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Ecology and microbiology of black band disease:

New insights into the etiology of an old coral disease

Thesis submitted by

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Statement on the contribution of others

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Yui Sato provided intellectual input in writing, and conducted field sample collection and preparation of samples for molecular analyses. David Bourne wrote the manuscript after Yui Sato’s intellectual input. Andrew Muirhead conducted laboratory work and data collection.


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Abstract

The prevalence of black band disease (BBD), a virulent coral disease characterized by a thick microbial mat that migrates across coral colonies causing rapid tissue loss, is generally low on Indo-Pacific reefs, but the destructive impact that the disease has had on Caribbean reefs highlights the urgent need to understand the role that the disease could play in the dynamics of coral populations on the Great Barrier Reef (GBR). In 2006, the first recorded epizootic of BBD on the GBR infected an inshore assemblage of corals in the genus Montipora at Pelorus Island, located in the central GBR region. Over the next 2.7 years, recurring summer outbreaks of BBD were monitored, with BBD abundance peaking in the warmest month in each of three summers. Mean maximum abundance of BBD reached 16±6 colonies per 100 m² in summer, affecting up to 10% of coral colonies (n=485 colonies monitored), and decreased to 0-1 colony per 100 m² in winter. Reappearance of BBD on previously infected colonies and continuous tissue loss after BBD signs had disappeared suggest that disease impacts are of longer duration than indicated by the presence of macroscopic disease signs. On average, BBD lesions caused the loss of 40% of tissue surface area per colony, and 5% of infections led to whole colony mortality. Overall, the BBD epizootic had a substantial impact on Montipora assemblages on this inshore reef in the central GBR, clearly establishing the virulent nature of the disease for GBR corals.

Rates of new infections and linear progression of lesions recorded during the field monitoring program were both positively correlated with seasonal fluctuations in seawater temperature and light, suggesting that seasonal increases in these environmental parameters enhance the virulence of the disease. To isolate the potential contributions of temperature and light, which both vary seasonally, I examined the relative impacts of these two environmental variables on the virulence of BBD under controlled aquarium conditions. Progression rates of BBD lesions on Montipora hispida colonies were compared among three controlled temperature (28.0, 29.0, 30.5°C) and two controlled light treatments (170, 440 µmol m² s⁻¹). BBD progression rates were greatest (5.2 mm d⁻¹) in the 30.5°C/high-light treatment and least (3.2 mm d⁻¹) in the 28°C/low-
light treatment. High light significantly enhanced BBD progression, whereas rates of disease progression did not vary significantly among temperature treatments, identifying the greater role of light in driving BBD dynamics within the temperature range examined. Greater BBD progression during the day compared to the night (by 2.2 - 3.6-fold across temperature and light treatments) corroborates my conclusion that light is the preeminent factor driving BBD progression at typical summer temperatures. Decreased photochemical efficiency of algal endosymbionts in the high-temperature/high-light treatments suggests that compromised health of the coral holobiont contributes to enhanced disease progression, highlighting the complexity of disease dynamics in host-pathogen systems responding to environmental variation.

The complex microbial consortium comprising BBD lesions, including cyanobacteria, sulfate-reducing bacteria, sulfide-oxidizing bacteria, marine fungi and other heterotrophic microorganisms, act together to produce highly concentrated sulfide and anoxic conditions beneath the BBD mat; conditions that are lethal to coral tissue. However, little is known about how this microbial community develops to form the complex microbial consortia of BBD in situ. While monitoring the BBD outbreak at Pelorus Island, I observed a less-virulent precursor stage, which I named ‘cyanobacterial patch’ (CP), and followed successional changes in microbial communities leading to the development of BBD from CP. CP lesions found on M. hispida colonies were macroscopically distinct from BBD lesions and preceded the onset of BBD in 19% of cases (n=262 CP lesions). Dominant cyanobacteria within CP lesions were morphologically and phylogenetically distinct from those dominating BBD lesions. Molecular analysis of cyanobacterial 16S ribosomal RNA (rRNA) coding genes confirmed shifts within cyanobacterial assemblages from Blennothrix/Trichodesmium spp.-related sequences dominating CP lesions to Oscillatoria sp.-related sequences, which were similar to those retrieved from other BBD samples worldwide, dominating BBD lesions. 16S rRNA gene clone libraries targeting Bacteria also demonstrated shifts in bacterial ribotypes during transitions from CP to BBD, with Alphaproteobacteria-affiliated sequences dominating in CP libraries, whereas gammaproteobacterial and cyanobacterial ribotypes were more abundant in BBD clone libraries.
Sequences affiliated with sulfur-cycling organisms were commonly retrieved from lesions exhibiting characteristic field signs of BBD. Since high sulfide concentrations have been implicated in BBD-mediated coral tissue degradation, proliferation of a microbial community actively involved in sulfur-cycling potentially contributes to the higher progression rates found for BBD compared to CP lesions.

To further characterize microbial community interactions contributing to BBD pathogenicity, I investigated the diversity of Bacteria, as well as previously-unexplored Archaea, associated with both BBD and CP microbial consortia, using high-throughput pyrosequencing. Profiles of bacterial 16S rRNA genes of BBD and CP illustrated patterns of community changes during BBD development that were consistent with those observed in the clone library-based study, confirming that bacterial ribotypes often observed in oxygen-depleted, sulfide-rich environments become relatively abundant during disease onset. Archaeal sequences retrieved from BBD were dominated (up to 94%) by a novel ribotype distantly affiliated to Euryarchaeotes, whereas CP-derived profiles indicated the presence of diverse archaeal assemblages affiliated to species across the Thaumarchaeota and Euryarchaeota, and were similar to communities reported from oxic marine environments. Although function(s) of BBD-associated Archaea are unknown due to the novelty of the 16S rRNA sequences, given organic- and sulfide-rich anoxic microenvironments within BBD lesions, BBD-associated Archaea may carry out methanogenesis and/or anaerobic methane-oxidation, syntrophically coupled with bacterial sulfate-reduction, thereby potentially enhancing the virulence of BBD.

Lastly, I developed a model of BBD pathogenesis based on my studies of the in situ development of BBD from CP. The model demonstrates that successional changes in key cyanobacterial and bacterial members of the initial CP microbial community lead to the development of a complex polymicrobial consortium that provides ideal conditions for sulfide production and the development of anoxic conditions as CP lesions transition into BBD. The changing nature of this microenvironment facilitates the growth of anaerobic Bacteria and Archaea, further developing the virulence of the microbial community. Knowledge derived from
this study provides new insights into the microbial ecology of BBD, contributing to a better mechanistic understanding of BBD pathogenesis that is vital for any future development of management strategies to mitigate the impacts of BBD on coral reef ecosystems.
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Chapter 1:

Background and general introduction

1.1 Researching single versus polymicrobial infectious diseases

An infectious disease is defined as any impairment that interferes with or modifies the performance of normal functions caused by living organism(s) residing in or on a host organism (Wobeser 2006). Identifying the causative agent (pathogen) of an infectious disease is a crucial step to understanding its etiology. Pathogens of most well-characterized infectious diseases have been identified by fulfilling Koch’s postulates, which involves isolating a pure culture of a biological agent unique to a disease, confirming disease reproduction by inoculating the isolated agent into a new host, and re-isolating the pathogen from the newly infected host (Wobeser 1994). A definitive identification of the causative agent for any disease significantly aids the development of methods and protocols for accurate diagnosis, treatment and control of infectious diseases.

An infectious disease is a consequence of complex interactions among a host, pathogen and the environment (Figure 1.1; Wobeser 1994). Environmental factors can affect both the susceptibility of hosts and the virulence of pathogens, and consequently they influence host-pathogen interactions, potentially shifting the host organism into a disease state (Figure 1.1). Most diseases are typically established by transmission and subsequent colonization of host tissues by a single pathogen, thus a single causative agent is involved (Figure 1.1; Munn 2004). Epidemiological studies of single pathogen-diseases enable tracking of infectious routes through host populations in public health control programs (Fraser et al. 2004). For example, transmission pathways and the immediate origin of a recent human influenza outbreak were identified by genetic analyses of the H1N1 virus responsible for the disease (Smith et al. 2009). However, approaches developed for single-pathogen diseases often do not supply sufficient etiological information for
polymicrobial diseases, which are caused by a mixed community of microorganisms. Polymicrobial diseases are being increasingly recognized, especially in human and animal disease research (Bakaletz 2004, Brogden et al. 2005, Sibley et al. 2008). For example, the etiologies of human diseases, including dental caries, periodontitis and cystic fibrosis (lower airway disease) have been studied extensively and their pathogenic mechanisms have been identified to be the result of complex interactions among polymicrobial communities (Sibley et al. 2006, Belda-Ferre et al. 2012). Pathogenesis (processes through which pathogenicity of a disease is developed) of polymicrobial diseases involves mechanisms whereby (1) one microorganism predisposes a host to colonization by other pathogenic microorganisms by creating new microbial niches in succession, or (2) interplay between two or more non-pathogenic microorganisms activates the virulence factor of a primary pathogen (Bakaletz 2004, Brogden et al. 2005, Sibley et al. 2008).

Polymicrobial diseases are similarly governed by interactions among a host, environmental factors, and a pathogenic microbial community, but they differ from single pathogen diseases in that complex interactions occur between more than one microbial agent.
Figure 1.2 Schematic model of interactions among a host organism (host), a mixed community of biological agents (pathogenic community) and environmental parameters (environment) governing the occurrence of a polymicrobial disease (right). Pathogenicity of the disease in another host system (left) results from either (1) successional development of the pathogenic community, or (2) transmission of the entire pathogenic community (purple arrows).

within the pathogenic community, each of which are also affected by environmental factors (Figure 1.2; Sibley et al. 2008). Onset of a polymicrobial disease requires either development of a pathogenic community in or on a host, or transmission of the entire pathogenic community (Figure 1.2); colonization by a single member of the pathogenic community may not be sufficient to cause the disease (Smith 1982). Fulfilling Koch’s postulates is thus not an effective way to study the etiology of polymicrobial diseases (Wobeser 1994). Instead, etiological aspects of polymicrobial diseases are best addressed by microbial ecology approaches (Kuramitsu et al. 2007), which consider interactions within the community of pathogens, as well as interactions between a host and the pathogen community, in conjunction with the effects of environmental factors on each of these interactions.

1.2 Coral disease as an identified threat to coral populations

Coral reefs currently represent one of the most vulnerable ecosystems on Earth, with
alarming rates of decline over the past century attributed to a number of factors, including coastal development, over-harvesting and pollution, and more recently, mass coral bleaching events and outbreaks of emerging diseases (Harvell et al. 1999, Hughes et al. 2003, Pandolfi et al. 2003, Bellwood et al. 2004, Baker et al. 2008, Carpenter et al. 2008, Hughes et al. 2010). Coral diseases are increasingly recognized as one of the major threats to reef ecosystems (Richardson 1998, Harvell et al. 1999, Willis et al. 2004, Weil et al. 2006) and have contributed to declines in coral populations, most notably in the wider Caribbean region (Green and Bruckner 2000, Gardner et al. 2003, Weil 2004). Although parameters contributing to increasing prevalence of coral disease are often unclear (Harvell et al. 1999, Porter et al. 2001, Sokolow 2009), global ocean warming (Harvell et al. 2002, Rosenberg and Ben-Haim 2002, Bruno et al. 2007, Harvell et al. 2007) and human-induced marine eutrophication (Weil 2004, Jordan-Dahlgren et al. 2005, Kaczmarsky et al. 2005, Kline et al. 2006) have both been implicated as major drivers of disease occurrence. These potential stressors reduce resistance of the coral host, enhance pathogen proliferation, and/or facilitate disease transmission (Harvell et al. 1999, Harvell et al. 2002). Current knowledge of most coral diseases is limited, and epizootic dynamics, host population impacts, causative agents, transmission modes and specific environmental drivers are poorly understood (Harvell et al. 1999). In particular, causative agents have not been conclusively identified for most coral diseases (Green and Bruckner 2000, Weil et al. 2006), which represents a major obstacle in the elucidation of coral disease etiology and the development of management strategies. Although there have been no reports of mass coral mortality in the Great Barrier Reef (GBR) region comparable to those observed in the wider Caribbean, results of a long term monitoring program on the GBR between 1995 and 2009 indicated that coral diseases accounted for 6.5% of the total loss of coral cover detected (Osborne et al. 2011). There is ample evidence that the consequences of disease outbreaks for coral populations will be intensified if global ocean warming progresses further (Harvell et al. 2002, Sokolow 2009). Increasing incidence of coral diseases on the GBR (Willis et al. 2004, Osborne et al. 2011) indicates that there is an urgent need to monitor and understand potential impacts of coral diseases even on the GBR, one of the world’s best managed coral reef
1.3 Black band disease in corals and population consequences

Black band disease (BBD) was the first coral disease to be reported, and since the first records of BBD in early 1970s, it has become known as one of the most virulent coral diseases thus far identified. BBD manifests as a bacterial mat forming a darkly pigmented band at the interface between apparently normal coral tissue and freshly exposed skeleton, which migrates across coral colonies causing rapid loss of coral tissue at the lesion interface (reviewed in Richardson 2004). BBD lesions have been reported to progress across infected colonies at rates up to 2 cm d\(^{-1}\) for Caribbean corals (Kuta and Richardson 1997). On average, however, BBD progression rates are typically in the order of 3 mm d\(^{-1}\) (Sutherland \textit{et al.} 2004), and they vary between host species (Sutherland \textit{et al.} 2004) and seasons (Boyett \textit{et al.} 2007) (also see Chapter 2). Rapid rates of BBD progression can result in death of entire coral colonies in relatively short time frames (Richardson 1996, Kuta and Richardson 1997), however, the disease is often arrested (disappearance of characteristic field signs) before the coral host dies completely, leaving partial mortality on coral colonies (Edmunds 1991, Porter and Meier 1992). A range of coral species of different families are known to be susceptible to BBD, including at least 42 Caribbean and 57 Indo-Pacific coral species (Sutherland \textit{et al.} 2004, Kaczmarsky 2006, Page and Willis 2006).

BBD outbreaks have decimated framework-building corals and contributed to ongoing loss of reef-building corals, particularly in the Caribbean (Edmunds 1991, Bruckner and Bruckner 1997, Goreau \textit{et al.} 1998, Green and Bruckner 2000, Sutherland \textit{et al.} 2004). Although BBD prevalence (proportion of infected colonies in a population) in this region is typically less than 5%, an exceptional event with 50% prevalence was reported for a population of \textit{Montastrea annularis} in the Florida Keys in 1993 (Green and Bruckner 2000). BBD infections have also been observed on Indo-Pacific coral reefs, although surveys indicate that the overall occurrence of BBD in coral
populations is low (Willis et al. 2004, Kaczmarsky 2006). In the Great Barrier Reef (GBR) region, BBD was first reported from 19 reefs in 1993 and 1994 (Miller 1996) and then sporadic cases of BBD (0.0% to 0.7% prevalence) were reported throughout the GBR reef systems in the summer of 2004 (Page and Willis 2006). The destructive consequences of BBD on coral populations recorded in the Caribbean highlight the urgent need for assessing the impacts of BBD on GBR coral populations and for evaluating the consequences of BBD outbreaks in this region. At the time of embarking on the present study (the summer of 2006), an epizootic of BBD was identified in an assemblage of species in the common scleractinian genus *Montipora* at Pelorus Island, located in the central inshore GBR region, providing an important opportunity to assess the impact of a BBD outbreak on an Indo-Pacific coral reef.

### 1.4 Microorganisms associated with black band disease

BBD is considered to be a polymicrobial disease (Frias-Lopez et al. 2004b). Identification of the primary causative agent of BBD has been difficult, but a tightly organized synergistic microbial consortium appears to act in concert to cause coral tissue necrosis (Carlton and Richardson 1995, Richardson 2004, Richardson et al. 2007). It is well-established that the microbial consortium is dominated by cyanobacteria and includes sulfate-reducing bacteria, sulfide-oxidizing bacteria, marine fungi and other heterotrophic microorganisms (Cooney et al. 2002, Frias-Lopez et al. 2002, Barneah et al. 2007, Sekar et al. 2008). These groups of microorganisms represent different functional roles (i.e. photosynthetic autotrophs, heterotrophs, and sulfur-cycling organisms) and are found consistently within BBD mats (Miller and Richardson 2011), but members of these groups can vary at a species level between host coral species, geographic locations and seasons (Frias-Lopez et al. 2002, Frias-Lopez et al. 2004b, Myers et al. 2007, Voss et al. 2007). While bacterial communities associated with BBD have been studied extensively, the possibility that hitherto unknown groups, like the Archaea, contribute to
BBD pathogenicity has not been explored.

The cyanobacteria and sulfide-oxidizing bacteria associated with BBD exhibit vertical migration within the microbial mat under changing light environments. High light elicits upward migration of sulfide-oxidizers through the cyanobacterial biomass (Richardson 1996, Viehman and Richardson 2002), resulting in dynamic vertical microgradients in oxygen and sulfide (Carlton and Richardson 1995, Richardson et al. 1997). The presence of highly concentrated sulfide and anoxic conditions underneath the BBD mat is lethal to coral tissue and may be essential to the pathogenicity of BBD (Richardson et al. 1997, Richardson et al. 2009). Other factors have also been proposed as etiological mechanisms of BBD, such as toxins produced by potentially pathogenic heterotrophic bacteria (Cooney et al. 2002, Sekar et al. 2008), cyanobacterial penetration of coral tissue (Barneah et al. 2007), cyanotoxin production (Richardson et al. 2007, Gantar et al. 2009), and Vibrio protease production (Arotsker et al. 2009). Currently, however, it is difficult to determine the extent to which each of these factors is involved in coral tissue breakdown and which factors are essential in the pathogenesis of BBD, mainly due to technical difficulties associated with teasing apart intricate synergistic interactions among members of the complex BBD consortium.

Complex interactions within BBD microbial consortia represent one of the greatest challenges in elucidating the etiology of BBD using conventional pathogen-determining criteria, such as Koch’s postulates. For example, although mono-cyanobacterial cultures have been obtained from BBD microbial mats (Richardson and Kuta 2003, Sussman et al. 2006, Myers and Richardson 2009, Rasoulouniriana et al. 2009, Glas et al. 2010), successful reproduction of BBD has not been accomplished through inoculation of a healthy coral. In contrast, microbial ecology-based approaches have revealed a complex structure of BBD microbial consortia (Frias-Lopez et al. 2002, Frias-Lopez et al. 2004a). Recent technological advances in massive parallel sequencing, such as 454 pyrosequencing, offer improved resolution of microbial profiling (e.g. Roesch et al. 2009, Andersson et al. 2010, da Jesus et al. 2010), which is directly applicable to studies of BBD etiology.
1.5 Environmental drivers of black band disease

Understanding potential links between environmental variables and the virulence of BBD is vital for identifying drivers of BBD outbreaks and predicting future epizootiological consequences for coral populations. Temporal and spatial patterns in BBD abundance have been documented in conjunction with a number of environmental parameters, including seawater temperature, water depth, solar irradiance, host population diversity and anthropogenic nutrients (Al-Moghrabi 2001, Kuta and Richardson 2002, Kaczmarsky et al. 2005, Rodriguez and Croquer 2008, Weil and Croquer 2009). In particular, seawater temperature has been suggested as a major driver of seasonal variability in BBD virulence because of strong positive correlations observed between seawater temperature and BBD abundance in the field (Antonius 1981, Edmunds 1991, Kuta and Richardson 1996, Bruckner et al. 1997, Borger 2005, Voss and Richardson 2006a, Rodriguez and Croquer 2008). This correlation is of particular concern given predicted increases in global ocean temperatures (e.g. Hansen et al. 2006) and increased host susceptibility driven by warmer seawater temperatures (Harvell et al. 2002). Light has also been suggested to be an important environmental factor governing distribution patterns of BBD (Kuta and Richardson 2002, Page and Willis 2006, Croquer and Weil 2009). BBD abundance decreases with water depth (Kuta and Richardson 2002) and water turbidity (Page and Willis 2006), suggesting that light availability is important for BBD associated microorganisms. Manipulative experiments have also indicated that strong light levels may be essential for enhanced virulence of BBD under warmer seawater temperatures (Boyett et al. 2007). Although the combined effect of light and seawater temperature is potentially a key to understanding etiological mechanisms of BBD, interactive effects of temperature and light levels on BBD activity have not been examined systematically and thus remain inconclusive. Determining important environmental factor(s) governing BBD etiology in situ is often challenging due to interactions among factors (Bruno et al. 2007, McClanahan et al. 2009, Williams et al. 2010), therefore experimental studies under controlled conditions are important to isolate the potential contributions of major environmental
factors that vary seasonally.

1.6 Knowledge gaps in the onset and development of black band disease

Although there have been extensive descriptive and fundamental etiological studies of BBD, key biological and ecological aspects leading to BBD epizootics have not been explored comprehensively. For example, how microbial communities associated with BBD become established on host corals, transmission mechanisms of the disease, and environmental factors that enhance progression and transmission of this disease remain to be elucidated (Voss and Richardson 2006a). Little is known about etiological processes during the early onset of BBD in situ and how they might predispose corals to infections by members of the complex BBD microbial community. Knowledge of transmission modes and the early developmental stages of the BBD microbial consortium is essential to the development of reef management strategies to mitigate the impacts of this disease.

In a pilot study that involved monitoring a BBD outbreak at Pelorus Island in the Palm Island Group in the central inshore GBR region, hitherto unreported lesions were observed to precede BBD infections in situ. Lesions found on encrusting and plating Montipora colonies were typically small patches (2 - 10 cm in diameter) and overgrew apparently healthy tissue, causing coral tissue loss at slower progression rates than those of BBD lesions. Preliminary microscopic observations revealed that the lesions are dominated by filamentous cyanobacteria and thus are henceforth referred to as “cyanobacterial patch(es) (CP)” (described in Chapter 4). Cyanobacterial filaments observed in CP are morphologically distinct from those observed in BBD, indicating the possibility that CP and BBD have different overall microbial communities. These observations suggest that CP predispose corals to BBD infection and that the overall microbial community within CP lesions changes into BBD-associated microbial consortia during the development of BBD. This in situ system provides a unique opportunity to investigate the pathogenesis of BBD.
Since little is known about how BBD is triggered and developed in situ, investigations of successional changes in microbial communities between CP and BBD will provide new insights into pathogenesis of BBD.

1.7 Study aims and objectives

This study aims to improve current understanding of the ecology and microbiology of BBD, with a particular focus on environmental factors that promote BBD outbreaks and etiological mechanisms that underpin the development of BBD. To accomplish these aims, my objectives are four-fold:

1. To examine temporal patterns in the abundance and severity of BBD in plots established during a BBD outbreak at Pelorus Island in the central inshore GBR.

   Long term monitoring of permanent plots encompassing coral populations with BBD-infections will enable evaluation of the impacts of the disease on GBR coral populations. In conjunction with time-series environmental data, this study examines major environmental drivers of BBD, enabling predictions of the frequency and severity of the disease under different climatic settings.

2. To clarify the roles of seawater temperature and light irradiance in determining virulence of BBD. A controlled aquarium experiment is used to examine progression of disease lesions under different temperature and light treatments. In combination with measurements of the overall health status of the coral host, this information provides further insights into the ecology of BBD in the ‘host – pathogens – environment’ model.

3. To investigate cyanobacterial patches as an early successional stage in the
development of BBD. Transitions in cyanobacterial species and composition of overall bacterial communities are characterized throughout the development of BBD from earlier identified lesions termed ‘cyanobacterial patch(es)’ (CP), using clone library-based molecular profiling techniques. Determining the relationship between CP and BBD and characterizing changes in bacterial communities during the development of BBD provide better understanding of mechanisms leading to disease onset.

4. To compare the diversity and interactions of Bacteria and Archaea in microbial mats between CP and BBD. Using high-throughput pyrosequencing, detailed profiles of Bacteria and Archaea are obtained and compared between CP and BBD. Resulting information contributes to a better mechanistic understanding of BBD etiology by identifying interactions of key microbial members leading to BBD pathogenesis.

1.8 Thesis structure

The four objectives listed above are addressed in Chapters 2 to 5. In Chapter 2, I document long term temporal patterns in BBD abundance and virulence, and assess impacts of the BBD outbreak observed in the central inshore GBR on assemblages of *Montipora* species. Seasonable variation in major environmental factors, namely seawater temperature and light, are also examined as potential environmental drivers of BBD in this chapter. In Chapter 3, effects of temperature and light on the virulence of BBD are systematically tested in a controlled aquarium experiment, clarifying the roles of these environmental factors in seasonal patterns of BBD documented in Chapter 2. In Chapter 4, I describe the natural onset of BBD by monitoring transitions of CP into BBD *in situ*. Successional changes in bacterial communities within disease lesions as CP develops into BBD are also characterized by molecular profiling of Bacteria in this chapter. Chapter 5 provides additional corroborative evidence for findings reported in Chapter 4.
and further identifies important bacterial members for BBD pathogenicity. Archaeal profiling in this chapter offers new insights into the microbial ecology of BBD by identifying an unexplored domain of life potentially playing important roles in BBD pathogenesis. Lastly, in Chapter 6, I discuss results obtained in the above chapters in an ecological context and synthesize discoveries from this complete body of work into a conceptual model of microbial mechanisms that underlie the development of BBD.
Chapter 2:

Dynamics of seasonal outbreaks of black band disease

The content of this chapter has been published as:


The published paper is attached as Appendix 2.1.

The entire chapter was written by Yui Sato, with co-authors providing intellectual direction in the design and implementation of the research and editorial inputs. Data collection and analyses, and production of tables and figures were conducted by Yui Sato.
Recurring summer outbreaks of black band disease on an inshore reef in the central Great Barrier Reef constitute the first recorded BBD epizootic in the region. In a 2.7 year study of 485 colonies of *Montipora* species, BBD affected up to 10% of colonies in the assemblage. Mean maximum abundance of BBD reached 16±6 colonies per 100 m$^2$ (mean ±SE, n=3 quadrats, each 100m$^2$) in summer and decreased to 0-1 colony per 100 m$^2$ in winter. On average, BBD lesions caused 40% tissue loss and 5% of infections led to whole colony mortality. BBD reappearance on previously infected colonies and continuous tissue loss after BBD signs had disappeared suggest that disease impacts are of longer duration than indicated by the presence of characteristic signs. Rates of new infections and linear progression of lesions were both positively correlated with seasonal fluctuations in seawater temperatures and light, suggesting that seasonal increases in these environmental parameters promote virulence of the disease. Overall, the impacts of BBD are greater than previously reported on the GBR and likely to escalate with ocean warming.
2.2 Introduction

Infectious diseases in reef building corals have emerged at an accelerating rate over the last few decades (Richardson 1998, Harvell et al. 1999, Willis et al. 2004) and have contributed to a decline in hard coral cover, most notably in the wider Caribbean region (Green and Bruckner 2000, Gardner et al. 2003, Weil 2004). Although causative agents for the majority of coral diseases are difficult to identify and parameters contributing to patterns of increasing prevalence are often unclear (Harvell et al. 1999, Porter et al. 2001), global ocean warming (Harvell et al. 2002, Rosenberg and Ben-Haim 2002, Bruno et al. 2007, Harvell et al. 2007) and human induced marine eutrophication (Weil 2004, Jordan-Dahlgren et al. 2005, Kaczmarsky et al. 2005, Kline et al. 2006) have both been implicated as major drivers of increasing disease occurrence. There is an urgent need, however, for longer term monitoring studies at small, detailed scales to more clearly elucidate links between environmental parameters and disease abundance.

Black band disease (BBD) is readily visible in the field and thus a good candidate for an intensive monitoring study. Macroscopic signs of the disease are a bacterial mat forming a black band that migrates across apparently healthy coral colonies, actively killing tissue and exposing skeleton (Richardson 2004). BBD has been reported from reefs throughout the Caribbean, Red Sea and Indo-Pacific (reviewed in Sutherland et al. 2004), affecting at least 42 Caribbean and 57 Indo-Pacific coral species (Sutherland et al. 2004, Kaczmarsky 2006, Page and Willis 2006). BBD has been a major contributor to declines in coral cover in Caribbean populations (reviewed in Green and Bruckner 2000). In the Great Barrier Reef (GBR) region, BBD was first reported from 19 reefs in 1993-1994 (Miller 1996). Low levels of BBD prevalence (0.0% to 0.7%) were reported throughout the GBR reef system in the summer of 2004 (Page and Willis 2006). Records of BBD abundance in yearly surveys on 48 reefs spanning the GBR between 1998 and 2004 also suggest that the disease typically remains at low background levels (Willis et al. 2004) and there have been no reports of destructive epizootics like those in the Caribbean. Although some investigations have
been multi-year and multi-seasonal, most estimates of BBD prevalence have been based on “snapshot” observations or infrequent surveys. However, population impacts of disease are best evaluated from continuous monitoring of individually recognized colonies. Given potentially rapid rates of tissue loss caused by BBD, the disease is a potential threat to Indo-Pacific coral populations and warrants monitoring even in well-managed reef systems like the GBR.

Clumped BBD distribution patterns and apparent spread of disease to neighboring colonies suggest that BBD is transmissible potentially through water movement and direct contact of colonies (Kuta and Richardson 1996, Bruckner and Bruckner 1997, Voss and Richardson 2006a), although specific transmission modes and mechanisms of band formation are still poorly understood. Understanding potential links between environmental fluctuations and BBD abundance is also an important component of epizootiological assessment and vital for identifying outbreak drivers. Seasonal patterns in BBD abundance have been documented in conjunction with a number of environmental parameters. For example, it has been suggested that seawater temperature is a major driver of seasonal variability of BBD since strong positive correlations between seawater temperature and BBD abundance have been demonstrated in field studies (e.g. Bruckner et al. 1997, Borger and Steiner 2005, Voss and Richardson 2006a, Rodriguez and Croquer 2008). Light intensity is also an important seasonal variable that may contribute to seasonal patterns in the dynamics of BBD. Evidence that water depth and turbidity are negatively correlated with disease abundance (Kuta and Richardson 2002, Page and Willis 2006) also suggests that the availability of light may govern occurrence of the disease. High light has been demonstrated to elicit an immediate behavioral response in the microbial community, causing upward migration of *Beggiatoa* spp. within the cyanobacterium dominated BBD mat and shifting vertical gradients of oxygen and sulfide, which contribute to pathogenesis (Carlton and Richardson 1995, Viehman and Richardson 2002). While these studies suggest that seasonal fluctuations in light intensity may affect the virulence of BBD, the role of annual photoperiod cycles in driving BBD outbreak dynamics has not been tested previously.

In the summer of 2006, large numbers of BBD infections were observed on laminar
corals in the genus Montipora on an inshore reef within the Palm Island group in the central GBR region. Given that no BBD had been observed in field studies over the previous 15 years at this site (BW, pers. observ.) or on adjacent reefs surrounding nearby islands in the Palm Island group in a recent survey (Page and Willis 2006), the sudden increase in BBD cases can be considered an epizootic (sensu Stedman 2000). Since first detecting BBD, I have monitored this site systematically for 2.7 years to document the dynamics of BBD in a Montipora assemblage. My main objectives of this chapter were: (1) to characterize seasonal and long term trends in the incidence (rate of appearance of new disease cases per unit time) and abundance of BBD infections (appearance of the characteristic disease signs) in a host assemblage, (2) to identify potential seasonal environmental factors driving BBD virulence, (3) to assess the consequences of BBD outbreaks for the host assemblage, and (4) to examine the frequency of direct and indirect transmission of BBD, assuming that BBD is transmitted through physical contact between colonies and/or through the water column. Monitoring of BBD dynamics in conjunction with seasonally varying environmental parameters, i.e. annual seawater temperature and light cycles, will help to identify which environmental factors play important roles in governing progression and potential transmission of the disease. Results are pertinent to other Indo-Pacific reef populations and will aid the development of possible management strategies to mitigate impacts of coral disease outbreaks.

2.3 Materials and methods

2.3.1 Study site and field surveys

In January 2006, laminar and encrusting colonies of Montipora hispida, M. aequituberculata and M. mollis were observed to have signs of BBD on reefs fringing the southeast corner of Pelorus Island (18°33´S, 146°30´E), in the central region of the Great Barrier
Reef Marine Park (Figure 2.1). The study site is located on the upper reef slope, where it is exposed to strong wave surges year round caused by predominantly southeasterly winds but minimal levels of terrestrial run-off or human impact. Three replicate 10m x 10m permanent quadrats, haphazardly placed at 5-10m intervals, were established at depths of 2.5-3.0m. Percentage cover of the dominant scleractinian corals inside the quadrats was approximately 33% for *Montipora* spp., 8% for *Acropora* spp., and 4% for *Porites* spp. (3% for other species). Observations of BBD were collected for the assemblage of laminar and encrusting species of *Montipora* because identification to the species level requires microscopic observation of coenosteal features on skeletal samples (Veron 2000) and extractive sampling within the permanent quadrats was avoided. From comparisons of field characteristics between colonies inside the quadrats with those outside that had been sampled and checked microscopically, the majority of species within the quadrats were *M. hispida*, followed by *M. aequituberculata* and *M. mollis* comprised a minor component of the assemblage. Each quadrat encompassed between 8 and 24 colonies with BBD lesions showing the characteristic dark band and exposed skeleton.

![Figure 2.1 Location of study site (circle) at Pelorus Island in the central Great Barrier Reef Marine Park (GBRMP) (arrow), where black band disease was monitored. Seawater temperature and surface light data were logged at Orpheus Island Weather Station (cross) throughout the study period.](image-url)
Locations of all BBD colonies inside the plots were mapped and marked with numbered tags attached to substratum near each colony to facilitate re-location in subsequent surveys. No coral species other than *Montipora* displayed BBD signs in the plots throughout the study.

Tagged colonies were photographed at an angle approximately perpendicular to the colony surface to follow progression of the disease band and the fate of tagged individuals through time. A 10cm scale was included in each photograph for size calibration. During subsequent surveys, the number of BBD infected colonies and the presence or absence of neighboring colonies in direct contact with BBD infected colonies were recorded and all newly developed BBD cases were mapped and tagged in the same manner. Field data collections were conducted approximately monthly between January 2006 and August 2008, except for a 2-3 month interval during winter when abundance of BBD remained low. The total number of colonies of all *Montipora* species inside each quadrat was also recorded to examine prevalence of the disease in the assemblage.

### 2.3.2 Measurements of disease progression and coral tissue loss

Disease progression and tissue loss due to BBD were measured from underwater photographs, taking advantage of the flat and easily discernable features of *Montipora* colonies, by superimposing pictures taken from two consecutive surveys (Appendix 2.2). To ensure the measured tissue loss was caused only by BBD, tissue loss that had potentially occurred after disappearance of BBD signs was excluded (i.e. measurement of tissue loss caused by BBD was conservative). The original size of BBD infected colonies was measured as the area of apparently healthy tissue at the time when the infection was first recorded. Percentage tissue loss due to BBD was calculated as the area of tissue remaining when signs of BBD disappeared divided by the original tissue area.
2.3.3 *Data analyses*

To assess the frequency of potential BBD transmission over time, the number of newly developed BBD cases per unit time (standardized as wk\(^{-1}\)) and an index representing the extent of infectiousness (infectiousness index) were calculated for each quadrat between two consecutive surveys. The infectiousness index was defined as the number of newly contracted BBD cases between two surveys, divided by the number of BBD cases observed in the previous survey and the length of the intervening time interval (weeks). The index indicates the average number of potential disease transmissions per unit time for each infected colony in the susceptible assemblage, assuming that (1) abundance of BBD pathogens in the area within and surrounding the quadrat was represented by the abundance of BBD infected colonies within the quadrat, and (2) waterborne transmission or direct contact of colonies were the sources of disease infection. When BBD was not present within the quadrat, the infectiousness index for the following survey was not calculated because the source of the infection could not be defined.

Seawater temperature data at a depth of 1m and surface light levels (photon flux density of photosynthetically active radiation) were measured every 30 minutes at the Orpheus Island Weather Station (Figure 2.1) throughout the study period (data obtained online from AIMS Weather Observing System <http://data.aims.gov.au/awsqaqc/do/advancedPlot.do>). Environmental data were used as indicators of seasonal fluctuations rather than as absolute measurements because there were minor hydrological differences between the weather station and study site. Patterns in the number of new BBD cases per unit time, the infectiousness index and linear progression rate of lesions were statistically compared against means for temperature and light data collected during the corresponding measuring period. The strengths of correlations and regressions between disease and environmental parameters were computed using a Pearson’s Product Moment Correlation \(r\) and the General Linear Model, respectively. To meet the assumptions of normality and homogeneity of residuals required for the analyses, disease parameters were transformed as \(X^{0.25}\). Relationships between living tissue area and linear
progression rate of BBD or tissue loss per unit time were also examined with the same analyses. Regressions were carried out with the statistical analysis package, Statistica (StatSoft, Tulsa, Oklahoma).

A G test was used to compare infection data between 2007 and 2008 to determine whether colonies previously infected with BBD had higher incidence of the disease than colonies that did not have BBD during at least the previous 12 months. Yates’ correction for continuity was applied in the G tests because the number of recurrences was limited.

2.4 Results

2.4.1 Temporal patterns in black band disease and environmental parameters

Overall, a total of 485 colonies of laminar and encrusting Montipora species were monitored for 2.7 years, providing evidence of recurring BBD outbreaks annually in summers between 2006 and 2008. Outbreaks were positively correlated with seawater temperature and light fluctuations (Figure 2.2a, b). Daily average seawater temperatures fluctuated between 20ºC and 30ºC, but did not exceed 30ºC throughout the study period, except for a short period in February 2006 when temperatures reached 30.4ºC (Figure 2.2a). Bleaching was not detected in Montipora species nor in any other coral during the 2.7 year period, providing corroborative evidence that temperatures did not exceed upper thermal thresholds for bleaching (Berkelmans and Willis 1999) at this site. Daily average light levels fluctuated extensively due to daily changing cloud cover, but overall, annual maximum levels occurred in November-December at approximately 710 µmol m$^{-2}$ s$^{-1}$ in both 2006 and 2007.

BBD abundance peaked at 16±6, 15±8 and 11±3 infected colonies per 100 m$^2$ in the summers of 2006, 2007 and 2008, respectively (mean ±SE, n=3 quadrats), during the period of maximum seawater temperature each year (January-February) (Figure 2.2b). The numbers of
Figure 2.2 Temporal patterns in (a) daily averages of seawater temperature and surface light levels, (b) abundance of black band disease (BBD) (infected colonies per 100 m$^2$), (c) number of new BBD infections per unit time (standardized as wk$^{-1}$), (d) infectiousness index of BBD (representing potential transmissibility), and (e) linear progression rate of BBD lesion. Data in (b)-(e) represent means ±SE. (c), (d) and (e) are plotted in the middle of the measurement period as on the horizontal axis. Numbers above plots are sample sizes for quadrats (d; quadrats, e; colonies).
BBD infected colonies decreased when seawater temperature started to decline (Figure 2.2a, b), and were stable at low levels between April and September. BBD was absent within the quadrats in July 2006, although BBD infections were observed locally outside the plots throughout the study. BBD was most prevalent in January 2007 when mean (±SE) prevalence reached 9.6±5.1% (n=3 quadrats each encompassing 157-168 colonies).

Occurrence of new BBD infections per unit time increased from October to January, reaching a mean peak of 2.0±1.0 and 2.4±1.2 colonies wk⁻¹ (n=3 quadrats) in 2007 and 2008, respectively, and was lowest (<0.4±0.3 colonies wk⁻¹) between March and August each year (Figure 2.2c). Increases in the number of new cases per unit time between October and January reflected an accelerating increase in the cumulative number of colonies infected, although this is not apparent from Figure 2.2b due to the concurrent disappearance of BBD signs on a number of colonies. The number of new infections per unit time was significantly and positively associated with both seawater temperature (r=0.4697, df=1, F=18.967, p<0.001, n=60 measurements) and light (r=0.5252, df=1, F=25.909, p<0.001, n=60 measurements) (Appendix 2.3 and Appendix 2.4). Variability among quadrats was also significant in the tests for both temperature (df=2, F=5.501, p=0.007) and light (df=2, F=6.010, p=0.004), reflecting consistently lower numbers of new BBD cases in one quadrat. However, patterns of increasing or decreasing new BBD cases with temperature and light were identical in all three quadrats.

The infectiousness index peaked at 0.62 in September 2006 and at 1.45 in October 2007 (Figure 2.2d), prior to January peaks each year in both BBD abundance and new BBD cases per unit time. There was a smaller peak in the infectiousness index of 0.67 in April 2008, which corresponded to a rise in light levels from an anomalous dip in March (Figure 2.2a). Overall, however, the index started to decrease in December-January, when light levels were declining from their annual maxima, yet seawater temperatures were still rising (Figure 2.2a, d). The index was not significantly associated with temperature (r=0.2072, df=1, F=2.908, p=0.097, n=38 measurements) but it was positively associated with light levels (r=0.5550, df=1, F=17.578, p<0.001, n=38 measurements). Variability among quadrats was not significant in the regression
models for either temperature (df=2, F=2.079, p=0.141) or light (df=2, F=2.278, p=0.118).

Mean (±SE) linear progression rate of BBD was greatest between December and February each year and reached a maximum of 3.7±0.1 mm d\(^{-1}\) in 2007 (maximum linear progression rates ranged from 3.0±0.3 mm d\(^{-1}\) in 2006 to 3.2±0.2 mm d\(^{-1}\) in 2008) (Figure 2.2e). Mean rates of linear progression were lowest between autumn and spring (<1.7 mm d\(^{-1}\)). Linear progression rates of the disease band were significantly and positively correlated with both temperature (r=0.4034, df=1, F=8.331, p=0.007, n=92 measurements) and light (r=0.6383, df=1, F=24.086, p<0.001, n=92 measurements). While sample size was small during winter due to the low abundance of BBD infections, colony effect was not significant in the regression models for either temperature (df=60, F=1.290, p=0.226) or light (df=60, F=1.287, p=0.227).

2.4.2 Reappearance of black band disease and probability of direct transmission

During BBD outbreaks in 2007 and 2008, recurrent BBD infections were observed on colonies that were deemed to be in remission because BBD signs had disappeared. In both 2007 and 2008, G tests indicated that the incidence of BBD on previously infected colonies was significantly higher than in the proportion of the assemblage that had no signs of BBD in the previous 12 months (Table 2.1). A total of 31% of recurrent BBD lesions appeared at the same site on colonies as the previous lesion (n=13 infections).

Most BBD lesions appeared for the first time on physically isolated colonies. Throughout the study period, only 3% of BBD infections (n=178 infections) appeared to be transmitted from a neighboring colony through direct contact.

2.4.3 Black band disease impacts on infected coral assemblage

The average area of tissue loss caused by a single BBD infection was 304 cm\(^2\) (n=57 infections), although 75% of disease cases caused less than 300 cm\(^2\) of tissue loss (Appendix 2.5).
Table 2.1 Comparison of black band disease (BBD) incidence between colonies with and without a history of BBD infection in the previous 12 months. Data were collected during BBD outbreaks in an assemblage of *Montipora* species throughout 2007 and 2008. Incidence of BBD infections was tested between the non-infected assemblage and previously infected assemblage using a G test with Yates’ continuity correction.

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<th>2007 not infected previously</th>
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<td>43</td>
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<td>$p$</td>
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Figure 2.3 Relationship between original tissue area of *Montipora* colonies and percentage tissue loss by black band disease.

While the average percentage tissue loss was 40% (n=57 colonies), 37% of BBD infected colonies lost more than 50% of their original tissue area. There was a negative relationship between original tissue area and percentage tissue loss, with smaller colonies suffering a larger percentage tissue loss, including death of entire colonies (Figure 2.3), although the largest area of tissue loss caused by one BBD lesion was >1,800 cm$^2$, which accounted for 65% of the original live tissue.
area of one large (2,900 cm\(^2\)) colony. However, no significant correlation was detected between linear rate of BBD progression (between January 2008 and February 2008) and remaining tissue area on the corresponding colony (\(r=-0.023, df=1, F=0.011, p=0.918, n=23\) colonies, tissue areas ranged from 48 cm\(^2\) to 2447 cm\(^2\)). Similarly, no correlation was detected between tissue area loss per unit time during the same period and original colony size (\(r=0.033, df=1, F=0.023, p=0.881, n=23\) colonies, colony size ranged from 48 cm\(^2\) to 7419 cm\(^2\)). Case fatality (number of infections that led to death, divided by the total number of infections) during the outbreak each year was approximately 5% (\(n=23, 57\) and 51 colonies in the 2006, 2007 and 2008 outbreaks, respectively) and only 3 BBD cases in 2007 remained until the next summer outbreak, thus the majority of infected colonies (90-95%) survived BBD infection. Most of the surviving colonies remained apparently healthy after signs of BBD disappeared. However, 7.6% of surviving colonies that had ceased to have any visible signs of disease subsequently died (\(n=105\) colonies). On average, such colonies had remaining tissue areas of 59 cm\(^2\) (\(n=8\) colonies).

2.5 Discussion

2.5.1 Prevalence and virulence of black band disease outbreaks

Annual summer outbreaks of BBD were observed between 2006 and 2008 in an assemblage of *Montipora* species on reefs surrounding Pelorus Island in the central inshore Great Barrier Reef region. The average prevalence of BBD peaked at 9.6%, which is the highest prevalence of BBD recorded on the GBR to date (cf. Dinsdale 2002, Page and Willis 2006) and constitutes the first report of a BBD epizootic (sensu Stedman 2000) in the region. Peak prevalence during the outbreak was greater than the previous highest record of BBD prevalence on an Indo-Pacific reef (7.8% for a Philippine population of *Montipora aequituberculata*; Kaczmarsky 2006), but comparable to BBD prevalences on some of the most severely affected

Maximum rates of BBD progression (3.7±0.1 mm d⁻¹) in my study are also comparable to rates on massive corals in the Caribbean (mean=3 mm d⁻¹; maximum=2 cm d⁻¹; Kuta & Richardson 1997). This study confirms that coral diseases like BBD, which have caused significant mortality in Caribbean coral assemblages (Green and Bruckner 2000), can also reach epizootic proportions on Indo-Pacific reefs.

2.5.2 Effects of seasonal fluctuations in seawater temperature and light on black band disease dynamics

My results indicate that seasonal fluctuations in both seawater temperature and light drive the occurrence of BBD infections and virulence of BBD lesions. The observed temperature-driven increases in BBD are potentially explained by host- and/or pathogen-responses to seasonal thermal fluctuations. High (but not anomalous) summer seawater temperatures cause stress to coral hosts (Fitt et al. 2001) and increase their susceptibility to disease infections by microorganisms such as fungal pathogens (Alker et al. 2001). Cyanobacterium species dominating the biomass of BBD bacterial mats (i.e. *Geitlerinema* species, formerly referred to as *Phormidium corallyticum*; Myers et al. 2007) have an optimal photosynthetic production rate at or above 30°C (Richardson and Kuta 2003). Other cyanobacterial species associated with BBD mats, such as strains closely related to an *Oscillatoria* species (recently described as *Roseofilum reptotaenium*; in press, L Richardson), have been shown to occupy the same ecological niche within BBD mats as *Geitlerinema* species (Myers and Richardson 2009) and have been detected worldwide (reviewed in Myers et al. 2007). Molecular analysis of cyanobacterial 16S rRNA gene sequences associated with BBD on *Montipora* species at the study site demonstrated 99% sequence similarity to that of the ubiquitous BBD *Oscillatoria*-related strain (see Chapter 4). Although an optimal temperature for the ubiquitous BBD *Oscillatoria*-related strain has not been reported thus far, the summer outbreaks of BBD reported here indicate that higher temperatures may also be
favorable for this strain. Increased cyanobacterial biomass under higher temperatures may be important in BBD pathogenesis by generating dynamic vertical micro-gradients of oxygen and sulfide, which have been implicated in coral tissue degeneration (Carlton and Richardson 1995, Richardson et al. 1997). Enhancement of BBD progression rates under higher temperatures on GBR corals has also been demonstrated experimentally (Boyett et al. 2007), which further supports the positive association between temperature and BBD virulence found in the present study. Importantly, positive correlations between BBD activity and seawater temperature suggest that warmer ocean conditions will lead to longer BBD outbreak events and more rapid tissue loss, thus more intense degradation of coral populations.

My results also support that light is an environmental driver of both linear progression and incidence of BBD in the Montipora host assemblage. Previous microbial studies have shown that BBD-associated cyanobacterial species are adapted to low light levels and known to have a ‘self-shading’ behavior under high light conditions (Kuta and Richardson 2002, Richardson and Kuta 2003). Clumping behavior of cyanobacteria has been suggested to contribute to the pathogenesis of BBD by providing anoxic conditions that favour other pathogenic community members, such as sulfate-reducing Desulfovibrio species (Kuta and Richardson 2002, Richardson and Kuta 2003, Myers et al. 2007), producing sulfide that causes coral tissue lysis (Richardson et al. 1997). Thus cyanobacterial clumping in response to high light may accelerate increase rates of disease progression. Evidence that coral-associated microbial communities vary with depth (Klaus et al. 2007) further corroborates conclusions that solar irradiance is a key factor structuring coral microbial communities and thus seasonally changing light levels may affect the virulence of BBD microbial communities. An experimental study also reported no difference in the probability of BBD transmission under different temperature regimes (Aeby and Santavy 2006). Therefore seasonally increasing light levels may be more important in the frequency of new BBD infections than seasonally rising seawater temperatures.

Identifying an independent effect of a specific environmental factor is often difficult in field studies because environmental variables are typically correlated with each other. In the
current study, however, seasonal patterns in light preceded seasonal patterns in seawater
temperatures by approximately 2 months. The significant association of my infectiousness index
with light but not with seawater temperature suggests that light plays an important role in driving
new infections. Boyett et al. (2007) also proposed that strong light enhances BBD progression
rates under elevated temperatures. Enriquez et al. (2005) describe the physical mechanism by
which high solar radiation synergistically exacerbates oxidative stress in heat-stressed corals, and a
number of studies have experimentally demonstrated that solar radiation increases damage to both
coral tissues and symbiotic algae experiencing thermal stress (e.g. Brown 1997, Lesser and Farrell
2004). Such synergistic effects of temperature and light may also contribute to the observed
seasonal patterns of BBD virulence. There is need for a manipulative experiment, with
temperature and light as independent variables, to unequivocally separate temperature and light
effects on BBD transmission and progression rates (see Chapter 3).

2.5.3 Potential source of black band disease infections

Apparent direct transmission of BBD between physically connected colonies was
recorded but was not the major mode of spread of the disease. Similar observations have been
recorded in previous studies (e.g. Kuta and Richardson 1996, Sutherland et al. 2004), although
specific mechanisms of the major transmission mode(s) are still unknown. One potential
transmission mechanism is transport of the BBD bacterial community by water movement,
considering that a developed BBD bacterial mat is easily sloughed off into the water column
(Richardson 2004). Bruckner et al. (1997) recorded spread of BBD infections over 3km in a
down-current direction, suggesting mechanical transport of BBD pathogens by water movement.
The study site at southeast Pelorus Island is constantly exposed to strong wave surges, therefore
discharge and local transport of BBD bacterial mats by water movement may be present.

Recurrence of BBD on previously infected colonies is common (Kuta and Richardson
1996, Bruckner and Bruckner 1997, Voss and Richardson 2006a, Rodriguez and Croquer 2008),
suggesting that colonies which survive BBD may act as reservoirs for pathogens. While my results suggest that 31% of recurrent lesions in the study may have been caused by residual pathogens, it cannot be distinguished whether recurrent BBD lesions observed at different positions on the host colony than previous infections were caused by pathogens from the water column or by pathogens remaining on the colony that were motile and present at visually undetectable levels. It is also possible that members of BBD-associated microbial communities present in either healthy coral tissues (Frias-Lopez et al. 2002, Klaus et al. 2007), dead coral skeleton (Frias-Lopez et al. 2002) or sediment on live coral (Richardson 1997) caused the disease in response to environmental or biological triggers (Rohwer et al. 2002). Additionally, both vectors (see Aeby and Santavy 2006) and reservoirs for BBD pathogens other than infected coral colonies (Richardson 1997) may play important roles in BBD transmission.

2.5.4 Impact of black band disease on coral assemblages

The present study demonstrates that small colonies are most likely to suffer whole colony mortality, as indicated by a negative correlation between original tissue area of colonies and percentage tissue loss caused by BBD infections. Two factors appeared to contribute to this pattern: (1) linear progression rate of the band was not dependent on host tissue area, and (2) most BBD infections started as small lesions and disappeared within a season, thus smaller colonies were more likely to lose larger proportions of live tissue area. However, an extensive (>1,800 cm²) tissue loss caused by one BBD lesion was recorded on one large (2,900 cm²) colony, indicating that a BBD lesion can potentially kill a substantial proportion of host tissue if environmental conditions (e.g. seawater temperature, light) are favorable for the disease. Moreover, the impact of BBD on host population dynamics is potentially larger than the apparent loss of tissue area because substantial tissue loss may result in cessation of reproductive activity regardless of colony age (Szmant-Froelich 1985). It is also important to note that the current study underestimated tissue loss by excluding mortality on tagged colonies when BBD signs disappeared between visits.
It is notable that, after the disappearance of visible BBD lesions on some small colonies, whole colony mortality nevertheless occurred. These observations suggest that coral health may be impaired even after BBD signs disappear and/or that continued tissue loss may be caused by BBD pathogens remaining at visually undetectable levels, potentially within the skeleton (Ainsworth et al. 2007). Photo-inhibition of symbiotic algae has been demonstrated in apparently healthy tissue areas of coral hosts near BBD lesions (Roff et al. 2008), suggesting that BBD affects the host before the band migrates over nearby tissues. Considering the small size of most colonies that suffered whole colony mortality after a BBD infection in my study, this potential distant impact of BBD before the band disappeared may have been lethal to tissues remaining on small colonies. Patterns of susceptibility to BBD infection for specific Montipora species are needed in future epizootiological studies to further validate colony size and mortality patterns found in the present study.

The ~3.5-fold higher incidence of BBD I observed on previously infected colonies, in comparison to colonies that had no previous history of BBD signs, accords with high probabilities of BBD recurrence reported for a Venezuelan population of Diploria strigosa (Rodriguez and Croquer 2008) and highlights the vulnerability of large colonies to recurrent infections. Although the source of pathogens in recurrent cases is unclear, the following hypotheses may contribute: (1) BBD may compromise a coral’s immune responses, which may include amoebocytes (Hildemann et al. 1977), melanin deposition (Palmer et al. 2008) and antibacterial chemicals (Koh 1997, Gochfeld et al. 2006, Ritchie 2006), increasing its susceptibility to recurring summer infections, and/or (2) although undetectable in the field, pathogens may remain on or within apparently healthy colonies and act as winter reservoirs, contributing to reappearance of BBD signs in the following summer. It is thus possible that the history of past disease infections is a colony-specific factor governing susceptibility to BBD. It has also been suggested that susceptibility to coral disease increases with decreasing colony size (Kramarsky-Winter 2004, Sutherland et al. 2004, Kaczmarsky et al. 2005). Therefore, while larger colonies are more likely to survive a BBD infection despite a potentially greater loss of tissue area, the surviving colonies have high
probability of recurrence of BBD, leading to further tissue loss in subsequent infections. Small colonies, on the other hand, have overall higher percentage tissue loss and may suffer further tissue loss after disappearance of BBD signs, potentially causing whole colony mortality. The case fatality was calculated at 5%; however, this study suggests that long term consequences of BBD on host coral population dynamics can be greater due to “post BBD infection” effects, particularly disease recurrence on large colonies and continuous tissue loss on small colonies.

2.5.5 Conclusions

The present study documents the first BBD epizootic on the GBR and highlights colony size-related patterns in mortality caused by BBD infections that have significant long-term implications for Indo-Pacific populations of *Montipora* species. Seawater temperatures and light levels were identified as environmental drivers governing the abundance and virulence of BBD, with light having a potentially greater role in facilitating new infections. The long-term nature of my study revealed the seasonally fluctuating nature of BBD dynamics, with infections increasing exponentially in summer and declining to low levels in winter. Therefore frequent reef monitoring should be encouraged to detect potential disease outbreaks that otherwise terminate, resulting in loss of important information relating to disease impacts. It is likely that warmer seawater temperatures predicted in association with global warming will exacerbate the impacts of BBD on Indo-Pacific reefs by increasing rates of tissue loss and the duration of outbreak events.
Chapter 3:

Effects of temperature and light on the progression of black band disease

The content of this chapter has been published as:


The published paper is attached as Appendix 3.1.

The entire chapter was written by Yui Sato, with co-authors providing intellectual guidance in the design and implementation of the research and editorial contributions to the paper. Experiments, data collection, data analyses and production of tables and figures were conducted by Yui Sato.
3.1 Abstract

Understanding environmental drivers of black band disease (BBD), a virulent disease affecting corals worldwide, is critical to managing coral populations. Field monitoring studies have implicated seasonally elevated temperature and light as drivers of annual BBD outbreaks on the GBR, but do not distinguish their relative impacts. Here I compare progression of BBD lesions on *Montipora hispida* among three controlled temperature (28.0, 29.0, 30.5°C) and two controlled light treatments (170, 440 µmol m⁻² s⁻¹) within normal seasonal ranges at the site. BBD progression rates were greatest (5.2 mm d⁻¹) in the 30.5°C/high-light treatment and least (3.2 mm d⁻¹) in the 28°C/low-light treatment. High light significantly enhanced BBD progression, whereas increases in disease progression under high temperatures were not statistically significant, identifying the greater role of light in driving BBD dynamics within the temperature range examined. Greater BBD progression during daytime compared with nighttime (by 2.2 - 3.6-fold across temperature and light treatments) corroborates my conclusion that light is the preeminent factor driving BBD progression at typical summer temperatures. Decreased photochemical efficiency of algal endosymbionts in the high-temperature/high-light treatments suggests that compromised health of the coral holobiont contributes to enhanced disease progression, highlighting the complexity of disease dynamics in host-pathogen systems responding to environmental changes.
3.2 Introduction

Environmental stressors associated with both global ocean warming and human activities appear to contribute significantly to trends of increasing abundance and severity of coral disease outbreaks over the past few decades, potentially by reducing resistance of the coral host, enhancing pathogen proliferation, and/or facilitating disease transmission (Harvell et al. 1999, Harvell et al. 2002). Given that coral disease studies repeatedly suggest that global ocean warming will lead to increasingly severe epizootics, with dire consequences for coral populations and associated coral reef communities (Harvell et al. 2002, Sokolow 2009), greater understanding of factors driving the dynamics of coral diseases is urgently required to enable development of appropriate reef management strategies.

Field observations have suggested that interactions among many environmental factors govern abundance of black band disease (BBD), including seawater temperature, water depth, solar irradiance, host population diversity and anthropogenic nutrients (Al-Moghrabi 2001, Kuta and Richardson 2002, Kaczmarsky et al. 2005, Rodriguez and Croquer 2008, Weil and Croquer 2009; Chapter 2). In particular, BBD abundance has been correlated with warm seawater temperatures, as documented in chapter 2 and other seasonal monitoring programs (Antonius 1981, Edmunds 1991, Kuta and Richardson 1996, Bruckner et al. 1997, Borger 2005, Voss and Richardson 2006a, Rodriguez and Croquer 2008, Zvuloni et al. 2009). Light has also been suggested as an important environmental factor governing distribution patterns of BBD (Kuta and Richardson 2002, Page and Willis 2006, Croquer and Weil 2009), as well as the dynamics of disease abundance and lesion progression (Chapter 2). Identifying seasonal environmental drivers of BBD is fundamental to predicting BBD epizootics and will underpin the development of potential management strategies to mitigate disease impacts. However, determining environmental factor(s) governing BBD etiology is difficult because of interactions among factors in situ (but see Bruno et al. 2007, McClanahan et al. 2009, Williams et al. 2010), thus controlled experimental
studies are important to isolate the potential contributions of major environmental factors that vary seasonally.

Using controlled experiments, Boyett et al. (2007) demonstrated that elevated water temperature enhances the progression of BBD on Acropora muricata. However, this thermal effect was absent under dim indoor conditions, and thus it was proposed that high light is also required for BBD virulence. Although both temperature and light may be important for BBD epizootic dynamics, relationships between BBD virulence and these two seasonally fluctuating environmental factors have not been examined systematically. In this chapter, I examine effects of temperature, light and the combination of these factors on BBD progression rate under controlled conditions and evaluate the importance of both parameters as environmental drivers in the progression of BBD. The two major objectives of this study are to quantify the influence of temperature and light on (1) progression rates of BBD lesions, and (2) photochemical efficiency of coral holobionts with and without BBD.

3.3 Materials and methods

3.3.1 Study site and sample collection

In January of 2008, samples of the common scleractinian coral Montipora hispida were collected for controlled experimental studies from a reef along the east coast of Pelorus Island (18°33’S, 146°30’E), which is located in the central inshore Great Barrier Reef (GBR) (see Chapter 2 for detailed site description). Thirty coral fragments with a distinctive band of BBD and 24 healthy fragments (uninfected controls), each approximately 10 cm × 10 cm in size, were collected using a hammer and chisel from 54 colonies (> 40 cm × 40 cm in size) of M. hispida at depths between 3 and 4 m. Colonies sampled were selected haphazardly after ensuring that each was at least 5 m away from other colonies. Fragments were placed in 70 L plastic aquaria filled
with freshly collected ambient seawater, separating healthy fragments from those with BBD into separate aquaria, and transported to Orpheus Island Research Station (OIRS) within 1 hour from time of collection.

Ambient seawater temperatures and light levels at the field site were measured at 3.5 m depth during the study period to determine levels that would be ecologically relevant for experimental aquarium studies. Ambient seawater temperatures were recorded throughout the experiment at 30 min intervals with an Odyssey temperature logger (Dataflow Systems, Christchurch). Maximum ambient daylight (photosynthetic available radiation, PAR) was measured using a LI-192SA underwater quantum sensor (LI-COR Inc., Lincoln, NE) at 1 min intervals around midday (1140hrs - 1220 hrs) on a day with clear weather during the study period and stored on a LI-COR LI-1000 data logger (LI-COR). In situ light measurements were available for only a short period of time because characteristically strong wave exposure at the study site prevented continuous deployment of light sensors and loggers for long-term monitoring. Therefore, the range in light levels experienced by the coral population sampled was estimated from diel patterns of surface irradiance recorded by a permanent weather station located in a nearby bay at Orpheus Island (data available online from AIMS Weather Observing System <http://data.aims.gov.au/awsqaqc/do/advancedPlot.do>). Differences in simultaneous bottom- and surface-light measurements were used to determine the range of light levels experienced by corals from long-term surface light records.

### 3.3.2 Experimental settings

Effects of temperature and light on BBD progression were compared among 6 experimental treatments using a combination of 3 temperatures (28.0°C, 29.0°C, and 30.5°C; each with maximum diel fluctuations of ±0.2°C) and 2 light levels (low: ranging 140 - 200 μmol m⁻² s⁻¹; high: ranging 400 - 480 μmol m⁻² s⁻¹) set at a 12 hr-light/12 hr-dark diel photoperiod. Treatment temperatures were all within the temperature range typically experienced at the study site during
summer, when BBD is abundant within local *Montipora* populations (see Chapter 2). To avoid thermal bleaching (loss of symbiotic algae and/or photosynthetic pigments) as an additional stress, which would confound interpretations of light versus temperature as drivers of BBD progression, the highest experimental temperature was set at 30.5 °C, which corals typically experience in summer without visual signs of bleaching (Chapter 2). Each temperature/light treatment was assigned 5 replicate BBD-infected and 4 healthy coral fragments. To prevent potential cross infection, each fragment was individually housed in a 2 L container, supplied with flow-through seawater (0.1 L min\(^{-1}\)) and aeration using thin (5 mm in diameter) tubing to provide water circulation without directly disturbing coral samples. Four or five containers assigned to the same treatment regime were placed in a 16 L water bath to ensure minimum variance in experimental conditions among fragments, while water contamination between sample containers was avoided by controlling water levels of the water bath. Incoming seawater, freshly pumped from the reef flat in front of OIRS, was passed through a series of inline filter cartridges that filtered the seawater down to 1 μm. Coral fragments were acclimatized for 24 hours at ambient seawater temperatures (daily range: 28.8 - 29.7 °C) and treatment light regimes (commencing with 12 hrs of darkness, followed by 12 hrs of the target light level). Temperatures for heated treatments were ramped at a rate of 0.5°C hr\(^{-1}\) until target temperatures were reached (day 0), and coral fragments were then maintained at treatment temperatures for 7 days (days 1 - 7). Waste water was pooled and processed with 0.3 μm filter units to remove potential bacterial contaminants.

### 3.3.3 Measurements of disease progression rates

Photographs of experimental coral fragments were taken every 12 hrs, coinciding with the end of experimental nighttime and daytime periods. *M. hispida* colonies possess a flat, plate-like morphology, which enables BBD lesion progression to be measured readily from digital photographs. Average linear progression rates of BBD lesions were measured at 5 random points within each colony, using the graphic software package, Canvas (ACD Systems, British
Columbia) as previously described (Appendix 2.2). Therefore, progression rate measurements on the same coral were assumed to be independent through time.

3.3.4 Photochemical efficiency measurement

The maximum quantum yield of photosystem II (Fv/Fm) for endosymbiotic algae associated with experimental corals was measured using a pulse amplitude modulated (PAM) fluorometer (Diving PAM, Heinz Walz GmbH, Eichenring). PAM fluorometry of coral-associated photoautotrophs has been extensively applied to assess physiological performances of corals (e.g. Jones and Hoegh-Guldberg 2001, Lesser and Gorbunov 2001, Jones and Kerswell 2003, Winters et al. 2006, Manzello et al. 2009), and the Fv/Fm ratios of coral symbionts are commonly used as an indicator of stress levels experienced by the coral holobiont (Jones 2004, Winters et al. 2006). At the end of nighttime periods on experimental days 2 - 7, measurements of Fv/Fm were made of apparently normal coral tissue for four randomly chosen BBD-infected fragments and four healthy fragments per experimental treatment. For diseased coral fragments, Fv/Fm was measured on coral tissue >5cm away from lesions to compare stress levels caused to host corals primarily by different temperature and light treatments regardless of position of the BBD lesion (Roff et al. 2008).

3.3.5 Data analyses

Average daily rates of BBD progression calculated over the duration of the experiment (days 1-7) were statistically compared among temperature and light regimes using a two-way ANOVA (n=5 colonies per temperature/light treatment). Mean daytime and nighttime progression rates for days 5-7 were compared using a three-way ANOVA to determine the effects of time period (day vs. night) in addition to temperature and light on BBD progression rates (n=5 colonies per treatment). Fv/Fm ratios at the end of the experiment (day 7), when potential accumulated
effects of the treatments would be greatest, were compared between fragments with and without BBD, and among temperature and light treatments with a three-way ANOVA (n=4 colonies per treatment). To meet the assumptions of normality and homogeneity of residuals required for the analyses, all parameters tested were square-root transformed. Data comparisons were carried out with the statistical analysis package, Statistica (StatSoft, Tulsa, OK) with an alpha value of 0.05.

3.4 Results

3.4.1 Temperature and light effects on the progression of black band disease

Mean linear rates of BBD progression varied among different temperature and light treatments, ranging from 3.1 mm d$^{-1}$ under the low-temperature (28.0°C)/low-light regime, to 5.2 mm d$^{-1}$ under the high-temperature (30.5°C)/high-light regime (Figure 3.1). BBD progression rates were significantly greater in high light compared with low light treatments (Table 3.1a), spreading at rates that were 12 - 41% greater than in the corresponding temperature treatments.

![Figure 3.1](image)

**Figure 3.1** Comparison of mean (±SE) linear progression rates of BBD lesions on the scleractinian coral *Montipora hispida* among three temperature and two light treatments between day 1 and 7 (n=5 colonies per treatment).
Table 3.1 Statistical results testing effects of temperature and light on BBD lesions affecting *Montipora hispida* fragments, and effects on photochemical efficiencies of host coral fragments with and without BBD infection. a Results of a two-way ANOVA testing temperature and light effects on average linear progression rates of BBD lesions measured between day 1 and 7, and results of three-way ANOVAs testing b effects of temperature, light and photoperiod of measurements (daytime vs. nighttime) on average linear progression rates of BBD lesions measured during either daytime or nighttime between day 5 and 7, and c effects of temperature, light and presence or absence of BBD on the maximum quantum yield of photosystem II (Fv/Fm) of symbiotic algae of *M. hispida* fragments. Asterisks indicate significant effects (significance level $\alpha=0.05$).

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<th>df</th>
<th>MS</th>
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supplied with low light (Figure 3.1). The effect of temperature on BBD progression was not significant, although there was a trend for increasing rate of mean progression with increasing temperature. In the field, diurnal temperature experienced by the coral population sampled fluctuated between 28.5°C and 29.7°C. Daily mean bottom light levels at the field site ranged between approximately 70 and 180 μmol m⁻² s⁻¹ throughout the study period, although maximum day light levels reached approximately 600 μmol m⁻² s⁻¹ around 1200 hrs. These ambient environmental measurements indicate that ranges in both experimental temperature and light
levels were similar to those experienced by the coral population in situ, and thus ecologically relevant to test the effects of temperature and light on BBD progression for this coral population. No signs of BBD or mortality were detected on any of the healthy (uninfected) experimental controls in any of the temperature/light treatments.

It is notable that mean BBD progression rates were nearly 3-fold greater in the daytime compared with nighttime experimental periods for all temperature and light treatments (i.e. 2.2 - 3.6 times greater in the daytime across the temperature/light treatments; Figures 3.2; p<0.001, Table 3.1b). Light had a significant effect on BBD progression rate throughout the diel photoperiod (Table 3.1b). In contrast, the range of experimental temperatures tested did not significantly affect BBD progression rate (Table 3.1b).

### 3.4.2 Temperature and light effects on photochemical efficiency of coral endosymbionts

The maximum quantum yields of photosystem II (Fv/Fm), measured as an indicator of stress experienced by each coral holobiont, were generally stable between days 2 and 7 within each temperature and light treatment, regardless of the presence or absence of BBD infection (Figure 3.3). However, Fv/Fm values measured for corals in the high-light treatments were
Figure 3.3 Transitions in the mean (±SE) maximum quantum yield of photosystem II (Fv/Fm) of symbiotic algae in (a) healthy *Montipora hispida* fragments, and (b) *M. hispida* fragments infected with BBD, under two temperature and three light treatments (n=4 colonies per treatment, LL; Low-light, HL; High-light).

consistently lower than values measured for corals in the low-light treatments. A three-way ANOVA comparing Fv/Fm ratios measured on day 7 indicated that the presence of a BBD lesion did not affect the photochemical efficiency of symbiotic algae, at least on apparently normal coral tissues >5 cm from the lesion, yet both light and temperature treatments did, with light having a greater impact than temperature (Table 3.1c). Fv/Fm ratios were lowest when diseased fragments were exposed to the high-light and 30.5°C-temperature treatment (Figure 3.3). No visual signs of coral bleaching were observed in any of the healthy or BBD-infected colonies throughout the experiment.

### 3.5 Discussion

3.5.1 Effects of temperature and light on the progression of black band disease

This study is the first attempt to differentiate the effects of temperature and light on the progression of black band disease, a virulent coral disease that has caused substantial mortality on both Caribbean and Indo-Pacific reefs. Controlled aquarium experiments revealed that high light
significantly enhances BBD progression rates but high temperature does not, when treatment
temperatures were within the normal summer range currently experienced at the study site. The
2.2- to 3.6-fold greater rates of BBD progression found in the daytime portion of the diel
photoperiod cycle than in dark periods further corroborate my conclusion that light is the
preeminent factor governing BBD progression within typical seasonal environmental ranges. My
results confirm patterns suggested from field-based studies, which have implicated light as a factor
that enhances progression of lesions and abundance of BBD (Kuta and Richardson 2002, Page and
Willis 2006, Croquer and Weil 2009, Chapter 2), and from an experimental study, which proposed
that high light is required for enhanced BBD progression rates on *A. muricata* in warmer
temperature treatments (Boyett *et al.* 2007). In most coral habitats in tropical and temperate
regions, peaks in the annual photoperiod cycle occur earlier than peaks in annual temperature
cycles, for example by approximately two months in the central GBR (Chapter 2). Thus it will be
important to include light as a factor in BBD outbreak models, not only because of the greater
impact of light on disease progression, but also because it is important to capture the earlier rise in
seasonal light levels for accuracy of model predictions. Nonetheless, there is ample evidence that
maximum and elevated summer temperatures increase BBD progression and abundance
Chapter 2), thus ocean warming remains a cause for concern, particularly if high temperatures
interact synergistically with high light, resulting in more severe BBD impacts on coral populations.

### 3.5.2 Possible mechanisms of increased disease progression rates under high light

Increased virulence of BBD pathogen(s) when experimental corals were exposed to
high light may have increased the progression of BBD lesions. Strong light affects migration
patterns of the cyanobacteria that dominate the BBD microbial consortium (Richardson 1996,
Viehman and Richardson 2002), with BBD cyanobacteria exhibiting strong negative phototaxis
(Viehman and Richardson 2002) and clumping behavior under high light. Clumping behavior
promotes self-shading and has been suggested to enhance anoxic conditions beneath the BBD mat, which, in turn, may favour the proliferation of sulfate-reducing bacteria and other anaerobic bacteria (Kuta and Richardson 2002, Richardson and Kuta 2003, Myers et al. 2007). Enhanced nighttime bacterial respiration and sulfide production by proliferating bacterial populations within the mat potentially further facilitate anoxic and sulfide-rich conditions, which are lethal to coral tissues (Richardson et al. 1997). Therefore, it is probable that the clumping response of cyanobacteria when exposed to high light represents an important mechanism by which light increases the virulence of BBD microbial mats, thereby enhancing lesion progression rates.

Diseases of corals, like all animal diseases, are the result of complex interactions between hosts, causative agents, and the environment (Work et al. 2008; Figures 1.1 and 1.2), thus it is also possible that high light compromised resistance of the coral host, in addition to increasing pathogen virulence. It is well established that strong light within diurnal fluctuations of natural solar irradiance is a source of stress for corals (Jones and Hoegh-Guldberg 2001, Lesser and Gorbunov 2001). Correspondingly, Fv/Fm ratios consistently indicated lower photochemical efficiency of coral holobionts in high-light compared with low-light treatments throughout the experimental period of the present study. This suggests that high light levels represented a source of stress that may have contributed to enhanced progression rates of BBD. Interestingly, consistently greater nighttime progression rates of BBD in high-light than in low-light treatments, despite the absence of light throughout the 12 hours preceding the nighttime measurement, suggest that high light during the day stresses the coral holobiont (and/or enhances pathogen virulence) for longer than the length of the daytime period. Also, measurements of photochemical efficiency suggest that light-induced stress may affect both BBD-infected and healthy colonies equally, although it is possible that further decreased Fv/Fm ratios might have been detected if measurements had been made closer (<2.5 mm) to the BBD band (see Roff et al. 2008). However, the lack of an effect of BBD infection on Fv/Fm values, when measurements were made at least 5 cm away from a BBD lesion, suggest that the photochemical efficiency of coral tissues distant to the lesion is unaffected by the BBD mat.
3.5.3 Potential effect of anomalously high temperatures on the progression of black band disease

The lack of a significant relationship between temperature and BBD progression found in this study is in contrast to significant positive correlations found in previous experimental and field studies (Boyett 2006; Chapter 2). However, the minimal difference in Fv/Fm ratios found among corals in the three temperature treatments under high light suggests that experimental temperatures represented minimal levels of stress to the coral holobiont. Higher (~32°C) temperatures in previous field and lab studies would have represented greater stress, thus it is likely that temperature becomes a driver when levels approach or exceed maximum summer temperatures. Anomalously warm seawater temperatures stress coral hosts through increased respiration rates of symbiotic algae and/or the coral itself (Fitt et al. 2001), deterioration of host defense mechanisms against microbial pathogens by reduced antibacterial activity in mucus (Ritchie 2006), and lower lysozyme-like- and prophenoloxidase-activities in coral tissue (Mydlarz et al. 2009). Furthermore, a cyanobacterial strain cultured from BBD lesions on Caribbean corals exhibited greater photosynthetic activity at higher temperatures and was photosaturated between 30°C and 37°C (Richardson and Kuta 2003). The dominant cyanobacterial strain of the BBD microbial mat isolated from corals at the study site (Pelorus Island) by Glas et al. (2010) also exhibited a positive relationship between growth rates and temperature up to 35°C. While the narrow thermal range tested in my study (28.0°C - 30.5°C) was appropriate for unraveling the effects of light and temperature within an ecologically relevant temperature range, the lack of a temperature effect does not negate the fact that maximum and anomalously high summer temperatures are likely to enhance BBD progression rates.

3.5.4 Conclusions

The complexity of interactions between the host, pathogen(s) and environmental factors
that lead to increased BBD pathogenesis is highlighted by results of PAM fluorometry, suggesting that health of the coral holobiont was compromised in experimental treatments that had the greatest rates of BBD progression. Corals possess a number of innate defense mechanisms against microbial pathogens, including mucus production, amoebocytes, antibacterial activity, melanin deposition, fluorescent proteins and lysozyme-like activity (Hildemann et al. 1977, Koh 1997, Gochfeld et al. 2006, Ritchie 2006, Gochfeld and Aeby 2008, Palmer et al. 2008, Mydlarz et al. 2009, Palmer et al. 2009, Reed et al. 2010), although little is known about the degree to which corals can cope with diseases and environmental fluctuations using these host defense systems (Ritchie 2006, Mydlarz et al. 2009, Mydlarz et al. 2010). In contrast, the responses of BBD pathogen(s) to environmental changes have been investigated extensively, particularly through physiological studies of cyanobacterial cultures isolated from BBD lesions (Richardson and Kuta 2003, Gantar et al. 2009, Myers and Richardson 2009). Nevertheless, further investigations of the complexity of microbial interactions that maintain the pathogenicity of the BBD mat (Richardson 2004, Richardson et al. 2009) are needed to elucidate links between changing environmental factors and members of the BBD microbial consortium.

In summary, the present study highlights the importance of light as an environmental driver of black band disease. Increased rates of BBD progression across coral colonies with increased light are likely related to both decreased resistance of the coral holobiont, as manifested by decreased photochemical efficiency of the coral endosymbionts, and increased virulence of BBD pathogens. Developing predictive models for coral diseases is becoming increasingly urgent in a globally changing climate (Williams et al. 2010). In addition to summer maximum seawater temperatures, light should be included as an important seasonal factor in BBD outbreak models, increasing the accuracy of BBD epizootic forecasts and contributing to the development of potential management strategies.
Chapter 4:

Successional changes in bacterial communities during the development of black band disease

The content of this chapter has been published as:


The published paper is attached as Appendix 4.1.

The entire chapter was written by Yui Sato, with co-authors providing intellectual guidance in the design and implementation of the research and editorial contributions to the paper. Data collection, data analyses and production of tables and figures were conducted by Yui Sato.
4.1 Abstract

Microbial communities associated with black band disease (BBD) have been well-characterized, but little is known about how these complex bacterial consortia develop. This study investigated successional changes in microbial communities leading to the development of BBD. Long-term monitoring of tagged corals throughout outbreaks of BBD in the central inshore Great Barrier Reef documented cyanobacterium-infected lesions, herein termed ‘cyanobacterial patch(es) (CP)’, which were macroscopically distinct from BBD and preceded the onset of BBD in 19% of cases. Dominant cyanobacteria within CP lesions were morphologically distinct from ones dominating BBD lesions. Clone libraries and T-RFLP analysis confirmed shifts within cyanobacterial assemblages, from Blennothrix/Trichodesmium spp.-affiliated sequences dominating CP lesions, to Oscillatoria sp.-related sequences, similar to those retrieved from other BBD samples worldwide, dominating BBD lesions. Bacterial 16S rRNA clone libraries also demonstrated shifts in bacterial ribotypes during transitions from CP to BBD, with Alphaproteobacteria-affiliated sequences dominant in CP libraries, whereas Gammaproteobacterial and cyanobacterial ribotypes were more abundant in BBD clone libraries. Sequences affiliated with organisms identified in sulfur cycling were commonly retrieved from lesions exhibiting characteristic field signs of BBD. Since high sulfide concentrations have been implicated in BBD-mediated coral tissue degradation, proliferation of a microbial community actively involved in sulfide cycling potentially contributes to the higher progression rates found for BBD compared to CP lesions. Results demonstrate how microbial colonization of indistinct lesions like CP may facilitate the onset of BBD, which has been proven to have ecological impacts on coral populations.
4.2 Introduction

Black band disease (BBD) has been termed a polymicrobial disease since a tightly organized, complex microbial consortium appears to act in concert to cause coral tissue necrosis (Richardson et al. 1997, Richardson 2004, Richardson et al. 2007). Currently, however, little is known about how bacterial pathogens form the black band on coral hosts or how changes in microbial communities lead to onset of the disease. Given the potential for BBD to cause substantial tissue loss in coral populations (reviewed in Green and Bruckner 2000; also see Chapter 2) and the likelihood that impacts of the disease will be exacerbated as seawater temperatures warm with predicted climate change (Bruckner et al. 1997, Kuta and Richardson 2002, Borger and Steiner 2005, Voss and Richardson 2006a, Rodriguez and Croquer 2008; Chapter 2), there is need for greater understanding of early stages in the onset of the disease if BBD is to be managed effectively.

It is well-established that the microbial consortium making up the black band is dominated by cyanobacteria, but also includes sulfate-reducing bacteria, sulfide-oxidizing bacteria, marine fungi and other heterotrophic microbes (reviewed in Richardson 2004). Molecular analyses of BBD-dominating cyanobacteria have retrieved bacterial 16S rRNA gene sequences affiliated with many cyanobacterial species/strains, such as a *Geitlerinema* sp. (Cooney et al. 2002, Myers et al. 2007) (formerly known as *Phormidium corallyticum*), *Trichodesmium* spp. (Frias-Lopez et al. 2002, Frias-Lopez et al. 2003), *Leptolyngbya* sp. (Myers et al. 2007) and an *Oscillatoria* sp., which has been most commonly detected in BBD samples collected from the wider Caribbean, Indo-Pacific and the Red Sea (reviewed in Myers et al. 2007, Myers and Richardson 2009) (Cyanobacteria represented by this *Oscillatoria* sp.-related ribotype was later isolated and described as *Pseudoscillatoria coralii* (Rasoulouniriana et al. 2009) and also described recently as *Roseofilum reptotaenium* (in press, L Richardson)). To what extent each of these cyanobacterial strains is involved in BBD pathogenesis is unclear, however there is emerging evidence that BBD-associated cyanobacteria vary between geographical and regional
locations and host coral species (Voss et al. 2007), yet each may occupy the same ecological niche in BBD microbial mats (Myers and Richardson 2009).

Pathogenesis of BBD is currently thought to involve light-associated, vertical micro-gradients in oxygen and sulfide (Carlton and Richardson 1995), accompanied by down-migration of the filamentous cyanobacteria when light levels are high (Richardson 1996, Viehman and Richardson 2002). Subsequent cyanobacterial penetration of coral tissue (Barneah et al. 2007) suggests that mechanical or chemical degeneration of coral tissue is also involved in pathogenesis. Additionally, genes for the biosynthesis of microcystin have been detected from Geitlerinema and Leptolyngbya sp. derived from BBD samples, and production of the toxin has been demonstrated with HPLC/MS and a protein phosphatase inhibition assay (Richardson et al. 2007). These findings indicate that the cyanotoxin may contribute to coral tissue necrosis caused by BBD. Desulfovibrio spp. are also thought to play a role in BBD pathogenesis through production and accumulation of sulfide, which is present in high concentrations under the BBD mat and can be lethal to coral tissue when combined with the anoxic microenvironment (Richardson et al. 1997). Sequences affiliated with other potential pathogenic bacteria, including Cytophaga sp., Clostridium sp., Campylobacter sp., Arcobacter sp. and an alphaproteobacterium responsible for juvenile oyster disease, have been retrieved from Caribbean BBD samples (Cooney et al. 2002, Frias-Lopez et al. 2002), though it is unknown whether these organisms play any role in the pathogenesis of BBD.

Little is known about how bacterial pathogens form the characteristic black band on host corals or about microbial communities during the early onset of BBD in nature. In one published observation, a new BBD infection on Diploria strigosa was documented as a small pigmented ring a few centimeter in diameter, which apparently had a BBD microbial mat already formed (Antonius 1981). Given that BBD-associated bacteria may be present on or within healthy coral tissues at background levels (Frias-Lopez et al. 2002), the disease band may be formed when environmental factors change and/or host coral health is biologically or physically compromised (Rohwer et al. 2002). For example, the BBD-associated cyanobacterium designated as CD1C11 is
commonly detected in healthy tissues of *Montastraea annularis* with culture-independent methods (Klaus et al. 2007). It has also been shown that predation of octocorals by corallivorous animals (e.g. the gastropod *Cyphoma gibbosum* and polychaete *Hermodice carunculata*) facilitates BBD infection in areas where BBD is present (Antonius 1985). Lack of knowledge about transmission modes and the early developmental stages of the BBD microbial consortium hinders development of reef management strategies to mitigate the impacts of this disease.

Recurring summer outbreaks of BBD were documented in the long term monitoring study of tagged corals in an assemblage of *Montipora* species on an inshore reef in the central GBR presented in Chapter 2. In some cases, actively expanding cyanobacterium-infected lesions, whose green or brown colorations were distinct from characteristic BBD lesions, were identified prior to the onset of BBD (Figure 4.1). I herein refer to these lesions as ‘cyanobacterial patch(es)’ or ‘CP’. Preliminary microscopic observations of CP indicate that they contain cyanobacteria that are morphologically distinct from ones observed in subsequent BBD lesions, suggesting that CP and BBD have different overall microbial communities. In this chapter, I investigate successional changes in microbial communities from early CP lesions to fully developed BBD lesions to document development of the complex bacterial consortia that characterize BBD. Seasonal patterns in abundance of CP and BBD were also documented to determine how often and when CP microbial communities develop into BBD microbial consortia. I compare the virulence of CP and BBD by recording progression rates of the lesions on host corals and by histological observations. In addition, I determine phylogenetic diversity of bacteria associated with CP and BBD lesions to examine changes in these microbial communities during the development of black band disease from cyanobacterial patches.
Figure 4.1 Transition in macroscopic signs of a disease lesion on the identical colony of *Montipora hispida* (colony 3; see Materials and methods) from: (a) a typical cyanobacterial patch (7 Oct. 2007), through (b) an intermediate stage between cyanobacterial patch and black band disease (25 Oct. 2007), to (c) a well-developed black band disease lesion (10 Nov. 2007). BBD, black band disease; CP, cyanobacterial patch; IM, intermediate stage between cyanobacterial patch and black band disease.
4.3 Materials and methods

4.3.1 Study site and field surveys

*Montipora* assemblages on an inshore reef at Pelorus Island in the central GBR were monitored between September 2006 and January 2009 to examine seasonal patterns in prevalences of CP and BBD. The study site is exposed to minimal levels of terrestrial run-off or human impact (see Chapter 2). In addition to the three previously described quadrats at the southeast corner of the island (18°33.6´S, 146°30.1´E; Chapter 2), three replicate 10m × 10m permanent quadrats were established at the northeast corner of the island (18°32.5´S, 146°30.0´E) in the same manner in May 2007. CP-infected colonies were individually tagged and monitored approximately monthly to determine if CP lesions developed into BBD. Progression rates of CP and BBD lesions were measured throughout the study period as described previously (Chapter 2, Appendix 2.2), and annual means and means for summer (December 2007–February 2008) and winter (June 2007–August 2007) were statistically compared between seasons and disease signs.

4.3.2 Sample collection

Samples of CP and BBD lesions were collected from five tagged colonies of *Montipora hispida* adjacent to the monitoring plots at depths of 2.5 – 3.0 m between August 2007 and February 2008. For bacterial examination, samples were collected at approximately 2 week intervals to monitor changes in microbial communities as CP lesions progressed into characteristic BBD lesions. To minimize sampling impacts on disease progression, a small (approximately 5 mm in diameter, 2 mm in depth) portion of the cyanobacterial mat and skeleton underneath the mat was collected from the front of each lesion, using a separate sterilized stainless steel chisel for each sample to avoid cross contamination. The samples were placed in individual 1.5 ml screw cap tubes underwater and kept on ice upon returning to the surface. After examining the
cyanobacterial morphology under a phase contrast microscope (BX41TF, Olympus Corporation, Tokyo, Japan) at a magnification of 400 times, excess seawater was removed and lesion samples were preserved in 100% ethanol at -20 °C. A small portion of coral tissue with attached skeleton was also sampled from two healthy colonies and preserved in the same manner. For histological observations, small (3 cm × 3 cm) diseased coral fragments were sampled from each of five CP- and BBD-infected colonies and immediately fixed in 10% seawater formalin. In addition, an ambient seawater sample (1 L) was collected from approximately 30 cm above the sampled colonies and immediately filtered through a 0.22 µm Millipore Sterivex filter unit (Sigma-Aldrich, St. Louis, MO, USA). The filter unit was kept on ice while transported to the laboratory before being stored at -20 °C.

4.3.3 Histological preparation

Formalin fixed samples were placed between sponge pads in histology cassettes to avoid detachment of the disease mat from the underlying coral tissue during decalcification in a 1% to 3% series of formic acid washes. Following paraffin wax embedding of decalcified samples, transverse sections of coral tissue (5 µm thickness) from the edge of each lesion were prepared. Sections were stained with hematoxylin and eosin to examine the extent of tissue structure degradation.

4.3.4 DNA extraction

DNA extraction from ethanol preserved lesions and coral tissue samples was performed with the PowerPlant® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) as described by the manufacturer with the following modifications. Following the initial preparation, 2 × 30 sec bead-beating cycles with a 30 sec interval were performed with a Mini-Beadbeater (Biospec Products, Bartleville, OK, USA). DNA from the seawater filter unit was isolated
according to the methods outlined in Schauer et al. (2000). The quality of extracted DNA was verified on 1% agarose gel stained with ethidium bromide and quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

4.3.5 **PCR amplification, cloning, and sequencing**

Cyanobacterial clone libraries were constructed from microbial mat samples derived from two tagged corals, each with characteristic CP lesions (colonies 1 and 3, sampled on 24 August and 7 October, respectively). Lesions on these two same colonies were repeatedly sampled as they visually transitioned into characteristic BBD lesions (fully developed BBD samples were collected on 22 September and 10 November, respectively). Cyanobacteria-specific PCR primers CYA106F (CGGACGGGTGAGTAACGCGTGA) and CYA781R [an equimolar mixture of CYA781R(a) (GACTACTGGGGTATCTAATCCCATT) and CYA781R(b) (GACTACAGGGGTATCTAATCCCTTT)] (Nubel et al. 1997) were used to amplify an approximately 700 bp region of the 16S ribosomal RNA (rRNA) gene. Each reaction mixture of 50 µl contained 0.2 µM of each primer, 0.2 mM of each dNTP, 1× PCR Reaction Buffer (TAQ DNA Polymerase kit, Scientifix, Clayton, Australia), 0.08% (w/v) bovine serum albumin, 1.25 U of TAQ DNA Polymerase (Scientifix) and 10 - 20 ng of template DNA. Amplification was performed with the initial melting at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were purified with gel extraction using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned with the T&A Cloning Kit (Real Biotech Corporation, Taipei, Taiwan) and TOP10F" Competent Cells (Invitrogen, Carlsbad, CA, USA). A total of 72 clones were randomly selected from each library and the insert product was PCR re-amplified with the M13 primers supplied with the T&A Cloning Kit. Operational taxonomic units (OTUs) were identified with restriction fragment length polymorphism using enzymes *MspI* and *RsaI* (New England BioLabs, Beverly, MA). Clones representative of each OTU group were sequenced with the M13 forward
primer (Macrogen Inc., Seoul, Korea). A cyanobacterial 16S rRNA gene clone library was also constructed from seawater sampled above the coral colonies using the method outlined above. Cloning and sequencing of a total of 96 randomly selected clones of this sample were performed at Australian Genome Research Facility Ltd. (AGRF: Brisbane, Australia).

Bacterial 16S rRNA gene clone libraries were constructed from microbial samples derived from 3 coral colonies (colonies 1 – 3), each with a characteristic CP lesion that was repeatedly sampled throughout its transition into BBD (lesions were sampled 24 August – 22 September, 7 October – 10 December, and 24 August – 23 November, respectively). PCR amplification of an approximately 1300 bp region was performed with bacterial specific forward primer 63F (CAGGCCTAACACATGCAAGTC) and reverse primer 1387R (GGGCGGWGTGTACAAGGC) (Marchesi et al. 1998). Each reaction mixture of 50 µl contained 0.2 µM of each primer, 0.2 mM of each dNTP, 1× Buffer (HotStarTaq DNA Polymerase Kit, Qiagen), 1 U of HotStarTaq DNA Polymerase (Qiagen) and 2-10 ng of template DNA. Amplification was performed with initial heating at 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) and ligated into the TOPO® TA cloning vector (Invitrogen). Cloning and sequencing of a total of 96 random clones per library using the M13 forward primer were performed by the AGRF.

4.3.6 T-RFLP analysis

Cyanobacterial community profiles of 5 lesions that visually changed from CP to BBD (lesions were sampled 24 August – 22 September, 7 October – 10 December, 24 August – 23 November, 25 October – 10 December, or 27 January – 15 February) were examined by terminal restriction fragment length polymorphism (T-RFLP). Cyanobacterial 16S rRNA genes were PCR amplified using the same reaction mixture and conditions as outlined for the cyanobacterial clone libraries, except that 0.1 µM CYA106F with the WellRED D4 fluorescent label at the 5’ end.
(Sigma-Aldrich) and 0.1 µM CYA781R were used as the primers. Labeled PCR products were quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific) and 75 ng of each product was digested with the restriction enzyme Rsal (New England Biolabs). Digested fragments were purified with the DyeEx 2.0 Spin Kit (Qiagen). Lengths of terminal restriction fragments (T-RFs) were determined and visualized using the CEQ8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA) with a size standard (600bp, Beckman Coulter) following the manufacturer’s instructions. Resulting T-RFLP profiles were compared with ones obtained from cyanobacterial clones representing each of the OTUs in the same manner.

4.3.7 Phylogenetic analysis of clone sequences

Retrieved cyanobacterial and bacterial 16S rRNA gene clone sequences were visualized and vector sequences were removed with the sequence analysis package Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were imported into the ARB software package (http://www.arb-home.de) (Ludwig et al. 2004), aligned against the Greengenes database (http://greengenes.lbl.gov) (DeSantis et al. 2006), followed by a manual correction of the alignment when necessary. Additional reference sequences not available in the Greengenes database at the time of analysis were identified from BLAST searches (Altschul et al. 1997) of sequences retrieved in this study, imported and aligned using ARB. Phylogenetic trees were constructed using the neighbor-joining (Jukes-Cantor correction) (Saitou and Nei 1987) algorithms implemented in ARB. The robustness of the inferred tree topologies was evaluated after 1000 bootstrap replicates of the neighbor-joining data. Partial sequences (~700 bp) were inserted into the tree without changing the tree topology by using the ARB parsimony interactive method.

All unique sequences retrieved from the present study have been deposited in the GenBank database under accession numbers GQ204788 - GQ204978.
4.4 Results

4.4.1 Field observations of cyanobacterial patches and black band disease

Cyanobacterial patch lesions on laminar and encrusting colonies of *Montipora* characteristically appeared either as small (1 - 5 cm in diameter), pale green/brown, semi-circular lesions at the edge of colonies or as round patches surrounded by apparently healthy coral tissue in the middle of colonies (Figure 4.1a: CP). CP lesions typically expanded radially across coral tissues and subsequently darkened at their edges (intermediate stage; Figure 4.1b: IM), leaving exposed skeleton that gradually became covered with algal turf behind the lesion front. Darkened edges of CP lesions visually changed into the dark microbial mat characteristic of BBD, which also migrated across coral colonies, killing tissue and leaving freshly exposed white skeleton (Figure 4.1c: BBD). From a total of 262 CP lesions recorded on *Montipora* colonies monitored between September 2006 and January 2009, 18.7% developed into the visually characteristic BBD lesions. The remaining CP lesions disappeared leaving only exposed coral skeleton covered with turf algae without an actively progressing microbial mat. These CP-derived BBD lesions accounted for 18.6% of a total of 263 BBD cases that were observed within this *Montipora* assemblage during the same period. Sources of infection for the remaining BBD lesions were unknown, except for 1.5% of infections that were apparently caused by direct contact with a BBD lesion on a neighboring colony.

The 2.3 year monitoring program documented that maximum prevalence of CP occurred between November and December each year, followed by a peak in BBD prevalence 40 - 50 days later (Figure 4.2). Specifically, characteristic BBD lesions developed from tagged CP lesions 62 ± 5 days (mean ± SE, n = 49 lesions) after the corresponding CP was first recorded. Annual mean (± SE) rates of linear progression for lesion fronts were 0.47 ± 0.03 mm d⁻¹ for CP (n = 55 lesions) and 2.38 ± 0.12 mm d⁻¹ for BBD (n = 65 lesions). Linear progression rates of BBD were significantly higher than those of CP lesions, regardless of the season (2-way
Figure 4.2 Temporal patterns in the prevalence of cyanobacterial patch and black band disease in a *Montipora* species assemblage on the east coast of Pelorus Island, central Great Barrier Reef. Plots and error bars represent mean prevalence ± SE. Numbers above or below plots indicate sample size (quadrats, note that 3 quadrats were established in May 2007 and were not accessible in April 2008). BBD, black band disease; CP, cyanobacterial patch.

Figure 4.3 Linear progression rates of cyanobacterial patch and black band disease on *Montipora* species during summer and winter. Columns and bars represent means ± SE. Numbers in brackets indicate sample size (lesions). BBD, black band disease; CP, cyanobacterial patch.
ANOVA, $F = 160.5$, df = 1, $p<0.001$: Figure 4.3). CP and BBD lesions both progressed more rapidly in summer than in winter.

4.4.2  *Microscopic observations of cyanobacterial patches and black band disease*

Histological examination revealed that microbial mats of both CP and BBD were dominated by cyanobacterial filaments, although the dominant cyanobacteria within the respective microbial mats were morphologically distinct (Figures 4.4a – b). Cyanobacterial filaments within CP lesions were typically two-fold greater in diameter ($9.0 – 9.2 \mu m$) than filaments in the BBD mat ($4.0 - 4.2 \mu m$). Both CP- and BBD-derived cyanobacteria demonstrated gliding motility. At the interface between the CP lesion and apparently healthy tissue, free body wall coral tissues (e.g. tentacles, oral discs, coenosarc) were degraded beneath the CP bacterial mat, however aboral structures (i.e. calicoblastic layer, lower mesenteries) appeared intact (Figure 4.4c). In contrast, tissue structure beneath the BBD mat at the interface area was severely disorganized from the oral to the aboral surface (Figure 4.4d). Trichomes penetrating into CP-infected coral tissue were observed at the base of apparently intact epidermis (Figure 4.4e), whereas BBD cyanobacteria were observed throughout the necrotic tissue mass including the gastrodermis (Figure 4.4f).
Figure 4.4 Microscopic images showing typical appearance of: (a) cyanobacterial filaments derived from cyanobacterial patches (CP) and (b) black band disease (BBD). Histological appearance of the interface between a microbial mat and tissues of the coral, Montipora hispida, for: (c, e) CP; and (d, f) BBD. CF, cyanobacterial filament; EP, epidermis; GA, gastrodermis; ZX, zooxanthella.

4.4.3 Molecular comparisons of cyanobacteria between cyanobacterial patches and black band disease

Cyanobacterial specific 16S rRNA gene clone libraries derived from microbial mats were distinct for CP and BBD lesions (Table 4.1). CP clone libraries (n = 2 lesions) were
Table 4.1 Compositions and identity of partial 16S rRNA gene sequences amplified with cyanobacterial specific primers (CYA106F/CYA781R) in clone libraries derived from microbial mats of cyanobacterial patch, black band disease on two Montipora hispida colonies and ambient seawater above the sampled colonies.

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>Accession Number</th>
<th>Closest relative</th>
<th>Identity</th>
<th>colony 1</th>
<th>colony 3</th>
<th>SW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CP</td>
<td>BBD</td>
<td>CP</td>
</tr>
<tr>
<td>CL35-CP-OTU1</td>
<td>GQ204788</td>
<td><em>Blennothrix</em> sp. PNG05-4</td>
<td>[EU253968]</td>
<td>98%</td>
<td>61</td>
<td>8</td>
</tr>
<tr>
<td>CL36-BBD-OTU1</td>
<td>GQ204789</td>
<td>Uncultured BBD cyanobacterium BB1S16S1-18</td>
<td>[EF433097]</td>
<td>99%</td>
<td>1</td>
<td>55</td>
</tr>
<tr>
<td>CL37-SW-OTU1</td>
<td>GQ204790</td>
<td>Uncultured <em>Synechococcus</em> sp. MC21</td>
<td>[DQ903987]</td>
<td>99-100%</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>CL35-CP-OTU3</td>
<td>GQ204791</td>
<td>Uncultured cyanobacterium 1DP1-B21</td>
<td>[EU780374]</td>
<td>99%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CL28-BBD-OTU3</td>
<td>GQ204792</td>
<td><em>Trichodesmium erythraeum</em> IMS101</td>
<td>[CP000393]</td>
<td>94%</td>
<td>1</td>
<td>49</td>
</tr>
<tr>
<td>CL37-SW-OTU2</td>
<td>GQ204793</td>
<td><em>Prochlorococcus marinus</em> MIT 9312</td>
<td>[CP000111]</td>
<td>99%</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>CL37-SW-OTU3</td>
<td>GQ204794</td>
<td>Uncultured <em>Plectonema</em> sp. C8</td>
<td>[DQ072876]</td>
<td>96%</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CL37-SW-OTU4</td>
<td>GQ204795</td>
<td><em>Synechococcus</em> sp. RCC307</td>
<td>[CT978603]</td>
<td>99%</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>CL37-SW-OTU5</td>
<td>GQ204796</td>
<td>Uncultured cyanobacterium A822</td>
<td>[EU283559]</td>
<td>98%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Non cyanobacterial sequences</td>
<td>-</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Sum</td>
<td>68</td>
<td>69</td>
<td>67</td>
<td>68</td>
<td>83</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CP, cyanobacterial patches; BBD, black band disease; SW, seawater sample.

*a*Sequences were aligned to the closest relative over 700bp using BLAST (Altschul et al. 1997). The similarity was calculated with gaps not taken into account.

*b*Labeling of colonies corresponds with materials and methods.

*c*Dates represent when each sample was collected.
dominated by sequences closely affiliated with a *Blennothrix* sp. (designated as CL35-CP-OTU1). In contrast, BBD libraries (n = 2 lesions) were dominated by sequences (designated as CL36-BBD-OTU1) closely related to ones previously retrieved from BBD lesions on Red Sea corals (BB1S16SI-18). This BBD dominant clone type, CL36-BBD-OTU1, also appeared in the CP libraries, although it represented only 1.5% of each library. Similarly, the CP dominant clone type, CL35-CP-OTU1, was retrieved from the BBD lesion of colony 1, representing 11.6% of the clone library. Ambient seawater collected above the diseased corals displayed a higher diversity of cyanobacterial ribotypes, although they were generally distinct from ribotypes retrieved from CP and BBD lesions, with only one sequence (CL37-SW-OTU1), related to an uncultured *Synechococcus* species MC21, shared with one CP library (colony 1).

T-RFLP profiles of amplified cyanobacterial 16S rRNA genes illustrated successional changes in dominating T-RFs from 343/344 to 149 nucleotide length fragments as CP changed into BBD (Figure 4.5). Five replicate coral colonies exhibited this identical T-RF pattern (including colonies 1 – 3, data not shown). T-RFLP analyses of representative cyanobacterial 16S rRNA gene clones confirmed that the 343/344 and 149 peaks corresponded to CL35-CP-OTU1 and CL36-BBD-OTU1, respectively, which also matched with theoretical lengths of T-RFs of CL35-CP-OTU1 and CL36-BBD-OTU1 digested with the *Rsa*I restriction enzyme.

Phylogenetic analysis of 16S rRNA gene partial sequences indicated that the dominant cyanobacterial sequences retrieved from the CP and BBD lesions were positioned in two distant groups in comparison with other reference cyanobacteria (Figure 4.6). CL35-CP-OTU1 formed a cluster related to *Trichodesmium* species, although it was more closely affiliated with a group including a *Blennothrix* species, an *Oscillatoria* sp. PAB-21, and PNG-50 retrieved from a BBD lesion on corals in Papua New Guinea. CL36-BBD-OTU1 and closely related BBD cyanobacterial sequences previously retrieved from the Red Sea (BB1S16SI-18), Palau (BMS1) and the Caribbean (128-56 and CD1C11) formed a distinct group.
Figure 4.5 Results of T-RFLP analyses of microbial communities associated with lesions on the coral, *Montipora hispida* (colony 3), showing cyanobacterial communities from: (a) cyanobacterial patch (CP) (7 Oct. 2007), (b) an intermediate stage between CP and black band disease (BBD) (25 Oct. 2007), and (c) BBD (10 Nov. 2007). Approximately 700 bp partial sequences of 16S rRNA genes were PCR-amplified with cyanobacterium-targeting primers and digested with the *Rsa*I restriction enzyme. Numbers above peaks indicate exact sizes of T-RFs. BBD, black band disease; CP, cyanobacterial patch; IM, intermediate stage between cyanobacterial patch and black band disease.
Figure 4.6 Phylogenetic neighbor-joining tree of approximately 700 bp partial 16S rRNA gene sequences of cyanobacteria derived from cyanobacterial patch (CL35-CP-OTU1) and black band disease (CL36-BBD-OTU1) with other reference sequences including cyanobacteria previously retrieved from black band disease samples (labeled with ‘BBD’). Bar indicates 0.05 change per base position.
4.4.4 Bacterial shifts during the transition from cyanobacterial patches to black band disease

The composition of bacterial 16S rRNA gene clone libraries derived from CP and BBD lesions sampled from the same colony showed distinctive phylogenetic shifts in higher bacterial taxonomic groups as the lesions transitioned from CP to BBD (n = 3 colonies, Appendix 4.2). In general, *Alphaproteobacteria* affiliated sequences were dominant in CP clone libraries (56.8 – 63.6% of libraries), followed by *Gammaproteobacteria* related sequences (26.1 - 33.0% of libraries). In contrast, BBD libraries were dominated by sequences affiliated to *Gammaproteobacteria* (23.0 – 57.0% of libraries). *Alphaproteobacteria* represented 30.1% of the BBD library of colony 1 but were only a minor constituent (5.5 -11.5%) of the other two replicate libraries. Cyanobacteria were also more commonly retrieved from BBD libraries than CP libraries, representing 57.7% of clones in the BBD library of colony 3. *Deltaproteobacteria* affiliated sequences, including *Desulfobacter* and *Desulfovibrio* spp., were retrieved from two BBD libraries, although one *Desulfovibrio* sp. affiliated sequence was also retrieved from one CP library (colony 2). *Epsilonproteobacteria* and *Deferribacteres* related sequences were retrieved only from BBD clone libraries.

The bacterial communities of disease lesions displayed successional changes during the transition from CP into BBD, as illustrated by clone libraries constructed for a lesion on colony 3 that was repeatedly sampled (Figure 4.7 and Appendix 4.3). Firstly, the libraries from the early stages of the CP lesion (CP1 and CP2) contained a limited number of cyanobacterial affiliated sequences. Even at the intermediate stage (IM), the relative number of cyanobacterial sequences represented only 7.3% of sequences, though interestingly, these cyanobacterial sequences shifted from those dominating the early CP libraries (CP1 and CP2) to sequences closely related to previously retrieved BBD cyanobacterial sequences (Appendix 4.3). When the characteristic BBD signs were first visually observed (BBD1), the relative number of cyanobacterial sequences increased to 42.6% and increased further as the lesion developed into a rapidly expanding BBD.
Figure 4.7 Comparison of clone library compositions of bacterial 16S rRNA genes amplified with bacterial specific primers (63F and 1378R) throughout the transition from cyanobacterial patch (CP1, 25Aug. 2007; CP2, 07Oct. 2007), intermediate stage (IM, 25Oct. 2007) and black band disease (BBD1, 10Nov. 2007; BBD2, 23Nov. 2007) from the identical lesion on a Montipora hispida colony (colony 3).

lesion (57.7% of BBD2 library). Secondly, coinciding with this increase in relative number of cyanobacterial affiliated sequences, Epsilonproteobacteria affiliated sequences were retrieved in the BBD1 and BBD2 libraries, although these represented only a small relative percentage of clones within the libraries (1.1 – 2.6%), while the relative abundance of Alphaproteobacterial sequences declined. Thirdly, Desulfovibrio spp. affiliated sequences first appeared in the IM library and subsequently formed small percentages (1.3 – 4.6%) of the BBD1 and BBD2 libraries. The identity of sequences retrieved from all clone libraries and their closest affiliations are compared in Appendix 4.4. Appendix 4.4 also illustrates the bacterial sequences retrieved from a healthy coral tissue sample (colony 4 - HC) and highlights the distinctiveness of this library, which shares only a few individual sequences with CP- and BBD-derived clone libraries.

4.5 Discussion

Field monitoring throughout seasonal outbreaks of BBD within an assemblage of
Montipora species in the central GBR confirmed that, in some cases, characteristic BBD develops from visually distinctive, cyanobacterium-infected lesions, herein named cyanobacterial patch(es) (CP). The 40- to 50-day earlier peak in the prevalence of CP compared to the peak in BBD prevalence, which roughly coincides with the average length of time for individually monitored CP lesions to develop into BBD, provides corroborative evidence that CP represents an early successional stage in the development of some BBD cases.

4.5.1 Cyanobacterial shift during the onset of black band disease derived from cyanobacterial patches

Histological and microbiological investigations indicated that CP and BBD lesions were comprised of morphologically and phylogenetically distinct cyanobacterial assemblages and that this shift occurred through successional changes in microbial assemblages correlated with field observations of disease signs. While cyanobacterial 16S rRNA gene clone libraries derived from CP lesions were dominated by *Blennothrix/Trichodesmium* spp.-affiliated sequences (CL35-CP-OTU1), libraries from BBD lesions were dominated by a phylogenetically distinct sequence (CL36-BBD-OTU1), which formed a tight cluster with sequences previously retrieved from BBD mats in the Red Sea (Barneah *et al.* 2007), Palau (Sussman *et al.* 2006), and the Caribbean (Cooney *et al.* 2002, Frias-Lopez *et al.* 2003). T-RFLP analyses of replicate samples (n = 5 colonies) all supported this successional change in dominant T-RFs, with peaks corresponding to CL35-CP-OTU1 in CP and CL36-BBD-OTU1 in BBD samples. Cyanobacterium-affiliated sequences in 16S rRNA gene clone libraries constructed with the bacterial specific 63F/1387R primers also support distinct CP and BBD cyanobacterial assemblages: i.e. Red Sea BBD cyanobacterium (BB1S16SI-18)-affiliated sequences dominated cyanobacterial portions of BBD libraries, while sequences affiliated with an *Oscillatoria* sp. PAB-21 appeared in CP libraries, which are phylogenetically close to *Blennothrix* sp. PNG05-4 (98% sequence identity).

Phylogenetically, the dominant cyanobacterial sequence derived from CP lesions (CL35-
CP-OTU1) clusters with *Trichodesmium* spp.-sequences and is closely related to a *Blennothrix (= Hydrocoleum)* sp.-affiliated sequence. *Hydrocoleum* spp. are the most common mat-forming benthic cyanobacteria in tropical oceans (Abed *et al.* 2003), and they contribute the major component of nitrogen fixation in tropic lagoons (Charpy *et al.* 2007). If this cyanobacterium within the CP mat functions similarly, it may play an important role in bacterial succession from CP to BBD by increasing local concentrations of fixed nitrogen and creating new niches for other bacteria utilizing these products. *Hydrocoleum* spp. are also morphologically and phylogenetically close to planktonic species of *Trichodesmium*, suggesting a common evolutionary origin (Abed *et al.* 2006). Interestingly, *Trichodesmium* spp. related 16S rRNA gene sequences have been retrieved previously from BBD bacterial communities as dominant cyanobacterial sequences, such as a *Trichodesmium tenue*-affiliated (93% sequence identity) sequence from the Caribbean (Frias-Lopez *et al.* 2002) and a sequence designated as PNG-50 from the Indo-Pacific (Frias-Lopez *et al.* 2003). Together, these results suggest that the ‘*Trichodesmium/Hydrocoleum* cluster’ (Abed *et al.* 2006) includes benthic cyanobacterial species that dominate pathogenic bacterial mats on live coral colonies such as BBD and CP. Sequences affiliated with planktonic species recovered from a seawater clone library, such as *Trichodesmium erythraeum* IMS101 and *Synechococcus* sp. MC21, also occurred in CP and BBD clone libraries. Since these sequences constitute only minor components in the CP and BBD clone libraries, it is unclear whether they are contaminants from the ambient water column or cyanobacteria playing specific roles in CP and BBD microbial communities.

Linear progression rates of BBD lesions were 5-fold greater than those of CP lesions, suggesting that the BBD bacterial community is more virulent than the CP community. Although trichomes of both CP- and BBD-dominating cyanobacterial morphotypes demonstrated gliding motility and penetration into coral tissues, BBD cyanobacteria were observed at deeper histological layers within coral polyps than CP cyanobacteria, suggesting their greater potential for mechanical or chemical destruction of coral tissues. However, it is unclear whether this is because of a greater ability of the BBD cyanobacteria to penetrate into coral tissue or because the coral
tissue was already degraded by other virulence factors associated with the BBD bacterial community. Higher biomass of cyanobacteria in the BBD mat, suggested by the greater representation of cyanobacterial affiliated sequences within BBD clone libraries, is also possibly an important factor that promotes faster progression of BBD lesions through formation of a dense microbial community that allows development of anoxic zones and consequently substantial diurnal changes between oxic and anoxic conditions (Carlton and Richardson 1995). A darkening in the color of lesions at the onset of characteristic BBD signs may be attributed to an increase in cyanobacterial densities. Furthermore, cyanobacterial toxins have been proposed to play a role in the pathogenesis of BBD (Richardson et al. 2007). The toxicity of cyanobacterial blooms of *Trichodesmium erythraeum* and *T. thiebautii*, phylogenetically close to CL35-CP-OTU1, has been reported from the GBR and Caribbean waters, respectively (Hawser and Codd 1991, Endean et al. 1993). However, the presence of toxins produced by cyanobacterial strains dominating CP and BBD lesions cultured from the study site was not detected (Glas et al. 2010), although further assessment of the role of toxins in the etiology of CP and BBD is required.

4.5.2 Changes in sulfur-cycling bacteria during the development of black band disease

Sequences involved in sulfur cycling appeared during successional changes in bacterial communities from CP into BBD, as illustrated by bacterial 16S rRNA gene clone libraries. In addition to anoxic conditions, the presence of highly concentrated sulfide at the base of the BBD mat has been demonstrated to cause coral tissue necrosis (Richardson et al. 1997) and there is clear evidence that *Desulfovibrio* spp. and other sulfide-reducing bacteria occupy microenvironmental niches within the BBD mat (Cooney et al. 2002, Frias-Lopez et al. 2002, Sekar et al. 2006, Viehman et al. 2006, Barneah et al. 2007, Sekar et al. 2008). This study retrieved sequences affiliated with *Deltaproteobacteria*, including *Desulfobacter* and *Desulfovibrio* spp. from BBD clone libraries. One *Desulfovibrio* sp.-affiliated sequence was detected in one CP library, suggesting that these organisms may already be present in the CP
bacterial mat. Interestingly, *Epsilonproteobacteria*- and *Deferribacteres*-affiliated sequences were retrieved only from BBD clone libraries. Epsilonproteobacterial sequences retrieved from BBD libraries were affiliated with *Candidatus Arcobacter sulfidicus* (94 – 97% sequence identity), a marine sulfide-oxidizing autotrophic bacterium that produces filamentous sulfur in sulfide-oxygen gradients (Wirsen *et al.* 2002). The *Deferribacteres* bacteria include iron-, manganese-, and nitrogen-reducing bacteria, found in anaerobic microenvironments (Greene *et al.* 1997, Myhr and Torsvik 2000). The sulfur cycle is often tied with other elemental cycles including nitrogen, iron, phosphorus and carbon within a microenvironment of highly diverse bacterial communities (Sievert *et al.* 2007). Thus, the presence of these *Epsilonproteobacteria* and *Deferribacteres* species, as well as *Gammaproteobacteria* and *Deltaproteobacteria*, within BBD libraries likely indicates that BBD lesions possess a more dynamic sulfur cycle with higher concentrations of toxic sulfide than CP lesions. Combined with higher cyanobacterial biomass, these bacterial element cycles may promote further anoxic sulfide rich microenvironments and hence faster lesion progression of BBD compared to CP. As the BBD band forms, diurnal fluctuations of sulfide and oxygen concentrations along the vertically stratified microenvironment are expected to occur (Carlton and Richardson 1995). Under these rapidly changing microenvironmental conditions, bacteria that can withstand such extreme fluctuations possibly become dominant in the microbial community (Sekar *et al.* 2008). Isolation and physiological characterization of CP- and BBD-dominating cyanobacterial cultures along with micro-sensor studies will verify microenvironmental regimes within the changing microbial mat from CP to BBD.

Sequences affiliated with bacteria involved in sulfur metabolism were, however, potentially under-represented in the clone library analyses. The bacterial specific primers used in this study (63F/1387R) match only 28% of *Desulfovibrio* species and less than 1% of *Epsilonproteobacteria* affiliated sequences that are available in public databases (assessed by primer comparison to the Ribosomal Database Project II). Sulfur-oxidizing *Beggiatoa* spp. have been reported as constituents of the BBD microbial mat (Richardson 1996, Viehman and Richardson 2002, Sekar *et al.* 2008), although ribotypes affiliated with *Beggiatoa* spp. were not
retrieved from CP or BBD clone libraries in the present study. This may be due to primer bias or limitations, bias in DNA preparation (Sekar et al. 2009), and/or because sequences of Beggiatoa strains specifically associated with BBD have not been deposited in public databases (Frias-Lopez et al. 2002). Nonetheless, all samples in this study were treated similarly and therefore major shifts in bacterial ribotypes are deemed to reflect the actual transition in bacterial compositions from CP into BBD. Further investigation of bacterial populations using probes and primers targeting specific bacterial groups and their functional genes (e.g. Barneah et al. 2007) will provide further insight into the development of the BBD bacterial consortium and roles of key members in the transition from CP to BBD.

4.5.3 Factors governing the onset of black band disease from cyanobacterial patches

Approximately 19% of CP lesions developed into BBD, however it is unclear what factors govern whether a CP lesion develops into BBD or loses the active microbial front. Overall, bacterial ribotype composition derived from a CP lesion that did not develop into BBD was similar to that of CP lesions which did develop into BBD (Y. Sato; unpublished data). Further investigations of intra-colony variation in bacterial members within CP lesions and of host immune responses are required to identify factors driving the development of BBD from CP lesions. Although BBD has been reported from throughout the GBR on multiple host species (Page and Willis 2006), CP lesions that develop into BBD have been observed only on Montipora species in the central inshore GBR. Therefore species- and location-specific disease mechanisms leading to the onset of BBD may exist. Previous reports have shown that BBD can be triggered from white band disease (Antonius 1985) on scleractinian corals despite the absence of a visible microbial mat in this case. The present study indicates that microbial lesions with a distinct bacterial community such as CP may develop into the characteristic BBD through successional changes in bacterial communities, but this may not be the only mode of BBD onset.
4.5.4 Conclusions

In summary, BBD lesions displayed faster band progression and hence greater virulence than CP lesions, potentially caused by distinct bacterial assemblages in the BBD microbial mat, including the dominant cyanobacterial and sulfur-cycle associated species. Formation of the characteristic BBD band may be facilitated by the bacterial community in CP lesions through alteration of microenvironmental conditions and creation of micro-ecological niches. Differences in bacterial composition also suggest that bacterial pathogen(s) that trigger the BBD infection may become less abundant during subsequent successional changes. This may be one reason why identifying the primary causative agent(s) of BBD has been difficult. Further investigations of key functional genes and their expressions at different disease stages in the CP-BBD system will provide valuable information to elucidate what role each bacterial member performs within the microbial communities and thus, which bacteria are key players in the development of BBD pathogenesis.
Chapter 5:

High-resolution profiling of Bacteria and Archaea associated with the pathogenesis of black band disease

The content of this chapter was submitted to the ISME Journal as a manuscript entitled;

“Microbial profiling identifies a novel archaeon associated with black band disease in corals”, by Sato Y, Willis BL, and Bourne DG.

The entire chapter was written by Yui Sato, with co-authors providing intellectual guidance in the design and implementation of the research and editorial contributions to the paper. Data collection, data analyses and production of tables and figures were conducted by Yui Sato.
5.1 Abstract

Reef corals are globally impacted by black band disease (BBD), caused by a microbial consortium that maintains anoxic, sulfide-rich microenvironments lethal to underlying coral tissues. Although bacterial communities associated with BBD have been studied extensively, the presence and roles of Archaea are unexplored. To understand microbial community interactions contributing to BBD pathogenicity, I investigated the diversity of Bacteria and Archaea associated with BBD and the less-virulent precursor disease state ‘cyanobacterial patch(es)’ (CP) on the coral *Montipora hispida*. Microbial profiles of BBD and CP were obtained using amplicon-pyrosequencing of 16S ribosomal RNA (rRNA) genes and illustrated community changes during the development of BBD from CP. Cyanobacterial sequences were dominated by *Oscillatoria* sp.-related ribotypes in BBD, whereas CP lesions contained *Blennothrix/Trichodesmium* spp.-related and *Oscillatoria* sp.-related ribotypes, confirming results of the clone library-based study (Chapter 4). In BBD, archaeal sequences were dominated (up to 94%) by a novel ribotype distantly affiliated to Euryarchaeotes, but CP archaeal profiles suggested the presence of diverse assemblages affiliated to species across the Thaumarchaeota and Euryarchaeota, and were similar to communities described for oxic marine environments. Although function(s) of BBD-associated Archaea are unknown due to the novelty of the 16S rRNA sequences, I present a model for potential metabolic processes within BBD microbial mats. Given organic- and sulfide-rich anoxic microenvironments within BBD lesions, I hypothesize that BBD-associated Archaea carry out methanogenesis and/or anaerobic methane-oxidation, syntrophically coupled with bacterial sulfate-reduction, thereby potentially enhancing the virulence of BBD. Discovery of this novel archaeal ribotype provides new insights into the microbial ecology of BBD.
5.2 Introduction

Previous studies have provided evidence that the microbial mat of BBD is comprised of cyanobacteria, sulfur cycle-related bacteria and other heterotrophic microorganisms that create anoxic, sulfide-rich microenvironmental conditions (Cooney et al. 2002, Frias-Lopez et al. 2002, Barneah et al. 2007, Sekar et al. 2008; Chapter 4), which underlie the pathogenicity of BBD (Carlton and Richardson 1995, Richardson et al. 1997, Richardson et al. 2009). In the central inshore reef study presented in Chapter 4, I documented the presence of cyanobacterial-dominated disease lesions, named ‘cyanobacterial patches’ (CP’s), which preceded the development of BBD infections in approximately 20% of cases found in an assemblage of corals in the genus of Montipora. Bacterial communities associated with CP undergo successional changes as the disease develops into BBD, providing a unique opportunity to study the development of BBD pathogenicity. The lower virulence of CP lesions (progression rates of CP were fivefold less than those of BBD) correlated with lower abundance of bacteria involved in sulfur-cycling, as determined using molecular finger-printing techniques (Chapter 4). Furthermore, results of recent collaborative studies demonstrated that, as bacterial communities transitioned from CP to BBD-characteristic communities, the abundance of sulfate-reducing bacteria (SRB) in lesions increase (Appendix A; Bourne et al. 2011) and concurrently, anoxic and sulfide-rich microenvironmental conditions form within disease mats (Appendix B; Glas et al. 2012). The virulence of BBD lesions (band-progression rates) was correlated with intensification of these microenvironmental chemical conditions (Appendix B), highlighting their importance in BBD pathogenicity. Although bacterial communities associated with BBD have been studied extensively (see meta-community analyses in Miller and Richardson 2011 for a recent review), the possibility that hitherto unknown groups, like the Archaea, contribute to the development of anoxic and sulfide-rich microenvironmental conditions in BBD lesions has not been explored.

The Archaea inhabit diverse environmental conditions and have important roles in nutrient-cycling (reviewed in Cavicchioli 2011), but their presence and roles in the development of
BBD lesions have not yet been documented. Archaeal populations may account for substantial proportions of microbial biomass in extreme conditions, including anoxic and sulfurous marine environments (Knittel et al. 2005, Schleper et al. 2005), as well as surface and deep seawater columns (Massana et al. 1997, DeLong et al. 1999, Karner et al. 2001). It is thus likely that anoxic and sulfide-rich environments in the BBD mat represent ideal niches for certain Archaea, which potentially have important roles in the microbial ecology of the BBD consortia. Therefore investigations of archaeal diversity within the BBD mat may provide further insights into BBD pathogenicity.

In this chapter, I profiled both bacterial and archaeal communities within BBD mats using amplicon-pyrosequencing of 16S ribosomal RNA (rRNA) coding genes, and directly compared them to communities within less-virulent lesions of the disease precursor stage, CP. This characterization of shifts in microbial communities during disease onset further identifies microbial members that are key to the virulence of BBD, and represents an important step for better mechanistic understanding of BBD pathogenicity.

5.3 Materials and methods

5.3.1 Study site and sample collection

Assemblages of the common scleractinian coral Montipora spp. were monitored along the east coast of Pelorus Island, located in the central inshore GBR (18°33’S, 146°30’E; see Chapter 2), between October 2007 and November 2009. Colonies of Montipora hispida naturally infected with CP were individually marked and development of the characteristic signs of BBD (see Chapter 4 for changes in macroscopic signs) was followed in situ at intervals of between 2 weeks and 1 month. Samples of microbial mats were collected from 3 coral colonies (colonies 1 – 3; Table 5.1), including the initial CP lesion stage and lesions characteristic of fully developed
BBD (total of 6 samples). Samples labeled with the same number (for example ‘CP1’ and ‘BBD1’) were collected from the identical lesion at different stages of development and hence at different time points (Table 5.1). A small portion of the microbial mat, approximately 5 mm in diameter, 2 mm in depth and including the surface of the underlying skeleton, was collected from the progressing front of each lesion using a separate sterilized stainless steel chisel for each sample to avoid cross contamination. Samples were immediately placed in individual 1.5 ml screw cap tubes underwater and kept on ice upon returning to the surface. Within 4 hrs of sampling, excess seawater was removed and lesion samples were preserved in 100% ethanol at -20 °C.

**Table 5.1** Microbial mat samples examined and number of obtained archaeal and bacterial 16S ribosomal RNA gene sequences in bacterial and archaeal diversity assays

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<th>Colony 2</th>
<th>Colony 3</th>
</tr>
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<td>CP BBD</td>
<td>CP BBD</td>
</tr>
<tr>
<td><strong>Sample name</strong></td>
<td>CP1 BBD1</td>
<td>CP2 BBD2</td>
<td>CP3 BBD3</td>
</tr>
<tr>
<td><strong>Bacterial pyrosequencing assay</strong></td>
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<tr>
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<td>4271</td>
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<tr>
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<td>4015</td>
<td>4271</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<td>3.7</td>
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</tr>
<tr>
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<td>70</td>
<td>1169</td>
<td>-</td>
</tr>
<tr>
<td>Non-archaeal reads</td>
<td>5020</td>
<td>5092</td>
<td>-</td>
</tr>
<tr>
<td>Shannon Index(^a) (archaea)</td>
<td>3.63</td>
<td>0.75</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Shannon Indices were calculated from the maximum number of either bacterial or archaeal sequences obtained from each sample in the respective assays.

\(^b\) Tag-encoded amplicon pyrosequencing assay using archaeal 16S rRNA gene -targeting primers, 340F (Gantner et al. 2011) and 915R (Stahl and Amann 1991).

\(^c\) Tag-encoded amplicon pyrosequencing assay using archaeal 16S rRNA gene -targeting primers, Arch349F and Arch806R (Takai and Horikoshi 2000).

Hyphens denote results that were not obtained. Abbreviations: CP; cyanobacterial patches, BBD; black band disease.
5.3.2 Sample preparation and sequencing

DNA was extracted from preserved lesion samples with the PowerPlant® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) as previously described in Chapter 4. Community compositions of bacterial 16S rRNA genes in samples CP1 – CP3 and BBD1 – BBD3 were examined using a tag-encoded amplicon pyrosequencing assay with bacterial-targeting universal primers Gray28F (5’-TTTGATCNGCTGCTCAG-3’) and Gray519R (5’-GTNTTACNGCGGCKGCTG-3’) (Ishak et al. 2011). Archaeal 16S rRNA gene compositions for each of the samples CP1, CP3, BBD1, and BBD3 was examined with two tag-encoded amplicon pyrosequencing assays using different sets of archaeal-targeting primers, namely 340F (5’-CCCTAYGGGGYGCASCAG-3’) (Gantner et al. 2011) plus 915R (5’-GTGCTCCCCCGCCAATTCCT-3’) (Stahl and Amann 1991) (assay 1), and Arch349F (5’-GYGCASCAGKCGMGAAW-3’) plus Arch806R (5’-GGACTACVSGGTATCTAAT-3’) (Takai and Horikoshi 2000) (assay 2). For the bacterial and archaeal amplicon pyrosequencing, PCR amplification and sequencing utilizing the Roche 454 FLX instrument and Titanium reagents (Roche, Branford, CT, USA) were performed at the Research and Testing Laboratory (Lubbock, TX, USA; detailed protocols are available at http://www.researchandtesting.com). Although archaeal 16S rRNA gene sequences from CP2 and BBD2 samples were PCR-amplified, concentrations of the amplified products were insufficient for downstream pyrosequencing. Consequently, CP2 and BBD2 were excluded from archaeal diversity assays.

5.3.3 Sequence analyses

The quality of amplicon pyrosequencing reads was first checked using 25 as a threshold average quality-score in the sequence analysis platform QIIME (Caporaso et al. 2010). Sequences potentially containing chimeric artefacts were removed using UCHIME (Edgar et al. 2011), with the SILVA 16S rRNA alignment (Pruesse et al. 2007) as a reference dataset. Clean 16S rRNA
sequences sharing more than 97% sequence identity were clustered into operational taxonomic units (OTUs) using UCLUST (Edgar 2010). Taxonomic assignment of representative sequences of OTUs was performed with a BLAST-based method implemented in QIIME, searching against the Greengenes 16S rRNA gene sequence database (Altschul et al. 1997, DeSantis et al. 2006) with an E-value threshold of 0.001. Sequences without hits were re-checked against the NCBI-nr database (http://www.ncbi.nlm.nih.gov/) for 16S rRNA gene sequences retrieved from uncultured Bacteria or Archaea using a BLAST search (Altschul et al. 1997) with the same E-value threshold. Sequences apart from archaeal 16S rRNA gene sequences (i.e. those annotated as bacterial 16S rRNA genes or genes other than 16S rRNA in the above database searches) were excluded from further analyses of archaeal diversity (Table 5.1). Indicators for the representativeness of sequencing effort and diversity of bacterial and archaeal 16S rRNA genes were computed as rarefaction curves of observed OTUs (200 replications per step) and the Shannon Index using the maximum number of sequences obtained in each sample, respectively, both implemented in QIIME. To examine phylogenetic relationships of archaeal 16S rRNA gene sequences, OTU sequences retrieved and relevant reference archaeal sequences were aligned against the SILVA database (Pruesse et al. 2007) using the SINA online aligner (http://www.arb-silva.de/aligner/). Phylogenetic trees of aligned sequences were constructed with the FastTree algorithm (Price et al. 2009; implementing Neighbour-Joining method with improved accuracy and efficiency), and visualized with the phylogenetic illustration software FigTree (Rambaut, unpublished; available at http://tree.bio.ed.ac.uk/software/figtree/). Archaeal and bacterial 16S rRNA gene OTU sequences identified in this study were deposited in the GenBank database under accession numbers JX021771 – JX022549. The raw pyrosequencing readings were submitted in Study SRP012616 in the NCBI Sequence Read Archive.

5.4 Results
5.4.1 Bacterial diversity

The bacterial pyrosequencing assay retrieved between 2419 and 4336 16S rRNA gene sequences per sample (Table 5.1), with an average length of 271 nucleotides. Diversity indices of bacterial 16S rRNA genes were greater in CP than in BBD lesions for colonies 2 and 3, whereas diversity indices were similar between CP and BBD for colony 1 (Table 5.1). Rarefaction curves for bacterial OTUs detected did not reach a plateau (Appendix 5.1), indicating that CP and BBD microbial mats potentially contained more bacterial ribotypes than detected.

A shift in the taxonomic composition of bacterial 16S rRNA gene sequences was detected as CP lesions developed into BBD (Figure 5.1; see Appendix 5.2 for a full list of the taxonomic affiliations of bacterial sequences detected in CP and BBD lesions). Cyanobacterial sequences

![Figure 5.1](image-url)  
*Figure 5.1* Relative composition of bacterial 16S rRNA gene affiliations obtained from amplicon-pyrosequencing of microbial-mat samples derived from cyanobacterial patch (CP1, CP2 and CP3) and black band disease lesions (BBD1, BBD2 and BBD3). See Table 5.1 for the total number of retrieved sequences for each sample.
accounted for a comparatively large proportion (CP; 19 - 86%, BBD; 58 - 91%) of sequence reads obtained for both CP and BBD samples (Figure 5.1). Within the Cyanobacteria, Oscillatoria sp.-affiliated sequences dominated in all three BBD samples (more than 97% of cyanobacterial sequences). In contrast, Trichodesmium sp.-affiliated sequences comprised the major proportion of cyanobacterial sequences in all CP samples (26 - 65% of cyanobacterial sequences), although Oscillatoria sp.-related sequences were also present in all CP samples and accounted for between 2 and 54% of total sequences. These cyanobacterial sequences had high sequence identity to Trichodesmium- and Oscillatoria-spp.-related ribotypes previously retrieved from the lesions of CP and BBD, respectively (more than 98% sequence identity over 285 nucleotides; Glas et al. 2010, Chapter 4). The relative abundance of alphaproteobacterial sequences decreased in colonies 2 and 3 as lesions developed from CP into BBD (Figure 5.1), whereas colony 1 showed a small increase (11% in CP to 17% in BBD). However, if sequences affiliated to bacterial groups other than Cyanobacteria were separately compared between CP and BBD samples, BBD samples had lower proportions of Alphaproteobacteria-affiliated sequences than those in CP samples in all three replicate colonies (41% in BBD1 vs. 77% in CP1, 0% in BBD2 vs. 59% in CP2, 45% in BBD3 vs. 77% in CP3; Appendix 5.2). In contrast, Epsilonproteobacteria-affiliated sequences accounted for less than 1% of sequences retrieved from CP lesions on colonies 1 and 2, though their relative abundance in BBD lesions increased to 7% and 29% of retrieved sequences, respectively (Figure 5.1). Only a small number of Epsilonproteobacteria-affiliated sequences were retrieved from CP- and BBD-lesions on colony 3 and the relative abundances of these sequences were comparable (2% in CP3 and 1% in BBD3). However, BBD samples had higher relative proportions of epsilonproteobacterial sequences than CP samples in all three replicate colonies if sequences affiliated to bacterial groups other than Cyanobacteria were separately compared. Within Epsilonproteobacteria, sequences affiliated to Arcobacter sp. increased from between 1 and 2% in CP to between 13 and 74% of retrieved sequences in BBD (cyanobacterial sequences excluded from comparison; Appendix 5.2). Sequences affiliated with sulfate-reducing bacteria (SRB) belonging to the families of Desulfobulbaceae, Desulfovibrionaceae, and
Desulfobacteraceae were detected in small relative abundances in both CP and BBD samples (Appendix 5.2). *Gammaproteobacteria*-affiliated sequences represented between 2 and 15% of the total sequences detected in CP and BBD samples (Figure 5.1), including sequences affiliated to *Thalassomonas* sp. and species in the families of Alteromonadaceae, Pseudoalteromonadaceae, Oceanospirillaceae, and Vibrionaceae (Appendix 5.2). Ribotypes detected in all CP samples but not in BBD samples included *Microscilla marina*, *Asticcacaulis excentricus*, and *Inquilinus* sp.-associated sequences (Appendix 5.2). Other *Bacteroidetes* - and *Firmicutes*-affiliated sequences occurred in CP and BBD samples, but did not show distinctive trends in relative abundances in comparisons between CP and BBD samples (Figure 5.1). These general trends in bacterial diversity and relative sequence abundance were consistent with a previous study based on sequencing of clone libraries (Chapter 4).

### 5.4.2 Archaeal diversity

The first archaeal amplicon-pyrosequencing assay (assay 1) generated between 1295 and 5355 archaeal 16S rRNA gene sequences with an average length of 343 nucleotides after the removal of contaminating bacterial sequences (Table 5.1). Archaeal sequences accounted for between 78% and 90% of the total sequences in assay 1, but comprised only a low proportion (between 1% and 49%) of sequences retrieved when a different primer set was used in assay 2 (Table 5.1), with sequences having an average length of 320 nucleotides. In particular, low numbers of archaeal reads were obtained for CP samples (43 and 70 reads) compared with BBD samples (1169 and 2484 reads). Diversity indices for archaeal 16S rRNA gene libraries were consistently higher for CP samples than for BBD samples in both assays (Table 5.1). Rarefaction curves of observed archaeal OTUs also indicated that CP samples were more diverse than BBD samples, although curves did not reach a plateau for any sample (Appendix 5.1), indicating that larger numbers of archaeal ribotypes can be expected with increased sequencing efforts. Archaeal OTUs identified in the CP samples were phylogenetically diverse and grouped into 3
clusters (clusters A, B, and C) within the Thaumarchaeota and Euryarchaeota (Figure 5.2).

**Figure 5.2** Phylogenetic positions of archaeal 16S rRNA gene sequences of all OTUs identified from cyanobacterial patch (CP) and black band disease (BBD) microbial mat samples, in relation to representative reference Archaea chosen from all archaeal phyla currently recognized (Brochier-Armanet et al. 2011). Square symbols represent OTUs obtained in assay 1 and closed circles represent OTUs obtained in assay 2. Blue, red, and yellow symbols represent OTUs that were CP-unique, BBD-unique, and shared between CP and BBD, respectively. Larger symbols indicate that the corresponding OTU comprised more than 1% of sequences in at least one library. Scale bar represents a phylogenetic distance of 0.1 nucleotide change per position. Local bootstrap support values are indicated at nodes where the values indicated more than 0.5.
Relative abundances of CP-sequences derived from assay 1 were 64% in cluster A, 3% in cluster B and 33% in cluster C (percentages averaged for CP1 and CP3). The relative abundances of sequence ribotypes retrieved in assay 2 were similar to assay 1 across these three clusters (A; 42%, B; 11%, C; 47%). OTU sequences within cluster A demonstrated close affiliation with Thaumarchaeotes, including *Cenarchaeum symbiosum*, *Nitrosopumilis maritimus*, *Candidatus (Ca.) Giganthauma karukerense*, *Ca. Giganthauma insulaporcus* and other uncultured Archaea belonging to Marine Group 1, which are commonly detected in diverse marine environments (DeLong 1992) (Figure 5.3; cluster A). OTUs from cluster B, which fell within the Euryarchaeota, were closely related to ribotypes of uncultured marine planktonic Archaea affiliated with Marine Group 2 (DeLong 1992, DeLong 1998). Two dominant ribotypes were retrieved from the CP3 sample in the two separate assays (Figure 5.3; assay1_OTU314 and assay2_OTU915) and fell within cluster C in the Euryarchaeota, which was distantly related to 16S rRNA gene sequences of anaerobic methanogens (*Methanobacterium beijingense*, *Methanogenium boonei*, *Methanosarcina mazei*), anaerobic methanotrophic Archaea (ANME1, ANME2, ANME3), and the chemo-organotrophic archaeon *Halobacterium jilantaiense*. These dominant OTUs (along with other retrieved sequences, for example assay2_OTU452) formed a paraphyletic sub-cluster termed ‘sub-cluster C-1’ (see Figure 5.3).

The majority of sequences derived from BBD samples (92% and 99% in assay 1 and 2, respectively; percentages averaged for BBD1 and BBD3) also fell into cluster C (Figure 5.2). The vast majority of these BBD-derived sequences within cluster-C (more than 99.9%) were tightly grouped in a sub-cluster termed C-2 (see Figure 5.3), which was phylogenetically distinct from sub-cluster C-1, sharing a maximum of 85% sequence identity (BLAST comparison of assay1_OTU314 vs. assay2_OTU591; Figure 5.3). A large number of sequences derived from CP samples also fell into sub-cluster C-2 (Figure 5.3; assay2_OTU872, assay2_OTU592, and assay1_OTU160). Within sub-cluster C-2, two archaeal OTUs, assay1_OTU160 and assay2_OTU592, dominated the BBD-sequences (Figure 5.3). These sequences were retrieved in both archaeal assays, which used different primer sets, and shared 361 identical nucleotides over
Figure 5.3 Phylogenetic relationships of archaeal 16S rRNA gene sequences of representative OTUs identified in microbial mat samples of cyanobacterial patches (CP) and black band disease (BBD) and reference archaeal sequences. Bold characters indicate OTUs identified in this study. OTUs accounting for more than 1% in one or more samples are indicated. Numbers in panels adjacent to OTUs indicate relative abundance (%) of the representative OTUs within a sample, obtained in pyrosequencing assay 1 (right column) and assay 2 (left column) of microbial mat samples for CP (blue: left; CP1, right; CP3) and BBD (red: left; BBD1, right; BBD3). Relative abundances of OTUs were categorized into three groups and indicated by filling patterns of panels (<5%; no filling, >5%; light shading, >10%; filled). Scale bar represents a phylogenetic distance of 0.1 nucleotide change per position. Cluster labels correspond to Figure 5.2.
362 positions (99.7% sequence identity), indicating that they were likely recovered from a single entity at a species level. These BBD-dominating archaeal 16S rRNA gene sequences and other closely related OTU-sequences in sub-cluster C-2 appeared to be novel archaeal ribotypes (Figure 5.3). They shared only 88% sequence identity with any known archaeal 16S rRNA sequence (a BLAST search against the NCBI-nr/nt database). The most closely related sequences at the time of study (June 2012) were retrieved from uncultured Archaea detected in deep sea hydrothermal vent water (Nercessian et al. 2003) and oxygen-depleted water in cold saline springs (Perreault et al. 2007).

5.5 Discussion

5.5.1 A novel archaeal ribotype dominates black band disease-associated archaeal communities

This study provides evidence that unique archaeal populations are members of the microbial consortium characteristic of black band disease lesions on the coral *Montipora hispida*. Results of archaeal 16S rRNA gene profiling indicate that as BBD lesions develop, the archaeal community shifts from being phylogenetically diverse in the early CP stage to a less diverse community dominated by a novel archaeon in mature BBD lesions. The potential functional role of the archaeal ribotype dominating BBD communities is of interest, but remains speculative as its 16S rRNA phylogenetic position is highly novel (sub-cluster C-2; Figure 5.2 and 5.3). Its ribotype is affiliated with an unknown group within the Euryarchaeota, but it is only distantly related to archaeal ribotypes retrieved from sulfurous environments with depleted oxygen levels (Figure 5.3; AF526960 and DQ521152, Nercessian et al. (2003) and Perreault et al. (2007)). Similarly, other retrieved sequences from both BBD and CP lesions within cluster C, including the majority of BBD-derived sequences, are distantly related to methanogenic- and methanotrophic-
Euryarchaeotes and other uncultured Archaea (EU731562, AJ296315 and GU190985; Figure 5.3) found in anoxic and sulfate-reducing environments (Boetius et al. 2000, Rudolph et al. 2001, D’Hondt et al. 2002, Robertson et al. 2009, Alperin and Hoehler 2010, Lloyd et al. 2010, Cavicchioli 2011). These phylogenetic relationships suggest that the archaeal sequences dominating BBD samples are derived from an archaeal species that can survive or thrive under the anoxic and high sulfide concentrations present in the BBD mat (Carlton and Richardson 1995, Appendix B).

The archaeon found to dominate BBD communities may obtain energy through methanogenesis (hypothesis 1), anaerobic methane-oxidation (hypothesis 2), and/or chemo-organotrophic metabolism (hypothesis 3). These functional roles are likely supported by the high influx of organic matter derived from necrotic coral tissue produced through rapid progression of BBD lesions (see Chapter 4). Various species of Euryarchaeotes produce methane using molecular hydrogen (H₂) plus carbon dioxide (CO₂), acetate (CH₃COO⁻), or other methylated compounds under strictly anaerobic conditions (Pace 1997, Simankova et al. 2003, Ma et al. 2005, Kendall et al. 2007, Cavicchioli 2011). In the presence of sulfate (SO₄²⁻), methanogens are typically outcompeted by sulfate-reducing bacteria (SRB) for these metabolic substrates due to the high affinity of SRB for their energy sources, including H₂, CH₃COO⁻, fatty acids and other carbon compounds (Sansone and Martens 1982, Holmer and Kristensen 1994). However, with a readily available influx of organic matter, methanogens and SRB can co-exist and produce methane and sulfide because fatty acids from the source of organic influx (such as necrotic coral tissue) are used by SRB as their major energy source, and H₂, CO₂ and CH₃COO become available for methanogenic Archaea (Holmer and Kristensen 1994). Methanogenesis co-occurring with sulfate reduction through this mechanism has been well documented in high organic-input environments (Holmer and Kristensen 1994). Figure 5.4 schematically illustrates the potential co-existence of sulfate-reducers (red arrows) and methanogenic Archaea (hypothesis 1; arrows with broken lines) in BBD, both utilizing organic matter derived from necrotic coral tissue.
Figure 5.4 Model of metabolic process within the microbial mat of black band disease, focusing on potential functions of Archaea (methanogenesis (hypothesis 1) and anaerobic methane-oxidation (hypothesis 2)) coupled to sulfate-reduction by sulfate-reducing bacteria (SRB) under high levels of organic input from lysing coral tissue. Necrotic coral tissues are decomposed by bacterial heterotrophs into sulfate ($SO_4^{2-}$), molecular hydrogen ($H_2$), acetate ($CH_3COO^-$), fatty acids, and other organic compounds. $H_2$, fatty acids, $CH_3COO^-$ and other organic compounds are utilized by SRB, during reduction of $SO_4^{2-}$ into sulfide ($H_2S$) (red arrows). $H_2$, $CO_2$, $CH_3COO^-$ and other methylated compounds are used by methanogenic Archaea in $CH_4$-production (hypothesis 1; arrows with broken lines). $CH_4$ is oxidized to bicarbonate ($HCO_3^-$) and water ($H_2O$) by methane-oxidizing Archaea (hypothesis 2; blue arrow) coupled with SRB in a syntrophic relationship (red bold arrow). Locations of components are not drawn to scale.

Alternatively, the dominant BBD archaeon is potentially an anaerobic methane-oxidizer (termed ‘ANME’; Hinrichs et al. 1999), utilizing methane as a carbon substrate through a syntrophic relationship SRB (reviewed in Knittel and Boetius 2009) (Figure 5.4; hypothesis 2). SRB serve as an electron sink with $SO_4^{2-}$ being utilized as the electron acceptor, and the net reaction of the ANME-SRB syntrophy is described in the following equation (Knittel and Boetius 2009): $CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O$. This potential coupling of methane-oxidation and sulfate-reduction is illustrated with a blue arrow (Archaea) and bold red arrow (SRB) in Figure 5.4. The possibility of the BBD-dominant archaeon being an ANME is supported by the increase in SRB observed during the development of BBD from CP (Appendix A). This proposed metabolic function is not mutually exclusive with the first hypothesis that the BBD-dominant archaeon is a
methanogen. Evidence based on microbial distributions and lipid-biomarkers associated with methane-cycling Archaea suggests that the same micro-organisms are involved in both anaerobic oxidation of methane and methanogenesis (Orcutt et al. 2005, Orcutt et al. 2008, Knittel and Boetius 2009), supporting this hypothesis.

A third possibility (hypothesis 3) is that the dominant BBD archaeon is an anaerobic or facultative aerobic chemo-organotroph with a metabolic mode similar to that observed in *Halobatreria* spp. (Yang et al. 2006), although potentially using SO$_4^{2-}$ as an electron accepter and scavenging carbon compounds available in the microbial mat of BBD (Cavicchioli 2011). Testing these hypotheses and identifying the biological capabilities of the BBD-dominating archaeon clearly requires further study, ideally involving enrichment, cultivation, and/or genome analyses of the target archaeon (Brochier-Armanet et al. 2011).

In contrast to BBD lesions, the phylogenetic affiliations of CP-associated archaeal ribotypes are widely distributed among 3 clusters formed within the Thaumarchaeota and Euryarchaeota (Figure 5.3; cluster A, B and C). Although the functional roles of Archaea belonging to Marine Group 1 and Marine Group 2 (within clusters A and B) are currently unclear due to a paucity of representative strains that have been cultured (Schleper et al. 2005, Brochier-Armanet et al. 2011), they have been observed as abundant members of microbial communities in marine water columns and sediments (DeLong 1998, Karner et al. 2001, Heijs et al. 2008, Durbin and Teske 2010, Belmar et al. 2011), as well as coral mucus (Kellogg 2004, Siboni et al. 2008). The microbial mat of the CP stage of disease development is characterized by oxic microenvironmental conditions with moderate oxygen-fluctuations between day and night (Appendix B), which is similar to environmental conditions from which these archaeal ribotypes have previously been recovered. Cluster A included ribotypes closely related to aerobic ammonia-oxidizers (*C. symbiosum* and *N. maritimus*), and thus CP-associated Archaea may aerobically utilize ammonium available through ammonification of organic compounds or produced by nitrogen-fixers present within the lesions. However, growth of aerobic ammonium oxidizers is generally inhibited by the presence of organic compounds (Konneke et al. 2005), therefore the
presence of these sequences may suggest low organic-input rates within CP lesions. In contrast, only a few ammonium oxidizer-affiliated sequences were retrieved from BBD, possibly due to the higher organic-influx present in BBD lesions. Other OTUs in cluster A were closely affiliated to Ca. G. karukerense and Ca. G. insulaporcus (assay1_OTU259 and assay2_OTU221; Figure 5.3). These Archaea were described from a sulfurous mangrove swamp, with sulfide-oxidizing bacteria found to be coating the surface of Ca. G. karukerense cells (Muller et al. 2010). It is unclear if these OTUs are typically associated with CP lesions because of inconsistency in their relative abundance among samples (Figure 5.3), although it is possible that they are associated with marginal concentrations of sulfide present in CP lesions (Appendix B) and CP-inhabiting sulfide-oxidizers (D. Bourne, Y. Sato, E. Botté and M. van der Zee; unpublished data). CP-derived archaeal sequences in cluster B are affiliated to Marine Group 2 Archaea, and closely related with a sequence detected from oxygenated sea surface water (DeLong 1992) and coral mucus (Kellogg 2004) (Figure 5.3). Although Marine Group 2 Archaea have been described throughout the oxygen-gradient (Belmar et al. 2011), archaeal ribotypes detected in CP samples are distantly related to sequences of Marine Group 2 Archaea retrieved from oxygen-depleted zones in the deep ocean (JF715335; Figure 5.3). This phylogenetic relationship suggests that CP-derived archaeal OTUs in cluster B represent facultative anaerobes that usually thrive in oxic conditions present in CP lesions (Appendix B).

Interestingly, many archaeal sequences derived from CP samples fall within cluster C and are identical to the dominant OTUs in BBD (Figure 5.3). These OTUs (assay1_OTU160 and assay2_OTU592) represented 7% and 11% of retrieved sequences in the CP1-sample, respectively. This result indicates that small archaeal populations characteristic of BBD can exist in CP and become dominant during the development of BBD. In contrast, the CP3-sample was dominated by two OTUs, assay1_OTU314 and assay2_OTU915, which represented 41% and 44% of retrieved sequences in assay 1 and assay 2, respectively (Figure 5.3). These sequences in cluster C are phylogenetically distinct from the BBD-dominating sequences, suggesting the presence of other novel types of Archaea in CP lesions, which may be outcompeted during the development of BBD.
lesions. This finding would also imply that the CP1 sample was at a later stage in the transition to BBD than CP3, which may explain the larger component of Oscillatoria sp.-related sequences in CP1 than CP3 (Figure 5.1).

Two different PCR-based pyrosequencing assays, using different primers but otherwise identical samples and protocols, support the generality of my findings. The primer set used for assay 1, which was selected to maximize the phylogenetic coverage of archaeal 16S rRNA sequences and selectively target Archaea over Bacteria, resulted in a higher number of archaeal reads than assay 2 (Table 5.1). This result highlights that specific primers target different archaeal groups but may also amplify bacterial sequences, leading to biases in amplicon-libraries. Nevertheless, consistency in the results provided by the two archaeal diversity assays provides corroborative evidence that archaeal assemblages change during the development of BBD and one archaeon dominates archaeal communities in BBD.

5.5.2 Shifts in bacterial communities from cyanobacterial patches to black band disease

A shift in the dominant cyanobacterial ribotype is the most distinctive change in bacterial communities during the development of BBD from CP. Cyanobacterial 16S rRNA gene sequences retrieved from CP and BBD samples are identical to previously identified dominant ribotypes affiliated to Blennothrix/Trichodesmium- and Oscillatoria-spp. in CP and BBD lesions, respectively (see Chapter 4). The Oscillatoria sp.-affiliated ribotype is also highly similar (99% sequence identity) to sequences recovered from BBD lesions collected in the Red Sea (Barneah et al. 2007, Arotsker et al. 2009), the Caribbean (Cooney et al. 2002, Frias-Lopez et al. 2002, Sekar et al. 2008) and Palau (Sussman et al. 2006) and a cyanobacterial culture of Pseudoscillatoria coralii isolated from BBD lesions in the Red Sea (Rasoulouniriana et al. 2009). Cyanobacterial sequences abundant in CP are affiliated with nitrogen-fixing Trichodesmium sp. (Charpy et al. 2007) and closely associated with Blennothrix sp.-related cyanobacteria, which are abundant in oligotrophic oceans (Abed et al. 2003).
Comparison of sequences retrieved from CP and BBD lesions excluding cyanobacterial sequences indicate that BBD lesions typically have lower relative abundance of Alphaproteobacteria and higher relative abundance of Epsilonproteobacteria than CP lesions, while sequences related to Gammaproteobacteria and SRB belonging to Deltaproteobacteria are present in both CP and BBD lesions. These trends are consistent with patterns observed in a previous study that characterized CP- and BBD-associated bacterial ribotypes through construction of clone-libraries and sequencing (Chapter 4). Epsilonproteobacterial sequences affiliated to Arcobacter sp. were abundant in BBD1 and BBD2, and Arcobacter spp. have previously been speculated to contribute to the occurrence of BBD, as they represent known human enteropathogens (Frias-Lopez et al. 2002). Arcobacter spp. have been also associated with cycling of enriched organic matter in sulfide-rich and oxygen-depleted environments (Campbell et al. 2006), which are similar to microenvironments found in the BBD lesions (Carlton and Richardson 1995, Appendix B).

Other bacterial species that are phylogenetically similar to Cytophaga spp., Clostridium spp., an alphaproteobacterium responsible for juvenile oyster disease (JOD), and Vibrio spp., have previously been detected in BBD lesions and suggested to be responsible for the virulence and occurrence of BBD in the Caribbean and the Red Sea (Cooney et al. 2002, Frias-Lopez et al. 2002, Barneah et al. 2007, Arotsker et al. 2009). In this study, relative abundances of Clostridium sp.-affiliated ribotypes and Thalassobius mediterraneus-affiliated sequences (most closely related to the sequence previously detected in BBD and affiliated to the JOD pathogen in Cooney et al. (2002)) substantially increased during the onset of BBD from CP in two cases. This suggests that they are potentially involved in the development of BBD virulence and thus further investigations of their functional roles in BBD etiology are merited. Conversely, sequences associated with Vibrio spp. did not show a consistent increase as CP lesions developed into BBD lesions, and Cytophaga sp.-affiliated sequences were not retrieved from CP or BBD samples, despite their sequence match with the primers used in the assay (checked against SILVA database). Sequences related to the sulfide-oxidizing bacteria Beggiatoa spp., microscopically described as abundant
members of BBD microbial communities (Ducklow and Mitchell 1979), were not detected in my pyrosequencing libraries, likely due to a mismatch of the reverse primer.

Some OTUs affiliated within the Alphaproteobacteria (Asticcacaulis excentricus-, and Inquilinus sp.) and Bacteroidetes (Microscilla marina) were only found in CP samples. A sequence identical to the Inquilinus sp.-related sequence has previously been retrieved from CP lesions (Chapter 4). Microscilla marina-affiliated sequences were highly similar (98% sequence identity) to the ribotype of a bacterial epibiont associated with Trichodesmium cyanobacteria (Mann et al., 2011, unpublished; available in NCBI-nr database), which indicates that cyanobacteria within the lesions may have a driving influence on other associated bacteria. Asticcacaulis excentricus-affiliated sequences were detected only in CP samples (up to 17% of sequences in CP3; Appendix 5.2) though they were highly similar (99% sequence identity) to bacterial ribotypes previously retrieved from BBD lesions affecting the Caribbean coral Siderastrea siderea (Sekar et al. 2008). This observation suggests the bacteria represented by this OTU do not have a role in the pathogenicity of BBD lesions affecting the coral Montipora hispida on the GBR, although they may be present in BBD lesions in other locations and on other host corals.

5.5.3 Conclusions

A 16S rRNA gene-targeted pyrosequencing approach has yielded a high resolution profile of microbial communities associated with CP and BBD lesions and provided further evidence that distinct shifts in dominant microbial communities occur during the onset of BBD. Members of the microbial consortia shift from organisms commonly found in oligotrophic oxic conditions in CP lesions to communities adapted to anoxic and sulfide-rich environments in BBD, potentially fuelled by high influx of organic matter from lysing coral tissue. A novel and dominant ribotype within the BBD-archaeal community is potentially involved in methane-cycling coupled to sulfate-reduction by SRB. If anaerobic oxidation of methane is present, sulfate-reduction may
be further enhanced through a syntrophic relationship between Archaea and SRB (Nauhaus *et al.* 2002, Nauhaus *et al.* 2005, Orcutt *et al.* 2008), thereby driving high sulfide concentrations and high virulence of BBD. This hypothesis can be tested by examining methane-production within the BBD mat by chemical detection, and the effects of both an inhibitor of methanogenesis (bromoethanesulfonate) on sulfate-reduction rates and an inhibitor of sulfate-reduction (molybdate) on methane-oxidation rates (Orcutt *et al.* 2008). While functional roles of Archaea associated with BBD and CP remain to be elucidated, my finding of a novel and dominant archaeal sequence provides new insights into the microbial ecology of black band disease by adding another important domain of life to the microbial consortium characteristic of this virulent coral disease.
Chapter 6:

General discussion

Research presented in this thesis significantly advances current understanding of the ecology, environmental drivers and etiology of black band disease (BBD), one of the most virulent diseases presently affecting scleractinian coral populations globally, and conclusively establishes that epizootics of the disease also occur on the Great Barrier Reef. In this concluding chapter, I synthesize the results presented in the preceding chapters to develop a broader view of disease ecology and a model of BBD pathogenesis, and highlight the wider implications of my research. I conclude by identifying future research directions that would further progress the field and provide the knowledge required to underpin the development of strategies to manage BBD.

6.1 Ecology of black band disease

Epizootics of BBD have been decimating Caribbean coral populations for over two decades (Edmunds 1991, Bruckner and Bruckner 1997, Green and Bruckner 2000), but destructive impacts of BBD epizootics had not been reported from the Indo-Pacific region before the present study. Although the disease is known to affect a wide range of Indo-Pacific species (~57 species; Kaczmarsky 2006, Page and Willis 2006), which occur throughout the GBR, prevalence of BBD on the GBR has typically been reported to be low (Miller 1996, Willis et al. 2004, Page and Willis 2006). More importantly, impacts of BBD on coral population dynamics had not been assessed by long term monitoring of coral loss previously. Documentation of recurring summer epizootics affecting up to 10% of colonies in an inshore assemblage of the coral genus *Montipora*, which caused loss of 40% of tissue surface area per colony and whole colony mortality in 5% of infections (Chapter 2), provides the first evidence that BBD epizootics also
occur on the GBR and cause substantial coral tissue loss over time. The impact that such tissue loss has on the fitness of host coral populations highlights the need for greater understanding of disease drivers and etiology of BBD on Indo-Pacific reefs.

The 20 year lag in the first reports of destructive BBD epizootics on the GBR versus in the Caribbean suggests that environmental conditions promoting BBD have developed much later on GBR reefs than on Caribbean reefs. Nutrients, pesticides and suspended sediments in terrestrial runoff entering the GBR lagoon have increased dramatically over the last 200 years due to large scale agricultural, coastal and mining developments, and consequently water quality in the GBR region has been altered (Brodie et al. 2012, Uthicke et al. 2012). Given evidence that nutrient enrichment can enhance the abundance and progression of BBD (Al-Moghrabi 2001, Kuta and Richardson 2002, Voss and Richardson 2006b), the increasing abundance of BBD on the GBR is consistent with a continuing decline in water quality in the GBR lagoon over the last decade (Thompson et al. 2011). The Queensland Government has set legislative requirements for water discharge into the GBR catchment area (Queensland Water Act; 2000) and recently implemented a Water Quality Protection Plan (Reef Plan; 2009) and Great Barrier Reef Protection Amendment (Reef Protection Package; 2009) to reduce the load of pollutants entering the GBR region. Furthermore, the Federal Government implemented incentive-based initiatives (Reef Rescue; 2007), calling for voluntary industrial partnerships to improve the water quality in the GBR lagoon. Outcomes of these water quality control efforts will be assessed in the near future (i.e. mostly by 2013), but continuation of measures to improve water quality will be key to achieving effective results because of time-lags expected in the ecosystem response of the GBR due to slow turnover of other ecosystems and processes impacting on reef water quality (e.g. wetlands, groundwater systems, sedimentation in catchment areas; Brodie et al. 2012). The recent BBD epizootic documented in my study provides additional evidence for the continuing need for strict controls on water quality in the GBR lagoon.

Increasing frequency and intensity of natural disasters (e.g. extreme weather conditions), presumably associated with global climate change, can have confounding effects on the
prevalence of coral disease on the GBR, as such events reduce the capacity of coral communities to resist other natural and anthropogenic stressors. In February 2011, category 5 tropical cyclone Yasi, the most severe cyclone system in the historical record, crossed over reefs in the central GBR region. The combination of direct physical damage to corals and water quality changes associated with flood plumes resulted in high mortality of GBR coral populations. Immediately after this cyclonic event, a large number of BBD cases were observed in a population of *Pavona* sp. in the vicinity of the study site for the first time in recorded history (Y. Sato; pers. observ.). The emergence of a new BBD outbreak following cyclone Yasi underscores the importance of extreme weather events and decreased water quality as drivers of BBD outbreaks. Given the likelihood of increasing frequency and intensity of natural disturbances with climate change, this observation further highlights the continuing need for improvement of water quality in the GBR region.

Future research priorities relating to the ecology of BBD should include the development of a model of BBD outbreak dynamics in coral populations, which in turn would enable predictions of the future consequences of the disease for GBR coral populations. In conventional epidemiological theory, the case reproductive number $R_0$ (defined as the average number of secondary infections produced when one individual infected with a disease is introduced into a susceptible host population; Anderson and May 1991) has been applied to studies of infectious disease dynamics as a key metric for predicting disease incidence and prevalence. However, application of the $R_0$ parameter assumes that a population is homogenously mixed by the movement of individuals throughout the population, and that a disease case is caused by a biotic agent with a measurable generation time (Anderson and May 1991). The sessile nature of corals violates the first assumption, and thus a modified epizootiological model suitable for sessile host populations needs to be developed for coral diseases including BBD. Furthermore, results of my study indicate that seasonal changes in environmental factors are important for the occurrence of BBD (Chapters 2 and 3), and that a suite of microorganisms involved in the production of anoxic and sulfide-rich microenvironments are key in the pathogenesis of BBD (Chapters 4 and 5). Given
that oxygen-consuming heterotrophic and sulfide-producing microorganisms are virtually ubiquitous in marine environments (Munn 2004), effects of environmental drivers on the dynamics of microorganisms associated with coral hosts may better explain the epizootiology of BBD outbreaks. An epizootiological model for BBD should therefore be developed using a theoretical framework based on the ecology of the disease-associated microbial community (such as illustrated in Figure 1.2), and focusing on environmental variables affecting both virulence of the microbial communities responsible for BBD pathogenesis and susceptibility of the host to the establishment of the BBD microbial consortia.

6.2 Environmental drivers of black band disease

Seasonal patterns in the abundance of BBD infections and rates of BBD progression in an assemblage of Montipora spp. in the central inshore GBR (Chapter 2) clearly implicate seasonally varying parameters (i.e. temperature and light) as the most likely environmental drivers of BBD dynamics at the study site. The positive correlation between abundance of BBD and seawater temperature found at the site is in accordance with findings of previous field studies of BBD in the Caribbean, Indo-Pacific and Red Sea regions (Antonius 1981, Edmunds 1991, Kuta and Richardson 1996, Bruckner et al. 1997, Borger 2005, Voss and Richardson 2006a, Rodriguez and Croquer 2008, Zvuloni et al. 2009). My aquarium-based experimental study provides corroborative evidence that temperature and light influence rates of BBD progression (Chapter 3). Importantly, observations from this study were the first to show that seasonal changes in light levels also contribute to in situ increases of BBD abundance and virulence. Previously, the importance of light for in situ dynamics of BBD abundance has only been suggested from spatial distribution patterns of BBD infections, which demonstrate greater abundance of BBD in shallower waters with less turbidity (Kuta and Richardson 2002, Page and Willis 2006). Results of the controlled aquarium experiment, in which BBD-infected corals were exposed to a range of
temperatures and light intensities that corals normally experience in summer (Chapter 3), confirm results from my field-based studies (Chapter 2), demonstrating that light is a significant driver of BBD progression. Moreover, they suggest that light has a greater influence on BBD virulence than seawater temperature within typical summer temperature ranges.

Accumulating evidence that thermal stress enhances BBD abundance and progression throughout reef regions globally (Antonius 1981, Edmunds 1991, Kuta and Richardson 1996, Bruckner et al. 1997, Borger 2005, Voss and Richardson 2006a, Rodriguez and Croquer 2008, Zvuloni et al. 2009; Chapters 2 and 3) highlights the likelihood that the impacts of BBD will intensify with warming oceans predicted by climate change models (Harvell et al. 2002, Hansen et al. 2006). Under an ocean warming scenario, the role of thermal stress as a driver of BBD abundance and progression, combined with the likelihood of disease recurrence and case fatalities shown in my study, predict elongated periods of active BBD outbreaks and greater rates of coral tissue death. Consequently, BBD outbreaks are likely to pose an even greater threat to coral populations in the future. However, our ability to explain and predict patterns of natural BBD outbreaks is still limited, largely due to complex combinations of environmental factors that vary temporally and spatially and interact each other (Kuta and Richardson 2002, Page and Willis 2006, Rodriguez and Croquer 2008). In the controlled aquarium experiment, I demonstrated that decreased host resistance (indicated by compromised photochemical efficiency of the coral holobiont in both high light and high temperature treatments) is an important factor to consider when interpreting studies of environmental drivers of BBD. Clearly, future studies of environmental drivers of BBD, and of any coral diseases, should place the disease system in the ‘environment – pathogen(s) – host’ model, such as illustrated in Figures 1.1 and 1.2 (see Section 1.1), to improve our mechanistic understanding of the links between disease occurrence and the environment. A model of BBD dynamics that captures environmental factors potentially influencing both the fitness of host corals and the virulence of pathogen(s) will provide further insights into environmental drivers of BBD.
6.3 Microbial community dynamics leading to establishment of the black band disease microbial consortium

Field patterns in the abundance of BBD and the precursor stage, ‘cyanobacterial patch(es)’ (CP), indicate that transitions from CP to BBD occur most actively immediately before the warmest months, typically between November and February, when ambient light levels are decreasing from a seasonal maximum whereas seawater temperatures are rising to a seasonal maximum (Chapters 2 and 4). The growth responses of cyanobacterial cultures, which were isolated from CP and BBD lesions from the current study site, to different temperatures and light levels (Glas et al. 2010), suggest a potential mechanism driving these seasonal patterns in the development of BBD from CP. Specifically, an increase in temperature (from 26°C to 35°C) reduced the growth of CP-derived cyanobacterial cultures but enhanced the growth of cyanobacterial cultures isolated from BBD. Interestingly, a decrease in light level (from 80 µmol m$^{-2}$ s$^{-1}$ to 10 µmol m$^{-2}$ s$^{-1}$) reduced the growth of the CP-dominating cyanobacterial strain, although a similar effect was not apparent on the BBD-derived dominant cyanobacterial strain (Glas et al. 2010). These cyanobacterial growth responses to different temperature and light treatments suggest that the BBD-dominating cyanobacterial strain out-competes the CP-associated cyanobacterial strain when temperature is increasing and light is decreasing, as observed in the field between November and February.

Molecular profiling techniques targeting bacterial 16S rRNA genes revealed that the cyanobacterial community undergoes a transition during BBD onset, from being dominated by a *Blennothrix/Trichodesmium* spp.-related strains in CP lesions, to communities dominated by an *Oscillatoria* sp.-related strain in BBD lesions (Chapter 4). Structurally, cyanobacteria are responsible for most of the biomass of the microbial consortia in CP and BBD lesions. Moreover, composition of the cyanobacterial community within the lesion likely affects the community structure of other microbial members potentially interacting with cyanobacteria (Chapters 4 and 5). Therefore, seasonal changes in the environment observed in my study between November and
February would have facilitated the development of BBD from CP by driving a shift in cyanobacterial communities, which in turn is likely to have affected other microbial members of the consortium. Other factors discussed in Chapter 2, including cyanobacterial growth in BBD lesions, clumping behavior of the BBD-associated cyanobacteria, and host stress levels, which increase under high seawater temperatures and light levels during summer, are also likely to have contributed to the transition from CP to BBD.

Changing environmental conditions are also likely to influence the metabolic activities of microbial members other than cyanobacteria that comprise the polymicrobial communities implicated in BBD pathogenicity (see e.g. Arotsker et al. 2009). Metatranscriptomic approaches that target transcriptomes of mixed microbial communities will help to elucidate how environmental factors, such as temperature and light, affect BBD-associated microorganisms at the consortium level. Advances in high-throughput sequencing technologies and bioinformatics, including the application of metatranscriptomic approaches to microbial ecology studies (e.g. Poretsky et al. 2005, Gilbert et al. 2008, Moran 2009), represent powerful tools to elucidate complex functional processes within the microbial consortia associated with BBD in relation to changing environments. Knowledge from such studies will further identify mechanisms underlying the links between environmental changes and BBD outbreaks.

6.4 Developing a model for black band disease pathogenesis

The discovery of the transition of lesions from CP into BBD provides a novel system to study the pathogenesis of BBD in the field. The relatively long period (approximately one month) over which CP lesions transition into BBD enabled successional changes in macroscopic features and the microbial composition of disease lesions to be documented. Here I develop a model of BBD pathogenesis by combining my results on changes in the cyanobacteria, bacterial (Chapters 4 and 5) and archaeal (Chapter 5) communities during the transition from CP to BBD, with results
of collaborative studies on: the molecular quantification of other important functional members of the microbial consortium, such as sulfate-reducing bacteria (SRB) (Appendix A; Bourne et al. 2011) and sulfide-oxidizing bacteria (SOB) (D. Bourne, Y. Sato, E. Botté and M. van der Zee; unpublished data); biogeochemical profiling of the disease mat (Appendix B; Glas et al. 2012); and a comparative metagenomic analysis of the whole microbial consortia associated with CP and BBD (E. Ling, Y. Sato and D. Bourne; unpublished data). This model identifies three microbial processes that underpin BBD pathogenesis, namely (1) formation of anoxic conditions within the microbial consortium associated with CP, (2) proliferation of sulfate-reducers and accumulation of sulfide within the lesion, and (3) intensification of anoxic and sulfide-rich conditions within the BBD mat through a positive feedback mechanism (Figure 6.1).

Consistent with microscopic observations, 16S rRNA gene-profiling techniques (T-RFLP analyses, clone libraries and amplicon-pyrosequencing) identified characteristic shifts in the cyanobacterial community during the development of BBD from CP. *Blennothrix/Trichodesmium* spp.-related cyanobacteria are dominant in CP lesions, while *Oscillatoria* sp.-related cyanobacteria dominate the BBD microbial mat (Chapters 4 and 5), with culture-based studies indicating that the transition is driven by seasonally increasing temperature and decreasing light (Figure 6.1a (1), (2); Section 6.3; Glas et al. 2010). Bacterial profiles obtained with amplicon-pyrosequencing and clone libraries targeting 16S rRNA genes suggest that the composition of heterotrophic bacterial communities within lesions also shifts during the onset of BBD (Chapters 4 and 5). Increased biomass derived from the thin cyanobacterial filaments characteristic of BBD lesions potentially provides more abundant microbial habitats for heterotrophic bacteria (Figure 6.1a (3)). In addition to a shift in the composition of cyanobacterial communities, increasing temperature may enhance metabolism and growth of heterotrophic microorganisms within lesions (Figure 6.1a (4), (5)). *In situ* profiling of oxygen throughout CP and BBD microbial mats using microsensors demonstrated that anoxic conditions are formed close to the base of the microbial mat during the onset of BBD (especially in darkness; Appendix B). These results suggest that increased abundance of oxygen-consuming heterotrophic bacteria produce spatially and temporally localized anoxic conditions...
Figure 6.1 A model of black band disease (BBD) pathogenesis, showing how cyanobacterial patch (CP) lesions transition into BBD as two major virulence factors, anoxia and sulfide deposition, develop within the BBD microbial mat, according to processes labeled sequentially (1-9) in two panels: (a) Early stages of microbial succession, i.e. 1) environmental changes (increasing temperature and decreasing light) drive transitions in dominant cyanobacterial species from a *Blennothrix/Trichodesmium* spp.-related strain (characteristic of CP) to an *Oscillatoria* sp.-related strain (characteristic of BBD), which 2) facilitates the formation of a tightly-clumped thick cyanobacterial mat, in turn causing 3) a shift in heterotrophic microbial communities as a consequence of the greater abundance of microbial habitats associated with increased cyanobacterial biomass. Also, 4) increased temperatures enhance growth of heterotrophs, and 5) increase the abundance and/or activities of heterotrophs. 6) Anoxic conditions produced locally by increased microbial respiration beneath and within the cyanobacterial mat generate necrotic tissue, increasing organic input to the microbial consortium, further stimulating microbial activities within the lesion, and 7) facilitating growth of anaerobic sulfate-reducers, and 8) suppressing growth of aerobic sulfide-oxidizers, resulting in 9) production and accumulation of sulfide.
(b) Late stages of microbial succession leading to fully developed BBD lesions, i.e. 1) sulfide produced by sulfate-reducers and desulfuration of coral tissue further increases organic input and proliferation of the microbial consortium, leading to 2) sulfide-rich and anoxic conditions that further drive 3) shifts in cyanobacterial communities to the BBD-associated cyanobacterium. 4) Increased biomass of BBD-associated cyanobacteria provides further microbial niches for anaerobic heterotrophs beneath the thickening cyanobacterial mat, and 5) increased habitats and organic input from necrotic coral tissue enable heterotrophs to proliferate further and enhance anoxic conditions. 6) Intensified anoxia increases organic input from necrotic tissue and further provides anaerobic niches available for sulfate-reducers. Steps (1) ~ (6) repeat in a loop until 7) conditions at the bottom of the microbial consortium are strictly anaerobic, causing 8) archaeal communities to transition into a community dominated by a strictly anaerobic Archaea characteristic of BBD. 9) Through syntrophic coupling of sulfate-reducers and BBD-associated Archaea, BBD-associated Archaea indirectly enhance the production of sulfide. Steps (1) ~ (9) repeat through a positive feedback loop, and the microbial community in the fully developed BBD lesion becomes highly virulent and causes substantial degeneration of coral tissue.

Microenvironments linked to their metabolism (Figure 6.1a (6)). Nighttime respiration of cyanobacteria may further facilitate production of anoxic microenvironments (Appendix B). Anoxia causes tissue necrosis (Richardson et al. 1997), providing a source of organic matter for heterotrophic bacteria in the microbial consortium and establishing a positive feedback loop that intensifies anoxic conditions within BBD lesions.

Anoxic conditions within the microbial consortium are essential for anaerobic SRB, which are implicated in the production of sulfide, the second virulence factor of BBD (Carlton and Richardson 1995, Richardson et al. 1997, Cooney et al. 2002, Frias-Lopez et al. 2002, Viehman et al. 2006, Barneah et al. 2007, Richardson et al. 2009). Profiles of bacterial 16S rRNA genes indicate that BBD lesions typically have higher relative abundance of bacteria involved in sulfur-cycling than in CP-associated bacterial communities (Chapters 4 and 5). Furthermore, a comparative study of metagenomes derived from CP- and BBD-associated microbial mats revealed that the relative abundance of genes involved in sulfate-reduction pathways (responsible for the production of sulfide) increases during BBD onset, and that the relative abundance of genes involved in sulfide-oxidation pathways (responsible for the degradation of sulfide) is greater in CP than in BBD (E. Ling, Y. Sato and D. Bourne; unpublished data). Similarly, quantitative PCR of the sulfate-reducing gene, dsrA, demonstrated that copy numbers of dsrA genes per total bacterial
16S rRNA coding genes increase when CP lesions transition into BBD (Appendix A). On the other hand, the *saxB* gene, which is involved in sulfide removal, decreases in relative abundance (i.e. copy number per total bacterial 16S rRNA coding genes) during the onset of BBD from CP (D. Bourne, Y. Sato, E. Botté and M. van der Zee; unpublished data). These molecular analyses of sulfur cycling-related genes indicate a relative increase in the abundance of sulfate-reducers and a relative decrease of sulfide-oxidizers during the course of BBD onset, presumably due to the formation of anoxic conditions within the disease lesion (Figure 6.1a (7), (8)). These changes in the relative abundance of bacterial genes involved in sulfur-cycling indicate that microbial consortia associated with BBD have a greater potential to produce sulfide, coupled with a lower potential to remove sulfide, compared to CP-associated microbial consortia. The accumulation of high sulfide levels within the lesions was confirmed during the onset of BBD from CP by *in situ* measurement of sulfide concentrations using microsensors (Appendix B; Figure 6.1a (9)). Highest sulfide concentrations within the BBD mat were observed at the interface between coral tissue and the microbial mat, highlighting the links between sulfide-production, coral tissue degeneration and accumulation of organic matter, which serves as the metabolic substrate for sulfate-reducers, occurring most actively at the tissue-lesion interface (Appendix B; Figure 6.1b (1)). Additionally, desulfuration of necrotic coral tissue (release of sulfide from organic compounds through microbial, chemical and coral derived-enzymic processes) may also contribute to the high sulfide level at the tissue-lesion interface (Weber *et al.* 2012; Appendix B).

Bacterial profiles indicate that the relative abundance of 16S rRNA genes that are found in anaerobic, organic-rich environments (e.g. sequences affiliated to *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Deferribacteres*) increases as anoxic and sulfurous conditions intensify during the onset of BBD from CP (Chapters 4 and 5). Cyanobacterial cultures isolated from BBD mats in the Caribbean demonstrate resistance to sulfide-rich and anoxic conditions (Richardson *et al.* 1997, Myers *et al.* 2007, Richardson *et al.* 2009), whereas the CP-associated cyanobacterial strain is phylogenetically similar to the aerobic cyanobacteria *Trichodesmium* and *Blennothrix* spp., which thrive in oligotrophic open oceans (Abed *et al.* 2003, Charpy *et al.* 2007).
These observations suggest that sulfide-rich and anoxic conditions formed within the microbial consortium likely drive successional changes in the composition of cyanobacterial communities observed during BBD onset (Figure 6.1b (2), (3)). Physiological studies of cyanobacterial strains isolated from CP and BBD lesions from the study site are underway to test their tolerance against sulfide and anoxia to examine this hypothesis.

Increased biomass of BBD-associated cyanobacteria creates a microenvironment where oxygen does not penetrate throughout the microbial mat (Appendix B), providing greater niches for anaerobic heterotrophic microorganisms (Figure 6.1b (4)) and thereby indirectly contributing to the depletion in oxygen in the microbial mat and consequential degradation of coral tissue (Figure 6.1b (5)). Given that anoxic conditions are favorable for the growth of both sulfate-reducers and anaerobic heterotrophs, the two virulence factors that degenerate coral tissue (i.e. anoxia and sulfide) are inextricably linked and continue to enhance each other through a positive feedback mechanism mediated by organic input from necrotic coral tissue (Figure 6.1b (1) – (6)). Sulfide concentrations and the extent to which oxygen is depleted within the microbial consortium are both shown to positively correlate with the progression of the lesion throughout the development of BBD from CP (Appendix B), underpinning the importance of these virulence factors for the pathogenesis of BBD. As sulfide-rich, anoxic conditions further intensify, the bottom of the BBD microbial mat becomes permanently anoxic (Appendix B), creating suitable conditions for strictly-anaerobic microorganisms, including the dominant Archaea observed in BBD (Chapter 5; Figure 6.1b (7), (8)). Phylogenetic analysis of the dominant archaeon associated with BBD suggests that this archaeon may enhance sulfide production through a syntrophic interaction with sulfate-reducers (Figure 6.1b (9)), resulting in a higher virulence of the microbial mat associated with fully developed BBD.

This BBD pathogenesis model, developed here from my studies of the CP – BBD in situ system, demonstrates that tightly coupled microbial consortia develop toxic (i.e. sulfide-rich and anoxic) microenvironments in succession, which in turn drive microbial succession in disease lesions further, resulting in the establishment of the BBD microbial consortia. Future studies
focusing on changes in microbial functional modes (e.g. autotrophy vs. heterotrophy) during BBD onset will provide deeper insights into etiological mechanisms underlying the pathogenesis of BBD. Metatranscriptomic approaches used to elucidate the functioning of other complex microbial consortia (e.g. Poretsky et al. 2005, Gilbert et al. 2008, Moran 2009) can be directly applied to functional studies of microbial consortia comprising CP lesions, lesions at intermediate stages and BBD lesions, and will provide fundamental information on the etiology of BBD. Stable isotope-tracing of key elements (e.g. C, N, S, P), as well as histological observations using fluorescent in situ hybridization will also be useful to locate key functions and microbial taxonomic groups that confer toxicity on the BBD microbial consortium during disease onset. Such efforts will identify key functional genes of BBD-associated microorganisms and their distribution in the disease mat, and will contribute to a better mechanistic understanding of the complex nature of BBD pathogenesis.

Microbial ecology processes similar to those demonstrated in the current CP-BBD model may be responsible for progression of other coral disease lesions. For example, formation of anoxic zones and high concentrations of sulfide on corals could be triggered by infestation of heterotrophic microorganisms at sites of coral injury or by sediment accumulating in depressions on the surfaces of corals and suffocating coral tissue (Weber et al. 2012). Weber et al. (2012) demonstrated that organic-rich sediment, but not organic-poor sediment, placed on coral surface initiates tissue mortality through production of low pH and anoxic conditions mediated by microorganisms, and subsequently through increased concentrations of hydrogen sulfide that accelerate tissue degradation. Factors that enhance microbial processes leading to formation of anoxic and sulfide-rich conditions should therefore be targeted if polymicrobial diseases like BBD are to be managed in coral populations. Such management measures for mitigating disease impacts should include reducing nutrients in terrestrial run-off, which has been shown to be implicated in BBD epizootics (Antonius 1981, Al-Moghribi 2001) and enhanced virulence of BBD (Voss and Richardson 2006b).
6.5 Concluding remarks

Knowledge derived from my studies provides new insights into the ecology and microbiology of BBD. Intense field monitoring of coral assemblages and microbial examination of disease lesions during *in situ* establishment of BBD provided a novel approach to elucidate the etiology and pathogenesis of BBD. A robust foundation built on comprehensive etiological knowledge is indispensable for successful management of any infectious disease (Anderson and May 1991, Fraser *et al.* 2004, Wobeser 2006). Successes in controlling major diseases, like smallpox, cholera, severe acute respiratory syndrome (SARS) in humans and foot-and-mouth disease and bovine spongiform encephalopathy (BSE) in domestic animals (e.g. Anderson *et al.* 1996, Ferguson *et al.* 2001, Anderson *et al.* 2004), attest to the importance of comprehensive knowledge of the ecology of a disease, including etiological mechanisms and infectious sources, for their management. Accordingly, the better mechanistic understanding of BBD pathogenesis provided by my study will hopefully contribute to the development of strategies for managing this disease.

As outlined above, controlling nutrient inputs that enhance the growth of microorganisms responsible for the formation of anoxia and high sulfide levels represents an important strategy for managing BBD in coral populations.

Results of my study provide further evidence that BBD is a polymicrobial disease, and thus strongly suggest that traditional approaches developed for etiological studies of single-pathogen diseases, such as fulfilling Koch’s postulates, are unsuitable for studying this coral disease. Instead, advances in the understanding of polymicrobial diseases, like BBD, require knowledge of (1) interactions within the community of pathogens, (2) interactions between the host and the pathogen community, and (3) the effects of environmental factors on each of these interactions. My studies highlight the importance of approaches based on microbial ecology concepts for understanding the etiology of BBD and for developing a model of BBD-epizootic dynamics. Research presented in this thesis provides the foundation upon which the full complexity of etiological mechanisms involved in BBD pathogenesis can be further explored in
future studies.
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Appendices

Appendix 2.1 Published paper (Chapter 2):

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Appendix 3.1 Published paper (Chapter 3):


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