Exploration of the Role of Microbiome Structure, Metabolism, and Modification in Black Band Disease Etiology

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

EXPLORATION OF THE ROLE OF MICROBIOME STRUCTURE, METABOLISM, AND MODIFICATION IN BLACK BAND DISEASE ETIOLOGY

A dissertation submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BIOLOGY by Patricia A. Waikel

2020
To: Dean Michael R. Heithaus
    College of Arts, Sciences and Education

This dissertation, written by Patricia A. Waikel, and entitled Exploration of the Role of Microbiome Structure, Metabolism, and Modification in Black Band Disease Etiology, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: September 18, 2020

The dissertation of Patricia A. Waikel is approved.

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Dean Michael R. Heithaus
    College of Arts, Sciences and Education

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Andrés G. Gil
    Vice President for Research and Economic Development
    and Dean of the University Graduate School

Florida International University, 2020
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DEDICATION

This dissertation is dedicated to my husband Jerry

and our furry family Diego, Olivia, Gator, Sophie, and Willow.
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ABSTRACT OF THE DISSERTATION

EXPLORATION OF THE ROLE OF MICROBIOME STRUCTURE, METABOLISM, AND MODIFICATION IN BLACK BAND DISEASE ETIOLOGY

by

Patricia A. Waikel

Florida International University, 2020

Miami, Florida

Professor Mauricio Rodriguez-Lanetty, Major Professor

The coral microbiome plays an integral role in coral health. Modification of the microbiome is thought to alter susceptibility to disease. Black Band Disease (BBD), is polymicrobial, mat forming, and affects reef building coral globally. Dominated by the cyanobacterium *Roseofilum reptotaenium*, it has been noted to increase in virulence with increasing temperatures, making BBD of particular concern in the face of climate change-induced warming seas. The active sulfur cycle of BBD makes dimethylsulfiniopropionate (DMSP), a widely available source of sulfur in the marine environment, of particular interest in the study of BBD. Traditional infection studies require field collection and subsequent maintenance of corals in aquaria, often including lengthy acclimation times, making the identification of a model system for studying BBD timely. I explored the role of DMSP metabolism in BBD, investigated the suitability of the tropical sea anemone *Exaiptasia pallida* as a model system for studying BBD, and examined modification of the host and pathogen microbiomes during a BBD challenge. These aims were accomplished by metagenomic analysis and bacterial challenges of *E. pallida* combined with high throughput 16S rRNA sequencing by Illumina MiSeq.
discovered that DMSP-metabolizing taxa and genes related to DMSP metabolism are present in BBD, suggesting DMSP metabolism by the BBD microbial consortium possibly influencing recruitment of pathogens and promoting the production of toxic microcystin and sulfide. I have demonstrated that the tropical anemone *E. pallida* is susceptible to BBD across a range of temperatures, symbiotic states, and symbionts hosted, making it a strong candidate model system for studying this disease. Further, susceptibility may be influenced by symbiotic state and fluctuation in virulence of BBD over time. Modification of host and pathogen microbiomes during BBD challenge studies revealed recruitment of taxa from the host microbiome to the disease consortium and loss of taxa from both, providing a foundation for future studies to focus on determining how these specific taxa influence virulence of the disease and susceptibility of the host. Taken together, these findings add to our understanding of the role of microbiome structure, metabolism, and modification in the etiology of BBD.
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Chapter 1: Introduction

Coral reefs are biologically diverse ecosystems that provide both physical and economic stability to coastal communities worldwide (Brander et al. 2007; van Beukering et al. 2007; Hoegh-Guldberg et al. 2011; van Zanten et al. 2014; Harris et al. 2018). Coral reefs serve as physical barriers that attenuate wave energy, thereby preventing coastal erosion and minimizing flooding (Ellif and Silva 2017). They provide habitat for fish as well as a variety of invertebrates that serve as food sources to humans (Moberg and Folke 1999). Reefs have been and continue to be a source for new drugs (Leal et al. 2013). Coral reef tourism, also important to local economies, is valued at 35.8 billion dollars globally per year (Spalding et al. 2017).

Coral reefs face many challenges including over-fishing, pollution, dredging, climate change, and disease (Aronson et al. 2003; Goldberg et al. 2004; Pandolfi et al. 2005; Zaneveld et al. 2016; Cunning et al. 2019). Coral reefs are in decline worldwide, in part, because of the increasing prevalence of coral disease and bleaching (Hughes et al. 2003; Bourne et al. 2009; Wada et al. 2018; Walton et al. 2018; Mohamed et al. 2019). As of 2004, corals of the Caribbean had been documented as being affected by at least 18 diseases (Sutherland et al. 2004). New cases continue to be documented, including a recent report of coral disease in the Republic of Maldives (Montano et al. 2012) and Stony Coral Tissue Loss Disease in the Florida Reef Tract (Precht et al. 2016; Walton et al. 2018; Weil et al. 2019; Muller et al. 2020) which has continued to spread rapidly throughout the Caribbean (Alvarez-Filip et al. 2019). Continued warming of the oceans, as a result of climate change, is predicted to increase the prevalence of disease (Sokolow; 2009; Lesser et al. 2015; Maynard et al. 2015).
Coral microbiome

Coral health is thought to be tied to the microorganisms that live in close association with coral tissues and the surface mucopolysaccharide layer (SML) (Ritchie 2006). The association of these microorganisms, including bacteria, archaea, viruses, and eukarya with coral, is well established (Bentis et al. 2000; Toller et al. 2001; Rohwer et al. 2001; Rohwer et al. 2002; Knowlton and Rohwer 2003; Wegley et al. 2004; Wilson et al. 2005; Davy et al. 2006; Rosenberg et al. 2007). The most notable of these associations is that of the endosymbiotic alga Symbiodiniaceae with corals (Muscatine and Porter 1977). These associations are so integral that Rohwer et al. (2002) proposed that a coral be considered, with all of its associated microorganisms, as a single organismal unit termed the holobiont. It has been further suggested that the holobiont be considered a unit of evolutionary selection (Rosenberg et al. 2007) indicating that these associations are essential for the animal’s survival.

Bacteria as beneficial members of the coral holobiont is proposed by the coral probiotic hypothesis which states that a dynamic relationship between symbiotic microorganisms and environmental conditions exists that results in selective advantage for the coral holobiont (Reshef et al. 2006). Evidence cited in support of this hypothesis includes the abundance and diversity of bacterial populations associated with corals and rapid changes of these populations in response to environmental changes as evidenced by studies of the microbial associates of healthy corals (Rohwer et al. 2001; 2002; Klaus et al. 2005; Littman et al. 2009; Morrow et al. 2012; Bourne et al. 2016; Glasl et al. 2016), as well as studies comparing the microbial associates of healthy coral with those of diseased coral (Bourne et al. 2008; Correa et al. 2009; Sunagawa et al. 2009; Thurber et
Despite the number of studies conducted, questions remain regarding how these microbial communities are structured and how their presence and interactions may contribute to disease onset and persistence.

**Black Band Disease of corals**

Black band disease (BBD), one of the first identified and best characterized of the coral diseases, is a polymicrobial, mat forming disease, in which cyanobacteria dominate (Antonius 1981). It forms a characteristic dark band that moves horizontally across coral (Rützler et al. 1983), lysing tissue in its wake. This disease is known to have a very active sulfur cycle including sulfate reduction (both assimilatory and dissimilatory) and sulfide oxidation (Sato et al. 2016). Steep sulfide/oxygen gradients are present with supersaturation of oxygen in the upper part of the band during the day and anoxia, combined with high levels of sulfide, at night (Carlton and Richardson 1995) which contribute to BBD pathogenicity. Additionally, the cyanobacterial toxin microcystin was shown to be produced by BBD cyanobacterial isolates (Gantar et al. 2009) including sulfide tolerant *Geitlerinema* and *Leptolyngbya* that have also been detected in molecular studies of BBD (Myers et al. 2007) and shown to play a role in BBD pathogenicity (Myers et al. 2007; Richardson et al. 2009). Sulfide, toxic to corals, is integral to initiation of BBD infections (Brownell and Richardson 2014).

The community structure of BBD is functionally similar to that of other marine microbial mats (Stal et al. 1985; Visscher et al. 2005; Prieto-Barrajas et al. 2018). In addition to photoautotrophs, sulfate reducing bacteria (SRB), sulfide oxidizing bacteria (SOB), and a variety of heterotrophs make up the mat community (Garret and Ducklow
Secondary metabolite synthesis, including antimicrobial compounds (Gantar et al. 2011) and lyngbic acid (Meyer et al. 2016) may further influence structure of the associated microbial consortium.

Infection studies with *Roseofilum reptotaenium* have successfully resulted in BBD onset in coral fragments (Stanić 2010) making this cyanobacterial member of BBD a suspected primary pathogen. However, SRB have been shown to be secondary and required pathogens for successful infection (Brownell and Richardson 2014).

Successional changes in bacterial communities have been documented on corals of the Great Barrier Reef (GBR) where cyanobacterial patches have appeared prior to onset of BBD (Sato et al. 2010). These changes include shifts in identity and abundance of cyanobacteria as well as an increase in sulfur cycling organisms. An increase in sulfate-reducing bacteria was also observed at the onset of BBD on the GBR (Bourne et al. 2011). These changes, combined with the high sulfide environment of the band, suggest a role for SRB in sulfur cycling within BBD as well as BBD pathogenesis.

Sulfur cycling within BBD is an important process which contributes to its pathogenicity (Richardson et al. 2009; Sato et al. 2016). Sulfur cycling bacteria including SRB and SOB are two of the most functionally important groups in the BBD mat (Brownell et al. 2014). The former is responsible for sulfide production within the mat which is thought to build up in the absence of oxygen during the night and the latter is integral in oxidizing sulfide during the day in the presence of oxygen. One potentially important source of sulfur input for BBD is dimethylsulfoniopropionate (DMSP).
Metabolism of dimethylsulfoniopropionate in coral-associated bacteria

Dimethylsulfoniopropionate is a widely available sulfur and carbon-containing compound in the marine environment. Much of its production is attributed to phytoplankton (Sievert et al. 2007). The metabolism of DMSP is of particular interest because one possible product is dimethyl sulfide (DMS), a volatile compound known to influence cloud formation when released to the atmosphere, impacting local climate (Charlson et al. 1987). This product is a result of the cleavage pathway of metabolism (Reisch et al. 2011) driven by several genes including $dddD$, $dddK$, $dddL$, $dddP$, $dddQ$, $dddW$, and $dddY$ (collectively referred to as the lyase genes) (Moran et al. 2012). The bulk of DMSP metabolism in the marine environment is attributed to the demethylation pathway (Raina et al. 2010) driven by the $dmdA$ gene (Howard et al. 2006). Substantial overlap between genera capable of metabolizing DMSP and genera associated with corals has been observed (Raina et al. 2009; Raina et al. 2010) implicating DMSP metabolism in structuring coral-associated bacterial communities. Additionally, chemo-attraction from natural populations of coral reef bacteria to DMSP has been observed (Tout et al. 2015), and a decrease in $dmdA$ gene abundance has been correlated with higher levels of DMSP in corals (Frade et al. 2015). A possible link between DMSP metabolism and coral disease was revealed by positive chemo-attraction of *Vibrio coralliilyticus*, a known coral pathogen, to DMSP, suggesting the bacterium might use DMSP as a chemical cue to locate a coral host (Garren et al. 2014). Dimethylsulfoniopropionate and/or its associated compounds have been detected previously in marine microbial mats (Visscher et al. 1991; Visscher and van Gemerden 1991; Jonkers et al. 1998). Given that BBD mats
contain similar microbial community composition to other marine microbial mats, DMSP may be important to the function of the microbial consortium.

New Cnidarian model to study coral disease

The study of a coral disease such as BBD can be complicated by the collection of corals and their subsequent maintenance in aquaria. Collection of corals can require special permits, SCUBA certification, and specialized tools for fragmenting. Rearing of corals in the lab requires costly lighting and highly specific parameters of water quality. Lengthy acclimation periods and inconsistent inocula contribute to a lack of well replicated studies of BBD in the laboratory. A model system for studying a coral disease such as BBD would be beneficial.

The tropical sea anemone *Exaiptasia pallida* has proven a good model system for studying cnidarian-dinoflagellate symbioses (Rädecker et al. 2018; Weis et al. 2008; Voolstra 2013). Several attributes make *E. pallida* a more favorable organism than corals for study in the laboratory including their small size, fast growth rate, minimal requirements for growth, and availability of clonal populations (Weis et al. 2008). Additionally, the ability of *E. pallida* to be maintained in an aposymbiotic state for extended periods of time, as well as its ability to be reinfected by multiple strains of Symbiodiniaceae, make it a good choice to serve as proxy for corals (Weis et al. 2008). *E. pallida* has also shown promise as a model for studying coral diseases. *Exaiptasia pallida* has been successfully infected with *Serratia marcescens* (Krediet et al. 2014), *Staphylococcus sciuri* (Divya et al. 2018) and *Vibrio coralliilyticus* (Zaragoza et al. 2014; Brown and Rodriguez-Lanetty 2015). Successful infection of *E. pallida* with coral
pathogens suggest it may be a good candidate model system for a polymicrobial infection such as BBD.

Dissertation research questions and organization

My dissertation seeks to answer the following overarching questions:

1) What role does dimethylsulfoniopropionate metabolism play in the initiation and persistence of Black Band Disease?

2) Is *Exaiptasia pallida* susceptible to Black Band Disease, and is this susceptibility affected by temperature, symbiotic state, or symbiont hosted?

3) How are the microbiomes of BBD and the host anemone modified by one another?

In the second chapter, I explore the possible role of DMSP metabolism in BBD through metagenomic analysis of polymicrobial BBD samples as well as metagenomic analysis of the suspected primary pathogen *Roseofilum reptotaenium* in support of answering the following questions:

1) Are genes associated with DMSP metabolism present in Black Band Disease?

2) Are genes associated with the demethylase pathway of DMSP metabolism in BBD more or less abundant than those associated with the cleavage pathway?

3) Are genes associated with DMSP metabolism present in *Roseofilum reptotaenium*?

4) Are genes associated with the demethylase pathway of DMSP metabolism in *Roseofilum reptotaenium* more or less abundant than those associated with the cleavage pathway?

5) Are both pathways of DMSP metabolism equally important in BBD or is one favored over the other?
In Chapter three, I examine the susceptibility of *E. pallida* to BBD of corals. Susceptibility was measured by defense-associated behaviors as well as mortality. Environmental factors including temperature, symbiotic state, and symbiont hosted were manipulated to determine if the factor had an effect on disease susceptibility. The following questions are addressed:

1) *Is E. pallida* susceptible to Black Band Disease of corals?
2) Does temperature influence the susceptibility of *E. pallida* to BBD?
3) Does nutritional status influence the susceptibility of *E. pallida* to BBD?
4) Does symbiotic state influence the susceptibility of *E. pallida* to BBD?

In Chapter four, I investigate the microbiomes of both BBD and *E. pallida* to understand the differences and similarities between the microbial communities associated with each. Additionally, I determine how those communities change over time during a disease challenge in support of answering the following questions:

1) Are there specific microbial taxa that overlap between BBD and *E. pallida* before and/or during infection?
2) What microbial taxa are unique to *E. pallida* and BBD before and/or during infection?
3) Does the microbial community of *E. pallida* change during infection with BBD?
4) Does the microbial community of BBD change during infection of *E. pallida*?
5) Does BBD recruit members of the *E. pallida* microbiome during infection?

Finally, in chapter five I summarize the conclusions of this dissertation and suggest future investigation of BBD utilizing the model system of *E. pallida*. 
References


Ziegler M, Seneca FO, Yum LK, Palumbi SR, and Voolstra CR (2017) Bacterial community dynamics are linked to patterns of coral heat tolerance. *Nature Communications*, 8:142
Chapter 2: Metagenomic insight into the role of dimethylsulfoniopropionate in black band disease of corals

Abstract

Dimethylsulfoniopropionate (DMSP), a widely available source of carbon and sulfur in the marine environment is produced predominantly by marine phytoplankton, including the dinoflagellate symbiont of corals (Symbiodiniaceae). Increasing interest in DMSP from coral researchers in recent years is the result, in part, of the association of microbial DMSP-metabolizing taxa with corals. The recent discovery of DMSP production by the coral animal and increasing production in response to thermal stress, as well as chemoattraction of the known coral pathogen Vibrio coralliilyticus to DMSP, suggests a role for DMSP in coral health and disease. This study shows the presence of taxa capable of DMSP metabolism in four metagenomes of black band disease (BBD) as well as cultured Roseofilum reptotaenium, the dominant cyanobacterial member of BBD. Additionally, genes related to DMSP metabolism including the demethylase gene (dmdA) and lyase genes (dddD, dddL) were present in the metagenomes while the lyase genes dddP, dddQ, dddW, and DddY were absent. Overall, a higher abundance of lyase genes was detected as compared to demethylase genes suggesting the cleavage pathway of DMSP metabolism may be favored in the diseased state. These data suggest a potential role for DMSP metabolism in the etiology and persistence of a coral disease.

Introduction

Dimethylsulfoniopropionate (DMSP) is an abundant source of carbon and reduced sulfur for bacteria in the marine environment (Kiene et al. 2000). Primarily produced by phytoplankton, DMSP is an important compound in the biogeochemical
sulfur cycle (Sievert et al. 2007). Metabolism of DMSP occurs through two competing pathways, demethylation and cleavage (Reisch et al. 2011; Curson et al. 2011). Demethylation is driven by the \textit{dmdA} gene (Howard et al. 2006) which accounts for the majority of DMSP metabolism in the marine environment (Raina et al. 2010). However, the cleavage pathway (driven by the lyase genes \textit{dddD, dddK, dddL, dddP, dddQ, dddW, or dddY}) is known to play a critical role in biogeochemical sulfur cycling (Moran et al. 2012). Dimethylsulfide (DMS), a volatile breakdown product of the cleavage pathway, links marine and atmospheric sulfur flux. It regulates local climate through cloud formation (Charlson et al. 1987) and is released from the oceans at an estimated rate of 13-40 Tg per year (Yoch 2002).

Recently, the role of DMSP in structuring coral-associated communities has been investigated, revealing a substantial overlap between coral-associated bacteria and bacteria known to metabolize DMSP (Raina et al. 2009). This recent study demonstrated that some coral-associated bacteria are able to grow using DMSP and/or related compounds as their sole energy source. Additional metagenomic analysis revealed that, of the bacterial genera implicated in DMSP/DMS metabolism, more than 65% have been found in association with corals (Raina et al. 2010).

The relationship between these marine bacteria and DMSP is further documented by studies that demonstrate positive chemotaxis of coral-associated bacteria to DMSP/DMS (Miller et al. 2004; Garren et al. 2014; Tout et al. 2015). The majority of the bacteria attracted to DMSP/DMS are capable of metabolizing one or both compounds. However, \textit{Vibrio coralliilyticus} (a known coral pathogen) is attracted to DMSP but is unable to metabolize it. This suggests that DMSP may serve solely as a
chemical cue to help the organism find a coral host (Garren et al. 2014). In addition to serving as a chemical cue for potential coral pathogens, DMSP may fuel coral diseases that have particularly active carbon and sulfur cycles such as black band disease.

Black band disease (BBD), one of the first identified and best characterized of the coral diseases, is a polymicrobial, mat forming disease in which cyanobacteria are the dominant members (Rützler et al. 1983). It consists of a characteristic dark band ranging from 1 mm to several centimeters in width and approximately 1 mm in thickness (Richardson 1996). As the band moves horizontally across a coral, it lysed tissue through the synergistic effects of the cyanobacterial toxin microcystin and sulfide (Miller and Richardson 2011). Toxic microcystin is produced by BBD cyanobacteria (Gantar et al. 2009; Myers et al. 2007; Richardson et al. 2009), and sulfide is produced by BBD sulfate reducing bacteria (SRB) (Viehman et al. 2006). The sulfide, in addition to being toxic to corals, is integral to initiation of BBD infections (Brownell and Richardson 2014).

The production of sulfide is linked directly to dissimilatory sulfate reduction, an important process in the active sulfur cycle of BBD in addition to assimilatory sulfate reduction and sulfide oxidation (Glas et al. 2012; Sato et al. 2016). Dimethylsulfiniopropionate provides an abundant source of reduced sulfur that may contribute to these processes (Kiene et al. 2000). These processes take place in the context of steep sulfide and oxygen vertical gradients. During the day, the upper part of the band is supersaturated with oxygen, while high levels of sulfide (40-400 μM) are present at the base (Carlton and Richardson 1995). The band is fully anoxic and sulfide rich at night, with observed levels of sulfide as high as 835 μM (Carlton and Richardson 1995). In addition to serving as a source of reduced sulfur, DMSP also provides an
abundant source of carbon which may be used in carbon transformational processes operating within the BBD community such as fermentation, and both aerobic and anaerobic respiration (Sato et al. 2016).

The microbial community structure of BBD is functionally similar to that of other marine microbial mats and mats in freshwater environments that contain sulfide (Bolhuis et al. 2014). Infection studies with unialgal cultures of Roseofilum reptotaenium (the dominant cyanobacteria of BBD) have successfully resulted in BBD onset in coral fragments (Casamatta et al. 2012) including development of the complex BBD community. Roseofilum reptotaenium is a suspected primary pathogen of BBD (Richardson et al. 2014), and SRB have been shown to be secondary and required pathogens for successful infection in the laboratory (Brownell and Richardson 2014). SRB have also been shown to increase at the onset of BBD on the Great Barrier Reef (Bourne et al. 2011) where BBD infection is preceded by cyanobacterial patches. These patches undergo successional changes in bacterial communities including shifts in identity and abundance of cyanobacteria as well as an increase in sulfur cycling organisms (Sato et al. 2010). These changes, combined with the high sulfide environment of the band, suggest a role for SRB in sulfur cycling within BBD as well as BBD pathogenesis.

The established relationship of DMSP with coral-associated bacteria, combined with recent evidence of DMSP synthesis by the coral animal (Raina et al. 2013), makes an examination of DMSP’s influence in structuring coral disease communities timely in adding to our current understanding of the role of bacteria in coral health and disease. The overlap of DMSP metabolizing bacteria with coral-associated bacteria, together with
the high levels of sulfide present in the band, strongly suggest that BBD would have bacteria capable of metabolizing DMSP. Similarly, the observed increase in DMSP production by corals in response to thermal stress (Raina et al. 2013) combined with evidence of elevated temperature as a driving force in BBD emergence (Miller and Richardson 2015) supports a potential link between DMSP and BBD. To date, no studies have specifically examined the role of DMSP in structuring coral disease communities. Here we investigate this possible link between DMSP and BBD by metagenomic analysis of BBD samples. A metagenomic approach facilitates screening the metagenomes for taxa capable of metabolizing DMSP as well as screening for genes related to DMSP metabolism. The presence of DMSP-metabolizing taxa and/or DMSP-metabolizing genes may reveal the genetic potential for DMSP metabolism in a coral disease.

Materials and methods

Sample collection of Black Band Disease (BBD)

Three samples of BBD were collected from the coral species Colpophylia natans at Horseshoe Reef in the Florida Keys, and one sample of BBD was collected from the coral species Pseudodiploria strigosa in Curaçao between June 2012 and August 2013 (Table 1). The band was aspirated using sterile 10mL needleless syringes. Samples were preserved in RNA Later and kept at -80 until extraction. A sample of Roseofilum reptotaenium (the dominant cyanobacterium of BBD) was obtained from a unialgal, but non-axenic lab culture maintained in marine BG11. This culture originated from a single filament of R. reptotaenium isolated from a BBD-infected colony of Pseudodiploria strigosa in the U.S. Virgin Islands that was used to successfully infect coral in the laboratory (Casamatta et al. 2012).
**Genomic DNA extraction and metagenomic sequencing**

Whole genomic DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) according to the manufacturers protocol. Paired end sequencing was run by Illumina HiSeq technology at the Microbiome Analysis Center of George Mason University. All samples were barcoded and run in a single lane with an insert size of 500bp. Resulting FASTQ sequence data were uploaded to the metagenomics rapid annotation using subsystems (MG-RAST) open source server (Meyer et al. 2008) under the project ID mgp11531. Quality control measures including dereplication, dynamic trimming, length filtering and ambiguous filtering were applied. A minimum quality score of 15 was required to pass quality filtering.

**Data analysis**

Metagenomes were screened using the functional search tool for genes related specifically to DMSP metabolism including *dmdA*, *dddD*, *dddL*, *dddP*, *dddQ*, *dddW*, and *dddY* using the MG-RAST subsystems annotation source with an E-value cutoff of 1e-5, minimum identity of 60%, and a minimum alignment of 15 amino acids. Relative abundance of each gene identified was determined within its respective subsystem and compared. Raw sequence abundance of the *dmdA* gene was compared to that of the *dddD* gene.

Analysis of 16S rDNA was carried out using the best hit classification for organismal abundance, with Ribosomal Database Project (RDP) as the annotation source, an E-value cutoff of 1e-5, minimum identity of 97%, and a minimum alignment of 50 base pairs. After sequences were assigned taxonomic designations, relative abundance of each was determined at the following taxonomic levels: phylum, class, order, and family.
Results

Metagenome summary

A total of 16,446,958; 10,993,951; 14,080,073; 20,062,787; and 8,513,163 reads were generated and uploaded to MG-RAST for the four BBD samples (BBD1, BBD2, BBD3, BBD4), and *R. reptotaenium* sample respectively. Post quality control, 13,666,627; 9,466,162; 12,989,966; 13,728,492; and 7,327,706 sequences remained with an average length of 141 bp and an average GC content of 47%. Of these, 6,600,640; 3,541,370; 8,472,536; 7,559,274; and 1,762,094 were predicted protein features while 118,311; 76,825; 109,226; 125,669; and 54,902 were predicted rRNA features.

Sulfur metabolism

The total numbers of sequences related to sulfur metabolism by subsystems annotation for metagenomes BBD1, BBD2, BBD3, BBD4, and *R. reptotaenium* were 55,411; 54,676; 38,803; 104,688; and 42,720 respectively. Within sulfur metabolism, the highest sequence abundance for all metagenomes was related to inorganic sulfur assimilation (ranging from 37%-66%), followed by thioredoxin-disulfide reductase (ranging from 13%-16%) (Figure 1).

For the category of sulfur oxidation-related sequences, the highest average number of sequences associated with this subsystem was related to cytochrome c-type biogenesis protein CcdA and sulfite oxidase (Figure 2). Additionally, several genes associated with the *sox* pathway were present in all metagenomes including *SoxA, SoxB, SoxC, SoxD, SoxF, SoxH*, and *SoxW*.

Sequences associated with DsrK and DsrP had the greatest average abundances within the subsystem of sulfate reduction (Figure 3). It should be noted that only 2
sequences were identified in the *R. reptotaenium* metagenome as being related to sulfate reduction by subsystems annotation. These account for the highest abundance shown as 50% for sequences related to DsrP and tRNA 2-thiouridine synthesizing protein E. Sequences related to the remaining functional annotations are absent from the *R. reptotaenium* metagenome.

**DMSP metabolism-related genes**

From the overall sulfur metabolism data, two subsystems associated specifically with DMSP metabolism (Release of DMS from DMSP and DMSP Breakdown) were identified with percent abundances ranging from 0.1-1.8% and 0.2-1.5% respectively (Figure 4). Release of DMS from DMSP included the lyase genes while DMSP Breakdown included the demethylase gene *dmdA*. The specific lyase genes detected and their percent abundances within the subsystem of Release of DMS from DMSP are summarized in table 2. Of the six lyase genes queried, only two were identified in the metagenomes (*dddD* and *dddL*). Of those detected, *dddD*-related sequences were more abundant than *dddL* in all four BBD metagenomes. The *R. reptotaenium* metagenome, however, was dominated by *dddL* related sequences. Raw sequence abundance associated with the demethylase gene (*dmdA*) was compared to the dominant lyase gene (*dddD*) for the four BBD metagenomes (Table 3). Overall, 962 sequences were classified as *dddD* as compared to 681 for *dmdA*. However, the proportion of *dmdA* to *dddD* sequence abundance within each metagenome varied.

**Organismal abundance**

A total of 7,955; 4,050; 10,104; and 9,802 sequences from metagenomes BBD1, BBD2, BBD3, and BBD4 respectively and 2,469 sequences from the *R. reptotaenium*
metagenome were identified as rRNA sequences using the RDP annotation source for organismal abundance. In the BBD metagenomes, 2% to 34%, <1% to 56%, 1% to 13%, and 8% to 56%, of the total sequences were most closely related to \( \textit{Bacteroidetes} \), \( \textit{Cyanobacteria} \), \( \textit{Firmicutes} \), and \( \textit{Proteobacteria} \) respectively (Figure 5). In the \( \textit{R. reptotaenium} \) metagenome, 12%, 56%, and 19% of the total sequences were most closely related to \( \textit{Bacteroidetes} \), \( \textit{Cyanobacteria} \), and \( \textit{Proteobacteria} \) respectively. No sequences related to \( \textit{Firmicutes} \) were identified within the \( \textit{R. reptotaenium} \) metagenome. A percentage of sequences for BBD1, BBD2, BBD3, BBD4 and \( \textit{R. reptotaenium} \) metagenomes could not be assigned a taxonomic designation including 17%, 22%, 36%, 23%, and 13% respectively. Additional phyla representing less than 1% of the total sequences included \( \textit{Actinobacteria} \) in BBD3 and \( \textit{R. reptotaenium} \), \( \textit{Deferribacteres} \) in BBD3, \( \textit{Fusobacteria} \) in BBD1 and BBD2, \( \textit{Planctomycetes} \) in \( \textit{R. reptotaenium} \), \( \textit{Spirochaetes} \) in BBD4, and \( \textit{Verrucomicrobia} \) in all BBD metagenomes.

Apart from \( \textit{Cyanobacteria} \), sequences related to \( \textit{Proteobacteria} \) dominated all of the metagenomes (Figure 6). Within the phylum \( \textit{Proteobacteria} \), the \( \textit{R. reptotaenium} \) metagenome was dominated by the class \( \textit{Alphaproteobacteria} \), followed by \( \textit{Gammaproteobacteria} \) with 91% and 9% of sequences respectively (Figure 6). A single sequence was most closely related to \( \textit{Deltaproteobacteria} \). The BBD metagenomes were dominated by \( \textit{Gammaproteobacteria} \) with 26 to 62%, followed by \( \textit{Alphaproteobacteria} \) with 17 to 47%, \( \textit{Deltaproteobacteria} \) with 4 to 42%, and \( \textit{Epsilonproteobacteria} \) with 2 to 12%. Additionally, BBD4 had 3 sequences associated with \( \textit{Betaproteobacteria} \).

The orders \( \textit{Alteromonadales} \) and \( \textit{Oceanospirillales} \) were highly represented within the class \( \textit{Gammaproteobacteria} \) and found within each of the metagenomes.
(Figure 7). *Vibrionales* was also found within each of the BBD metagenomes. These three orders were the most highly represented in BBD2, BBD3, and BBD4. *Enterobacterales* was more highly represented than *Oceanospirillales* in BBD1. *Alteromonadales* was the most highly represented order in the *R. reptotaenium* metagenome, followed by *Thiotrichales*, and *Oceanospirillales*.

Within the class of *Alphaproteobacteria*, the order *Rhodobacterales* was the most highly represented order across all BBD metagenomes while *Rhizobiales* was the most highly represented order in the *R. reptotaenium* metagenome (Figure 8). Within the order *Rhodobacterales*, 21 genera within the family *Rhodobacteraceae* were identified among the metagenomes (Figure 9). Members of the genus *Ruegeria* were found within each of the metagenomes. This genus was the most highly represented among the BBD metagenomes and the second highest in the *R. reptotaenium* metagenome. Members of the *Pannonibacter* genus were the most highly represented in the *R. reptotaenium* metagenome. *Pannonibacter* and *Sagittula* were the only identified genera within *Rhodobacteraceae* that were present in the *R. reptotaenium* metagenome but absent from all of the BBD metagenomes.

**Discussion**

Four major bacterial groups have been established as being functionally important in BBD including cyanobacteria, sulfide-oxidizers, sulfate-reducers, and heterotrophs (Sato et al. 2016). This study supports the presence and importance of these groups as evidenced by 16S rDNA identification of a diverse group of heterotrophic bacterial taxa as well as abundant functional genes that are associated with metabolic processes.
important to the BBD microbial consortium including sulfide oxidation and sulfate reduction. Many of these bacterial taxa are capable of DMSP and/or DMS metabolism.

Evidence of major bacterial groups and their metabolism in BBD

Black band disease is known to have a very active sulfur cycle including sulfate reduction (both assimilatory and dissimilatory) and sulfide oxidation (Glas et al. 2012; Sato et al. 2016). Steep sulfide/oxygen gradients are present with supersaturation of oxygen in the upper part of the band during the day and anoxia, combined with high levels of sulfide, at night (Carlton and Richardson 1995) which contribute to BBD pathogenicity. Inorganic sulfur assimilation was the most highly represented category of sulfur metabolism and the presence of sulfate reduction-associated complexes is consistent with other metagenomic and metatranscriptomic studies of BBD (Arotsker et al. 2016; Sato et al. 2016; Meyer et al. 2017). The presence of sulfur oxidation-related sequences agrees with Sato et al. (2016) metagenomic and transcriptomic data but is in contrast to the Meyer et al. (2017) metagenomic study, which failed to detect any genes associated with the sulfur oxidation pathway. The Arotsker et al. (2016) metatranscriptomic study fails to address the presence or absence of sulfur oxidation transcripts. The current metagenomic study identified a suite of sox genes including SoxA, SoxB, SoxC, SoxD, SoxF, SoxH, and SoxW. The current study further shows that the abundance of sulfur oxidation-related sequences is greater than the abundance of sequences related to sulfate reduction for all metagenomes, particularly the R. reptotaenium metagenome. This might suggest a greater importance for sulfur oxidation as compared to sulfate reduction in BBD or may simply reflect a greater abundance of SOB present in the metagenomes. Metagenomic evidence from Sato et al. (2016)
corroborates the higher abundance of sulfide oxidation-related sequences as compared to sulfate reduction-related sequences in BBD. However, the metatranscriptomic data indicates a much greater abundance of transcripts related to sulfate reduction than those related to sulfur oxidation in BBD. The study further demonstrates that both sequences and transcripts related to sulfate reduction increase in BBD as compared to cyanobacterial patches, while sequences and transcripts related to sulfur oxidation decrease in BBD when compared to cyanobacterial patches. This suggests that sulfur oxidation may be less important once BBD is fully established. Cyanobacterial patches have not been observed as precursors to BBD in the Caribbean. The absence of nearly all sulfate reduction-associated complexes from the *R. reptotaenium* metagenome, and a marked increase in these complexes in the BBD metagenomes, is in agreement with an increase in SRB as BBD develops (Sato et al. 2016; Bourne et al. 2011).

Diverse groups of heterotrophic bacteria are found associated with BBD (Cooney et al. 2002; Friaz-Lopez et al. 2002; Miller and Richardson 2011; Arotsker et al. 2016; Meyer et al. 2016; Meyer et al. 2017). Findings of the current study also support this assertion with an abundance of Gammaproteobacteria including members of the order *Vibrionales* (Figure 7) and an abundance of *Alphaproteobacteria* including members of the order *Rhodobacterales* (Figures 8). Members of these orders are well-known metabolizers of DMSP and DMS (Raina et al. 2010). Ten different bacterial phyla were represented across the five metagenomes. The top four phyla identified (Figure 5), were in agreement with a recent metagenomic study of BBD (Meyer et al. 2017). Within the phylum *Proteobacteria*, the order *Rhodobacterales* had more than 20 different genera represented (Figure 9).
DMSP metabolism-related genes detected in BBD

Two subsystems identified within the sulfur metabolism data were associated specifically with the metabolism of DMSP (DMSP Breakdown and Release of DMS from DMSP). These subsystems include genes associated with the competing DMSP metabolism pathways of demethylation and cleavage respectively. Data from this study indicate that genes associated with both pathways are present in the BBD and R. reptotaenium metagenomes. Specifically, sequences associated with the genes dmdA, dddD, and dddL were identified in the metagenomes. To our knowledge, this is the first study to identify genes specific to DMSP metabolism within BBD metagenomes.

Sequences related to dmdA were detected in all of the metagenomes. This is not surprising as demethylation related sequences are the most abundantly represented DMSP metabolism genes in marine metagenomes (Moran et al. 2012). To date, dmdA is the only gene that has been identified as mediating the primary step in the demethylation pathway of DMSP metabolism (Howard et al. 2006). This gene codes for the production of DmdA demethylase, a glycine cleavage T-protein that utilizes methyl-accepting tetrahydrofolate (THF) as a co-factor (Johnston et al. 2016). The presence of this gene suggests the potential of the BBD consortium to metabolize DMSP by first generating methyl-mercaptopropionate (MMPA). Methyl-mercaptopropionate is further metabolized to methanethiol (MeSH) and acetate. Methanethiol is readily assimilated into the sulfur-containing amino acids methionine and cysteine but may also be oxidized by MeSH-oxidase to produce H₂S, H₂O₂, and formaldehyde (Reisch et al. 2011). The production of H₂S by oxidation of MeSH may directly contribute to the build up of sulfide, a major toxin associated with BBD that contributes to coral tissue lysis. Acetate can be utilized as
a carbon source by aerobic organisms (including a diverse group of heterotrophs within the BBD bacterial community) through the citric acid cycle.

A recent transcriptomic analysis of BBD did not reveal DMSP metabolism-related transcripts (Arotsker et al. 2016). There are several possible explanations for this. The first is that genes related to DMSP metabolism may not have been present in the sample. This is unlikely given that bacteria capable of metabolizing DMSP have been found throughout the marine environment, both in association with corals and as members of the BBD community. Another possibility is that these genes may have been present but were not being actively transcribed at the time of sampling. The wide availability of DMSP in the marine environment, combined with the number of organisms known to metabolize it, make this scenario unlikely as well. The most likely explanation is that the transcripts were simply not detected because of low abundance or bias. Additionally, this study analyzed a single sample of BBD. Quantitative polymerase chain reaction (qPCR) experiments targeting DMSP specific transcripts in BBD could help confirm this. Interestingly, the average number of reads in the present study was 10-fold higher than the average number of reads in the transcriptomic analysis. Increasing the depth of sequencing may reveal DMSP-related transcripts. Additionally, enrichment of the BBD community with DMSP would likely increase the abundance of transcripts above the level of detection.

A recent study examined the abundance of the *dmdA* gene (associated with the demethylase pathway) in healthy coral mucus (Frade et al. 2016) and found, for the most part, a decrease in *dmdA* abundance for those corals producing higher levels of DMSP. It may then be possible that, as temperature increases, increased production of DMSP in
response to thermal stress may cause a shift in the dominant pathway of DMSP metabolism. As temperature increases are often associated with the onset of disease, this could explain the overall dominance of the lyase gene \textit{dddD} to \textit{dmdA} in the BBD metagenomes.

Of the lyase genes queried, \textit{dddD} and \textit{dddL} were the only two detected. The \textit{dddD} gene codes for the production of DddD lyase, an acetyl CoA-transferase with no known co-factor. \textit{DddD} catalyzes a reaction resulting in the production of 3-hydroxypropionate (3-HP), acetate, and DMS (Johnston et al. 2016). The \textit{dddL} gene codes for the production of DddL lyase, a cupin that results in the production of acrylate and DMS (Johnston et al. 2016). While both \textit{dddD} and \textit{dddL} mediated cleavage result in the production of DMS, they differ in production of 3-HP and acrylate respectively. 3-HP can be further metabolized to acetate or reduced to form propionate. Acrylic acid can be used as a sole carbon source by some bacteria including vibrios (Raina et al. 2009) and, along with DMS, has been implicated as an antimicrobial (Sieburth, 1960).

The \textit{dddW} gene codes for DddW lyase, a cupin that utilizes Fe(II) as a co-factor, and produces acrylate and DMS (Johnston et al. 2016). The absence of \textit{dddW} and \textit{dddY} is not surprising given the former was rare and restricted to a single site while the latter was completely absent from available marine metagenomes (Raina et al. 2010). Interestingly, \textit{dddP} and \textit{dddQ} were more frequently represented than \textit{dddD} and \textit{dddL} in marine metagenomes (Moran et al. 2012). Additionally, \textit{dddP} has been found in \textit{Aspergillus sydowii}, the primary pathogen of Aspergillosis in sea fans (Kirkwood et al. 2010). The presence of \textit{dddD} may be indicative of organisms capable of using DMSP as their sole carbon source (Curson et al. 2011).
A recent study indicated an absence of DMSP lyase genes in 5 BBD metagenomes (Meyer et al. 2017). This is in contrast to the current study as well as a recent draft genome of *R. reptotaenium* (Buerger et al. 2016) in which the *Alphaproteobacteria* A01-B was identified in association with a unialgal culture of *R. reptotaenium* and found to have the *dddL* gene present with a length of 226 aa (Genbank Accession number OJJ11322). Additionally, Buerger et al. (2016), identified a *dmdA* gene associated with the same *Alphaproteobacteria* species, with a length of 373 aa (Genbank Accession number OJJ09738). Curiously, Meyer et al. (2017) did not specifically address the presence or absence of the demethylase gene in BBD.

The greater number of sequences associated with *dddD* overall as compared to *dmdA* among the BBD metagenomes suggests that the cleavage pathway may be more functionally important than *dmdA* and the demethylation pathway in BBD. This implies that the presence of BBD could contribute to a greater amount of DMS being released during the metabolism of DMSP than it might otherwise be in a healthy coral. This DMS could be further degraded by marine bacteria or released to the atmosphere where it may influence local climate through the production of cloud condensation nuclei. As corals face the threat of changing climate, including higher sea surface temperatures, favoring of the cleavage pathway may be particularly significant in mitigating thermal stress at the local level. There was, however, some inconsistency in the dominant pathway within the metagenomes suggesting there may be variability across BBD samples.

*Recruitment of potentially pathogenic organisms by DMSP*

Dimethylsulfiniopropionate has been shown to be a potent chemoattractant for some marine microbes (Seymour et al. 2010) including those associated with corals (Tout
et al. 2015). Specifically, it has been shown to attract *Silicibacter*, a member of the Roseobacter clade (Miller et al. 2004) as well as the coral pathogen *Vibrio coralliilyticus* (Garren et al. 2014). Members of the Roseobacter clade, including *Silicibacter*, have been detected previously in BBD clone libraries (Cooney et al. 2002; Sekar et al. 2008; Miller and Richardson 2011). Roseobacter clade member, *Roseovarius crassostreae* (*Aliiroseovarius crassostreae*), a known pathogen of Roseovarius Oyster Disease (Boettcher et al. 2005), has also been found in association with BBD (Cooney et al. 2002; Sekar et al. 2008). A recent draft genome of *A. crassostreae* (Kessner et al. 2016), identified the demethylase gene *dmdA*, indicating that this pathogen can metabolize DMSP. The establishment of DMSP as a strong chemoattractant to coral-associated bacteria and, in particular, to a coral pathogen, suggests that bacteria associated with the BBD consortium, specifically those of the Roseobacter clade, may be attracted to DMSP and therefore potentially recruited to a healthy or diseased coral in response to DMSP production. In the diseased state, which is often accompanied by elevated temperatures, increased DMSP production by the coral holobiont may exacerbate the recruitment of these potential pathogens.

*Role of DMSP metabolism in production of BBD toxins*

*Cyanobacteria* dominate BBD communities with regard to biomass (Taylor 1983) and to date, all isolates from BBD tested, including *R. reptotaenium*, are capable of producing microcystin (Gantar et al. 2009), a cyanotoxin known to cause liver damage in humans (Dawson 1998) and tissue lysis in corals (Richardson et al. 2009). Although microcystin does not contain sulfur, methionine, a sulfur containing amino acid, is a necessary component in the cyanotoxin’s biosynthesis (Arment and Carmichael 1996).
When DMSP is metabolized via the demethylation pathway, MeSH is produced and can be incorporated into the sulfur-containing amino acid methionine (essential to the production of microcystin). Environmental factors including inorganic phosphorous, irradiance level, and sulfur have been shown to influence microcystin production (Jahnichen et al. 2011). Specifically, sulfur limitation has been shown to decrease microcystin production in the cyanobacterium Microcystis aeruginosa (Long 2009). If limitation of sulfur decreases microcystin production, it is possible that an increase in the availability of sulfur in the form of DMSP may be readily used in the biosynthesis of microcystin, thereby increasing its production. The role of methionine in toxin production is not limited to microcystin. Although MeSH resulting from DMSP catabolism via the demethylation pathway may be incorporated into sulfur-containing amino acids, it can also be oxidized by MeSH-oxidase to produce H$_2$S, H$_2$O$_2$, and formaldehyde (Reisch et al. 2011). In addition to sulfide production by SRB such as Desulfovibrio sp., the production of H$_2$S by oxidation of MeSH may directly contribute to the build up of sulfide, a major toxin associated with BBD that contributes to coral tissue lysis.

Metabolism of DMSP via the demethylation pathway may also indicate a role for DMSP metabolism specifically in onset of the disease as sulfide has been shown to be important to initiation of BBD (Brownell and Richardson 2014).

The diversity and composition of the BBD microbial consortium has been examined previously (Cooney et al. 2002; Frias-Lopez et al. 2002; Frias-Lopez et al. 2004; Voss et al. 2007; Miller and Richardson 2011; Meyer et al. 2017). However, this is the first study to focus specifically on DMSP-metabolism associated organisms within BBD. Marine bacteria belonging to the Gammaproteobacteria class dominated the
DMSP-metabolizing isolates in one study of healthy corals (Raina et al. 2009). This same class of bacteria was shown to respond rapidly to DMSP enrichment (Vila-Costa et al. 2010). In a meta-analysis of 16S rRNA gene clone libraries from BBD (Miller and Richardson 2011), *Alphaproteobacteria* were the most diversely represented group including several genera implicated in DMSP metabolism (Raina et al. 2010): *Roseovarius, Roseobacter, Silicibacter, Sulfitobacter, and Ruegeria*. Additionally, DMSP-metabolizing genera including *Cytophaga, Desulfovibrio, Clostridium, and Pseudoalteromonas* were detected. Of these taxa, *Roseovarius, Roseobacter, Silicibacter, Desulfovibrio, Sulfitobacter, and Ruegeria* were present in the BBD and *R. reptotaenium* metagenomes.

**Possible sources of DMSP in BBD**

While the presence of DMSP has not been directly measured in BBD, possible sources of introduction include the water column, the coral animal and/or its associated symbionts, or production of DMSP by organisms within the band. Recent identification of the *dsyB* gene, implicated in biosynthesis of DMSP by some marine bacteria including *Labrenzia aggregata* (Curson et al. 2017), suggests that perhaps bacterial members of the BBD consortium may provide a source of DMSP. Interestingly, members of the genus *Labrenzia* were detected in two of the four BBD metagenomes and the *Roseofilum* metagenome. However, it is unclear if the presence of these organisms could provide a significant source of DMSP given low sequence numbers as well as a lack of detection in two of the BBD metagenomes.
Conclusion

Overall, our results indicate that DMSP metabolizing genes are present in both BBD and the cyanobacterial culture of *R. reptotaenium*. While DMSP metabolizing genes have been found previously in coral-associated bacteria, this is the first study to focus on their presence in the BBD community. The presence of these genes, combined with the presence of several taxa known to metabolize DMSP, suggests the potential for DMSP to be metabolized by the BBD microbial consortium, thereby providing a source of carbon and sulfur that may influence the initiation and/or persistence of the disease. Further, DMSP may influence recruitment of pathogens and its metabolism may promote the production of toxic microcystin and sulfide.
Table 2-1 Sources of samples analyzed in this study.

<table>
<thead>
<tr>
<th>Metagenome</th>
<th>ID</th>
<th>Location</th>
<th>Coral Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBD1</td>
<td>4602080.3</td>
<td>Florida Keys</td>
<td>*Colpophy whole</td>
</tr>
<tr>
<td>BBD2</td>
<td>4602083.3</td>
<td>Curacao</td>
<td><em>Pseudodiploria strigosa</em></td>
</tr>
<tr>
<td>BBD3</td>
<td>4602084.3</td>
<td>Florida Keys</td>
<td>*Colpophy whole</td>
</tr>
<tr>
<td>BBD4</td>
<td>4602085.3</td>
<td>Florida Keys</td>
<td>*Colpophy whole</td>
</tr>
<tr>
<td><em>R. reptotaenium</em></td>
<td>4602082.3</td>
<td>Unialgal Culture</td>
<td>*Pseudodi whole</td>
</tr>
</tbody>
</table>

Table 2-2 Relative abundance of DMSP lyase genes detected in BBD and *R. reptotaenium* metagenomes

<table>
<thead>
<tr>
<th>Metagenome</th>
<th>dddD</th>
<th>dddL</th>
<th>dddP</th>
<th>dddQ</th>
<th>dddW</th>
<th>dddY</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBD1</td>
<td>100%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BBD2</td>
<td>92%</td>
<td>8%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BBD3</td>
<td>95%</td>
<td>5%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BBD4</td>
<td>96%</td>
<td>4%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>R. reptotaenium</em></td>
<td>9%</td>
<td>91%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations: ND, not detected

Table 2-3 Sequence abundance comparison of *dmdA* and *dddD* in BBD metagenomes

<table>
<thead>
<tr>
<th>Metagenome</th>
<th>dmdA (demethylase)</th>
<th>dddD (lyase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBD1</td>
<td>46</td>
<td>94</td>
</tr>
<tr>
<td>BBD2</td>
<td>81</td>
<td>57</td>
</tr>
<tr>
<td>BBD3</td>
<td>192</td>
<td>544</td>
</tr>
<tr>
<td>BBD4</td>
<td>362</td>
<td>267</td>
</tr>
<tr>
<td>Total</td>
<td>681</td>
<td>962</td>
</tr>
</tbody>
</table>
Figure 2-1 Percent abundance of sequences related to sulfur metabolism across all metagenomes
Figure 2-2 Percent abundance of sequences related to sulfur oxidation across all metagenomes
Figure 2-3 Percent abundance of sequences related to sulfate reduction across all metagenomes
Figure 2-4 Relative abundance of BBD and *R. reptotaenium* sequences related to DMSP metabolism-associated subsystems
Figure 2-5 Bacterial community composition by phylum based on 16S rRNA sequence abundance for four different BBD metagenomes and one R. reptotaenium metagenome
Figure 2-6 Bacterial community composition of Proteobacteria based on 16S rRNA sequence abundance for four different BBD metagenomes and one *R. reptotaenium* metagenome
Figure 2-7 Bacterial community composition of Gammaproteobacteria based on 16S rRNA sequence abundance for four different BBD metagenomes and one *R. reptotaenium* metagenome.
Figure 2-8 Bacterial community composition of Alphaproteobacteria based on 16S rRNA sequence abundance for four different BBD metagenomes and one *R. reptotaenium* metagenome
Bacterial community composition of *Rhodobacterales* based on 16S rRNA sequence abundance for four different BBD metagenomes and one *R. reptotaenium* metagenome.

**Figure 2-9** Bacterial community composition of *Rhodobacterales* based on 16S rRNA sequence abundance for four different BBD metagenomes and one *R. reptotaenium* metagenome.
References


Chapter 3: Mortality and defensive behavior of the tropical sea anemone *Exaiptasia pallida* in response to infection by Black Band Disease of corals.

Abstract

Black Band Disease (BBD) is a polymicrobial, mat forming disease, affecting more than 64 species of reef building coral worldwide. It presents as a dark band separating denuded coral skeleton from apparently healthy tissue and can migrate up to 1 cm per day, quickly devastating entire coral colonies. Virulence of this disease has been shown to increase at elevated temperatures. Traditional infection studies require field collection of coral and subsequent maintenance in aquaria, often including lengthy acclimation periods prior to the initiation of a study. The tropical sea anemone *Exaiptasia pallida* is well established as a model system for studying cnidarian-dinoflagellate symbiosis (including that of corals) and has gained momentum in recent years as a model for studying coral disease. *E. pallida* has been successfully infected with opportunistic coral pathogens, including *Vibrio* spp. and *Serratia marcescens*. To assess the potential of *E. pallida* as a model system to study this polymicrobial disease, we examined the susceptibility of *E. pallida* to BBD. We further examined the potential effect of temperature, symbiotic state, and symbiont type hosted on susceptibility. To accomplish this, we challenged *E. pallida* with BBD collected from an infected coral colony of *Montastraea cavernosa*. A total of 5 BBD challenge experiments were carried out. In response to infection, anemone behaviors indicative of defense were observed including tentacle retraction, locomotion, detachment from substrate, and ejection of acontia. Our results indicate that *E. pallida* is susceptible to BBD infection across a range of temperatures, symbiotic state, and symbiont type hosted, supporting the potential use of
*E. pallida* as a model system for studying BBD. This would obviate the need for field collection and difficult maintenance of corals in aquaria, making the study of BBD more accessible to researchers and promoting advances in understanding the etiology of this devastating coral disease.

**Introduction**

Black Band Disease (BBD) is a polymicrobial (Carlton and Richardson 1995), mat forming disease, affecting more than 64 species of reef building coral worldwide (Sutherland et al. 2004). Discovery of the disease in novel geographic locations and susceptibility of additional species have been reported (Aeby et al. 2015; Lewis et al. 2017). The mat is dominated by filamentous, phycoerythrin-rich cyanobacteria (Rützler et al. 1983) which provide physical structure to the band, in part through exopolysaccharide production. The disease presents as a dark band separating denuded coral skeleton from apparently healthy tissue (Rützler et al. 1983) and can migrate at rates up to 1 cm per day, quickly devastating entire coral colonies. The presence of BBD on the reef has been positively associated with temperature (Kuta and Richardson 2002). Stratification between oxic and anoxic microenvironments within the band (Carlton and Richardson 1995), resulting from biogeochemical conditions, has been linked to increased virulence of this disease (Glas et al. 2012). Additionally, virulence has been shown to increase at elevated temperatures (Kuehl et al. 2011). However, BBD has been observed at temperatures below 20° C (Kuta and Richardson 1996).

Black band disease was first observed in 1976 and described by Antonius (1981). Since that time, several field studies have been conducted worldwide to determine the prevalence of BBD (Edmunds 1991; Kuta and Richardson 1996; Page and Willis 2006;
Sato et al. 2009; Montano et al. 2013; Lewis et al. 2017). Although the prevalence of BBD on reefs is often found to be <1% (Edmunds 1991; Kuta and Richardson 1996; Montano et al. 2013), the rate at which BBD has been observed to migrate (Miller et al. 2012) combined with its affinity for major reef building corals (Rützler et al. 1983; Kuta and Richardson 1996) make it of particular concern. Studies examining the microbial composition of BBD using traditional microbiological methods as well as molecular methods have been undertaken in an effort to understand the etiology and pinpoint a primary pathogen (Cooney et al. 2002; Sekar et al. 2006; Myers et al. 2007; Arotsker et al. 2009; Miller and Richardson 2011). However, Koch’s postulates have yet to be fulfilled for any of the proposed primary pathogens.

Traditional infection studies with BBD and its associated cyanobacteria require field collection of coral and subsequent maintenance in aquaria (Voss and Richardson 2006; Richardson et al. 2009; Kuehl et al. 2011; Casamatta et al. 2012; Brownell and Richardson 2014; Richardson et al. 2014). These studies lack consistency in inoculation of coral fragments and experimental parameters. For example, one study that examined the role of sulfate reducing bacteria in BBD described the amount of inoculum qualitatively as a “small amount” (Brownell and Richardson 2014). Other studies have described varying amounts of inoculum used in challenge studies including 0.25mL (Voss and Richardson 2006), 0.5 cm² (Kuehl et al. 2011), 3-4mm in diameter (Richardson et al. 2009), and 5mm in diameter (Casamatta et al. 2012; Richardson et al. 2014). Additionally, lengthy acclimation periods are required prior to the initiation of a study such as Brownell and Richardson (2014) which cited an 8-week acclimation period after collection of coral fragments and a further 7-day acclimation period after being
transferred to experimental aquaria. The establishment of a model system for the study of BBD would eliminate lengthy acclimation periods and provide a consistent source of similarly sized organisms that can facilitate standardization of inoculation.

The tropical sea anemone *Exaiptasia pallida* is well established as a model system for studying cnidarian-dinoflagellate symbioses (including that of corals) (Weis et al. 2008; Voolstra 2013; Rädecker et al. 2018). This system has gained momentum in recent years as a model for studying coral disease (Krediet et al. 2014; Zaragoza et al. 2014; Divya et al. 2018). *Exaiptasia pallida* has been successfully infected with opportunistic coral pathogens including *Vibrio* spp. (Zaragoza et al. 2014), *Serratia marcescens* (Krediet et al. 2014), and *Staphylococcus sciuri* (Divya et al. 2018). There are several advantages of using *E. pallida* in place of corals including their small size, fast growth rate, minimal requirements for growth, and availability of clonal populations (Weis et al. 2008). An additional advantage is that *E. pallida* can be maintained in an aposymbiotic state and is able to be reinfected by different species of Symbiodiniaceae (Weiss et al. 2008; Leal et al. 2015).

The goal of the present study is to determine if *E. pallida* is susceptible to BBD infection. Further investigation of whether that susceptibility is affected by temperature, an environmental factor well established as influencing the onset and progression of BBD infection (Kuta and Richardson 2002; Kuehl et al. 2011), symbiotic status, and symbiont type hosted were undertaken to provide additional support for *E. pallida* as a model system for studying BBD of corals.
Materials and methods

Collection and culture of BBD

Black band disease was collected from an infected colony of Montastraea cavernosa located at East Turtle Shoals in the Florida Keys, USA (N24’ 43.195’ W80’ 55.163’). A sterile needleless syringe was used to aspirate biomass from the infected coral’s surface. The syringe was capped, floated in ambient seawater, and protected from light during transport to shore. The BBD culture was transferred to Marine BG11 and maintained in the laboratory until infection studies were initiated.

E. pallida stock cultures

Stock cultures of clonal CC7 E. pallida hosting Symbiodinium linucheae were maintained in the laboratory at ambient temperature (~25° C). Anemones were fed twice per week with recently hatched Artemia nauplii with water changes 6-12 hours post feeding. Aposymbiotic E. pallida were obtained from a stock established by the methods detailed in Medrano et al. (2019) including a combination of repeated cold treatments and 3-(3,4_Dichlorophenyl)1-1 dimethylurea (DCMU). Anemones had been maintained as aposymbiotic for more than one year at the time the current study was initiated. Stock cultures of E. pallida hosting Durusdinium trenchii or Breviolum minutum, were obtained from aposymbiotic stocks previously exposed to free living cultures of D. trenchii and B. minutum respectively as described by (Medrano et al. 2019). These stocks had also been maintained for more than one year prior to the initiation of this study.

Challenge of E. pallida with BBD

A total of 5 BBD challenge experiments were conducted on different dates, including two that examined the effect of temperature, two that examined the effect of
symbiosis, and one that examined the effect of symbiosis and symbiont type on the susceptibility of *E. pallida* to BBD (Table 1). For each experiment, single individual anemones were obtained from stock cultures maintained in the laboratory and transferred to individual wells in a 6-well cell culture plate. For experiment 1 (Table 1) examining the effect of temperature, anemones were acclimated for 72 hrs. at 25°, 27°, and 32° C prior to inoculation. For the subsequent temperature-related experiment, as well as all other experiments, anemones were transferred to individual wells and allowed to acclimate at ambient room temperature (25° C) for ~ 3hrs prior to inoculation and placement at appropriate temperatures for a particular study. The two temperature-related experiments were carried out at ambient lab temperature (25° C), and in water bath-style incubators maintained at 27° C and 32° C. All other experiments were carried out at 27° C in a water bath-style incubator. For each experiment, an amount of uninoculated control anemones equal to the number of experimental (inoculated) anemones were used per treatment. Experimental anemones were inoculated with equal amounts of BBD biomass (~4mm in diameter). Experimental and control anemones were observed and photographed daily for 10 days post-infection, noting behavior and mortality.

*Statistical Analysis*

The chi-square test for association or Fisher’s exact test was conducted to determine if a relationship exists between BBD infection and mortality for each experiment. Survival distributions for each experiment were generated using the Kaplan-Meier method (Kaplan and Meier 1958) and compared with the log rank test. Pairwise comparisons were made with Bonferroni correction if a statistically significant difference
was detected when more than two groups were compared. Statistical analyses were run using SPSS version 23.0 statistical software (IBM Corp. Armonk, NY).

**Results**

A total of 174 anemones were utilized across 5 experiments examining the susceptibility of *E. pallida* to BBD. Of those, half (87) were challenged with BBD while the other 87 served as controls. Mortality was observed in 99% of the BBD-challenged anemones within the 10-day post-infection observation period. A single BBD-challenged anemone survived up to 17 days post-infection. All control anemones survived and remained visually healthy beyond the duration of the experiments. The specific effects of temperature, symbiotic state and symbiont types are presented below.

*Effect of Temperature on the anemone susceptibility to BBD*

In the first temperature experiment, mortality was first observed in the 27° treatment at 4 days post-infection. At the end of the 10 day observation period, 100% mortality was observed in both the 25° and 32° BBD-challenged groups. A single anemone in the 27° group remained alive 17 days post-infection. A statistically significant association was detected between infection with BBD and mortality (Fisher exact Test, N=18, p < 0.001). To determine if temperature had an effect on mortality of BBD-infected anemones, Kaplan-Meier survival distributions were generated (Figure 1) and tested by log rank, revealing no statistically significant difference in mortality ($X^2 = 1.441, df = 2, p = 0.487$).

In the second temperature experiment, mortality was first observed at 48hrs in the 32° treatment and after 6 days, 100% mortality of infected anemones was observed across all temperatures while control anemones remained alive and visually healthy. Chi square
test for association revealed a statistically significant association between infection status and mortality ($X^2 = 36, \text{df} = 1, p < 0.001$). This association was strong ($\Phi = 1.00, p < .001$). Kaplan-Meier survival distributions were generated (Figure 2), followed by a log rank test revealing no significant difference in survivability across temperatures ($X^2 = 5.866, \text{df} = 2, p = 0.053$). No further pairwise comparisons were warranted.

**Effect of Symbiotic State susceptibility of *E. pallida* to BBD**

In the first of two experiments comparing symbiotic anemones to aposymbiotic anemones, a significant association between BBD infection and mortality of anemones was detected ($X^2 = 24, \text{df} = 1, \Phi = 1, p < 0.001$). Kaplan-Meier survival analyses showed no significant difference between mortality of BBD-challenged aposymbiotic anemones and BBD-challenged symbiotic anemones hosting *S. linucheae* ($X^2 = 4.767, \text{df} = 1, p = 0.029$). Tentacle retraction was observed in a single BBD-challenged aposymbiotic anemone within 24 hrs. By 48 hrs., all BBD-challenged anemones regardless of symbiotic state exhibited tentacle retraction. However, detachment and acontia ejection were only observed in the symbiotic BBD-challenged group. Mortality was first observed at 72 hrs. in the symbiotic BBD-challenged group. After 6 days post-infection, 100% mortality was observed across all BBD-challenged anemones (Figure 3).

In the second experiment comparing symbiotic to aposymbiotic anemones, a significant association of BBD and mortality of anemones was also detected ($X^2 = 48, \text{df} = 1, \Phi = 1, p < 0.001$, Figure 4a). Moreover, Kaplan-Meier survival analyses detected a significant difference between mortality of BBD-challenged aposymbiotic anemones and BBD-challenged symbiotic anemones hosting *S. linucheae* ($X^2 = 20.277, \text{df} = 1, p <0.001$, Figure 4b). By 24 hrs., tentacle retraction was observed in both BBD-challenged
groups as well as one aposymbiotic control. By 48 hrs., locomotion was observed in both challenge groups. Mortality was first observed 4 days post-infection in the BBD-challenged aposymbiotic group. By day 10, 100% mortality was observed across all BBD-challenged anemones.

*Effect of symbiotic type on the anemone susceptibility to BBD*

In the experiment comparing symbiont types hosted, a significant association between BBD and mortality of anemones was detected ($X^2 = 48$, df = 1, $\Phi = 1$, $p < 0.001$, Figure 5a). Moreover, Kaplan-Meier survival analyses showed no significant differences in mortality of BBD-challenged anemones across symbionts hosted. ($X^2 = 4.438$, df = 3, $p = 0.218$, Figure 5b). No further pairwise testing was warranted. Mortality was first observed at 48 hrs. post-infection in all BBD-challenged groups. By 72 hrs. post-infection, 100% mortality was observed in BBD-challenged anemones hosting *B. minutum* and *S. linucheae* as well as aposymbiotic anemones. A single anemone hosting *D. trenchii* survived to day 4 post-infection.

**Discussion**

*Behavioral response supports susceptibility of E. pallida to BBD*

Behaviors consistent with defense including detachment from substrate, locomotion, tentacle retraction, darkening of tissue, and ejection of acontia (Edmunds et al. 1976) were observed in anemones challenged with BBD. Tentacle retraction was the most documented behavior in response to BBD-challenge. Tentacle retraction and darkening of tissue has been observed in previous bacterial challenges (Krediet et al. 2014; Zaragoza et al. 2014). Darkening of tissue is suggestive of melanin production, a well-known innate immune response in invertebrates including corals (Palmer and
Acontia ejection and retraction were not specifically examined during this study. However, the presence of completely ejected acontia in the wells of BBD-challenged anemones from each experiment suggests that complete ejection as well as ejection followed by retraction was utilized for defense. This is in contrast to a previous study by Lam et al. (2017), where *E. pallida* was repeatedly probed with a plastic dropper to induce acontia ejection followed by periods of rest during which acontia were observed to retract. Perhaps this difference is caused, in part, by the nature of the disease challenge, whereby no periods of rest occur. Once a well containing a single anemone is inoculated, the cyanobacteria and associated microbes presumably continue their advance. This is supported by the observation of cyanobacterial filaments spreading across the surface of the liquid as well as the plastic surface of the well.

One key difference is that coral polyps are non-mobile (i.e., fixed to substrate) whereas *E. pallida* polyps have the ability to locomote and move away from infectious agents. Although no controlled taxis experiments were conducted, negative taxis was observed in several anemones in response to the advance of cyanobacterial filaments. This could be observed most prominently by red circles left behind by phycoerythrin-rich *Roseofilum reptotaenium* after an anemone (whose pedal disc was previously surrounded by cyanobacterial filaments) moved (Figure 6). This may indicate that anemones are responding negatively to physical contact by the filaments or a chemical (produced by the cyanobacteria and/or associated organisms) such as microcystin or sulfide.
Acclimation at elevated temperatures may provide temporary protection of E. pallida from BBD

Exaiptasia pallida demonstrated susceptibility to BBD at all three temperatures tested (25°, 27°, and 32° C) while controls remained alive and apparently healthy. This suggests that BBD can be studied using E. pallida across a wide range of temperatures in the laboratory. Interestingly, when allowed to acclimate for 72 hrs. prior to inoculation, the 32° treatment had the longest post-infection time to mortality as compared to the 25° and 27° C treatments. This seems counter-intuitive as BBD is thought to be more virulent at higher temperatures (Boyett et al. 2007). One explanation is that heat shock proteins may have been upregulated during the initial acclimation period that provided a protective effect prior to inoculation with BBD. Induction of heat shock proteins in E. pallida has been shown at elevated temperatures ranging from 29 – 35° C (Black et al. 1995). Expression levels of a heat shock protein of the same size class (hsp70) in Acropora millepora increased in response to microbial challenge with Vibrio coraliilyticus and Alteromonas sp., suggesting the possible involvement of this heat shock protein in the immune response and stress response of corals (Brown et al. 2013). Similarly, heat shock protein 70 was the most highly up-regulated protein identified in a study challenging E. pallida with V. coraliilyticus (Brown and Rodriguez-Lanetty 2015).

In the second temperature experiment, where no temperature acclimatization occurred prior to BBD inoculation, the synergistic effect of temperature stress combined with microbial challenge may have outpaced any benefit conferred by increased heat shock protein production. Although BBD is typically associated with water temperatures greater than 27.5° C, it has been found to occur at lower temperatures such as 26.0° C
and has been observed in the field at temperatures as low as 20° C (Kuta and Richardson 1996). One study, examining the susceptibility of *Montastraea faveolata* to BBD, found that temperature did not influence the initiation of the disease when coral fragments were exposed to BBD at temperatures ranging from 23 to 29.5° C (Aeby and Santavy 2006). Optimal photosynthetic capability for BBD-associated cyanobacteria has been observed at or above 30° C with moderate photosynthetic capability observed at 25° and 28° C (Kuta and Richardson 1996; Richardson and Kuta 2003). Elevated temperatures have been shown to enhance production of quorum sensing molecules by vibrios associated with BBD (Bhedi et al. 2017).

*The presence of symbionts may confer resistance of E. pallida to BBD*

While both symbiotic and aposymbiotic *E. pallida* were susceptible to BBD, we detected a significant difference in survivability between aposymbiotic and symbiotic *E. pallida* (hosting *S. linucheae*) indicating that the algal symbiont may confer some degree of resistance to the anemone. This suggests that BBD infection can be studied under both symbiotic and aposymbiotic conditions in the laboratory. The benefit of studying BBD in the context of an aposymbiotic anemone is two-fold. First, it allows experiments examining the role of bleaching in susceptibility to BBD to be conducted. This is important as there is conflicting evidence regarding bleaching and disease susceptibility in corals. Merselis et al. (2018) proposes that bleaching may provide the coral with temporary resistance to disease while Muller et al. (2018) suggests that bleaching results in the loss of disease resistance in corals. Second, unlike corals, *E. pallida* can be maintained in the aposymbiotic state for extended periods of time allowing for studies.
that focus on the possible role of the symbiont hosted in disease susceptibility. Presumably, the long-term survival of aposymbiotic anemones suggests that the stress of bleaching is transient in this organism unlike in corals where prolonged bleaching leads to death. The cycling of nutrients has been shown to differ between aposymbiotic and symbiotic anemones (Rädecker et al. 2018).

Curiously, while a significant difference in susceptibility to BBD was detected between aposymbiotic and symbiotic anemones hosting *S. linucheae* in the symbiotic state experiments, no significant difference in BBD susceptibility was detected between aposymbiotic and symbiotic anemones hosting *S. linucheae* during the symbiont type experiment. One interesting difference to note between the two types of experiments is the number of days to mortality. In the symbiotic state experiments (Table 1, Experiments 3 and 4), mortality was first observed at days 4 and 6 post-infection for aposymbiotic and symbiotic anemones respectively while mortality was first observed at day 2 post-infection for both aposymbiotic and symbiotic anemones in the symbiont type experiment (Table 1, Experiment 5). By day 3 post-infection 100% of the aposymbiotic and symbiotic anemones hosting *S. linucheae* were dead while 100% mortality of aposymbiotic anemones and symbiotic anemones was not observed until days 7 and 10 respectively during the symbiotic state experiment. One possible explanation for this difference in mortality is that the virulence of BBD may fluctuate. This could be the results of successional changes within the band as well as sulfur cycling resulting in production of microcystin and sulfide. Successional changes have been observed in cyanobacterial patches that appear to be precursors to BBD infection in corals on the Great Barrier Reef (Sato et al. 2010). Changes including a shift in the dominant
cyanobacteria as well as an increase in sulfide-reducing bacteria and decrease in sulfide-oxidizing bacteria have been observed and correlated with an increase in virulence (Sato et al. 2010; Bourne et al. 2011; Bourne et al. 2013). The development of an anoxic microenvironment that is sulfide-rich has also been linked to virulence (Glas et al. 2012; Sato et al. 2017). The longer time to mortality suggests that BBD may have been less virulent during the symbiotic state experiment and that perhaps there is a threshold at which the advantage conferred by the photosymbiont is overwhelmed by virulence of the disease, resulting in the lack of significant difference in survivability between aposymbiotic and symbiotic anemones. Future studies should monitor indicators of virulence over time, including changes to the population of sulfide-oxidizing and sulfate-reducing bacteria as well as levels of oxygen and sulfide. These could be measured at the start of a challenge experiment and followed at intervals throughout the infection process.

Effect of symbiont type hosted on susceptibility of E. pallida to BBD

Exaiptasia pallida was susceptible to BBD across all symbiont types hosted. E. pallida hosting D. trenchii had the longest post-infection time to mortality while anemones hosting S. linucheae and B. minutum were the first to experience mortality. It is possible that the presence of D. trenchii provided some temporary resistance to disease for the anemone. One study examining the potential effect of symbiont type on the health and disease of scleractinian corals found that members of the genus Breviolum (ITS-2 types B1 and B5a) were among the most commonly detected symbiont types in diseased corals while D. trenchii (ITS-2 type D1a), found in a small fraction of diseased corals, may provide disease resistance to its host (Correa et al. 2009). Additionally, colonies of Colpophyllia natans and Diploria strigosa, infected with BBD, hosted B. minutum (ITS-2
type B1) and all BBD-infected colonies that were sampled lacked *D. trenchii* (Correa et al. 2009). This further supports the idea that *D. trenchii* may provide some protection to its host specifically from BBD. *Exaiptasia pallida* hosting *B. minutum* experienced mortality at a faster rate than the other groups with 67% mortality by 48 hrs. Anemones hosting *B. minutum* or *D. trenchii* had similar photosynthetic rates (Gabay et al. 2018).

**Benefits of using *E. pallida* as a model system for studying BBD**

A working model system for studying polymicrobial coral disease, especially one dominated by a cyanobacterium, may become increasingly important as coral reefs worldwide face climate change and the potential emerging diseases that will accompany it. Seasonal variability of BBD makes sample collection difficult during cool months. In warm months, when BBD is more prevalent on reefs, hurricanes are expected to increase in number and intensity with continued warming of sea surface waters, making sample collection equally difficult.

Advantages of using *E. pallida* as a model system include their small size, fast growth rate, hardiness and availability, making them easier to maintain than corals in the laboratory (Weis et al. 2008). Collection of corals often requires special permits, SCUBA certification, tools for fragmenting, and expensive lighting. Anemones can be maintained in small bowls or aquaria with simple lighting. Additionally, clonal populations can be maintained to provide genetic uniformity. The availability of *E. pallida* from sources such as Carolina Biological make them a widely available and affordable model organism that can easily be propagated from a single stock culture. Utilizing *E. pallida* to study a disease such as BBD allows us to examine onset and mortality at the individual polyp level whereas most studies are focused on band establishment and its movement across a
colony in the field or a fragment in the lab. It will also facilitate the standardization of
BBD challenge experiments including acclimation time, amount of BBD inoculum, as
well as other experimental parameters.

**Conclusion**

My results indicate that *E. pallida* is a strong candidate model system for studying
BBD in the laboratory. Support for this includes susceptibility of *E. pallida* to BBD
across a range of temperatures, symbiotic states, and symbionts hosted as evidenced both
by defensive behavioral responses, and ultimately, mortality. Our data further suggest
that this susceptibility may be influenced by symbiotic state and that fluctuation in
virulence of BBD over time may modulate this susceptibility.
Table 3-1 Summary of Black Band Disease challenge experiments with *E. pallida*

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Sample Size</th>
<th>Acclimation Temp. (°C)</th>
<th>Acclimation Time (hours)</th>
<th>Exp. Temp. (°C)</th>
<th>Variable Tested</th>
<th>Symbiont Type(s) Hosted by <em>E. pallida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>25, 27, and 32</td>
<td>72</td>
<td>25, 27, and 32</td>
<td>Temperature</td>
<td><em>S. linucheae</em></td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>25</td>
<td>3</td>
<td>25, 27, and 32</td>
<td>Temperature</td>
<td><em>S. linucheae</em></td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>25</td>
<td>3</td>
<td>27</td>
<td>Symbiont Presence</td>
<td><em>S. linucheae, aposymbiotic</em></td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>25</td>
<td>3</td>
<td>27</td>
<td>Symbiont Presence</td>
<td><em>S. linucheae, aposymbiotic</em></td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>25</td>
<td>3</td>
<td>27</td>
<td>Symbiont Type</td>
<td><em>S. linucheae, B. minutum, D. trenchii, aposymbiotic</em></td>
</tr>
</tbody>
</table>
Fig. 3-1 Susceptibility of *Exaiptasia pallida* to Black Band Disease (BBD) of corals across three temperatures (25°, 27°, and 32°) with 72hr. acclimation. A) Survival of control and BBD-challenged anemones across three temperatures (25°, 27°, and 32°). N = 3 per group. B) Kaplan-Meier analysis of survivability in anemones challenged with BBD across three temperatures (25°, 27°, and 32°). N=3 per temperature ($X^2 = 1.441, df = 2, p = 0.487$).
Fig. 3-2 Susceptibility of *Exaiptasia pallida* to Black Band Disease (BBD) of corals across three temperatures (25°, 27°, and 32°) without acclimation. A) Survival of control and BBD-challenged anemones across three temperatures (25°, 27°, and 32°). N = 6 per group. B) Kaplan-Meier analysis of survivability in anemones challenged with BBD across three temperatures (25°, 27°, and 32°). N=6 per temperature ($X^2 = 5.866$, df = 2, p = 0.053)
Fig. 3-3 Susceptibility of *Exaiptasia pallida* to Black Band Disease of corals across symbiotic state. A) Survival of control and BBD-challenged anemones across symbiotic state (aposymbiotic and symbiotic hosting *S. linucheae*) at 27°C. N = 6 per group. B) Kaplan-Meier analysis of survivability in anemones challenged with BBD across symbiotic state (aposymbiotic and symbiotic hosting *S. linucheae*). N=6 per treatment ($X^2 = 4.767$, df = 1, $p = 0.029$).
A. Survival of control and BBD-challenged anemones across symbiotic state (aposymbiotic and symbiotic hosting *S. linucheae*) at 27°C. N = 12 per group.

B. Kaplan-Meier analysis of survivability in anemones challenged with BBD across symbiotic state (aposymbiotic and symbiotic hosting *S. linucheae*). N=12 per treatment ($X^2 = 20.277$, df = 1, p < 0.001).

**Fig. 3-4** A) Survival of control and BBD-challenged anemones across symbiotic state (aposymbiotic and symbiotic hosting *S. linucheae*) at 27°C. N = 12 per group. B) Kaplan-Meier analysis of survivability in anemones challenged with BBD across symbiotic state (aposymbiotic and symbiotic hosting *S. linucheae*). N=12 per treatment ($X^2 = 20.277$, df = 1, p < 0.001).
A. Survival of control and BBD-challenged aposymbiotic and symbiotic anemones hosting different members of Symbiodiniaceae (S. linucheae, B. minutum, D. trenchii) at 27° C. N = 6 per group.

B. Kaplan-Meier analysis of survivability in anemones challenged with BBD across aposymbiotic and hosted symbiont types (S. linucheae, B. minutum, D. trenchii). N=6 per group ($\chi^2 = 4.438$, df = 3, p = 0.218).

**Fig. 3-5** A) Survival of control and BBD-challenged aposymbiotic and symbiotic anemones hosting different members of Symbiodiniaceae (S. linucheae, B. minutum, D. trenchii) at 27° C. N = 6 per group. B) Kaplan-Meier analysis of survivability in anemones challenged with BBD across aposymbiotic and hosted symbiont types (S. linucheae, B. minutum, D. trenchii). N=6 per group ($\chi^2 = 4.438$, df = 3, p = 0.218).
Fig. 3-6 Infection of *Exaiptasia pallida* with Black Band Disease at 27° C. Representative control and BBD-infected anemones are shown at days 1-5 post-infection.

Fig. 3-7 Interaction of Black Band Disease microbial mat with *Exaiptasia pallida*
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Chapter 4: Dynamic alteration of host and pathogen microbiomes during Black Band Disease challenge of the tropical sea anemone *Exaiptasia pallida*

Abstract

The microbiome has been investigated across a broad spectrum of organisms from honeybees to humans. Corals are known to have a particularly diverse microbiome including bacteria, fungi, archaea, viruses, and single-celled eukaryotes. It is thought that the microbiome plays a major role in maintaining health. Changes, in particular to the bacterial community, may contribute to disease susceptibility. Black Band Disease (BBD), a polymicrobial, mat-forming disease capable of quickly devastating entire coral colonies, is unique in that it has an established microbiome of its own including the dominant cyanobacterium *Roseofilum reptotaenium*, sulfate reducing bacteria, and a diverse group of heterotrophic bacteria. While BBD has been studied extensively since its discovery more than thirty-five years ago and much has been learned about the roles of individual organisms or groups of organisms and their effects on coral tissue, little is known about the interaction of BBD with the host microbiome and how modification of the host microbiome by pathogens may contribute to disease progression. This study used the model Cnidarian system *Exaiptasia pallida* to examine the alterations of host and pathogen microbiomes during a BBD challenge. Sequence comparison of the V3-V4 hypervariable regions of the 16S rRNA gene revealed distinct bacterial communities between BBD and *E. pallida* microbiomes at the outset of the experiment. Bidirectional effects were observed between the host and pathogen microbiomes. While the microbial communities of *E. pallida* and BBD control samples (unchallenged) changed little over time, when exposed to BBD, the microbiome of *E. pallida* experienced significant
changes. Resulting shifts in the microbial community of the host in response to bacterial challenge provide insight into specific taxa that may drive dysbiosis, leading to altered health states, and increased susceptibility to disease.

**Introduction**

Microbiome refers to the collection of microorganisms associated with a specific environment. The role that the microbiome plays appears to be far reaching with implications ranging from influencing truffle aroma (Vahdatzadeh et al. 2015), to enhancing plant growth (Eltlbany et al. 2019), to maintaining health (Zarco et al. 2012; Thomas et al. 2017) to prompting disease (Ordovas et al. 2006; Yeoh et al. 2013; Bhatt et al. 2017). The microbiome has been explored in many organisms ranging from honeybees (Hamdi et al. 2011) to humans (Turnbaugh et al. 2007). Microbiome exploration in humans has been divided further into microbiomes specific to different parts of the body including gut (Zhu et al. 2010), hand (Hospodsky et al. 2014), reproductive tract (Franasiak et al. 2015), and nasal (Koskinen et al. 2018) among others. Understanding how shifts in these microbiomes lead to dysbiosis and ultimately changes in the health of an organism is a major area of ongoing research (Lloyd et al. 2018; Saltzman et al. 2018; Xu et al. 2018).

The holobiont includes the coral animal and all of its associated microbiota including bacteria, fungi, archaea, viruses, and single-celled eukaryotes including the photosymbiont members of Symbiodiniaceae (Rohwer et al. 2002; LaJeunesse and Thornhill 2011). These associates compose the microbiome of corals (van Oppen and Blackall 2019). The microbiomes of corals have also been investigated extensively (Bourne et al. 2005; Wegley et al. 2007), including their Symbiodiniaceae component
Microbiomes have been shown to be distinct between coral tissue and mucus (Chiu et al. 2012; Apprill et al. 2016). Welsh et al. (2017), showed that coral microbiomes change in response to bacterial challenge. Additionally, changes in the coral microbiome have been shown in response to environmental stressors such as thermal stress (Pootakham et al. 2018).

*Exaiptasia pallida* is a tropical sea anemone that is well established as a model system for studying cnidarians (Weis et al. 2008; Voolstra 2013; Radecker et al. 2018). *E. pallida* has been successfully infected with multiple coral pathogens (Krediet et al. 2014; Zaragosa et al. 2014; Brown et al. 2015; Divya et al. 2018) including BBD (Chpt. 3). The microbiome of *E. pallida* has been investigated (Roethig et al. 2016) and recently characterized by a global study across populations of both wild and lab-reared anemones (Brown et al. 2017). The microbiome of *E. pallida* has also been shown to change in response to thermal stress (Ahmed et al. 2019).

Black Band Disease (BBD) is composed of a mat-forming, polymicrobial consortium (Carlton and Richardson 1995) which affects several species of reef building corals globally (Sutherland et al. 2004). The microbiome of BBD has been well studied (Cooney et al. 2002; Voss et al. 2007; Sekar et al. 2008; Sato et al. 2017) and is dominated by the filamentous cyanobacterium *Roseofilum reptotaenium* (Rutzler et al. 1983; Taylor 1983; Casamatta et al. 2012) which produces microcystin (Myers et al. 2007; Gantar et al. 2009), a toxin responsible for coral tissue lysis (Richardson et al.
Sulfate-reducing bacteria such as *Dsulfovibrio sp.* are known to be an important group within the BBD microbiome, most notably for their production of sulfide which has been shown to work in synergy with microcystin to degrade coral tissue (Richardson et al. 2009). Additionally, they are secondary and required pathogens for successful BBD infection in the laboratory (Brownell and Richardson 2014) and have been noted to increase during the transition from cyanobacterial patches to BBD infection on the Great Barrier Reef (Bourne et al. 2011).

A diverse group of heterotrophic bacteria have also been linked to BBD (Cooney et al. 2002; Sekar et al. 2006; Miller et al. 2009; Meyer et al. 2017). Vibrios, in particular, have been suggested as an important group of heterotrophs in the BBD microbiome (Arotsker et al. 2009) and most are considered opportunistic pathogens (Munn et al. 2015).

Microbiomes shift in response to environmental changes. Thermal stress and bacterial challenge are two environmental changes that have been shown specifically to cause shifts in coral microbiomes. Understanding how these shifts drive dysbiosis, ultimately affecting host health, is an important component in understanding disease etiology. Studying shifts in the microbiome at the onset of disease and through its progression may provide insight into the drivers of dysbiosis and further elucidate microbial interactions between host and pathogen.

This study examined the bacterial component of the microbiomes of both BBD and *E. pallida* through sampling of DNA and subsequent sequencing of the V3-V4 hypervariable regions of the 16S rRNA gene over the course of 8 days during a disease challenge to determine the composition of the *E. pallida* and BBD microbiomes, if the
microbiome of one is modified in the presence of the other, and how that modification may contribute to onset and persistence of the disease.

Materials and methods

Collection and culture of Black Band Disease microbial mat

A single sample of BBD was collected in June of 2017 from a Montastraea cavernosa coral head (Figure 1) located at East Turtle Shoals in the Florida Keys, USA (N24° 43.195’ W80° 55.163’). The BBD microbial mat was aspirated from the surface of the coral using a sterile 10 mL needleless syringe under SCUBA, capped, floated in ambient seawater, and protected from light during transport to the lab. The BBD sample was transferred to and maintained in marine BG11 media in the laboratory until August of 2018 when the disease challenge was initiated.

Culture and maintenance of Exaiptasia pallida sea anemones

Stock cultures of clonal CC7 E. pallida (hosting Symbiodinium linucheae microalgae) were maintained in the laboratory at ambient temperature (~25° C). Anemones were fed twice per week with recently hatched Artemia nauplii. Anemones were periodically cut in half with a sterile razor blade to stimulate asexual growth and thus maintain the stock population and animal size required for experimentation.

Experimental design of Black Band Disease challenge on sea anemones

To determine the effect of the interaction of host and microbial disease mat on the microbiomes of both host and pathogen consortia, we set up experimental and control groups as follows. Individual anemones were removed from stock cultures and transferred to six well plates. Control anemones remained in wells without the introduction of BBD biomass. Experimental anemones were inoculated by placing equal
amounts of BBD biomass (~ 4mm in diameter) into each well. The BBD biomass introduced to the experimental anemones served also as the experimental BBD samples. Control BBD samples (~ 4mm in diameter) were placed in six well plates in the absence of anemones. The disease challenge was carried out in a water bath-style incubator maintained at 27° C.

*Time-course sampling and collection processing*

*E. pallida* and BBD samples were collected in triplicate from each treatment group (control and experimental) at the following times during the experiment: 0hrs, 48 hrs. (2 days), 96 hrs. (4 days), and 192 hrs. (8 days). Whole anemones were removed with sterile forceps, flash frozen in liquid nitrogen, and stored at -80° C. Samples of BBD were collected using a sterile transfer pipet, flash frozen in liquid nitrogen and stored at -80° C.

*Total DNA extraction for subsequent bacterial DNA amplification*

Whole genomic DNA was extracted from anemone and BBD samples using the FastDNA™SPIN Kit for Soil (MP Biomedicals) according to the manufacturer’s instructions. Due to low quantity, anemone DNA was precipitated prior to PCR by adding 12 µl NH₄OAc and 10 µl of 4 µg/µl glycogen to 98 µl of DNA template. 250 µl of 100% EtOH was added and samples were allowed to precipitate at -20° C overnight. Samples were then centrifuged (~ 20,000 rcf) for 30 min. at 4° C, washed twice with ice cold 70% EtOH, followed each time by 10 min of centrifuging at 4° C and removal of supernatant, allowed to air dry for 10 min, and diluted in DES.
Bacterial 16S rDNA amplification and Illumina MiSeq sequencing

The V3-V4 region of the 16S rRNA gene was amplified using 341F and 805R primers with Illumina overhang adapters added. Each reaction was composed of 12.5 µL 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 5 µL forward primer, 5 µL reverse primer, and 2.5 µL of template DNA for a total reaction volume of 25 µL. PCR conditions were as follows: 1 cycle of 95° for 3 min., followed by 30 cycles of 95° for 30 sec., 55° for 30 sec., and 72° for 30 sec. A final elongation step was performed at 72° for 5 min. Products were electrophoresed on a 1% agarose gel to confirm product size and estimate quantity. The presence of an off-target amplicon in some of the anemone samples prompted gel extraction of all anemone samples. Gel extractions were carried out using the Monarch® DNA Gel Extraction Kit (New England BioLabs) according to manufacturer’s instructions.

PCR products were cleaned up using AMPure XP Beads (New England Biolabs). Samples were indexed in 50 µL reaction volumes including 5 µL of DNA, 5 µL of Nextera XT Index Primer 1 (N7xx), 5 µL of Nextera XT Index Primer 2 (S5xx), 25 µL of 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), and 10 µL PCR grade water. PCR conditions were as follows: 1 cycle of 95° for 3 min., followed by 10 cycles of 95° for 30 sec., 55° for 30 sec., and 72° for 30 sec. A final elongation step was performed at 72° for 5 min. A second clean up with AMPure XP Beads (New England Biolabs) was performed.

Resulting libraries were quantified using the Qubit 3 fluorometer (Invitrogen) with the Qubit dsDNA HS Assay Kit (Invitrogen). Samples were then normalized and pooled. A 15% PhiX control was spiked-in and the resulting mixture denatured using
0.2N NaOH. The pooled sample was loaded onto a MiSeq v3 reagent cartridge (Illumina) and 2x300 paired-end sequencing was run on a MiSeq FGx sequencer (Illumina) at the Florida International University Forensic DNA Profiling Facility.

**Microbiome statistical analysis**

Forward and reverse read quality of the resulting FASTQ files were assessed using Q values. Based on poor quality of the reverse reads, only forward reads were used for subsequent analysis with Quantitative Insights into Microbial Ecology 2 (QIIME2) 2018.2 (Bolyen et al. 2018). Sequences were denoised, trimmed, and quality filtered (including removal of phiX and chimeric sequences) with DADA2 (Callahan et al. 2016). A cutoff of Q20 was used to determine trimming of the first 14 bases and a truncation value of 270 bp. A feature table was then generated from resulting data.

A multiple sequence alignment was generated using mafft (Katoh et al. 2002) and fasttree2 (Price et al. 2010). Samples were then rarefied using a sampling depth of 15,192. Rarefaction eliminated one replicate sample of the anemone control at 192 hours post infection from downstream analysis. Alpha diversity metrics were generated including Shannon’s diversity index, observed OTUs, Faith’s Phylogenetic Diversity and Pielou’s Evenness. Beta diversity was calculated using weighted UniFrac distance.

Taxonomy was assigned to sequences using a pre-trained Naïve Bayes classifier trained to the V3-V4 region of the 16S rRNA gene using the Greengenes_99 database v. 13.8.

Differential abundance testing was conducted by Analysis of composition of microbiomes (ANCOM) (Mandal et al. 2015). Analyses were conducted to compare all anemone and BBD samples; anemone and BBD baseline samples; anemone and BBD
192 hours post infection samples; anemone baseline, 48 hrs., and 192hrs post infection experimental samples; and BBD baseline, 48 hrs., and 192 hrs. post infection experimental samples.

**Results**

*Overview of 16S rRNA sequence output*

A total of 9,726,793 sequences were generated from 21 anemone and 21 BBD samples. After denoising and quality filtering, including the removal of phiX reads and chimeric sequences, 6,315,949 sequences remained. A minimum of 6,015; maximum of 363,097; and median of 130,706 sequences remained per sample. A total of 4,216 unique features were detected. Rarefaction at a sampling depth of 15,192 resulted in the removal of one replicate sample of anemone control 192 hours post infection (AC_192). Rarefaction curves suggest that sampling at this depth was sufficient to capture diversity of all samples (Fig. 2).

*Alpha and Beta Diversity*

Differences in alpha diversity, including species richness and species evenness, were observed between sample types and between timepoints. A significant difference in species richness ($p = 1.37 \times 10^{-7}$) was observed between sample types of anemone and BBD (Fig. 3A). Species richness was higher in the anemone samples than in the BBD samples. While all anemone samples had greater phylogenetic diversity than those of BBD, the greatest phylogenetic diversity was observed in the experimental anemone samples. Additionally, the variability between anemone samples was greater than that of BBD samples. A significant difference in species richness ($p = 5.96 \times 10^{-4}$) was also observed across all timepoints (Fig. 4). Species richness increased in both experimental anemone
and experimental BBD samples over the 8-day challenge. The highest level of richness was observed in the experimental anemone sample at 192 hours post-infection and the lowest level was observed in the baseline BBD samples at 0 hours. Pairwise comparisons revealed significant differences in species richness between baseline anemone samples and experimental anemone samples at 48 hrs. (p = 0.049) and 192 hrs. (p = 0.049) post infection. Significant differences were also observed between baseline BBD samples and experimental BBD samples at 48 hrs. (p = 0.049), 96 hrs. (p = 0.049), and 192 hrs. (p = 0.049) post infection. Although no significant difference was observed (p = 0.116) for species evenness by sample type (Fig. 3B), anemone samples, overall, appeared to have a higher level of species evenness than BBD samples. A significant difference in species evenness (p = 0.0148) was observed by timepoint (Fig. 5) with the baseline anemone samples at 0 hours having the highest evenness and the BBD control samples at 48 hours post infection having the lowest evenness. Species evenness increased in the experimental BBD samples while decreasing in the experimental anemone samples. Pairwise comparisons revealed significant differences between baseline anemone samples and experimental anemone samples at 48 hrs. (p = 0.049) and 192 hrs. (p = 0.049) post infection. Significant differences in species evenness were also observed between baseline BBD samples and experimental BBD samples at 48 hrs. (p = 0.049) 96 hrs. (p = 0.049), and 192 hrs. (p = 0.049) post infection.

Differences in beta diversity were observed for both sample types and timepoints. Significant differences in beta diversity were detected between sample types (p = 0.001). Principle coordinate analysis revealed relatively tight clustering of BBD samples as compared to anemone samples (Fig. 6A). A significant difference was also detected
between timepoints (p = 0.001). Relatively tight clustering of the control and experimental BBD samples was observed across all time points (Fig. 5B). Principle coordinate analysis showed tighter clustering of BBD samples as compared to those of *E. pallida*. This tight clustering was apparent not only among the replicates of each time point but across all BBD samples (both experimental and control) from 0 hours through 192 hours post infection. In contrast, the anemone samples displayed a lack of tight clustering across timepoints as well as across replicates of individual timepoints.

Principle coordinate analysis also shows increasing dissimilarity among the experimental samples with the highest dissimilarity among the experimental anemone samples 192 hours post infection. These samples are also highly dissimilar when compared to the anemone baseline samples.

**Major representative taxa**

The dominant phyla for the baseline anemone samples included *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Acidobacteria*, and *Planctomycetes*. While Proteobacteria also dominated the baseline BBD samples, *Cyanobacteria* was the second most abundant phylum followed by *Bacteroidetes* and *Planctomycetes*. Specific taxa within the phylum *Proteobacteria* including *Octadecabacter sp.*, *Idiomarina sp.*, and *Alcanivorax dieselolei* were also identified as being present in the baseline BBD samples but absent from the anemone baseline samples (Table 2). Percent abundance of *Verrucomicrobia* and *Acidobacteria* decreased while *Bacteroidetes* and *Proteobacteria* increased in the experimental anemone samples over time (Fig. 7). In contrast, percent abundance of *Verrucomicrobia*, *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* all increased in the experimental BBD samples (Fig. 7).
Analysis of Composition of Microbiome (ANCOM)

The number of sequence variants differing significantly in abundance between sample types (anemone and BBD) across all timepoints was 23 (Table 1). Taxa included 2 sequences of unidentified BRC1 and one sequence each of unidentified Phycisphaerales, Cyanobacteria, Rhodospirillaceae, Cohaesibacteraceae, Flavobacteriaceae, Oceanospirillales, Alphaproteobacteria, Hyphomicrobiaceae, Opitutae, and ZB2. Additional taxa included two unknown species of Spirulina; one sequence each of unknown species of Octadecabacter, Halomonas, Congregibacter, Idiomarina, Francisella, Pseudomonas and Ralstonia; Alcanivorax deiselolei and Burkholderia tuberum. Notably, unidentified Oceanospirillales, Spirulina sp., and Alcanivorax dieselolei were present in the BBD samples but absent from all anemone samples.

Five sequence variants were significantly different in abundance between baseline anemone and baseline BBD samples (Table 2). Taxa included one unidentified BRC1, one unidentified Cyanobacteria, one unknown species of Octadecabacter, one unknown species of Idiomarina, and Alcanivorax deiselolei, all of which were present in the BBD samples but absent from the anemone samples.

A single sequence variant identified as Alcanivorax dieselolei was significantly different in abundance for BBD samples when comparing baseline and experimental samples at 48 and 192 hours post infection (Table 3). A single sequence variant identified at the family level as Microbacteriaceae was significantly different in abundance for E. pallida samples when comparing baseline and experimental samples at 48 and 192 hours post infection (Table 3).
Several sequence variants differed significantly between experimental anemone and experimental BBD samples 192 hours post infection (Table 4). Taxa included one sequence each of unidentified A4b, Cyanobacteria, Ellin6513, Pirellulaceae, Rhodobacteraceae, Chitinophagaceae, BD7-11, Phycisphaeraceae, and 0319-6G20; two unknown species of Stenotrophomonas, one unknown species each of Salinispora, Ralstonia, Fluvicola, Muricauda, Thalassospira, Oleibacter, and Burkholderia; Thalassospira xiamenensis, Burkholderia bryophila, Lewinella cohaerens, and Burkholderia tuberum.

**Discussion**

This study showed that the interaction of sea anemones (host) and black band disease microbial mat exert bidirectional effects that drive changes in the composition and community structure of the original microbiomes of both host and pathogen consortia. These changes include recruitment of members of the phyla Verrucomicrobia, Proteobacteria, Bacteroidetes, and Planctomycetes from the sea anemone microbiome to that of the BBD mat; significant changes in the abundance of several taxa; and loss of Alcanivorax dieselolei and Microbacteriaceae from the BBD microbiome and anemone microbiome respectively. Moreover, the major changes of bacterial assemblages were detected within the microbiome of *E. pallida* as a result of the interaction with the BBD mats.

The bacterial assemblages of the BBD mat samples were relatively similar throughout the experiment as compared to the anemone samples suggesting the microbiome of the BBD sample is more stable than that of the anemone over time. This is likely, in part, due to the individual samples of BBD originating from a single laboratory
culture. This is in contrast to the anemone samples that, while taken from a single stock culture, were separate individuals with individual microbiomes. Dissimilarity can be observed in the baseline anemone samples, suggesting that the microbiomes associated with individual anemones (even those within the same timepoint and treatment) are highly variable. As a result, the anemone samples were more variable than the BBD samples from the start of the experiment.

The microbiomes of BBD and *E. pallida* are distinctly different in composition. While both are dominated by Proteobacteria, the BBD microbiome contains a notable amount of Cyanobacteria which is nearly absent in the *E. pallida* microbiome. This is expected as the BBD consortium is known to be dominated by Cyanobacteria (Rutzler et al. 1983; Casamatta 2012; Meyer et al. 2016). Further, 5 sequence variants were identified as being significantly different in abundance between baseline anemone and BBD samples including an unidentified cyanobacterium (Table 2). The significant difference in these abundances is owing entirely to the absence of these sequences from the anemone samples. In addition to an unidentified cyanobacterium, a sequence identified as belonging to the phylum *BRC1* and sequences belonging to the genera *Octadecabacter* and *Idiomarina* as well as *Alcanivorax dieselolei* were present in the baseline BBD samples but absent in the baseline anemone samples.

*Modification of the BBD microbiome by E. pallida*

Despite the high level of similarity among BBD samples, several changes to the BBD microbiome were detected in the experimental samples when exposed to *E. pallida* suggesting modification of the BBD microbiome by the anemone host. Increases in the relative abundance of specific taxa within the phyla *Verrucomicrobia, Proteobacteria,*
Bacteroidetes, and Planctomycetes were observed as well as an increase in species richness and a significant increase in species evenness (p = 0.049).

One possible explanation for these changes is that taxa from the anemone host microbiome may be recruited to the BBD consortium. Several sequence variants, belonging to the aforementioned taxonomic groups, were present in control and experimental E. pallida samples but absent from BBD baseline and control samples. These same sequence variants were, however, detected in BBD experimental samples suggesting that exposure to E. pallida was required for their presence in BBD.

E. pallida had notable amounts of Verrucomicrobia as compared to the BBD microbiome. Interestingly, Verrucomicrobia increased in the BBD experimental samples over time but not in the BBD control samples suggesting that perhaps members of this phylum were initially recruited to the BBD consortium within the first 48 hours and then increased in relative abundance at 96 and 192 hours post infection. Verrucomicrobia are a diverse and globally distributed group (Freitas et al. 2012) known for their ability to degrade polysaccharides (Martinez-Garcia et al. 2012). They have been found to be enriched in disease samples including those of BBD (Sekar et al. 2006) as well as diseased tissues of the Red Sea sponge Crella cyathophora (Gao et al. 2015) and have been implicated as indicators of environmental disturbance in riverine microbial communities (Balmonte et al. 2016).

Although a marked increase in Verrucomicrobia was observed in the experimental BBD samples, within this phylum, a single sequence belonging to the genus Persicirhabdus was identified as being present in all anemone samples and BBD experimental samples but absent from BBD baseline and control samples. While this
supports the idea that recruitment is occurring, the increased abundance of this organism alone cannot explain the overall increase in *Verrucomicrobia*. In addition to recruitment, the increased availability of polysaccharides provided by the presence of the host anemone may have contributed to an increase in overall abundance of this phylum.

Within the phylum *Bacteroidetes*, three sequences belonging to the class *BME43*, the family *Flammeovirgaceae*, and the genus *Fluviicola* respectively were identified as being present in all anemone samples but only experimental samples of BBD. Members of this phylum have previously been implicated in the pathogenicity of BBD (Cooney et al. 2002). Members of *BME43* have been found in association with the brown seaweed *Fucus vesiculosus* as part of the normal epibacterial community (Mensch et al. 2016). Members of *Flammeovirgaceae* have been found in association with the cyanobacterium *Geitlerinema*, isolated from a BBD sample (Den Uyl et al. 2016), as well as BBD samples (Klaus et al. 2011). Members of this family have also been found in anemones (Ahmed et al. 2019, Yoon et al. 2012), sea squirts (Yoon et al. 2011), and marine sediment (Yoon et al. 2007).

Within the phylum *Proteobacteria*, six sequences identified taxonomically as belonging to the order *Kiloniellales*, the family *Rickettsiaceae*, the genera *Cohaesibacter*, *Hyphomonas*, and *Nautella*, and *Thalassospira xiamensis* respectively were detected in all anemone samples but only experimental samples of BBD.

Within the phylum *Planctomycetes*, two sequences belonging to the families *Phycisphaeraceae* and *Pirellulaceae* respectively were identified as being present in all anemone samples and BBD experimental samples.
Modification of *E. pallida* microbiome by BBD

Species richness increased significantly in the experimental anemone samples over the course of the BBD challenge experiment with the highest species richness recorded for samples taken 192 hours post infection. Dominant phyla within the bacterial communities of *E. pallida* have been shown to include *Proteobacteria, Bacteroides, Firmicutes*, and *Cyanobacteria.* (Brown et al. 2017). Genera previously found to be common among *E. pallida* include *Vibrio, Nautella, Ruegeria, Marinobacter, Lentisphaera,* and *Flaviobacterium* (Brown et al. 2017).

A particularly interesting finding in the present study is that *Alcanivorax dieselolei* was identified as the only sequence variant differing significantly in abundance across the experimental BBD samples based on ANCOM analysis. This organism was not detected in any of the anemone samples and by 192 hours post infection, *A. dieselolei* could no longer be detected in the experimental BBD samples. The control BBD samples, however, still contained this organism suggesting that something specific to the presence of the anemone caused the decline of *A. dieselolei* in the BBD microbiome.

*A. dieselolei*, initially isolated from oil-contaminated water (Liu and Shao 2005), is an alkane-degrading bacterium that has been found in a variety of habitats including deep sea sediments (Liu and Shao 2005), bilge water (Santisi et al. 2015), sandy beaches (Shin et al. 2019), and in the mucus of *Palythoa caribaeorum* (Campos et al. 2015). It is interesting that this organism was found in association with the coral *Montastreae cavernosa* in the Florida Keys. Although this organism is typically found in association with oil-contaminated waters, Cyanobacteria, which dominate BBD in volume, are capable of producing hydrocarbons from fatty acids (Coates et al. 2014) which could
provide this bacterium with its obligate carbon source. Perhaps a change in cyanobacterial abundance and or metabolism, triggered by the presence of the anemone, contributed to the demise of *A. dieselolei*. The abundance of Cyanobacteria progressively declined in the experimental BBD samples while it remained relatively stable in the 48 hour control samples before declining in the 96 and 192 hour control samples.

A single sequence variant belonging to the family *Microbacteriaceae* differed significantly in abundance across the experimental *E. pallida* samples. By 192 hours post infection, this sequence variant was no longer detected in either the control or experimental anemone samples. However, no significant difference in abundance was detected among the control anemone samples. This was likely due to low levels of detection at 48 and 96 hours post infection in the control anemone samples whereas this sequence variant was not detected in experimental samples by 48 hours post infection. The family *Microbacteriaceae* belongs to the order *Actinomycetales*. Members of this order are known for their production of a diverse range of antimicrobial compounds (Subramani et al. 2019). While the absence of this sequence variant from the control samples did not seem to affect the health of the anemones adversely, its role may be contextual. The presence of this organism in a healthy anemone may help to prevent growth of potentially pathogenic organisms. Its absence, especially in the presence of disease challenge, may make the anemone more vulnerable to infection by potentially pathogenic organisms, and ultimately death.

A particularly puzzling finding was that the dominant cyanobacterium of BBD, *Roseofilum reptotaenium*, was not readily detected by the molecular methods employed in the current study despite being visually confirmed by microscopic observation. The
most abundant cyanobacterium detected was classified as *Spirulina*. A BLAST search using this sequence showed 100% identity to an uncultured bacterium clone Contig14H02 (HM768659) from a previous study of BBD (Janse et al. 2011) and 98% identity to *Spirulina sp.* (KU951804). One possible explanation for the lack of detection is that the pre-trained classifier database (last updated in May of 2013) did not contain a reference for *R. reptotaenium*. At the time of analysis, this was the most recent update. *R. reptotaenium* was characterized in 2012 (Casamatta et al. 2012) and the first sequences published were deposited to NCBI on September 19th 2012, less than a year before the database was updated. Additionally, the first 16S sequences submitted to NCBI under this nomenclature were partial sequences *Roseofilum reptotaenium* 100-1 (HM048872) and *Roseofilum reptotaenium* 101-1 (EU743965) with lengths of 381bp and 225 bp respectively. This assertion is supported by a previous study of BBD that utilized the same Greengenes v. 13.8 database and failed to detect *R. reptotaenium* (Meyer et al. 2016). The presence of *R. reptotaenium* was confirmed through direct comparison of full-length sequences generated by Sanger sequencing to sequences of *R. reptotaenium* in the NCBI database. The most abundant cyanobacterial OTU in the Meyer et al. 2016 study was classified as *Leptolyngbya*. *Leptolyngbya* was not detected in the present study. However, it should be noted that this previous study targeted the V6 region of the 16S rRNA gene whereas the present study targeted the V3-V4 region and the classifier used was trained specifically to the V3-V4 region of the 16S rRNA gene.

Another possible explanation for the absence of *R. reptotaenium* from the MiSeq data is the presence of primer bias. Primer bias has previously been noted in Illumina MiSeq studies (Pinto and Raskin 2012; Kennedy et al. 2014; Elbrecht and Leese 2015;
Schirmer et al. 2015; O’Donnell et al. 2016). Discrepancies between visual confirmation of large amounts of filamentous cyanobacteria and molecular evidence of cyanobacteria in BBD samples have previously been reported (Cooney et al. 2002; Frias-Lopez et al. 2002; Frias-Lopez et al. 2003; Richardson and Kuta 2003; Hadaidi et al. 2018). Future studies of BBD should employ microscopic methods to confirm the presence of cyanobacteria and validation studies should be conducted to determine the best primer set or sets to use when comparing microbiomes containing cyanobacteria.

**Conclusion**

Interactive effects of host and pathogen microbiomes were observed. While the BBD mat microbiome remained relatively stable throughout the 8-day challenge, the *E. pallida* host microbiome was significantly altered. Dysbiosis of the *E. pallida* host microbiome, including the loss of Microbacteriaceae may have increased the vulnerability of the anemones to pathogenic BBD and may be critical for disease development. The loss of *A. dieselolei* from the BBD mat microbiome indicates a potential shift in the metabolism of the BBD consortium, possibly signaling an increase in virulence. It is unclear, however, exactly how these alterations contributed to disease susceptibility of the anemone and pathogenicity of BBD. Future work should examine the metabolic interactions of the organisms identified in this study.
Figure 4-1 BBD-infected *Montastraea cavernosa* at East Turtle Shoals, Florida Keys USA. Photo credit: Cynthia Lewis
Figure 4-2 Rarefaction of BBD and anemone microbiome samples (Sampling Depth = 15,192) across all timepoints. A single replicate of AC_192 was excluded.
Figure 4-3 Comparison of species richness and evenness of anemone and black band disease (BBD) microbiome samples by sample type. A) Comparison of species richness (PD=Phylogenetic Diversity) of anemone and BBD samples by sample type ($p = 1.37 \times 10^{-7}$). B) Comparison of species evenness by Pielou’s Evenness of anemone and BBD samples by sample type ($p = 0.112$)
Figure 4-4 Comparison of species richness across all anemone and black band disease samples by time point (p = 5.96 e^{-4})
Figure 4-5 Comparison of species evenness for anemone and black band disease samples by time point (p = 0.0148)
Figure 4-6 Beta diversity measurements of *E. pallida* and black band disease (BBD) microbiomes. A) Principle Coordinate Analysis of anemone and BBD samples by sample type using Weighted Unifrac (p = 0.001). B) Principle Coordinate Analysis of anemone and BBD samples by time point using Weighted Unifrac (p = 0.001).
**Figure 4-7** Bacterial community composition by Phylum based on 16S rRNA sequence abundance for control and experimental anemone and BBD samples at 0, 48, 96, and 192 hours post infection.
Table 4-1 Analysis of composition of microbiome (ANCOM) results for sequence variants that differ significantly in abundance between anemone and BBD samples across all timepoints. Increasing $W$ values indicate an increase in significance.

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108
Table 4-2 Analysis of composition of microbiome (ANCOM) results for sequence variants that differ significantly in abundance between baseline anemone and BBD samples. Increasing $W$ values indicate an increase in significance.

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Table 4-3 Analysis of composition of microbiome (ANCOM) results for sequence variants that differ significantly in abundance between baseline and experimental BBD samples at 48 and 192 hours post infection and baseline and experimental anemone samples at 48 and 192 hours post infection.

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Table 4-4 Analysis of composition of microbiome (ANCOM) results for sequence variants that differ significantly in abundance between experimental anemone and BBD samples 192 hours post infection. Increasing $W$ values indicate an increase in significance.

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<td>Group</td>
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<td>BBD</td>
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Chapter 5: Conclusions and synthesis

The overarching goal of this dissertation was to explore aspects of microbiome structure, metabolism, and modification in the etiology of black band disease (BBD) of corals and to determine suitability of the sea anemone Exaiptasia pallida as a model system for studying BBD. This was accomplished through metagenomics analysis, bacterial challenges of E. pallida, and high throughput 16S rRNA sequencing by Illumina MiSeq and subsequent bioinformatics analysis.

Chapter two examined the potential role of dimethylsulfoniopropionate (DMSP), an abundant source of carbon and sulfur in the marine environment (Sievert et al. 2007), in BBD development and persistence and presented metagenomic evidence for the role of DMSP in BBD pathogenicity. Genera implicated in DMSP metabolism including Roseovarius, Roseobacter, Silicibacter, Desulfovibrio, Sulfitobacter, and Reugeria (Raina et al. 2009) were shown in metagenomes of both BBD as well as Roseofilum reptotaenium, the dominant cyanobacterial member of BBD and proposed primary pathogen (Cassamatta et al. 2012). Several genes associated with the metabolism of DMSP have recently been discovered (Moran et al. 2012). Of these, three were detected including the demethylase gene dmdA and the lyase genes dddD and dddL. However, lyase genes including dddP, dddQ, dddW, and dddY were not detected. Results also showed a higher abundance of lyase genes as compared to demethylase genes suggesting the cleavage pathway of DMSP may be favored over that of the demethylation pathway.

While the role of DMSP in structuring coral associated communities has been examined previously (Raina et al. 2010; Tout et al. 2015), this is the first study to examine the role of DMSP in structuring a disease community, specifically that of BBD.
The role of DMSP in BBD etiology warrants further investigation. DMSP levels have yet to be measured in BBD. It will be important to measure these levels and monitor if and how these levels change over the course of an infection to confirm and fully understand the role of DMSP in structuring the BBD microbial community. Testing chemotactic responses of BBD consortium members including *R. reptotaenium* to DMSP will contribute to a better understanding of how potentially pathogenic organisms are recruited to the coral surface and/or BBD consortia.

Additional studies that focus on the role of DMSP metabolism in production of BBD toxins should be conducted. Tracing of isotopic sulfur in BBD enriched with labeled DMSP could help elucidate whether DMSP contributes significantly to the production of sulfide. This toxin has been shown to be integral to the initiation of BBD infections (Brownell and Richardson 2014) and to work synergistically with microcystin to lyse coral tissue. The effect of DMSP on microcystin production should also be examined. DMSP enrichment of BBD cultures coupled with enzyme linked immunosorbent assay (ELISA) to measure potential increases in microcystin production should be employed.

Chapter three examined the susceptibility of *E. pallida* to BBD. Susceptibility was indicated by observed behavioral responses in *E. pallida* consistent with defense, including tentacle retraction, locomotion, detachment from substrate, ejection of acontia (Edmunds et al. 1976) and ultimately mortality when exposed to BBD. Susceptibility was shown across a range of temperatures, symbionts hosted, and symbiotic state supporting the potential use of *E. pallida* as a model system for studying BBD in the laboratory.
Chapter four examined the potential modification of microbiomes of *E. pallida* and BBD during a disease challenge. Changes were observed in both BBD and *E. pallida* with regard to species richness. While the BBD microbiome remained relatively stable as compared to *E. pallida*, there is evidence that members of the *E. pallida* microbiome may be recruited to the disease consortium though it is not clear if these organisms specifically contributed to pathogenicity. While significant changes in species richness were observed in *E. pallida*, and the microbiomes grew increasingly dissimilar over the course of the experiment, there is no evidence that members of the BBD consortium were recruited to the *E. pallida* microbiome. The most notable changes included the loss of *Alcanivorax dieselolei* from the BBD microbiome and the loss of Microbacteriaceae from the *E. pallida* microbiome.

The disease challenges presented in both chapters three and four used behavioral responses and ultimately mortality as indicators of disease susceptibility. The underlying mechanisms that elicited defensive behavioral responses and death in the anemones was not specifically investigated. Future studies should address these mechanisms, including potential for microcystin and/or sulfide to play a significant role in behavioral responses and mortality as these are two known virulence factors of BBD that are thought to work synergistically to lyse coral tissue. Metabolomics studies will aid in identifying additional compounds produced by *R. reptotaenium* and/or other members of the BBD consortium that may be impacting the anemone. Additional challenge studies using *R. reptotaenium*, the proposed primary pathogen of BBD which has been used to successfully infect corals in the laboratory (Cassamatta et al. 2012) will further shed light on potential recruitment
of organisms from the host microbiome or environment if studies are run in a seawater flow through environment.

While several advantages to using *E. pallida* as a model system to study corals are well known, including small size, fast growth rate, minimal growth requirements, and availability of clonal populations (Weiss et al. 2008), additional benefits of studying BBD in *E. pallida* were realized. Coral fragments often have stowaways residing in the underlying skeleton including crabs or sea urchins that can persist in aquaria unnoticed by researchers until they have reached a certain size (personal observation). Each of these organisms has the potential to influence the microbiome of the coral. By using lab-reared clonal populations of *E. pallida*, this possibility is eliminated. The ability of *E. pallida* to locomote, unlike individual polyps of coral which remain fixed, allowed visual observation of negative taxis by *E. pallida* and positive taxis of *R. reptotaenium* in relation to one another during the challenge studies. Controlled taxis studies in the context of the BBD bacterial challenge could confirm whether *R. reptotaenium* is positively chemotactic to *E. pallida* and by extension to coral. This type of study could also confirm whether *E. pallida* is negatively chemotactic to BBD or more specifically *R. reptotaenium*. Additional chemotaxis studies using purified microcystin and/or sulfide could further inform whether the putative negative chemotaxis observed in this study is specific to known toxins produced by the microbial consortium.

One possible limitation could be that because *E. pallida* has a soft body, lacking hard skeleton, the way in which BBD interacts with this organism differs from its interaction with scleractinian corals. On the surface, this seems to suggest that *E. pallida* may not be a suitable model system for studying BBD. However, this difference in
interaction can be seen as a benefit in that it allows us to test whether the anoxic environment created at the interface of coral and BBD is a necessary component in disease etiology as it is presumably absent in the *E. pallida* challenges. An additional difference between corals and *E. pallida* is that corals have been shown to produce DMSP in the aposymbiotic state (Raina et al. 2013) while aposymbiotic anemones have not (Van Alstyne et al. 2009). This difference would permit analysis of the effect of DMSP production on BBD formation and persistence that could not be achieved using coral fragments.

Evidence suggests that the emergence of BBD globally has been driven by higher temperatures (Miller and Richardson 2015). Increases in DMSP production by corals have also been observed at increasing temperatures (Raina et al. 2013). As temperatures continue to rise in response to climate change, increasing DMSP production by corals may trigger changes in the microbiomes of the coral and/or the BBD consortium including recruitment of potential pathogens. This could be the result of chemotaxis toward DMSP as seen previously in marine microbes associated with corals (Tout et al. 2015) including a known coral pathogen (Garren et al. 2014). The current study may support this in that positive taxis of *R. reptotaenium* towards *E. pallida* was observed in the challenge studies and several taxa were recruited to the BBD microbiome from the host anemone. Future studies carried out using the cnidarian model system of *E. pallida* will be critical in confirming whether DMSP plays a major role in the etiology of BBD and if so, contribute to the development of interventions to mitigate the effects of this destructive coral disease.
References


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PUBLICATIONS AND PRESENTATIONS


Waikel PA, Gillevet PM, Richardson LL, (2016). Potential role of dimethylsulfoniopropionate in black band disease etiology and persistence, presented at the 13th International Coral Reef Symposium


Waikel PA, Gillevet PM, Richardson LL, (2015). Potential role of dimethylsulfoniopropionate in black band disease of corals, presented at the 17th Florida International University Biomedical and Comparative Immunology Symposium


