Towards a Better Understanding of White Band Disease: Transmission, Causes, and Effects

by Sarah A. Gignoux-Wolfsohn

B.A. in Biology and French, Wesleyan University

A dissertation submitted to

The Faculty of
the College of Science of
Northeastern University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

April 11, 2016

Dissertation directed by

Steven V. Vollmer, PhD
Associate Professor of Marine and Environmental
Science
Dedication

I would like to dedicate this dissertation to the great female scientists on whose shoulders I stand and the many more who will come after me, and to my family and friends, the best cheerleaders I could ask for.
Acknowledgements

First, thank you to my advisor, Steve Vollmer, and my committee members: Geoff Trussell, Jon Grabowski, Win Chai, and Melissa Garren for the guidance and advice.

A huge thank you to Felicia Aronson, whose moral, physical, and intellectual support and input were instrumental in the completion of this dissertation.

I would like to thank the many people who have helped me over the last five years: Sarah Donelan, for years of science-laced friendship and experiments; Rebecca Certner for many rounds of edits and discussions of semantics; Tarik Gouhier for advice, code, and explanations of all things statistics; Jen Davidson for help with fieldwork and analyses, Kara Wiggin for help with lab work and organizing my ideas, and both for believing in the end of the week; Bill Precht for many discussions of white band disease; Lara McGrath for her wisdom; Emily Jones, Molly Roberts, Bobby Murphy, Nathaniel Chu, Hannah Nelson, Caitie Kuempel, and Chris Marks for help with field work; Sarah Kopac for editing and pep-talks; and Efren Bonner for italicizing all those periods.

Thank you to the staff of STRI Bocas del Toro: Plinio Gondola, Sebastian Castillo, Eric Brown, and Arnulfo Record for their understanding, patience, and logistical help. Thank you also to the staff of the Marine Science Center, especially Sonya Simpson, Heather Sears, Laura Evangelista, and Roberto Valdez for continually solving my problems and making me smile. I would like to acknowledge the support of the Three Seas Program, which enabled much of this research, and Liz Bentley Magee for all her help. Chapter 2 was funded by a grant from the NSF Biological Oceanography program to Steve Vollmer. I would also like to thank the many contributors to our Experiment.com campaign, who funded Chapter 4 of this dissertation. Thank you for believing in the importance of this research.

Thank you to Erik for teaching me math and computers and for hardening my resolve.
Lastly, thank you to my family: my Mom for encouraging my love of science from a young age, my Dad for copy-editing every paper I have ever written, and my sister for allowing me to practice lecturing and grading since 1992 and for always giving me much-needed perspective.
Abstract of Dissertation

This dissertation focuses on the Caribbean coral disease white band disease (WBD). Over the past forty years, WBD has been responsible for much of the destruction of the two previously dominant Caribbean acroporid corals Acropora cervicornis and A. palmata. WBD can be transmitted by direct contact and using sterile gauze soaked in disease homogenates and can be stopped by administration of antibiotics, suggesting an infectious, bacterial pathogen. The devastation caused by WBD and the other 20 described coral diseases worldwide necessitates a better understanding of disease transmission, the potentially pathogenic bacterial communities associated with diseased corals, and the potentially beneficial bacterial communities associated with healthy corals.

The first study explores biologically relevant methods of transmission of WBD using infection experiments in aquaria. I first exposed healthy fragments of A. cervicornis to corallivorous snails belonging to two species: Coralliophila abbreviata and C. caribaea. The snails had previously been exposed to healthy corals, diseased corals, or no corals. I found that C. abbreviata elicited disease signs regardless of previous coral consumed, showing that these snails are not just a vector but also a reservoir for WBD. These disease signs were transmissible by direct contact to fragments of healthy corals, demonstrating that this WBD phenotype is not simply a response to predation, but is the result of an infectious disease. In contrast, I found that C. caribaea never transmitted WBD regardless of previous exposure. I then placed intact and lesioned corals in aquaria and exposed them to either homogenates of corals displaying disease signs or control homogenates. I found that corals were only able to contract WBD through the water when they had previously been lesioned. The findings from this first study show that WBD can be transmitted by C. abbreviata and through the water column to injured corals, two methods that are likely responsible for its spread across the
Caribbean.

The second and third studies in this dissertation used next-generation high-throughput sequencing of the 16S rRNA gene to profile the bacterial communities associated with healthy and diseased *A. cervicornis*. In the first of these 16S studies, I sequenced both field-collected and tank-exposed diseased and healthy corals. The field-collected corals were collected from four sites during two different years. I found that disease state had a significant effect in structuring the bacterial communities of *A. cervicornis* in both datasets. In the field-collected corals, site had a significant effect on the bacterial communities of both healthy and diseased corals. I further identified OTUs that were consistently associated with diseased or healthy corals across both datasets. The majority of the disease-associated OTUs belonged to the orders *Flavobacteriales, Alteromonadales, and Oceanospirillales*. In the second of the 16S studies, I performed a large fully-crossed infection experiment using corals from two different sites inoculated with either a dose homogenate of corals exhibiting WBD signs or a healthy (control) homogenate. Corals were sampled before inoculation, after inoculation before corals showed disease signs, and after corals displayed disease signs. Using generalized linear mixed effects models, I was then able to separate OTUs based on their abundance in infected and control corals through time. I separated OTUs into four groups: colony-specific residents, primary responders, primary colonizers, and secondary OTUs.
# Table of Contents

Dedication iii  
Acknowledgements iv  
Abstract of Dissertation vi  
Table of Contents viii 
List of Tables ix 
List of Figures xi 

Chapter 1: Introduction 1

Chapter 2: White band disease transmission in the threatened coral, *Acropora cervicornis* 25

Chapter 3: Identification of candidate coral pathogens on white band disease-infected staghorn coral 38

Chapter 4: Experimental infection of *Acropora cervicornis* leads to changes in populations of resident and colonizing bacteria 71

Chapter 5: Conclusions 107
List of Tables

Table 2.1  White band disease transmission in corals exposed to the snails *C. abbreviata* and *C. caribaea* that were previously fed healthy or diseased corals, or were starved 36

Table 2.2  White band disease transmission in corals fed on by *C. abbreviata* versus experimental lesion controls 36

Table 2.3  White band disease transmission in corals (with and without lesions) inoculated with healthy or diseased coral homogenates 36

Supplementary Table 3.1  Infection rate for corals in tank-based infection experiment 65

Supplementary Table 3.2  Number of corals collected from each site 65

Supplementary Table 3.3  Significantly different OTUs between diseased and healthy for the field and tank datasets Available from publisher

Table 3.1  Diversity of OTUs 66

Table 3.2  Results of PERMANOVA based on Bray-Curtis dissimilarities of the relative abundance of OTUs on field-collected *A. cervicornis* in response to disease state, site, and year 66

Table 3.3  Results of PERMANOVA based on Bray-Curtis dissimilarities of the relative abundance of OTUs on field-collected healthy *A. cervicornis* in response to site and year 67

Table 3.4  Results of PERMANOVA based on Bray-Curtis dissimilarities of the relative abundance of OTUs on field-collected diseased *A. cervicornis* in response to site and year 67

Table 3.5  Significantly associated OTUs in tank and field-collected datasets 68

Supplementary Table 4.1  Mean diversity of bacterial communities associated with groups of coral 101

Supplementary Table 4.2  List of OTUs belonging to each designated group Available upon request
Supplementary Table 4.3  
**Significantly different OTUs**
Available upon request

Table 4.1  
**PERMANOVA of Bray-Curtis dissimilarity between samples collected at times two and three**

102
## List of Figures

<table>
<thead>
<tr>
<th>Figure 2.1</th>
<th>White band disease and predation in <em>Acropora cervicornis</em></th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3.1</td>
<td>nMDS plots of dissimilarities between samples</td>
<td>69</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Plots of the log2 fold abundance change of each OTU by the mean of normalized counts</td>
<td>69</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Taxonomic classification on the level of order of OTUs that are significantly more or less abundant in diseased corals compared to healthy across both tank-exposed and field-collected corals</td>
<td>70</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td><em>Endozoicomonas</em> are colony-specific resident bacteria of healthy corals</td>
<td>103</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Abundance of resident <em>Endozoicomonas</em> in high colonies at times two and three</td>
<td>104</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Mean abundance of primary responders belonging to selected families across time</td>
<td>104</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Mean abundance of primary colonizers belonging to selected families across time</td>
<td>105</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Mean abundance of secondary colonizers belonging to selected families on dosed corals that became diseased at time three</td>
<td>106</td>
</tr>
</tbody>
</table>
Chapter 1:
Introduction
Marine diseases

Marine organisms are currently experiencing an unprecedented rise in both the number and duration of disease epizootics (Burge et al., 2014). The increase in disease outbreaks is largely attributed to elevated water temperatures resulting from climate change, which intensify epizootics by simultaneously influencing the hosts, pathogens, and the environment (Harvell et al., 2002; Sokolow 2009). The effects of climate change are coupled with pollution and other anthropogenic factors (Harvell et al., 1999), which exacerbate disease severity. As we are now firmly in the Anthropocene age, in which humans are the dominant geophysical force, diseases, marine and otherwise, are expected to spread and increase (Harvell et al., 1999; Jackson 2008; Maynard et al., 2015; Steffen et al., 2007). This upsurge of new and more detrimental diseases could have large and potentially unforeseeable consequences on productive marine ecosystems, fisheries, and our global health (Lafferty et al., 2015).

When Dr. Robert Paine coined the term “keystone species,” he demonstrated how removal of the predatory *Pisaster ochraceus* sea star can lead to dramatic changes in species composition and physical appearance of the west coast intertidal system (Paine 1969; Paine 1974). Epizootics can play this role of the researcher, rapidly exterminating a single species or multiple species, and consequently profoundly changing the affected ecosystem. In recent years we have witnessed the fulfillment of Dr. Paine’s manipulative experiments on the west coast of North America, where the spread of the viral sea star wasting disease is currently decimating populations of *P. ochraceus* and other sea stars (Hewson et al., 2014; release 2013). In the Caribbean, an unknown pathogen which swept through populations of *Diadema antillarum* in the 1980s (Lessios et al., 1984)
elucidated this species’ role in maintaining a balance of macro algae and coral; once Diadema populations were removed, coral reefs became overgrown with macroalgae leading to the death of a large percentage of coral and associated fauna across the Caribbean (Lessios 1988; Liddell and Ohlhorst 1986). A better understanding of marine diseases that affect ecologically important species is imperative if we are to avoid future epizootics that bring about ecosystem-wide change.

Coral disease

Scleractinian corals are foundation species, providing the habitat (coral reefs) for many other organisms. These ecosystem engineers are responsible for creating some of the most diverse and productive ecosystems on the planet. Coral reefs provide humans with numerous ecosystem services: fish and invertebrates that rely on corals are a large source of protein, the flora and fauna surrounding reefs generate tourism revenue for developing nations, (Costanza et al., 1997; Moberg and Folke 1999) and reefs provide a buffer from incoming storms for low-lying coastal communities (Villanoy et al., 2012). Outbreaks of coral disease are being reported with alarming and increasing frequency around the globe (Harvell et al., 1999; Harvell et al., 2004; Hughes 1994; Maynard et al., 2015). In spite of the far-reaching consequences of diseases on these ecologically and economically important species, coral diseases are still poorly understood.

There are currently over 20 described coral diseases, all characterized by one of the four general signs of coral disease: tissue loss, skeletal anomalies, change in color, and bleaching (reviewed in: Bourne et al., 2009; Richardson 1998; Rosenberg and Ben-Haim 2002). Some of these diseases are found across the world and others in certain
locations, some are considered species-specific and others affect multiple species. Whether similar disease signs across oceans have the same etiology is unknown (Willis et al., 2004b). Furthermore, the pattern of the disease sign is very strongly influenced by the host species, making it difficult to distinguish between species-specific diseases and diseases whose diagnostic signs differ between species (Bourne et al., 2014; Willis et al., 2004a).

Coral disease signs can be indistinguishable from signs of abiotic stressors, further complicating identification of infectious diseases. For example, tissue loss from disease can look very similar to tissue loss due to sedimentation (Nugues and Roberts 2003) or predation (Rotjan and Lewis 2008). The observation of characteristic disease signs in the field is therefore not enough to confirm that the affected coral harbors an infectious disease. Abiotic stressors such as increased temperature and nutrient inputs can also increase the progression, severity, and spread of coral diseases, further complicating our ability to identify infectious diseases (Ben-Haim et al., 2003c; Bruno et al., 2003; Bruno et al., 2007; Tout et al., 2015; Voss and Richardson 2006). These interactions between abiotic stressors and infectious coral diseases have led some to conclude that all coral diseases are opportunistic infections, caused by members of the healthy coral microbiome, which can become pathogenic when the coral host is exposed to abiotic stressors (Lesser et al., 2007). While opportunistic infections are likely a large part of coral stress, the fact that some coral diseases can be transmitted to healthy corals free from stress negates the idea that environmental stress is the only cause of coral disease signs.
Coral disease transmission

An understanding of coral disease transmission will not only greatly improve our ability to distinguish between coral disease and abiotic stress, but also help us to better understand the causes of both. Transmission through the water column is the most logical non-vector transmission method; its existence has been inferred from patterns of disease spread for multiple diseases (Jolles et al., 2002; Zvuloni et al., 2009), and it has been confirmed for white plague disease (Clemens and Brandt 2015) and Vibrio-induced bleaching (Garren et al., 2014). Several coral diseases have been shown to be transmitted through vertebrate and invertebrate vectors (Antonius and Riegl 1997; Nicolet et al., 2013; Raymundo et al., 2010; Rosenberg and Falkovitz 2004; Rypien and Baker 2009; Sussman et al., 2003). One example in the Caribbean is the corallivorous snail Coralliophila abbreviata which is a vector for multiple tissue loss diseases (Clemens and Brandt 2015; Williams and Miller 2005). Confirmation of transmission methods for all described coral diseases will allow us to separate studies about disease signs caused by abiotic stressors from studies of infectious coral diseases, and help determine methods to control disease spread across reefs and oceans.

White band disease

White band disease (WBD), the focus of this dissertation, is a devastating coral disease affecting the two Caribbean acroporid corals: the staghorn coral, Acropora cervicornis and the elkhorn coral, A. palmata. WBD was first reported in 1979, and is cited as having led to the destruction of up to 95% of these two species (Aronson and Precht 2001). WBD has been divided into two types (Type I and II) based on gross
disease signs. WBD type I manifests itself as a moving front of necrotic tissue that rapidly progresses up a healthy coral leaving behind a band of bare, white skeleton (Gladfelter 1982). WBD type II has a margin of bleached tissue between the skeleton and healthy tissue (Ritchie and Smith 1998).

Whether the WBD signs seen across the Caribbean have different etiologies from the other white syndromes is unknown, since the characteristic band may result from the branching nature of these species. WBD is temperature-dependent: climate-driven changes in sea surface temperatures including increases in thermal minima and breaching thermal maxima have been shown to drive the increased disease incidence seen in recent years (Randall and van Woesik 2015). WBD can be transmitted by direct contact between corals, and through gauze soaked with disease homogenate.

**Koch’s postulates and coral diseases**

Koch’s postulates, which have historically been used to determine disease causation by a single pathogen, state: 1) The pathogen must be abundant in all infected organisms and absent from healthy organisms; 2) The pathogen must be isolated and cultured from a diseased organism; 3) The cultured pathogen must then be able to induce disease signs in a healthy organism and finally; 4) The pathogen must be re-isolated from that organism.

Attempts to apply these postulates to coral diseases have been marginally successful. The most well understood coral pathogens are multiple strains of *Vibrio*, which cause white syndromes in the Pacific (Arboleda and Reichardt 2010; Sere *et al.*, 2015; Sussman *et al.*, 2008; Ushijima *et al.*, 2012; Ushijima *et al.*, 2014; Zhenyu *et al.*, 2014).
2013). *Vibrio* have also been shown to cause bacterial bleaching in the Pacific (Ben-Haim *et al.*, 2003a; Ben-Haim *et al.*, 2003b). A strain of *V. shiloi* was found to induce bleaching in *Oculina patagonia* (Kushmaro *et al.*, 1997; Kushmaro *et al.*, 2001), but several years later, the same strains of *V. shiloi* no longer produced disease signs (Reshef *et al.*, 2006). This change in infectiousness prompted the development of the coral probiotic hypothesis, which states that changes in coral-associated microbial communities can enable corals to rapidly adapt to diseases and become resistant to previously infectious pathogens (Reshef *et al.*, 2006), thereby acting as a version of an adaptive immune system for the coral host. Attempts to fulfill Koch’s postulates for Caribbean coral diseases have been less successful than for Pacific diseases. Koch’s postulates have been partially fulfilled for multiple diseases (*e.g.*, Cervino *et al.*, 2008; Lesser and Jarett 2014; Patterson *et al.*, 2002; Richardson *et al.*, 1998), but attempts to identify putative pathogens on other diseased corals and repeat infection with previously identified pathogens commonly fail (Apprill *et al.*, 2013; Cardenas *et al.*, 2012; Closek *et al.*, 2014; Joyner *et al.*, 2015; Sutherland *et al.*, 2016).

Multiple factors contribute to the difficulties surrounding confirmation of coral disease etiologies using the traditional Koch’s postulates. First, coral diseases are in a near constant state of flux and disease signs are unreliable, as described in a recent review by Sutherland *et al.* (2016) as the “moving target” of coral disease etiology (Sutherland *et al.*, 2016). Second, as the coral probiotic hypothesis states, pathogens are acting on hosts that already house a suite of bacteria, which can change at a rapid rate and may help corals resist infection (Reshef *et al.*, 2006). Third, recent studies have shown that corals have a robust immune system, and some have genetic resistance to diseases, emphasizing
the importance of the host in our ability to infect with a putative pathogen (Brown and Rodriguez-Lanetty 2015; Burge et al., 2013; Libro et al., 2013; Libro and Vollmer 2016; Vollmer and Kline 2008). Lastly, marine bacteria have historically been difficult to culture using standard microbiology techniques designed to select for human pathogens, many of the coral pathogens that have been identified are pathogens that are easily culturable and usually associated with humans (Sutherland et al., 2016).

**White band disease etiology: What is currently known?**

A complete understanding of the etiology of WBD remains elusive, but multiple studies have shed light on putative causes of the disease. Kline and Vollmer (2011) used antibiotics and filtration to demonstrate that WBD transmission via homogenates of diseased corals is stopped by antibiotic treatment and filtration smaller than 0.2 um, indicating that WBD is a bacterial disease (Kline and Vollmer 2011). This finding was recently corroborated by a study that demonstrated that disease progression can be arrested by administration of antibiotics, but not by anti-ciliate compounds (Sweet et al., 2014).

Peters et al. (1983) first identified a possible cause of WBD as basophilic ovoid bodies containing gram-negative rod-shaped bacteria in the tissue of *A. cervicornis* and *A. palmata* in Bonaire. These bodies were detected in both corals exhibiting disease signs and apparently healthy corals. Casas et al. (2004) then used culture-independent techniques to identify a Rickettsia-like organism (RLO) associated with both diseased and healthy *A. cervicornis*, which may be the same bacteria seen in the initial ovoid bodies. Interestingly, the RLO was not identified in samples of *A. cervicornis* from
before the major WBD outbreak in the 1980s, indicating that the RLO may have been a cause of historical WBD outbreaks, but is now tolerated by the host. Another possibility is that the RLO may be involved in WBD by compromising infected corals before another pathogen or factor elicits the actual disease signs.

Using culture-dependent techniques, Ritchie and Smith (1998) isolated *V. charchariae* from WBD type-II corals, but not from healthy corals (Ritchie and Smith 1998). Signs of WBD type-II were later produced in corals inoculated with a strain of *V. charchariae* in Puerto Rico and three of Koch’s postulates were fulfilled (Gil-Agudelo et al., 2006). Whether this pathogen is responsible for WBD throughout the Caribbean is unclear, and efforts to replicate these results for WBD type-I in Panama were unsuccessful, suggesting the two types may have different etiological agents (Gignoux-Wolfsohn and Vollmer, unpublished data). *V. charchariae* was also identified as a putative pathogen by Sweet et al. (2014) as it was absent in antibiotic-treated corals where disease progression had stopped but abundant in untreated diseased corals. This study also identified two other putative pathogens in the same manner: *Lactobacillus suebicus* and *Bacillus* sp. as well as a species of ciliate that is consistently associated with diseased corals but does not seem to be the main cause of the disease (Sweet et al., 2014).

**The healthy coral microbiome**

Corals are not isolated organisms, but rather, “holobionts,” complicated symbioses consisting of the coral animal, endosymbiotic dinoflagellates (*Symbiodinium* sp.), other microbes including archaea and bacteria, and viruses (Bourne et al., 2009). This thesis will focus on the coral bacterial microbiome, and the diverse, complex
bacterial communities associated with the coral animal (Rohwer et al., 2002; Sunagawa et al., 2010). Many of the bacteria found on corals are uncultivable with conventional culture techniques (Zilber-Rosenberg and Rosenberg 2008). Culture-independent methods such as cloning allowed us to begin to see some of the true diversity of coral-associated bacterial communities. With the help of next-generation high-throughput sequencing, we are able to capture a much more accurate profile of these communities, and understand their true diversity. Corals represent an under-explored microbial habitat, which when sequenced, consists of a majority of sequences that cannot be identified past broad taxonomic classifications (Sunagawa et al., 2010).

Multiple studies have shown that coral species contain significantly distinct microbiomes, even when colonies are living sympatrically on the same reef (Kvennefors et al., 2010; Morrow et al., 2012; Roder et al., 2014a; Sunagawa et al., 2010). It has also been suggested that coral microbiomes may have evolved with their hosts, as frequently the similarities of microbiomes follow the phylogenetic patterns of their host species (Roder et al., 2014b; Bayer et al., 2013b; Sunagawa et al., 2010). This pattern of more closely related corals having more similar microbiomes supports what is known as the hologenome theory of evolution. This theory considers symbionts as genetic units undergoing selection, with the host transmitting symbionts between generations and the composition of symbionts affecting the fitness of the host (Zilber-Rosenberg and Rosenberg 2008). The host is thereby not merely evolving via changes to its genome, but rather changes to its entire “hologenome” consisting of the host genome and the genomes of its symbionts. This theory encompasses the coral probiotic hypothesis discussed above.
In addition to host species, healthy coral microbiomes are strongly affected by location. This pattern has been shown on both large (Kvennefors et al., 2010; Morrow et al., 2012; Roder et al., 2014b) and small (Littman et al., 2009; Pantos et al., 2015) spatial scales. Some of these geographic differences can be correlated to differences between near-shore and far-shore sites, suggesting that anthropogenic influences such as agricultural run-off and other pollutants may be affecting bacterial assemblages (Morrow et al., 2012; Roder et al., 2015).

Coral microbiomes have also been shown to vary seasonally, likely in response to fluctuations in temperature, dissolved oxygen, and rainfall (Kimes et al., 2013; Li et al., 2014; Littman et al., 2009; Roder et al., 2015). Interestingly, Li et al. (2014) found that these seasonal variations occur not only in the surface mucus layer (SML) of the coral but also in the tissue and the skeleton, indicating that seasonal changes affect the microbiome of the whole coral animal, not just the more transient mucus-associated bacteria. Temporal changes of the microbiome are compounded by replacement of the SML in a cyclical manner, with new mucus containing different bacterial communities from aged mucus (Glasl et al., 2016).

Although the existence of a beneficial symbiotic coral microbiome has long been assumed, efforts to identify a “core” coral microbiome taxonomically have yielded surprisingly few consistent results. A recent study by Ainsworth et al. (2015) showed that only 0.09% of OTUs were present in 90% of corals of a single species (Ainsworth et al., 2015). This finding suggests that perhaps the majority of the beneficial symbiotic microbiome is not conserved at the OTU level, but rather is transient at least partially due to environmental influences. If we subscribe to the coral probiotic hypothesis, and
consider bacteria as more rapidly adaptable elements of the coral holobiont, the lack of conservation of a microbiome across species and environments follows: a beneficial microbiome will vary depending on the environment and the fitness of corals in that environment. Studies showing a consistent core microbiome are often biased by small sample sizes.

While specific OTUs may not be consistently associated with healthy corals, certain taxa are repeatedly found on healthy corals, demonstrating that there may be redundancy in which OTUs can play the same beneficial roles. Bacteria belonging to the genera *Alteromonas* and *Roseobacter* are transmitted from parent corals to larvae and form specific associations with coral tissues at an early time point in development (Apprill *et al.*, 2012; Ceh *et al.*, 2013). Many groups of bacteria have been associated with healthy corals by comparing their abundance in healthy and diseased corals, these include: *Halomonas* (Meyer *et al.*, 2015), *Vibrionaceae* (Cardenas *et al.*, 2012; Closek *et al.*, 2014), *Enterobacteriaceae* (Closek *et al.*, 2014; Kellogg *et al.*, 2014; Roder *et al.*, 2014a) (Cardenas *et al.*, 2012), *Streptococcaceae* (Kellogg *et al.*, 2014), (Roder *et al.*, 2014a; Roder *et al.*, 2014b), and *Burkholderiales* (Cardenas *et al.*, 2012; Roder *et al.*, 2014a). The most widespread healthy-associated group of bacteria are bacteria in the genus *Endozoicomonas* (Order: *Oceanospirillales*, Family: *Hahellaceae*, sometimes referred to as *Endozoicomonaceae*). *Endozoicomonas* have been found to dominate the microbiomes of over 10 species of both hard and soft coral across multiple oceans (Bayer *et al.*, 2013a; Bayer *et al.*, 2013b; Apprill *et al.*, 2013; Bourne and Munn 2005; Jessen *et al.*, 2013; Kvennefors *et al.*, 2010; Morrow *et al.*, 2012; Morrow *et al.*, 2015; Roder *et al.*, 2015; Sere *et al.*, 2013; Speck and Donachie 2012; Sunagawa *et al.*, 2010; Yang *et al.*,
The immense diversity of bacterial communities associated with healthy corals and the influence of factors other than disease on these microbiomes complicate our ability to understand the effects of disease on healthy microbiomes, and even more so our attempts to identify the etiological agents of these diseases. Coral microbiomes, even when healthy, are clearly dynamic and complex communities, each bacterium interacting with both the host and its bacterial associates in numerous ways.

**The diseased coral microbiome**

These studies attempting to culture coral disease pathogens largely exist in a one-pathogen-one-disease framework and do not capture the full complexity of disease-associated bacterial communities. High throughput DNA sequencing has enabled us to sequence many more strains of bacteria than can be cultured, further calling into question the requirement of isolating a pathogen in pure culture in order to prove the etiology of a disease (Fredericks and Relman 1996). As our understanding of infectious diseases broadens across systems, we see that many diseases are not caused by a single pathogen. Rather, diseases can be caused by many species working together with the help of environmental factors and infection by a pathogen may be contingent on the pre-existing bacterial communities (Burge *et al.*, 2014; Byrd and Segre 2016). For these reasons, culture-independent examination of bacterial communities associated with diseased corals is imperative for a full understanding of coral disease etiology.

High-throughput sequencing of diseased coral-associated bacterial communities has revealed their full diversity. Frequently, these studies do not corroborate the results
of previous culture-dependent studies of diseases, which showed disease communities dominated by a handful of putative pathogens (Cardenas et al., 2012; Joyner et al., 2015; Sutherland et al., 2016; Apprill et al., 2013; Closek et al., 2014). Several studies have found a significant difference between the microbiomes of diseased corals and those of healthy corals across diseases (Closek et al., 2014; Croquer et al., 2013; Meyer et al., 2015; Roder et al., 2014a; Roder et al., 2014b; Sunagawa et al., 2009; Sweet et al., 2013). This distinction between diseased and healthy bacterial communities is frequently characterized by an increase in diversity in diseased corals, which may be either the cause or the effect of disease (Closek et al., 2014; Cooney et al., 2002; Croquer et al., 2013; Meyer et al., 2014; Pantos and Bythell 2006; Roder et al., 2014a; Sere et al., 2013; Sunagawa et al., 2009; Sweet et al., 2013). Comparisons of diseased and healthy corals have revealed taxa that are more abundant in diseased corals and several taxa have been found to be associated with multiple coral diseases. These taxa may be common secondary colonizers of already infected corals, or may be the causative agents of multiple diseases. Interestingly, multiple studies have noted that taxa that are associated with diseased corals are also found on healthy corals, supporting the idea that coral pathogens may be opportunistic and only become pathogenic when exposed to an external influence (Closek et al., 2014; Meyer et al., 2015). The use of high-throughput sequencing to profile WBD-associated bacterial communities will give us a more complete understanding of WBD than both previous low-throughput culture-independent studies and culture-dependent studies. This type of profiling will also allow us to compare WBD bacterial communities to those of other coral diseases, determining taxa which are unique to WBD-infected corals and common coral disease colonizers.
Dissertation objectives

The goal of this dissertation is to better understand the etiology and ecology of WBD as well as the ecology of WBD-associated bacterial communities using transmission experiments and culture-independent high-throughput sequencing. In Chapter 2, I will confirm modes of transmission of WBD via a corallivorous snail and through the water column. In Chapter 3, I will determine the effects of site, year, and disease state on the bacterial communities of A. cervicornis collected from the field, and identify putative WBD pathogens which are consistently associated with diseased corals collected from the field and infected in tanks. Finally, in Chapter 4, I will identify colony-specific resident bacteria of healthy A. cervicornis, and examine the timing and site-specificity of the establishment of disease-associated bacterial communities by infecting corals in tanks in a fully crossed experiment.
References


Glasl B, Herndl GJ, Frade PR (2016). The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance. *The ISME Journal*.


Lesser MP, Jarett JK (2014). Culture-dependent and culture-independent analyses reveal
no prokaryotic community shifts or recovery of Serratia marcescens in Acropora palmata with white pox disease. *FEMS microbiology ecology*.


Climatic Change 112: 493-505.


Chapter 2:
White Band Disease transmission in the threatened coral, *Acropora cervicornis*

Abstract

The global rise in coral diseases has severely impacted coral reef ecosystems, yet often little is known about these diseases, including how they are transmitted. White band disease (WBD), for example, has caused unparalleled declines in live *Acropora* cover, spreading rapidly throughout the Caribbean by unknown means. Here we test four putative modes of WBD transmission to the staghorn coral *Acropora cervicornis*: two animal vectors (*Coralliophila abbreviata* and *C. caribaea*) and waterborne transmission to intact and injured coral tissues. Using aquarium-based infection experiments, we determine that *C. abbreviata*, but not *C. caribaea*, acts as both a vector and reservoir for transmission of the WBD pathogen. We also demonstrate waterborne transmission to injured, but not intact staghorn coral tissues. The combination of transmission by both animal vectors and through the water column helps explain how WBD is spread locally and across the Caribbean.
Introduction

Coral reefs have experienced unprecedented declines due to a global rise in disease epizootics over the last 30 years (Bourne et al., 2009; Harvell et al., 2004; Ward and Lafferty 2004). There are now more than 20 described coral diseases (Sutherland et al., 2004), many of which are linked to increasing ocean temperatures and anthropogenic development (Harvell et al., 1999). Despite the negative impacts of these diseases, we often lack key information about their etiology and ecology (Lesser et al., 2007). Few coral pathogens have been identified and little is known about how most coral diseases are transmitted, making efforts to manage outbreaks difficult.

White band disease (WBD) is a prime example of a devastating coral disease that is poorly understood (Aronson and Precht 2001; Gil-Agudelo et al., 2006; Ritchie and Smith 1998). WBD is a host-specific disease that infects both Caribbean Acropora species (Aronson and Precht 2001) – the staghorn coral Acropora cervicornis and the elkhorn coral A. palmata – and is identified by a progressing band of dying white tissue (Gladfelter 1982). Since it was first reported in 1979 (Gladfelter 1982), WBD has led to the destruction of up to 95% of Caribbean Acropora cover (Aronson and Precht 2001; Bythell et al., 1993) and resulted in the listing of both species as threatened under the US Endangered Species Act (Hogarth 2006) and as critically endangered on the IUCN Red List (Aronson et al., 2010). Transmission experiments using filtered homogenates to isolate bacteria combined with antibiotic treatments demonstrate that WBD is infectious and caused by bacteria (Kline and Vollmer 2011). While a specific WBD pathogen has not been isolated, *Vibrio* and *Rickettsiales* bacteria have both been associated with the disease (Casas et al., 2004; Gil-Agudelo et al., 2006; Ritchie and Smith 1998).
WBD is highly transmissible experimentally, either via direct contact between diseased and healthy corals (Vollmer and Kline 2008) or through application of gauze soaked in diseased tissue homogenate to a healthy coral (Kline and Vollmer 2011). Natural modes of transmission, however, are poorly understood. Two putative methods of WBD transmission are animal vectors and waterborne transmission. The corallivorous snail Coralliophila abbreviata is a vector for “rapid tissue loss” in A. cervicornis (Williams and Miller 2005). Other coral diseases can be transmitted through the water column (Zvuloni et al., 2008) and transmission can be enhanced by prior injury (Aeby and Santavy 2006; Page and Willis 2007).

Here we investigate the potential for WBD transmission via two putative snail vectors and the water column. First, we tested if C. abbreviata and C. caribaea (two common corallivorous snails found on A. cervicornis (Miller 1981)) function as a vector and/or reservoir for WBD by exposing healthy A. cervicornis fragments to snails fed on healthy, diseased, or no corals (i.e. starved). Second, we tested the potential for waterborne transmission of WBD by adding diseased coral homogenates to closed-circuit aquaria containing intact and injured (i.e. lesioned) A. cervicornis fragments.

Methods

Healthy and diseased (active WBD) A. cervicornis fragments (6cm in length), and the snails C. abbreviata and C. caribaea were collected from Cayo Corales (9° 15’ 16” N, 82° 7’ 40” W) in Bocas del Toro, Panama in February 2012. Organisms were transported to the Smithsonian Tropical Research Institute in separate buckets. Coral fragments were cable-tied to plastic louver and acclimated in flow-through aquaria for
three days prior to each experiment.

In order to test whether *C. abbreviata* and *C. caribaea* are capable of acting as a vector and reservoir for WBD, both species of snails were starved for three days and then randomly fed healthy *A. cervicornis* tissue (Healthy), diseased *A. cervicornis* tissue (WBD), or nothing (Starved) for three days. Snails were then placed in individual cages each containing one healthy coral fragment. Four cages were randomly placed into nine 500L flow-through aquaria. Predation scars and WBD progression were measured (length x width) twice daily for five days. Once fragments exhibited signs of predation, the snails were removed from the cages. This allowed us to isolate predation (i.e. feeding) from disease progression.

A subsequent experiment was conducted to confirm that the WBD transmitted by *C. abbreviata* was not a response of the coral to mechanical damage. To do this, the first experiment was repeated with three cages containing one coral fragment and one *C. abbreviata*, and one cage containing a coral fragment that had been experimentally lesioned by removing ~7.5 mm² of coral tissue with an airbrush and 0.2 µm filtered seawater (Figure 1d). WBD resulting from either snail predation or lesion controls was monitored as above. To confirm that WBD caused by *C. abbreviata* was due to a transmissible pathogen, fragments that had been fed on by *C. abbreviata* and contracted WBD were grafted to experimentally lesioned healthy corals and monitored.

To test waterborne WBD transmission, twelve replicate fragments of five genotypes were collected and placed in six closed-circuit 500L aquaria containing a water circulation pump. Each aquarium contained two fragments of each of the five genotypes; one fragment per genotype was left intact and the other fragment was lesioned. Six
fragments of healthy and six fragments of diseased *A. cervicornis* were individually vortexed in 50 mL plastic conical tubes filled with 15 mL 0.2 µm filtered seawater and 3mm glass beads, and combined to make our separate diseased and healthy inoculates. Aquaria were then randomly inoculated with either a diseased or healthy homogenate.

Transmission data comparing presence and absence of WBD in each experiment were analyzed using Fisher’s exact tests (Sokal and Rohlf 1995).

**Results**

When transmission was compared between *C. abbreviata* and *C. caribaea*, predation by *C. abbreviata* caused significantly higher rates of disease transmission (9 out of 19 fragments) than predation by *C. caribaea*, which failed to transmit WBD (Fisher’s exact test, p=0.0046; Table 1). In contrast, prior feeding treatments (healthy, diseased, and starved) had no significant effect on WBD transmission rates by *C. abbreviata* (Fisher’s exact test, p=1; Table 1).

When WBD transmission was compared between *C. abbreviata* predation and lesion controls (mimicking predation), transmission was significantly higher in fragments fed on by *C. abbreviata* (21 out of 23) than lesion control fragments (1 out of 9) (Fisher’s exact test, p < 0.001; Table 2). When fragments with WBD transmitted by *C. abbreviata* were grafted to healthy corals with lesions, 43 percent (4 out of 9) of the grafted fragments contracted WBD, demonstrating that WBD resulting from snail predation is transmissible by multiple means.

When waterborne transmission was tested, 6 out of 15 lesioned fragments inoculated with disease homogenate contracted WBD (Fisher’s Exact test, p=0.017;
Table 3), while none of the intact (not lesioned) fragments inoculated with disease homogenate showed signs of WBD. No WBD was transmitted to fragments inoculated with a healthy homogenate.

**Discussion**

We tested four possible modes of White Band Disease transmission in *A. cervicornis*: predation by *C. abbreviata*, predation by *C. caribaea*, waterborne transmission to intact coral fragments, and waterborne transmission to lesioned coral fragments. Transmission was highest in corals bitten by *C. abbreviata*, verifying that *C. abbreviata* acts as a vector for WBD. WBD was transmitted by *C. abbreviata* regardless of whether snails fed upon healthy corals, diseased corals, or were starved, indicating that *C. abbreviata* is able to retain the disease pathogen for at least two weeks and act as a reservoir for WBD. In contrast, *C. caribaea* did not transmit WBD to *A. cervicornis*, suggesting that not all corallivorous snails are capable of transmitting WBD. Waterborne WBD transmission only occurred when corals were injured (i.e. lesioned), indicating that tissue injury facilitates WBD transmission in corals.

Previous experiments by Williams and Miller (Williams and Miller 2005) show that *C. abbreviata* acts as a vector, but not a reservoir for “rapid tissue loss” in *A. cervicornis*. Their experiments differ from our results in that only snails that had fed on disease caused tissue loss. They did not test the possibility of the snails being a reservoir by starving their snails. Our results indicating that *C. abbreviata* acts as a reservoir for WBD is similar to the well-characterized *Vibrio-Oculina* disease system where the fireworm, *Hermodice carunculata*, acts as a vector and winter reservoir for the *Vibrio*
shiloi bleaching pathogen on the Mediterranean coral *Oculina patagonica* (Sussman *et al.*, 2003). WBD prevalence also varies seasonally, and is highest in summer months (Libro and Vollmer, unpublished data). Thus, *C. abbreviata* may act like *H. carunculata*, and house the WBD pathogen during the winter and other periods of low WBD prevalence. Another corallivorous snail, *Drupella cornus*, has been associated with multiple coral diseases in the Indo-Pacific and Red Sea (Antonius and Riegl 1997; Onton *et al.*, 2011), but no explicit tests of its potential as a vector or reservoir have been performed to our knowledge.

It is unclear why *C. abbreviata*, but not *C. caribaea*, can transmit WBD given that both are corallivores of *A. cervicornis*. Failure of *C. caribaea* to transmit WBD could be due to a combination of factors. *Coralliophila caribaea* consumed less coral tissue than *C. abbreviata* (unpublished data) and thus, may not have acquired enough pathogen for transmission or produced a large enough lesion on healthy corals to allow the pathogen to infect the coral. An alternate and potentially more interesting possibility is that *C. caribaea* is inhospitable to the WBD pathogen.

In terms of waterborne transmission, our data indicate that WBD is transmissible through the water column only if the coral has been injured (i.e. lesioned). The potential for injury and thus infection seems high given the many ways that tissue injury can occur in nature, including competition, mechanical damage, or corallivory by snails, damselfish, butterflyfish, fireworms and other taxa. Evidence for waterborne WBD transmission helps explain how WBD spread across the Caribbean since direct contact between healthy and diseased corals occurs between neighbors and *C. abbreviata* generally do not travel long-distances (Williams and Miller 2005).
Understanding the nature of WBD transmission provides insights into how the disease might be managed and controlled. While controlling waterborne WBD transmission will prove difficult, management of *C. abbreviata* populations may be a practical method of reducing WBD incidence, especially in heavily impacted *Acropora* populations. More research is needed to assess the viability of *C. abbreviata* population control as a management strategy, to identify the WBD pathogen, and to understand pathogen dynamics within *C. abbreviata*.

**Acknowledgements**

We thank F. Aronson, N. Chu, E. Jones, E. Roberts, the Three Seas Program, and the Smithsonian Tropical Research Institute for field and logistical support. Members of the Vollmer lab provided valuable comments. Grant funding was provided by the NSF Biological Oceanography program to SV (OCE 0751666). Collection permits were provided by Autoridad Nacional del Ambiente (ANAM#SE/A-1-12).

**Author contributions**

SAGW, CJM and SVV designed the experiments. SAGW and CJM conducted the fieldwork, experiments, and analyzed the data. SAGW, CJM, and SVV co-wrote, reviewed, and edited the manuscript.
References


Table 2.1. White Band Disease transmission in corals exposed to the snails *C. abbreviata* and *C. caribaea* that were previously fed healthy or diseased corals, or were starved.

<table>
<thead>
<tr>
<th>Laboratory Feeding Treatment</th>
<th>n</th>
<th>WBD transmission in unbitten corals</th>
<th>WBD transmission in bitten corals</th>
<th>Total % WBD Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. abbreviata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>6</td>
<td>-</td>
<td>3 / 6</td>
<td><strong>50.0%</strong></td>
</tr>
<tr>
<td>WBD</td>
<td>6</td>
<td>-</td>
<td>2 / 6</td>
<td><strong>33.3%</strong></td>
</tr>
<tr>
<td>Starved</td>
<td>9</td>
<td>0 / 2</td>
<td>4 / 7</td>
<td><strong>44.4%</strong></td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>0 / 2</td>
<td>9 / 19</td>
<td><strong>42.9%</strong></td>
</tr>
<tr>
<td><em>C. caribaea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>6</td>
<td>0 / 5</td>
<td>0 / 1</td>
<td><strong>0.0%</strong></td>
</tr>
<tr>
<td>WBD</td>
<td>6</td>
<td>0 / 2</td>
<td>0 / 4</td>
<td><strong>0.0%</strong></td>
</tr>
<tr>
<td>Starved</td>
<td>3</td>
<td>0 / 1</td>
<td>0 / 2</td>
<td><strong>0.0%</strong></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>0 / 8</td>
<td>0 / 7</td>
<td><strong>0.0%</strong></td>
</tr>
</tbody>
</table>

Table 2.2. White Band Disease transmission in corals fed on by *C. abbreviata* versus experimental lesion controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th># WBD</th>
<th>% Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. abbreviata</em></td>
<td>23</td>
<td>21</td>
<td>91.3%</td>
</tr>
<tr>
<td>Lesion</td>
<td>9</td>
<td>1</td>
<td>11.1%</td>
</tr>
</tbody>
</table>

Table 2.3. White Band Disease transmission in corals (with and without lesions) inoculated with healthy or diseased coral homogenates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th># WBD</th>
<th>% Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Healthy Homogenate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lesion</td>
<td>15</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>No Lesion</td>
<td>15</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td><strong>Diseased Homogenate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lesion</td>
<td>15</td>
<td>6</td>
<td>40.0%</td>
</tr>
<tr>
<td>No Lesion</td>
<td>15</td>
<td>0</td>
<td>0.0%</td>
</tr>
</tbody>
</table>
Figure 2.1. White band disease and predation in *Acropora cervicornis*. WBD on *A. cervicornis* in the field (A) and experimentally transmitted in the laboratory (B), snail predation by *Coralliophila abbreviata* (C), and a lesion mimicking snail predation (D).
Chapter 3:
Identification of candidate coral pathogens on white band disease-infected staghorn coral

Abstract

Bacterial diseases affecting scleractinian corals pose an enormous threat to the health of coral reefs, yet we still have a limited understanding of the bacteria associated with coral diseases. White band disease is a bacterial disease that affects the two Caribbean acroporid corals, the staghorn coral *Acropora cervicornis* and the elkhorn coral *A. palmata*. Species of *Vibrio* and *Rickettsia* have both been identified as putative WBD pathogens. Here we used Illumina 16S rRNA gene sequencing to profile the bacterial communities associated with healthy and diseased *A. cervicornis* collected from four field sites during two different years. We also exposed corals in tanks to diseased and healthy (control) homogenates to reduce some of the natural variation of field-collected coral bacterial communities. Using a combination of multivariate analyses, we identified community-level changes between diseased and healthy corals in both the field-collected and tank-exposed datasets. We then identified changes in the abundances of individual operational taxonomic units (OTUs) between diseased and healthy corals. By comparing the diseased and healthy-associated bacteria in field-collected and tank-exposed corals, we were able to identify 16 healthy-associated OTUs and 106 consistently disease-associated OTUs, which are good candidates for putative WBD pathogens. A large percentage of these disease-associated OTUs belonged to the order *Flavobacteriales*. In addition, two of the putative pathogens identified here belong to orders previously suggested as WBD pathogens: *Vibronales* and *Rickettsiales*.

Keywords: white band disease/ *Acropora cervicornis*/ *Flavobacteria*/ coral disease
**Introduction**

Over the past few decades, coral reefs have experienced an unprecedented rise in the prevalence and impacts of coral disease epizootics (Harvell *et al.*, 1999; Harvell *et al.*, 2004) with especially severe impacts on Caribbean coral reefs (Goreau *et al.*, 1998). In spite of these impacts and a significant increase in scientific research, we still lack critical information about the etiology and ecology for most of the ~20 described coral diseases (Lesser *et al.*, 2007; Mouchka *et al.*, 2010; Sutherland *et al.*, 2004). The need for a better understanding of coral diseases has escalated with an increasing amount of data tying the rise in coral disease prevalence and increased pathogen virulence to warming ocean temperatures (Burge *et al.*, 2014; Harvell *et al.*, 1999).

A major roadblock for coral disease research is the difficulty of isolating and culturing coral disease pathogens (Richardson 1998; Rosenberg *et al.*, 2007). As a result, the discovery of putative pathogens has relied heavily on identifying bacteria that are strongly associated with diseases using genetic techniques such as clone-based 16S rRNA gene sequencing (Barneah *et al.*, 2007; Casas *et al.*, 2004; Closek *et al.*, 2014; Cook *et al.*, 2013; Cooney *et al.*, 2002; Croquer *et al.*, 2013; de Castro *et al.*, 2010; Frias-Lopez *et al.*, 2002; Frias-Lopez *et al.*, 2004; Kimes *et al.*, 2013; Pantos *et al.*, 2003; Sato *et al.*, 2009; Sekar *et al.*, 2006; Sweet and Bythell 2012) and, more recently, high-throughput 16S rRNA gene sequencing and high-throughput microarrays (Apprill *et al.*, 2013; Cardenas *et al.*, 2012; Garcia *et al.*, 2013; Lesser and Jarett 2014; Roder *et al.*, 2014b; Sunagawa *et al.*, 2009). High-throughput 16S rRNA gene sequencing of the coral
microbiome has revealed a higher diversity of bacteria than previously thought; in a survey of 16S gene sequencing studies across seven Caribbean coral species, Sunagawa et al. (2009) predict that each individual coral harbors several thousand operational taxonomic units (OTUs). This high diversity makes identifying putative pathogens in the coral microbiome more difficult.

One of the most destructive coral diseases to date is white band disease (WBD), a host-specific disease that affects the two Caribbean acroporid species: *Acropora cervicornis* (staghorn coral) and *A. palmata* (elkhorn coral). Since it was first observed in 1979 (Gladfelter 1982), WBD has caused unprecedented Caribbean-wide mass die-offs of these critical reef-building species (Aronson and Precht 2001), resulting in the recent listing of both species as endangered under the US Endangered Species Act (Sobeck 2014). WBD is infectious and can be transmitted by direct contact between corals, through the water column to an injured coral, and by the corallivorous snail *Coralliophila abbreviata* (Gignoux-Wolfsohn et al., 2012; Vollmer and Kline 2008). Previous work confirms that WBD is caused by a bacterial pathogen (Kline and Vollmer 2011; Sweet et al., 2014) and multiple putative pathogens have been identified (Casas et al., 2004; Gil-Agudelo et al., 2006; Ritchie and Smith 1998; Sweet et al., 2014). Ritchie and Smith (1998) isolated a strain of *Vibrio* from WBD-infected *A. cervicornis* that was similar to *Vibrio charchariae* in both metabolism and morphology (Ritchie and Smith 1995; Ritchie and Smith 1998) and Gil-Agudelo et al. (2006) were then able to elicit WBD signs in a healthy coral with a putative pathogen similar to *V. charchariae* (Gil-Agudelo et al., 2006). Sweet et al. (2014) used a combination of culture independent and culture dependent antibiotic experiments to identify three candidate WBD pathogens: *V.*
charchariae, Lactobacillus suebicus, and Bacillus sp. Casas et al. (2004) used culture-independent 16S rRNA sequences to identify a novel Rickettsiales-like bacterium associated with A. cervicornis fragments that were collected after the outbreak of WBD, but which was absent from museum samples collected prior to the outbreak. Because independent studies have identified different putative WBD pathogens, it is possible that multiple pathogens (either singularly or in combination) could elicit WBD signs.

In this study, we used Illumina 16S rRNA gene sequencing to compare the bacterial communities of healthy and diseased (WBD infected) A. cervicornis collected in the field from four sites in two different years. We then conducted tank-based infection experiments to identify differences in the bacterial communities of infected, exposed but asymptomatic, and healthy control corals, and compared these data to the two years of field data. Non-metric multidimensional scaling (nMDS) and PERMANOVA were used to characterize community-level changes in the coral microbiome due to disease state, site, and year. Multi-factor negative binomial generalized linear models (GLMs) were then used to quantify significant changes in individual OTU abundances between diseased and healthy corals in the field and tank datasets. Those OTUs that were strongly and consistently associated with disease in both datasets are the most likely WBD pathogens.

Materials and Methods

Field collections

Collection permits were provided by Autoridad Nacional del Ambiente (ANAM#SE/A-1-12) for sampling of the protected species Acropora cervicornis.
Seventy-nine one cm fragments of *A. cervicornis* (30 healthy and 49 diseased) were collected from the field in the summers of 2009 and 2010 from four sites in Coral Cay, Bocas del Toro, Panama approximately 500 m apart. WBD interfaces were tagged with cable ties prior to collection and only samples with actively progressing WBD were sampled from the field. Diseased coral samples were cut at the interface of living tissue and dead skeleton. Healthy samples were taken from completely asymptomatic coral colonies. Corals were transported from the field in separate containers and placed in one ml of DNA buffer for preservation (Fukami *et al.*, 2004). Seventy-nine samples were extracted: 49 diseased and 30 healthy. Numbers of corals collected from each site are available in supplementary Table 3.1.

**Tank-based infection experiment**

In February of 2012, 36 healthy five cm fragments of *A. cervicornis* from six colonies were collected, transported back to the wet lab at the Smithsonian Tropical Research Institute, and cable-tied to plastic louver. One fragment from each colony (six total fragments/tank) was randomly placed in six flow-through 20 L aquaria with a koralia nano powerhead (Hydor USA Inc., Sacramento, CA, USA) to acclimate for one day. A disease homogenate was made by first vortexing six five cm coral fragments with active WBD signs in separate falcon tubes with glass beads and 15 mL filtered seawater (after Kline and Vollmer 2011) and then pooling together the separate homogenates. A healthy (control) homogenate was made in the same manner using coral fragments from colonies that did not show signs of disease. Flow was stopped and three tanks were each dosed with 30 mL of diseased coral homogenate and the other three were dosed with 30
mL of the healthy homogenate. Healthy coral fragments were then monitored every two hours for disease signs. As infected corals exhibited signs of WBD (after 40 hours), they were removed from the experimental tanks and one cm of coral tissue at the disease interface was preserved in DNA buffer. Healthy fragments were all sampled on day four of the experiment. Number of corals infected in each tank is available in supplementary Table 3.2. Total DNA was extracted from 19 fragments from this experiment: seven dosed with disease homogenate and contracted WBD (DD), seven were dosed with healthy homogenate and remained healthy (HH), and five were dosed with disease homogenate but remained healthy or asymptomatic (DH).

16S library preparation

DNA was extracted from samples preserved in guanidine thiocyanate DNA buffer (Fukami et al., 2004) using the Agencourt DNAdvance bead extraction kit (Agencourt Bioscience Corporation, Beverly, MA, USA) a blank DNA extraction was performed with each round of extractions. The V6 hypervariable region of the ribosomal small subunit 16S gene was chosen as the target due to its short length but high sensitivity to diversity, making it a good candidate for Illumina sequencing which has short read lengths but high sequencing depth (Barriuso et al., 2011; Caporaso et al., 2012; Youssef et al., 2009). The V6 region was amplified with custom barcoded primers that consist of a region that anneals to the V6 region of interest, followed by a unique five basepair barcode, and the Illumina sequencing adapter (Gloor et al., 2010):

\[
V6-L \ [5’-CGGTCTCGGCATTCTGCTGAACCGCTCTCCGATCTnnnnnACRACAC]
\]
Barcodes differed by two or more basepairs to reduce incorrect barcode calling. A separate, 40 µl PCR reaction for each sample was performed with a unique combination of primers: 5 µl each 4mM primer, 8µl standard Taq buffer (New England Biolabs, Ipswich, MA, USA), 0.8µl dNTPs, 20 µl diH2O, 0.5 µl Taq DNA polymerase (NEB) for the following cycle: 94°C for 2m, 28 cycles of: 94°C for 15s, 55°C for 15s, 72°C for 30s, followed by 72°C for 1m. A negative control and blank was amplified with each set of reactions. PCR reactions were pooled and amplified with the Illumina primers: OLJ139 [5’AATGATACGGCGACCACCAGATCTACACTCTTTCCCTACACGA3’] OLJ140 [5’CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCCTGCTGAAC3’] in a 40 µl reaction: 8 µl Phusion buffer (NEB), 0.8 µl dNTPs, 0.5 µl Phusion HI fidelity Taq (NEB), 20.2 µl diH2O, 0.5 µl DNA (previous PCR product), for the following cycle: 98°C for 2m, 12 cycles of: 98°C for 1m, 55°C for 1m, 72°C for 1m, and finally 72°C for 5m. Final PCR products were cleaned using the DNAmpure beads (Agencourt), concentration and length were verified using the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA) and sequenced using paired-end 150 basepair sequencing on the Illumina Hiseq 2000 at Tufts University.
**Bioinformatics**

Paired reads were overlapped using FLASH (Magoc and Salzberg 2011). Basepairs with a Phred score <q20 were trimmed. Sequences were assigned sample IDs using a custom Python script (available at: https://github.com/eholum/BioStuffs). Samples were then run through QIIME v.1.7.0 (Caporaso et al., 2010). Briefly, OTUs were chosen based on 97% sequence identity using the open reference OTU picking workflow using UCLUST against the Greengenes database (DeSantis et al., 2006). Taxonomy of each OTU was assigned using BLAST against the Greengenes database and those samples that corresponded to chloroplast DNA in order or lower taxonomic levels were excluded as algal symbiont contamination. OTU counts were then normalized using the size factors method from the R package DESeq2 (Anders and Huber 2010) prior to statistical analyses to account for differences in library size (an alternative to rarefaction) and OTUs observed fewer than 10 times were removed in order to reduce undue bias of low abundance OTUs. Statistical analyses were run both with and without low abundance OTUs and results did not change significantly.

**Statistical analyses**

Dissimilarity between samples was visualized using non-metric multidimensional scaling (nMDS) using the metaMDS function in the R package Vegan (Jari Oksanen and O'Hara 2013), and community level differences between the three factors (disease state, site, year) were tested by PERMANOVA of Bray-Curtis dissimilarities using the Adonis function and by fitting the factors to the nMDS using the function envfit. Diversity metrics were calculated using the functions rarefy and diversity in Vegan. Significant
differences in the abundance of individual OTUs were identified using a multifactorial negative binomial GLM, implemented in the R package DESeq2 (Anders and Huber 2010). The data were fit to a negative binomial distribution and significantly different OTUs (p-value adjusted by FDR <0.05) between diseased and healthy samples were determined using the Wald test for significance of GLM terms (Anders and Huber 2010). Analyses were run separately for the field and tank-exposed data. In the field data, two separate GLMs were run for site (disease state x site) and year (disease state x year) and OTUs that differed significantly in either were considered to be transient OTUs. Disease-associated OTUs were compared between both datasets to identify OTUs with consistent and strong associations to WBD. Transient OTUs that were present in the list of disease-associated OTUs were placed in a second tier of disease-associated OTUs, leaving only those OTUs that were consistently disease-associated and did not differ due to site or year in tier 1. R scripts for statistical analyses are available at https://github.com/sagw/R-scripts.

Nucleotide sequence accession numbers

Sequences from this study have been deposited in the SRA database under biosample accession numbers SAMN03785223- SAMN03785320.

Results

16S v6 rRNA gene sequencing from the field-collected corals yielded 10,849,364 reads across 79 samples (49 diseased and 30 healthy). The data from tank-exposed corals contained 2,954,558 reads across 19 samples (seven exposed to disease that contracted
disease (DD), seven exposed to healthy that remained healthy (HH), and five exposed to disease that remained healthy (DH)). Read length varied from 57-137 bp. After clustering reads from both datasets at 97% similarity, 43,304 OTUs were identified. The field data contained 32,347 OTUs while the tank infection data had 18,195 OTUs. In all, 7,408 of these OTUs were shared between the two datasets. In both datasets, diseased corals exhibited elevated OTU diversity and richness, but OTU diversity was only significantly higher in diseased corals from the field (Table 1).

PERMANOVA detected strong, community-level differences in the coral bacterial communities in the field as well as in the tank-exposed corals. In the field-collected corals, the coral microbiome differed significantly due to disease state ($R^2 = 0.10, p = 0.001$), site ($R^2 = 0.12, p = 0.001$, and year ($R^2 = 0.019, p = 0.011$) as well as all combinations of interactions (Table 2). Disease state and site had similar and strong levels of influence on the bacterial community ($R^2$ of 0.10 and 0.12, respectively). To confirm the effects of the primary factors (disease state, site, and year) on OTU abundance, the factors were fit to the nMDS using envfit. Disease state ($R^2 = 0.39, p=0.001$) and site ($R^2=0.26, p=0.001$) had significant effects, with disease state having a greater influence on the bacterial community. These results corroborate the strength of the effects of site and disease state on the bacterial communities as demonstrated by PERMANOVA, the differences are most likely due to the reduction in dimensional space by nMDS.

On the nMDS plot, diseased and healthy corals separated along nMDS1 while corals separated by site along nMDS2 (Fig. 1a). PERMANOVA and nMDS analyses using presence/absence data (not shown) gave the same results as the abundance data,
indicating that significance was not driven solely by differences in abundance of OTUs. To further explore the strength of the effects of site on diseased and healthy corals, we split the data from diseased and healthy corals and ran two separate PERMANOVAs. These PERMANOVAs indicated that site had a larger effect on the bacterial communities of healthy corals ($R^2=0.28$, $p=0.001$, Table 3) than on those of diseased corals ($R^2=0.13$, $p=0.001$, Table 4).

PERMANOVA on the dataset from tank-exposed corals also indicated a significant effect of disease state ($R^2=0.29$, $p=0.007$), which was supported by the nMDS plot, where diseased and healthy corals separated along nMDS1 (Fig. 1b). Interestingly, the corals that were exposed to disease but remained asymptomatic (DH) clustered more closely on the nMDS plot with healthy (HH) corals than diseased (DD) corals.

The negative binomial GLM on the field data comparing the abundance of OTUs across disease state and year identified 1,363 individual OTUs that differed significantly due to disease state, 20 OTUs that differed significantly due to year, and 66 OTUs that differed significantly due to the interaction of disease state and year (Table 5). The majority of the OTUs that differed due to disease state (1,012 or 74%) were significantly more abundant on diseased corals than healthy corals (Fig. 2a). In the tank-infected corals, the negative binomial GLM comparing disease-exposed corals that contracted disease (DD) with healthy-exposed (i.e. control) corals (HH) identified 521 OTUs associated with disease state, the majority of which (n = 494; 95%) were more abundant on the disease-infected corals (Fig. 2b).

By comparing disease and healthy-associated OTUs in the field-collected dataset to those in the tank-exposed dataset, we identified 106 consistently disease-associated
OTUs (i.e. more abundant in diseased corals) and 16 consistently healthy-associated OTUs (i.e. more abundant in healthy corals) and classified them by order (Fig. 3). We further narrowed the list of consistently disease- or healthy-associated OTUs by dividing them into two tiers: tier 1 consists of those OTUs that differed significantly by disease state but not by site or year, and tier 2 consists of those OTUs that differed by disease state as well as by site or year (i.e. were significantly different in the DESeq model ~disease state:site or ~disease state:year). We identified 12 tier 2 OTUs: seven associated with diseased corals and two associated with healthy corals. Disease-associated tier 2 OTUs consisted of seven OTUs that differed by year (two belonging to Flavobacteriales, two Rhodobacterales, one Alteromonadales, one Oceanospirillales, one unidentified) and two OTUs that differed by both year and site (one belonging to the phylum Tenericutes and one unidentified). Healthy-associated tier 2 OTUs consisted of two OTUs that differed by site (belonging to the orders Burkholderiales and Pseudomonadales) and one OTU that differed by both year and site (Saprospirales).

The five bacterial orders with the greatest number of disease-associated OTUs in tier 1 were: Flavobacteriales with 27 OTUs, Alteromonadales with 13 OTUs, Oceanospirillales with eight OTUs, Campylobacterales with seven OTUs, and Rhodobacterales with four OTUs (Fig. 3). The three bacterial orders with multiple healthy-associated OTUs were: Flavobacteriales with four OTUs and Stramenopiles with two OTUs. Complete taxonomic information for all significantly different OTUs can be found in Supplementary Table 3.
Discussion

When *Acropora cervicornis* is infected with white band disease, its microbiome changes drastically, with an increase in hundreds of disease-associated OTUs, rather than just a few potential pathogens. The shift from a healthy to a diseased coral microbiome was marked by elevated microbial diversity and richness, a pattern that is consistent across other coral diseases (Apprill *et al.*, 2013; Closek *et al.*, 2014; Croquer *et al.*, 2013; Pantos *et al.*, 2003; Roder *et al.*, 2014b; Sere *et al.*, 2013; Sunagawa *et al.*, 2009). We also detected strong site-specific variation in the microbiomes of all corals. Interestingly, the site-specificity of the bacterial communities was strongest on healthy corals, and thus contraction of WBD appears to reduce this site specificity. Because we identified a large number of disease-associated OTUs, the observed differences in the bacterial communities of diseased and healthy corals are not likely the results of a change in the abundance of a single primary pathogen, but are also due to changes in opportunistic pathogens, secondary colonizers, and saprophytic bacteria. Many of the OTUs that are more abundant on diseased corals are also present on healthy corals. This could be because some apparently ‘healthy’ corals are asymptomatic, but harbor subclinical infections of disease-causing bacteria. Alternatively, these OTUs may be ‘pathobionts’ (Chow *et al.*, 2011): microbes that are present on the host but become pathogenic in certain hosts due to environmental conditions or host susceptibility. In all, we identified 97 consistently disease-associated bacteria across space and time belonging to 17 different orders of bacteria; the most abundant and noteworthy are discussed below.

*Flavobacteriales* made up the largest percentage (27 OTUs, 27%) of our disease-associated OTUs and are therefore likely involved in the WBD etiology. *Flavobacteria*
are well-known pathogens of fish (Bullock et al., 1986; Starliper 2011). White band disease shares some notable characteristics with flavobacterial diseases in fish. Two common flavobacterial fish pathogens, *Flavobacteria psychrophilum* and *F. columnaris*, both cause open lesions and tissue necrosis (Bullock et al., 1986; Davis 1922; Starliper 2011), similar to WBD. Furthermore, infection of salmonids with *F. columnaris* can be characterized by simultaneous infection with other strains of *Flavobacteria* (Tiirola et al., 2002), suggesting that one of the 27 WBD-associated *Flavobacterales* OTUs identified here may be the primary pathogen. The similarities between WBD and columnaris disease are strengthened by our finding that *Pseudomonadales*, which is an antagonist of *F. columnaris* (Tiirola et al., 2002), was consistently associated with healthy corals.

Lower abundances of *Pseudomonadales* in an initially healthy coral may allow strains of *Flavobacteria* to infect and proliferate and thereby cause disease. If the etiology of WBD is in fact similar to that of columnaris disease, then one of the 27 *Flavobacterales* OTUs that are associated with WBD may be a keystone pathogen. Keystone pathogens cause shifts in the natural bacterial community of their host, which lead to a diseased state, making it harder to detect the primary pathogen (Hajishengallis et al., 2012; Zwart et al., 2011). Because *Flavobacterales* are also found on healthy corals, they may be pathobionts or opportunistic pathogens. In addition, multiple strains of *Flavobacterales* may elicit the same characteristic disease signs in *A. cervicornis*, or they may work together to cause WBD. Understanding the time-course of infection with strains of *Flavobacterales* will help to elucidate what roles the different flavobacterial species are playing in the diseased coral. *Flavobacteria* have been largely overlooked as potential pathogens in coral diseases, yet species of *Flavobacteria* have been previously associated
with both black band disease and white plague disease (Cooney et al., 2002; Frias-Lopez et al., 2002; Jones et al., 2004; Sunagawa et al., 2009). Flavobacteria may therefore be more important in coral disease as a whole than previously thought.

The next most abundant orders of disease-associated OTUs—*Alteromonadales* (13 OTUs), *Oceanospirillales* (eight OTUs), *Campylobacterales* (eight OTUs), and *Rhodobacterales* (four OTUs)—are not likely candidates for a primary WBD pathogen. While they have all been associated with corals and in some cases coral diseases, none of these orders include well-characterized coral pathogens, but instead seem to play either a beneficial, opportunistic, or secondary role in the diseased coral. *Alteromonadales* have been associated with both white plague disease (Roder et al., 2014b; Sunagawa et al., 2009), and yellow band disease (Croquer et al., 2013), but have also been identified as “resident” coral bacteria (Allers et al., 2008; Bourne and Munn 2005; Ceh et al., 2013; Ritchie 2006). The only known pathogen in this order is *Thalassomonas loyana*, the causative agent of a white plague-like disease, but members of the *Alteromonadales* order are not well-known pathogens in other animals (Thompson et al., 2006). Members of the order *Oceanospirillales* have not been previously associated with any coral or other animal diseases (Morrow et al., 2012; Mouchka et al., 2010; Rohwer et al., 2002; Sharp et al., 2012), but have been recently been shown to be commonly associated with corals potentially as beneficial symbionts (Bayer et al., 2013; Sere et al., 2013). Some members of *Oceanospirillales* are well-known for their role in the bacterial bloom following the Deepwater Horizon oil spill (Hazen et al., 2010; Mason et al., 2012) and their ability to degrade Dimethylsulfoniopropionate, hydrocarbons, and amino acids (Jensen et al., 2010; Raina et al., 2009; Raina et al., 2010). Members the order *Campylobacterales* are
zoonotic pathogens which are commensal in marine mammals and birds (Lee and Newell 2006; Stoddard et al., 2005). While strains of *Campylobacterales* have been associated with coral diseases previously, including black band disease (Frias-Lopez et al., 2002), white syndrome, brown band disease (Sweet and Bythell 2012), and white plague (Sunagawa et al., 2009), their role in the etiologies of these diseases remains unclear. Given that *Campylobacter* are frequently found in sewage (Moreno et al., 2003), they may be introduced to the coral microbiome via human sewage deposition into the ocean. *Rhodobacteraceae*, our fifth most common disease-associated order, have also been associated with many coral diseases in multiple species across a variety of locations (Mouchka et al., 2010; Roder et al., 2014a; Roder et al., 2014b; Sunagawa et al., 2009), but have not been identified as a pathogen in corals or other animals. One of the six disease-associated *Rhodobacteraceae* identified here was present in every diseased and healthy coral sampled across both datasets, but increased in abundance on diseased corals, similar to previously described changes in abundance of strains of *Rhodobacter* in response to disease in other corals (Sunagawa et al., 2009). The disease-associated OTUs belonging to these four orders are likely either 1) secondary opportunistic colonizers, which take advantage of a diseased coral’s weakened state (Sunagawa et al., 2009); 2) beneficial symbionts, which increase in an effort to combat the primary pathogen; or 3) saprophytic bacteria, which are attracted to the increased nutrients of a dying coral.

In addition to these bacterial orders with multiple disease-associated OTUs, we identified two previously proposed WBD pathogens among the disease-associated OTUs: one belonging to the family *Vibrionaceae* and one to *Rickettsiaceae*. Species of *Vibrio* have been confirmed as pathogens in other coral diseases (Cervino et al. 2008, Ushijima
et al. 2012), most notably *V. corallilyticus* in *Pocillopora damicornis* tissue lysis (Ben-Haim *et al.*, 2003). *Vibrios* are of special interest as pathogens given their well-characterized role in human diseases such as cholera (Butler and Camilli 2005). Ritchie and Smith (1998) first isolated a strain of bacteria from diseased corals that was identified as *Vibrio charchariae* through morphological and metabolic methods. Gil-Agudelo *et al.* (2006) were then able to isolate and genetically identify a strain of *Vibrio charchariae* from diseased corals in Puerto Rico, which elicited signs similar to WBD in healthy corals (Gil-Agudelo *et al.*, 2006; Sweet *et al.*, 2014). While more research is needed to confirm the relationship of the strain identified here to *Vibrio charchariae*, our identification of a consistently white band disease-associated *Vibrio* using culture-independent methods supports the findings of these previous studies. We also identified five unclassified gammaproteobacteria associated with diseased corals, which may be *Vibrio* and warrant further investigation.

Members of the order *Rickettsiales* are well-characterized pathogens in marine invertebrates (Friedman *et al.*, 2000). As obligate intracellular parasites, pathogenicity of *Rickettsia* can only be determined using genetic or histological methods, not in culture (Walker 1996). *Rickettsia*-like organisms have been associated with diseased Caribbean acroporids in both histological and genetic studies (Casas *et al.*, 2004; Peters *et al.*, 1983). Casas *et al.* (2004) identified a coral-associated *Rickettsiales* sequence (called CAR-1a) on *A. cervicornis* collected after the outbreak of WBD, which was absent on museum specimens predating disease. CAR-1a was common on both diseased and healthy *A. cervicornis*, suggesting that it is likely an opportunistic pathogen, which only becomes pathogenic under certain conditions. Similarly in our study, *Rickettsiaceae* was
found on more than 90% of all *A. cervicornis* both from the field (92% on healthy; 97% on diseased) and tanks (100% of corals). While more information is needed to determine if the *Rickettsiaceae* observed here is the previously detected CAR-1a strain, it does display similar patterns of abundance.

Given the large number of WBD-associated OTUs, including two previously suggested putative pathogens as well as many strains of *Flavobacteria*, we should consider the possibility that multiple bacterial species could elicit WBD signs either singularly or as a consortium. Coral diseases such as yellow band disease and black band disease are caused by a consortium of bacteria (Cervino *et al.*, 2008; Cooney *et al.*, 2002). In WBD, multiple disease-associated bacteria may be able to cause disease signs in slightly different combinations. Alternatively, one or more of these putative pathogens may actually be secondary colonizers, bacteria that are only able to colonize the host after the primary colonizer (or pathogen) has infected and altered the host environment.

When looking at other factors that contribute to coral microbiome variability, we found that site had as large an effect on the composition of the coral microbiome as disease. While the collection sites were only two to six km apart, this finding is similar to the effects of site on the bacterial communities of other species of coral (Apprill *et al.*, 2013; Garcia *et al.*, 2013; Kvennefors *et al.*, 2010; Littman *et al.*, 2009; Roder *et al.*, 2014b; Sere *et al.*, 2013). Interestingly, we found that site had an effect on both healthy and diseased coral bacterial communities. However, site had less of an effect on diseased coral-associated bacterial communities, indicating that WBD reduces the natural site-specificity of coral-associated bacteria. We identified 5 OTUs in our list of disease- or healthy-associated OTUs that differed due to site and disease state belonging to the
orders: *Burkholderiales, Pseudomonadales, Sapropirales, Tenericutes*, and unidentified bacteria. Further investigation into OTUs that differ due to disease and site will require larger sample sizes from each site. The site-specific variation of diseased coral-associated bacteria is likely complicating our search for primary coral disease pathogens.

In examining WBD-associated bacterial communities, we have identified multiple previously proposed WBD pathogens, and propose strains of *Flavobacteriales* as new putative WBD pathogens. Further work should examine the possibility that multiple strains are involved in causing WBD. To separate the cause of the disease from the effects, the timing of colonization of diseased corals by the disease-associated bacteria must be examined. While culturing of pathogens is still required to confirm etiology, this culture-independent genetic analysis of diseased individuals will greatly inform future culture-based experiments. Understanding diseased and healthy bacterial communities on lower-level organisms such as corals can aid our understanding of more complex bacterial communities such as those in the human gut. Ultimately, a better characterization of coral-associated bacterial communities will inform our quest to understand and mitigate the current rise in coral diseases.

**Acknowledgements**

We would like to thank members of the Vollmer lab and the Three Seas program for help with sample collection, especially F. Aronson, S. Libro, and E. Hemond; G. Gloor and D. Carter for providing barcodes and PCR troubleshooting; T. Gouhier for help with statistical analyses; E. Holum for help with Bioinformatics; J. Davidson and K. Wiggin for help with analyses; and M. Garren, R. Certner, F. Aronson, and S. Kopac for valuable comments on the manuscript.
References


Davis HS (1922). *A new bacterial disease of fresh-water fishes*, vol. XXXVIII.


Ritchie KB (2006). Regulation of Microbial Populations by Coral Surface Mucus and


Supplementary Information

**Supplementary Table 3.1. Infection rate for corals in tank-based infection experiment**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Tank</th>
<th>N</th>
<th># WBD</th>
<th>% WBD</th>
<th># taken for 16S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseased</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tank 1</td>
<td>6</td>
<td>4</td>
<td>67%</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Tank 2</td>
<td>6</td>
<td>5</td>
<td>83%</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Tank 3</td>
<td>6</td>
<td>2</td>
<td>33%</td>
<td>4</td>
</tr>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tank 4</td>
<td>6</td>
<td>0</td>
<td>0%</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Tank 5</td>
<td>6</td>
<td>0</td>
<td>0%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Tank 6</td>
<td>6</td>
<td>1</td>
<td>17%</td>
<td>1</td>
</tr>
</tbody>
</table>

**Supplementary Table 3.2. Number of corals collected from each site**

<table>
<thead>
<tr>
<th>Disease state</th>
<th>Year</th>
<th>Site</th>
<th># corals collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>2009</td>
<td>CK4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Popa</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>CK4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Popa</td>
<td>4</td>
</tr>
<tr>
<td>Diseased</td>
<td>2009</td>
<td>CK4</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Popa</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>CK4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CK6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Popa</td>
<td>4</td>
</tr>
</tbody>
</table>

**Supplementary Table 3.3. Significantly different OTUs between diseased and healthy for the field and tank datasets.** (Attached)
Table 3.1. Diversity of OTUs. Bolded text indicates significantly different values as determined by Welch’s two sample t-test.

<table>
<thead>
<tr>
<th>Field</th>
<th>Tank</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>H</td>
<td>DD</td>
<td>DH</td>
<td>HH</td>
</tr>
<tr>
<td>Number of samples</td>
<td>49</td>
<td>30</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Number of OTUs</td>
<td>26,579</td>
<td>9,240</td>
<td>11,754</td>
<td>5,821</td>
<td>5,493</td>
</tr>
<tr>
<td>Simpson Diversity</td>
<td>0.84</td>
<td>0.64</td>
<td>0.89</td>
<td>0.87</td>
<td>0.78</td>
</tr>
<tr>
<td>Shannon diversity</td>
<td>3.08</td>
<td>2.36</td>
<td>3.77</td>
<td>3.29</td>
<td>2.82</td>
</tr>
<tr>
<td>Rarefied richness</td>
<td>542.42</td>
<td>308.00</td>
<td>1,679.14</td>
<td>1,164.20</td>
<td>784.71</td>
</tr>
</tbody>
</table>

Table 3.2. Results of PERMANOVA based on Bray-Curtis dissimilarities of the relative abundance of OTUs on field-collected *A. cervicornis* in response to disease state, site, and year.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease_state</td>
<td>1</td>
<td>3.22</td>
<td>3.22</td>
<td>10.96</td>
<td>0.10</td>
<td>0.001</td>
</tr>
<tr>
<td>Site</td>
<td>3</td>
<td>3.78</td>
<td>1.26</td>
<td>4.27</td>
<td>0.12</td>
<td>0.001</td>
</tr>
<tr>
<td>Year</td>
<td>1</td>
<td>0.617</td>
<td>0.62</td>
<td>2.09</td>
<td>0.019</td>
<td>0.011</td>
</tr>
<tr>
<td>Disease_state:Site</td>
<td>3</td>
<td>1.60</td>
<td>0.53</td>
<td>1.81</td>
<td>0.05</td>
<td>0.001</td>
</tr>
<tr>
<td>Disease_state:Year</td>
<td>1</td>
<td>0.68</td>
<td>0.68</td>
<td>2.32</td>
<td>0.022</td>
<td>0.005</td>
</tr>
<tr>
<td>Site:Year</td>
<td>3</td>
<td>1.74</td>
<td>0.58</td>
<td>1.98</td>
<td>0.06</td>
<td>0.001</td>
</tr>
<tr>
<td>Residuals</td>
<td>64</td>
<td>18.83</td>
<td>0.29</td>
<td>NA</td>
<td>0.60</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>31.13</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
<td>NA</td>
</tr>
</tbody>
</table>
**Table 3.3.** Results of PERMANOVA based on Bray-Curtis dissimilarities of the relative abundance of OTUs on field-collected healthy *A. cervicornis* in response to site and year.

<table>
<thead>
<tr>
<th>Df</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>3</td>
<td>2.80</td>
<td>0.93</td>
<td>3.69</td>
<td>0.28</td>
</tr>
<tr>
<td>Year</td>
<td>1</td>
<td>0.54</td>
<td>0.54</td>
<td>2.13</td>
<td>0.054</td>
</tr>
<tr>
<td>Site:Year</td>
<td>2</td>
<td>0.73</td>
<td>0.37</td>
<td>1.45</td>
<td>0.074</td>
</tr>
<tr>
<td>Residuals</td>
<td>23</td>
<td>5.82</td>
<td>0.25</td>
<td>NA</td>
<td>0.58</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>9.89</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3.4.** Results of PERMANOVA based on Bray-Curtis dissimilarities of the relative abundance of OTUs on field-collected diseased *A. cervicornis* in response to site and year.

<table>
<thead>
<tr>
<th>Df</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>3</td>
<td>2.35</td>
<td>0.78</td>
<td>2.40</td>
<td>0.13</td>
</tr>
<tr>
<td>Year</td>
<td>1</td>
<td>0.79</td>
<td>0.78</td>
<td>2.41</td>
<td>0.042</td>
</tr>
<tr>
<td>Site:Year</td>
<td>3</td>
<td>1.36</td>
<td>0.46</td>
<td>1.39</td>
<td>0.074</td>
</tr>
<tr>
<td>Residuals</td>
<td>43</td>
<td>14.04</td>
<td>0.33</td>
<td>NA</td>
<td>0.76</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>18.54</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
</tr>
</tbody>
</table>
**Table 3.5.** Significantly associated OTUs in tank and field-collected datasets.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Factor</th>
<th>Level</th>
<th>Significantly associated OTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field</td>
<td>Disease state</td>
<td></td>
<td>1,363</td>
</tr>
<tr>
<td></td>
<td>Disease state</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diseased</td>
<td>1,012</td>
<td>(74%)</td>
</tr>
<tr>
<td></td>
<td>Healthy</td>
<td>351</td>
<td>(26%)</td>
</tr>
<tr>
<td></td>
<td>Year</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Disease state * Year</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Tank</td>
<td>Disease State</td>
<td></td>
<td>521</td>
</tr>
<tr>
<td></td>
<td>Disease state</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diseased</td>
<td>494</td>
<td>(95%)</td>
</tr>
<tr>
<td></td>
<td>Healthy</td>
<td>27</td>
<td>(5%)</td>
</tr>
</tbody>
</table>
Figure 3.1. nMDS plots of dissimilarities between samples (a) Field collection samples, showing clustering according to disease state. “Healthy” “Diseased” and site names denote the centroids of each group and ellipses are 95% confidence ellipses. (b) Tank-exposed samples, showing clustering according to disease state. “Healthy” and “Diseased” labels denote the centroids of each disease state and ellipses are 95% confidence ellipses.

Figure 3.2. Plots of the log2 fold abundance change of each OTU by the mean of normalized counts. Significantly more or less abundant OTUs are in red (a) Field-collected corals by year + disease state (b) Tank-exposed corals by final disease state.
**Figure 3.3.** Taxonomic classification on the level of order of OTUs that are significantly more or less abundant in diseased corals compared to healthy across both tank-exposed and field-collected corals. Tier 1 consists of those OTUs that did not differ significantly across site and year, tier 2 consists of OTUs that differed significantly due to disease state and site and/or year.
Chapter 4:
Experimental infection of *Acropora cervicornis* leads to changes in populations of resident and colonizing bacteria.
Abstract

White band disease (WBD) is currently decimating populations of the endangered staghorn coral, *Acropora cervicornis* and elkhorn coral, *A. palmata* across the Caribbean. Since it was first reported in 1979, WBD has killed 95% of these critical reef-building Caribbean corals. WBD is an infectious bacterial disease that can be transmitted either through the water column or by a corallivorous snail. While we have previously shown that WBD-infected and healthy corals have significantly different microbiomes, we cannot identify which microbes cause disease and which are secondary responders. In order to better understand the microbes that are playing these roles, we performed a fully-crossed infection experiment. We inoculated healthy corals from ten colonies originating from two different sites with a dose made of corals exhibiting signs of WBD or a control inoculant from healthy corals from both sites. We sampled every coral at three time points: before inoculation, after inoculation, and when the coral showed signs of disease. We then performed 16S rRNA next-generation high-throughput sequencing. We identified multiple strains of *Endozoicomonas* that dominated the microbiomes of four colonies at the initial time point and subsequently decreased when those corals displayed disease signs. We also identified OTUs that were more abundant in the diseased corals than the control corals at both time points after inoculation and separated primary responders (present in healthy corals at the beginning of the experiment) from primary colonizers (originating in the dose).
Introduction

Since the first infectious coral diseases were reported in the 1970s (Dustan 1977), the number of described coral diseases and their impacts have increased rapidly (Weil, 2004; Harvell et al., 2004; Weil and Rogers, 2011), contributing to the collapse of coral reef ecosystems (Aronson and Precht 2001; Hughes 1994). This increase in disease has been seen across marine systems and is largely attributed to the synergistic effects of climate change and anthropogenic stressors (Harvell et al., 1999; Bruno et al., 2003; Bruno et al., 2007; Randall and van Woesik 2015). As the effects of these forces become more acute, we will likely continue to see an increased prevalence of marine diseases and the emergence of new diseases (Burge et al., 2014). Of the 20+ described coral diseases, the majority lack a consistently identified causative agent (reviewed in Sutherland et al., 2016). Much of the difficulty in identifying pathogens of corals and other marine animals and plants comes from the lack of understanding of the dynamics of the microbial communities associated with these underexplored systems (Sunagawa et al., 2010).

The healthy coral bacterial microbiome is diverse (Bourne et al., 2009; Sunagawa et al., 2010) and dynamic, differing across location (Kvennefors et al., 2010; Pantos et al., 2015; Roder et al., 2014b), time (Kimes et al., 2013; Li et al., 2014; Littman et al., 2009; Roder et al., 2015), and host species (Morrow et al., 2012; Roder et al., 2014a). An understanding of the role of disease-associated bacteria in coral disease will be greatly advanced by a better characterization of the healthy coral microbiome, how it varies across individuals, and how it changes in response to environmental stressors and when a disease develops.
White band disease (WBD) is an infectious coral disease currently decimating populations of the two species of Caribbean Acropora coral and the hybrid (Acropora cervicornis, A. palmata, and A. prolifera) (Aronson and Precht 2001; Gignoux-Wolfsohn et al., 2012). WBD is characterized by a front of necrotic tissue (and sometimes a zone of bleached tissue) followed by a band of white skeleton, which proceeds rapidly from the base to the tip of the coral colony (Gladfelter 1982). WBD has spread throughout the Caribbean by transmission through the water column and by the corallivorous snail Coralliophila abbreviata (Gignoux-Wolfsohn et al., 2012). Multiple studies have confirmed that WBD is a bacterial disease (Certner and Vollmer 2015; Kline and Vollmer 2011; Sweet et al., 2014) and Vibrio charariae has been shown to elicit WBD Type II signs in A. cervicornis in Puerto Rico (Gil-Agudelo et al., 2006), but a complete understanding of the dynamics of disease-associated bacteria and whether there is a single primary WBD pathogen across the Caribbean is unknown. A Rickettsiales-like organism (RLO), which may be compromising the host, has been associated with both (apparently) healthy and diseased A. cervicornis across the Caribbean (Casas et al., 2004; Peters et al., 1983). Further work is needed to understand how these and other bacteria that differ between diseased and healthy corals interact and contribute to the creation of WBD signs.

Previously, we used culture-independent high-throughput 16S rRNA gene sequencing to profile the bacterial communities of WBD-infected and healthy A. cervicornis and found that disease-associated bacterial communities were significantly different from those of healthy corals, exhibiting an increase in microbial diversity (Gignoux-Wolfsohn and Vollmer 2015) consistent with studies of other coral diseases
(e.g. Closek et al., 2014; Roder et al., 2014a; Sunagawa et al., 2009). In that study we found few OTUs that were consistently associated with healthy corals, suggesting that a more dynamic and holistic view of healthy bacterial communities is needed to identify resident microbes (Ainsworth et al., 2015). A static comparison of the microbiomes of diseased and healthy corals does not provide an understanding of where disease-associated OTUs originate and at what point in the disease progression they become more abundant. Furthermore, collection of corals displaying WBD signs in the field is problematic since it is impossible to know the history of the signs observed, how long the disease has been infecting a given coral, and whether or not the infection is currently active. In this study, we aim to first identify resident OTUs of healthy A. cervicornis (colony-specific residents) and determine how their abundance changes over time as the coral contracts disease. Second, we aim to better characterize disease-associated OTUs by infecting corals in tanks and separating the OTUs that originate in the infectious dose (colonizers) from those that are a priori present on the corals (responders) and determining the timing of their increased abundance on infected corals (primary vs. secondary).

Materials and Methods

Tank infection experiment

An infection experiment was set up in July 2014, using Acropora cervicornis from two sites (CK4 and CK14) 600 m apart in Coral Cay, Bocas del Toro, Panama (factor: “site”). At each site, five colonies of A. cervicornis (> 10m apart) were sampled and 12 five-cm fragments were collected from each colony (“colony”). Fragments were
brought to the Smithsonian Tropical Research Institute, cable-tied to plastic louver, and placed in 12 closed 50-L tanks with a koralia nano powerhead (Hydor USA Inc., Sacramento, CA, USA). Throughout the experiment, DI water was added to maintain salinity and volume.

Each tank contained one fragment from each colony for a total of ten fragments per tank (“tank”). Corals were sampled as they were placed in tanks (time one) in the following manner: two polyps from the middle of each fragment were removed using sterile forceps and placed in 200 µl of guanidine thiocyanate DNA Buffer (Fukami et al., 2004). Forceps were flame sterilized in between corals. To create the six doses, three 5-cm fragments were taken from the disease interface of each of three colonies exhibiting signs of active WBD at CK4 and CK14, for a total of 18 fragments. The corals were homogenized by shaking each fragment in a separate falcon tube with sterile glass beads and 15 mL filtered seawater until no tissue remained on the skeleton (Kline and Vollmer 2011). Fragments from the same colony were then pooled to create six doses, three from each site (“inoculant site”). 200 µl of each dose was centrifuged and preserved in 500 µl of DNA buffer. Control inoculants were created in the same manner as above, except fragments were taken from colonies exhibiting no signs of WBD.

Prior to inoculation, corals were injured using an airbrush (Gignoux-Wolfsohn et al., 2012). Six tanks were then inoculated with 30 mL of the dose, the “dose” level of treatment “inoculant,” and six tanks were inoculated with 30 mL of control inoculant, the “control” level of treatment “inoculant”. Corals were left for 10 hours after inoculation and then sampled as described above (time two). When dosed corals began showing disease signs (beginning at 22 hours post inoculation), they were sampled and removed
from the experiment along with the corresponding control fragments (time three). Sampling continued in this manner until 60 hours post inoculation when all remaining fragments were sampled. The “final disease state” of a coral was determined based on whether or not that coral ultimately showed disease signs, so even though a sample collected at time two came from a healthy-looking coral, if that coral displayed disease signs at time three, the final disease state for that sample was characterized as “diseased.” Hereafter, dosed corals whose final disease state was diseased will be referred to as “dosed diseased.” Two of the 60 control corals died over the course of the experiment and were removed from subsequent analyses.

16S library preparation

DNA was extracted from samples preserved in DNA buffer using the Agencourt DNAdvance bead extraction kit (Agencourt Bioscience Corporation, Beverly, MA, USA) with the addition of PEB buffer. A blank DNA extraction was performed with each round. The V6 hyper-variable region of the 16S gene was chosen as the target due to its short length and high sensitivity to species-level diversity (Barriuso et al., 2011) (Caporaso et al., 2012; Youssef et al., 2009). The V6 region was amplified with custom barcoded primers consisting of a region that anneals to the V6 hyper variable region followed by a unique five-base pair barcode, which differed by two or more base pairs, and then the Illumina sequencing adapter (Gloor et al., 2010):

V6-L [5’ACACTCTTTCCCTACACGACGCTCTTCCGATCTnnnnnCWACGCGARG AACCTTACC3’]

V6-R [5’CGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCTnnnnnACRACA]
In parallel, a separate, 40-µl PCR reaction was performed for each sample with a unique combination of primers: 5 µl each 4mM primer, 8µl standard Taq buffer (New England Biolabs, Ipswich, MA, USA), 0.8µl dNTPs, 20 µl diH20, 0.5 µl Taq DNA polymerase (NEB) for the following cycle: 94°C for 2m, with 28 cycles of: 94°C for 15s, 55°C for 15s, 72°C for 30s, ending with 72°C for 1m. A negative control and blank were amplified with each set of reactions. The concentrations of PCR products were quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA) in order to determine the volume of each product to add to the pool. The pool of all PCR products was then amplified with the following Illumina primers:

OLJ139 [5’AATGATACGGCGACCACCCGAGATCTACACTCTTTCCCTACACGA3’]
OLJ140 [5’CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCCTGC TGAAC3’] in a 40-µl reaction: 8 µl Phusion buffer (NEB) 0.8 µl dNTPs, 0.5 µl Phusion HiFidelity Taq (NEB), 20.2 µl diH20, 0.5 µl DNA (previous PCR product), for the following cycle: 98°C for 2m, 12 cycles of: 98°C for 1m, 55°C for 1m, 72°C for 1m, and finally 72°C for 5m. Final PCR products were cleaned using the DNAmpure beads (Agencourt). Concentration and length were verified using the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA) and sequenced using paired-end 150 base pair sequencing on an Illumina HiSeq2000.

**Bioinformatics**

Paired reads were overlapped using FLASH (Magoc and Salzberg 2011). Sequences were then demultiplexed, quality filtered, and trimmed using a custom
python script available at:

https://github.com/sagw/Python_scripts/blob/master/SD1/SD1_demultiplex.py

Using Qiime 1.9.0, 97% OTUs were picked using the open reference OTU picking method and taxonomy was assigned using BLAST against the SILVA database (version July 2015)(Quast et al., 2013). Chimeras were detected and removed using UCHIME (Edgar et al., 2011). Further details of bioinformatics can be found here:
https://github.com/sagw/Notebooks/tree/master/SD1_notebooks.

Statistical Analyses

Significance of treatments on transmission of disease was calculated using a binomial distribution mixed-effects generalized linear model (function GLMMPQL in the MASS package) (Venables and Ripley 2003) and a type-III ANOVA table using the Wald chi-square test (function Anova in the Car package) (Fox and Weisberg 2011), with the fully crossed fixed effects “site” (origin of the coral), “inoculant” (disease dose or control inoculant), and “inoculant site” (origin of the inoculant), with tank included as a random effect. Fixed-effects formula: ~ site * inoculant * inoculant site.

Normalized counts of OTUs were calculated using a modified version of the sizefactors method in the R package DESeq2 (Love et al., 2014; McMurdie and Holmes 2014). The significance of the community level effects was tested using PERMANOVA of Bray-Curtis dissimilarities with the Adonis function of Vegan (Jari Oksanen and O'Hara 2013). Two PERMANOVAS were performed: one for samples collected at time one examining the effects of the colony the coral was collected from, formula: ~ colony, and one for samples from times two and three examining the effect of the factor “final
disease state” (whether the coral showed signs of disease at time three) and the fully crossed factors “inoculant” (disease dose or control inoculant), “site” (origin of the coral), “time” (whether the sample was collected at time two or three), and “inoculant site” (origin of the inoculant), (formula: ~ final disease state + inoculant * site * time * inoculant site). Diversity metrics were calculated using the diversity functions in Vegan (Jari Oksanen and O'Hara 2013).

To evaluate changes in abundance of individual OTUs, abundance data for each OTU were fit to quasipoisson mixed-effects generalized linear models (function GLMMPQL in the MASS package) (Venables and Ripley 2003). GLMMs for time one and times two and three samples were performed separately in the same manner as the PERMANOVAs. GLMMs for time one samples tested the effects of the fixed effect “colony”, fixed-effect formula: ~ colony and the random effect “tank,” formula: ~1|tank. GLMMs for times two and three samples examined the same fixed effects as the PERMANOVA, fixed effect formula: ~ final disease state + site * inoculant * time * inoculant site and the random effects “time” and “tank,” random effect formula: ~1|tank/time. Significance of effects was then determined by calculating a type-III ANOVA table using the Wald chi-square test (function Anova in the Car package) (Fox and Weisberg 2011) and significantly different OTUs (p-value adjusted by FDR <0.05) were determined for each main effect and interaction. OTUs were then grouped according to significance of GLMM terms and post-hoc calculated means and the mean abundance of a subset of OTUs for each group was plotted using ggplot2 (Wickham 2009).

We identified colony-specific healthy residents as OTUs that differed
significantly by “colony” at time one and by “final disease state” at times two and three, with a higher abundance in control corals than dosed diseased corals. The mean of each OTU identified as *Endozoicomonas* was then calculated for each colony and percent *Endozoicomonas* composition was calculated as a mean of the percent of the total microbiome for each sample belonging to a given colony. “High” colonies contained greater than 40% *Endozoicomonas* in the total microbiome.

We identified OTUs as primary colonizers if they: 1) were more abundant in the dose than the control inoculant; 2) differed significantly by “final disease state”; 3) were more abundant in dosed diseased corals than control corals at both times two and three; and 4) did not differ significantly by “colony,” “site,” and “inoculant x inoculant site.” These OTUs were grouped by family and means were calculated for families with the largest number of OTUs or previously associated with WBD. First the corals were divided by “time,” “inoculant,” and “final disease state,” and then the mean abundance of all the OTUs within a family was calculated for the resulting groups of corals.

We identified OTUs as primary responders if they 1) were absent from the dose; 2) were present in time-one corals; 3) differed significantly by “final disease state” in time-two and three corals; 4) were more abundant in dosed diseased corals than controls; and 5) did not differ significantly by “colony,” “site,” or “site x inoculant.” Secondary OTUs differed significantly by “final disease state” and were more abundant in dosed diseased corals than control corals at time three but not time two. Means of these OTUs were calculated in the same manner as for primary colonizers.

All sequences were deposited to the SRA database under (accession numbers pending). Further specifics of analyses can be found here: https://github.com/sagw/R-
Results

Transmission

The dose from site CK4 was more infectious than that from CK14, (GLMM, P<0.001, pseudo R²=0.94), causing disease signs in 28/30 corals (93% transmission) compared to 15/30 corals inoculated with dose from CK14 (50% transmission).

Community-level effects

Two hundred and seventy five samples were sequenced yielding 65,413,553 overlapped reads, averaging 237,867 reads per sample. After clustering reads at 97% similarity and removing chimeras and chloroplast sequences, 97,933 OTUs were produced. The final disease state of the corals had a significant effect on the Shannon diversity (ANOVA, F_{1,272}=52.37, P<0.001) and rarefied richness (ANOVA, F_{1,272}=27.95, P<0.001) with the highest diversity in dosed diseased corals at time three (Supplementary Table 4.1). Within samples collected at time one, “colony” had a significant effect on bacterial community structure (PERMANOVA, F_{9,81}=1.8, P=0.001, R²=0.18), explaining 18% of the variation. For samples collected at times two and three, the main effects of “final disease state,” “timepoint,” “inoculant,” “inoculant site,” and all interactions of the latter three had significant effects on the coral-associated bacterial communities, explaining between 1 and 4.3% of the variation (Table 1).

Colony-specific residents
One hundred and seventy five OTUs were identified as colony-specific residents of healthy corals (Supplementary Table 4.2), with 139 belonging to the genus *Endozoicomonas* (family *Hahellaceae*), which dominated the microbiomes of four of the ten colonies (marked as “High” in Figure 1) and were present at lower abundances in the other colonies at time one. For these “High” colonies, when we compared the abundance of the resident *Endozoicomonas* in the dosed corals to the control corals at times two and three, we found a lower abundance in dosed corals that contracted disease and a higher abundance in dosed corals that remained healthy (Figure 2).

*Primary responders*

Two hundred and seventy two OTUs were identified as primary responders (present in time one corals), (Supplementary Table 4.2). Only one family of primary responders (*Alteromonadaceae*, 24 OTUs) exhibited a higher abundance in all dosed corals at time two than time three, although they were also present in low abundance in control corals at time two. At time three, *Alteromonadaceae* was also the only family with a higher abundance in dosed corals that contracted disease than dosed corals that stayed healthy. The family *Rhodobacteraceae* (18 OTUs) was the only family that was consistently absent from control corals throughout time, with a higher abundance in dosed corals regardless of final disease state at times two and three. At time three, OTUs belonging to the family *Cryomorphaceae* (22 OTUs) not only had the largest increase in abundance in dosed corals as compared to time two, but also had the largest increase in abundance in control corals. The families *Flavobacteriaceae* (26 OTUs) and *Saprospiraceae* (20 OTUs) both increased in time-three-dosed corals compared to
controls, which had a slight increase in abundance compared to time two (Figure 3).

*Primary colonizers*

Two hundred and sixty-five OTUs were classified as primary colonizers (originating in dose), (Supplementary Table 4.2). All of these OTUs were absent from control corals (mean abundance=0) except for OTUs belonging to *Flavobacteriaceae* (22 OTUs), which were detected in controls at time three. *Vibrionaceae* (one OTU) was the only family to have a higher abundance in dosed corals at time two than time three. *Alteromonadaceae* (22 OTUs), *Campylobacteraceae* (25 OTUs), *Francisellaceae* (38 OTUs), *Rickettsiaceae* (5 OTUs), and *Pasteurellaceae* (26 OTUs) all exhibited the same pattern—a higher abundance in dosed corals that became diseased than dosed corals that stayed healthy at both times two and three, with a larger difference between diseased and healthy corals at time three. *Francisellaceae* exhibited the highest abundance in dosed corals that became diseased at time three and *Pasteurellaceae* exhibited the highest abundance of any family in these corals at time two.

*Secondary OTUs*

One thousand nine hundred and six OTUs increased secondarily (at time three), (Supplementary Table 4.2). Forty-four of these OTUs were identified as secondary responders (present in time 1 corals) and 222 were secondary colonizers (present in dose). All of these secondary OTUs were also significant for the interaction of “site” x “inoculant” x “inoculant site” x “timepoint,” and in the dosed corals that developed disease at time three, 1,676 OTUs were more abundant in corals from CK4 inoculated
with dose from CK14 and 230 OTUs were more abundant in corals from CK14 inoculated with dose from CK4. The mean abundances for all of these secondary families were less than one (Figure 5). *Francisellaceae* made up the majority of these secondary OTUs on corals from CK14 dosed with CK4 (55 OTUS), and had the second highest mean abundance after *Methylococcaceae*. For corals from CK14 dosed with CK4, the most abundant family of secondary OTUs with the highest number of OTUs was *Campylobacteraceae* (358), 132 of which were secondary colonizers coming from the CK4 dose.

**Discussion**

Our results suggest that there are factors that consistently contribute to the characteristic diseased coral microbiome: a reduction in resident OTUs associated with the host coral colony (colony specific residents), an increase in abundance of already present OTUs (primary responders), and colonization by foreign bacteria (primary colonizers). By identifying these groups of disease-associated OTUs taxonomically, we are able to make conclusions about the functional roles of specific bacteria in the coral bacterial community.

We identified multiple strains of *Endozoicomonas* as colony-specific residents of healthy corals, which decreased in corals prior to displaying disease signs, before being reduced to nearly undetectable levels once the coral displayed the disease phenotype. These findings suggest that these *Endozoicomonas* may be beneficial symbionts of healthy corals that decrease in diseased corals either because they can only survive in healthy tissues or because they are out-competed by disease-associated bacteria. We saw
an increase in these colony-specific residents in corals that were exposed to the dose but remained healthy until the end of the experiment, suggesting that they may help the coral fight off infection by increasing host fitness or directly interacting with the pathogen. Our hypothesis that *Endozoicomonas* are beneficial coral symbionts is consistent with previous studies, where *Endozoicomonas* were found to dominate the healthy microbiomes of over 10 species of both hard and soft coral (e.g., Apprill *et al.*, 2013; Bayer *et al.*, 2013; Jessen *et al.*, 2013; Klaus *et al.*, 2011; Roder *et al.*, 2015; Yang *et al.*, 2010) with lower abundances in corals displaying disease signs (Klaus *et al.*, 2011; Meyer *et al.*, 2014). This is the first study, however, to show that only certain healthy colonies within a species are consistently dominated by *Endozoicomonas*, though previous studies have found that *Endozoicomonas* do not dominate the microbiomes of all species of coral, even when living sympatrically (Morrow *et al.*, 2012; Morrow *et al.*, 2015). The colony-specificity of resident bacteria seen here could be due to the host genetics and/or the environment, as colonies are likely genetically unique, but are also in different locations on the reef. The increase in abundance of *Endozoicomonas* seen in corals that resist infection coupled with the colony-specificity of these OTUs suggest that they may be contributing to the disease resistance previously seen in certain genotypes of *A. cervicornis* (Libro and Vollmer 2016; Vollmer and Kline 2008). Abundance of *Endozoicomonas* correlates with favorable environmental conditions, and colonies with high *Endozoicomonas* abundance may therefore live in locations more favorable for the host (Roder *et al.*, 2015). A recent study found that removal of *Endozoicomonas* from the surface mucus layer of corals using antibiotics made corals more susceptible to bleaching and necrosis, suggesting that *Endozoicomonas* may be important for coral fitness (Glasl
et al., 2016). *Endozoicomonas* have also been found in the endodermal tissues of some coral species and may be vertically transmitted through generations as beneficial endosymbionts (Bayer et al., 2013).

After dosing, some corals developed disease signs, altering their bacterial communities, which resulted in the more diverse disease-associated bacterial communities found in other coral disease studies (e.g. Closek et al., 2014; Croquer et al., 2013; Meyer et al., 2015; Sweet et al., 2013). There was also, however, an effect of exposure to disease on the bacterial community regardless of the final disease state, emphasizing the importance of the disease history of the coral in structuring its microbial communities and the problem with using corals appearing to be healthy without extended observation as controls. If colonies are truly resistant to disease (i.e., decrease the pathogen load or prohibit infection), the changes in their microbiome upon dose exposure are not the result of infection by a pathogen. If, however, these corals are merely tolerating the pathogen(s), then the OTUs that increase with exposure to dose may be pathogenic.

Recently, it has been posited that coral disease signs are not caused by primary pathogen(s), but by opportunistic pathogens or “pathobionts” present on healthy corals that become pathogenic and increase in abundance due to external stimuli (Chow et al., 2011; Lesser et al., 2007). Our results confirm the presence of already present bacteria that increase in abundance opportunistically in response to disease exposure. However, our finding that this increase occurred in all dosed corals regardless of final disease state makes these primary responders unlikely to be the sole cause of the disease.

Furthermore, primary responders belonging to the families *Flavobacteriaceae,*
Cryomorphaceae, and Saprospiraceae (all within the phylum Bacteroidetes) increased in control corals at time three, likely in response to the general stress of the host caused by the tank environment. Flavobacteriaceae have been previously associated with multiple coral diseases across oceans (e.g. Apprill et al., 2013; Frias-Lopez et al., 2002; Ng et al., 2015; Roder et al., 2014b) and members of this family are known to cause disease in fish (Starliper 2011) but also dominate the healthy microbiomes of other marine organisms (Apprill et al., 2014). Cryomorphaceae are in the same order (Flavobacteriales) as Flavobacteriaceae, and while we previously suggested that Flavobacteriales may contain a putative WBD pathogen (Gignoux-Wolfsohn and Vollmer 2015) the primary responders identified as Flavobacteriales here do not show abundance patterns consistent with a primary pathogen. We previously identified Saprospiraceae as being inconsistently associated with both diseased and healthy corals (Gignoux-Wolfsohn and Vollmer 2015). This family does not contain known marine pathogens, but is made up of commonly found marine bacteria involved in the breakdown of complex carbon molecules (Krieg et al., 2011).

The only family of primary responders that was most abundant before any corals displayed disease signs was Alteromonadaceae, suggesting that these OTUs may have increased as an initial response to any sort of perturbation of the corals existing microbiome. If Alteromonadaceae are acting as defensive symbionts of the host coral, then their decrease in time three control corals to undetectable levels could be because the control microbial communities did not contain pathogens, whereas the smaller decrease in dosed corals at time three could be a result of the coral successfully fighting off infection (healthy) or dying (diseased). The complicated role of Alteromonadaceae in
both the health and disease of corals suggested by our data has been previously suggested by their associations with the following: healthy coral larvae (Ceh et al., 2013), healthy adult corals (Cardenas et al., 2012), corals infected with multiple diseases (Frias-Lopez et al., 2002; Roder et al., 2014a; Roder et al., 2014b; Sunagawa et al., 2009), and both healthy and diseased corals in our previous study of WBD (Gignoux-Wolfsohn and Vollmer 2015).

*Rhodobacteraceae*, another prominent family of primary responders, have been associated with multiple coral diseases (including WBD) in over ten species of corals across many oceans (summarized in: Mouchka et al., 2010 see also: Cardenas et al., 2012; Gignoux-Wolfsohn and Vollmer 2015; Klaus et al., 2011; Ng et al., 2015; Roder et al., 2014a; Roder et al., 2014b). Our finding that primary responders belonging to *Rhodobacteraceae* responded to the dose regardless of final disease state is consistent with the idea that *Rhodobacteraceae* are not specific pathogens of a particular coral disease, but rather common opportunists responding to a stressed host (Glasl et al., 2016; Mouchka et al., 2010). The response of already present taxa identified above clearly contributes to the establishment of the characteristic diseased microbiome, but their role in disease remains unclear; they may be beneficial symbionts, opportunists taking advantage of the compromised host, or they merely may be responding to the introduction of foreign bacteria and/or their secondary metabolites.

While we cannot rule out the involvement of the aforementioned primary responders in the final establishment of disease, in this study, the development of disease signs occurred only in dosed corals, meaning that the dose must contain bacteria that are infectious. We were able to identify several OTUs present in the dose but not the control
inoculant, which subsequently colonized dosed corals that contracted disease. These primary colonizers are the most likely putative primary pathogens in this experiment. Since there were multiple taxa within this group, infection may be reliant on interactions between multiple groups of bacteria originating in the dose, consistent with a recent study showing that quorum sensing is used by bacteria causing WBD (Certner and Vollmer 2015).

Two of these families also contained multiple OTUs that were primary responders—Flavobacteriaceae and Alteromonadaceae. These Flavobacteriaceae followed a very similar pattern to that of the primary responders. Given that we do not know where the bacteria in the dose originated, it is possible that these Flavobacteriaceae were responders on the diseased corals used to create the dose. The OTUs identified as Alteromonadaceae here followed a different pattern from those in the primary responders group, which was identical to that of the primary colonizers identified as Campylobacteraceae, Francisellaceae, and Rickettsiaceae—they all colonized corals prior to displaying disease signs, and then proliferated once the coral succumbed to disease. Campylobacteraceae (order Campylobacterales) have been associated with multiple coral diseases in both the Caribbean and Pacific (e.g., Roder et al., 2014a; Sunagawa et al., 2009; Sweet and Bythell 2012; Sweet et al., 2013), and were associated with diseased corals in our previous study of WBD (Gignoux-Wolfsohn and Vollmer 2015). Members of the family Campylobacteraceae can be commensal, but are also sometimes zoonotic pathogens (Lee and Newell 2006; Stoddard et al., 2005). While they are not uniquely associated with WBD, the pattern of colonization seen here suggests Campylobacteraceae may contribute to the development of WBD signs. In contrast,
members of the family *Francisellaceae* have not been previously associated with coral diseases, but are common marine bacteria (Duodu et al., 2012), which can be intracellular pathogens of Atlantic cod (Wangen et al., 2012) and humans (Sjostedt 2006) and also endosymbionts of ciliates (Schrallhammer et al., 2011). Further research is needed to understand the role *Francisellaceae* are playing in WBD, but the large increase in infected corals over time seen here makes them promising candidate pathogens.

*Rickettsiaceae* have been previously associated with both healthy and diseased *A. cervicornis* (Casas et al., 2004; Gignoux-Wolfsohn and Vollmer 2015; Peters et al., 1983), and while a comparatively low number of primary colonizers were identified as *Rickettsiaceae* in this study, their increased abundance in corals exhibiting WBD signs is consistent with involvement in WBD etiology. Strains of *Vibrionaceae* have been shown to cause multiple coral diseases (e.g. Sere et al., 2015; Sussman et al., 2008; Ushijima et al., 2012; Zhenyu et al., 2013), including WBD type-II in Puerto Rico (Gil-Agudelo et al., 2006). The OTU identified as *Vibrionaceae* in the primary colonizers may be a primary pathogen of dosed corals infecting at the initial time point, which is then outcompeted by opportunists and other bacteria once the coral develops disease signs, but its reduction in corals displaying disease signs is not suggestive of a classic primary pathogen.

Finally, primary colonizers in the family *Pasteurellaceae* colonized dosed corals in the greatest abundance of any family before corals showed disease signs and increased when corals displayed disease signs, returning to zero in dosed corals that remained healthy. This pattern of colonization and the fact that *Pasteurellaceae* were the most abundant family in the dose suggests that *Pasteurellaceae* were the most likely candidate
primary pathogens, proliferating in diseased corals and likely being destroyed by the host immune response in resistant corals. *Pasteurellaceae* have not been previously associated with coral disease, but they are common pathogens of animals including humans (Frey and Kuhnert 2002; Johnson and Rumans 1977). *Pasteurella piscicida*, the cause of pasteurellosis (Janssen and Surgalla 1968; Romalde 2002), was reclassified as *Photobacterium damselae* (family: *Vibrionaceae*) (Gauthier et al., 1995). It is possible that these OTUs are misclassified as *Pasteurellaceae* given the limitations of databases and sequence length.

Corals exhibiting disease signs were the most diverse, but the majority of the OTUs increasing secondarily were not consistent across individual fragments. The only consistency in secondary OTUs was in corals that were inoculated with dose from a foreign site, suggesting adaptation of the bacterial community to the local disease-associated bacteria.

In conclusion, the diseased coral microbiome is dependent on the environmental and disease history of the infected coral, the origin of the disease, and the timing of disease progression. While we identified some consistent actors in the diseased coral microbiome, our factors did not explain the majority of the variation between samples, indicating that substantial work is still needed to understand the complex interactions of bacteria with the host coral. Our results underscore the importance of incorporating time into future studies of coral disease and the need to observe the behavior of individual bacterial strains rather than summarizing changes in communities only by higher-level taxonomy. Future work will focus on more clearly defining bacterial function on diseased corals.
Acknowledgements

We would like to thank H. Nelson for help with sample collection; T. Gouhier for help with statistical analyses; E. Holm for help with Bioinformatics; M. Garren, R. Certner, and S. Kopac for valuable comments on the manuscript. This project was funded by an experiment.com crowd-funding campaign: https://experiment.com/projects/what-is-killing-caribbean-corals-investigating-a-devastating-coral-disease.
References


Glasl B, Herndl GJ, Frade PR (2016). The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance. The ISME journal.


Youssef N, Sheik CS, Krumholz LR, Najar FZ, Roe BA, Elshahed MS (2009). Comparison of species richness estimates obtained using nearly complete fragments and


Supplementary Information

Supplementary Table 4.1. Mean diversity of bacterial communities associated with groups of coral.

<table>
<thead>
<tr>
<th>Time, Inoculant, Final disease state</th>
<th>Shannon</th>
<th>SE</th>
<th>Richness</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculant, Dose, Diseased</td>
<td>3.83</td>
<td>0.12</td>
<td>400.40</td>
<td>47.77</td>
</tr>
<tr>
<td>Three, Dose, Diseased</td>
<td>4.18</td>
<td>0.19</td>
<td>402.57</td>
<td>21.38</td>
</tr>
<tr>
<td>Two, Dose, Diseased</td>
<td>3.62</td>
<td>0.15</td>
<td>333.42</td>
<td>15.28</td>
</tr>
<tr>
<td>Three, Dose, Healthy</td>
<td>3.16</td>
<td>0.35</td>
<td>326.96</td>
<td>35.85</td>
</tr>
<tr>
<td>Two, Dose, Healthy</td>
<td>3.16</td>
<td>0.39</td>
<td>309.52</td>
<td>41.93</td>
</tr>
<tr>
<td>Inoculant, Control, Healthy</td>
<td>3.09</td>
<td>0.33</td>
<td>279.55</td>
<td>19.63</td>
</tr>
<tr>
<td>One, Control, Healthy</td>
<td>2.13</td>
<td>0.12</td>
<td>224.43</td>
<td>13.47</td>
</tr>
<tr>
<td>Three, Control, Healthy</td>
<td>3.26</td>
<td>0.19</td>
<td>345.90</td>
<td>20.47</td>
</tr>
<tr>
<td>Two, Control, Healthy</td>
<td>3.00</td>
<td>0.13</td>
<td>274.16</td>
<td>12.14</td>
</tr>
</tbody>
</table>

Supplementary Table 4.2. List of OTUs belonging to each designated group. (Available upon request)

Supplementary Table 4.3 Significantly different OTUs. P values determined by GLMMs.
Table 4.1. PERMANOVA of Bray-Curtis dissimilarity between samples collected at times two and three. Only significant effects (p<0.05) are shown.

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>Sums of Sqs</th>
<th>Mean Sqs</th>
<th>F Model</th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final disease state</td>
<td>1</td>
<td>2.03</td>
<td>2.03</td>
<td>5.61</td>
<td>0.028</td>
<td>0.001</td>
</tr>
<tr>
<td>Inoculant</td>
<td>1</td>
<td>0.88</td>
<td>0.88</td>
<td>2.44</td>
<td>0.012</td>
<td>0.001</td>
</tr>
<tr>
<td>Time</td>
<td>1</td>
<td>3.07</td>
<td>3.07</td>
<td>8.50</td>
<td>0.043</td>
<td>0.001</td>
</tr>
<tr>
<td>Inoculant site</td>
<td>1</td>
<td>1.03</td>
<td>1.03</td>
<td>2.86</td>
<td>0.014</td>
<td>0.001</td>
</tr>
<tr>
<td>Inoculant x time</td>
<td>1</td>
<td>0.89</td>
<td>0.89</td>
<td>2.46</td>
<td>0.012</td>
<td>0.001</td>
</tr>
<tr>
<td>Inoculant x inoculant site</td>
<td>1</td>
<td>1.23</td>
<td>1.23</td>
<td>3.41</td>
<td>0.017</td>
<td>0.001</td>
</tr>
<tr>
<td>Time x inoculant site</td>
<td>1</td>
<td>0.93</td>
<td>0.93</td>
<td>2.57</td>
<td>0.013</td>
<td>0.001</td>
</tr>
<tr>
<td>Inoculant x time x inoculant site</td>
<td>1</td>
<td>0.73</td>
<td>0.73</td>
<td>2.03</td>
<td>0.010</td>
<td>0.001</td>
</tr>
<tr>
<td>Residuals</td>
<td>162</td>
<td>58.51</td>
<td>0.36</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>178</td>
<td>72.163</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1. *Endozoicomonas* are colony-specific resident bacteria of healthy corals. a) Mean abundance of each resident OTU within each colony at time one. Colonies with greater than 40% of their total microbiome consisting of *Endozoicomonas* at time one are labeled “High”. b) Percent of total microbiome for each colony at time one that is identified as *Endozoicomonas* or other taxa.
Figure 4.2. Abundance of resident *Endozoicomonas* in high colonies at times two and three. Y-axis is the difference between dosed corals and control corals at each time point; a negative value denotes a lower abundance in dosed corals than controls, and a positive value denotes a higher abundance in dosed corals than controls. Means were calculated for corals exhibiting different final disease states (diseased or healthy) and then control means were subtracted.

Figure 4.3. Mean abundance of primary responders belonging to selected families across time. OTUs are grouped by family, and the size of the points denotes how many OTUs belonged to that family.
Figure 4.4. Mean abundance of primary colonizers belonging to selected families across time. OTUs are grouped by family, and the size of the points denotes how many OTUs belonged to the specified family. Inset is the mean abundance for OTUs in that family in the inoculants (dose and control). Arrows signify time of inoculation. Error bars denote standard error.
Figure 4.5. Mean abundance of secondary colonizers belonging to selected families on dosed corals that became diseased at time three. Dosed corals are separated by the site of origin of the dose and the site of origin of the corals. OTUs are grouped by family, and the number of OTUs in each group is noted on the top of the mean abundance bar.
The results presented in this thesis provide a picture of an infectious Caribbean coral disease that manifests as a complex disease-specific coral microbiome. This diseased microbiome consists of bacteria that may cause disease as well as bacterial populations that change as a result of the disease.

In our studies of microbial communities associated with diseased and healthy corals, we observed many taxonomic groups that changed abundance in similar patterns, *i.e.* increasing or decreasing in diseased or healthy corals at the same time. This trend implies cooperation between individual bacteria that are taxonomically related potentially causing WBD signs. This hypothesis is supported by a recent paper showing the ability of quorum sensing molecules to elicit WBD signs when added to a healthy bacterial community (Certner and Vollmer 2015). More work is needed to understand how these bacteria are interacting with each other both within and across taxonomic groups.

We also observed, however, that taxonomic groups could contain OTUs that change in abundance in different ways. For example, in Chapter 3, the taxonomic order with the largest number of OTUs consistently associated with both diseased and healthy corals was *Flavobacteriales*. In Chapter 4, we further identified OTUs belonging to *Flavobacteriaceae* as both primary responders and primary colonizers. These findings underscore why studies grouping OTUs by broader taxonomy and then looking for patterns are problematic.

Ultimately, identifying changes in the microbiome using 16S is limited in that this method only identifies changes in abundance of genetically distinct bacterial species, it does not allow us to find functional similarities of bacteria across taxa. Bacteria can horizontally transfer genes across species, and when these genes confer pathogenicity
they are often referred to as “pathogenicity islands” (Faruque and Mekalanos 2003). The infectious bacteria in the dose may not be closely related, but may share a pathogenicity island that allows them to infect healthy corals. This is difficult to determine as an inability to culture coral-associated bacteria limits our understanding of their genomes. Tools such as RNASeq of the metatranscriptome (mRNA of every bacteria on a given coral) can shed light on how these communities are functioning as a whole (e.g. Vega Thurber et al., 2009), but the limitations of sequencing length, sample number, and the complex nature of these communities make assembly of these transcriptomes difficult (Celaj et al., 2014). As technology improves, metatranscriptome sequencing of these communities will hopefully replace 16S sequencing and give a more complete and realistic idea of not just which bacteria are present, but which are metabolically active, and how they are functioning.

As part of this dissertation, I performed multiple transmission experiments that all produced disease signs that are consistent with those of white band disease, proving that this phenotype can be transmitted between corals. Whether all experiments were the result of the same etiological agent(s) is unknown. Furthermore, it is possible that the infection experiments do not have the same cause as all of the field-collected samples sequenced in Chapter 3. It is likely that, across the Caribbean, multiple infectious and non-infectious agents, which may work both in tandem and/or individually, cause these common disease signs.

It is also possible that other tissue-loss diseases, or white syndromes, may be caused by some of the same etiological agents as WBD. This is especially likely given the recent paper by Clemens et al. (2015) demonstrating that white plague disease can be
transmitted by *Coralliophila abbreviata* and through the water column to *Orbicella annularis* (Clemens and Brandt 2015). Since *O. annularis* and *A. cervicornis* often live sympatrically, the tissue loss seen on these two species caused by *C. abbreviata* may be the result of the same pathogen housed in the snail. Further work is needed to determine if this is the case, and if *C. abbreviata* may be a vector for white syndromes between species.

Our understanding of coral diseases is impeded not just by our lack of understanding of host-microbe interactions, but also by how microbes are distributed and located on a reef. Our work identifying foreign microbes that are introduced and resident microbes that increase or decrease in response to disease helps shed light on this question. In addition, our finding that coral microbiomes exhibit different changes when exposed to geographically different disease-associated bacterial communities points to local adaptation of these communities to disease. This local adaptation may explain a large amount of the heterogeneity seen between the microbiomes of diseased corals. Adaptation to disease may also explain different rates of infection of corals seen in the separate transmission experiments performed in each chapter.

Future work should focus on confirming the role *Endozoicomonas* plays in coral function as a beneficial symbiont. *Endozoicomonas* could potentially be used as either a marker of coral health, or as a mechanism of increasing coral fitness. Furthermore, how *Endozoicomonas* are distributed among colonies on a reef could indicate resistant genotypes or favorable locations for coral survival.

One potential conservation application of this work could be controlling the spread of the disease by altering *C. abbreviata* distribution. Further research is needed to
understand the role of *C. abbreviata* within the coral reef ecosystem, and especially its natural predators, which are poorly characterized (Baums *et al.*, 2003).

This work has brought us closer to understanding the etiological agent of WBD, which in turn brings us closer to identifying infected corals and isolating them. Due to the notorious difficulty of distinguishing between biotic and abiotic disease, conservation work does not currently focus on coral disease as an infectious disease needed to be controlled in the same way that terrestrial diseases are managed (*e.g.*, Smith and Wilkinson 2003; Van Buskirk and Ostfeld 1995). If we can understand the etiology of infectious diseases, we can work to develop a method to stop outbreaks and identify specific factors that increase infectious disease spread and pathogenicity. Furthermore, identification of the etiological agents of coral diseases may identify zoonotic pathogens that originate on land. Understanding the effects putative human pathogens have on corals may help reveal their functioning in humans. Furthermore, controlling these bacteria in the marine environment may limit the potential for zoonoses.
References


