoalteromonas*, *Silicibacter*, and *Vibrio*, and 342.42 corresponding to *Alteromonas*, *Dietzia*, *Ferrimonas*, *Pseudoalteromonas*, *Roseivivra*, *Shewanella*, *Thalassomonas*, and *Vibrio* (Appendix 3).

To determine species-level differences among the selected bacterial colonies, additional cloning and sequencing or pyrosequencing would be needed. For all three tissue types, all other OTUs from selected bacterial colonies were found in much lower amounts (Table 16).

These data suggest that no single bacterium is dominant in any of the tissue types. Regarding the diseased-margin tissue, the results indicate that, if this is a bacterial disease, there is a more complex community responsible for WPt-like disease signs. The results presented here would be consistent with a hypothesis that the loss of coral tissue at FGB was not WPt and may well not represent a bacteria-caused disease or at least not a disease with a single-species etiology.

#### **FGBNMS Bacterial Diversity: 16S rRNA Sequencing Data**

The 16S rRNA gene sequencing data support the findings of the LH-PCR analysis. The gene sequencing data for the 288 bacterial colonies selected represents a fairly diverse set of culturable bacteria, with a total of 21 different genera identified. Again there was a high degree of similarity among the three tissue types. For all three tissue types, *Vibrio* was the most common genus selected from the culturable bacterial samples. *Vibrio* represented 53%, 49%, and 57% of the bacterial colonies selected for the D, HD, and H samples, respectively (Figures 13–15). Two other genera were identified in all three tissue types, but in much lesser quantities—*Pseudoalteromonas*, representing 17%, 12%, and 18% of the bacterial colonies selected for the D, HD, and H samples, respectively, and *Micrococcus*, representing 10%, 5%, and 7% of the of the bacterial

colonies selected for the D, HD, and H samples, respectively (Figures 13–15). The remaining 18 genera were less prominent (Figure 12). These data support the hypothesis that no single bacterium is dominantly associated with any one of the tissue types and raise the possibility that multiple pathogens and/or other environmental factors may be causing WPII disease signs in various coral species.

*Thalassomonas*, the genus of the proposed etiological agent for the WP-like disease signs observed on the Eilat coral reef in 2002, was found among the selected bacterial colonies. However, it was found in only one selected colony from an apparently healthy coral tissue sample from West Bank Site 106. Therefore, it is highly unlikely that the suspect WPII disease observed at the FGBNMS was caused by the same etiological agent described by Barash et al. 2005 and Thompson et al. 2006.

Because some bacteria may not be culturable [Amann et al. report that less than 1% of all environmental bacteria are culturable in some low-nutrient cases (Amann et al. 1995), while Cook reports that 27% of OTUs from a whole bacterial community can be matched to OTUs associated with the culturable bacterial community (Cook 2006)], it is possible that the etiological agent for suspect WPII in the FGBNMS coral colonies was not cultured. *A. coralicida*, however, is culturable on the medium employed here. So, it seems clear that the tissue loss observed at FGBNMS was not caused by the same agent as described in Richardson et al. (1998a).

Finally, it is also possible that sampling bacteria from growth plates resulted in some bias. In each case, care was taken to sample the eight most morphologically distinct cultured bacterial colonies associated with each triplicate dilution series and the 24 most

morphologically distinct culturable bacterial colonies within the triplicate dilution series for each core sample. However, comparing the coral colony morphology observed in this study with OTU and gene sequence results indicate that morphological characteristics are not necessarily a good predictor of differences in OTUs or gene sequence results (Appendix 3). Given these constraints, colony selection was likely sufficiently complete to provide a reasonably reliable description of the culturable community for comparison of differences in culturable bacterial community among coral tissue types.

Additional analysis (genomic extracts) using whole coral bacterial community samples would help verify the results presented. Comparisons of whole bacterial community LH-PCR fingerprints with these culturable community fingerprints and 16S rRNA gene sequencing results would provide invaluable information as to the value of directly investigating the culturable community, as described here. Obviously, the benefit of culturing bacteria is to be able to later test their metabolic characteristics. However, missing important bacteria that might not be culturable could seriously bias overall understanding of the coral bacterial community, whether in the healthy or diseased state.

### **FGBNMS Bacterial Diversity (LH-PCR Data): Coral Species and Site-Specific Comparisons**

Surprisingly little variation was noted in diversity of the selected bacterial isolates among either the three coral species/morphotypes or four sampling sites, with most variations seen in the less abundant OTUs. The most abundant OTUs identified were common to all three species/morphotypes and all four sample sites. Of the 29 different

OTUs identified, 12 (41%) were common to *Colpophyllia* and *Montastraea*, representing 88% (308 OTUs) of all OTUs identified among all species/morphotypes, and 9 (31%) were common to all four sampling sites, representing 79% (278 OTUs) of all OTUs identified among the four sampling sites. For all three species/morphotypes and all four sampling sites, the most common OTU was 352.74. Differences were seen in the relative abundance of the remaining OTUs in each species/morphotype and sampling site. However, as noted previously, most of the variations were seen among the least common OTUs.

Such similarity in the composition of culturable bacterial communities among the different sampling sites is surprising, given (1) the significant differences in total bacterial abundance found among the sample sites in this study, (2) the potential for environmental differences among the sampling sites to impact bacterial abundance and diversity, and (3) the fact that different coral species were sampled and research has shown that different coral species are associated with distinct bacterial species (Rohwer 2001 and 2002). Because little is known about the normal variations that exist in bacterial populations associated with physiological differences in coral colonies, species, and morphotypes or those due to possible variations in environmental conditions at different sampling sites, these results provide a very important insight. While the importance of differences among the culturable bacterial populations is not yet understood, nor their causes discerned, the data reported here provide a unique window into this important biotic ecosystem.

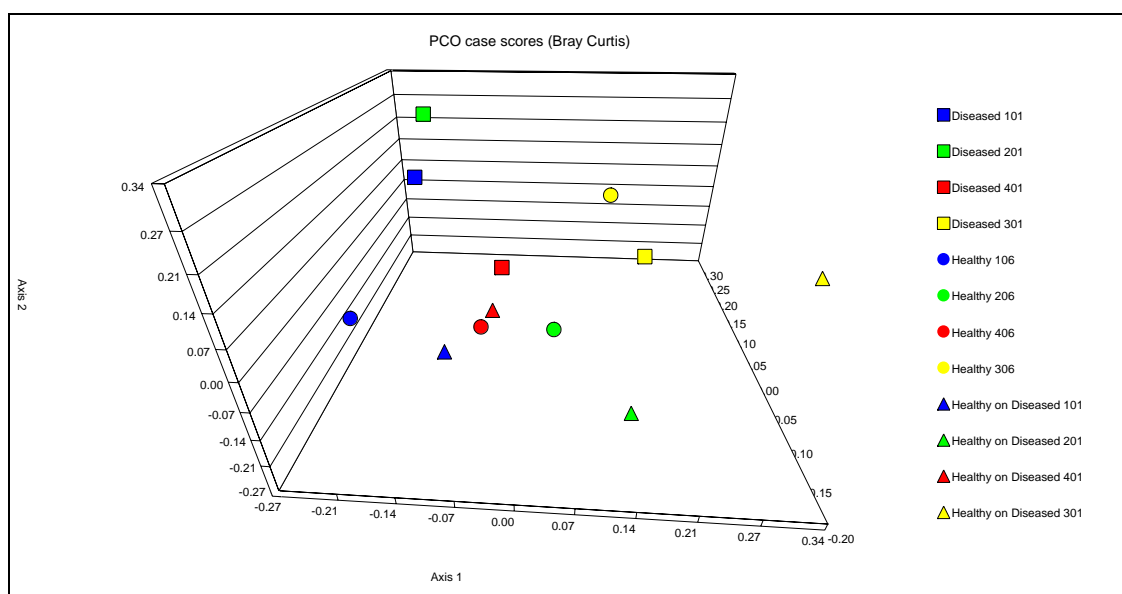
Clearly, bacterial diversity among the three tissue types by both species and sampling site indicates that these variations may be due, at least in part, to coral species- and site-specific factors. For both *C. natans* (West Bank 101/106) and *M. franksii* (West Bank 201/206), a similar pattern in OTU diversity was observed. The highest number of different OTUs in both species were found on the D tissue (14 OTUs in each), intermediate on the HD tissue (9 and 10 OTUs, respectively), and lowest on the H tissue (8 and 9 OTUs, respectively) (Table 15). *M. faveolata* (East Bank 301/306 and 401/406) differed from both *C. natans* and *M. franksii*, with the highest number of unique OTUs on the HD (16 OTUs) and H (16 OTUs) tissue samples and the lowest on the D tissue (13 OTUs) (Table 15). Site-specific differences were seen among the *M. faveolata* coral pairs sampled from East Bank 301/306 and 401/406. At East Bank 301/306 the greatest number of unique OTUs were found in the H tissue (12 OTUs), followed by both the D and HD tissues (which contained 10 OTUs each). At the East Bank 401/406 site, the greatest number of unique OTUs were found in both the H and D tissues (10 OTUs each) and the least found in the HD tissue (9 OTUs) (Table 14).

Principal coordinate analysis (PCO) of the LH-PCR fingerprint data, combining both the amplicon lengths and the relative (selection-linked) abundance of each OTU for each tissue type at each sample site, indicates that “selected” OTUs clustered by sample site (Figure 16) rather than by tissue type or coral species/morphotype. This clustering of data was strongest for sample sites West Bank 101/106 (*C. natans*), East Bank 301/306 (*M. faveolata*) and East Bank 401/406 (*M. faveolata*) and less pronounced for West Bank 201/206 (*M. franksii*). The clustering of data suggests that even though there is little

diversity among the OTUs from the various sample sites and tissue types, sample site appears to play a role in bacterial diversity in the coral microflora.

Based on reports that coral species have evolved distinct microbial communities (Ritchie and Smith 1997, 2004), one would anticipate that PCO would indicate clustering of data among East Bank 301/306 and 401/406 (*M. faveolata*) that would be distinct from West Bank 101/106 (*C. natans*) and West Bank 201/206 (*M. franksii*) data. However, no such clustering was seen. Differences in site location (e.g., differences in micro-climate and environment) potentially play a larger role in impacting bacterial species diversity among the various tissue types than do species-specific factors (e.g. differences in the nutrient composition of the coral surface mucus layer).

These data suggests that coral species-specific factors and potentially minute differences in micro-climate and environment are impacting bacterial diversity among tissue types. Because this work focused on data from selected bacterial colonies that grew on marine agar, future analysis of the whole bacterial community (genomic extracts) from these samples would help determine whether the culturable bacterial community accurately represented the overall diversity of the bacterial community.



**Figure 16:** Three-Dimensional Principal Coordinate Analysis of the LH-PCR Data. The Analysis Combines Both the Amplicon Lengths and Relative Abundances of Each OTU from Each Tissue Type at Each Sample Site from the FGBNMS.

### Testing for Presence of *A. coralicida*

The genetic fingerprint created for each bacterial isolate sampled from the FGBNMS were screened against a genetic fingerprint obtained from a pure culture of *A. coralicida*. No match for the *A. coralicida* (amplicon length 313.1 base pairs), was found among the FGBNMS samples, even though isolates were selected based on morphological characteristics of *A. coralicida* to focus on likely candidates. Not finding *A. coralicida* among the bacterial isolates selected raises the possibility that *A. coralicida* might not be the etiological agent causing the suspect WPII disease signs at FGBNMS. It is possible that another etiological agent is causing the suspect WPII-like disease signs at FGBNMS, or maybe that there are a variety of etiological agents that cause WPII-like

signs in coral colonies. At the same time it seems possible that the lesions observed at FGBNMS may not be caused by a bacterial disease but rather by some other agent in that environment.

## CONCLUSION

Culturable bacterial populations associated with coral from the Flower Garden Banks National Marine Sanctuary were large, averaging more than  $1 \times 10^9$  CFU/g of coral tissue. Culturable bacterial abundance was significantly different in healthy and diseased coral tissue for three of the four sample sites as well as for all three coral species/morphotypes. In *Montastraea* there were more culturable bacteria in the diseased-margin tissue samples, while there were more bacteria in the healthy tissue of *Colpophyllia*. Variation in culturable bacterial abundance among tissue types may be at least partially dependent on species-specific and/or site-specific factors at the FGBNMS.

The results of the LH-PCR data indicate that the amplicon length (313.1 base pairs) for *Aurantimonas coralicida*, the putative etiological agent for white plague type II, was not present among any of the selected bacterial samples. Little variation was observed in the OTU data among the various tissue types. Of the 29 unique OTUs identified among all the samples, two were found in a majority of the samples. One amplicon length, 352.74, was the most abundant OTU at all sample sites, in all species, and in all tissue types. A second amplicon length, 342.42, featured predominantly in two of the four sample sites and for one of the three coral species/morphologies sampled. The remaining 27 unique OTUs were much lesser prevalent among selected colonies,

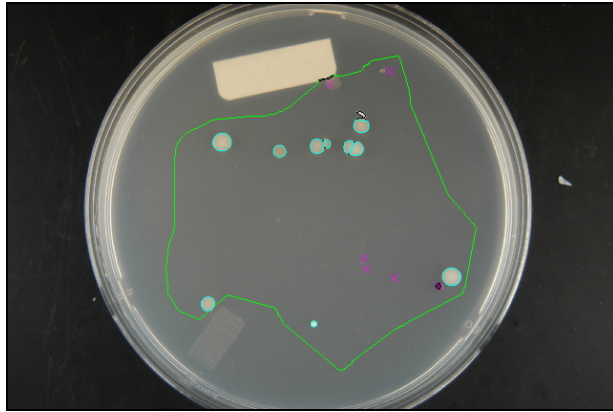
suggesting that most of the variability (in the selected colonies of the culturable community) occurs among the less abundant organisms. The 16S rRNA gene sequencing indicated that, among all samples, the majority of the bacteria growing in marine agar are members of the genus *Vibrio* while *Pseudoalteromonas* and *Micrococcus* were the next most abundant culturable genera respectively. As in the LH-PCR results, most of the variability in the bacterial colonies was found among the less abundant organisms.

## Appendix 1

### **BioQuant Nova Prime (Version 6.90.10) Image Analysis Software: Conducting Counts of Bacterial Colony Forming Units using Various BioQuant Arrays**

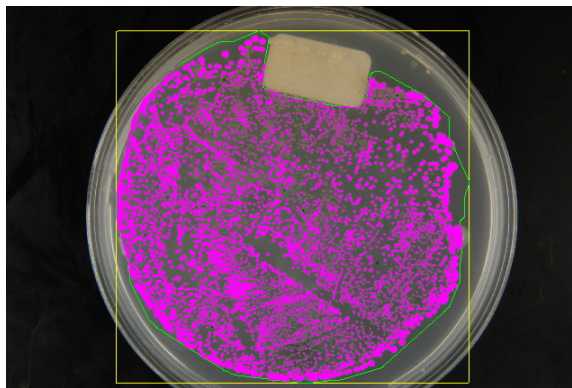
Depending on the size, uniformity, and density of bacterial colonies on the spread plates in the photos, three types of BioQuant analyses and/or a combination of these analyses were used to enumerate the bacterial colonies in each plate photograph: (1) trace-based array counts, (2) pixel-based array counts, and/or (3) manual counts. Each type of analysis is briefly explained below.

1. **Trace-based Array Counts.** To enumerate larger, solitary, and defined colony forming units (CFUs), trace-based arrays were used. Trace-based arrays first threshold (i.e., highlight pixels of interest) and then outline and enumerate each individual colony on the plate. The array consists of measurements taken from the shape of the preview tracing drawn around an object.



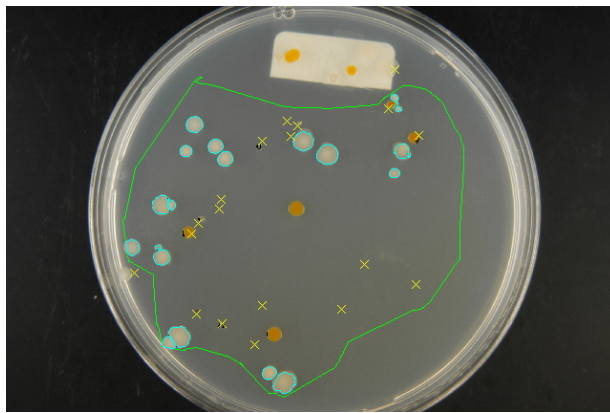
**Figure A-1:** Example of a Trace-based Array Count

2. **Pixel-based Array Counts.** To enumerate small, overlapping CFUs of fairly uniform size, pixel-based arrays were used. Pixel-based arrays are created by first approximating the number of pixels in an average-sized bacterial colony. The entire plate is then thresholded, and divided by the number of pixels in an average colony to approximate the total number of individual colonies on the plate.



**Figure A-2:** Pixel-based Array Count

3. **Manual Counts.** To enumerate collections of overlapping, non-uniform CFUs and CFUs that could not be thresholded (i.e., the color of the colony cannot be easily thresholded by BioQuant; this often occurs with darker colored bacterial colonies), manual counts were used. Manual counts consist of manually counting, marking with an “x” and enumerating individual colonies.



**Figure A-3: Manual Count**

## Appendix 2

### One-way ANOVA Analysis Results from Culturable Abundance Count Data

Table A-1: One-way ANOVA of culturable bacterial abundance for all D, HD, H tissue samples from the FGB in May 2005 plated on half-strength Marine Agar 2216

Source of Variation	Sum of Squares	df	Mean Square	F	P
Between Groups	8.01E+18	2	4.01E+18	1.914	0.153
Within Groups	2.07E+20	99	2.09E+18		
Total	2.15E+20	101			

Table A-2: One-way ANOVA of culturable bacterial abundance for all tissue samples from Sites 101/106, 201/206, 301/306, and 401/406 from the FGB in May 2005 plated on half-strength Marine Agar 2216

Source of Variation	Sum of Squares	df	Mean Square	F	P
Between Groups	1.06E+20	3	3.59E+19	31.56	0.000
Within Groups	1.09E+20	98	1.12E+18		
Total	2.15E+20	101			

Table A-3: One-way ANOVA of culturable bacterial abundance for all tissue samples from the three coral species sampled (i.e., *C. natans*, *M. franksii*, and *M. faveolata*) from the FGB in May 2005 plated on half-strength Marine Agar 2216

Source of Variation	Sum of Squares	df	Mean Square	F	P
Between Groups	9.35E+19	2	4.68E+19	38.02	0.000
Within Groups	1.22E+20	99	1.23E+18		
Total	2.15E+20	101			

Table A-4: One-way ANOVA analysis of culturable bacterial abundance for D, HD, H tissue samples from the FGB West Bank Sample Site 101/106 in May 2005 plated on half-strength Marine Agar 2216

<b>Source of Variation</b>	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
Between Groups	2.26E+19	2	1.13E+19	8.25	.002
Within Groups	3.28E+19	24	1.37E+18		
Total	5.54E+19	26			

Table A-5: One-way ANOVA analysis of culturable bacterial abundance for D, HD, H tissue samples from the FGB West Bank Sample Site 201/206 in May 2005 plated on half-strength Marine Agar 2216

<b>Source of Variation</b>	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
Between Groups	8.12E+17	2	4.06E+17	5.06	.015
Within Groups	1.92E+18	24	8.02E+16		
Total	2.74E+18	26			

Table A-6: One-way ANOVA analysis of culturable bacterial abundance for D, HD, H tissue samples from the FGB East Bank Sample Site 301/306 in May 2005 plated on half-strength Marine Agar 2216

<b>Source of Variation</b>	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>Sig.</b>
Between Groups	4.43E+19	2	2.22E+19	77.87	.000
Within Groups	5.40E+18	19	2.84E+17		
Total	4.97E+19	21			

Table A-7: One-way ANOVA analysis of culturable bacterial abundance for D, HD, H tissue samples from the FGB East Bank Sample Site 401/406 in May 2005 plated on half-strength Marine Agar 2216

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.66E+17	2	1.33E+17	2.22	.131
Within Groups	1.37E+18	23	5.98E+16		
Total	1.64E+18	25			

Table A-8: One-way ANOVA analysis of culturable bacterial abundance for all D, HD, H tissue samples from the *C. natans* coral samples from the FGB in May 2005 plated on half-strength Marine Agar 2216

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.26E+19	2	1.13E+19	8.25	.002
Within Groups	3.28E+19	24	1.37E+18		
Total	5.54E+19	26			

Table A-9: One-way ANOVA analysis of culturable bacterial abundance for D, HD, H tissue samples from the *M. franksii* coral samples from the FGB in May 2005 plated on half-strength Marine Agar 2216

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8.12E+17	2	4.06E+17	5.06	.015
Within Groups	1.92E+18	24	8.02E+16		
Total	2.74E+18	26			

Table A-10: One-way ANOVA analysis of culturable bacterial abundance for D, HD, H tissue samples from the *M. faveolata* coral samples from the FGB in May 2005 plated on half-strength Marine Agar 2216

<b>Source of Variation</b>	<b>Sum of Squares</b>	<b>df</b>	<b>Mean Square</b>	<b>F</b>	<b>P</b>
Between Groups	2.75E+19	2	1.38E+19	17.13	0.000
Within Groups	3.61E+19	45	8.03E+17		
Total	6.36E+19	47			

### Appendix 3

**Description of Bacterial Colony Morphology, with Corresponding OTU and Genus-level Identification of Selected Bacterial Colonies from the FGBNMS, May 2005, Plated on Half-Strength Marine Agar 2216**

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
<b>Diseased Coral Tissue Samples</b>					
golden orange, opaque, circular, entire	332.82	335.59		<i>Alteromonas</i>	99%
translucent with opaque white center, circular, entire	348.88	350.11		<i>Alteromonas</i>	100%
off-white, opaque, irregular, undulate	342.42			<i>Dietzia</i>	100%
small, cream, opaque, round, entire	339.67			<i>Dietzia</i>	100%
small, off-white, opaque, round, entire	347.97	352.74		<i>Ferrimonas</i>	97%
small, yellow, opaque, round, entire	341.68			<i>Ferrimonas</i>	92%
small, black, circular, entire	332.82			<i>Micrococcus</i>	99%
white, opaque, round, entire	334.26			<i>Micrococcus</i>	100%
large, off-white, opaque, round, entire	352.74			<i>Micrococcus</i>	99%
beige, round, entire	352.74			<i>Micrococcus</i>	100%
large, beige, opaque center, translucent edge, round, entire	352.74			<i>Photobacterium</i>	100%
medium, beige, opaque, round, entire	352.74			<i>Pseudoalteromonas</i>	100%
translucent with opaque white center, irregular, undulate	342.42			<i>Pseudoalteromonas</i>	84%
white, opaque, circular, entire	332.82	342.42		<i>Pseudoalteromonas</i>	99%
translucent with opaque white center, circular, entire	311.69	352.74		<i>Pseudoalteromonas</i>	100%

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
very small, bright white, round, entire	334.26	335.59		<i>Pseudoalteromonas</i>	83%
medium, beige, opaque, round, entire	340.79			<i>Pseudoalteromonas</i>	98%
small, white, opaque, round, entire	340.79			<i>Vibrio</i>	96%
small, cream colored, opaque, round, entire	334.26			<i>Vibrio</i>	100%
medium, translucent with opaque white center, irregular, undulate	352.74			<i>Vibrio</i>	94%
small, white, translucent with opaque white center, irregular, undulate	337.40			<i>Vibrio</i>	87%
small, reddish brown, translucent, round, entire	335.59			<i>Vibrio</i>	99%
medium, black, circular, entire	352.74			<i>Vibrio</i>	99%
large, translucent with opaque brownish-beige center, regular, entire	352.74			<i>Vibrio</i>	95%
medium, black, circular, entire	352.74			<i>Vibrio</i>	97%
medium, white, opaque, round, entire	310.43			<i>Vibrio</i>	98%
large, white, opaque, round, entire	352.74			<i>Vibrio</i>	100%
large, beige, translucent, round, entire	335.59			<i>Vibrio</i>	93%
large, white, opaque, round, entire	352.74			<i>Vibrio</i>	95%
small, either white or yellow, round, entire	341.68			<i>Vibrio</i>	100%
large, yellow, opaque, round, entire	337.40			<i>Vibrio</i>	100%
medium, white, opaque, round, entire				<i>Vibrio</i>	95%
small, either white or yellow, round, entire	337.98			<i>Vibrio</i>	83%
small, white, opaque, round, entire - darker center and "halo" edging	352.74			<i>Vibrio</i>	95%
small, white, opaque, round, entire	334.26	352.74		<i>Vibrio</i>	97%
medium, white, opaque, irregular, undulate	352.74			<i>Vibrio</i>	100%

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
small, white, opaque, round, entire - possible raised surface	352.74			<i>Vibrio</i>	100%
small, white, opaque, round, entire - darker center and "halo" edging	345.24			<i>Vibrio</i>	91%
small, beige, opaque, round, entire - darker center and "halo" edging	352.74			<i>Winogradskyella</i>	96%
<b>Healthy Coral Tissue Samples</b>					
very small, bright white, round, entire	335.59	352.74		<i>Erythrobacter</i>	100%
small, round, dark red, entire	352.74	354.95	335.59	<i>Listonella</i>	83%
small, light yellow, opaque, round, entire	336.32			<i>Micrococcus</i>	84%
large, yellow, opaque, round, entire	337.98	339.67		<i>Micrococcus</i>	100%
medium, translucent with opaque white center, irregular, undulate	352.74			<i>Micrococcus</i>	99%
small, golden yellow, opaque, round, entire	336.32			<i>Nocardioides</i>	100%
flesh colored	327.00			<i>Paracoccus</i>	84%
large, white, opaque, round, entire, with slightly darker center	342.42			<i>Pseudoalteromonas</i>	85%
large, white, opaque, round, entire - darker center	342.42			<i>Pseudoalteromonas</i>	88%
large, white, opaque, round, entire - with slightly darker center	342.42			<i>Pseudoalteromonas</i>	89%
large, white, opaque, round, entire	342.42			<i>Pseudoalteromonas</i>	81%
small, white, round, entire	338.73			<i>Pseudoalteromonas</i>	100%
very small, bright white, round, entire	342.42			<i>Pseudoalteromonas</i>	80%
small, black, circular, entire	334.26			<i>Pseudoalteromonas</i>	83%
small, round, dark brown, entire,	335.59			<i>Pseudoalteromonas</i>	92%
small, flesh-colored, opaque, round, entire	311.69	352.74		<i>Shewanella</i>	100%
small, dark red, opaque, irregular	352.74			<i>Shewanella</i>	100%

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
very small, brown, opaque, round, entire	342.42			<i>Shewanella</i>	99%
medium, white, round, entire	352.74			<i>Silicibacter</i>	87%
small, white, opaque, round, entire	348.88			<i>Staphylococcus</i>	100%
golden orange, opaque, circular, entire	342.42			<i>Thalassomonas</i>	91%
small, reddish brown, opaque, round	342.42			<i>Vibrio</i>	99%
large, off-white, opaque, irregular, undulate	334.26			<i>Vibrio</i>	95%
small, brown beige, round, entire, opaque, with darker center	337.40			<i>Vibrio</i>	95%
large, white, opaque, round, entire	352.74			<i>Vibrio</i>	100%
small, orange-yellow, opaque, round, entire	352.74			<i>Vibrio</i>	100%
small, transparent, round, entire - with off-white center	352.74			<i>Vibrio</i>	95%
small, beige, opaque, round, entire	352.74			<i>Vibrio</i>	100%
large, white, opaque, round, entire	352.17			<i>Vibrio</i>	94%
large, white, opaque, round, entire - darker center and "halo" edging	352.74			<i>Vibrio</i>	94%
small, orange-yellow, opaque, round, entire	352.74			<i>Vibrio</i>	97%
small, golden yellow, opaque, round, entire	352.74			<i>Vibrio</i>	93%
large, white, opaque, round, entire - rings	352.74			<i>Vibrio</i>	96%
large, white, opaque, round, entire	352.17			<i>Vibrio</i>	95%
medium, beige, opaque, round, entire	352.74			<i>Vibrio</i>	91%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	100%
medium, beige, opaque, round, entire	352.74			<i>Vibrio</i>	99%
medium, beige, opaque, round, entire - with rings?	352.74			<i>Vibrio</i>	100%
small, white, opaque, round, entire - possible raised surface	352.74			<i>Vibrio</i>	99%

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
large, beige, opaque, irregular, undulate	352.74			<i>Vibrio</i>	100%
small, brown with white border, round, entire	350.11			<i>Vibrio</i>	100%
medium, brown, opaque, round, entire				<i>Vibrio</i>	99%
small, black, circular, entire	352.17			<i>Vibrio</i>	100%
large, bright yellow, opaque, circular, entire	352.74			<i>Vibrio</i>	95%
large, bright yellow, opaque, circular, entire	337.40			<i>Vibrio</i>	100%
large, off-white, opaque, irregular, undulate	352.74			<i>Vibrio</i>	100%
<b>Apparently Healthy Tissue Samples from Diseased Coral</b>					
medium, off-white/beige, opaque, round, entire	352.74			<i>Alteromonas</i>	100%
large, opaque off-white center, irregular, undulate	342.42	352.74		<i>Alteromonas</i>	100%
medium, beige, opaque, round, entire	352.74			<i>Alteromonas</i>	99%
cream-colored, opaque, round, entire	342.42			<i>Ferrimonas</i>	96%
medium, orange-yellow, opaque, round, entire	352.74			<i>Janibacter</i>	87%
medium, yellow, opaque, round, entire	339.67			<i>Kytococcus</i>	100%
small, bright yellow, round, opaque	340.79			<i>Kytococcus</i>	100%
medium, off-white, opaque, round, entire	342.42	356.03		<i>Micrococcus</i>	98%
large, white, opaque, round, entire	352.74			<i>Micrococcus</i>	99%
medium, off-white, opaque, round, entire -- darker center	352.74			<i>Pseudoalteromonas</i>	97%
medium, black, opaque, round, entire	335.59			<i>Pseudoalteromonas</i>	94%
medium, white on outside and golden inside, round, entire	335.59	337.40		<i>Pseudoalteromonas</i>	100%
small, dark with white edging, round, entire	332.82	334.26	342.42	<i>Pseudoalteromonas</i>	84%
small, brown, irregular, lobate				<i>Pseudoalteromonas</i>	97%

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
small, yellow, opaque, round, entire	352.74			<i>Pseudoalteromonas</i>	80%
medium, white, opaque, round, entire	337.40			<i>Psychrobacter</i>	100%
large, round, translucent, undulate	337.98			<i>Psychrobacter</i>	100%
translucent	341.68			<i>Psychrobacter</i>	100%
medium, yellow, opaque, round, entire	342.42			<i>Roseivirga</i>	100%
beige	341.68			<i>Shewanella</i>	100%
medium, white, opaque, round, entire	342.42			<i>Shewanella</i>	100%
medium, dark red, opaque, round, entire	335.59			<i>Tenacibaculum</i>	100%
white, opaque, circular, entire	352.74			<i>Vibrio</i>	89%
small, black, circular, entire	342.42			<i>Vibrio</i>	96%
medium, white, opaque, round, entire	341.68			<i>Vibrio</i>	86%
medium, off-white/beige, opaque, round, entire	352.74	347.05		<i>Vibrio</i>	84%
medium, dark orange with white border, opaque, round, entire	335.59	352.74		<i>Vibrio</i>	83%
medium, white, opaque, round, entire	352.74	354.06		<i>Vibrio</i>	98%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	100%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	98%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	93%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	99%
large, round, entire, translucent				<i>Vibrio</i>	98%
beige, round, entire	352.74			<i>Vibrio</i>	94%
large, white, opaque, round, entire	352.74			<i>Vibrio</i>	99%
medium, white, opaque, round, undulate	350.11			<i>Vibrio</i>	93%

<b>Description</b>	<b>OTU 1</b>	<b>OTU 2</b>	<b>OTU 3</b>	<b>Genus</b>	<b>% ID</b>
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	95%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	98%
medium, white, opaque, round, entire	352.74			<i>Vibrio</i>	99%
medium, white, opaque, irregular, lobate	352.74			<i>Vibrio</i>	84%
large, translucent with opaque white center, irregular, undulate	352.74			<i>Vibrio</i>	100%
medium, white, opaque, round, entire	334.26			<i>Vibrio</i>	95%
red, opaque, round, entire	352.17	337.40		<i>Vibrio</i>	98%

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