THE ECOLOGY AND MICROBIOLOGY OF BLACK BAND DISEASE AND BROWN BAND SYNDROME ON THE GREAT BARRIER REEF

Thesis submitted by
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THESIS DEDICATION

This thesis is dedicated to my loving parents, Buddy and Pat Boyett, for their emotional, inspirational, and financial support.

PUBLICATIONS ARISING FROM THIS THESIS

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ABSTRACT

The overall objective of this study was to investigate the progression and transmission of black band disease (BBD) and brown band syndrome (BrB) on the Great Barrier Reef. Specifically, my aims were to: 1) examine variations in the natural progression and transmission of BBD and BrB between different months of the year and reef sites (Chapter 2); 2) investigate the effect of elevated temperature on the progression of BBD and BrB using experimental aquarium manipulations (Chapter 3); 3) histologically examine the microorganisms associated with BBD (Chapter 4); and 4) investigate the microorganisms associated with BrB using microbiological and molecular techniques (Chapter 5).

The *in situ* rate of progression and transmission of black band disease (BBD) on the coral *Acropora muricata* was measured and compared between seasonal field studies at Lizard Island on the Great Barrier Reef (GBR). BBD progressed along and transmitted between coral branches at a significantly faster rate during the austral summer month of January as opposed to the cooler months of July and May. The 2-3°C increase in seawater temperatures and 650 $\mu E/m^2/s$ rise in light intensities measured between the months of January and July/May suggest that elevated temperatures and light intensities are positively correlated to the progression and transmission of BBD. Temperature manipulations within closed experimental tanks were also performed to determine the effect of elevated temperatures on the rate of BBD progression. Increased rates of BBD progression within the higher temperature treatment (32°C) during summer clearly indicate that elevated temperatures near the upper thermal limits of corals promote the progression of BBD, possibly due to a combination of increased virulence of the pathogen and increased host susceptibility at these higher temperatures. However, the lack of increased progression within elevated temperature treatments (29°C and 31°C) during May trials, raise the question concerning other factors that may also be required to promote the progression of BBD.

The *in situ* rate of spread of brown band syndrome (BrB) on *Acropora muricata* branches was compared between two reefs in the northern and central sectors of the

Great Barrier Reef (GBR). The rate of spread of the syndrome was 2.3 times faster on a lagoon reef at Lizard Island (Horseshoe Reef) than on the reef flat at Davies Reef. Although a combination of parameters is most likely responsible for this variation, the most obvious difference between the two reef sites was the degree of water circulation. Experimentally elevated temperatures in aquarium experiments did not influence the progression of BrB at Lizard Island suggesting that temperature on its own does not enhance the progression of this syndrome. Future research should experimentally investigate whether or not the combination of elevated temperatures and high light intensities, commonly associated with summer months, enhance the progression of this syndrome.

The microbial consortium of BBD isolated from *Acropora elseyi, A. florida, A. muricata, A. nasuta, Pocillopora verrucosa,* and *Porites* spp. at Lizard Island consisted of five different taxa of cyanobacteria. Based on morphological characteristics, two of these cyanobacteria taxa appear to belong to the genus *Oscillatoria*, two may be in the Order *Nostocales*, and one of these taxa resembles the morphological features of *Phormidium corallyticum*. However, these predictions are based solely on histological features and further molecular identifications are required before these species can be formally classified. Morphological and molecular studies indicated that the microorganisms associated with BrB on five acroporid colonies from Davis Reef consisted of a newly identified ciliate species and an array of associated bacteria. Analysis of 18S rDNA sequence data confirmed the ciliate as a new species belonging to the Class Oligohymenophora, Subclass Scuticociliatia. Isolation of BrB bacterial species detected a potentially pathogenic strain (HB-8) which was closely affiliated by 16S rDNA comparisons with *Vibrio fortis* strains. Six out of 12 acroporid branches inoculated with this potentially pathogenic strain reached 100% mortality after 48 hours, however the macroscopic signs (brown band) of the syndrome were not observed. This indicates that there are likely two phases of BrB including a tissue necrosis phase, which may be caused by the bacterium strain HB-8, and a ciliate phase which causes the characteristic brown band of the syndrome.

In summary, the rate of progression and spread of BBD and BrB on the Great Barrier Reef appear to be dependent on the response of both the coral host and the disease

pathogen to changing environmental conditions. The higher *in situ* rates of BBD progression and transmission during the summer month of January and the higher rates of BBD progression within temperature treatments experimentally elevated to near the upper thermal limits of corals likely reflect that environmental conditions detrimental to the coral host may simultaneously increase the virulence of the coral pathogen while reducing the coral's immunity. However, it is important to note that the mechanisms causing mortality (i.e. ingestion of tissue versus tissue necrosis) may also have an influence on disease progression. Consequently, in order to fully understand the mechanisms and parameters involved in the progression and transmission of coral diseases, coral disease research should continue to investigate these diseases using an ecological and microbiological approach.

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CHAPTER 1.0

GENERAL INTRODUCTION

1.1. PREVALENCE OF CORAL DISEASE

Over the last 20 years, the prevalence of marine diseases has increased globally and reduced populations of several ecologically and commercially important species including scallops, clams, pilchards, herrings, seals, and oysters (Peters 1997, Harvell *et al.* 1999). Although reports of coral diseases did not appear until the early 1970's (Antonius 1973), there has been increasing evidence that the frequency and prevalence of diseases infecting scleractinian corals has also proliferated in the last three decades. Between 1973-1999, coral disease has been documented in more than 102 species of coral across 54 countries, however 66% of the 155 reports reviewed were from the Caribbean region (Bruckner 2002). In the Caribbean, coral disease epizootics have resulted in reduced coral abundance (Aronson and Precht 1997, Porter *et al.* 2001), diminished reproductive potential (Edmunds 1991, Edmunds 2000, Kuta and Richardson 1997), and possible phase-shifts in the community composition of coral reefs (Aronson and Precht 2001). If the prevalence of coral diseases on the Great Barrier Reef (GBR) is comparable to disease prevalence in the Caribbean, then coral disease may have important implications for the health of the GBR.

Willis *et al.* (2004) reported that the mean disease prevalence during the summer of 2003 on 8 reef sites within the northern and southern sectors of the GBR (8.97 ± 0.79%) was greater than the mean disease prevalence over the past four years at 28 reef sites in the Caribbean (5.38 ± 1.2%) (Weil 2004). Furthermore, they observed eight disease states on the GBR including diseases that have caused major impacts within Caribbean reef communities, such as black band disease and possibly white syndrome (if it is found to include some of the Caribbean white diseases) (Willis *et al.* 2004). As a result, coral disease research has become an area of high priority along the Great Barrier Reef where disease was initially believed to have little impact on coral communities (Willis *et al.* 2004).

1.2. CORAL DISEASE TERMINOLOGY

Although there are multitudes of definitions for coral disease, it is widely accepted that a disease is, "any deviation from or interruption of the normal structure or function of any body part, organ, or system that is manifested by a characteristic set of signs and whose etiology, pathology, and prognosis may be known or unknown" (Peters 1997, Borneman 2001, Parnell and Richardson 2002). Diseases in scleractinian corals have been characterized by detectable signs, identifiable causative agents or pathogens, and/or consistent alterations of the coral's structural composition (Peters 1997, Parnell and Richardson 2002). Parasites or infections from bacterial, fungal, protozoan, or viral pathogens may cause disease (Peters 1997, Borneman 2001). In this study, the term *syndrome* is used for maladies where the causative agent has not been identified while the term *disease* is used for maladies where the causative agent has been identified. *Progression* is used to describe the movement of a disease band along interconnected coral branches within a colony, while *spread* and *transmission* are defined as the passage or transfer of an infectious disease between colonies.

1.3. POTENTIAL CAUSES OF CORAL DISEASE

It has become increasingly evident that physical properties of the environment may affect the progression and transmission of coral diseases (Santavy and Peters 1997, Bruckner 2002). Coral reef ecosystems are continually exposed to increasing levels of sedimentation, nutrient enrichment, and ocean warming (Acosta 2001). The stress resulting from these events influences a coral's susceptibility to disease infection (Bak and Criens 1981, Porter *et al.* 2001). Although these conditions may decrease host resistance and increase the virulence of coral pathogens (Bruckner and Bruckner 1997, Harvell *et al.* 1999), few studies have experimentally investigated the links between environmental parameters and the progression and spread of coral disease. Alker *et al.* (2001) investigated the role of elevated temperatures in enhancing the growth of the fungal pathogen, *Aspergillus sydowii*. They found that growth rates of four *A. sydowii* strains displayed significant interactions with temperature (Alker *et al.* 2001). All strains were capable of growing between 22-36°C with significantly faster growth rates at 30°C (Alker *et al.* 2001). An experimental approach was also used to

link the severity of aspergillosis and yellow band disease to nutrient enrichment (Bruno *et al.* 2003). Under nutrient concentrations 2 to 5 times greater than normal levels, coral tissue lysis in infected colonies was significantly greater than in control treatments (Bruno *et al.* 2003). However, in the majority of studies, linkages between coral disease and environmental factors are based on correlative data comparing disease prevalence between seasons (Antonius 1985, Edmunds 1991, Willis *et al.* 2004) or locations (Porter *et al.* 2001, Kuta and Richardson 2002). In all these cases, coral epizootics appear to be promoted by two main conditions, climate variability and anthropogenic pollution.

Increasing atmospheric concentrations of greenhouse gases are expected to have substantial impacts on the world's climate over the next few decades. Land and ocean surface temperatures have experienced consistent and large-scale warming trends throughout the 20th century, with mean surface air temperatures having risen by 0.6°C on a global scale in the last 100 years (Gitay *et al.* 2002). The warmest year recorded took place in 1998 while the 1990's have been considered the warmest decade (Gitay *et al.* 2002). Tropical seawater temperatures have also increased by approximately 1°C over the past century and are expected to rise a further 1-2°C per century (Hoegh-Guldberg 1999). Since the mid-1970's, El Nino Southern Oscillations (ENSO) have occurred more frequently with greater intensity (Gitay *et al.* 2002). El Nino events are commonly associated with high temperature increases capable of heating vast areas of the ocean to above-average temperatures (Gitay *et al.* 2002). Although a direct link between these Southern Oscillation Events and disease has not been identified, the rising sea-surface temperatures associated with these events may have important consequences for the virulence of disease pathogens.

In the Caribbean, 97% of coral disease has been documented in locations of medium to high anthropogenic impacts (Green and Bruckner 2000). In general, it seems that human activities have increased the transport of pathogens across the globe and anthropogenic influences such as nutrient enrichment, terrestrial run-off, sewage outfalls, and sedimentation have degraded environments and inflicted sources of stress on many sessile organisms (Green and Bruckner 2000, Garrison *et al.* 2003). Several studies infer that poor water quality is a possible cause of stress in corals (Porter *et al.* 2001, Garrison *et al.* 2003). Since stress increases the susceptibility of

organisms to disease infection (Rapport and Whitford 1999), pollution may be a significant factor responsible for the increasing amount of disease observed within coral reef communities. It has been suggested that water eutrophication resulting from sewage and terrestrial run-off may promote the progression and spread of black band disease (BBD) (Porter *et al.* 2001). Furthermore, increases in BBD have been reported in industrialized areas and species usually resistant to the disease have been infected in polluted waters (Edmunds 1991, Bruckner and Bruckner 1997).

The prevalence of coral disease is continually increasing on a global scale (Bruckner 2002). On the GBR, increases in the prevalence of coral disease have primarily been correlated with the summer season (Willis *et al.* 2004). However, it is difficult to isolate the effect of a single environmental parameter based on correlations between seasons and disease prevalence without considering additional factors, such as temperature, water circulation, turbidity, and light, that might be involved. These factors may interact synergistically to increase the overall impact on the prevalence of coral disease. By using experimental manipulations to isolate a parameter's influence on the progression and transmission of disease, the nature of the relationship between a single parameter and disease prevalence may be determined. By investigating the factors believed to promote the prevalence of coral diseases on the GBR, management strategies can be directed to reduce the potential causes of coral disease in an effort to control the increasing frequency of disease outbreaks in coral communities.

1.4. PATHOGENS AND VECTORS

A wide range of microorganisms including fungi, bacteria, cyanobacteria, and protozoans have been identified to associate with both healthy and diseased corals (Peters 1997, Richardson *et al.* 1997, Rohwer *et al.* 2001, Cooney *et al.* 2002, Frias-Lopez *et al.* 2002, Patterson *et al.* 2002, Rohwer *et al.* 2002, Rohwer and Kelley 2004). Currently, pathogens associated with coral diseases have only been identified for 7 out of 22 Caribbean diseases (BBD, white plague type II, white band type II, white pox, aspergillosis, algal tumors, and red band disease) and 5 diseases in other regions of the world (two types of bacterial bleaching, skeletal eroding band, pink spot, and poritid pox) (reviewed by Weil 2004). Koch's postulates have only been fulfilled for four coral diseases (bacterial bleaching, white plague type II,

aspergillosis, and white pox) (Kushmaro *et al.* 1996, reviewed by Weil 2004). In order to identify a disease pathogen as the causative agent, the fulfillment of these postulates is essential. This is particularly difficult in coral hosts because the replication of natural reef conditions in aquariums is quite difficult (Richardson 1998, Sutherland *et al.* 2004). Furthermore, initial stages of infections are virtually unknown and it is nearly impossible to prove that re-isolated pathogens from newly infected corals are not caused by water contamination (Richardson 1998, Sutherland *et al.* 2004). The limited information on the causative agents of coral disease prevents current research from determining potential disease reservoirs and vectors. Disease reservoirs have only been identified for BBD (Richardson 1997) and aspergillosis (Weir *et al.* 2000) while the only known disease vector is the Mediterranean fireworm, *Hermodice carunculata*, which harbors the pathogen (*Vibrio shiloi*) responsible for bacterial bleaching in *Oculina patagonica* (Sussman *et al.* 2003).

To date, the causative agents of coral diseases on the GBR remain undescribed except for the disease condition known as skeletal eroding band (Antonius and Lipscomb 2001). Environmental parameters promoting the prevalence of coral diseases likely influence the health of the coral host in addition to the virulence and ecology of the coral pathogen(s) (Bruckner 2002, Weil 2004). Consequently, the combination of a microbiological and ecological approach to the study of coral diseases is essential.

1.5. JUSTIFICATION OF STUDY SPECIES

Approximately 29 coral diseases have been described in the scientific literature (Green and Bruckner 2000), yet only a handful of these have been experimentally examined. In fact, the wide array of diseases named solely from macroscopic field signs has led to confusion and uncertainty regarding their validity (Richardson 1998, Green and Bruckner 2000). In Caribbean coral reef communities, high mortality and reduced reproductive outputs have occurred over the past decade due to the increasing prevalence of black band disease (BBD) (Edmunds 1991, Porter *et al.* 2001, Bruckner 2002). Characterized by a dark band positioned between exposed skeleton and seemingly healthy tissue (Bak and Laane 1987, Kuta and Richardson 1997, Porter *et al.* 2001), BBD has been recorded in 26 different countries on 42 different species

within the genera *Favia*, *Favites*, *Diploria*, *Montastrea*, *Colpophyllia*, *Acropora*, *Agaricia*, *Montipora*, *Porites*, *Gorgonia*, and *Siderastrea* (Green and Bruckner 2000). The prevalence of BBD has been positively correlated with the summer season on the Great Barrier Reef (Willis *et al.* 2004) and in the Caribbean (Kuta and Richardson 2002) thereby justifying it as good candidate for this study. Moderate increases in the prevalence of brown band syndrome (BrB) during the summer have also been recorded on the Great Barrier Reef (Willis *et al.* 2004). In the field, the syndrome is characterized by a brown zone preceded by healthy tissue and followed by exposed white skeleton (Willis *et al.* 2004). In some cases, a white zone is observed between the brown band and healthy tissue (Willis *et al.* 2004). Due to its rapid rates of progression which reach up to 9 cm/day, BrB has recently been identified as a potential threat to the Great Barrier Reef (Nash 2003, Willis *et al.* 2004) making it a second candidate and focus of this study.

1.6. SPECIFIC OBJECTIVES

Currently, we know very little about rates of progression and spread of coral disease on the Great Barrier Reef. The research presented in this thesis examines the progression and spread of two coral maladies on the GBR (black band disease and brown band syndrome) using an ecological and microbiological approach. This will provide insights into the biotic and abiotic factors promoting the progression and transmission of coral disease. The prevalence of coral disease on the GBR is highest during the summer (Willis *et al.* 2004), therefore we would expect a positive relationship between the progression and transmission of coral disease and high seawater temperatures. Detecting the correlations and links between environmental parameters, such as elevated temperature, and the progression of coral disease will supply an important baseline study for future disease research in this region to build upon. Specifically, my aims are:

1. **To determine rates of natural progression and transmission of black band disease and brown band syndrome (Chapter 2).** Determining the rates at which these pathogens destroy coral tissue and how quickly they can infect adjacent healthy coral branches will provide insights into the impacts these diseases may have on GBR coral populations. Determining differences in the

progression and spread of disease between months and locations will help to elucidate the links between disease and varying environmental parameters and will enhance current understanding of the potential abiotic factors promoting the increasing prevalence of diseases on the GBR.

2. **To investigate the effect of elevated seawater temperatures on the progression of black band disease and brown band syndrome (Chapter 3).** This study will give an insight into the role of temperature in determining the virulence of disease pathogens, specifically the potential for global warming to enhance the progression and transmission of disease. Using an experimental approach, this study will isolate potential links between increasing temperatures and coral disease. Manipulations under aquarium conditions will control for additional biological and environmental parameters that may be influencing the progression and spread of coral disease in the field such as grazing, nutrient concentrations, water circulation, salinity, and turbidity.

3. **To investigate the histopathology of black band disease on the Great Barrier Reef by distinguishing between the various microorganisms inhabiting infected corals (Chapter 4).** In this chapter, I will identify the different types of cyanobacteria found within the microbial consortium of black band disease using morphological characteristics and measurements. The histology of black band disease will be compared between coral species to determine if the consortium is identical among taxa. Identifying the microorganisms associated with black band disease on the GBR will provide the first step to understanding the potential causative agents of this disease.

4. **To identify members of the microbial community associated with brown band syndrome and determine the potential causative agents of this syndrome (Chapter 5).** The investigation of the microorganisms involved in brown band syndrome is the first step to discovering the pathogen(s) involved in the progression and spread of this syndrome. The baseline information provided in this chapter is essential to furthering biological and ecological understanding of BrB. Identifying the causative agents will allow us to

determine how environmental changes may impact the progression and spread of this syndrome.

CHAPTER 2.0

PROGRESSION AND SPREAD OF BLACK BAND DISEASE AND BROWN BAND SYNDROME ON THE GREAT BARRIER REEF

2.1. ABSTRACT

The focus of this chapter is to examine variations in the natural progression and spread of black band disease (BBD) and brown band syndrome (BrB) between different months of the year and reef sites. The progression and spread of black band disease significantly varied between months with greater rates of progression and transmission observed during the austral summer month of January 2004. The environmental factors most likely to be implicated given these sampling times were elevated temperatures and increased light intensities. Average seawater temperatures in January were more than 2-3°C greater than average seawater temperatures during May and July while light intensities were 650 μE/m^2/s higher during January than May. Significant variations in the rate of progression for brown band syndrome were observed between two sites in the northern and central sectors of the Great Barrier Reef. The progression of this syndrome was 2.3 times faster at Horseshoe Reef, Lizard Island than on the reef flat at Davies Reef. A variety of parameters is most likely responsible for this variation including the degree of water circulation which varied between reef sites. Net flow velocities within lagoon locations at Lizard Island have been reported to average 17.1-25.3 cm/sec while net flow velocities on the reef flat at Davies reef averaged 35 cm/sec.

2.2. INTRODUCTION

Reports of disease epizootics affecting marine organisms have increased dramatically in the last 20 years (Harvell *et al.* 1999, Bruno *et al.* 2003). Compared to terrestrial environments, the spread of pathogens in marine environments can be extremely rapid (McCallum *et al.* 2004). In Australian pilchards, the herpes virus spread across populations at rates greater than 10,000 km/year while the morbillivirus spread throughout seal and dolphin populations at rates of 3,000 km/year (McCallum *et al.* 2004). The spread of epizootics in sedentary invertebrates, such as *Diadema*, have been reported to be as high as 2,800 km/year (McCallum *et al.* 2004). If coral pathogens are capable of spreading at comparable rates, epizootics may have considerable consequences for coral communities. Currently, little information exists on the spread of disease among coral populations or the progression across coral colonies, especially on the Great Barrier Reef (GBR). The majority of studies have focused on comparisons of disease prevalence among years, seasons, and locations (Porter *et al.* 2001, Weil 2004, Willis *et al.* 2004) while rates of disease spread and progression are generally reported for a single month, site, or coral colony (Dinsdale 1994). Knowledge of how environmental factors such as elevated temperature and light will influence rates of disease progression and spread will enhance current understanding of the impact of disease on coral populations and the likelihood of disease epizootics as a consequence of predicted climate change (Gitay *et al.* 2002).

Approximately eight coral disease syndromes have been described on the Great Barrier Reef (Willis *et al.* 2004). Two of these disease syndromes, black band disease (BBD) and brown band syndrome (BrB), will be addressed in this study. In the Caribbean, an increasing prevalence of BBD has been reported in numerous locations (Bruckner 2002). The incidence of BBD in the Florida Keys increased from 7 to 28 stations and the number of species exhibiting the disease tripled from 11 to 36 species within a 2-year period (Porter *et al.* 2001). Coral species infected with BBD included *Montastrea annularis*, *M. cavernosa*, *Colpophyllia natans*, and *Siderastrea siderea*. Off the north coast of Jamaica, BBD infections spread 3 km over a 19-month period infecting 5.2% of massive coral populations (Bruckner *et al.* 1997). Infections were dispersed throughout the survey sites and in most locations, the disease infected 0.5 to 1.0% of susceptible colonies (Bruckner and Bruckner 1997). Outbreaks of BBD in the

U.S. Virgin Islands infected 0.3% of susceptible colonies, including *Montastrea annularis* and *M. cavernosa*, between September 1988 and November 1988 (Edmunds 1991). On Caribbean reefs, the progression of BBD has been reported to destroy coral tissue at a rate of several centimeters per week (Richardson 1997) progressing across a single colony at rates as high as 6.2 mm per day with a mean of 3.1 mm per day (Rutzler and Santavy 1983). It has the capacity to eliminate small corals in days to weeks and is highly infectious, commonly spreading from one colony to the next (Bruckner *et al.* 1997). The spread of BBD within and between coral colonies at these rates has caused considerable mortality within Caribbean scleractinian coral populations. As a result, many studies have recognized black band disease as a potential threat to Caribbean coral communities and a phenomenon requiring immediate attention (Bruckner 2002).

Recently, symptoms of BBD have been recorded on five major families in the GBR including the *Pocilloporidae*, *Acroporidae*, *Poritidae*, *Faviidae*, and *Mussidae* (Dinsdale 1994, Willis *et al.* 2004). To date, BBD has been observed in very low abundances on the GBR with 0.01 (Heron Island) to 1.7% (Lizard Island) of surveyed colonies showing characteristic signs of infection (Willis *et al.* 2004). The prevalence of BBD at Lizard Island was comparable to data obtained from Caribbean reefs (Willis *et al.* 2004). According to a recent review of Caribbean diseases by Weil (2004), the average prevalence of black band disease at the community level is approximately 0.2-6%. However, it is important to note that even at this low prevalence, BBD has caused considerable mortality and possible phase shifts in Caribbean coral reef communities. In order to understand how this occurs, it is important to investigate the rate at which BBD progresses within coral colonies and transmits between coral colonies during different months of the year. To date, one *in vitro* study has reported rates of progression for BBD on the GBR, however these rates were only obtained during one month of the year over a 4-day period (Dinsdale 1994). In this aquarium study, Dinsdale (1994) reported that BBD progressed at a rate of 12.5 mm/day across an *Acropora millepora* colony, moving a total linear distance of 5 cm over the 4-day period. In addition, it had been observed that the disease was capable of transmitting to uninfected colonies when the diseased area was in contact with a nearby healthy colony. These observations suggest that BBD on the GBR is infectious and progresses at rates greater than those recorded in the Caribbean (12.5

mm/day versus 6.2 mm/day) (Dinsdale 1994, Richardson 1997). Consequently, BBD may be a future threat to the Great Barrier Reef and understanding the variations in the rates of progression and spread of BBD is an important step in determining how this disease will affect coral reef communities.

Although BrB has only recently been observed along the Great Barrier Reef, Palau, Japan, and the Marshall Islands, it has been reported to progress along coral branches at extremely rapid rates (Nash 2003, Willis *et al.* 2004). The rate of tissue loss on acroporid colonies at Davies Reef ranged from 0.3 to 9 cm/day suggesting that the progression of this syndrome may be highly variable and extremely quick in some cases (Nash 2003). Willis *et al.* (2004) reported a low prevalence of BrB with less than 1% of surveyed colonies infected with the syndrome, however Nash (2003) reported a higher prevalence on John Brewer Reef where approximately 10.04% of scleractinian colonies displayed signs of BrB during May 2003. In the latter case, staghorn acroporids encompassed 100% of infected colonies (Nash 2003). An epidemic of a syndrome which progresses this rapidly could result in significant damage to coral assemblages, therefore monitoring the progression and transmission of this syndrome and, in particular, the consequences along the GBR is extremely important.

Currently, we know very little about rates of progression and spread of coral disease on the GBR. Given that disease has resulted in substantial reductions in coral abundance (Kuta and Richardson 1996) and shifts in the composition of coral communities in the Caribbean (Aronson and Precht 2001), establishing baseline data for the GBR should be a primary goal. The aims of this chapter are to measure the natural progression and transmission of BBD and BrB. These measurements will determine the rates at which pathogens associated with these two diseases destroy coral tissue within a colony (rate of progression) and how quickly it can infect adjacent healthy coral colonies (rate of transmission/spread). By quantitatively measuring variability in these rates for BBD during different months, correlations between high water temperatures and the progression and transmission of disease may be established. By measuring the rate of progression and transmission for BrB at two reef sites in the central and northern sectors of the GBR, we will gain insights into the variability that occurs between locations.

2.3. MATERIALS AND METHODS

2.3.1. STUDY SITE DESCRIPTION

Measurements of the natural rate of progression and transmission of black band disease were conducted at Horseshoe Reef off the coast of Lizard Island, Australia (14° 40'S 145° 28'E) during July 2003, January 2004, and May 2004 (Figure 2.1). These months were selected because there were significant differences between the average seawater temperatures during July 2003/May 2004 (25.7˚C ± 0.5 and 27˚C ± 0.5) and January 2004 (30˚C ± 0.5). Field studies of natural rates of progression and transmission of brown band syndrome took place at Horseshoe Reef off the coast of Lizard Island in May 2004 and Davies Reef (18° 49.86'S 147° 38.2'E), a mid-shelf location in the central GBR, during December 2003 (Figure 2.1). These reefs were chosen to represent the central (Davies) and northern (Lizard Island) sectors of the GBR.

2.3.2. SAMPLING DESIGN

During each month, the rate of progression for BBD was measured on 3-10 branches from three *Acropora muricata* colonies (formerly known as *A. formosa*) depending on the number of available branches displaying signs of BBD (Figure 2.2). Measurements were taken from the same three colonies during each month. Rates of transmission were measured during January and May 2004 on three branch pairs consisting of one *in situ* diseased branch and one healthy branch fragment from each of 2-3 *A. muricata* colonies (Figure 2.3). Healthy branch fragments were cable tied to *in situ* diseased branches in order to minimize the spread of black band disease to healthy colonies. As a control, three pairs of healthy branch fragments cable tied to *in situ* healthy branches were monitored for signs of tissue lysis. Once again, measurements were taken from the same three colonies during each month. The rate of progression for BrB was measured on 2-10 branches of 4-5 *A. muricata* colonies depending on the number of branches displaying characteristic signs of the syndrome (Figure 2.4). Rates of transmission of BrB between branches were measured during December 2003 on ten branch pairs (consisting of a healthy branch fragment tied to an *in situ* diseased branch) from each of three *A. muricata* colonies (Figure 2.5). As a control, 6 pairs of healthy branch fragments cable tied to *in situ* healthy branches were monitored during the transmission trial for signs of tissue death.

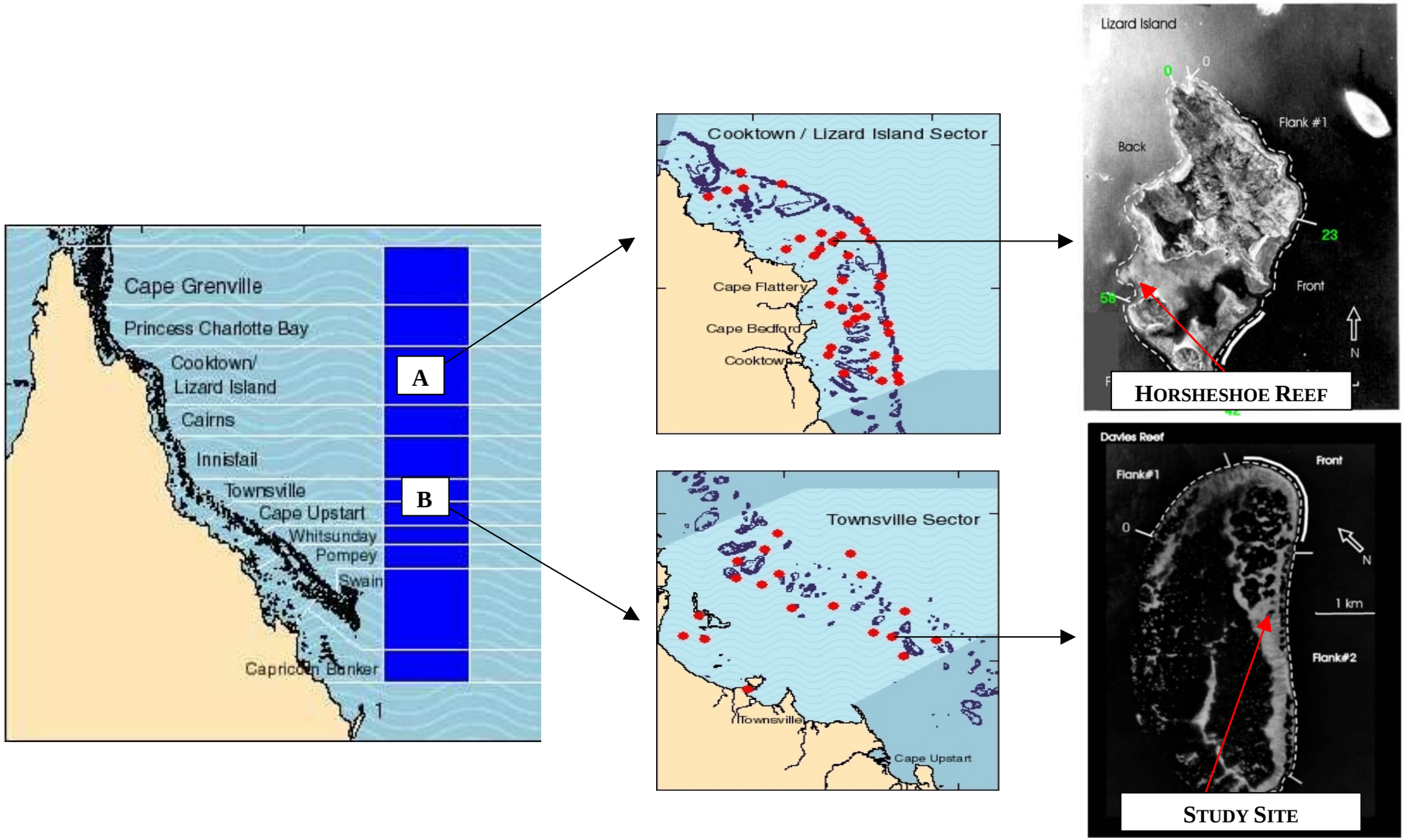

Figure 2.1. Study sites for field measurements of black band disease and brown band syndrome. (a) Horseshoe Reef, off the coast of Lizard Island, Australia (b) Davies Reef, a midshelf reef in the Great Barrier Reef Marine Park (www.aims.gov.au images).

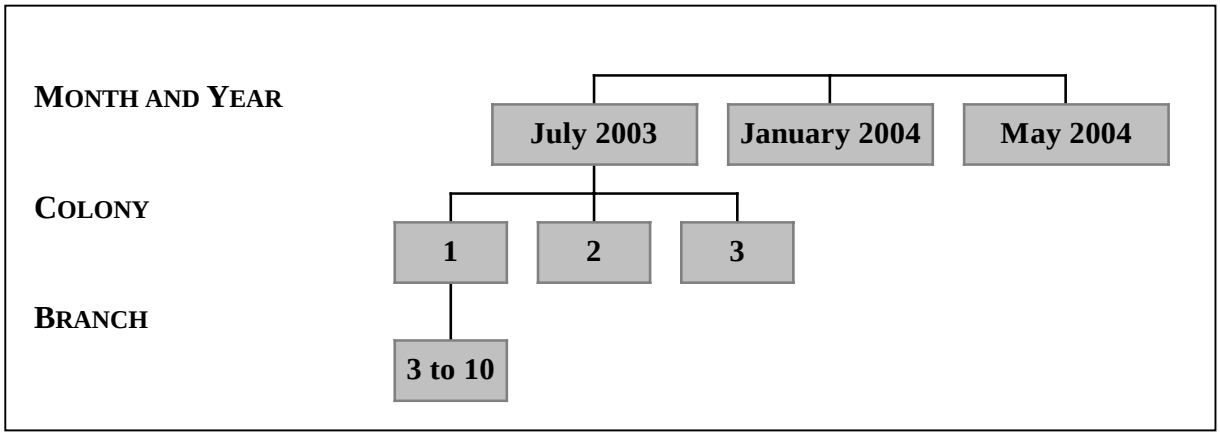

FIGURE 2.2. Sampling design for measuring the rate of progression, band width, and circumference for black band disease on *Acropora muricata*. The band width was measured during 2004 only.

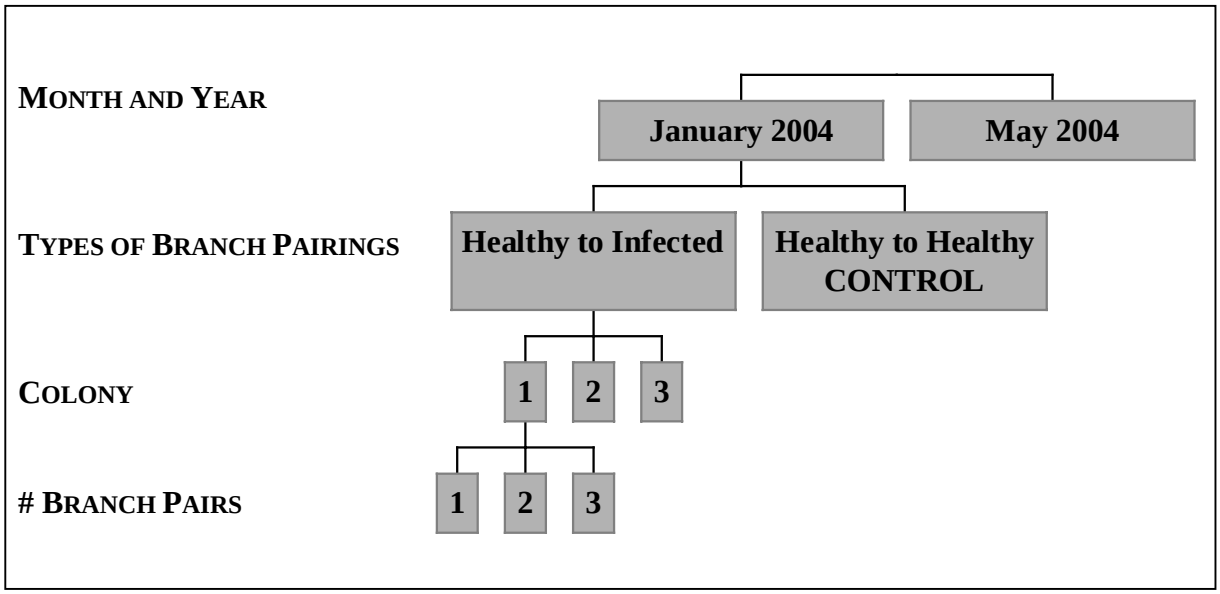

FIGURE 2.3. Sampling design for measuring the transmission of black band disease between branches of *Acropora muricata*.

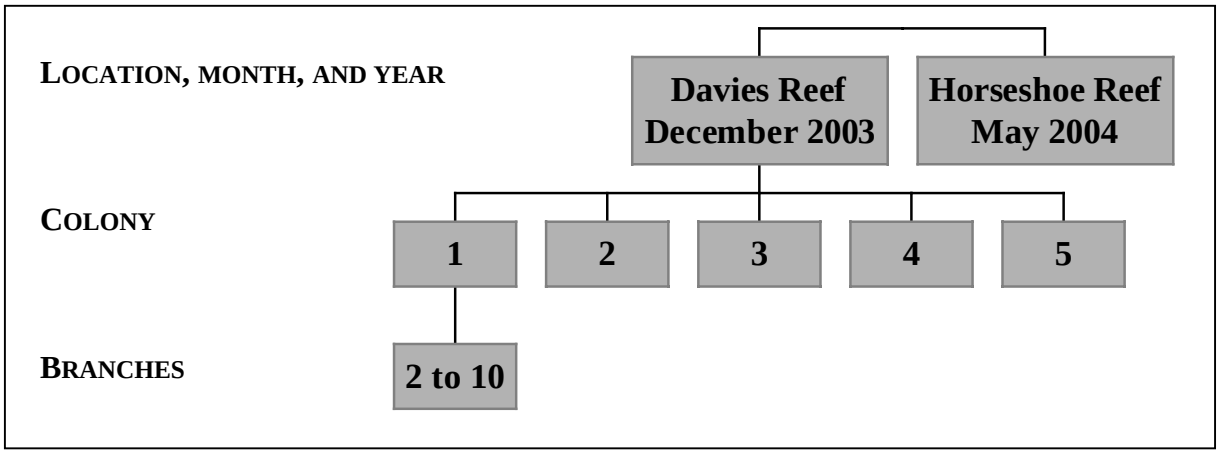

FIGURE 2.4. Sampling design for measuring the rate of progression and band width for brown band syndrome on *Acropora muricata*.

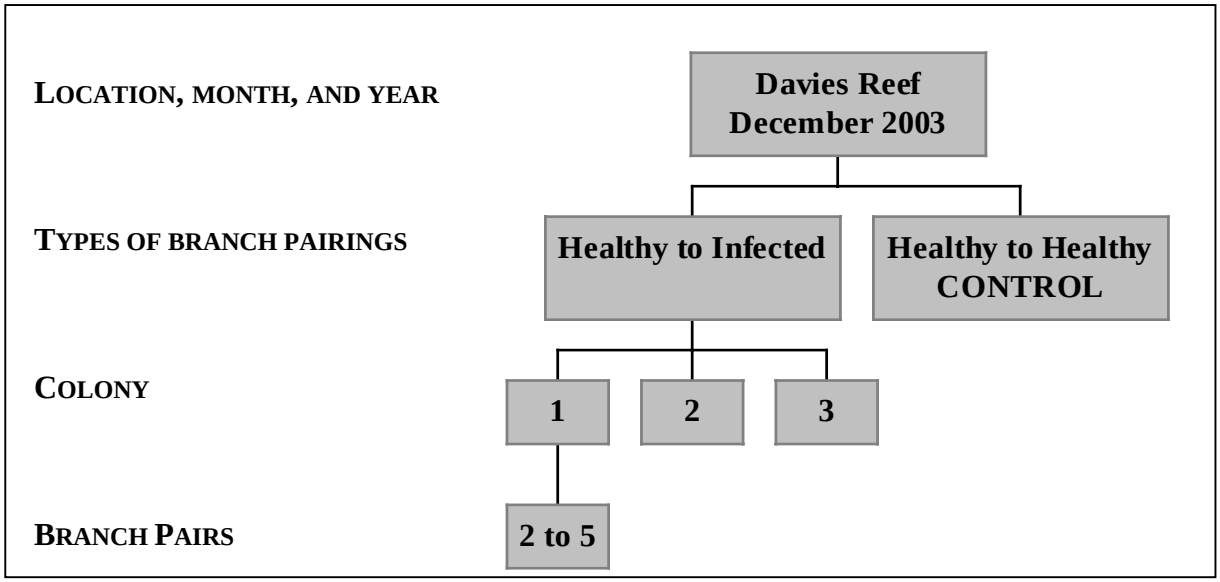

FIGURE 2.5. Sampling design for measuring the transmission rate of brown band syndrome between branches of *Acropora muricata*.

2.3.3. RATE OF PROGRESSION

To measure the rate of disease progression along a branch, cable ties were secured to exposed skeleton approximately 2 cm away from the disease band, marked with numbered flagging tape, and photographed with a Nikon Coolpix 5000 (Figure 2.6). Using a flexible measuring tape, the distance from the cable tie to the healthy tissue, width of the disease band, and circumference of the branch near the disease band were measured and recorded every 24 hours for no less than 3 days.

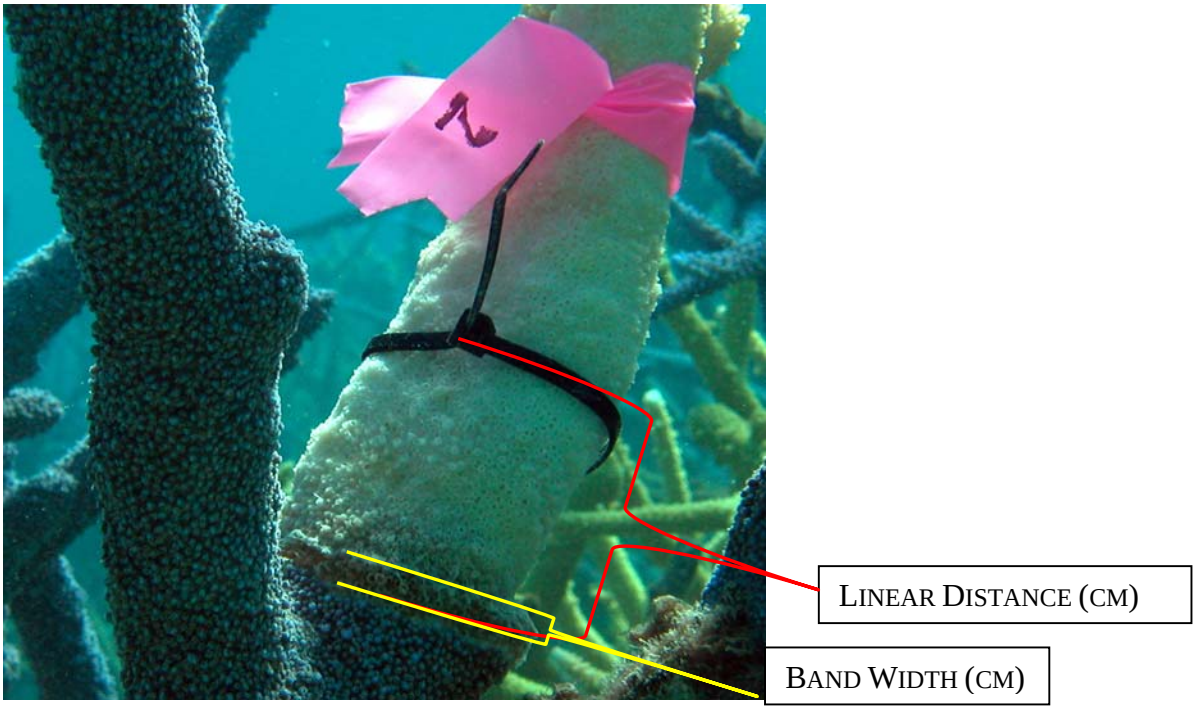

FIGURE 2.6. Photographic diagram showing brown band syndrome and depicting the tagging method and measurements taken on each branch for both disease states. Photograph taken by Eric Matson.

2.3.4. RATE OF TRANSMISSION

The rate of transmission was measured by removing branch fragments from healthy colonies and attaching them to *in situ* diseased branches using a cable tie and numbered flagging tape. As a control, removed healthy branch fragments were also attached to *in situ* healthy branches in the same manner. Cable ties were secured to exposed skeleton about 2 cm away from the disease band on each infected branch in order to measure the rate of progression. The distance between the cable tie and healthy tissue and the width of the disease band was measured using a flexible measuring tape. In addition, the time taken for the healthy branch fragment to show

macroscopic signs of the disease was recorded. The pairs of healthy and diseased branches were observed and measured every 24 hours for no less than 3 days.

2.3.5. STATISTICAL ANALYSES

To assess if the rate of progression for black band disease and brown band syndrome varied during different months and locations, a nested GLM Repeated Measures Analysis of Variance was conducted using a Type IV Sum of Squares to compensate for missing values (Zar 1999, Scheiner and Gurevitch 2001, Quinn and Keough 2002). The significance value was set at 0.05. A third nested Repeated Measures Analysis of Variance was used to compare differences in the band widths of black band disease during different months of the year. Furthermore, variations between the rate of progression of black band disease and brown band syndrome were detected using a nested Repeated Measures Analysis of Variance.

Nested One-way Analysis of Variance tests were used to compare differences in the band width of BrB between reef sites. If missing values were present, a Type IV Sum of Squares was used. Significance values remained at 0.05. Variations in the band width of brown band versus black band diseases were analyzed with the same statistical tests but band width data were log transformed to satisfy assumptions of normality and equal variances. Nested One-way Analysis of Variance tests were also used to assess differences between the rate of progression along *in situ* diseased branches cable tied to healthy branch fragments and controls (a single *in situ* diseased branch). One-way Analysis of Variance tests were performed to compare differences in BBD infection times between months. Log transformations were used for transmission data to satisfy the equal variances assumption.

2.4. RESULTS

2.4.1. PROGRESSION AND TRANSMISSION OF BLACK BAND DISEASE

The rate of progression of BBD at Horseshoe Reef was significantly different between months ($p < 0.05$, Table 2.1). Bonferroni post-hoc tests detected significantly greater rates of progression during the month of January as opposed to July ($p = 0.004$) and May ($p = 0.004$). BBD progressed at a rate 1.7 and 2.4 times faster in January 2004 than it did in July and May respectively (Figure 2.7). There was not a significant difference in the rate of progression between coral colonies nested within months. The average surface area of tissue loss (cm^2) was significantly greater during the warmer month of January with two times more tissue death recorded than during the month of July ($p < 0.05$, Table 2.1) (Figure 2.8). The loss of tissue surface area between months was significantly different between coral colonies (Table 2.1). The width of the black band did not show significant differences between January and May ($p > 0.05$, Table 2.1). Furthermore, variations in band width were not observed between colonies. Average band width in January was 0.412 ± 0.042 cm while average band width in May was 0.368 ± 0.067 (Figure 2.9).

TABLE 2.1. Statistical results for the rate of progression, surface area of tissue loss, and band width of black band disease between months (July, January, and May). A nested General Linear Model Repeated Measure test (RM) was used to compare the rate of progression of BBD between months and colonies nested within months.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	RM	months	2	31.568	$p < 0.05$
		colonies	5	1.225	0.316
Surface Area (cm^2)	RM	months	1	8.227	$p < 0.05$
		colonies	4	7.840	$p < 0.001$
Band Width (cm)	RM	months	1	0.1908	$p > 0.05$
		colonies	4	1.622	0.198

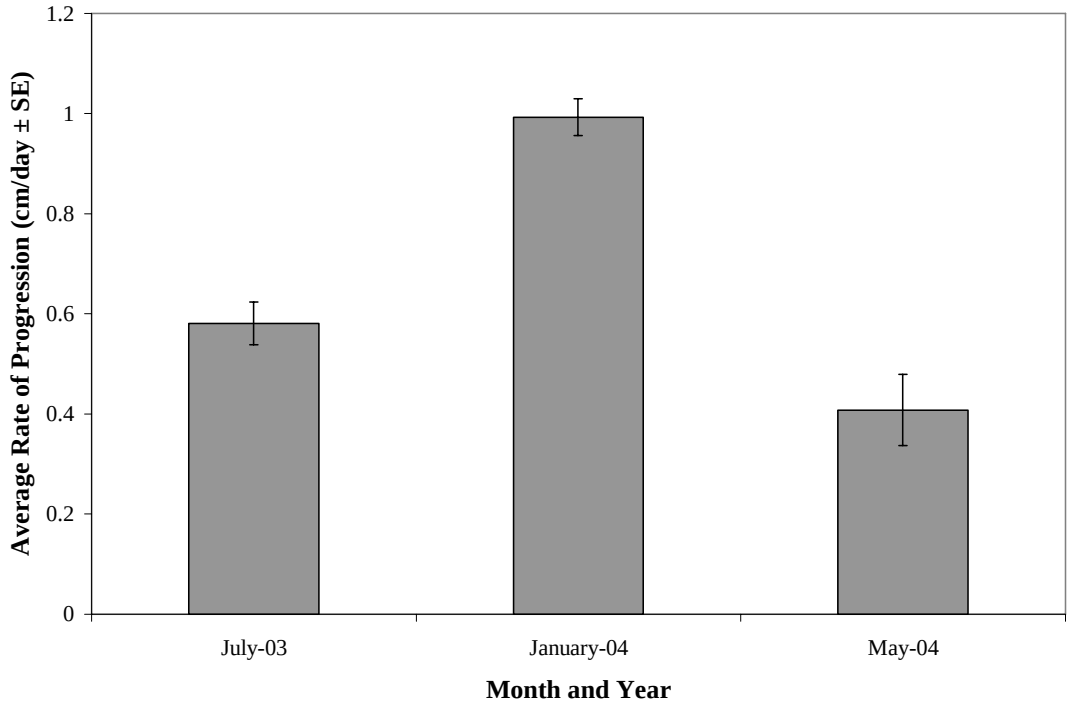

FIGURE 2.7. Average linear rate of progression (measured as cm/day ± SE) of black band disease on *Acropora muricata* across time. July 2003 and May 2004 seawater temperatures averaged 25.7˚C ± 0.5 and 27˚C ± 0.5 respectively, while January 2004 seawater temperatures averaged 30˚C ± 0.5.

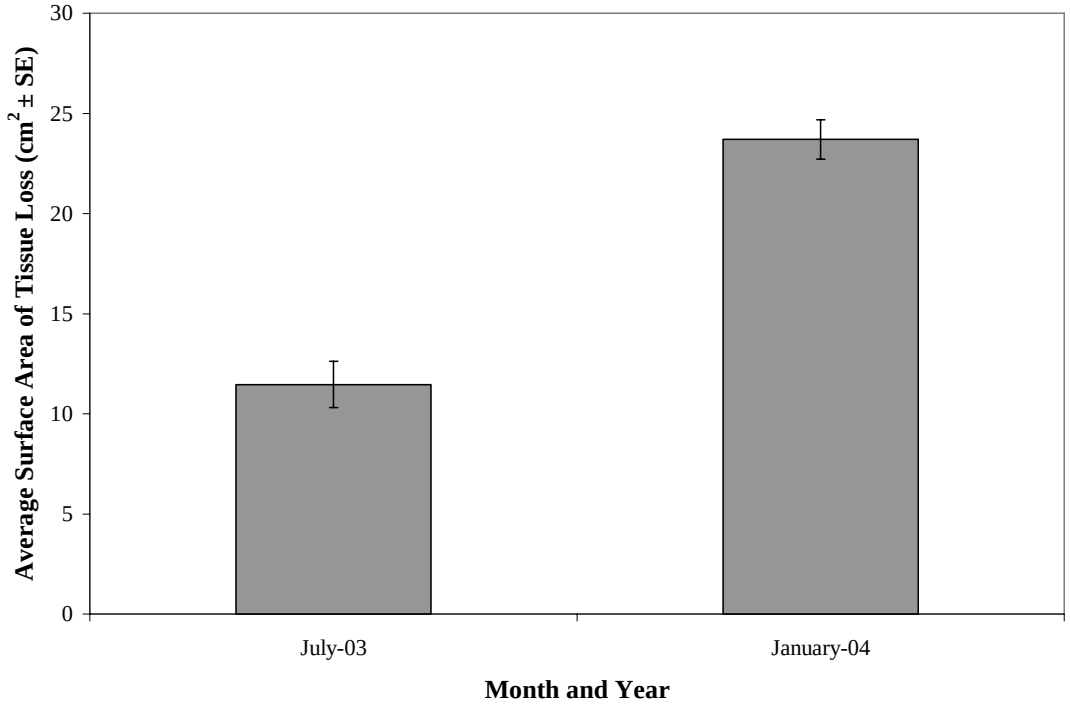

FIGURE 2.8. Average surface area of tissue loss (measured as cm^2 ± SE) of black band disease on *Acropora muricata* across time. July 2003 seawater temperatures averaged 25.7˚C ± 0.5, while January 2004 seawater temperatures averaged 30˚C ± 0.5.

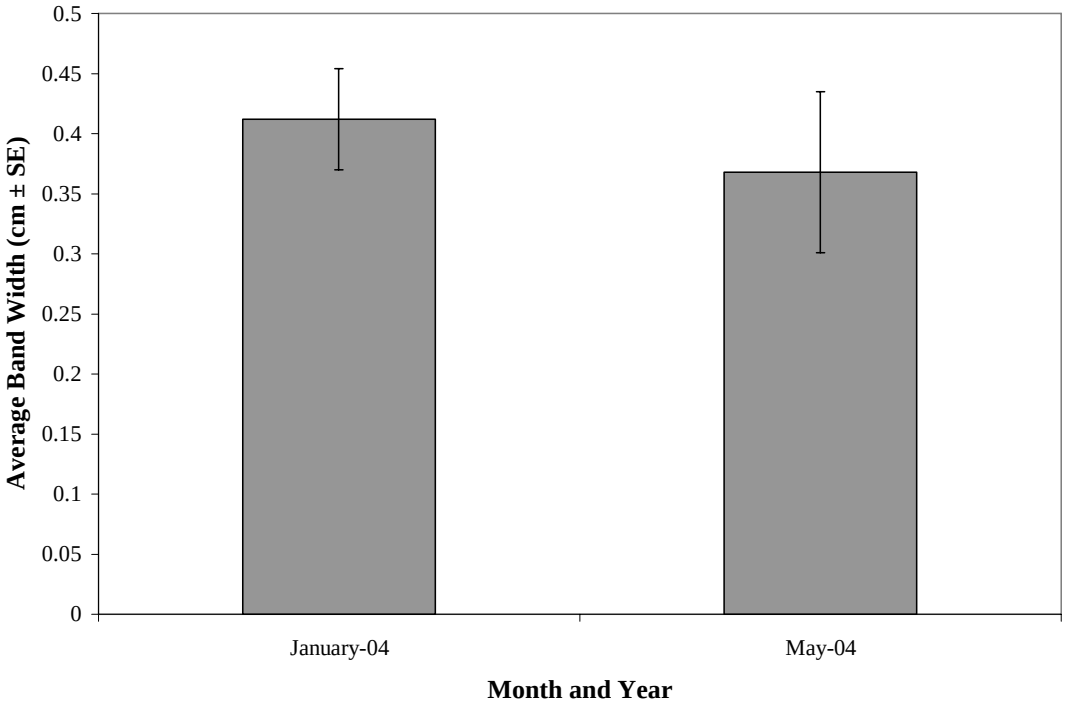

FIGURE 2.9. Average band width (measured as cm ± SE) of black band disease on *Acropora muricata* across time. January and May 2004 seawater temperatures were measured at 27˚C ± 0.5 and 30˚C ± 0.5 respectively.

The rate of transmission of black band disease was significantly greater during the month of January than during May 2004 ($p < 0.001$, Table 2.2). During January, healthy branch fragments displayed characteristic signs of infection 1.2 days quicker than during the month of May (Figure 2.10). Inducing infection by cable tying a healthy branch fragment to an *in situ* diseased branch caused the progression of BBD along the *in situ* diseased branch to slow down. Compared to controls (a single *in situ* diseased branch), the progression of BBD along *in situ* diseased branches used to induce infection was significantly slower ($p < 0.01$, Table 2.2). Over a 3-day period, BBD progressed a total linear distance of 2.7 ± 0.268 cm along *in situ* diseased branches cable tied to healthy branch fragments while controls progressed a total linear distance of 4.5 ± 0.213 cm (Figure 2.11).

TABLE 2.2. Statistical results for time to infection between months (January and May) and the rate of progression along *in situ* black band diseased branches used to induce the infection of healthy branches (experimental transmission pairings) and single *in situ* diseased branches (controls). A One-way Analysis of Variance test (ANOVA) was used to compare the time to infection between months and a general linear model nested Univariate Analysis of Variance (NANOVA) was used to compare differences in the rate of progression between branches and branches nested within colonies.

Dependent Variable	Test	Factors	df	F value	Significance
Time to Infection [$\log_{10}$(days)]	ANOVA	months	1	58.495	$p < 0.001$
Rate of Progression (cm/day)	NANOVA	transmission pairs vs. *in situ* controls	1	48.446	$p < 0.01$
		colonies	4	0.594	0.669

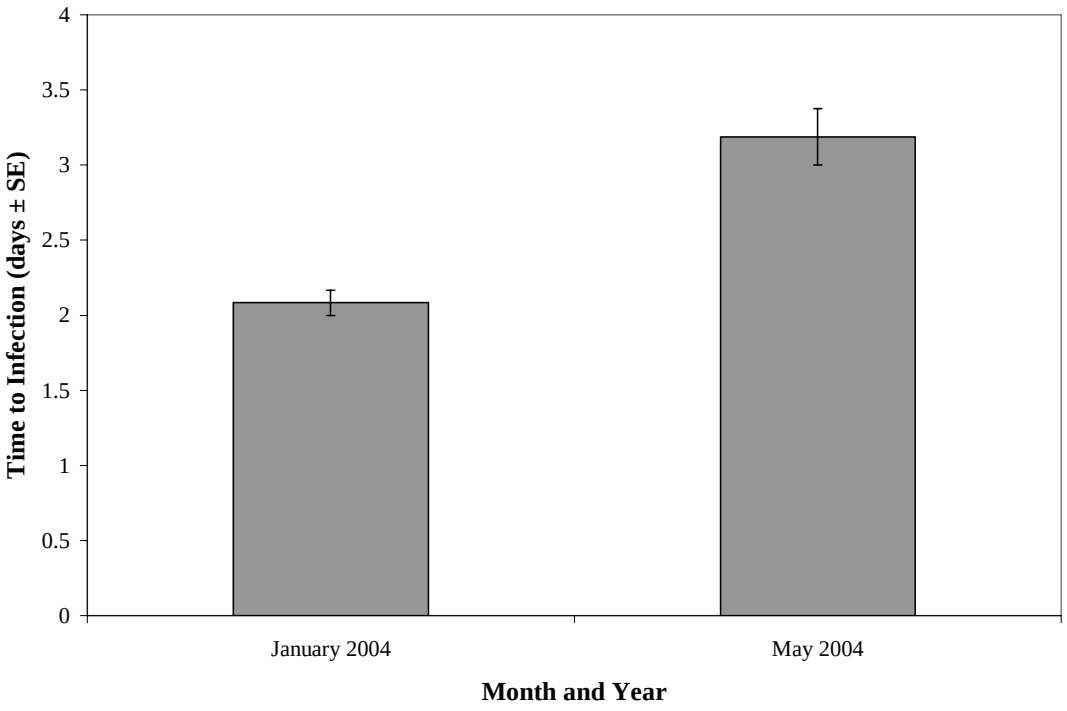

FIGURE 2.10. Time taken for *in situ* black band diseased branches to infect healthy branch fragments (measured in days ± SE) on *Acropora muricata* across time. January and May 2004 seawater temperatures were measured at 30˚C ± 0.5 and 27˚C ± 0.5 respectively.

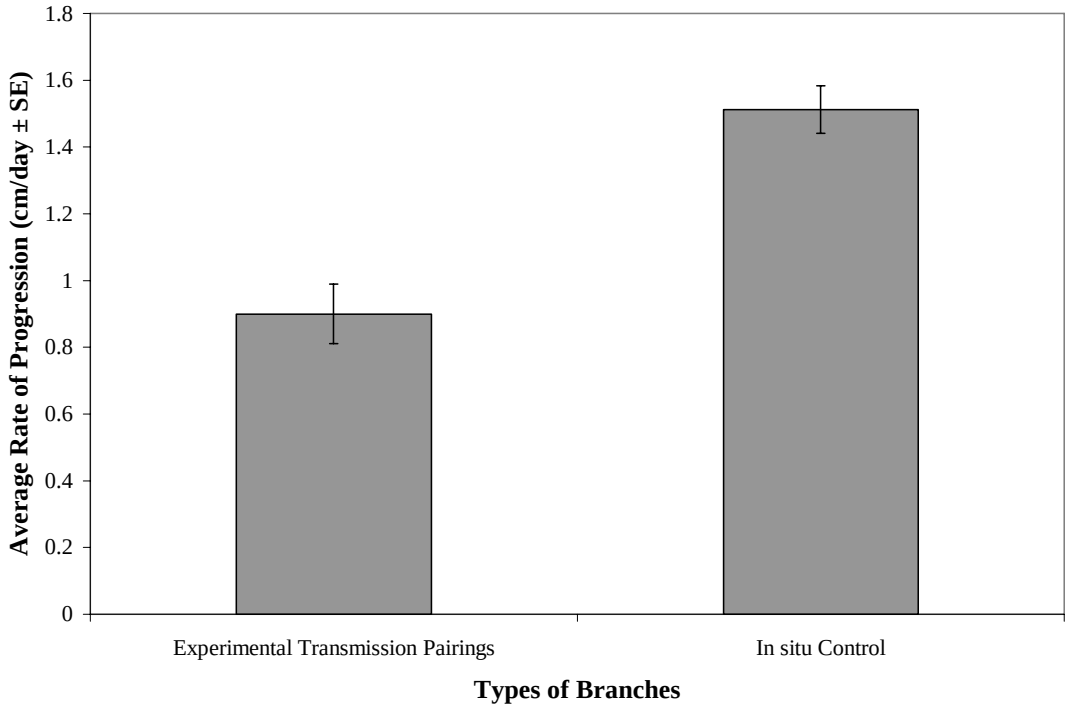

FIGURE 2.11. Average rate of progression (measured as cm/day ± SE) of black band disease along *in situ* diseased *Acropora muricata* branches cable tied to healthy branch fragments (experimental transmission pairings) and single *in situ* diseased branches (control) on Horseshoe Reef during January 2004.

2.4.2. PROGRESSION AND TRANSMISSION OF BROWN BAND SYNDROME

Significant differences in the rate of progression of brown band syndrome were observed between Horseshoe and Davies Reef ($p < 0.01$, Table 2.3). Compared to Davies Reef (average rate of 0.902 ± 0.275 cm/day), brown band syndrome progressed 2.3 times faster at Horseshoe Reef (2.104 ± 0.348 cm/day) (Figure 2.12). The progression of the syndrome showed no significant variations between colonies at both locations. There was a significant difference in the width of the brown band between Horseshoe and Davies Reef ($p < 0.05$, Table 2.3). The width of the disease band was 2.4 times greater at Horseshoe Reef than at Davies Reef (Figure 2.13). Furthermore, band widths varied significantly between colonies ($p < 0.05$, Table 2.3).

At Davies Reef, brown band syndrome infected 50% of branch pairs. The 4 healthy branches that were infected showed characteristic signs of the syndrome after an average of 1.7 days. Compared to controls (a single *in situ* diseased branch), inducing the infection of healthy branch fragments by cable tying them to *in situ* diseased

branches did not slow the rate of progression along the *in situ* diseased branch ($p > 0.05$, Table 2.4). The rate of progression along single *in situ* diseased branches *and in situ* diseased branches with an attached healthy branch fragment was 0.796 ± 0.166 and 0.732 ± 0.218 cm/day respectively (Figure 2.14).

TABLE 2.3. Statistical results for the rate of progression and band width of brown band syndrome between reef sites (Davies and Horseshoe Reef). A nested General Linear Model Repeated Measure test (RM) was used to compare the rate of progression of BrB between sites and colonies nested within sites. A general linear model nested Univariate Analysis of Variance test (ANOVA) was used to compare the band width of BrB between sites and colonies nested within sites.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	RM	sites	1	19.834	$p < 0.01$
		colonies	6	0.371	0.888
Band Width (cm)	ANOVA	sites	1	8.079	$p < 0.05$
		colonies	8	2.858	0.023

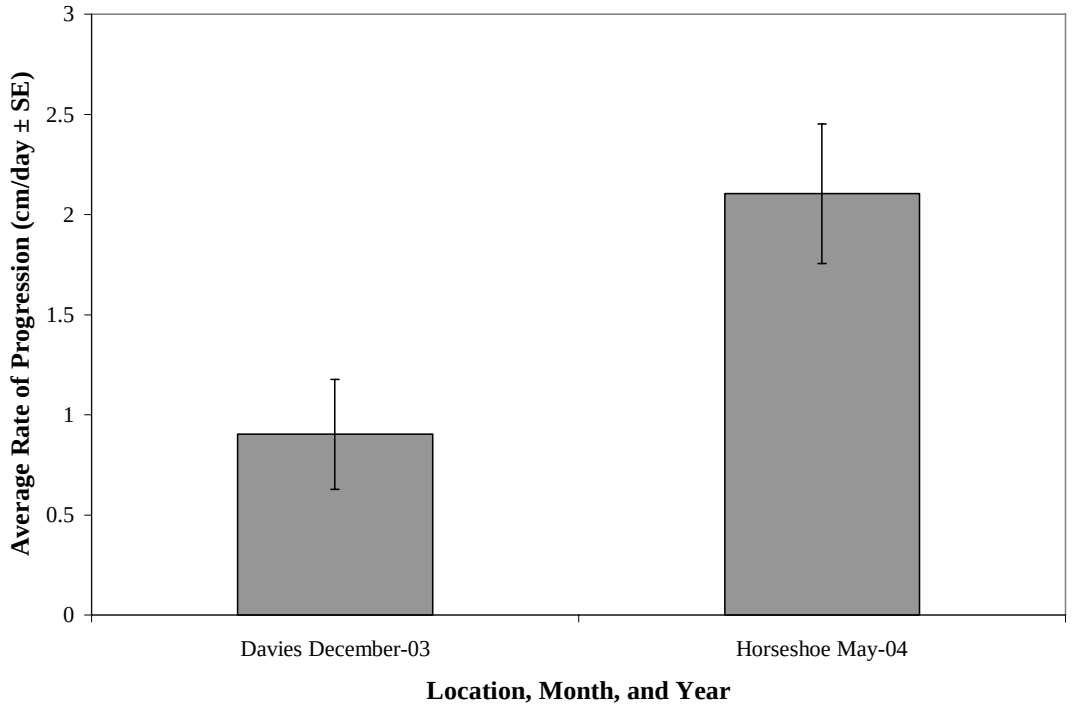

FIGURE 2.12. Comparison of the average rate of progression (measured as cm/day ± SE) of brown band syndrome on *Acropora muricata* on two reefs (Davies and Horseshoe Reef) across time. December 2003 and May 2004 seawater temperatures were measured at 27.3˚C ± 0.5 and 27˚C ± 0.5 respectively.

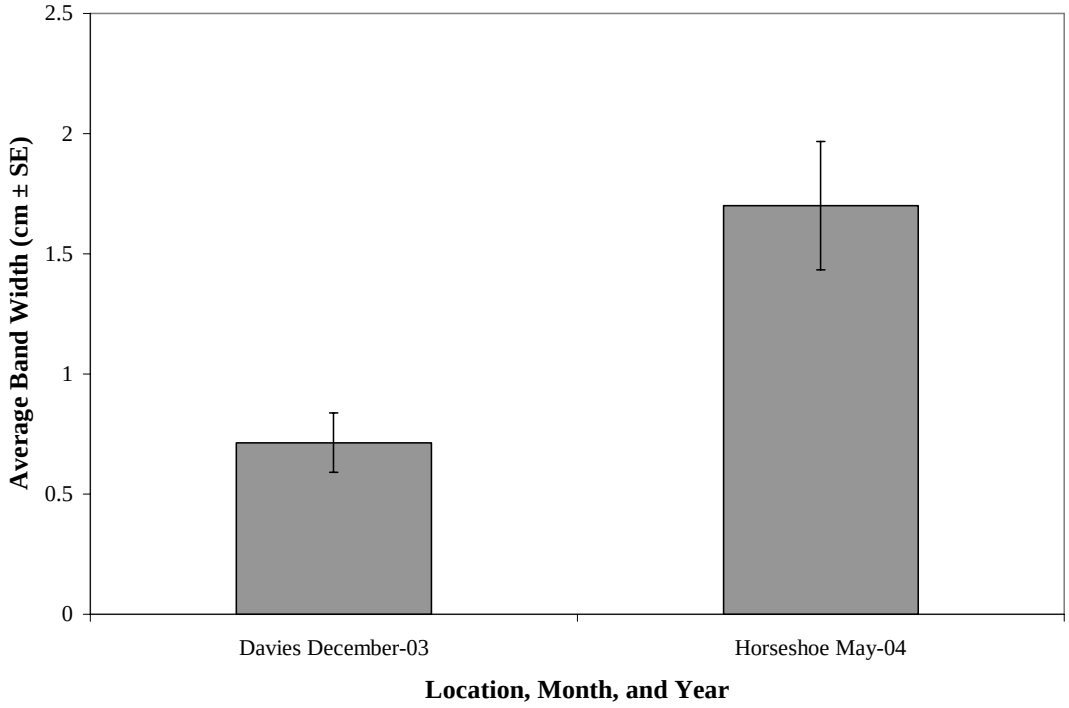

FIGURE 2.13. Average band width (measured as cm ± SE) of brown band syndrome on *Acropora muricata* on two reefs (Davies and Horseshoe Reef) across time. December 2003 and May 2004 seawater temperatures were measured at 27.3˚C ± 0.5 and 27˚C ± 0.5 respectively.

TABLE 2.4. Statistical results for the progression of brown band syndrome along *in situ* diseased branches used to induce the infection of healthy branches (experimental transmission pairings) and single *in situ* diseased branches (controls). NANOVA stands for the nested General Linear Model Univariate Analysis of Variance.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	NANOVA	transmission pairs vs. *in situ* controls	1	0.112	p > 0.05
		colonies	3	0.475	0.704

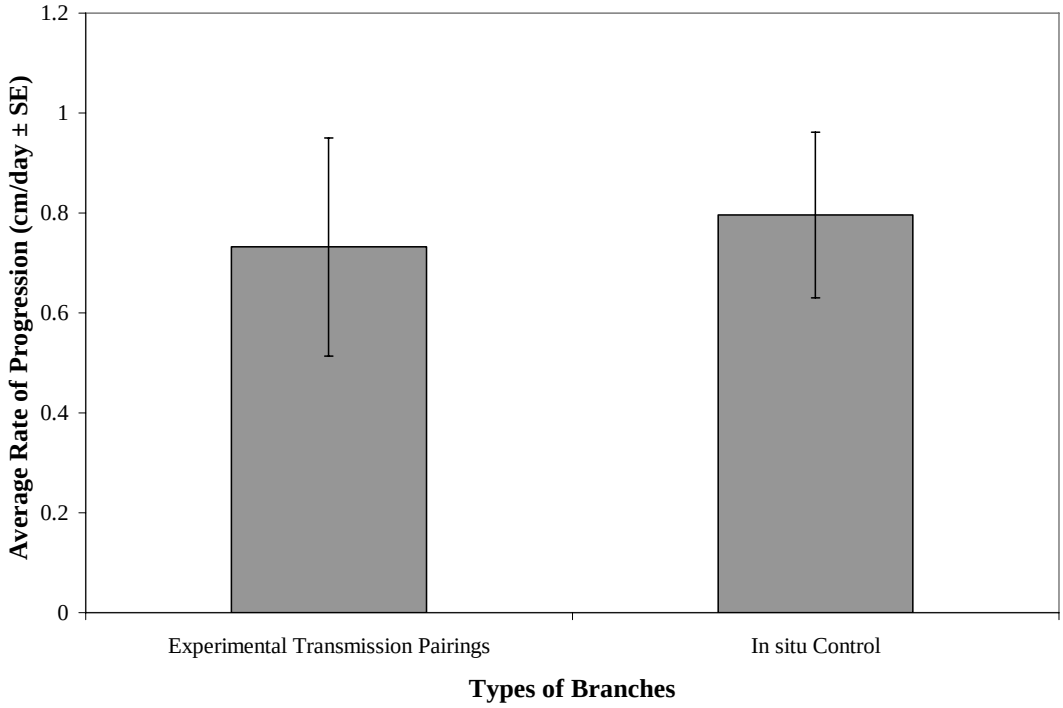

FIGURE 2.14. The rate of progression (measured as cm/day ± SE) of brown band syndrome along *in situ* diseased *Acropora muricata* branches cable tied to healthy branch fragments (experimental transmission pairings) and single *in situ* diseased branches (control) on Davies Reef during December 2003.

2.4.3. BLACK BAND DISEASE VERSUS BROWN BAND SYNDROME

During May 2004, there was a significant difference in the rate of progression between black band disease and brown band syndrome at Horseshoe Reef, Lizard Island ($p < 0.05$, Table 2.5). Brown band syndrome (average rate of 2.104 ± 0.334 cm/day) progressed considerably faster along coral branches than black band disease (average rate of 0.387 ± 0.309 cm/day) (Figure 2.15). There were no significant variations between colonies of BBD and BrB. Band widths were significantly different between the two maladies with brown band syndrome exhibiting a mean band width 5 times greater than black band disease ($p < 0.05$, Table 2.5, Figure 2.16).

TABLE 2.5. Comparative statistical results for the rate of progression and band width of black band disease and brown band syndrome during May 2004. A nested General Linear Model Repeated Measures (RM) was used to compare the rate of progression between diseases and colonies nested within diseases. A nested General Linear Model Univariate Analysis of Variance (ANOVA) was used to detect differences in the width of disease bands between diseases and colonies nested within diseases.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	RM	diseases	1	24.417	$p < .05$
		colonies	4	0.582	0.682
Band Width [$\log_{10}$(cm)]	NANOVA	diseases	1	17.768	$p < .05$
		colonies	6	1.707	0.197

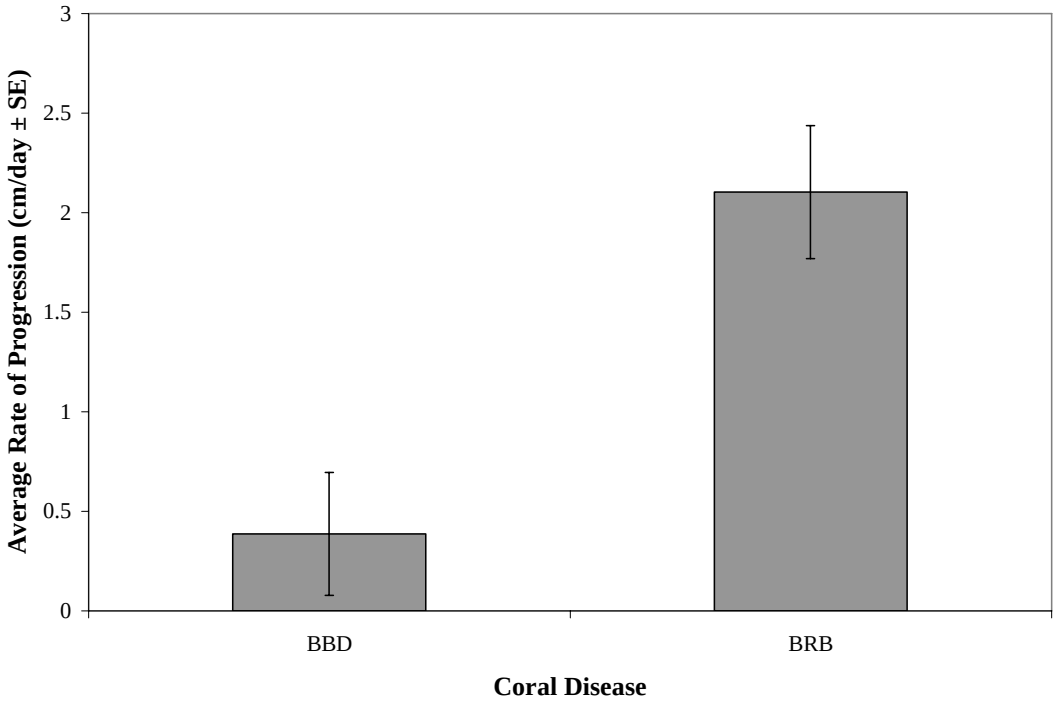

FIGURE 2.15. Comparison of the May 2004 average rate of progression (measured as cm/day ± SE) between black band disease (BBD) and brown band syndrome (BrB) on *Acropora muricata* at Horseshoe Reef, Lizard Island.

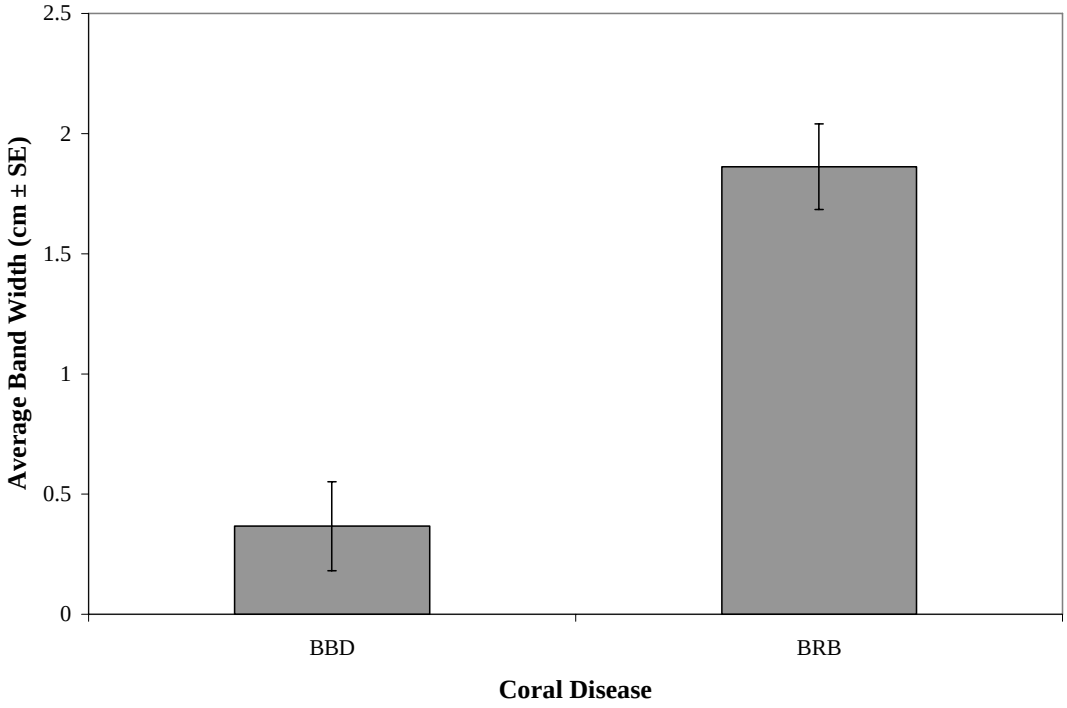

FIGURE 2.16. Comparison of the May 2004 average band width (measured as cm ± SE) between black band disease (BBD) and brown band syndrome (BrB) on *Acropora muricata* at Horseshoe Reef, Lizard Island.

2.5. DISCUSSION

The progression and transmission of black band disease on the Great Barrier Reef significantly varied between sampling times (Table 2.1, Table 2.2). During the month of January, BBD progressed along coral branches 2.4 times faster than it did during the months of July and May. Compared to the month of May, BBD was capable of transmitting from *in situ* diseased branches to healthy branch fragments 1.2 days quicker during January. Given these sampling times, the factors most likely to be implicated for these variations include seawater temperatures and light intensities. Ocean temperatures over approximately two weeks in January averaged 30°C ± 0.5 while average temperatures over two weeks in July and May reached 25.7 °C ± 0.5 and 27°C ± 0.5 respectively. In addition to elevated temperatures, light intensity data collected from the Australian Institute of Marine Science weather station on Agincourt Reef (16° 2'S 145° 49'E) showed that light intensities were 1.6 times greater during the month of January (~1750 $\mu E/m^2/s$ compared to 1100 $\mu E/m^2/s$ in May). Due to the 2-3°C increase in seawater temperatures and 650 $\mu E/m^2/s$ rise in light intensities between the months of January and July/May, it can be assumed that elevated temperatures and light intensities are positively correlated to the progression and spread of BBD.

Tropical reefs are often exposed to warm seawater temperatures and high levels of ultraviolet light (UV) (Veron 1986, Hoegh-Guldberg 1999). These parameters have been intensified by global warming and the depletion of the stratospheric ozone layer (Jokiel and Coles 1977, Baker 1999). During the summer, increasing temperatures and ultraviolet (UV) radiation are often reported as a source of stress for coral communities (Siebeck 1988, Lesser 1996, Hoegh-Guldberg 1999). High ocean temperatures have been identified as a major cause of mass bleaching events in the GBR (Jokiel and Coles 1990, Hoegh-Guldberg 1999, Berkelmans and Oliver 1999, Berkelmans and Willis 1999, Lough 2000, Berkelmans 2004) while high light intensities have been observed to intensify the damage caused by elevated temperatures (Coles and Jokiel 1978). During long-term growth experiments in Hawaii, corals exposed to high levels of light and sub-lethal temperatures exhibited a substantial loss of zooxanthellae, increased mortality, and reductions in growth rates (Coles and Jokiel 1978). If the combination of elevated temperatures and light

intensities has the ability to reduce coral health, then the positive correlation between these parameters and the progression and transmission of BBD may involve the response of the coral and the pathogen(s) to these adverse conditions.

Disease epidemics have been linked to adverse conditions (Porter and Tougas 2001, Kuta and Richardson 2002), suggesting that increased temperature and UV may enhance a coral's susceptibility to disease infections (Porter and Tougas 2001). For example, the highest prevalence of new black band disease infections on Jamaican coral reefs was recorded during the summer months (July to September) where 33% of colonies displayed characteristic signs of infection (Bruckner and Bruckner 1997). In comparison, the disease was observed on 17.6% of colonies during the winter months (October to December) (Bruckner and Bruckner 1997). Significant relationships have also been reported between the prevalence of black band disease and high water temperatures up to 29 to 30°C (Kuta and Richardson 2002). On the Great Barrier Reef, the prevalence of black band disease increased eight-fold from 11 to 95 observed cases during the summer month of January 2003 (Willis *et al.* 2004). These are just a few of the numerous studies that have reported an increased prevalence of black band disease during the summer months (Bruckner and Bruckner 1997, Willis *et al.* 2004). Furthermore, the growth rate and activity of *Phormidium corallyticum*, the cyanobacterial pathogen of black band disease in the Caribbean, has been reported to increase at higher temperatures with an optimum of 28 to 30°C (Richardson and Kuta 2003). This suggests that elevated temperatures enhance the virulence of BBD giving it the ability to progress among and spread between colonies at a faster rate. Consequently, increased virulence of the pathogen would lead to faster rates of spread and progression and therefore a higher prevalence of BBD during the summer months.

Comparable to reports from the Caribbean, black band disease at Lizard Island was highly infectious and capable of spreading between adjacent coral colonies in 2-3 days depending on the month sampled. The progression of BBD along an infected branch was diminished when a healthy branch fragment was cable tied to it. This suggests that when the microbial community associated with BBD transmits to an adjacent healthy branch, the pathogen's concentration may be reduced or its activity may be suppressed by the anti-microbial response of the new host colony thereby

causing its progression along the original branch to slow down. The ability of black band disease to transmit to neighboring healthy coral branches allows the disease to progress throughout multiple regions of a single colony. On Jamaican coral reefs, BBD transmitted to neighboring *Montipora annularis* colonies and infected multiple regions on each colony (Bruckner and Bruckner 1997). Since black band disease is capable of spreading throughout colonies of the massive Caribbean species *M. annularis* and the branching *A. muricata* colonies at Lizard Island, perhaps the transmission of black band disease is comparable among different coral morphologies.

Significant differences in the progression of brown band syndrome were observed between the two reef sites (Table 2.3). BrB progressed 2.3 times faster at Horseshoe Reef, in the northern sector of the GBR, than at Davies Reef, in the central sector of the GBR. Sampling times at the two reef sites occurred during December 2003 (Davies Reef) and May 2004 (Horseshoe Reef, Lizard Island). During these sampling times, ambient seawater temperatures over a two week period averaged 27.5˚C ± 0.5 at Davies Reef and 27˚C ± 0.5 at Horseshoe Reef. Since temperature differences were minimal, it can be assumed that this parameter would have little impact on the variations in rate of progression observed between the two sites. On the other hand, light intensities did vary greatly between sampling times with 1.3 times higher light intensities measured at Davies Reef. Weather stations operated by the Australian Institute of Marine Science recorded average light intensities of 1400 µE/m^2/s at Davis Reef in December 2003 and 1100 µE/m^2/s at Agincourt Reef during May 2004. Although increased light has been identified as a possible enhancer of coral disease (Kuta and Richardson 2002), higher rates of progression were observed at Horseshoe Reef, Lizard Island than at Davies Reef. This discrepancy may be the result of three factors: (1) light may not affect the virulence of BrB pathogens, (2) only a combination of elevated temperatures and light can impact the progression of BrB, while light on its own cannot, or (3) light levels may have been diminished by turbidity. In any case, the increased light levels at Davies Reef did not seem to promote the progression of the brown band syndrome.

In addition to variations between sampling times, it is important to consider differences between the sites themselves. Horseshoe Reef is a sheltered patch reef

near the lagoon on the leeward side of Lizard Island. Wave exposure at this site is minimal most of the year, but larger swells can occur during southwesterly winds in the summer (personal observation). Fulton and Bellwood (2005) have measured net flow velocities at 17.1-25.3 cm/sec and average wave heights of approximately 0.18 m during rough sea conditions for two lagoon locations at Lizard Island. In contrast, sampling sites at Davies Reef were located on the windward reef flat where flow velocities averaged 35 cm/sec (maximum flow rates to 60 cm/sec) and wave heights reached 2 m (Sebens and Done 1992). Heavy wave action on shallow coral reefs commonly leads to increased turbidity. The presence of suspended particulate matter in the water column has the ability to filter out harmful ultraviolet radiation associated with high light intensities. Furthermore, aquarium observations from this study have observed the dislodgement of BrB ciliates, which are implicated as potential causative agents of BrB (see Chapter 5), whenever high water flow and/or aquarium powerheads were used. If high water circulation is capable of dislodging the ciliates associated with BrB under aquarium conditions, then we would expect smaller disease band widths at exposed locations where flow velocities are greater. The average width of the brown band measured at Davies reef was significantly smaller than the width of the disease band at Horseshoe Reef (Table 2.3). Since smaller disease bands would have lower concentrations of potentially pathogenic microorganisms, higher water flow may lead to reductions in the rate of disease progression. The slower rate of progression and smaller band widths measured at Davies Reef support this theory. Although this suggests that high water flow and wave exposure may have important implications for the progression and spread of disease, it is important to note that the differences in sampling times may be involved in the variations observed. Future studies should aim to complete measurements during the same month of the year in order to adequately determine any variations in the progression of BrB between coral reefs.

The rapid rates of progression observed at Horseshoe Reef may account for the high prevalence of BrB reported at Lizard Island (Willis *et al.* 2004), however additional data are needed to support this hypothesis. Currently, the prevalence of brown band syndrome has only been compared between the northern and southern sectors of the GBR (Willis *et al.* 2004) while comparisons between the central and northern sectors of the GBR remain undescribed. Willis *et al.* (2004) found twice as many cases of

brown band syndrome in the southern sector of Heron Island than on two northern sector reefs. In the central GBR, only one study has reported the prevalence of BrB (Nash 2003). In this study, BrB was not observed at the Palm Islands, an inshore reef, yet it infected 10.4 ± 1.07% of scleractinian colonies on John Brewer Reef, a mid-shelf reef (Nash 2003). Although the prevalence of BrB is known for regions in both the northern and central GBR, it is difficult to compare because prevalence surveys took place during different months and years. Consequently, any regional differences between the central and northern/southern sector surveys could not be compared without considering annual and seasonal variations. In the future, studies examining regional differences in the prevalence or progression of coral disease should aim to complete surveys during the same month of the year in order to avoid possible variations due to seasonal patterns.

The average time taken for brown band syndrome to transmit from *in situ* diseased branches to healthy branch fragments was 1.7 days, therefore this new syndrome is highly infectious and capable of progressing within and spreading between coral colonies. The progression of BrB along *in situ* diseased branches cable tied to healthy branch fragments was comparable to the rate of progression measured for single diseased branches (controls) (Table 2.4), suggesting that this syndrome is capable of infecting adjacent healthy branches while maintaining its rate of progression along the original infected branch. Differences in disease progression along *in situ* diseased branches of BBD and BrB cable tied to healthy branch fragments may be a result of the unique microbial communities associated with each disease state. The anti-microbial response of the new coral host to the BrB pathogen may be ineffective or minimal compared to the anti-microbial response of the new coral host to the pathogen of BBD. Consequently, pathogen concentrations may remain unchanged allowing the progression of the syndrome to continue at its previous rate.

Compared to black band disease, the average rate of progression for brown band syndrome was 5.4 times greater at Lizard Island during May 2004 (Table 2.5). To date, brown band syndrome has been documented as one of the fastest progressing syndromes worldwide (Willis *et al.* 2004) with rates of tissue loss as high as 9 cm/day on Davies Reef (Nash 2003). BrB has been observed to affect a range of coral hosts, including acroporids, pocilloporids, and faviids. The extremely rapid progression of

this syndrome could result in high mortalities within scleractinian coral communities, therefore future investigations should continue to monitor the status of brown band syndrome, in addition to other known GBR maladies, on a seasonal and regional basis. Furthermore, research should focus on the identification of pathogens and vectors that promote the progression and transmission of these syndromes. This will provide an important step to understanding how future environmental conditions may impact their behavior.

In conclusion, the progression and transmission of coral disease on the Great Barrier Reef varies across time and between reefs. Monthly variations detected in the progression and transmission of black band disease may be the result of a combination of environmental parameters including elevated temperatures and light intensities. Variations in the progression of brown band syndrome between Davies and Horseshoe Reef may be due to the higher water circulation and flow rates recorded for Davies Reef, however it is important to note that additional factors, such as turbidity, light, and time are probably involved. Consequently, it is important to monitor these factors and begin to eliminate those which are not influencing the ecological behavior of the disease. The increasing prevalence of coral diseases on the Great Barrier Reef cannot be prevented until the factors promoting their progression and transmission are determined.

CHAPTER 3.0

EFFECT OF INCREASING TEMPERATURES ON THE PROGRESSION OF BLACK BAND DISEASE AND BROWN BAND SYNDROME

3.1. ABSTRACT

During the summer, elevated temperatures associated with global warming have been correlated with an increased prevalence of coral disease on the Great Barrier Reef (GBR) and throughout the Caribbean. The objective of this chapter is to investigate the effect of elevated temperatures on the progression of black band disease (BBD) and brown band syndrome (BrB) using experimental aquarium manipulations. Increased rates of BBD progression within the higher temperature treatment (32°C) during summer clearly indicate that elevated temperatures near the upper thermal limits of corals (Berkelemans & Willis 1999) promote the progression of BBD, possibly due to a combination of increased virulence of the pathogen and increased host susceptibility at these higher temperatures. However, the lack of increased progression within elevated temperature treatments (29°C and 31°C) during May trials, raise the question concerning other factors that may also be required to promote the progression of BBD. The progression of BBD within the 31°C treatment during May was approximately two-thirds the rate of BBD progression within the 30°C treatment in January, where light intensities were 28 times higher. This suggests that high light intensities in combination with elevated temperatures may further enhance BBD progression. Significant differences were not detected in the progression of BrB between experimental temperature treatments (27°C, 28.5°C, 30.5°C) in May, suggesting that temperature on its own does not enhance the progression of this syndrome. Future research should experimentally investigate how the combination of elevated temperatures and high light intensities commonly associated with summer months impact BrB in order to determine whether or not the combination of these two parameters have the ability to enhance the progression of this syndrome (as was the case for BBD).

3.2. INTRODUCTION

Coral reef ecosystems are continually exposed to deteriorating environmental conditions resulting from overfishing, sedimentation, nutrient enrichment, and ocean warming (Acosta 2001). The current trend toward a warming climate has the ability to modify biological characteristics of marine populations leading to a subsequent increase in their susceptibility to disease (Harvell *et al.* 1999). Reports suggest that climatic alterations associated with global warming will influence the health and productivity of marine systems by increasing the virulence of marine pathogens and reducing host resistance (Harvell *et al.* 1999, Rosenberg and Ben-Haim 2002). A coral disease model presented by Peters (1997) and reproduced from Warren (1991) predicts that several stresses including elevated temperatures, reduced water quality, increased nutrients and toxins, and higher storm frequencies may inhibit the coral's immune system (Peters 1997, Porter and Tougas 2001). Consequently, the coral's resistance to infection becomes diminished, thereby increasing the likelihood of a disease epizootic (Peters 1997, Porter and Tougas 2001).

Land and ocean temperatures have consistently increased throughout the 20th century due to increasing greenhouse gas concentrations (Karl and Trenberth 2003). Emission scenarios predict that globally averaged surface temperatures will rise between 1.4 to 5.8°C between 1990 and 2100 (Gitay *et al.* 2002). Land regions are predicted to warm more than oceans, however seawater temperatures are still expected to rise at a rate of 1-2°C per century (Hoegh-Guldberg 1999). On the Great Barrier Reef (GBR), sea surface temperatures have increased by approximately 0.6°C (Lough 2000) over the period 1903 to 1999 and are predicted to rise 1-3°C by the year 2100 (Lough 1999). Since many corals may already be living near their thermal maxima (Berkelmans and Willis 1999), elevated temperatures may be classified as an important source of stress in these sessile organisms and a potential enhancer of coral disease (Kuta and Richardson 2002, Rosenberg and Ben-Haim 2002).

Although it is widely thought that, "coral reefs have been adversely affected by rising sea surface temperatures (Gitay *et al.* 2002)", few studies have conclusively isolated the effect of elevated temperatures predicted by global warming on coral disease. It has been suggested that global warming is correlated with the increased activity of

disease pathogens in marine organisms in general (Harvell *et al.* 1999, Harvell *et al.* 2001), but there have only been two experimental studies that have specifically tested the effect of elevated temperatures on disease activity in reef corals. Experimental studies by Torren *et al.* (1998) and Banin *et al.* (2000) demonstrated that elevated seawater temperatures promote the expression of virulence genes by *Vibrio shiloi*, a pathogen responsible for the bleaching of *Oculina patagonica* in the Mediterranean. Elevated seawater temperatures have also been observed to increase infection and lysis in a pathogenic bacterium isolated from *Pocillopora damicornis* in Zanzibar (Ben-Haim and Rosenberg 2002). According to these results, infection and lysis of corals was extremely rapid between 27-29°C (Ben-Haim and Rosenberg 2002). Exposure to 26°C resulted in a much slower rate of tissue lysis while lysis did not occur at 25°C (Ben-Haim and Rosenberg 2002). There is also strong correlative evidence from field surveys that elevated temperatures increase the progression and disease activity in coral disease syndromes though such studies cannot discount potential contributions from other factors, such as turbidity, nutrient enrichment, or salinity, to the increased activity. For example, the first appearance of black band and white plague diseases in the Red Sea coincided with unusually high sea surface temperatures (27°C) in the summer of 2001 (Rosenberg and Ben-Haim 2002). Also, studies in the Caribbean suggest that virulence of the causative agent of black band disease, *Phormidium corallyticum*, is temperature dependent (Porter *et al.* 2001), although this species is not necessarily the sole causative agent of black band disease (Frias-Lopez *et al.* 2003, Richardson and Kuta 2003). In Jamaica, the highest prevalence of new black band disease infections occurred during the warm water months between July and September (33%) while the lowest number of new infections was observed between October and December (17.6%) (Bruckner and Bruckner 1997). A recent study in the Florida Keys found that water temperatures at sites with a high prevalence of black band disease were significantly greater than temperatures at sites where disease was absent (Kuta and Richardson 2002). At two sites on the Great Barrier Reef, the prevalence of five diseases including BBD increased up to 20 fold in summer (Willis *et al.* 2004). Although correlative studies are not conclusive, they suggest that elevated temperatures may have important implications for increasing the virulence of disease pathogens and the prevalence of diseases on coral reefs. In order to further explore the impact of elevated temperatures on the progression and spread of coral disease, experimental temperature

manipulations that control for the influence of other factors, such as turbidity and salinity, on the progression of coral disease are required.

The overarching objective of this study is to determine the potential impact of increasing sea-water temperatures on the progression of coral diseases along the GBR, particularly black band disease and brown band syndrome. These diseases were chosen because their prevalence in field surveys has commonly been correlated with elevated seawater temperatures in the Caribbean and the GBR (Bruckner and Bruckner 1997, Porter *et al.* 2001, Kuta and Richardson 2002, Willis *et al.* 2004). Past surveys at Lizard Island have reported eight-fold increases in black band disease incidence and moderate increases in the prevalence of brown band syndrome from 1 to 12 cases during the summer months (Willis *et al.* 2004). Although these studies provide correlations between environmental factors and coral disease, experimental approaches are needed to determine which factors are impacting the increasing prevalence and progression of the disease. In the field, it becomes difficult to determine the impact of a single environmental parameter, such as temperature, without taking into account the additional factors involved. Although temperature may influence the progression of coral disease, other environmental parameters such as water motion, salinity, sedimentation, nutrients, or light intensity may be involved in the process as well.

The aim of this study is to isolate the effect of elevated seawater temperatures on the progression of black band disease and brown band syndrome using experimental aquarium manipulations. Specifically, I will expose corals infected with black band disease and brown band syndrome to elevated temperature treatments and compare the rates of coral tissue loss between treatments by measuring the linear progression of the disease front. This study will give an insight into the influence of temperature on the virulence of disease pathogens and specifically the potential for global warming to enhance the progression and transmission of disease.

3.3. MATERIALS AND METHODS

3.3.1. STUDY SITE

Experiments to test the impact of elevated temperatures on the rate of progression of black band disease and brown band syndrome were set-up on flow-through seawater tables at Lizard Island Research Station, Australia (14° 40'S 145° 28'E) (Figure 2.1). Experiments were run during January and May 2004 because significant ambient seawater temperature differences measured over a two-week period with a digi-therm thermometer were detected during these two months. Ambient seawater temperatures averaged 30°C ± 0.5 during January while average ambient seawater temperatures were 27°C ± 0.5 in May.

3.3.2. FIELD CONTROL SAMPLING DESIGN

As controls for the experimental temperature treatments (see 3.3.3), measurements of the natural rate of progression of black band disease and brown band syndrome were conducted at Horseshoe and No. 2 Reefs off the coast of Lizard Island. During January and May 2004, the progression of black band disease was measured on 3-10 branches from three *Acropora muricata* colonies depending on the number of branches displaying signs of the disease (Figure 3.1). Rates of progression for brown band syndrome were measured on 2-3 branches from five *A. muricata* colonies (Figure 3.1). Once again, the number of measured branches depended on how many branches displayed signs of the disease. All measurements were taken at a depth of approximately 5 meters at high tide.

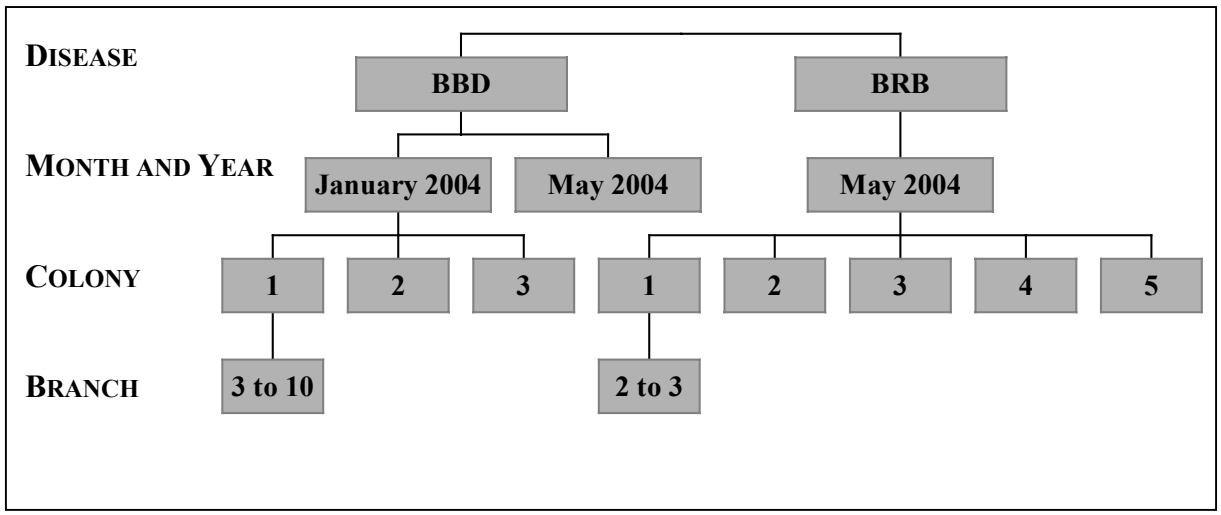

FIGURE 3.1. Sampling design for measuring the natural rate of progression and band width for black band disease (BBD) and brown band syndrome (BrB) on *Acropora muricata*.

3.3.3. AQUARIUM SETUP – BLACK BAND DISEASE

Acropora muricata branches infected with black band disease were collected from Horseshoe Reef and placed in 35-litre plastic aquaria where they were acclimated to aquarium conditions for at least 12 hours. Subsequently, water temperatures were raised 0.3°C every hour until desired temperatures were reached. Seawater temperatures experienced by local reef flat corals commonly change at rates more than 1°C per hour during spring tides (Berkelmans and Oliver 1999, Berkelmans and Willis 1999). This rate of temperature increase is less than the 0.3°C per hour rate used in this study. Furthermore, coral branches collected for experiments in this study were approximately 10 cm in length as per the conditions of the Great Barrier Reef Marine Park permit, therefore it was important to reach desired elevated temperature treatments before the disease progressed along the entire coral branch. Temperatures were monitored using a digi-therm thermometer accurate to 0.2°C. Each 390 x 305 x 300 mm plastic aquarium was supplied with a constant flow of ambient seawater and one Aquaclear 301 powerhead capable of pumping 570 liters of seawater per hour. Glass Via Aqua 250-watt heaters with external temperature controls were used to increase seawater temperatures above ambient temperature.

Diseased coral branches were subjected to elevated temperature treatments in increments of 2°C in order to accurately distinguish between treatment temperatures plus or minus a standard error. In January 2004, ambient seawater temperatures averaged 30°C ± 0.5, therefore diseased corals were exposed to two temperature treatments (30°C ± 0.5 control and 32°C ± 0.8). Berkelmans and Willis (1999) have reported that bleaching of *Acropora muricata* may occur when water temperatures reach only 2-3°C higher than average seawater temperatures during the summer (January) therefore temperatures were not elevated above 32°C in order to avoid bleaching of the diseased corals. During May 2004, ambient seawater temperature averaged 27°C ± 0.5, therefore corals were subjected to three temperature treatments (27°C ± 0.5 control, 29°C ± 0.5, and 31°C ± 0.5). Once again, temperatures were increased by increments of 2°C in order to justify comparisons between January and May experiments. Temperatures were not elevated above 31°C in order to prevent biased results due to coral bleaching. Previous bleaching studies with *Pocillopora damicornis* showed that during the winter, 65% of *P. damicornis* colonies exposed to 32°C showed signs of bleaching after 5 days (Berkelmans and Willis 1999). In addition, the bleaching threshold of *P. damicornis* was 1°C higher than the bleaching threshold of *A. muricata* in the summer (Berkelmans and Willis 1999).

In January 2004, three replicate ambient temperature treatments and 6 replicate elevated temperature treatments were randomly assigned to tanks. Since diseased corals are already stressed and highly sensitive to further changes, such as elevated temperature stress, six replicate tanks were used for the 32°C treatment in order to increase sampling size. Five diseased coral branches were placed within each tank (Figure 3.2). May 2004 temperature trials were performed with three replicate tanks for each of the three treatments and five infected coral branches within each tank (Figure 3.3).

Experimental manipulations to test the impact of elevated temperatures on the progression of black band disease took place on aquaria bench tops exposed to full sunlight (daily light levels averaged ~1400 to 1500 $\mu E/m^2/s$ between 10am-3pm over a 2-week period) during the month of January while May trials took place in a temperature-controlled aquarium room (set at 27°C, light levels averaged ~50 $\mu E/m^2/s$ for 12 hours) under artificial lighting supplied by sun-glow aquarium bulbs. By

repeating the experiment at these two locations, I was able to investigate the effect of elevated temperatures and increased light intensities on the progression of black band disease.

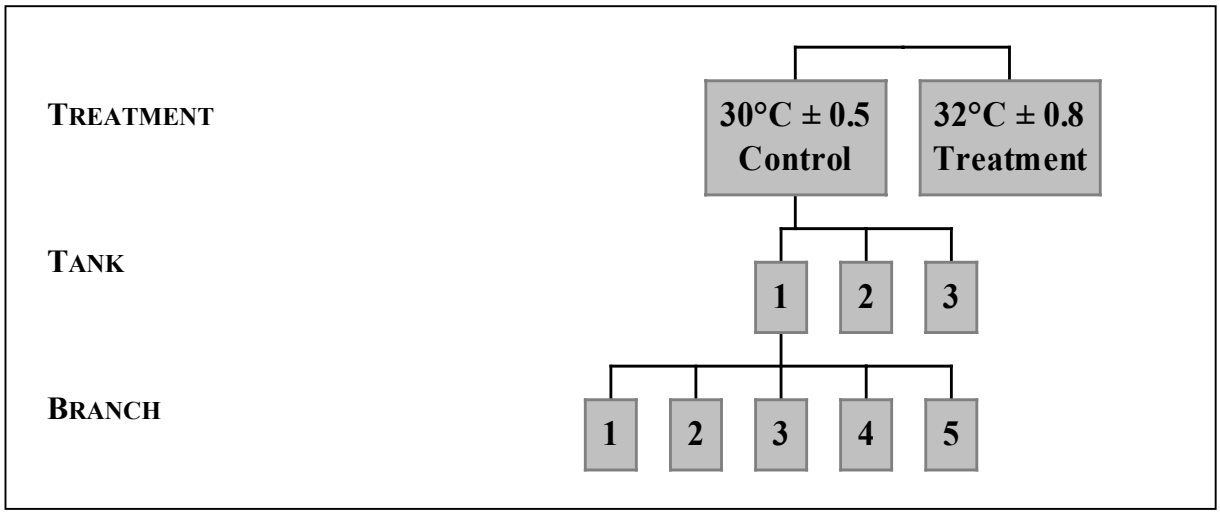

FIGURE 3.2. Sampling design to determine the effect of increasing temperatures on the rate of progression of black band disease in *Acropora muricata* during January 2004.

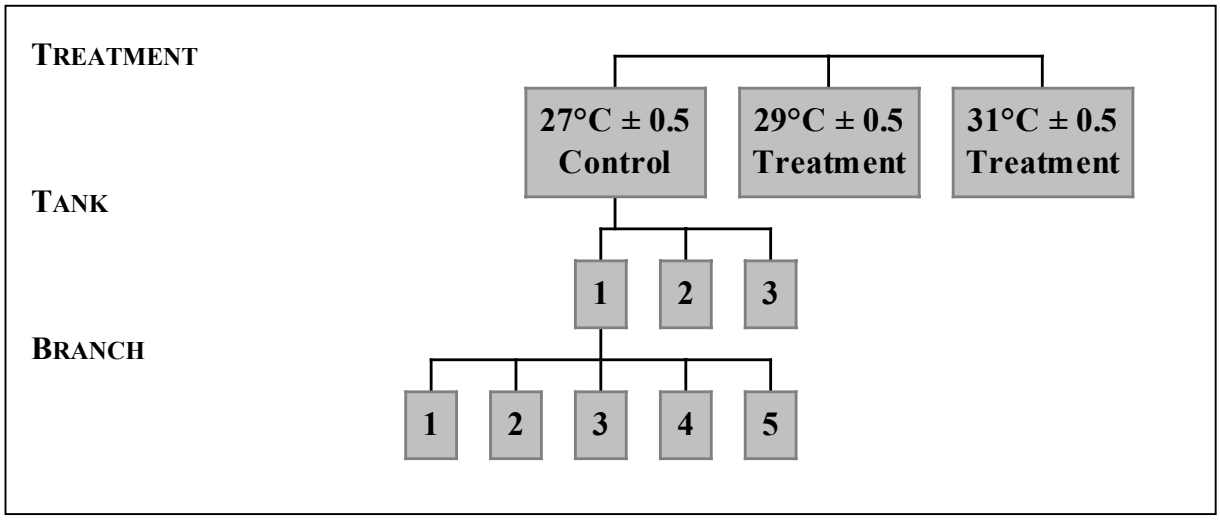

FIGURE 3.3. Sampling design to determine the effect of increasing temperatures on the rate of progression of black band disease in *Acropora muricata* during May 2004.

3.3.4. AQUARIUM SETUP - BROWN BAND SYNDROME

Acropora muricata branches infected with brown band syndrome were collected from two reefs, Horseshoe and No. 2. Diseased branches were placed directly in aquaria and allowed to acclimate to aquarium conditions for at least 6 hours. Water temperatures were gradually raised 0.5°C every hour until desired temperatures were reached. Since highly variable temperature differences up to 1°C per hour are often experienced by corals inhabiting reef flat areas (Berkelmans and Oliver 1999), this rate of temperature increase was justifiable. In addition, brown band syndrome can progress up to 9 cm/day (Nash 2003) as opposed to the 1.25 cm/day rates observed for BBD (Dinsdale 1994), therefore it was important for diseased corals to acclimate to temperature treatments before the syndrome progressed along the entire coral branch. Aquariums were set-up the same as described above for black band disease experiments in section 3.3.3. Since Aquaclear 301 powerheads were observed to dislodge the ciliates associated with brown band syndrome, each plastic aquarium was given a single air stone to maintain water circulation instead of a powerhead. It was assumed that the combination of the air stone and constant flow of ambient seawater maintained oxygen levels equal to ambient conditions.

Diseased coral branches were subjected to elevated temperature treatments in increments of 1.5°C to distinguish between treatment temperatures plus or minus a standard error. Since ambient seawater temperatures during May 2004 averaged 27°C ± 0.5, coral branches were subjected to 3 temperature treatments (27°C ± 0.5 ambient control, 28.5°C ± 0.5, and 30.5°C ± 0.8) and constantly monitored using a digi-therm thermometer accurate to 0.2°C. Temperature treatments did not exceed 30.5°C in order to prevent coral mortality and avoid coral bleaching. Previous pilot studies observed that corals infected with brown band syndrome experienced tissue sloughing when slowly exposed (0.15°C per hour) to temperatures as high as 31°C for more than 24 hours, therefore temperature treatments were lowered. Temperature treatments were randomly assigned to tanks and each treatment consisted of three replicate tanks with 5 branches in each tank (Figure 3.4).

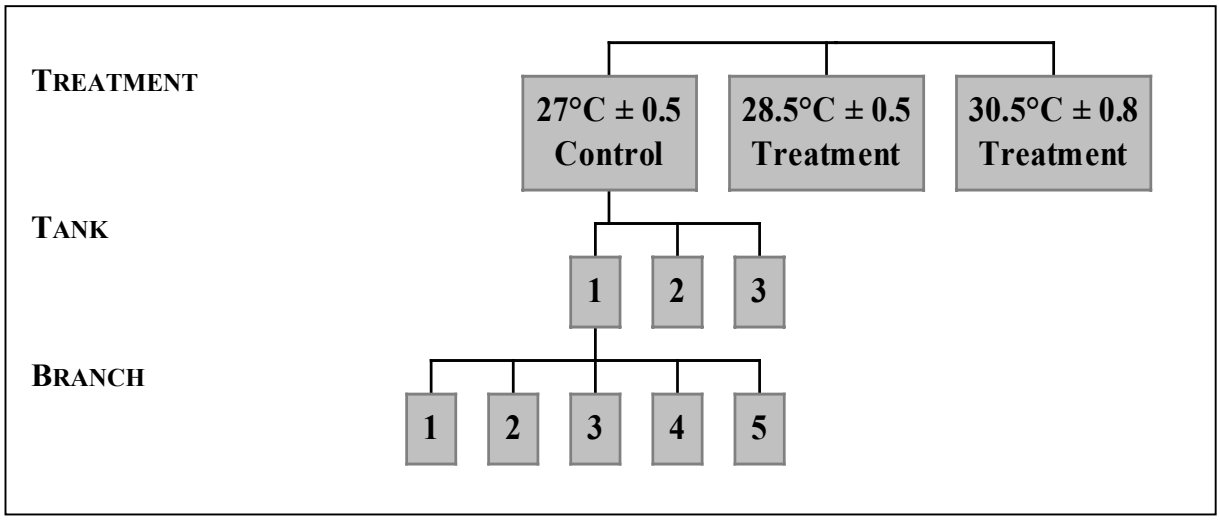

FIGURE 3.4. Sampling design to determine the effect of increasing temperatures on the rate of progression of brown band syndrome in *Acropora muricata* during May 2004.

3.3.5. RATE OF PROGRESSION

The rate of disease progression along branches infected with black band disease or brown band syndrome in the aquarium and the field was measured by securing cable ties to the exposed skeleton away from the disease band (see Figure 2.6). The distance from the cable tie to the healthy tissue interface and the width of the disease band was measured using a flexible measuring tape and calipers every 12-24 hours for no less than 3 days.

3.3.6. STATISTICAL ANALYSES

Statistical differences in the progression of black band disease and brown band syndrome subjected to elevated temperature treatments were analyzed using a nested GLM Repeated Measures Analysis of Variance (Zar 1999, Scheiner and Gurevitch 2001, Quinn and Keough 2002). The significance value was set at 0.05 and a Type IV Sum of Squares was used to account for missing values in the data. Missing values in the data occurred when the disease had progressed along the entire length of the coral branch. Whenever significant differences in the progression of black band disease were observed, Bonferroni post hoc tests were used to detect which treatments were responsible for the variation. A third Repeated Measures Analysis of Variance test

was also used to compare differences in the width of the disease band for BBD between temperature treatments. Once again, if significant variations were observed, Bonferroni post hoc tests were used to distinguish which treatments were responsible for the variation. Since measurements of brown band width displayed heterogeneity of variances, a nonparametric Kruskal Wallis test was used to compare BrB band widths between the three treatments and between the field control and ambient tanks. Significance values were set at 0.05.

3.4. RESULTS

3.4.1. EFFECT OF TEMPERATURE ON THE PROGRESSION OF BBD IN JANUARY 2004

During January 2004 trials, the rate of progression of BBD on branches at ambient temperature averaged 0.920 ± 0.072 cm/day while the maximum rate of progression of BBD at elevated temperatures was 2.6 cm/day. The rate at which BBD progressed along coral branches of *Acropora muricata* differed significantly between treatments ($p < 0.05$, Table 3.1). Post hoc tests revealed that the progression of black band disease was significantly greater in 32°C treatment tanks than ambient tank controls ($p = 0.003$) and field controls ($p = 0.021$) while the progression of BBD along branches showed no significant differences between tanks at ambient temperature and field controls ($p = 1.0$). On branches exposed to 32°C treatments, BBD progressed 1.3 times faster than on experimental controls held in tanks at ambient temperatures (30°C) (Figure 3.5). There were no differences in the rate of BBD progression between tanks ($p > 0.05$, Table 3.1).

The width of the disease band was significantly different between treatments ($p < 0.001$, Table 3.1). Post hoc tests detected significantly greater band widths at 32°C as opposed to 30°C tanks ($p = 0.025$) and field controls ($p = 0.000$) (Figure 3.6). The width of the black band on branches in the field did not differ significantly with the width of the black band on branches in ambient tanks ($p = 0.088$). There were no differences observed in band widths between tanks ($p > 0.05$, Table 3.1).

TABLE 3.1. Statistical results for the rate of progression and band width of black band disease at two experimental temperature treatments (30°C and 32°C) and a field control during January 2004. A nested General Linear Model Repeated Measures test (RM) was used to compare the progression of BBD and band width between treatments and tanks nested within treatments.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	RM	treatments	2	4.231	$p < 0.05$
		tanks	6	1.579	0.192
Band Width (cm)	RM	treatments	2	31.796	$p < 0.001$
		tanks	9	0.646	0.751

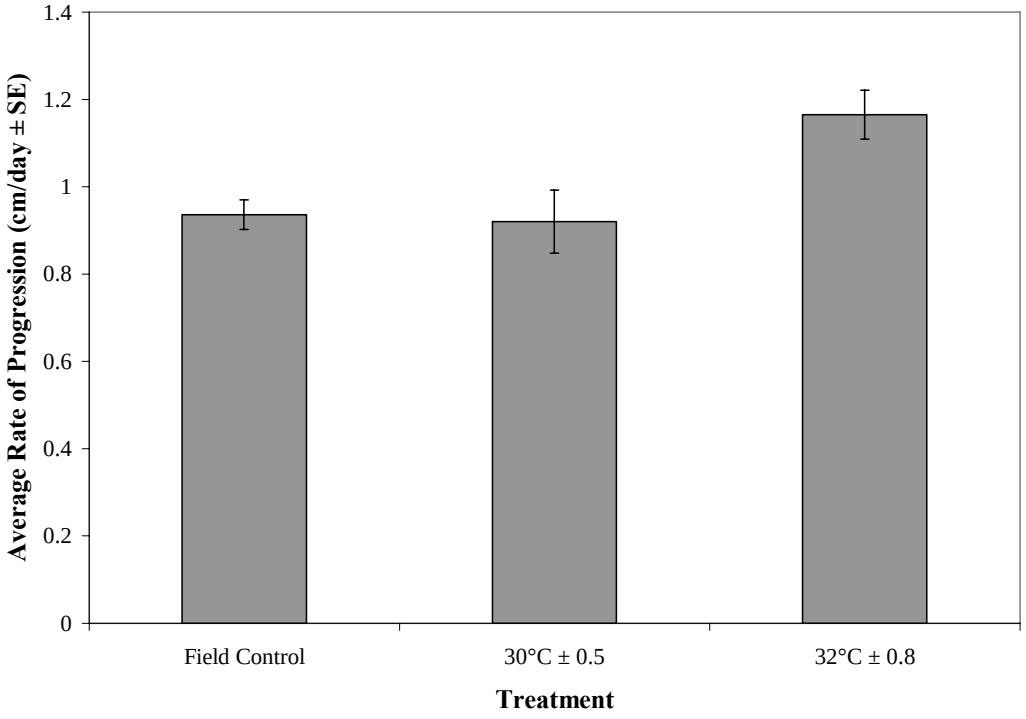

FIGURE 3.5. The average rate of progression (cm/day ± SE) of black band disease on branches of *Acropora muricata* in experimental temperature treatments [ambient (30˚C) and elevated (32˚C)] and a field control (30˚C) at Horseshoe reef, Lizard Island in January 2004.

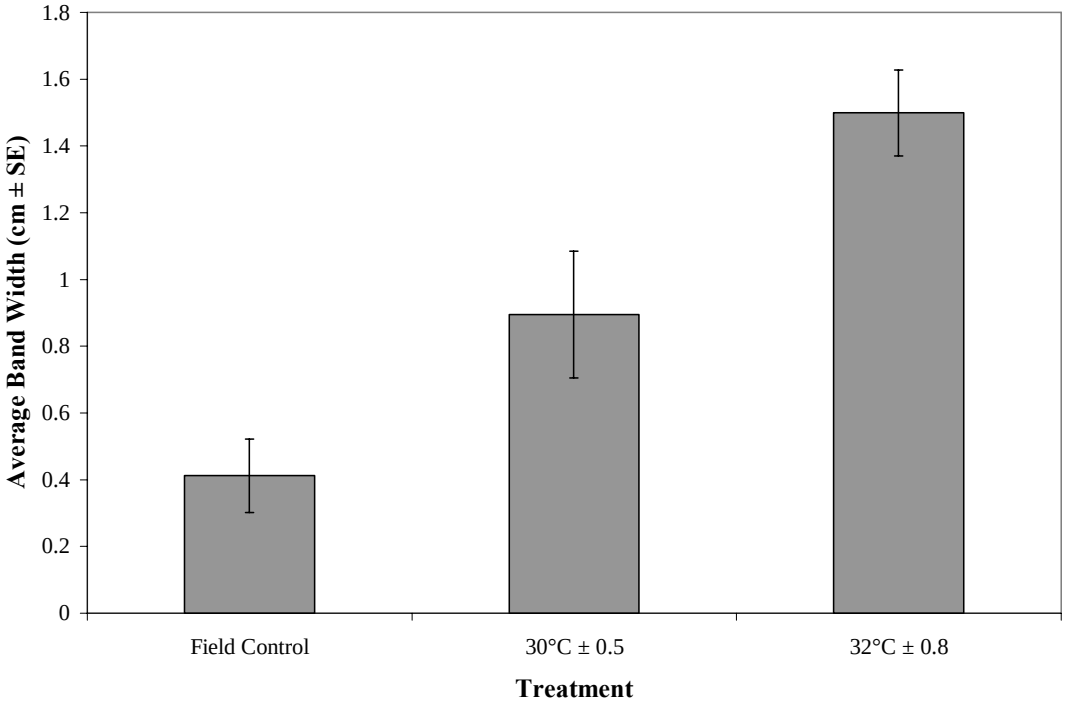

FIGURE 3.6. The average band width (cm ± SE) of black band disease on *Acropora muricata* in January 2004 on branches acclimated to two experimental temperature treatments [ambient (30˚C) and elevated (32˚C)] and a field control (30˚C) at Horseshoe reef, Lizard Island.

3.4.2. EFFECT OF TEMPERATURE ON THE PROGRESSION OF BBD IN MAY 2004

The progression of black band disease along *Acropora muricata* branches at ambient temperature averaged 0.370 ± 0.057 cm/day while the maximum rate of progression of BBD at elevated temperatures was 1.35 cm/day along a coral branch exposed to the 29°C treatment. Although the maximum rate of progression at elevated temperatures was 3.6 times faster than the average progression of black band disease at ambient temperature (0.370 ± 0.057 cm/day), the average rate at which BBD progressed along coral branches of *A. muricata* was not significantly different between treatments ($p > 0.05$, Table 3.2, Figure 3.7). The mean rate of progression of BBD in ambient tank controls was comparable to field controls. Coral branches exposed to 31°C displayed the highest averages while branches within ambient tanks exhibited the lowest averages. There were no differences in the rate of progression of black band disease between tanks ($p > 0.05$, Table 3.2).

Under ambient temperatures, the width of the black band averaged 0.522 ± 0.086 cm (Figure 3.8). There were no differences observed in the width of the disease band between treatments ($p > 0.05$, Table 3.2) or tanks ($p > 0.05$, Table 3.2). Band widths were greatest within the 31°C treatment, however these were only about 0.03 cm wider than band widths on diseased corals in ambient tanks (Figure 3.8).

TABLE 3.2. Statistical results for the rate of progression and band width of black band disease at three experimental temperature treatments (27°C, 29°C, and 31°C) and a field control during May 2004. A nested General Linear Model Repeated Measures test (RM) was used to compare the progression of BBD and band width between treatments and tanks nested within treatments.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	RM	treatments	3	1.721	$p > 0.50$
		tanks	7	.613	0.742
Band Width (cm)	RM	treatments	3	0.648	$p > 0.50$
		tanks	8	1.339	0.255

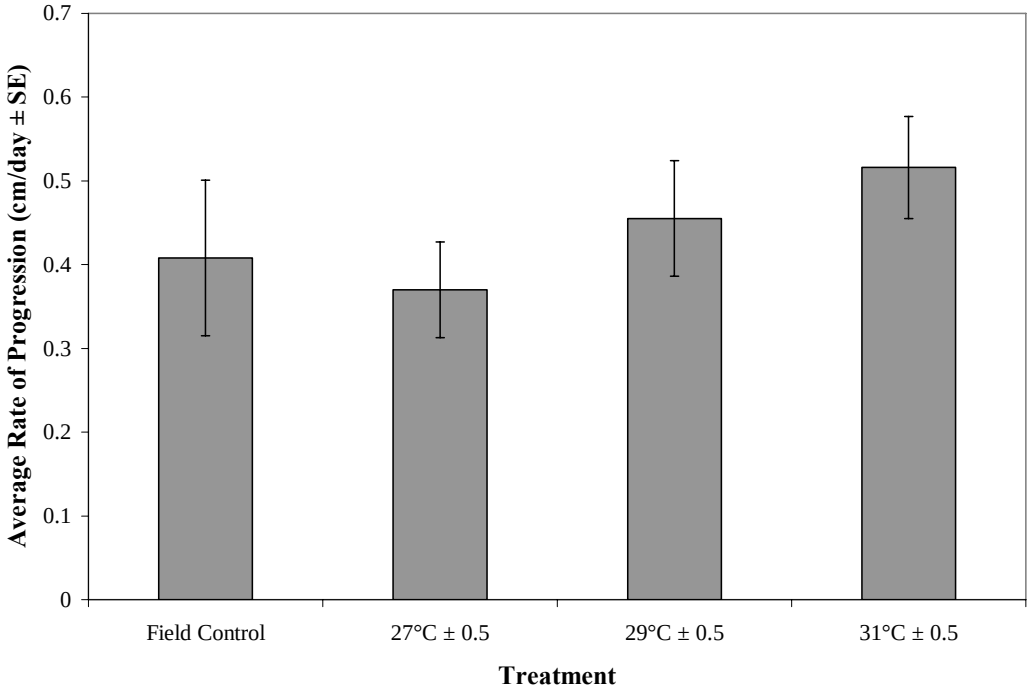

FIGURE 3.7. Comparison of the average rate of progression (cm/day ± SE) of black band disease on branches of *Acropora muricata* acclimated to three experimental temperature treatments [ambient (27˚C) and two elevated (29˚C and 31˚C)] and a field control (27˚C) at Horseshoe reef, Lizard Island during May 2004.

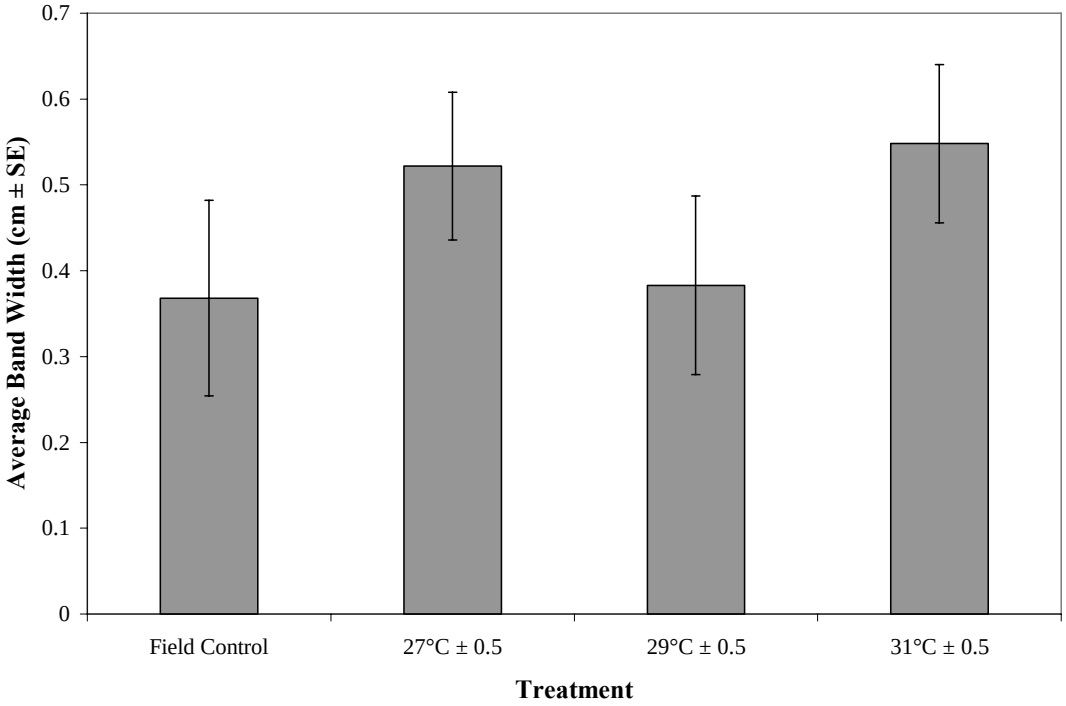

FIGURE 3.8. Average band width (cm ± SE) of black band disease on *Acropora muricata* branches acclimated to three experimental temperature treatments [ambient (27˚C) and two elevated (29˚C and 31˚C)] and a field control (27˚C) at Horseshoe reef, Lizard Island in May 2004.

3.4.3. EFFECT OF TEMPERATURE ON THE PROGRESSION OF BRB IN MAY 2004

Overall, the rate of progression of brown band syndrome did not differ between the three experimental treatments ($p > 0.05$, Table 3.3) and differences were not detected in the progression of brown band syndrome between branches in the field and those within ambient temperature tanks ($p > 0.05$, Table 3.3). In the 28.5°C treatment, BrB progressed along coral branches at rates up to 9 cm/day while maximum rates of progression along corals kept at ambient temperatures (27°C) were only 5.7 cm/day. However, the average progression of the syndrome at 28.5°C was only ½ cm quicker than the average progression of the syndrome at ambient temperatures (Figure 3.9). There were no differenced detected in the progression of brown band syndrome between tanks ($p > 0.05$, Table 3.3).

Average band widths of brown band syndrome were significantly different across temperature treatments ($p < 0.05$, Table 3.4) with the greatest band widths detected in the 28.5°C treatment (Figure 3.10). The average width of disease bands in ambient tanks was approximately 0.8 cm greater than the width of the disease bands on branches in the field (Figure 3.10).

TABLE 3.3. Statistical results for the rate of progression of brown band syndrome at three experimental temperature treatments (27˚C, 28.5˚C, and 30.5˚C) and a field control during May 2004. A nested General Linear Model Repeated Measure test (RM) was used to compare the rate of progression of BrB between temperature treatments and tanks nested within treatments.

Dependent Variable	Test	Factors	df	F value	Significance
Rate of Progression (cm/day)	RM	treatments	3	0.461	$p > 0.50$
		tanks	8	1.432	.219

TABLE 3.4. Statistical results for the band width of brown band syndrome at three temperature treatments (27˚C, 28.5˚C, and 30.5˚C) and a field control during May 2004. A nonparametric Kruskal Wallis test (KW) was used to compare difference in band widths between treatments.

Dependent Variable	Test	Factors	df	$\chi 2$ value	Significance
Band Width (cm)	KW	treatments	3	8.729	0.013

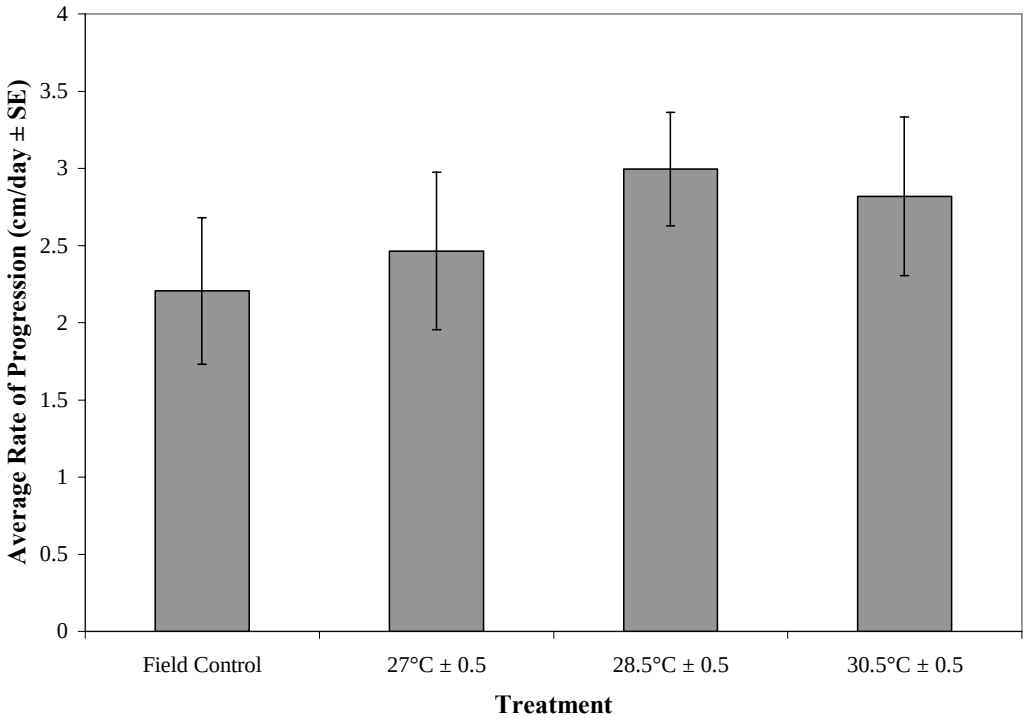

FIGURE 3.9. Average rate of progression (cm/day ± SE) of brown band syndrome on branches of *Acropora muricata* exposed to three experimental temperature treatments [ambient (27˚C) and two elevated (28.5˚C and 30.5˚C)] and a field control (27˚C) at Horseshoe Reef, Lizard Island in May 2004.

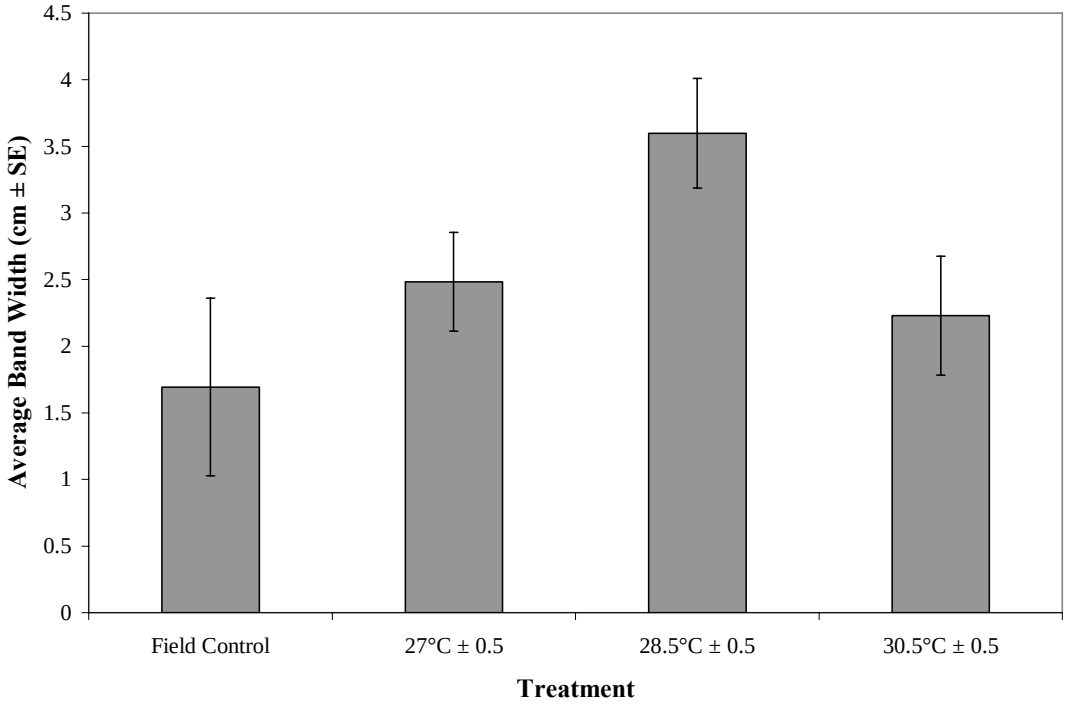

FIGURE 3.10. Comparison of the average band width (cm ± SE) of brown band syndrome on *Acropora muricata* acclimated to three temperature treatments [ambient (27˚C) and two elevated (28.5˚C and 30.5˚C)] and a field control (27˚C) at Horseshoe reef, Lizard Island in May 2004.

3.5. DISCUSSION

Increased rates of BBD progression within the higher temperature treatment (32°C) during summer (Table 3.1) clearly indicate that elevated temperatures near the upper thermal limits of corals (Berkelemans and Willis 1999) promote the growth and spread of BBD. However, the lack of increased progression within elevated temperature treatments (29°C and 31°C) during May trials (Table 3.2), raise the question concerning other factors that may also be required to promote the progression of BBD. In May, light intensities in the field were 22 times greater than those in the lab (1100 $\mu E/m^2/s$ versus 50 $\mu E/m^2/s$) yet rates of BBD progression did not vary between lab and field controls. This implies that light does not have a major impact on the progression of BBD at cooler temperatures. However, the average rate of progression of BBD within the 31°C treatment in May was approximately two-thirds the average rate of BBD progression within the 30°C treatment in January suggesting that the higher light intensities measured during January trials (1400 $\mu E/m^2/s$ vs. 50 $\mu E/m^2/s$) may interact with elevated temperatures to increase BBD progression. Previous reports have suggested that temperature is an important seasonal factor influencing the prevalence of disease (Richardson and Kuta 2003) however, results from experimental trials demonstrate that potentially a combination of high light intensities and elevated temperatures may have a greater influence on the rate of BBD progression than elevated temperatures alone.

High light intensities have been reported to intensify the adverse effects of elevated temperatures (Coles and Jokiel 1978). Reduced growth rates, decreased carbon fixation, high mortality rates, and the additional loss of zooxanthellae have been observed in *Pocillopora damicornis* colonies exposed to sub-lethal temperatures and high light levels (Coles and Jokiel 1978). Furthermore, ultraviolet radiation is capable of damaging DNA, proteins, and membrane lipids of corals and their symbiotic zooxanthellae (Lesser 2000). As a result, photosynthetic and calcification rates are diminished (Jokiel and York 1982, Lesser 2000). It has been suggested by several studies that high ultraviolet radiation causes considerable stress in coral communities (Coles and Jokiel 1978, Lesser 2000), especially if the corals are unable to produce mycosporine-like amino acids (MAA) (Shick et al. 1995) due to limited energy-budgets as a result of disease (Oren et al. 2001). Stress has been implicated as a

possible enhancer of coral disease and speculated to reduce the coral's resistance to disease infections (Peters 1997, Porter and Tougas 2001). If this is the case, then the speed at which the disease progresses may depend on the response of the coral to stressful conditions, such as elevated temperatures and high light intensities.

In addition to the coral's immune response to increasing levels of stress, the physiological ecology of the pathogen responsible for black band disease may also have an influence on the progression of this disease. Black band disease is comprised of a consortium of microorganisms including cyanobacteria (Rutzler and Santavy 1983, Frias-Lopez *et al.* 2003, Richardson 2004), a multitude of heterotrophic bacteria (Garrett and Ducklow 1975), marine fungi (Ramos-Flores 1983), sulfate-reducing bacteria (*Desulfovibrio*), and sulfide-oxidizing bacteria (*Beggiatoa*; Ducklow and Mitchell 1979, Antonius 1985). The majority of cyanobacteria are photoautotrophic, therefore light has been named as the most important factor governing their growth (Sinha *et al.* 2001). Although extreme light intensities and ultraviolet radiation can cause stress in these microorganisms, they have developed five adaptation strategies to limit its effect (Sinha *et al.* 2001). These include the production of MAA's, quenching agents (carotenids), and repair mechanisms (photoreactivation) as well as their ability to migrate to more favorable locations and alter their phycobiliprotein composition (Sinha *et al.* 2001). Moderate doses of ultraviolet-B (UV-B) have been observed to induce the vertical migration of the cyanobacterium, *Microcoleus chthonoplastes* in Solar Lake, Egypt (Bebout and Garcia-Pichel 1995). This species resided in the deeper layers of the microbial mat where exposure to high light conditions was limited. Surface layers filtered the UV-B providing an optimum light environment within deeper layers of the consortium (Bebout and Garcia-Pichel 1995). These conditions increased the photosynthetic output of the cyanobacteria thereby enhancing their growth rates (Bebout and Garcia-Pichel 1995). The ability of cyanobacteria to minimize stress while increasing growth and productivity suggests that these microorganisms are capable of thriving under high light intensities. As a result, elevated temperatures and light intensities have the ability to increase the growth rates of aquatic cyanobacteria (Bebout and Garcia-Pichel 1995) in addition to reducing coral health (Coles and Jokiel 1978).

In the Caribbean, the primary pathogen of black band disease was originally identified as the cyanobacterium, *Phormidium corallyticum* (Rutzler and Santavy 1983), however this early result has been disputed since more recent molecular studies have not been able to isolate this particular cyanobacterium (Frias-Lopez *et al.* 2003). Increasing water temperatures have been reported to promote the growth of *P. corallyticum* (Richardson and Kuta 2003). In fact, maximum photosynthetic efficiency has been reported at temperatures equal to and greater than 30˚C (Richardson and Kuta 2003). Richardson and Kuta (2003) also noted that the cyanobacteria cells multiplied in response to light resulting in a dense mat which shaded cells in the lower layers. This self-shading behavior, known as cyanobacterial clumping, enhanced anaerobic microzones within the consortium, resulting in the enrichment of sulfate reducers (Richardson and Kuta 2003). As a result, sulfide accumulated and stable anaerobic and sulfide-rich zones were formed. It was suggested that these areas may be harmful to adjacent coral tissues and eventually result in tissue death (Richardson and Kuta 2003). Furthermore, under high conditions this clumping behavior allowed the cyanobacteria to attain desirable light levels required for optimal photosynthesis and growth (Sinha *et al.* 2001). Increases in the width of the black band with temperature on experimental corals in January are consistent with the cyanobacterial clumping observed by Richardson and Kuta (2003) under high light conditions. If pathogens of black band disease on the GBR are similar to the clumping, cyanobacterial pathogens observed in Caribbean reefs, then the significantly greater band widths observed in January may be attributed to higher light intensities. However, since maximum photosynthesis is attained at elevated temperatures and optimal photosynthesis is achieved by cyanobacterial clumping at high light intensities (Richardson and Kuta 2003), the increased progression of BBD cyanobacteria is probably due to a combination of both high temperatures and high light intensities.

A combination of increasing seawater temperatures and high light levels are commonly associated with the summer season (Lough 1999, Lough 2001) and the summer season is often correlated with an increased prevalence of coral disease (Kuta and Richardson 2002, Willis *et al.* 2004). For example, increasing ocean temperatures and high light intensities associated with summer months have been correlated with a higher prevalence of black band disease (Antonius 1985, Kuta and Richardson 1996),

white plague (Rosenberg and Ben-Haim 2002), and dark spot diseases (Gil-Agudelo and Garzon-Ferreira 2001). On the Great Barrier Reef, a higher prevalence of black band disease, white syndrome, skeletal eroding band, and black necrosing syndrome were observed during the summer as opposed to the winter (Willis *et al.* 2004). The faster rate of progression of BBD at elevated temperature treatments and high light intensities during January experiments is consistent with previous correlations between the increased prevalence of coral disease and summer months. This suggests that the higher prevalence of black band disease during the summer may be a result of the increased progression of the disease among and between coral colonies. In other words, the faster a disease progresses, the more abundant it becomes. This hypothesis is only valid to a point because the disease may be so virulent that it quickly infects and kills all available hosts subsequently reducing its prevalence.

The rate of progression of brown band syndrome did not differ between experimental temperature treatments during May (Table 3.3). The syndrome progressed fastest on experimental branches exposed to 28.5°C, whereas tissues of several diseased corals exposed to higher temperatures sloughed and died. Although this suggests that a temperature threshold may exist for the microorganisms associated with brown band syndrome, the high mortality rates observed for diseased corals exposed to temperatures exceeding 28.5°C may also be linked to the coral's stress response to the combination of high temperature conditions and the disease itself. Recent coral disease surveys have reported a higher prevalence of brown band syndrome during summer months (Willis *et al.* 2004), but faster rates of progression have been reported during cooler months in October and May (Nash 2003). At Davies Reef, brown band disease progressed across individual acroporid colonies at a rate of approximately 3.76 cm/day during October 2002 and 0.5 cm/day during February/April 2003 while the rate of progression was measured at around 1.92 cm/day on John Brewer Reef during May 2003. Although these results suggest that brown band disease progresses more quickly during cooler months (Nash 2003), data were not obtained for the coolest and warmest months of the year and measurements were taken at two different locations. Consequently, in addition to seasonal variations, differences between sites may be influencing the progression of brown band syndrome. Several environmental factors vary on a seasonal and regional basis including light, turbidity, and water circulation (Lough 2001). As a result, any combination of these factors could be

influencing the progression and prevalence of brown band syndrome. To date, the micro-organisms associated with brown band syndrome have not been identified, however field observations suggest that the brown band includes a variety of bacteria and an unknown ciliate (Willis *et al.* 2004). Future investigations should focus on identifying the bacteria and ciliate species associated with brown band syndrome. Only after the microorganisms associated with this syndrome are identified can we determine why experimentally elevated temperatures during May had no impact on the rate of progression of brown band syndrome.

In summary, the enhanced progression and transmission of BBD on the GBR during the summer month of January is due to a combination of environmental factors including elevated temperatures and high light intensities. Brown band syndrome, on the other hand, did not show any significant variations in the rate of progression among experimental temperature treatments even though a higher prevalence of BrB has been reported during the summer (Willis *et al.* 2004). This could be because: (1) elevated temperatures in combination with additional factors, such as high light intensity, reservoir or vector abundance, and water circulation, are required to enhance the progression of the syndrome or (2) the progression of brown band syndrome is not dependent on temperature but may be influenced by other parameters which vary on a seasonal basis. Branches exposed to temperatures higher than 28.5°C did experience higher mortality rates, suggesting there may be a temperature threshold beyond which the pathogens of this disease cannot survive. However, it could be related to the stress response of the coral as well. The rate at which BBD and BrB progress and transmit within coral communities seems to be dependent upon the response of the coral host and the disease pathogen(s) to variations in environmental parameters. Future investigations should identify and describe the microorganisms associated with coral diseases on the GBR because their ecology plays an important role in the processes determining their progression and transmission. Furthermore, researchers should aim to isolate the effect of light intensities under different temperatures on the progression of black band and brown band diseases using an experimental approach to determine the contribution of this factor to the progression of coral disease.

CHAPTER 4.0

HISTOPATHOLOGICAL EXAMINATION OF THE CYANOBACTERIAL CONSORTIUM ASSOCIATED WITH BLACK BAND DISEASE ON THE GREAT BARRIER REEF

4.1. ABSTRACT

The primary focus of this chapter is to examine the microorganisms associated with black band disease on the Great Barrier Reef using histological techniques. The microbial consortium of black band disease isolated from *Acropora elseyi, A. florida, A. muricata, A. nasuta, Pocillopora verrucosa*, and *Porites* spp. consisted of five different taxa of cyanobacteria in addition to an array of algae and fungi. Each cyanobacterial species exhibited distinct mean trichome widths and unique morphologies. According to morphological characteristics, two of these taxa may belong to the genus *Oscillatoria*, two may be in the Order *Nostocales*, and one of these taxa resembles the morphological features of *Phormidium corallyticum*. These conclusions are based solely on histological features. Further molecular identifications are required before these species can be formally classified.

4.2. INTRODUCTION

Although initial observations of bacteria associated with corals emerged in the 1900's (Duerden 1902), it wasn't until the early 1970's that the first disease (black band disease) was observed on a scleractinian coral (Antonius 1973). The causative agent was described as a consortium of microorganisms including cyanobacteria, a multitude of heterotrophic bacteria (Garrett and Ducklow 1975), marine fungi (Ramos-Flores 1983), sulfate-reducing bacteria (*Desulfovibrio*), and sulfide-oxidizing bacteria (*Beggiatoa*; Ducklow and Mitchell 1979, Antonius 1985). This complex disease community creates a microbial black mat that horizontally migrates across the colony's surface at 1 mm to 1 cm per day (Carlton and Richardson 1995), giving rise to the characteristic black band after which the disease is named.

Although early studies suggested that the primary pathogen responsible for black band disease (BBD) is the cyanobacterium *Phormidium corallyticum* (Rutzler and Santavy 1983), recent molecular studies have reported the presence of additional cyanobacteria species inhabiting the black mat in some cases (Frias-Lopez *et al.* 2003) and notably, the absence of *P. corallyticum* in other cases (Cooney *et al.* 2002, Frias-Lopez *et al.* 2003). Therefore, further studies of BBD in different global reef regions are necessary in order to clarify the role of *P. corallyticum* in the etiology of black band disease.

The presence of *Phormidium corallyticum*, a gliding, filamentous cyanobacterium previously known as *Oscillatoria submembranacea*, in black bands can be readily ascertained through histological studies because of the distinct morphology of its trichome (Santavy and Peters 1997), which are the chains of cells without an investing sheath (Bergey *et. al* 2001). Approximately 4.2 µm wide and 4.0 µm in length (Rutzler and Santavy 1983), cells are isodiametric and the movable filaments of the cyanobacterium exhibit one rounded end and one narrow, distinctly tapered end (Rutzler and Santavy 1983). The cell wall of the cyanobacterium is Gram-negative and often contains an envelope-like capsule, known as a sheath, exterior to the outer membrane (Graham and Wilcox 2000). In *P. corallyticum*, this sheath may be absent or distinct (Rutzler and Santavy 1983). Furthermore, sheaths may be branched

exhibiting one to several trichomes (Rutzler and Santavy 1983). *P. corallyticum* contains high levels of the photosynthetic pigment, phycoerythrin (Richardson and Kuta 2003) and is widely distributed throughout saltwater and freshwater regions within both temperate and tropical climates (Humm and Wicks 1980).

Early studies suggested that *Phormidium corallyticum's* ability to consume live coral tissue might be related to its role in nitrogen fixation (Dinsdale 1994). It was thought that the photosynthetic capabilities of the cyanobacterium might allow it to fix nitrogen during anoxic periods, for example, at night or at the base of bands during the day (Dinsdale 1994, Richardson and Kuta 2003). Lack of evidence of nitrogen fixation has refuted this theory in more recent studies that have examined the potential of *P. corallyticum* to fix nitrogen using the acetylene reduction technique (Richardson and Kuta 2003). Consequently, Richardson and Kuta (2003) suggest that the entire microbial community acts as a pathogenic consortium, where the microorganisms within the consortium utilize nutrients released from the lysis of coral tissue as they migrate across the surface of the colony (Carlton and Richardson 1995). If this is the case, black band disease may be initiated by other members of the consortium even though *P. corallyticum* may subsequently become the dominant component of the microbial community (Richardson 1997, Cooney *et al.* 2002). Clearly, further investigations are required to determine the causal role of each member in the microbial community and to determine if there is more than one pathogen capable of producing the black band that is the macroscopic signature of the disease.

Other bacteria associated with black band disease, such as the sulfide-oxidizing bacteria *Beggiatoa* spp., appear as distinct bands that migrate vertically within the disease line (Richardson 1996, Richardson and Kuta 2003). These bacteria accumulate elemental sulfur as refractive, intracellular granules, which are visible in the 1-4 µm wide *Beggiatoa* spp. filaments and cause them to appear white along the disease line (Richardson 1996, Richardson and Kuta 2003). The reduced sulfur compounds and elemental sulfur are used as sources of energy to drive CO_2 assimilation (Sorokin 1999). The elemental sulfur taken up by the *Beggiatoa* spp. bacteria is produced by the sulfate-reducing bacteria, *Desulfovibrio* spp. (Sorokin

1999), which is also associated with the black band and produces large concentrations of sulfide at the base of the microbial mat (Sorokin 1999).

My aim in this study is to investigate the histopathology of black band disease on the Great Barrier Reef by distinguishing between the various microorganisms inhabiting infected corals. In particular, the different types of cyanobacteria living in the microbial consortium will be observed and described based on their morphological characteristics and measurements. The position of the cyanobacteria within the coral's tissues will be assessed in order to gain an understanding of their role in black band disease infections. The histology of black band disease will be compared between various coral species to determine whether or not the consortium is identical among taxa. These observations will provide an insight into the histopathology of black band disease and provide a baseline for future genetic investigations.

4.3. MATERIALS AND METHODS

Specimens of *Acropora elseyi, A. florida, A. muricata, A. nasuta, Pocillopora verrucosa,* and *Porites* species were collected from Lizard Island, Australia in January 2003 by Dr. Bette Willis and Cathie Page. Five healthy and five diseased specimens were examined for each coral species. All specimens were fixed in a 10% seawater formalin solution for at least 48 hours and then rinsed in tapwater prior to subsampling. A diamond saw was used to separate samples into healthy and diseased portions, and isolate the interface between the two. Selected specimens were embedded in 3% agar prior to decalcification in order to preserve the original conformation of the diseased tissues. These samples remained in 70% alcohol overnight. All samples were placed in sectioned mesh trays and the sample within each square of the tray was labeled for future reference. Trays were placed in a 22 liter nally bucket filled with a 5% formalin/3% formic acid solution for 2-3 weeks until completely decalcified.

After the specimens were decalcified, they were rinsed with tap water for a minimum of 4 hours and placed in 25 ml vials containing 70% alcohol. Next, samples were sliced using a gem blade into 4-5 mm thick sections and placed in plastic cassettes for histological processing. Tissues were processed overnight in a Shandon Hypercentre. Using the Shandon Histocentre 2, processed tissues were embedded in hot paraffin wax and cooled. Tissue blocks were cut with a micrometer into 5 µm wax sections, which were placed in a water bath for ease of attaching to glass slides. Sections mounted on slides were dried in an oven overnight. Slides were stained with 3 different stains. Staining with Picro Gomori allows zooxanthellae and coral tissues to be easily distinguished from the various microorganisms inhabiting the disease band. A Periodic Acidic stain was used to detect any fungi present in the microbial mat while a Gram stain was used to observe gram-negative cyanobacteria (Bergey *et al.* 2001) which stain red in color. After each stain, slides were covered with a cover slip using DPS and dried in an oven overnight.

Each specimen was examined microscopically and photographed to morphologically identify the microorganisms present within healthy and diseased coral tissues. The

mean diameter of 5-10 randomly chosen cyanobacteria within each taxon was measured using a calibrated micrometer eyepiece and the morphological features including apical cell shape and internal structure were recorded for the future identification of each cyanobacteria taxon.

4.4. RESULTS

4.4.1. DESCRIPTION OF OBSERVED CYANOBACTERIA

Five cyanobacteria taxa, referred to here as cyanobacteria taxa A-E and distinguished primarily by differences in trichome width (Table 4.1), were observed in histological sections. Approximately 17% of *Porites* spp. specimens (5 out of 30) contained all five cyanobacteria taxa (Figure 4.1). All five taxa had linear morphologies consisting of a chain of cells whose nuclei stained red with Picro Gomori. Key features that differentiate each of the morphological types are highlighted in the descriptions below.

Cyanobacteria Taxon A: With a mean cell diameter of approximately 7.182 ± 0.295 µm, Cyanobacteria Taxon A had the second widest trichome of the five morphological types (Figure 4.2). This microorganism appeared to penetrate the epidermis, embedding itself within the tissues of the gastrodermis. Externally, this cyanobacterium displayed a linear morphology with a rounded apical cell, while internal structures appeared segmented into equal subdivisions (Figure 4.3). Ten observed specimens of Cyanobacteria Taxon A appeared blue in the Gram stain (Figure 4.4), indicating that either this taxon is a gram-positive bacterium and not a cyanobacterium or the cells were not exposed to the de-colorization process for a long enough time frame before they were counterstained with nuclear fast red dye. Due to similar morphological features between the Phylum *Cyanobacteria* and this taxon, the latter is most likely the case. Additional gram staining was performed to support this classification. Five stained specimens showed an outer red sheath and inner blue segmentation, suggesting that the thin external sheath of the cyanobacterium is gram-negative and the blue stain is a result of the nuclei or zooxanthellae inside the cyanobacterium. Cyanobacteria Taxon A commonly occurred in combination with Cyanobacteria Taxa B, C, D, and E and was observed in *Acropora elseyi*, *A. florida*, A. *muricata, A. nasuta*, *Pocillopora verrucosa*, and *Porites* species.

TABLE 4.1. Summary of morphological characteristics and location within coral tissues of five cyanobacteria taxa associated with black band disease in Lizard Island, Australia.

Cyanobacteria	Coral Host Species	Stain		Trichome Width (µm ± SE)	Apical Cell Shape	Cell Shape	Trichome Morphology	Sheath	Internal Structure	Location within Coral Tissues
		Picro Gomori	*Gram*							
A	*A. elseyi, A. florida, A. muricata, A. nasuta, P. verrucosa, Porites spp.*	Red	Outer - Red (Barely Visible) Inner - Blue	7.182 ± 0.295	Round	wider than long disc-like	cylindrical straight	slightly visible	segmented into equal subdivisions	penetrated the epidermis and embedded within the gastrodermis
B	*A. elseyi, A. florida, A. muricata, A. nasuta, P. verrucosa, Porites spp.*	Red	Outer - Red Inner - Blue	11.466 ± 0.381	Round	wider than long disc-like	cylindrical straight	slightly visible	segmented with dark speckles in each segment	embedded within the gastrodermis and mesentaries
C	*Porites spp.*	Red	Red	4.158 ± 0.214	not observed	longer than wide or isodiametric	cylindrical straight	not observed	segmented with dark speckles in each segment	embedded within the gastrodermis and mesentaries
D	*A. elseyi, A. nasuta, Porites spp.*	Red	Red	2.352 ± 0.116	not observed	spherical	uniseriate	not observed	2 rows of circular cells	penetrated the epidermis and embedded within the gastrodermis
E	*A. elseyi, A. florida, A. nasuta, P. verrucosa, Porites spp.*	Red	Red	2.016 ± 0.254	not observed	spherical	uniseriate	not observed	single row of circular cells which may be falsely branched	embedded within the gastrodermis

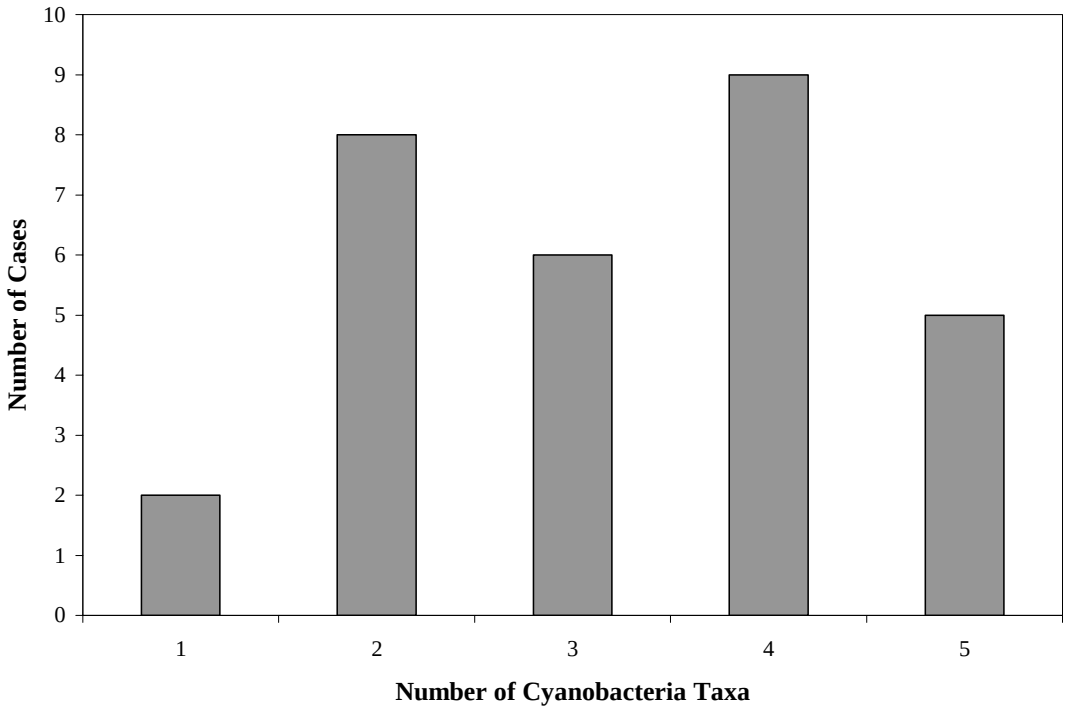

FIGURE 4.1. The number of cases the consortium contains 1, 2, 3, 4, and all 5 of the cyanobacteria taxa associated with black band disease on the Great Barrier Reef (N = 30 coral specimens).

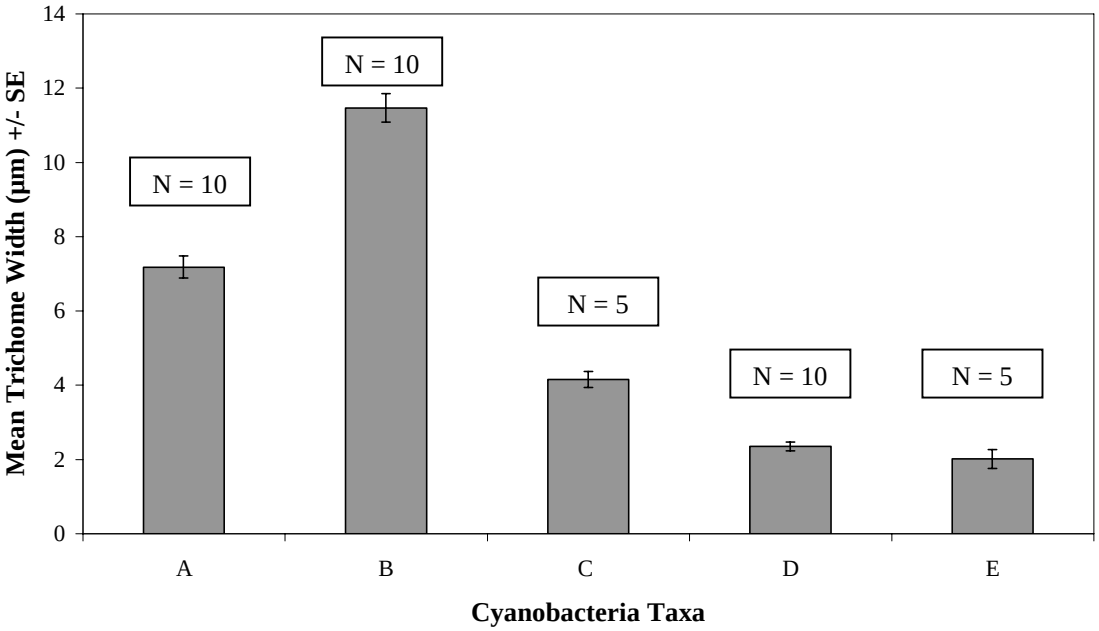

FIGURE 4.2. Mean trichome width (µm ± SE) of the different cyanobacteria taxa associated with black band disease on the Great Barrier Reef.

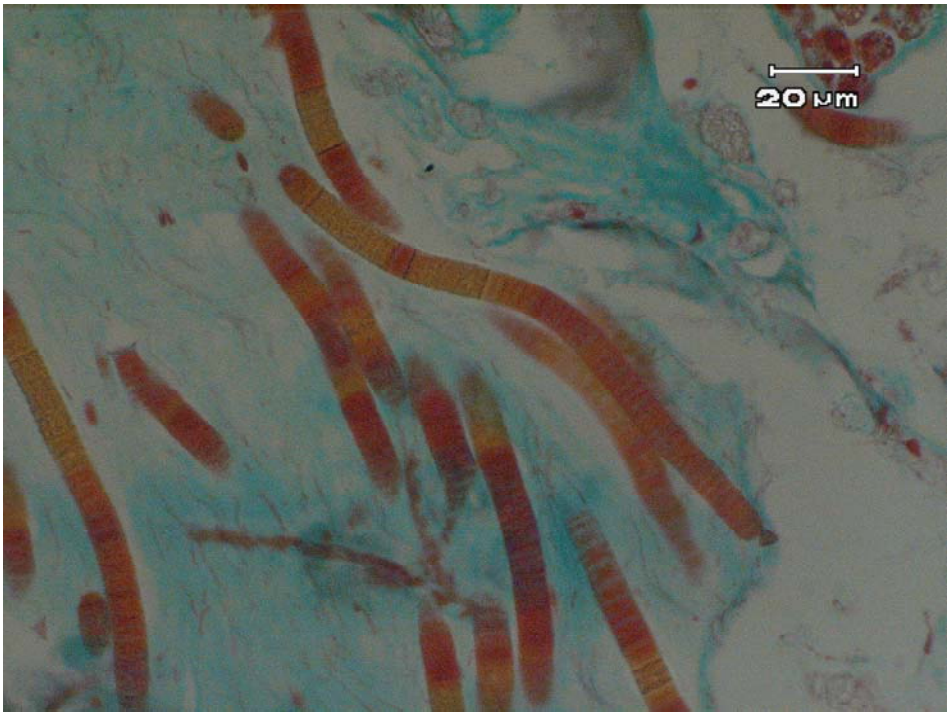

FIGURE 4.3. Histological photograph of black band disease on the coral *Pocillopora verrucosa*. The photograph was taken within the gastrodermal tissue layer of the coral polyp. Cyanobacteria Taxon A appears red in the Picro Gomori stain.

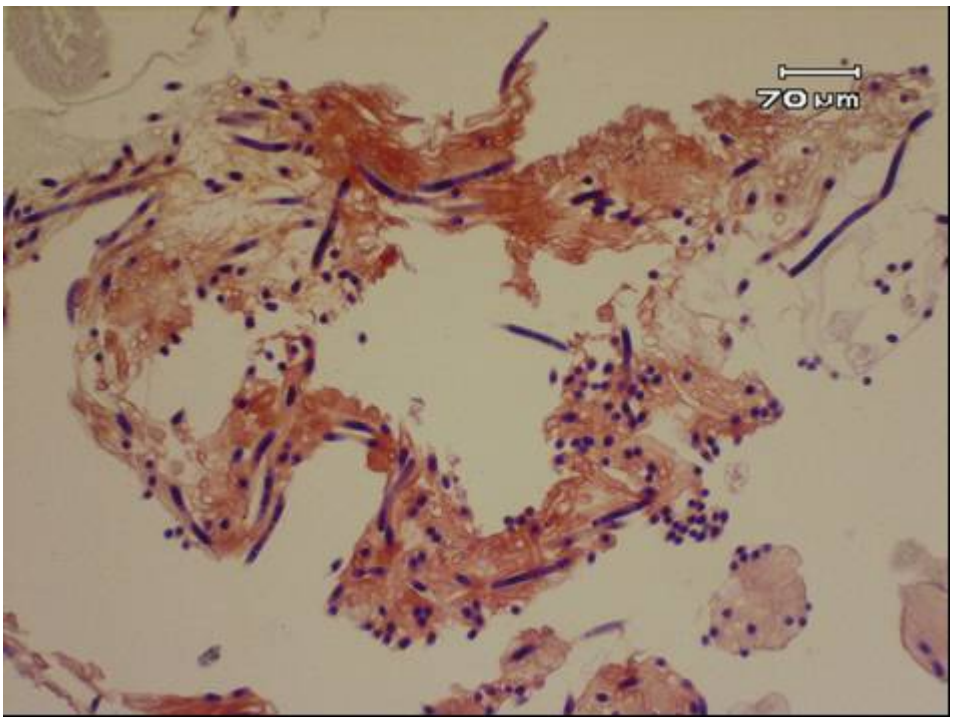

FIGURE 4.4. Histological photograph of black band disease on the coral *Acropora florida*. The photograph was taken within the gastrodermal tissue layer of the coral polyp. Cyanobacteria Taxon A appears blue in the Gram stain.

Cyanobacteria Taxon B: This taxon had the widest trichome (mean cell diameter of 11.466 ± 0.381 µm) of the five cyanobacteria taxa. Similar to Cyanobacteria Taxon A, apical cell shape was rounded, a sheath surrounded the trichome, and internal structures were segmented (Figure 4.5). However unlike Taxon A, darkened speckles could be seen within each segment. These speckles might represent nuclei and/or ingested zooxanthellae in combination with coral tissues. Commonly occurring with Cyanobacteria Taxa A, C, D, and E, this cyanobacterium was observed within the coral tissues of *Acropora elseyi*, *A. florida*, A. *muricata*, *A. nasuta*, *Pocillopora verrucosa*, and *Porites* species, particularly in the gastrodermis region. All 10 specimens appeared red in the Picro Gomori stain. In the Gram stain, the outer sheath of the cyanobacteria was red, suggesting Cyanobacteria Taxon B is gram negative.

Cyanobacteria Taxon C: Mean trichome diameters of Cyanobacteria Taxon C (4.158 ± 0.214 µm) were half to approximately a third of the size of those of Taxa A and B (Figure 4.5). This cyanobacterium was observed only in *Porites* colonies and occurred in combination with Cyanobacteria Taxa A, B, and E. All five specimens stained red in the Gram stain, identifying it as a gram-negative cyanobacterium. Terminal cells were not observed and there was no apparent sheath. Cells were longer than wide (i.e. isodiametric) and internal structures had a speckled appearance which, once again, might represent nuclei within the cells of the cyanobacteria and/or ingested zooxanthellae and coral tissues.

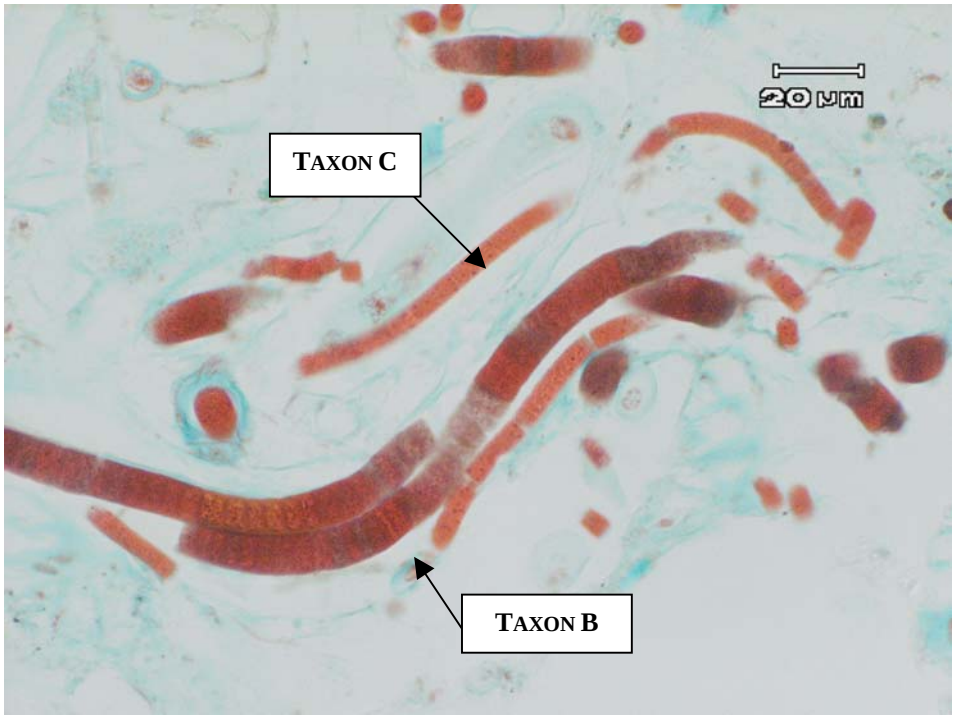

FIGURE 4.5. Histological photograph of black band disease on the coral *Porites* spp. The photograph was taken within the gastrodermal tissue layer of the coral polyp. Cyanobacteria Taxa B and C appear red in the Picro Gomori stain.

Cyanobacteria Taxon D: Cyanobacteria Taxon D had the second smallest mean cell diameter of 2.352 ± 0.116 µm (Figure 4.6). The microorganism was comprised of two rows of internal circular cells, parallel to each other. A sheath was not apparent and branching was not observed. Specimens stained red in both the Picro Gomori and Gram stains indicating the taxon is a gram-negative cyanobacterium. The cyanobacteria were observed within the tissues of *Acropora elseyi*, *A. nasuta*, and *Porites* species alongside Cyanobacteria Taxa A, B, and E.

Cyanobacteria Taxon E: Instead of two rows of circular cells, Cyanobacteria Taxon E contained a single row of discoid or disc-like cells which reduced its mean cell diameter to 2.016 ± 0.254 µm, the smallest width of the five taxa. Cyanobacteria Taxon E generally displayed a linear morphology, however in some cases the specimen appeared forked. This characteristic, known as false branching, often occurs within particular cyanobacteria taxa when the filament breaks apart near a dead cell and one piece emerges and continues to grow through the mucilage sheath (Lynn and Corliss 1991). Observed throughout the skeletal space, this cyanobacterium appeared red in both Picro Gomori and Gram stains, indicating this taxon is gram-negative. Its

presence was observed in conjunction with Cyanobacteria Taxa A, B, C, and D within *Acropora elseyi*, *A. florida*, *A. nasuta*, *Pocillopora verrucosa*, and *Porites* species.

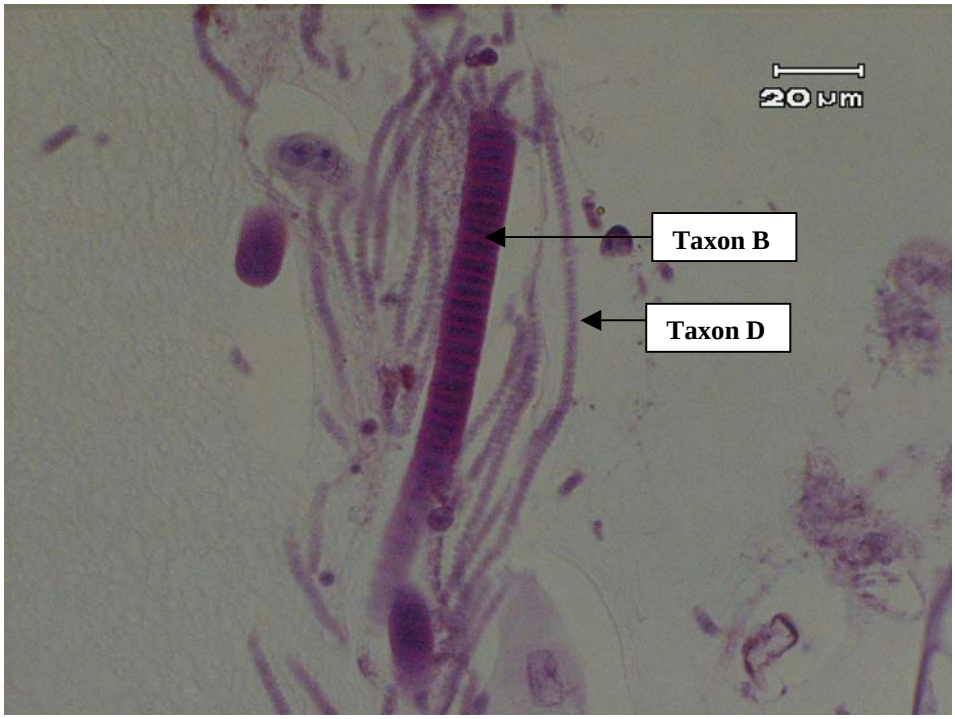

FIGURE 4.6. Histological photograph of black band disease on the coral *Porites* spp. The photograph was taken within the gastrodermal tissue layer of the coral polyp. Cyanobacteria Taxon D appears reddish-purple in the Gram stain.

4.4.2. DESCRIPTION OF ADDITIONAL MICROORGANISMS WITHIN THE CONSORTIUM

A possible endolithic algae with a diameter of 0.2394 ± 0.021 µm was observed throughout regions of the skeletal space in *Acropora elseyi*, *Pocillopora verrucosa*, and *Porites* species (Figure 4.7). It exhibited a linear morphology, appearing stringy and filamentous with a rounded end. Internal structures could not be differentiated, however the nuclei of the algae stained red in the Picro Gomori stain. An unknown fungus was observed within the tissues of a *Porites* species (Figure 4.8), however its presence was only recorded in two specimens. Although the hyphae could be easily distinguished, the fungus could not be identified based on morphological characteristics alone.

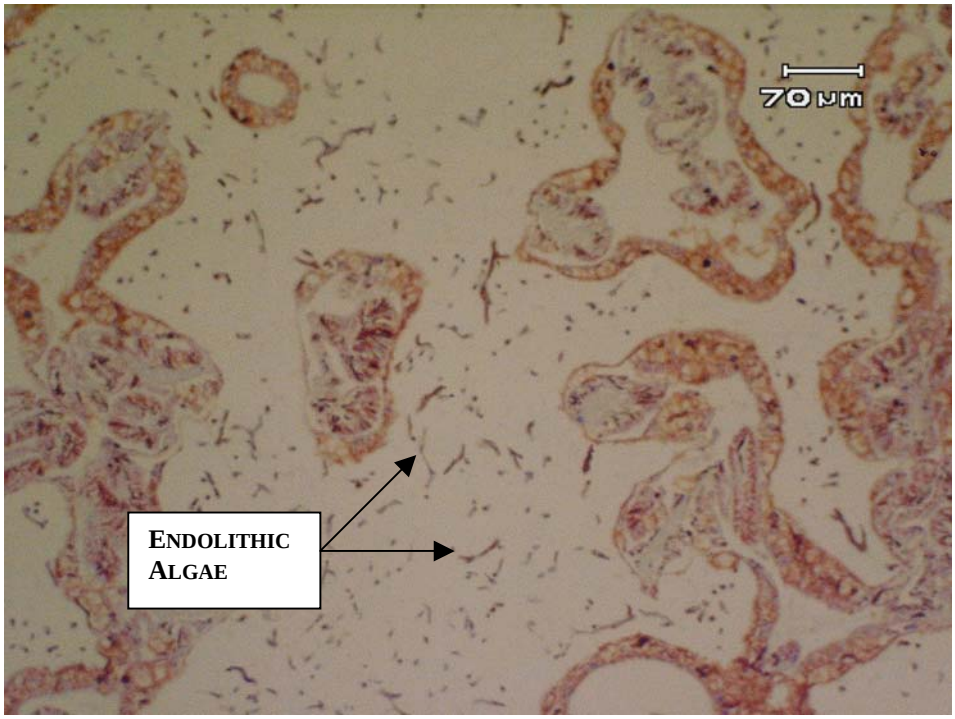

FIGURE 4.7. Histological photograph of black band disease on the coral *Porites* spp. Possible endolithic algae appears red and stringy in the Picro Gomori stain.

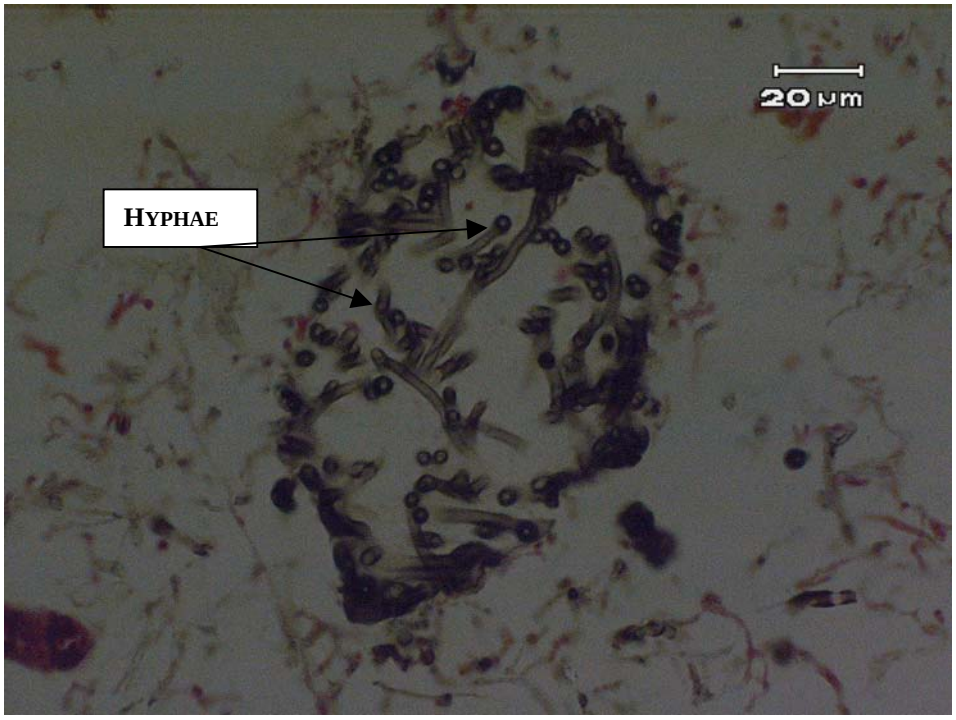

FIGURE 4.8. Histological photograph of black band disease on the coral *Porites* spp. depicting the hyphae of the unknown fungus.

4.5. DISCUSSION

In this study, I found that the consortium of microorganisms comprising black band disease included at least five different cyanobacteria taxa. Only three out of five cyanobacteria taxa were observed within tissues of all five coral species (*Acropora elseyi*, *A. florida*, *A. nasuta*, *Pocillopora verrucosa*, and *Porites* species) examined. The morphological characters that were most useful for classifying the cyanobacteria into different taxa were trichome diameter and to a lesser extent, cell shape and internal structure. This descriptive classification system based on morphological characters provides a preliminary assessment of the diversity of cyanobacterial species present in black band disease on the Great Barrier Reef. However, molecular studies will be required to more precisely identify the five taxa.

The presence of gram-negative cell walls in all five taxa supports their inclusion in the Phylum Cyanobacteria (Bergey *et al.* 2001). Although the internal structures of taxa A and B stained blue in the Gram stain, the outer sheath appeared a light red indicating that both cyanobacteria have a gram-negative cell wall. Furthermore, a number of other morphological features suggest these taxa are best classified within the Phylum Cyanobacteria. These include the fact that they had a filamentous growth form, a trichome morphology, and in some cases an outer membrane or sheath, all common morphological characteristics of the Cyanobacteria (Bergey *et al.* 2001). It is possible that the blue coloration in the Gram stain was an anomaly because the de-colorization process was not complete prior to counterstaining with nuclear fast red dye.

Histologically, the morphologies of taxa A and B closely resemble species within the genus *Oscillatoria*. Both taxa exhibit the rounded apical cells, filamentous morphology, and cylindrical trichomes measuring over 4 µm in diameter that are characteristic of the genus (Bergey *et al.* 2001). The disc-like cells of these taxa are also common traits of the *Oscillatoria* genus (Bergey *et al.* 2001). Although the 4 µm trichome diameter and segmented appearance of taxon C closely resemble *Phormidium corallyticum*, the cyanobacterium commonly associated with black band disease (Richardson and Kuta 2003), the rounded and pointed tips on opposite ends of

filaments, which are characteristic of *P. corallyticum,* were not observed. Consequently, Cyanobacteria Taxon C may belong to either the genus *Phormidium* or *Oscillatoria*. The uniseriate trichomes with diameters ranging from 2 to 14 µm observed in taxa D and E suggest that they may be species within the Order Nostocales (Bergey *et al.* 2001). False branching and tapering trichomes with thin, colorless apical cells were observed in specimens of both these taxa. These characteristics are also commonly observed within the Order Nostocales (Bergey *et al.* 2001). Generally, the morphological characteristics used in cyanobacteria taxonomy include cell structure (unicellular or filamentous), reproduction (budding, binary fission or multiple fission), trichome shape, and the presence or absence of heterocysts (Rippka *et al.* 1979). However, further molecular studies are required to test their efficacy as useful taxonomic predictors. To date, there are few taxonomic studies on marine cyanobacteria (Rutzler and Santavy 1983, Santavy and Peters 1997, Frias-Lopez *et al.* 2002, Frias-Lopez *et al.* 2003), especially those associated with corals. Therefore, it is possible that the cyanobacteria taxa examined in this study are new species.

Three of the cyanobacteria taxa (A, B, C) were observed to invade the epidermal layer of coral tissues and enter the gastrodermal cells of *Acropora elseyi*, *A. florida*, *A. nasuta*, *Pocillopora verrucosa,* and *Porites* spp. Cellular integrity near infected areas appeared less structured than apparently healthy cells and tissues further from infected sites. Observations of cyanobacteria penetrating gastrodermal cells corroborates Dinsdale's (1994) histopathological study, in which the author concluded that *Phormidium corallyticum* infects corals by penetrating the epidermis and spreading into the gastrodermal regions. The author's suggestion that the cyanobacteria utilize nutrients released by cells undergoing tissue necrosis and cell lysis for growth (Dinsdale 1994) is consistent with observations of reductions of cellular structure and densest growth of filaments near the necrosing front.

The presence of at least five cyanobacteria in the black band mat in this study provides further evidence that black band disease involves a consortium of cyanobacteria (Richardson 1997, Frias-Lopez *et al.* 2003). *Oscillatoria*, *Spirulina*, *Lyngbya*, *Arthrospira*, and *Phormidium* species have all been recorded within

diseased colonies of the Caribbean species, *Montastraea annularis*, *M. cavernosa*, and *Colpophyllia natans* (Richardson 1997). In contrast, several molecular techniques have failed to detect large abundances of cyanobacteria species in black band diseased corals of the Caribbean (Frias-Lopez *et al.* 2002). For instance, a molecular study using clone libraries suggests that the microbial mat consists of eight bacterial divisions and 13% unknowns, however cyanobacteria sequences comprised only 0-4% of the black band disease mat (Frias-Lopez *et al.* 2002). Furthermore, recent 16sRNA analyses have failed to detect the presence of *Phormidium* within some colonies infected by black band disease in the Netherlands Antilles and the northern coast of New Britain, Papua New Guinea (Frias-Lopez *et al.* 2003). Although the microbial community comprising black band disease appears to vary geographically (Dinsdale 1994, Richardson 1997, Frias-Lopez *et al.* 2003), cyanobacteria contribute to the black mat on all colonies. On the Great Barrier Reef, the microbial community associated with BBD has not been fully described, however my observations and those of Dinsdale (1994) suggest that cyanobacteria comprise a significant portion of the black mat.

Future studies investigating the discrepancies between the microorganisms comprising the mat of black band disease should aim to use a histological and microbiological approach. The combined utilization of these two techniques will provide information on the morphological characteristics and the genetic sequence of each species. This will provide a more stable approach than previous methods that rely on morphological characteristics and descriptive investigations alone and resolve the differences in results between the two techniques. In addition, these techniques may be used to characterize the microorganisms responsible for other coral diseases found on the Great Barrier Reef including white syndrome, brown band, and skeletal eroding band. Although this study focuses on the presence of cyanobacteria within the disease band, future investigations should assess the role of bacteria, fungi, and algae observed within the black mat because these microorganisms may also have a significant role in the progression, spread, and prevalence of black band disease.

In conclusion, the microbial consortium of black band disease consisted of at least five different taxa of cyanobacteria in addition to a wide array of algae and fungi. One

of these taxa resembled the morphological features of *Phormidium corallyticum*, however the characteristic apical cell shape of *P. corallyticum* was not observed. Two of the cyanobacteria taxa may be species within the genus *Oscillatoria* and two additional taxa may belong to the Order Nostocales. The presence of 4 to 5 cyanobacteria taxa on approximately 50% of examined coral specimens, including five different coral species from Lizard Island in the northern section of the Great Barrier Reef, indicate that these cyanobacteria taxa often comprise a microbial consortium. However, further behavioral and microbiological studies are needed to determine the role of these cyanobacteria in the progression of black band disease.

CHAPTER 5.0.

INVESTIGATION OF THE MICROORGANISMS ASSOCIATED WITH BROWN BAND SYNDROME FOR DETERMINATION OF THE CAUSATIVE AGENT(S)

5.1. ABSTRACT

In this chapter, my primary goal is to observe and characterize the ciliate found within brown band syndrome (BrB) using microbiological and molecular approaches. Secondly, I will investigate the potential bacterial pathogens that may cause the onset of this disease syndrome. Morphological and molecular studies indicated that the microorganisms associated with BrB on five acroporid colonies from Davis Reef consisted of a newly identified ciliate species and an array of associated bacteria. Analysis of 18S rDNA sequence data confirmed the ciliate as a new species belonging to the Class Oligohymenophora, Subclass Scuticociliatia and not *Helicostoma nonatum*, the ciliate believed to cause brown jelly syndrome in aquarium conditions. A potentially pathogenic bacterial strain (HB-8), which was closely affiliated by 16S rDNA comparisons with *Vibrio fortis* strains, was also isolated from the brown band. Six out of 12 acroporid branches inoculated with this potentially pathogenic strain reached 100% mortality after 48 hours, however the macroscopic signs (brown band) of the syndrome were not observed. This suggests that there are likely two phases of BrB including a tissue necrosis phase, which may be caused by the bacterium strain HB-8, and a ciliate phase which causes the characteristic brown band of the syndrome.

5.2. INTRODUCTION

Disease epizootics have become a major threat to coral reef ecosystems world-wide (Peters 1997, Harvell 1999, Rosenberg and Ben-Haim 2002, Weil 2004). Although reports of newly emerging syndromes continue to multiply, causative agents for the majority of these syndromes have not been identified. A wide range of microorganisms including fungi, bacteria, cyanobacteria, and protozoans have been identified to associate with both healthy and diseased corals (Peters 1997, Richardson *et al.* 1997, Rohwer *et al.* 2001, Cooney *et al.* 2002, Frias-Lopez *et al.* 2002, Patterson *et al.* 2002, Rohwer *et al.* 2002), however pathogens associated with coral diseases have only been identified for 7 out of 22 Caribbean diseases and 5 diseases in other regions of the world (Weil 2004). Although the fulfillment of Koch's postulates is essential before a potential disease pathogen can be identified as the causative agent, they have only been fulfilled for four coral diseases to date (bacterial bleaching, white plague type II, aspergillosis, and white pox; Kushmaro *et al.* 1996, Nagelkerken *et al*. 1997a, Nagelkerken *et al*. 1997b, Patterson *et al.* 2002, Denner *et al.* 2003, reviewed by Weil 2004). Even in cases where an organism has clearly been observed in association with the syndrome, the question as to whether it is a primary or secondary pathogen remains.

The appearance of brown band syndrome (BrB) was first noted during 2002 coral disease surveys on the northern and southern sectors of the Great Barrier Reef (GBR) (Willis *et al*. 2004). Although microscopic investigations of BrB have observed the presence of an unknown ciliate within the band (Willis *et al.* 2004), the microbial community associated with this syndrome remains undescribed. Protozoan infections often exist on corals subjected to aquarium conditions (Borneman 2001). For example, the consumption of coral tissue by the ciliate, *Helicostoma nonatum*, produces a brown jelly-like substance on infected coral colonies (Borneman 2001). Although it has been suggested that the ciliate found within BrB may be related to *H. nonatum* (Willis *et al.* 2004), conclusive phenotypic and genotypic characterization of the ciliate has not been performed. Given the high rates of tissue loss (up to 6 cm/day) measured in the field during active BrB infections (see chapter 2), this is one of the most virulent syndromes identified on the Great Barrier Reef to date. Consequently, further microbiological and molecular investigations of the microbial community

associated with BrB are merited and a necessary first step in determining the causative agent(s) involved and their roles as primary or secondary pathogens.

Protozoans are often identified within microbial communities associated with scleractinian corals (Toller *et al.* 2002) but few studies have addressed their role as primary or secondary invaders. A protozoan belonging to the phylum Apicomplexa has been observed to inhabit *Montastrea annularis* colonies in the Caribbean (Toller *et al.* 2002). Even though this protozoan is related to a group of highly parasitic organisms (Coccidians), the nature of its interaction with corals is currently unknown (Toller *et al.* 2002). It is suggested that, in general, coral pathogens may initially be benign microbes that only become pathogenic when the coral's health is compromised (Toller *et al.* 2002). Although ciliated protozoans are rarely classified as pathogenic parasites (Lynn and Corliss 1991), one study has linked a GBR disease condition with the ciliate, *Halofolliculina corallasia* (Antonius and Lipscomb 2001). Known as Skeletal Eroding Band (SEB), this syndrome has been characterized by an advancing mass of ciliates whose pericytostomial wings are encased within flask-like black loricae, creating a black band between the coral's healthy tissue and exposed skeleton (Antonius and Lipscomb 2001). The 1 mm to 10 cm wide band is preceded by small, black dots, which are clusters of black loricae produced by advancing propagules settling on live tissue (Antonius and Lipscomb 2001). Progressing at a rate greater than 1 mm per day, SEB is most prevalent in sheltered, shallow environments where up to 5% of any given coral species may be infected (Antonius and Lipscomb 2001). In addition to corals, marine ciliates have also been observed to form associations with symbiotic zooxanthellae. Approximately 50-800 dinoflagellates in the genus *Symbiodinium* clade-C lineage have been observed within the ciliate, *Maristentor dinoferus*, on Guam coral reefs (Lobban *et al.* 2002). Interestingly this species of dinoflagellate has also been detected within coral tissues (Lobban *et al.* 2002). Zooxanthellae have been observed within BrB ciliates but their origins (engulfed with coral tissue or acquired elsewhere) have not been identified (Willis *et al.* 2004). Until recently, interactions between protozoans and corals have remained virtually unexplored, with only a handful of studies examining these coral-protozoan associations (Antonius and Lipscomb 2001, Lobban *et al.* 2002). Further research is necessary to determine whether protozoans, such as the ciliate observed within BrB, represent primary or secondary pathogens.

Signs of BrB have been reported on three coral families including the Acroporidae, Pocilloporidae, and Faviidae (Willis *et al.* 2004), but its prevalence is variable. On three reefs in the northern GBR, less than 1% of coral colonies surveyed (12-24 cases per reef) displayed BrB signs (Willis *et al.* 2004). Higher abundances of BrB were observed at John Brewer Reef in the central GBR, where 10.04% ± 1.07 of scleractinian colonies were infected with the syndrome (Nash 2003). Rates of progression of BrB on branching acroporids have also been investigated in the central (Nash 2003) and northern sectors of the GBR (see chapter 2.0) and found to be highly variable. In the central GBR, the rate of linear tissue loss varied from 0.3 to 9 cm/day (Nash 2003) while it varied from 0.3 to 6.1 cm/day in the northern GBR (chapter 2.0). Compared to coral diseases on Caribbean reefs, BrB has the potential to progress extremely fast across coral colonies. These rapid rates of progression may result in significant impacts on GBR coral communities, especially on reefs like John Brewer where prevalence is higher. Given the potential ecological impact of the disease on 3 major families of corals on the Great Barrier Reef, studies investigating the organisms involved in these processes are warranted and will enhance our understanding of this syndrome.

My first objective is to describe the ciliate found within brown band syndrome using microbiological (scanning electron microscopy) and molecular approaches (polymerase chain reactions of 18S rDNA and 16S rDNA and clone libraries). My second objective is to investigate potential bacterial pathogens associated with disease lesions that may precede the ciliate infection and lead to the onset of the syndrome. Potential pathogens will be identified molecularly. The results from this chapter will provide important information regarding the microorganisms involved in brown band syndrome and provide insights into the pathogen(s) causing its progression and transmission.

5.3. MATERIALS AND METHODS

5.3.1. STUDY SITE AND FIELD COLLECTIONS

Samples from 10 colonies of the coral genus, *Acropora*, exhibiting symptoms of BrB were collected from Davies Reef, in the central sector of the GBR (18° 49.86'S 147° 38.2'E) (see Figure 2.1, Chapter 2) during December 2003. Portions of the brown band were either removed with a scalpel or airbrushed (80 psi) with 5 mL artificial seawater (ASW) to remove coral tissue and associated microbes from the coral skeleton. Samples were aliquoted into 1.5 mL Eppendorf tubes and stored at -80°C for later DNA analyses. Ciliate samples were collected for scanning electron microscopy (SEM) using sterilized pipettes to dislodge the ciliates from coral tissues. Samples used for bacterial plating and isolation experiments were taken from near the advancing front of the disease lesion encompassing the white tissue area between the brown band and healthy coral tissue as well as the brown band ciliate mass (see Figure 2.6, Chapter 2).

5.3.2. MICROSCOPIC CHARACTERIZATION OF CILIATES

Coral specimens, the brown band ciliate mass characteristic of the syndrome, and individual ciliate microorganisms were observed and photographed using a compound microscope (Olympus Vanox AH-2) and an Olympus digital camera (C-5050Z 5 Megapixel 3x Zoom). Ciliates were removed from 5 coral colonies and fixed immediately in Bouin's fixative solution [15 parts saturated, aqueous picric acid ($C_6H_3N_3O_7$), 5 parts formalin, 1 part glacial acetic acid] (Foissner 1991). Fixed ciliates were placed on a 0.22 μm filter paper and dehydrated with a 25%, 50%, 75%, and 100% ethanol series. Fixed and dehydrated samples were coated with gold and photographed in the SEM facility (JEOL JSM 5410LV) at James Cook University.

5.3.3. CULTURE-BASED BACTERIAL PLATING AND ISOLATION

To determine whether bacteria present in the disease front might compromise the health of the coral tissue facilitating potential secondary infection by ciliates, the presence of distinct groups of bacteria in such regions was investigated. One-cm^2 portions of diseased coral tissue were ground in sterile ASW with a sterilized mortar and pestle for culture-based analyses. Crushed samples were suspended in 9 mL of sterile ASW and vortexed for 10 minutes. Ten-fold serial dilutions were prepared to

10^{-4} and 100 µL of each dilution was spread-plated on Marine Agar 2216 (Difco Laboratories, Detroit, USA) and thiosulfate citrate bile sucrose (TCBS, Oxoid, Basingstoke, UK) media. These media are designed for isolation and enumeration of heterotrophic marine bacteria and *Vibrio* organisms respectively (Bolinches *et al.* 1988). Plates were incubated at 27°C for approximately 72 hours in order to isolate prospective heterotrophic marine bacteria and *Vibrio* species. Following incubation, dominant bacterial colony morphotypes were streak-plated on Marine Agar 2216 to obtain pure cultures for preliminary coral infection trials in an attempt to identify the potential disease causing organism. A total of 10 isolates were randomly selected from amongst the dominant colonies on the plates and used in subsequent preliminary coral infection studies (see section 5.3.12).

5.3.4. CULTURE OF BrB CILIATE

Culture media was prepared in sterilized 200 mL glass flasks using 1 mL of F/2 trace metal solution per 1 L of filtered sterilized seawater. Media was covered with wool gauze and foil to prevent contamination. Ciliates were either transferred to cultures using sterilized pipettes or 1-cm^2 portions of infected coral tissue near the brown band were removed with a scalpel and placed in cultures. Either the algae, *Isochrysis galvana* (T.150), or coral tissues were provided as a food source for the ciliate microorganisms. To promote growth, cultures were incubated at room temperature on a shaker and transferred to new sterilized media every 2 weeks.

5.3.5. EXTRACTION AND PURIFICIATION OF CILIATE DNA

The extraction of ciliate DNA from 5 acroporid colonies collected from Davies Reef was accomplished using a modified version of an UREA extraction buffer protocol (Asahida *et al.* 1996). Samples were frozen in liquid nitrogen and crushed three times with a sterilized plastic pestle. Next, 0.5 mL filtered and sterilized lysis buffer (8.4 g urea, 1.25 mL of 5 M NaCl, 2 mL of 1 M Tris, 0.8 mL of 0.5 M EDTA, 2 g sarcosine, 9 mL sterilized H_20) was added to the sample, re-ground, and shaken in a 37°C incubator for 5 minutes. Afterwards, 0.5 mL of phenol:chloroform:isoamylalcohol was added and the samples shaken an additional 5 minutes at 37°C. The aqueous phase was removed and extracted with an equal volume of chloroform/iso-amyl alcohol (24:1). Again, the aqueous phase was removed and 50 µL of sodium acetate (3 M) added along with an equal volume of isopropanol. DNA was pelleted (13,000 x

g for 15 minutes) and washed with 70% ethanol. DNA was recovered in 50 µL sterile milli-Q water, quantified using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech), and stored at -20°C until required.

5.3.6. EXTRACTION AND PURIFICATION OF BACTERIAL DNA

Genomic bacterial DNA was isolated using a Wizard Genomic DNA Isolation Kit (Promega Corporation, USA) and is briefly outlined below. Bacteria were cultured in marine broth and incubated overnight at 37°C. 1 mL of culture was placed in a 1.5 mL microcentrifuge tube and centrifuged at 13,000 rpm for 2 minutes to pellet the cells. The supernatant was removed, cells were re-suspended in 600 µL of Nuclei Lysis Solution, and incubated for 5 minutes at 80°C before being cooled to room temperature. 3 µL of RNase Solution was added to the cell lysate and mixed by gently inverting the tube. The sample was incubated at 37°C for approximately 45 minutes and combined with 200 µL of Protein Precipitation Solution. The cell lysate was vortexed at high speed for 20 seconds, incubated on ice for 5 minutes, and centrifuged for 3 minutes at 13,000 rpm. The resultant supernatant was transferred to a sterile 1.5 mL tube with 600 µL of isopropanol, mixed by inversion, and centrifuged at 13,000 rpm for 2-minutes. The supernatant was removed and 600 µL of 70% ethanol was added and mixed by inversion. The tube was again centrifuged for 2 minutes at 13,000 rpm to pellet the DNA and remove the ethanol. The DNA pellet was air-dried for 10 minutes and combined with 100 µL of DNA Rehydration Solution. Following 1-hour incubation at 65°C, the DNA was stored at 2-8°C before PCR and subsequent sequencing.

5.3.7. PCR AMPLICATION OF 18S RDNA AND 16S RDNA

Universal bacterial primers 27F and 1492R (Lane 1991) were used for amplification of the 16S rDNA from all bacteria. Universal Eukaryotic primers 18S-6-CIL-V and 18S-1511-CIL-R (Fried *et al.* 2002) were used for amplification of the 18S rDNA from ciliated protozoans and other eukaryotic organisms associated with the sample including dinoflagellates and the coral animal itself.

PCR amplifications were conducted on a GeneAmp® model 9700 temperature cycler (Perkin Elmer Cetus). Reactions took place in a total volume of 50 µL including 1 µL of template DNA, 5 µL of 10 x PCR Buffer, 3 µL of $MgCl_2$, 2.5 µmol of each

deoxyribonucleoside triphosphate, 10 pmol of each primer, 1 U of *Taq* DNA polymerase, and 37.75 µL of filter sterilized H_2O.

The temperature cycling for the PCR reaction was as follows: 27F/1492R: 1 cycle at 95°C for 5 min; 30 cycles at 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min; and 1 final cycle at 95°C for 1 min, 54°C for 1 min and 72°C for 10 min. 18S-6-CIL-V/18S-1511-CIL-R: 1 cycle at 95°C for 3 min; 30 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and 1 final cycle at 95°C for 1 min, 58°C for 1 min, and 72°C for 7 min. PCR products were checked on 1% ethidium bromide stained agarose gels and correct size bands identified against a 1kb DNA standard (Fermentas).

5.3.8. CONSTRUCTION AND RESTRICTION ENZYME ANALYSIS OF 18S rDNA CLONE LIBRARIES

A PCR product of the correct size (~ 1.8kb) derived from airbrushed tissue of BrB corals using an UREA extraction buffer protocol (see section 5.3.5) was cloned into the plasmid vector of the TOPO TA Cloning® Kit following the manufacturer's protocol (Invitrogen). Ligated vector and insert were transformed into competent *E. coli* cells again using the methods of the manufacturer with recombinant transformations selected by blue and white screening. White colonies were streaked on ampicillin LB plates and incubated at 37°C overnight. Forty-eight randomly selected clones were colony picked into individual wells of a 96-well plate with 100 µL of sterilized water. PCR was used to re-amplify the 18S rDNA insert gene of each clone according to the methods outlined previously and using primers 18S-6-CIL-V/18S-1511-CIL-R. Amplified PCR products were run on a 0.8% agarose gel with 1kb DNA marker to verify the presence of the correct size amplified DNA band. Amplified products were separately digested with 7.5 U of the restriction endonucleases *HhaI* and *HaeIII* (Promega) for 3 hours at 37°C. The resulting fragments were analyzed on 3% high quality ethidium bromide stained agarose gels and the restriction patterns compared. Clones having identical restriction patterns were grouped into OTU (Operational Taxonomic Unit) groups. Representative clones of OTU groups were re-grown in ampicillin-LB broth overnight at 37°C. Plasmid DNA was purified with a Mini-Prep Qiagen Kit according to the manufacturer's instructions.

5.3.9. SEQUENCING OF 18S RDNA PLASMID DNA

Purified plasmids representative of inserts of six unique OTU groups were sequenced using the Dynamic ET Dye (Amersham Biosciences) sequencing kit and reactions analyzed on a MegaBACE DNA Analysis System (Amersham Biosciences) located at the Advanced Analytical Center, James Cook University. Template DNA concentrations were determined with the GeneQuant spectrophotometer (Pharmacia). Sequencing cycling conditions were as follows: 35 cycles of 20 seconds at 95°C, 15 seconds at 50°C, and 3 minutes at 60°C. Partial sequences were performed with the M13-forward primer specific for the primer site located on the TOPO-TA cloning vector. Complete 18S rDNA sequences were accomplished using conserved Eukaryotic primers NS3, NS4, NS5, and NS7 (Lane 1991). Excess nucleotide and primer were removed from the sequencing mix using G50 sephadex columns (Pharmacia) according to the manufacturer's instructions.

5.3.10. SEQUENCING OF BACTERIAL ISOLATES 16S RDNA

Amplified bacterial 16S rDNA PCR products were purified with the QIAquick® PCR purification kit (Qiagen). Direct sequencing of PCR products was performed as outlined above. Primers used for sequencing included the conserved bacterial primers 339F, 732F, and 1492R.

5.3.11. SEQUENCE ALIGNMENTS AND PHYLOGENETIC ANALYSES

Ciliate and bacteria sequences were checked and compared to available sequences within the GenBank database (Altschul *et al.* 1997). Sequence alignments and phylogenetic comparisons were performed using the ARB software package (Ludwig *et al.* 2004). Tree phylogenies were constructed using evolutionary distance (Jukes and Cantor model), maximum parsimony (ARB and DNAPARS), and maximum likelihood analyses (ARB and fastDNAml) of aligned GenBank sequences (Ludwig *et al.* 2004). Missing sequence data and uncertainties in all near-complete sequences were omitted with a generated filter. Using the ARB parsimony algorithm feature, partial clone sequences were filtered with complete sequences and added to the overall tree without causing modifications to the tree topology. The confidence of branch points was then determined using a strict consensus rule applied to the results of the three analysis methods. The phylogeny presented is based on the evolutionary distance analysis.

5.3.12. PRELIMINARY INFECTION TRIAL

Ten dominant and morphologically distinct bacterial isolates cultured from BrB diseased samples were tested against coral tissues in preliminary infection trials. Cultures were grown in Marine Agar Broth (Difco) to a high density. Approximately $1x10^9$ bacterial cells were concentrated by centrifugation and washed twice in sterile ASW in preparation for addition to coral infection experiments. Twelve 2 L beakers containing 1.5 L filtered seawater were placed within two Nally bins, provided with an airstone, and maintained at room temperature (~25°C). Two healthy branches of *Acropora muricata* were placed in each beaker. Coral branches were allowed to acclimate for 24 hours before inoculation. Bacterial cells from each isolate were injected into a single beaker at a final concentration of ~1 x 10^6 cells mL^{-1} and monitored for the next 3-5 days along with two control beakers (no bacteria added). Coral death was monitored by percent tissue loss. Bacterial isolates observed to cause rapid tissue loss were used in further replicated infection trials. Further replicated experiments testing the bacterial isolate which appeared to necrose coral tissues were performed with three control beakers (no bacterial addition) and three treatment beakers (bacterial addition). Once again, two healthy branches were placed in each beaker and allowed to acclimate 24 hours. Aquaria set-up and bacterial concentrations were identical to the previous trial.

5.3.13. INFECTION TRIALS

Infection trials were performed within a temperature-controlled aquarium facility. The experimental setup consisted of three Nally bins placed side by side, each containing 6 glass beakers (Figure 5.1). Two healthy coral branches were placed in each beaker along with a single air stone. Healthy *Acropora muricata* branches were embedded in plastic poly-pipe using non-toxic putty and placed in 3-liter glass beakers with 2.5 liters of 0.22 µm filtered seawater. A constant supply of flow-through seawater (from a single source) at ambient temperature circulating around the beakers within each Nally bin maintained the filtered seawater at ambient temperature. Aquaria were exposed to a 12-hour light – 12 hour dark photoperiod regime (Sun-glow fluorescent aquarium lights). After healthy corals were placed in aquaria, they were allowed to acclimate to aquarium conditions for 24 hours. Following acclimation, the filtered seawater was injected with either ~1 x 10^6 cells mL^{-1} concentration of the most virulent bacterium (HB-8), a control bacterium (~1 x 10^6 cells mL^{-1}) that produced no

tissue lysis (HB-7), or no bacteria. Coral death was estimated as percent tissue lysis every 24 hours for the next 6 days. Filtered seawater was exchanged every 3 days in each beaker.

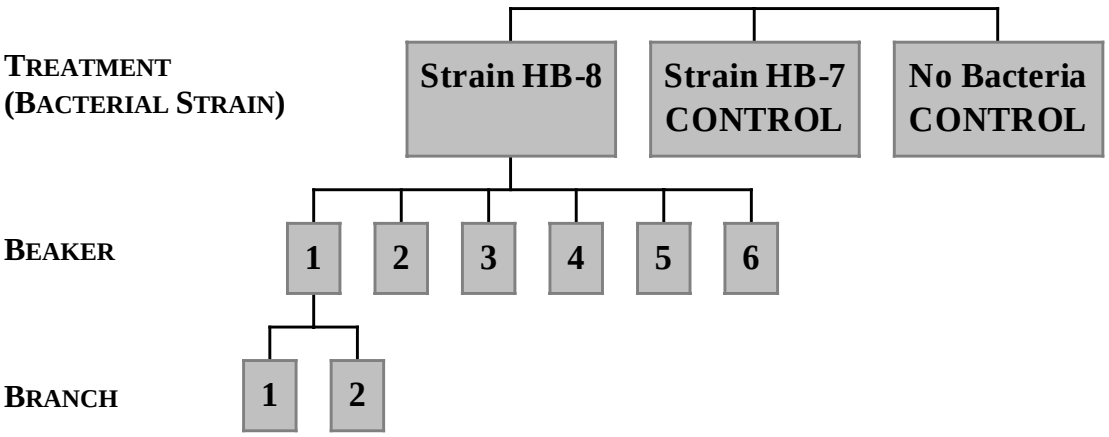

FIGURE 5.1. Experimental design for controlled aquarium infection trials investigating the effect of the most virulent bacteria (HB-8) isolated from brown band syndrome on healthy acroporid branches collected from Davies Reef, Australia.

5.4. RESULTS

5.4.1. CILIATE IDENTIFICATION

The ciliate had a tube-shaped, elongated morphology, which was rounded at both the posterior and apical ends (Figure 5.2). The length of the ciliate varied from 100-300 µm while the width fluctuated from 20 to 50 µm depending on available food sources. The oral apparatus appeared to be differentiated from somatic ciliature and located in the buccal cavity on the ventral side. Ciliation appeared uniform around the elongated organism, with the exception of three distinct cilia in the caudal region (Figure 5.3.A). On whole coral samples, the mass of ciliates glided over the exterior of the coral samples and into the cavities and polyps of the coral animal. Within cultures supplemented with coral tissue, the ciliates were free-living, however they became shorter and more rounded at the posterior end.

The ciliate appeared to ingest portions of the coral tissue as a potential nutrient source. This observation was supported by the obvious appearance of zooxanthellae within the ciliate (Figure 5.3.B). Fixed ciliate samples have been sent to the Laboratory of Prof. Wilheim Foissner in Vienna, Austria, for definitive phenotypic classification of this potentially novel protozoan.

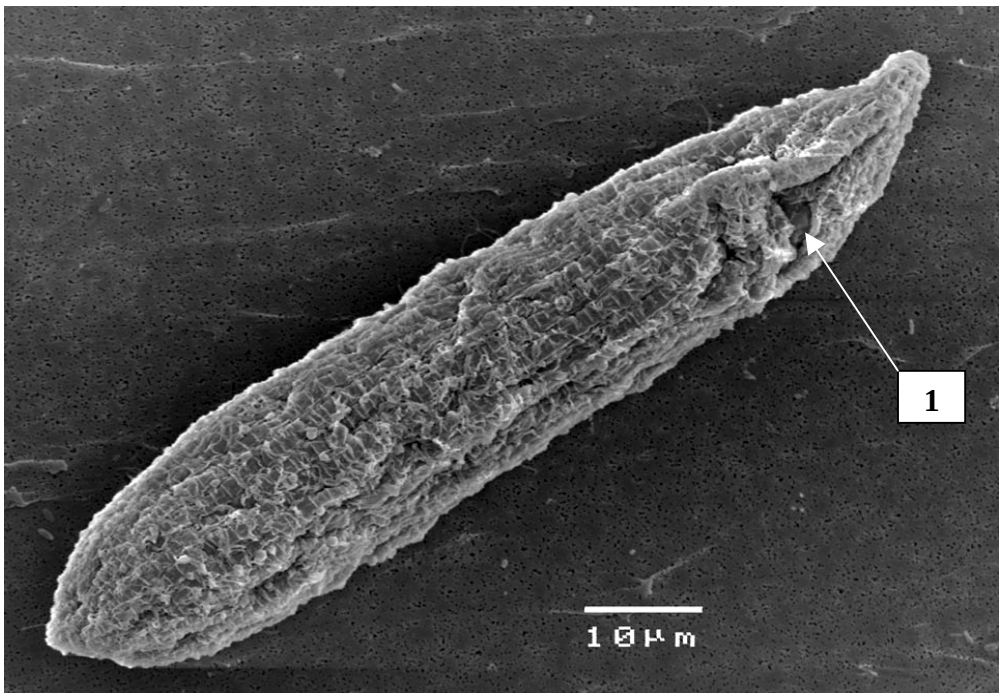

FIGURE 5.2. Scanning electron micrographs of a brown band ciliate showing its external morphology and the buccal cavity (1) on the ventral side. Photos taken by Dr. Kevin Blake, JCU.

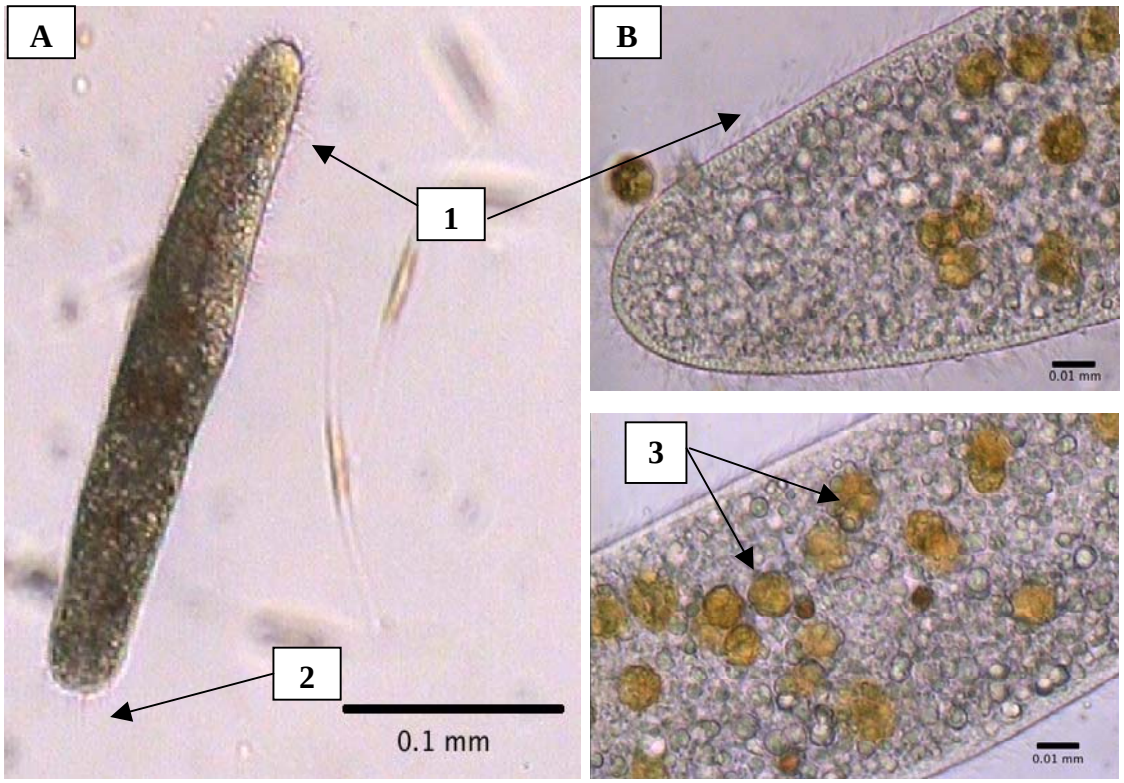

FIGURE 5.3.A. - 5.3.B. Living specimen of a brown band ciliate showing (1) uniform ciliation, (2) 3 distinct caudal cilia, and (3) zooxanthellae within the ciliate. Photos taken by Neal Young, AIMS.

5.4.2. CLONE LIBRARY CONSTRUCTION AND CILIATE CLONE IDENTIFICATION

Universal Eukaryotic primers were used to amplify the 18S rDNA sequences of a coral sample infected by a mass of BrB ciliates. 18S rDNA sequences derived from zooxanthellae and coral animal tissues were likely to be amplified along with the ciliate 18S rDNA. From a total of 48 clones, 6 different restriction enzyme patterns were observed and clones grouped into their respective Operational Taxonomic Unit (OTU) groups (Figure 5.4). One clone from each OTU group was sequenced. OTU groups 1-5 were affiliated with zooxanthellae sequences. OTU group 6 affiliated with 18S rDNA sequences of other ciliates and was concluded to be the organism associated with BrB.

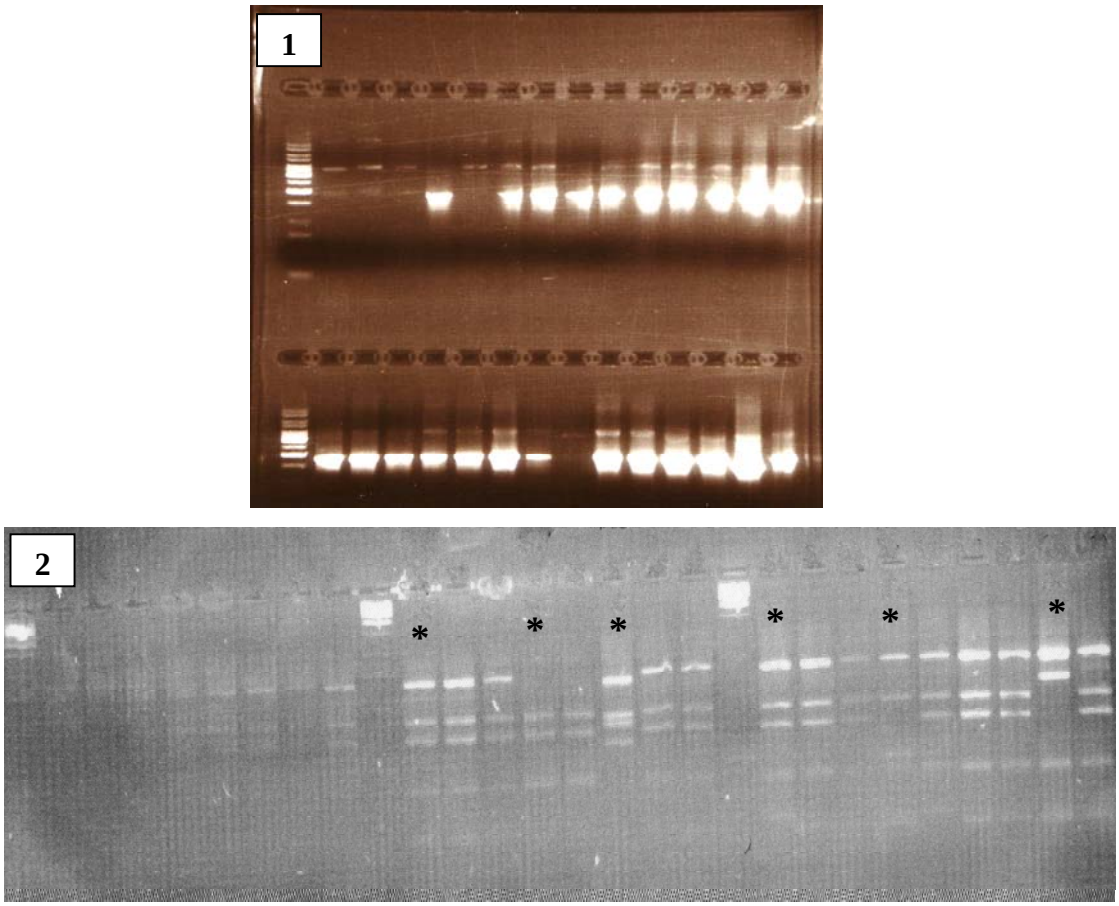

FIGURE 5.4. 18S RDNA gels of (1) Amplified PCR products clones #6-33, (2) *Hha-I* Restriction Enzyme Digests – 6 OTU groups (*) identified after RFLP analyses.

5.4.3. 18S rDNA SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES

The 18S rDNA sequence obtained from the BrB ciliate was compared to other closely related sequences in the GenBank database. The representative organisms showing highest sequence identity are shown in Table 5.1. The BrB ciliate sequence showed 95% identity to the 18S rDNA sequence of the ciliate, *Parauronema longum*. This represented a total of 1673 out of 1749 base pairs being identical to the *P. longum* species. According to phylogenetic comparisons, the unknown ciliate was closely related to ciliates belonging to the Class Oligohymenophora Subclass Scuticociliatia (Figure 5.5). These include the scuticociliates, *Schizocaryum dogieli*, *Cohnilembus verminua*, *Anophyroides haemophila*, *Pseudocohnilembus marinus*, and *Uronema marinum.* The zooxanthellae observed within the ciliate were closely related to *Symbiodinium* species (98-99% sequence identity), especially those within the clade-C lineage (Table 5.2).

TABLE 5.1. Phylogenetic affiliations of 18S rDNA sequences retrieved from OTU group 6 clones cultured from *Acropora* coral samples infected with brown band syndrome.

OUT	% similarity[a]	Relative & database Accession number	Taxonomic description	bp
6	95%	*Parauronema longum* (AY212807)	Scuticociliatia	1673/1749
	94%	*Schizocaryum dogieli* (AF527756)	Scuticociliatia	1656/1751
	94%	*Cohnilembus verminua* (Z22878)	Scuticociliatia	1616/1719
	94%	*Anophyroides haemophila* (U51554)	Scuticociliatia	1569/1664
	94%	*Miamiensis avidus* (AY550080)	Scuticociliatia	1188/1263
	93%	*Pseudocohnilembus marinus* (Z22880)	Scuticociliatia	1084/1160
	93%	*Metanophrys similis* (AY314803)	Scuticociliatia	1362/1453
	93%	*Paranophrys magna* (AY103191)	Scuticociliatia	1017/1083
	91%	*Uronema marinum* (Z22881)	Scuticociliatia	1551/1687

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

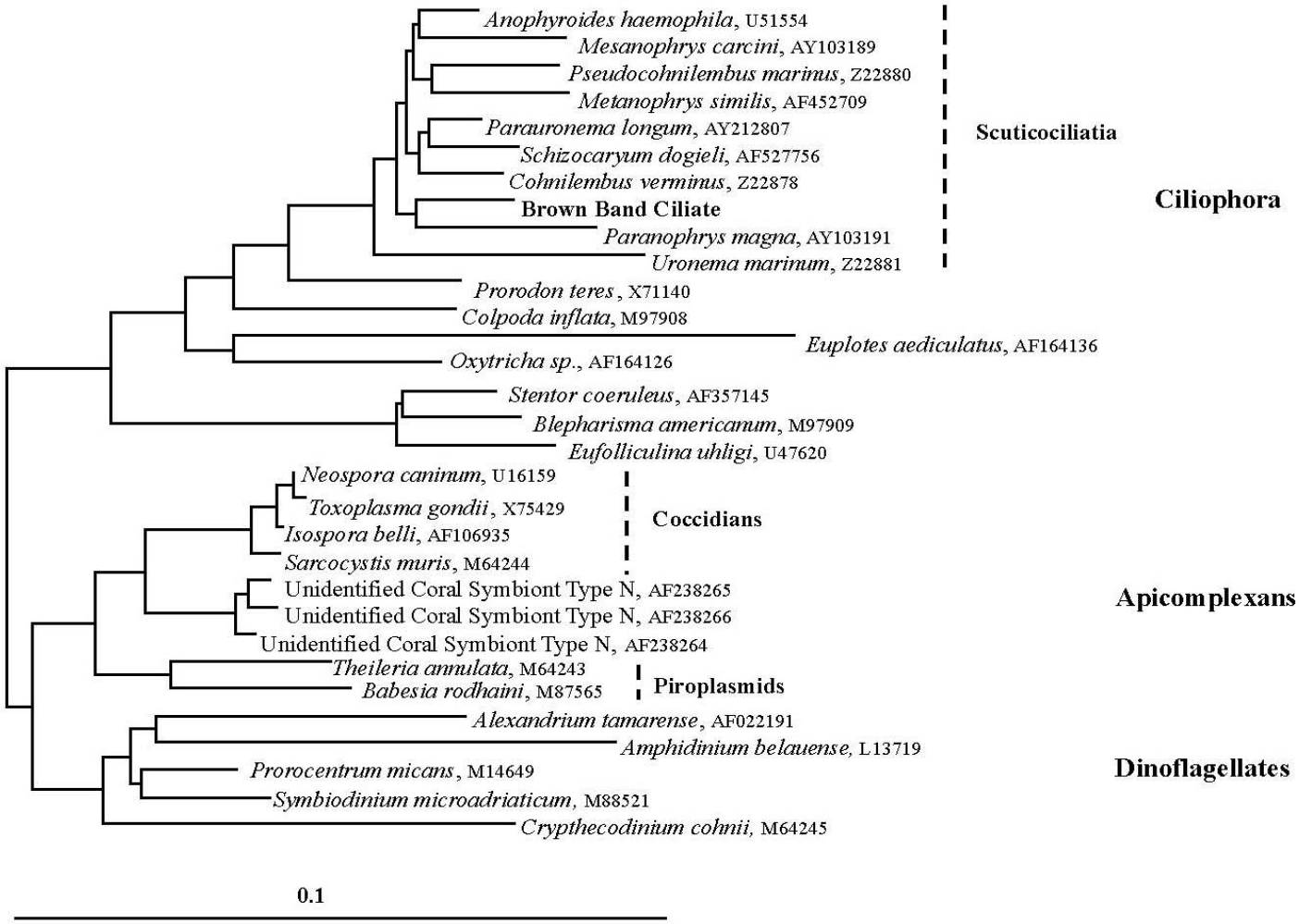

FIGURE 5.5. Phylogenetic tree showing the relationship between the BrB ciliate and selected reference ciliates based on 18S rDNA sequences listed in GenBank. Complete reference sequences were used to initially construct the tree and partial sequences were added later with the special algorithm in the ARB software package (Ludwig *et al.* 2004). These additions did not result in changes in the overall tree topology. The sequenced BrB ciliate is denoted in bold face type while the bar represents 10% estimated sequence divergence. The outgroup used in the construction of this tree was the dinoflagellate, *Crypthecodinium cohnii*.

TABLE 5.2. Phylogenetic affiliations of 18S rDNA sequences retrieved from OTU groups 1-5 clones cultured from *Acropora* coral samples infected with brown band syndrome.

OUT	% similarity[a]	Relative & database accession number	Taxonomic description	bp
1	99%	*Symbiodinium* sp. (AB016539)	Zooxanthellae	612/615
2	99%	*Symbiodinium* sp. Type C (AF238258)	Zooxanthellae	320/322
3	99%	*Symbiodinium* sp. (AB016539)	Zooxanthellae	525/528
4	99%	*Symbiodinium* sp. Type C (AF238258)	Zooxanthellae	324/326
5	98%	*Symbiodinium* sp. OTch-2 (AB085912)	Zooxanthellae	345/351

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

5.4.4. ISOLATION OF BACTERIA ASSOCIATED WITH BRB CORALS

A total of 10 dominant and unique bacterial colony morphologies were obtained on Marine Agar and TCBS media from 5 acroporid colonies infected with BrB. These organisms were observed at high Colony Forming Unit (CFU mL^{-1}) numbers in more than 50% of replicate plates. Colonies were streak-plated on new media until individual colony morphologies could be detected. The isolates were given strain numbers HB-1 to HB-10.

5.4.5. PRELIMINARY INFECTION TRIALS

Preliminary experimental inoculations revealed that the addition of one bacteria strain, designated as HB-8, to aquaria containing healthy corals resulted in tissue sloughing within 2-3 days. No tissue sloughing or coral death was observed in inoculation treatments involving other bacteria or in the controls, which were maintained without addition of bacteria. Strain HB-8 was isolated on TCBS agar indicating the bacterium is likely to be a *Vibrio* or *Alteromonas/Pseudoalteromonas* strain. In the subsequent replicated infection experiment, tissue sloughing and death again occurred within 2-3 days in the HB-8 treatment but not in the controls (no bacterial addition). Consequently, bacterium strain HB-8 was identified as a potential bacterial pathogen that may contribute or lead to the onset of necrosis and possibly facilitate secondary infections by BrB ciliates.

5.4.6. PHYLOGENETIC IDENTIFICATION OF BACTERIAL STRAIN HB-8

The complete 16S rDNA gene of strain HB-8 was sequenced and compared against other bacterial sequences listed in the GenBank database. The closest matching organisms and their sequence similarities are presented in Table 5.3. This organism showed closest sequence similarity (99%) to *Vibrio fortis* strain LMG21562 where 1037 out of 1041 base pairs were identical between the two bacterial species. A 16S rDNA phylogenetic tree of closely related *Vibrio* strains is depicted in Figure 5.6.

TABLE 5.3. Phylogenetic affliliation of the 16S rDNA sequence retrieved from bacterium strain HB-8 isolated from *Acropora* coral samples infected with brown band syndrome.

Sample	% similarity[a]	Relative & database accession number	Taxonomic description	bp
HB-8	99%	*Vibrio fortis* LMG 21557T (AJ514916)	γ-Proteobacteria	1034/1041
	99%	*Vibrio fortis* LMG21562 (AJ514915)	γ-Proteobacteria	1037/1041
	99%	*Vibrio fortis* LMG 21558 (AJ514913)	γ-Proteobacteria	1034/1041
	99%	*Vibrio fortis* LMG 21566 (AJ514917)	γ-Proteobacteria	1034/1041
	99%	*Vibrio fortis* LMG 20547 (AJ316202)	γ-Proteobacteria	1034/1041
	99%	*Vibrio pelagius* CECT 4202T (AJ293802)	γ-Proteobacteria	1033/1041

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

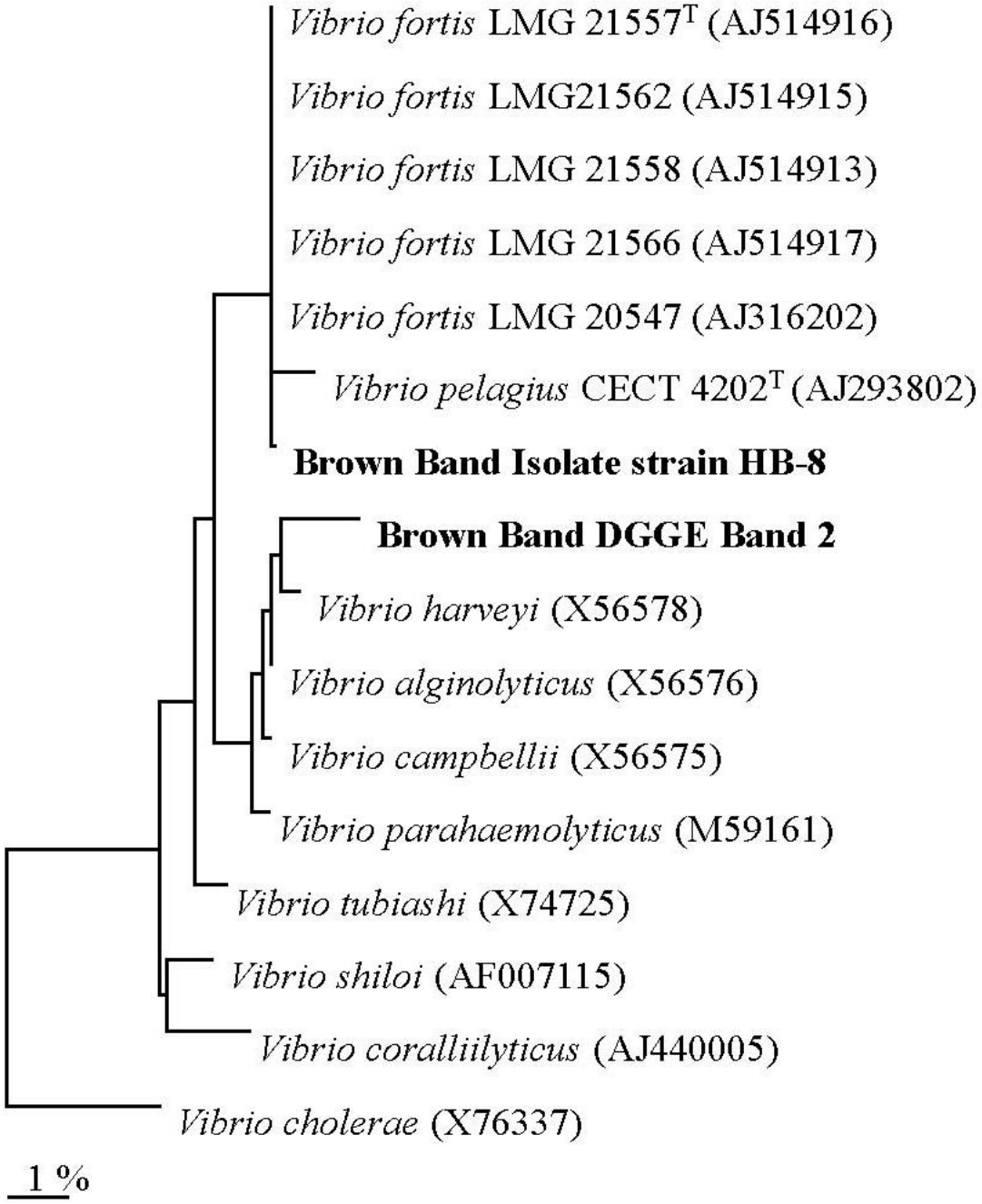

FIGURE 5.6. Phylogenetic tree exhibiting the relationships between the 16S rDNA sequence bacterium strain HB-8 and chosen reference *Vibrio* sequences listed in the GenBank database. Complete sequences were used to construct the tree and did not result in changes in the overall tree topology. The sequenced BrB strain HB-8 is denoted in bold face type while the bar represents 1% estimated sequence divergence. The outgroup used in the construction of this tree was the bacterium *Vibrio cholerae*.

5.4.7. INFECTION TRIALS

Infection trials to test the hypothesis that bacterial strain HB-8 causes tissue necrosis which may subsequently facilitate infection by BrB ciliates in *Acropora* corals, indicated that this strain causes necrosis of 50% of experimental fragments within 48 hours (Figure 5.7). Healthy corals exposed to bacterium strain HB-8 lost 50% of their tissue before any tissue loss was observed on corals in either of the control treatments (control 1: bacterial strain (HB-7); control 2: no bacterial addition) (Figure 5.7). Whereas >50% of corals exposed to HB-8 bacteria died within 3 days, corals in control tanks didn't show signs of mortality until after 3-4 days (Figure 5.8). On 8 out of 12 coral branches exposed to bacterial strain HB-8, tissue loss began near the base of the branch and progressed upward to the branch tips. Necrosis within the two control treatments differed with tissue sloughing off the entire length of the coral branch. Once tissue sloughing began, both coral branches within the beaker reached 100% mortality in approximately 12 hours.

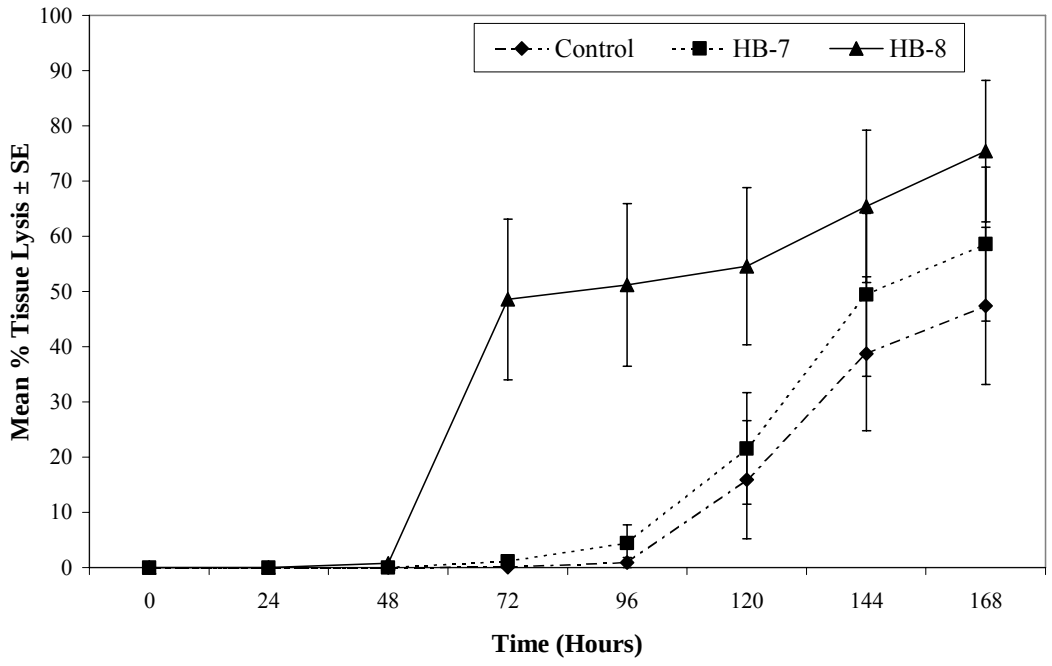

FIGURE 5.7. Average percent tissue loss ± SE in coral branches exposed to a bacterium strain HB-8, control bacterial strain HB-7, and a control with no bacterial addition across time (hours).

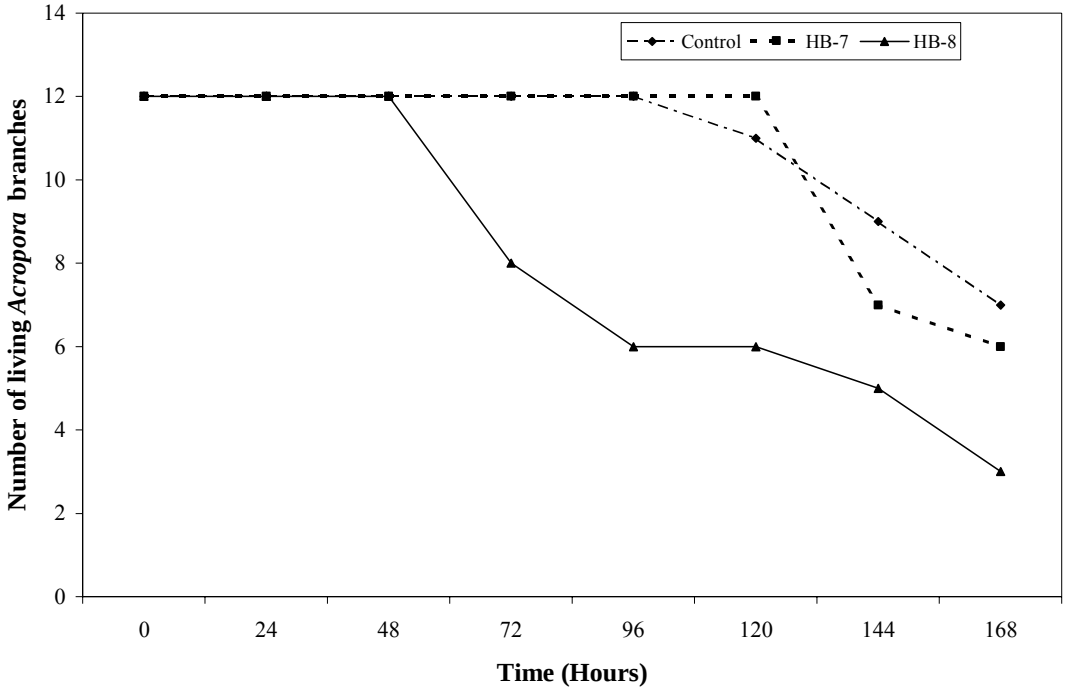

FIGURE 5.8. The survival of healthy *Acropora* branch fragments exposed to a bacterium strain HB-8, control bacterial strain HB-7, and a control with no bacterial addition across time (hours).

5.5. DISCUSSION

Morphological and molecular studies indicate that the protozoan associated with brown band syndrome on five acroporid colonies from Davies Reef, in the central section of the Great Barrier Reef, is a new species of ciliate (Foissner pers. comm.). Phylogenetic comparisons of 18S rRNA gene sequences indicate that the ciliate isolated from BrB-infected corals is closely related to protozoans in the Class Oligohymenophora, Subclass Scuticociliatia (Figure 5.5). Further morphological and molecular research is required to provide taxonomic descriptions of this new species and to determine if the ciliate found to be associated with BrB is the same throughout the distributional range of the syndrome.

Although ciliates belonging to the Scuticociliatia subclass are abundant in marine habitats and often observed as endosymbionts in several marine invertebrates including echinoids, crustaceans, polychaetes, and bivalve mollusks (Lee and Capriulo 1990), their impact on coral health is unknown. Ciliates found within this group may be bacterivorous, algivorous, carnivorous, omnivorous, or even histophagous (Lee and Capriulo 1990), yet they have not previously been reported to be carnivorous on corals. They have rarely been classified as pathogenic parasites (Lynn and Corliss 1991) within coral communities, however this may reflect a lack of study on associations between ciliates and corals. To date, only two ciliated protozoans have been implicated in the death of coral tissue. These are *Helicostoma nonatum*, the ciliate responsible for brown jelly in aquaria (Borneman 2001), and *Halofolliculina corallasia*, the heterotrich ciliate associated with skeletal eroding band (Antonius and Lipscomb 2001). These previous reports of ciliates causing tissue mortality corroborate the finding that ciliates may be pathogenic on corals.

Little is known about the feeding behavior of the brown band ciliate, yet numerous zooxanthellae have been observed within its membranes (Figure 5.3.B). Whether or not the ciliate is ingesting these zooxanthellae along with live coral tissue (carnivorous), dead coral tissue (histophagous), or acquiring them from elsewhere (algivorous) is a question that needs to be addressed. Furthermore, we cannot rule out the possibility that these zooxanthellae may form a symbiotic association with the ciliate. These symbiotic relationships have previously been reported on corals

(Lobban *et al.* 2002). Clarification of whether the ciliate feeds on coral tissue as a nutrient source or secondarily engulfs it while feeding on zooxanthellae will help to determine if the ciliate is a primary agent of tissue mortality or merely scavenging on necrotic tissue following infection by a primary pathogen.

A number of dominant bacterial isolates were obtained from culture-based analyses of BrB affected corals. Preliminary infection trials of corals identified one bacterial species, HB-8, which compromised the health of corals leading to tissue sloughing and coral death. A further infection trial demonstrated that after 48 hours, 100% mortality occurred in half of the corals infected with bacterium strain HB-8 whereas tissue necrosis in controls (10% mortality in a single branch) didn't occur until after 72 hours. Sequence alignments of the 16S rRNA gene of this species revealed that it was closely related to microbes within the genus *Vibrio* (Figure 5.6). In particular, strain HB-8 was closely grouped with a newly defined marine cluster of *Vibrio* isolates, *Vibrio fortis* (Thompson *et al.* 2003). *Vibrio* species have long been identified as major disease causing organisms in the marine environment (Kushmaro *et al.* 1996, Rosenberg and Ben-Haim 2002). In addition, species belonging to this genus have been described as coral pathogens in the Mediterranean, Indian Ocean, and the Red Sea. For example, inoculation experiments in the Mediterranean have determined that coral bleaching in *Oculina patagonica* is caused by the pathogenic bacterium, *Vibrio shiloi* (Kushmaro *et al.* 1996, Rosenberg and Ben-Haim 2002). Koch's postulates have been fulfilled for a second bacterium (*Vibrio coralliilyticus),* suspected of causing tissue lysis in *Pocillopora damicornis* (Ben-Haim and Rosenberg 2002). Healthy corals inoculated with less than 30 bacteria ml^{-1} displayed signs of infection and tissue lysis after 3-5 days and within two weeks, coral tissue was completely destroyed (Ben-Haim and Rosenberg 2002). Furthermore, the pathogen was highly contagious, spreading from infected to healthy corals in approximately 2 to 4 days (Ben-Haim and Rosenberg 2002). According to these examples, *Vibrio* species may be pathogenic in coral species, however future inoculation experiments and the fulfillment of Koch's postulates are essential before the *V. fortis*-related bacteria identified in this chapter can be confirmed as the pathogen inducing tissue mortality leading to invasion by BrB ciliates.

In conclusion, the characteristic macroscopic signs of the brown band syndrome have been attributed to a newly identified ciliate species of the Class Oligohymenophora, Subclass Scuticociliatia. Morphological studies to definitively classify this ciliate species are ongoing. However, analysis of 18S rDNA sequence data confirms it as a new species and not *Helicostoma nonatum*, the ciliate suggested to cause brown jelly syndrome on corals in aquariums (Borneman 2001). Several bacterial species associated with the BrB were also isolated with one in particular, strain HB-8, causing tissue necrosis that may precede ciliate infection. Although it is hypothesized that the brown band ciliate secondarily settles on necrotic coral tissue, further inoculation studies are necessary to confirm this theory. Confirmation of strain HB-8 as the primary pathogen inducing tissue necrosis cannot be concluded until Koch's postulates are fulfilled, however inoculation experiments suggest that the causative agent of tissue necrosis preceding brown band syndrome on five acroporid colonies at Davies Reef may be a *Vibrio* species closely related to the *Vibrio fortis* strain.

CHAPTER 6.0

GENERAL DISCUSSION

6.1. OVERALL SUMMARY

Natural rates of progression and transmission of black band disease (BBD) on *Acropora muricata* at Lizard Island were significantly greater during the warmer month of January as opposed to July and May (see Chapter 2). Experimental studies exposing coral branches infected with BBD to experimentally elevated seawater temperatures (32˚C) in summer enhanced the progression of BBD. However, the lack of increased progression in higher temperature treatments (29˚C and 31˚C) during May imply that other factors, such as high light intensities, may also be required to promote the progression of BBD (see Chapter 3). In Lizard Island samples, BBD was comprised of five morphologically distinct cyanobacterial species and a wide array of endolithic algae and fungi (see Chapter 4). Cyanobacteria, such as *Phormidium corallyticum*, which have been identified as possible pathogens of BBD in the Caribbean (Rutzler and Santavy 1983, Richardson 1997, Frias-Lopez *et al.* 2003), often achieve maximum photosynthetic efficiency at elevated temperatures and optimal photosynthesis by cyanobacterial clumping at high light intensities (Richardson and Kuta 2003). Consequently, the increased progression of BBD during January may be the result of increased virulence of the pathogen at these higher temperatures and light intensities, although increased host susceptibility from elevated temperature stress may also play a role.

In comparison, brown band syndrome (BrB) progressed along, and transmitted between, *Acropora muricata* branches 5.4 times faster than BBD (see Chapter 2), with the greatest rates of progression occurring at the calmer, less exposed Horseshoe Reef. The progression of BrB at Lizard Island was not influenced by experimentally elevated temperatures under low light intensities (~50 µE/m^2/s for 12 hour intervals), however the effect of combining elevated temperatures and high light intensities on the progression of this syndrome was not determined (see Chapter 3). High light

intensities combined with elevated temperatures might influence the progression of this syndrome since photosynthetic zooxanthellae were observed within the ciliate associated with BrB (see Chapter 5). Morphological and molecular studies indicate that the characteristic brown coloration of BrB on *Acropora* colonies at Davies Reef was caused by a ciliated protozoan in the Class Oligohymenophora, Subclass Scuticociliatia (see Chapter 5). However, it is not clear whether the ciliate is a primary pathogen feeding directly on coral tissue or whether it invades secondarily after some other agent causes tissue necrosis. Ten dominant bacterial isolates were obtained from culture-based analyses of BrB affected corals. Preliminary inoculation trials revealed that half of the healthy corals exposed to the dominant culturable bacterial strain (HB-8), reached 100% mortality two days before any of the control corals (i.e. either exposed to a non-necrosing bacterial strain or maintained without bacterial addition) displayed any signs of tissue lysis. According to 16S rDNA comparisons, strain HB-8 was closely affiliated with several strains of *Vibrio fortis*. *Vibrio* species have long been identified as major disease-causing organisms in the marine environment and as coral pathogens in the Mediterranean (Rosenberg and Ben-Haim 2002). In combination, these results suggest that a close relative of *V. fortis* may infect corals, causing tissue necrosis that may facilitate secondary infection by the BrB ciliate.

6.2. HOST VERSUS PATHOGEN INFLUENCES ON PROGRESSION AND SPREAD OF CORAL DISEASE

The progression and transmission of black band disease and brown band syndrome on the Great Barrier Reef may depend on a variety of responses from both the coral host and the disease pathogen. For instance, the rate at which a disease progresses and spreads may depend upon the response of the coral to the pathogen itself as well as the response of the coral and pathogen to changing environmental conditions, such as elevated temperatures, increased light intensities, and high water circulation. Adverse changes in the environment that are detrimental to the coral host may simultaneously increase the virulence of the coral pathogen while lowering host resistance (Torren *et al.* 1998, Banin *et al.* 2000, Alker *et al.* 2001, Hayes *et al.* 2001, Harvell *et al.* 2001). Since both responses dictate the rate at which these diseases progress among and spread between coral colonies, understanding the progression and transmission of

black band disease and brown band syndrome requires an approach which considers both the coral's (ecological) and pathogen's (microbiological) perspective.

6.2.1. PROGRESSION AND SPREAD OF DISEASE FROM THE PERSPECTIVE OF THE CORAL HOST

Corals are extremely sensitive to changing environmental conditions, such as elevated temperatures (Coles and Jokiel 1978, Brown *et al.* 1994, Hoegh-Guldberg 1999, Berkelmans 2004), increased sedimentation (Peters 1984), and reduced water quality (Roberts 1993, Coles and Ruddy 1995). Consequently, the stress associated with prolonged exposure to these adverse conditions may reduce the coral's health (Peters 1984, Hoegh-Guldberg 1999). Coral bleaching, for instance, involves the loss or expulsion of the coral's symbiotic zooxanthellae and is a stress response of the coral to unfavorable conditions, such as above-average seawater temperature, reduced salinity, or increased light intensities (Jokiel and Coles 1977, Jokiel and Coles 1990, Brown 1997, Berkelmans and Oliver 1999, Berkelmans and Willis 1999, Hoegh-Guldberg 1999, Lough 2000, Berkelmans 2004). Since a substantial portion of the coral's nutrition and photosynthetic energy comes from the symbiotic relationship between the coral host and its photosynthetic microalgal endosymbionts, the loss of these zooxanthellae have the potential to reduce the coral's health (Coles and Jokiel 1978, Lesser 1996). In addition to diminishing the health of corals, stress can play an influential role in a coral's susceptibility to disease infection by weakening its immunity to pathogenic microorganisms (Bak and Criens 1981, Bruckner and Bruckner 1997, Porter *et al.* 2001). After the coral is infected, the disease can be considered an additional stress increasing the colony's vulnerability to further disturbance events including secondary disease infection, predator attacks, and overgrowth by potential competitors such as algae (Rapport and Whitford 1999). Examples of previously infected colonies becoming re-infected by the same and different coral diseases have been reported throughout the Caribbean region (Antonius 1981, Antonius 1985, Kuta and Richardson 1996, Bruckner and Bruckner 1997). For example, BBD was observed to re-infect colonies of *Colpophyllia natans* in the Florida Keys (Kuta and Richardson 1996) and a re-occurrence of BBD on Jamaican reefs in 1995 and 1996 was observed in 39 corals (11.4%) previously infected with the disease between 1992 and 1993 (Bruckner and Bruckner 1997). In summary, environmental stress has the ability to reduce coral health (Peters 1984) thereby

making it more susceptible to disease infections. If infected, the disease may lower the colony's resistance against further disease infections and diminish the coral's ability to maintain colony integrity (Bak and Laane 1987, Rapport and Whitford 1999). As a result, the response of the coral to changing environmental conditions influences the rate of progression and spread of coral diseases.

The faster rates of progression and transmission of BBD that I observed in my field studies and experimental temperature manipulations in January support the idea that environmental stress can increase a coral's susceptibility to disease infections and enhance the advancement of the disease along infected branches (see Chapter 2, Chapter 3). Experimentally elevated temperatures (32˚C) and high light intensities (~1400 μE/m^2/s) during January may have stressed corals, reduced coral health, and increased their vulnerability to BBD; hence the more rapid progression and transmission of the disease in both the lab and field studies during the austral summer month of January.

6.2.2. PROGRESSION AND SPREAD FROM THE PERSPECTIVE OF THE PATHOGEN

Although the response of the pathogen to changing environmental conditions may also affect the rates at which the disease progresses among branches and transmits between colonies, it is likely that the mechanism causing mortality (i.e. ingestion of tissue versus tissue necrosis) also has an influence on the progression and transmission of the disease. The significantly different rates of progression and transmission measured for BBD and BrB are consistent with this idea. Comparisons of the rate of progression between these two diseases on *Acropora muricata* branches at Lizard Island during May 2004 showed that BrB progresses significantly faster along diseased coral branches than BBD (see Chapter 2). Since measurements were taken on the same coral species at the same location during May, the most obvious difference between these two diseases is the microbial communities associated with each disease and their etiology. BBD is comprised primarily of cyanobacteria believed to utilize nutrients released from the lysis of coral tissue (Carlton and Richardson 1995, Richardson and Kuta 2003) (see Chapter 4) while BrB consists of various bacteria species, one strain in particular (HB-8) which I observed to cause tissue necrosis, and a ciliate that may come in secondarily to ingest the coral tissue, zooxanthellae, and/or bacteria (see Chapter 5). Therefore, although differences in the

progression and transmission of BBD and BrB may primarily reflect differences in the mechanisms by which these pathogens cause mortality, the pathogen's response to environmental parameters may vary as well.

Although some conditions adversely affect corals, they may also increase the virulence of some marine pathogens (Rosenberg and Ben-Haim 2002). For example, elevated temperatures have been reported to promote the expression of virulence genes by *Vibrio shiloi*, a bacterium identified as the causative agent of a coral bleaching disease in the Mediterranean (Torren *et al.* 1998, Banin *et al.* 2000). Up to a point, elevated temperatures have also been shown to enhance growth rates of the fungal pathogen, *Aspergillus sydowii*, responsible for aspergillosis in Caribbean sea fans (Alker *et al.* 2001). Although the cyanobacteria, *Phormidium corallyticum* (Porter *et al.* 2001, Kuta and Richardson 2002, Richardson and Kuta 2003), has recently been shown not to be the sole causative agent of black band disease in the Caribbean (Frias-Lopez *et al.* 2003, Richardson and Kuta 2003), it has been demonstrated that its virulence increases with high seawater temperatures. In addition to elevated temperatures, degraded environmental conditions resulting from human activities such as nutrient enrichment, terrestrial run-off, sewage outfalls, and sedimentation increase the virulence of pathogens associated with BBD (Edmunds 1991, Bruckner and Bruckner 1997, reviewed in Rosenberg and Ben-Haim 2002), white band (Porter *et al.* 2001), white plague (Porter *et al.* 2001), yellow band (Bruno *et al.* 2003), and aspergillosis (Bruno *et al.* 2003). Consequently, the progression and transmission of disease can be highly dependent on the response of the pathogen to altered environmental parameters. If environmental conditions enhance the virulence of pathogens, we could expect the disease to progress along and between coral hosts at a faster rate. An example of this was observed at Horseshoe Reef where the rate of progression of brown band syndrome was significantly faster than at Davies Reef (Chapter 2). In this case, several environmental conditions may have enhanced the virulence of the BrB pathogen at Horseshoe Reef including water circulation, light intensity, and temperature. BrB progressed fastest at the calmer Horseshoe Reef as opposed to the more exposed Davies Reef. Under aquarium conditions, high water circulation was capable of dislodging the ciliates associated with BrB, therefore the slower rates of progression of BrB at the exposed Davies Reef may involve the inability of the ciliates to move between coral branches and colonies without being

flushed away by high water motion. Temperatures at these two reefs were within 0.5˚ of each other, but light intensities were 1.3 times higher at Davies Reef. Light has often been identified to increase the virulence of coral diseases (Kuta and Richardson 2002), yet in this case higher rates of progression of BrB were measured at Horseshoe Reef where light intensities were lower. In summary, a combination of factors have the ability to influence the progression and transmission of coral disease whether it is by increasing the virulence of the pathogen or influencing the behavior of the microorganisms involved.

6.3. CONCLUSIONS

In conclusion, changes in environmental conditions, such as elevated temperature and increased light levels, may stress coral communities, subsequently decreasing coral health and immunity to disease infections. Once infected, further stress caused by prolonged exposure to elevated temperatures and the pathogenic microorganisms associated with the disease may increase the vulnerability of the coral to the advancement of the disease between colonies and/or progression of the disease within colonies. The greater natural rates of progression and transmission measured during the warmer month of January, the increased rates of progression observed in experimental studies exposing BBD-infected coral branches to experimentally elevated seawater temperatures and high light intensities, and the unique microbial communities and mechanisms causing mortality between BBD and BrB lead to my conclusion that the variations in the progression and transmission of coral disease are a combined response of both the coral and the pathogen(s). Environmental conditions enhancing the prevalence, progression, and transmission of coral disease are likely decreasing coral health and immunity while simultaneously increasing the growth rate and virulence of coral pathogens. In order to fully understand the mechanisms and parameters involved in the progression and transmission of coral diseases, future research must aim to include an ecological and microbiological approach to the study of coral diseases.

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