The effects of disease and stress on the microbial community of the sponge *Ianthella basta*

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Abstract

Sponges form a highly diverse and ecologically significant component of benthic communities. Despite their ecological importance, threats to populations and communities, including disease dynamics in sponges, remain relatively unexplored. There have been severe disease epidemics in sponges from the Caribbean and the Mediterranean; however, evidence of extensive sponge mortalities have not been reported in the Great Barrier Reef (GBR) or Torres Strait, northeastern Australia. This thesis describes a disease-like syndrome affecting the sponge *Ianthella basta*, a sponge that is common to both regions. The prevalence of disease as well as a comprehensive analysis of the microbial communities associated with affected and healthy sponges was determined. Manipulation of environmental parameters, including temperature and sedimentation were undertaken to establish potential causes of the disease-like syndrome. In addition, the microbial community associated with tissue regression of *I. basta* is also reported.

Disease prevalence surveys were conducted in the Palm Islands on the central GBR and Masig Island, Torres Strait. Symptoms of the disease-like syndrome affecting *I. basta* included discolored, necrotic spots leading to tissue degradation, exposure of the skeletal fibers and disruption of the choanocyte chambers. Sponges were assigned to pre-determined disease categories using tissue necrosis and the presence of brown spot lesions as a proxy of health. Sponges with brown spot lesions were present at all sites with 43% and 66% of *I. basta* exhibiting disease-like symptoms in the Palm Islands and Torres Strait, respectively. Sponges from Torres Strait also showed a greater incidence of significant and extensive necrosis in comparison to sponges from Palm Island (11.5 vs. 6%).

A comprehensive comparison of bacteria, viruses, fungi and other eukaryotes was performed in healthy and diseased *I. basta* using multiple techniques to ascertain the role of microbes in the disease process. A low diversity of microbes was observed in both healthy and diseased sponge communities, with all sponges dominated by an *Alphaproteobacteria*, a *Gammaproteobacteria* and a Thaumarchaea. Bacterial cultivation, community analysis by Denaturing Gradient Gel Electrophoresis (DGGE) (Bacteria and Eukarya), sequencing of 16S rRNA clone libraries (Bacteria and Archaea) and direct visual assessment by electron microscopy failed to reveal any putative pathogens. In addition, infection assays could not establish the syndrome in healthy sponges even after direct physical contact with affected tissue. These results suggest that microbes are not responsible for the formation of brown spot lesions and necrosis in *I. basta*.

Elevated temperatures and other anthropogenic factors are increasingly being linked with disease in marine organisms. Manipulative experiments were undertaken to ascertain the potential role of environmental stressors (temperature and sedimentation) in the formation of brown spot lesions and necrosis in *I. basta*. In addition, the effects of elevated temperature, sedimentation and antibiotic exposure on the microbial community of *I. basta* were quantified. Neither elevated temperatures nor increased sedimentation induced the formation of brown spot lesions, indicating they are not responsible for the syndrome. However, sponges exposed to 32˚C developed substantial discoloration and deterioration of their tissues, resulting in death after eight days. The decline in sponge heath was accompanied by a shift in the microbial community, with higher diversity observed within the *Alpha-* and *Gammaproteobacteria*. Though this shift occurred, the microbial community was stable in sponges exposed to this temperature (32˚C) for the first four days of the experiment. No shifts in the microbial community of *I. basta* were observed with increased sedimentation

and antibiotic exposure, with all dominant symbionts present (*Alphaproteobacteria*, *Gammaproteobacteria* and Thaumarchaea). Overall, these results indicate *I. basta* has a stable microbial community.

The physiological stress response of tissue regression observed in heat-stressed *I. basta* was further explored. Following collection and transportation, tissues of *I. basta* often regress leaving visible subdermal gaps between primary fibers. Changes in the bacterial communities associated with each tissue state were also assessed using DGGE. Six necrotic specimens of *I. basta* and 12 healthy sponge explants were collected at Orpheus Is., northeastern Australia and transported to aquarium facilities at the Australian Institute of Marine Science. After 12 h both the healthy explants and necrotic sponges displayed substantial tissue contraction with visible gaps evident between sponge skeletal fibers. However, within 72 h all sponges recovered to their original states. To quantify the level of tissue regression and subsequent recovery, a photo of each sponge was taken and images were compared using an integrated density measurement of Image Tool for Windows (UTHSCA). Histological samples were taken from sponge explants to compare cellular organization during this regression and recovery process. The integrated density of the sponge tissue effectively doubled within 72 h (increasing by 92%), confirming tissue recovery in *I. basta*. Histological analysis of the explants revealed that sponges affected by tissue regression had significantly fewer choanocyte chambers [(0.4 ± 0.1 vs. 4.5 ± 0.7) (mean ± S.E)] in regressed and recovered tissues respectively, and more densely packed granulated cells than recovered sponges. Principal Component Analysis of the DGGE data showed consistent patterns of microbial symbionts in both regressed and recovered sponges.

This thesis represents a systematic approach to investigating disease in sponges, which can be used as a template for future research. Results obtained in this thesis contribute

valuable baseline data on a disease-like syndrome affecting a common marine sponge and provide evidence that microorganisms are unlikely the causative agent. In addition, disease-like symptoms were never induced in response to temperature or sedimentation stress, indicating an environmental origin of the syndrome is also unlikely.

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Chapter 1: General Introduction

Sponge Biology & Ecology

Sponges are the oldest extant Metazoans with fossils dating back to the Precambrian, approximately 600 million years ago (Li et al. 1998). They form a diverse and significant component of benthic communities, with an estimated 19,179 extant species described worldwide (Van Soest et al. 2008) occupying both freshwater and marine habitats from the poles to the tropics. Sponges are integral components of marine ecosystems, playing important roles in bioerosion and consolidation, calcification and benthic-pelagic coupling processes associated with their immense filtering capabilities (Diaz & Ruetzler 2001; Bell 2008). Indeed, their filtering capacity has also been implicated in the bioconversion of picoplankton into substantial amounts of allochthonous nitrogen and phosphorous, which contributes to the success of coral reef communities in nutrient poor waters (Richter et al. 2001). Through their complex symbioses, sponges are a key component of microbial processes such as primary production (Wilkinson 1983), nitrification (Bayer et al. 2008; Mohamed et al. 2008a; Southwell et al. 2008a; Southwell et al. 2008b; Steger et al. 2008; Off et al. 2010; Schläppy et al. 2010), anammox (Hoffmann et al. 2009; Mohamed et al. 2010), methane oxidation (Vacelet et al. 1996) and sulfate reduction (Hoffmann et al. 2005).

Sponges possess a body plan that is characterized by a lack of true tissues and organs. This requires them to rely on a very diverse assortment of highly specialized cells, which drive the important physiological processes of water circulation, feeding and reproduction. The outer cell layer of the sponge (pinacoderm) is made up of squamous pinaocyte cells, whilst the inner surface (choanoderm) is made up of flagellated choanocyte cells. The layer in between these, the mesohyl, supports a skeletal matrix of collagen fibers and spicules for most groups of sponges. The mesohyl is the functioning layer of the sponge where nutrient

transfer, metabolism, reproduction and cell communication occurs. In addition, the specialized amoeboid cells (archaeocytes), which are capable of differentiating into any other cell type (Müller 2006), are located in the mesohyl as well as the vast majority of symbiotic microorganisms (Wilkinson 1978a).

Sponge Microbiology

Sponge-microbe associations have existed for 600 million years (Wilkinson 1984), making them one of the most ancient of all metazoan-microorganism symbioses. Sponges commonly host a vast array of microorganisms, and in some species microbes can comprise up to 40-60% of the total sponge biomass (Vacelet & Donadey 1977). However, not all sponges harbor high abundances of microorganisms. These sponges, often referred to as low microbial abundance (LMA) sponges, occur in similar environments to their high microbial abundance (HMA) sponge counterparts (Hentschel et al. 2006) and it is unclear whether LMA sponges once contained higher numbers of microbes and subsequently lost them, or if the microorganisms in LMA sponges are phylogenetically similar those in HMA sponges (Taylor et al. 2007). Sponge-microbe associations have been described for sponges from tropical, temperate and Antarctic regions, revealing a diverse range of heterotrophic bacteria, cyanobacteria, archaea and fungi (Preston et al. 1996; Webster & Hill 2001; Webster et al. 2001c; Hentschel et al. 2002; Margot et al. 2002; Taylor et al. 2004; Webster et al. 2004; Webster et al. 2010; Lee et al. 2011).

Advances in sequencing technology over the last decade have greatly expanded our knowledge of microbial diversity in sponges. To date, 25 bacterial phyla and both major lineages of Archaea (*Crenarchaeota* and *Euryarchaeota*) have been reported from sponges (Taylor et al. 2007; Webster et al. 2010). Of those, the most frequently recovered 16S rRNA sequences from sponge-microbe studies belong to the *Proteobacteria, Acidobacteria,*

Actinobacteria, Bacteroidetes and *Chloroflexi* (Hentschel et al. 2006). Importantly, microbial communities in sponges are distinctly different from those in the surrounding seawater and sediment (Webster et al. 2001a; Hentschel et al. 2002; Taylor et al. 2004; Taylor et al. 2005) and sponges in different oceans often host the same microorganisms (Hentschel et al. 2002).

Sponge-derived 16S rRNA sequences from 16 different bacterial phyla and the *Crenarchaeota* and *Euryarchaeota* often form monophyletic, sponge-specific sequence clusters (Taylor et al. 2007). Of these, the *Cyanobacteria, Chloroflexi* and *Actinobacteria (Acidimicrobiaceae)* contained the largest numbers of sponge-specific clusters. For instance, 75% of the *Cyanobacteria* and 62% of the *Chloroflexi* 16S rRNA sequences analyzed fell into sponge-specific clusters. Six different sponge-specific clusters were also identified within the *Actinobacteria*, with host sponges from the Caribbean, Indonesia, the Red Sea and the South China Sea (Taylor et al. 2007). There is also a novel, sponge-specific candidate phylum reported from several sponges, *Poribacteria* (Fieseler et al. 2004).

The relationship between sponges and microorganisms are complex with examples of parasitism, mutualism and commensalism. The host sponge may benefit from the presence of the symbiotic microorganisms through increased structural rigidity (Wilkinson 1978b), digestion and recycling of insoluble collagen (Wilkinson 1979), direct incorporation of organic matter from seawater (Wilkinson 1980), and the microbial production of secondary metabolites that are utilized by the sponge (Unson et al. 1994; Blewley & Faulkner 1998). The benefits to the microbes in the association remain less clear; however, refuge and nutrient provision are proposed advantages (Sarà 1971). Apart from phototrophic sponges, which gain their carbon nutrition from their cyanobacterial symbionts, the majority of sponges utilize microorganisms filtered from the water column as a food source. In fact, bacteria and eukaryotic microalgae can satisfy the total food requirement for sponges

(Reiswig 1975; Topcu et al. 2010). The pumping ability of sponges is central to this style of feeding. Sponges can filter immense quantities of seawater (Reiswig 1974; Vogel 1977), facilitating the acquisition of up to 96% of the bacterial cells present in the water column (Reiswig 1971b; Yahel et al. 2007). There are significant knowledge gaps regarding sponge feeding ecology, with ambiguity still surrounding the ability to actively select food. While some sponges show no differential retention efficiency for picoplankton groups (Pile et al. 1996; Duckworth et al. 2006; Pile & Young 2006; Topcu et al. 2010), others exhibit specific selectivity of cell types, particularly in oligotrophic environments. For example, *Callyspongia* sp. from Western Australia actively retains 87% of *Synechococcus* cells over heterotrophic bacteria, (Hanson et al. 2009) while selective feeding on three pathogenic bacterial species occurs for *Hymeniacidon perlevis* (Maldonado et al. 2010). Similarly, the common Great Barrier Reef (GBR) sponge, *R. odorabile*, selectively retained picoeukaryotes with high efficiencies (92%), irrespective of the season (Bannister 2008).

Sponge Disease

While sponge-microbe associations can benefit the host in many ways, microorganisms as parasites and pathogens have direct negative effects on the host. Parasitic diatoms occur in several different Antarctic sponge species (Bavestrello et al. 2000; Cerrano et al. 2000) with tissue degradation in areas of dense diatom aggregations on the hexactinellid sponge, *Scolymastra joubini* (Cerrano et al. 2000). While bacteria or fungi are suspected to be responsible for the majority of past sponge disease outbreaks (Lauckner 1980; Webster 2007), there is little empirical evidence that unequivocally support this and, in most cases the microbe(s) responsible remains unidentified. The sole example where a disease is directly related to the presence of a microbe is for the GBR sponge, *Rhopaloeides odorabile*, where

the *Alphaproteobacteria* strain (NW4327) degrades the collagenous spongin fibers resulting in the death of the sponge (Webster et al. 2002).

Disease outbreaks, regardless of etiology have far reaching impacts on sponge populations, particularly for slow growing sponges (reviewed in Webster 2007). An epidemic in the Caribbean in 1938 affected 70-95% of the total sponge population (Galstoff 1942). The etiological agent responsible for this outbreak was never identified; however, fungi were always present in the diseased tissues (Glastoff 1942). In a similar large scale impact, the once very lucrative Mediterranean sponge fishery has been impacted by disease. The industry produced approximately 100 tons sponge biomass per annum (Josupeit 1990) until the 1980's when diseases in commercial sponge stocks contributed to the collapse of the sponge fishery, with diseased sponges exhibiting white spots and brittle skeletons following infection from the suspected bacterial pathogen (*Oscillatoria* sp.) (Gaino et al. 1992; Vacelet 1994).

More recently, there have been reported disease outbreaks affecting the giant barrel sponge, *Xestospongia muta*, in Belize and the Caribbean (Paz 1997; Nagelkerken et al. 2000). In both locations, sponges display bleaching (cyclic and fatal) and sponge orange band (SOB), with the color change being attributed to a photosynthetic cyanobacteria (Cowart et al. 2006; López-Legentil et al. 2010). However, the question still remains as to whether or not this cyanobacterium is the etiological agent of disease. Disease-like syndromes have also been recently reported in a variety of *Aplysina* spp. from multiple locations including the Caribbean (Gochfeld et al. 2007), Panama (Wulff 2007) and Slovenia (Webster et al. 2008b). Diseased sponges from the Caribbean display rust colored leading edges (bands) and, at times, necrotic tissue, termed *Aplysina* red band syndrome (ARBS) (Olson et al. 2006). Similar to SOB syndrome, the band coloration has been attributed to a filamentous

cyanobacterium, yet as with other studies the etiological agent is unresolved (Olson et al. 2006). *Aplysina* sponges from Slovenia showed different symptoms including black patches with white necrotic tissue, and/or exposed skeletal fibers (Webster et al. 2008b), with a clear shift in microbial populations between diseased and healthy sponges. Notably, a single *Desulfovibrio* strain was detected in all diseased sponges and was absent from healthy controls (Webster et al. 2008b).

The majority of sponge disease reports have been restricted to the Caribbean and Mediterranean; however, there was a report of disease in *Ianthella basta* from Papua New Guinea (PNG). Over the course of four years (1996-2000), there was a 90% increase in disease lesions in this species with the causative agents proposed to be *Bacillus* and *Pseudomonas* strains, though this was not confirmed (Cervino et al. 2006). To date, there are no known records of widespread sponge mortalities associated with disease on the Great Barrier Reef although over the last decade; anecdotal reports of sponge disease have grown substantially (Webster 2007). Despite the increased observations of sponge disease, there is a notable lack of baseline data establishing the extent of disease, making it difficult to ascertain whether the increased reports of disease are simply an artifact of increased awareness. Of key importance is that the etiological agents and/or environmental factors responsible for the disease-like syndromes remain unknown. This is not surprising given the difficulty involved in unequivocally assigning etiological agents by traditional medical microbiology methods (e.g. confirming Koch's postulates).

Sponges and Environmental Stress

Increasingly, researchers are identifying the link between disease in marine taxa and climate change or other anthropogenic factors (Harvell et al. 1999; Harvell et al. 2002). The Intergovernmental Panel on Climate Change (IPCC) has predicted that surface seawater

temperatures will increase between 1-5 ˚C by 2100 (IPCC 2001). In addition, coastal marine systems are increasingly being exposed to rising levels of nutrients, sediments and pollutants from terrestrial runoff (Fabricius 2005). For instance, on the Great Barrier Reef terrigenous sediment and turbidity rates have increased five to ten fold since the beginning of European settlement (McCulloch et al. 2003). It is plausible these predicted rises in seawater temperature, and increased terrestrial runoff, will enhance the severity and frequency of disease outbreaks in marine organisms.

Several studies show that elevated seawater temperatures has negative effects on marine organisms by increasing pathogen virulence, transmission and host susceptibility (Toren et al. 1998; Banin et al. 2001a; Banin et al. 2001b; Ben-Haim & Rosenberg 2002; Kuta & Richardson 2002; Banin et al. 2003; Jones et al. 2004). Sponges can be negatively affected by elevated seawater temperatures. Mass mortalities in sponge communities have occurred in the north-western (Perez et al. 2000; Cerrano et al. 2001; Laubier et al. 2003) and east Mediterranean (Warn 2000), as well as the West Indies (Vicente 1989) due to elevated temperatures. The significance of elevated seawater temperatures on sponge-microbe associations has not been fully explored with two studies establishing the effect of thermal stress on symbionts in sponges. Denaturing Gradient Gel Eelectorphoresis (DGGE) analysis revealed a loss of symbionts in *Halichondria bowerbanki* exposed to thermally stressful temperatures (Lemoine et al. 2007). Similarly, a microbial community shift and complete loss of a dominant culturable symbiont occurs in the GBR sponge *R. odorabile* following exposure to elevated temperature treatments (Webster et al. 2008).

While relatively little is known about the impacts of elevated temperatures on sponge-microbe associations, there are direct impacts of temperature on the sponge host, particularly at the molecular level. For example, heat shock proteins, particularly Hsp70, are induced by

elevated temperatures in a number of sponge species (Muller et al. 1995; Koziol et al. 1996; Krasko et al. 1997; Wiens et al. 1998; López-Legentil et al. 2008). In a qPCR analysis of heat stressed *R. odorabile*, a rapid down-regulation of most genes (actin related protein, ferritin, calmodulin and Hsp90) occurred at 31 and 32°C within 24 h. This indicates that sponges are capable of a rapid molecular shut-down in response to thermal stress. An increased expression of Hsp40 and Hsp90 in sponges at 32°C demonstrates an activation of the heat shock response system, and is consistent with their role as chaperones for directing degraded proteins to proteolysis (Pantile & Webster 2011). In addition, sponges respond to environmental stressors by reducing their tissues, forming reduction bodies and gemmules (Francis 1984). Tissue reduction or gemmule formation in sponges occurs in response to colder temperatures (Fell 1974), polluted waters (Imsiecke et al. 1996), bacterial infection (Böhm et al. 2001) and handling stress (Luter et al. 2011). At a cellular level, tissue reduction causes a reduction in the number of choanocyte chambers (Thoms et al. 2008; Luter et al. 2011), which may compromise the functional ability of the sponge.

Sedimentation caused by runoff and dredging is one of the largest factors contributing to coral reef degradation worldwide (Rogers 1990; Fabricius 2005). Increased sediment loads lead to the reduction of light available for photosynthesis and smother benthic organisms. Of particular importance to filter-feeding organisms, such as sponges, is the potential for increased suspended sediments to clog inhalant canals, leading to decreased feeding and respiration abilities (Gerrodette &Flechsig 1979). The effect of sedimentation on sponge physiology is not well documented, but there are very clear impacts on pumping rates (Reiswig 1971a; Gerrodette & Flechsig 1979; Bannister 2008). Beyond establishing decreased pumping rates, there is a single study that has investigated the effect of sediment exposure on metabolism. Respiration rates of the GBR sponge *R. odorabile* were increased

by 35% when exposed to fine clay sediments over a course of 4 days (Bannister 2008). In addition to the limited understanding of how increased sedimentation may impact sponge physiology, there is also a paucity of knowledge on the impacts of sedimentation on sponge-microbe associations or sub-lethal responses of the sponge host. It is likely that terrigenous sediments carry foreign microbes that sponges encounter during routine filter feeding, thereby altering their inherent microbial consortia. In addition, terrigenous inputs act as a reservoir for pathogens (Harvell et al. 2007).

Thesis outline

The principal theme of this thesis is to quantify the causes and impacts of sponge disease in Torres Strait and the Great Barrier Reef. There is a notable lack of baseline data on the prevalence of disease in sponge species worldwide. In addition, even less is known about etiological agents that are involved in disease processes. To address these topics, *Ianthella basta* (Verongida: Ianthellidae) (Pallas, 1776) (Fig. 1.1) is chosen as a model study species.

Fig. 1.1 The sponge *Ianthella basta* (Pallas, 1776)

This sponge species was chosen as it is a widely distributed sponge throughout the Indo-West Pacific (Bergquist & Kelly-Borges 1995; Hooper & Van Soest 2002; Fromont 2004) and there was a previous report of disease in this species from Papa New Guinea (Cervino et al. 2006). *I. basta* has a fan-like morphology reaching heights of approximately 1 m and is found in a variety of color morphs including yellow, brown, green, blue, and purple. This group of sponges is also characterized by their lack of spicules, with the skeleton comprising the bulk of the sponge (Bergquist & Kelly-Borges 1995; Hooper & Van Soest 2002).

I. basta therefore provides the opportunity to further investigate disease in sponges beginning with establishment of baseline prevalence data followed by identification of plausible etiological agents. Therefore:

Chapter 1 provides background for the thesis with a general introduction into sponge biology, microbiology and disease.

Chapter 2 explores the prevalence of a disease-like syndrome in *I. basta* populations from Masig Island, Torres Strait and the Palm Islands, GBR. Tissue necrosis and the presence of brown spot lesions were used as a proxy to assess sponge health. In addition, transmission electron microscopy (TEM) was used to evaluate sponge health on a cellular level, revealing high levels of cell degradation and disruption of choanocyte chambers in sponges displaying disease-like symptoms.

Chapter 3 expands on the prevalence of the disease-like syndrome affecting *I. basta* populations by attempting to identify putative pathogens and determining how disease affects the symbiotic microbial population of the sponge. The role microorganisms may play in the formation of brown spot lesions and tissue necrosis was explored using multiple techniques including Denaturing Gradient Gel Electrophoresis (DGGE), 16S rRNA clone libraries,

culture-based methods and TEM. In addition, the potential role of eukaryotic organisms and viruses were also examined. No shifts in the microbial community between healthy and diseased sponges could be detected, nor was a single microbe found to be responsible for the disease-like syndrome.

Chapter 4 explores an environmental origin of the disease-like syndrome described in previous chapters (2 & 3). Manipulative laboratory experiments are used to assess the effects of the environmental stressors of elevated temperature, sedimentation, and antibiotic exposure on the microbial community of *I. basta*. In addition, this chapter explores the stability in the microbial community associated with *I. basta* across a latitudinal gradient.

Chapter 5 explores the physiological stress response, tissue regression, displayed in *I. basta* as a result of collection and transportation. The microbial community composition of sponges displaying tissue regression was also examined.

In Chapter 6, the results of the previous chapters are discussed and main conclusions are outlined.

Chapter 2: Prevalence of Tissue Necrosis and Brown Spot Lesions in *Ianthella basta* Populations[1]

2.1 Introduction

Marine sponges are extremely diverse with an estimated 19,179 described species found worldwide (Van Soest et al. 2008). They can comprise a significant component of benthic communities aiding in functional roles such as bioerosion, consolidation and benthic–pelagic coupling processes (Diaz & Ruetzler 2001; Bell 2008). Despite their ubiquitous nature and ecological importance, information on health and disease in this group are only just becoming available (reviewed in Webster, 2007). Importantly, developing our understanding of disease in marine sponge species will contribute to the conservation and management of populations.

Macroscopic features such as tissue color, necrosis and degradation have served as a basis of health in sessile invertebrates, such as corals and is predominantly centered around disease diagnostics (Ainsworth et al., 2007 and references therein). One of the key signs of reduced health in sessile organisms is evidence of necrosis and tissue degradation and this can arise from multiple stimuli including: physical damage due to abiotic factors (Wulff 2006b), predation (Knowlton & Highsmith 2005), chemical defense (Lopez-Victoria et al. 2006) and disease (Richardson 1998; Webster et al. 2002; Weil et al. 2006; Webster et al. 2008b). Enhancing our understanding of disease dynamics in a wide range of marine taxa is a current research focus, particularly with the apparent links between disease prevalence and climate change in both marine and terrestrial systems (Harvell et al. 2002).

[1] Chapter 2 adapted from Luter HM, Whalan S, Webster NS (2010) Prevalence of tissue necrosis and brown spot lesions in a common marine sponge. Marine and Freshwater Research 61: 484-489.

Causative agents of disease in marine organisms include bacteria, fungi, viruses, protozoans and a variety of metazoan parasites (Kinne 1980). In sponges, bacteria and fungi are the most commonly reported pathogens (reviewed in Webster, 2007); although in most studies the etiological agents of disease have not been empirically determined. Consequently, very little is known about disease processes such as infection, transmission and virulence mechanisms associated with disease in sponges, but there is clear evidence of the impacts of disease on sponge populations (Cervino et al. 2006; Wulff 2007).

One of the earliest records of sponge disease was an epidemic in 1938, which caused widespread sponge mortality throughout the Caribbean (Galstoff 1942). Massive sponge mortalities have also occurred in the Mediterranean, drastically reducing outputs of economically important sponge fisheries (Gaino et al. 1992; Vacelet 1994). More recently, there have been reported disease outbreaks affecting the giant barrel sponge, *Xestospongia muta*, in Belize and the Caribbean (Nagelkerken et al. 2000), *Ianthella basta* in Papua New Guinea (PNG) (Cervino et al. 2006) and a variety of *Aplysina* spp. from the Caribbean, Panama and Slovenia (Olson et al. 2006; Gochfeld et al. 2007; Wulff 2007; Webster et al. 2008b). To date, there are no known records of widespread sponge mortalities on the Great Barrier Reef (GBR); although, over the last decade anecdotal reports of sponge disease have grown substantially (Webster 2007). Despite the increases in observations of sponge disease, there is a notable lack of baseline data establishing the extent of disease, making it difficult to ascertain whether the increased reports of disease are simply an artifact of increased awareness.

This study aimed to test the hypothesis that tissue necrosis and brown spot lesions affected a large proportion of the *I. basta* population over a wide geographic gradient. *I. basta* was selected as the model species given its widespread distribution throughout the Indo

Pacific region (Hooper & Weidenmayer 1994; Hooper & Van Soest 2002; Fromont 2004), a previous report of disease affecting this sponge in PNG (Cervino et al. 2006) and preliminary observations of brown spot lesions affecting this sponge on the GBR.

2.2 Materials and Methods

2.21 Study Species and Field Sites

I. basta (Pallas, 1776) is a Verongid sponge from the family Ianthellidae. Similar to other Verongids, *I. basta* lack spicules and are known for their oxidizing pigments. *I. basta* is a fan-like sponge reaching heights of approximately 1 m and is found in a variety of color morphs (yellow, brown, green, blue, or purple), though purple and yellow are the dominant morphs surveyed in this study.

Baseline surveys were conducted in the Palm Islands, GBR in September 2008 and Masig Island, central Torres Strait in June 2008. Twelve sites around the Palm Islands and 7 sites around Masig Island were surveyed for *I. basta* (Table 1). Sites within each location were separated by a minimum of 200 m. At each site, three 20 x 4-m transects were surveyed, each separated by at least 20 m to retain statistical independence. All transects were laid on sloping reef between 12-15 m, a depth range where *I. basta* is commonly observed (Cervino et al. 2006). To establish the extent of disease in *I. basta* populations, sponges were assigned to one of five categories based on the pathology of disease, in addition to the percentage of tissue affected by disease. Class 0 (healthy), no evidence of tissue necrosis; class 1 (minor), <10% tissue necrosis; class 2 (moderate), between 10 and 25% tissue necrosis; class 3 (significant), between 25 and 75% tissue necrosis; and class 4 (extensive), >75% tissue necrosis.

A one-way analysis of variance (ANOVA) was used to examine differences in *I. basta* abundance between sites at each location, separately. Data was log (x+1) transformed prior to the analyses to meet the assumptions of ANOVA (Zar 1999). Differences in the prevalence of sponges in each tissue necrosis category between sites at each location were analysed using the Kruskal-Wallis test. All statistical analyses were performed using Statistica 8 (StatSoft 2002).

Table 2.1 GPS coordinates of study sites in the Palm Islands and Masig Island, Torres Strait.

Palm Island Sites			Torres Strait Sites		
	S	E		S	E
1	18° 33.617'	146° 29.077'	1	9° 44.980'	143° 23.955'
2	18° 36.878'	146° 29.990'	2	9° 44.648'	143° 23.955'
3	18° 37.045'	146° 29.081'	3	9° 44.764'	143° 24.370'
4	18° 37.299'	146° 29.274'	4	9° 43.972'	143° 26.802'
5	18° 37.629'	146° 29.553'	5	9° 44.663'	143° 24.970'
6	18° 40.615'	146° 30.577'	6	9° 44.744'	143° 24.439'
7	18° 39.576'	146° 31.071'	7	9° 44.260'	143° 25.275'
8	18° 41.879'	146° 30.408'			
9	18° 42.291'	146° 30.591'			
10	18° 39.997'	146° 31.529'			
11	18° 40.129'	146° 31.583'			
12	18° 42.754'	146° 31.393'			

2.22 Transmission Electron Microscopy (TEM)

To qualitatively assess sponge health at the cellular level, small *I. basta* tissue samples (~1 mm diameter) from healthy sponges and sponges displaying visible signs of necrosis and brown spot lesions were collected for observation using transmission electron microscopy (TEM). Samples were fixed for 24 h in 2.5% gluteraldehyde in fresh filtered sea water and subsequently placed in filtered artificial seawater and stored at 4˚C until further processing. Fixed tissue was placed in a 1% osmium tetroxide solution (prepared in 0.1 M cacodylate buffer) for 3.5 h and subsequently dehydrated in a graded acetone series (50, 60, 70, 80, 90 and 100%). Tissue was then embedded in Epon resin, sectioned with an ultramicrotome and

stained with 5% uranyl acetate in 50% methanol, followed by Reynolds lead citrate. Ultrathin sections of 60 nm were mounted on Formvar coated copper grids and viewed using a JEOL 1010 operated at 80kV. Images were captured using a SoftImaging Megaview III digital camera.

2.3 Results

In total, 157 and 156 *I. basta* were surveyed at sites around the Palm Islands and Torres Strait respectively. At the Palm Islands the average abundance of *I. basta* ranged from 0.667 to 10.667 sponges per 80 m^2 and abundance varied significantly between sites ($F_{11, 24} = 11.056$; $P < 0.001$) (Fig. 2.1A). In Torres Strait, average abundances ranged between 0.667 to 19.667 *I. basta* per 80 m^2 and this also varied significantly between the 7 sites ($F_{7, 16} = 16.691$; $P < 0.001$) (Fig. 2.1B).

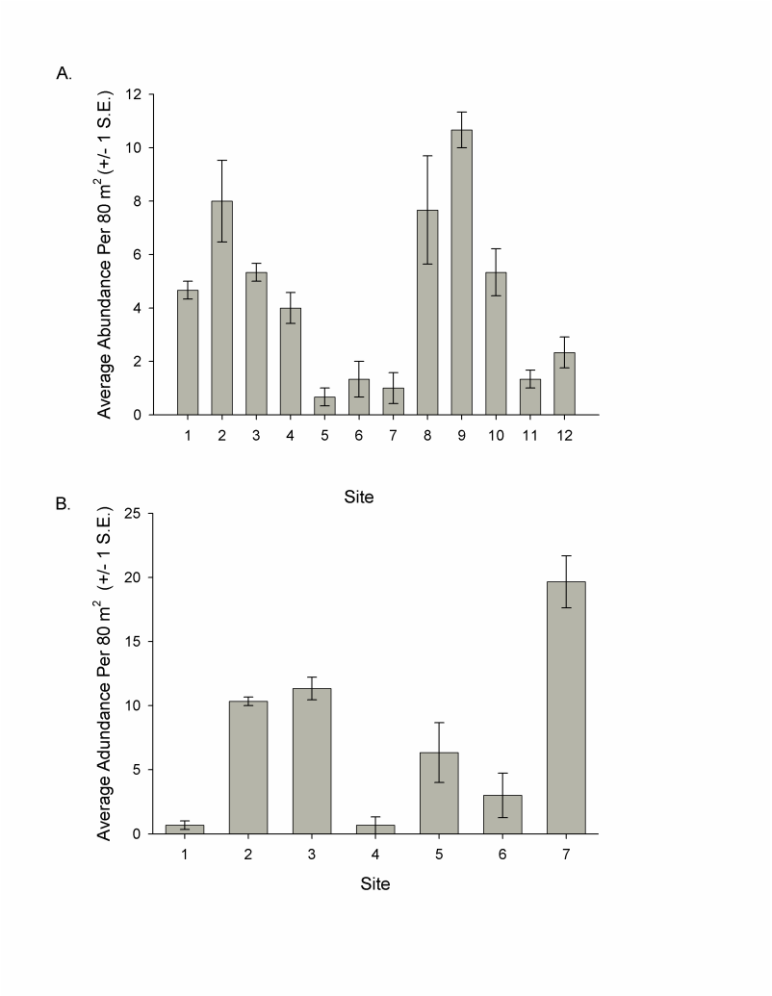

Fig. 2.1 Mean abundance of *I. basta* among sites in (A) the Palm Islands and (B) Masig Island, Torres Strait.

I. basta displaying signs of disease from the Torres Strait and the Palm Islands included discolored, necrotic spots in which sponge skeletal fibers were just becoming apparent (Fig. 2.2B). In addition, some *I. basta* also exhibited high levels of tissue degradation with completely exposed skeletal fibers (Figure 2.2C) and, in some cases, increased fouling (Fig. 2.2D). Electron micrographs from healthy sponges revealed complete choanocyte chambers comprised of healthy cells and little cellular degradation (Fig. 2.2E). In contrast, choanocyte chambers in sponges with necrotic brown spots and exposed fibers were often incomplete with high levels of cellular degradation and debris within the remnants of the chambers (Fig. 2.2F).

Overall, a relatively large percentage of sponges showed visible signs of necrosis. For example, almost half (43%) of the *I. basta* from the Palm Islands and 66% of the *I. basta* from the Torres Strait exhibited necrosis (Fig. 2.3A). With the exception of Fantome site 1 from the Palm Islands (in which only three sponges were surveyed), all sites contained sponges which exhibited visible signs of necrosis. Sponges falling within the minor (<10% tissue necrosis) and moderate (10-25% tissue necrosis) categories dominated at both locations with the prevalence of sponges in these categories ranging from 25-54% in the Palm Islands and 33-63% in Torres Strait. Sponges showing significant (25-75% tissue necrosis) and extensive (>75% tissue necrosis) levels of necrosis were less common, with a higher incidence (5-44%) noted in Torres Strait (Fig. 2.2B). Only two extensively necrosed sponges were recorded in the Palm Islands and 5 in Torres Strait. In general, sites from both locations with higher *I. basta* abundances contained sponges belonging to the full suite of tissue necrosis categories. There was no significant difference between the prevalence of sponges in each of the tissue necrosis categories between sites in the Palm Islands (K-W test, $H = 18.041$; d.f. = 11; $P = 0.081$) or between sites in Torres Strait (K-W test, $H = 12.130$; d.f. = 6; $P = 0.059$).

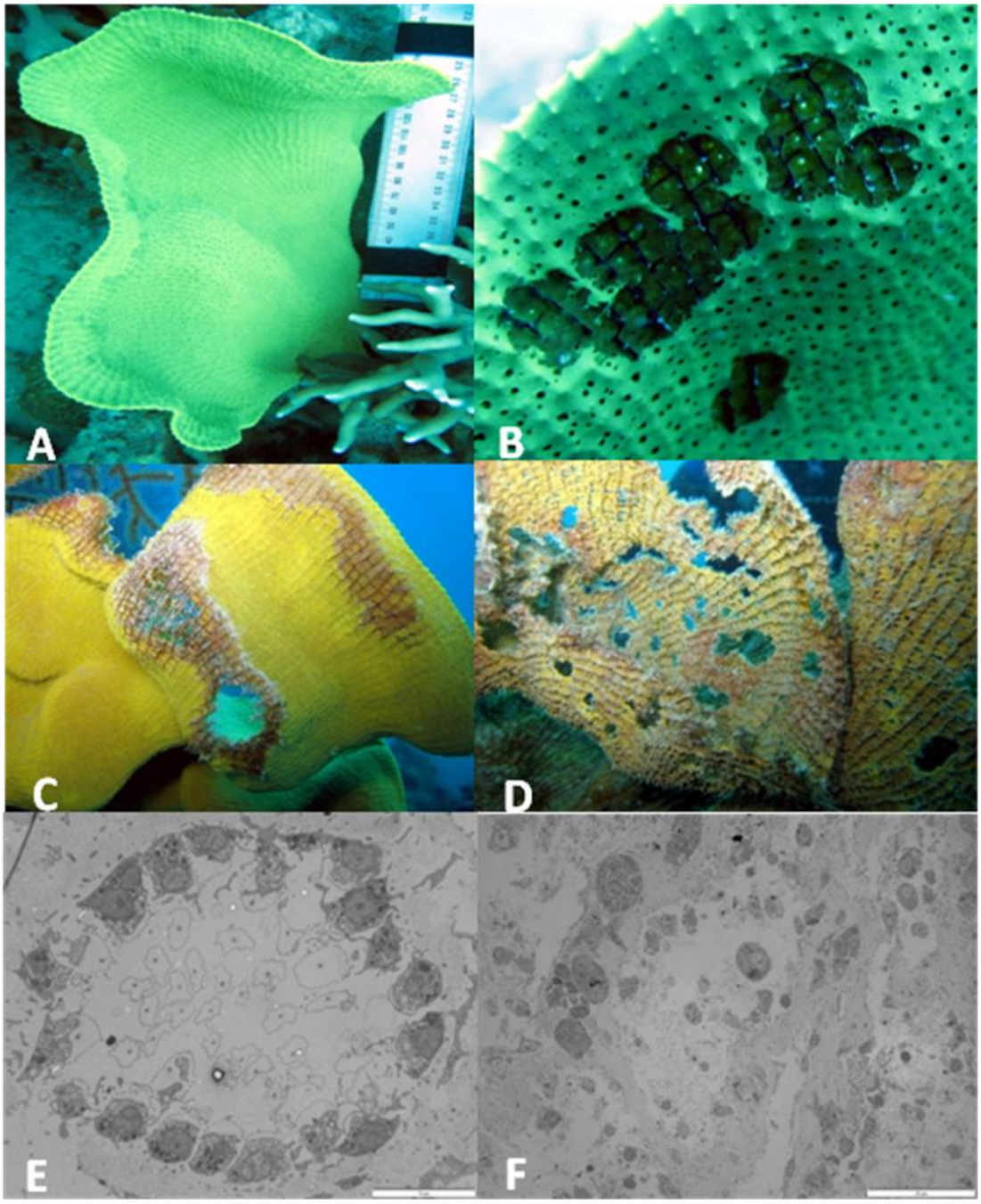

Fig. 2.2 *In situ* photographs of *Ianthella basta*, depicting varying levels of necrotic tissue. (A) Healthy sponge. (B) Brown spot lesions, with fibers just becoming apparent. (C) Increased necrosis with exposed fibers and tissue degradation. (D) Extensive necrosis. (E) Choanocyte chamber of a healthy sponge (scale bar=10 µm). (F). Remnants of a choanocyte chamber of a sponge with brown spot lesions (scale bar=20 µm).

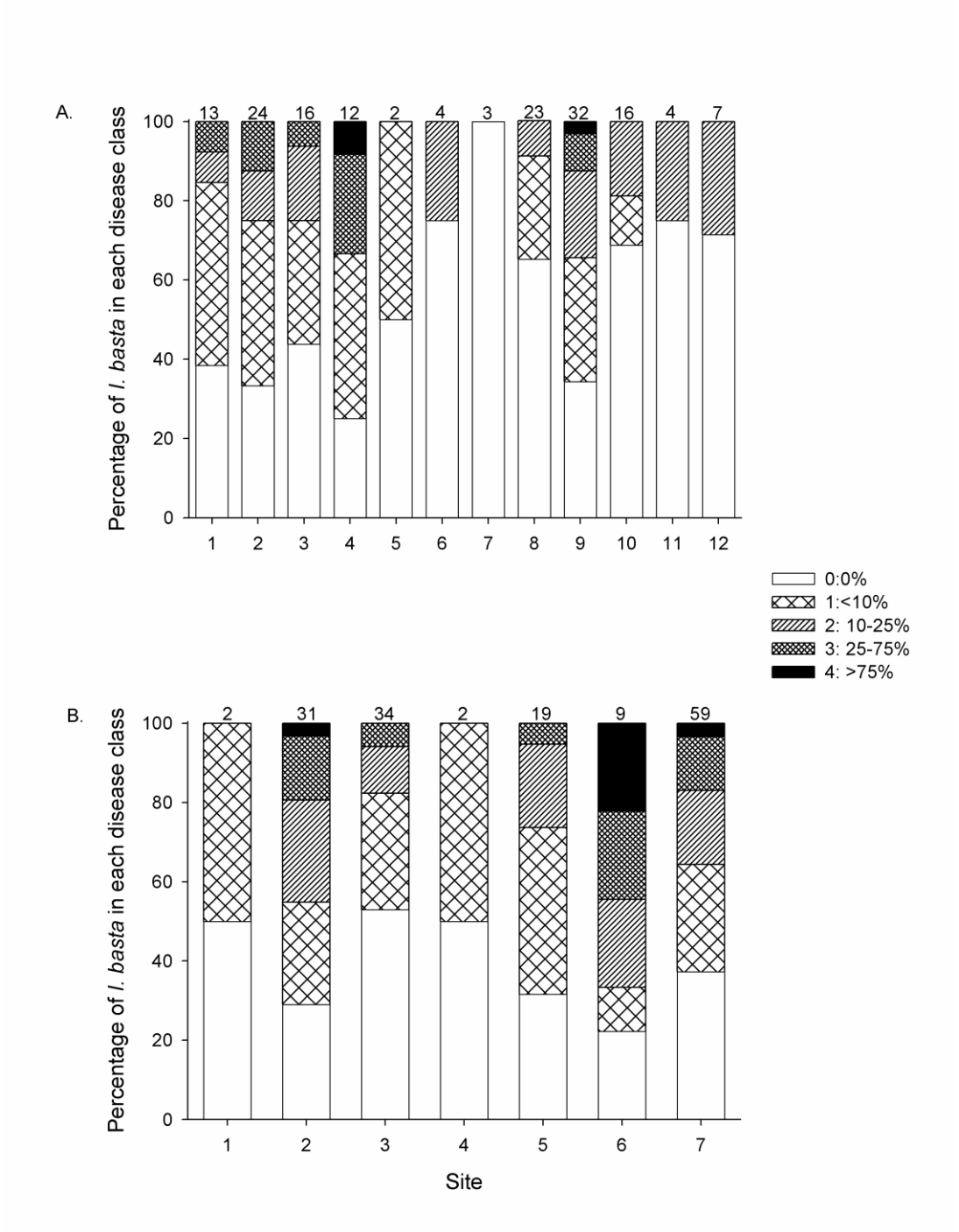

Fig. 2.3 Prevalence of disease in *I. basta* populations from (A) the Palm Islands and (B) Torres Strait, broken into disease classes based on the percentage of the individual displaying signs of disease. Sample size for each site noted at the top of the bar.

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2.4 Discussion

The number of sponges displaying signs of necrosis in *I. basta* populations from Torres Strait and the Palm Islands was higher than previous reports of disease syndromes in other sponge species. Disease affected 6-15% of the *Aplysina fulva* population in Panama over the course of a 14 year study period (Wulff 2007), 3.2- 12.2% of the *Aplysina* sp. population from the Caribbean (Olson et al. 2006) and 41.5% of the *A. aerophoba* population at a single site in Slovenia (Webster et al., 2008). Discrepancies in levels of disease prevalence among different sponge populations may be further complicated by variability in the sponge morphology (body size and growth form have been determined to influence the spread and visibility of disease) and the virulence of the pathogenic agents (Wulff 2006c). In addition, highly necrosed / dead sponges are not easy to detect as bare skeletal fibers rapidly disintegrate. For this reason, regular monitoring is essential to accurately quantify the prevalence of sponge disease.

In contrast to the massive mortalities observed during the Mediterranean (Vacelet 1994) and Caribbean (Galstoff 1942) sponge disease outbreaks, relatively few *I. basta* from the GBR and Torres Strait suffered extensive necrosis. However, given the occurrence of diseased sponges from all but one of the surveyed sites spanning a large longitudinal gradient, it appears that tissue necrosis is widespread among *I. basta* populations. A widespread distribution has previously been documented for black band disease (BBD) in corals from the GBR, occurring in 73.7% of the surveyed sites and spanning three longitudinal sectors of the GBR (Page & Willis 2006). However, in both corals and sponges, the variability in gross pathological symptoms and the lack of identified etiological agents makes it difficult to ascertain the geographical range of specific disease outbreaks.

This is one of two studies reporting disease-like syndromes in *I. basta* communities in the Indo Pacific; though, the macroscopic features in this study differed from that in Cervino et al. (2006), where they observed lesions developing within a biofilm and tangled mat of filamentous cyanobacteria. Consistent with the finding that a putative pathogen identified in Papua New Guinea *I. basta* was unable to cause disease in *I. basta* from the Great Barrier Reef (Negri et al. 2009), our initial microbial assessments (unpublished data) indicate that this is not the same disease syndrome as that reported by Cervino and colleagues. While some necrosed *I. basta* from this study displayed increased fouling; necrotic spots, exposed sponge fibers and tissue degradation were more common. Though the causative agent in this study remains to be identified, high levels of cellular degradation and dismemberment of choanocyte chambers in sponges with brown spot lesions indicate the sponge health is compromised. Choanocytes play important functional roles in sponge maintenance, being responsible for the production of water currents through the canal and the entrapment of potential food particles (Simpson 1984). Thus, significant degradation of these structures would compromise the overall health of the sponge.

There was no significant difference in the level of tissue necrosis between sponges at either location, with both the Palm Islands and Torres Strait containing sponges belonging to all disease categories. The only exception was a single site in the Palm Islands (n= 3 sponges) where no necrotic sponges were observed. This is most likely due to the low sponge abundance at this site rather than differing levels of anthropogenic or terrigenous input. This study assessed the health of *I. basta* populations by the level of tissue necrosis and it is important to acknowledge that necrosis may actually arise from a range of stressors in addition to infectious agents. These could include environmental factors (Bruno et al.

2003; Jones et al. 2004), physical damage (Wulff 2006b), predation (Knowlton & Highsmith 2005) and competitive interactions (Lopez-Victoria et al. 2006). Given the current interest in marine diseases there is a clear need to establish baseline health surveys for ecologically important species which will increase our understanding of disease prevalence and the biotic / abiotic factors that contribute to disease.

In conclusion, a large percentage of the *I. basta* population in the GBR and Torres Strait displayed signs of necrosis, although <10% of the sponge tissue is affected in the majority of cases. Tissue necrosis was widespread among this species, being documented in all but one of the sites surveyed, though healthy communities still persist at each location. This study contributes critical data on the prevalence of tissue necrosis and brown spot lesions in a common and ecologically important sponge species and will assist construction of a global sponge disease database and establishment of regular sponge disease monitoring programs. Future research is urgently required to elucidate the microbial communities associated with diseased and healthy *I. basta* and detect specific putative pathogens and their possible sources.

Chapter 3: Exploring the Role of Microorganisms in the Disease-like Syndrome Affecting *Ianthella basta* Populations[2]

3.1 Introduction

Sponges harbor a highly diverse range of microorganisms including representatives from 28 bacterial phyla and both major lineages of the *Archaea* (Taylor et al. 2007; Webster et al. 2010). Microorganisms can comprise up to 40% of sponge biomass; although sponges with more developed aquiferous systems and looser mesohyl often have lower microbial abundances (Vacelet & Donadey 1977). Some sponge-microbe associations may be considered symbiotic (Taylor et al. 2007), while others are non-specific and may include potentially pathogenic microorganisms (Cervino et al. 2006; Webster et al. 2008b).

Diseases of marine organisms have been attributed to bacteria, fungi, viruses, protozoans and a variety of metazoan parasites (Kinne 1980). In sponges, bacteria and fungi are the most commonly reported pathogens but the exact etiological agents are rarely identified and little is known about the disease processes (Webster 2007). In the past decade there has been an increase in reports of sponge disease around the globe including the Caribbean, Panama, Papua New Guinea and Slovenia (Kinne 1980; Nagelkerken et al. 2000; Cervino et al. 2006; Olson et al. 2006; Wulff 2007; Webster et al. 2008b). Disease-like symptoms in sponges may also arise from environmental stressors (Bruno et al. 2003; Jones et al. 2004), physical damage (Wulff 2006b), predation (Knowlton & Highsmith 2005) or competitive interactions (Lopez-Victoria et al. 2006).

[2] Chapter 3 adapted from Luter HM, Whalan S, Webster NS (2010) Exploring the role of microorganisms in the disease-like syndrome affecting the sponge *Ianthella basta*. Applied and Environmental Microbiology 76: 5737-5744.

Since 2006, two studies have reported a disease-like syndrome in the sponge *Ianthella basta* which is commonly distributed in Papua New Guinea (Cervino et al. 2006) and along the Great Barrier Reef (Luter et al. 2010b). A large percentage of *I. basta* from the Torres Strait and the Palm Islands in the Great Barrier Reef were found to exhibit signs of disease which included discolored, necrotic spots and exposed skeletal fibers (Luter et al. 2010b). In sponges affected by this syndrome there was a high level of cellular degradation and debris within the remnants of the choanocyte chambers. In Papua New Guinea, *I. basta* exhibited high mortality between 1996-2000 with the affected sponges exhibiting mottled brown lesions, rotted tissue and large holes (Cervino et al. 2006). The etiological agent of disease in *I. basta* was not unequivocally ascertained in either study.

Previous research using 454 tag pyrosequencing has assessed the microbial community in *I. basta* and reported high diversity with 1099 operational taxonomic units (OTUs) at 95% sequence similarity (Webster et al. 2010). However, most of this diversity was composed of rare organisms represented by only one or a few sequences. The community was dominated by the *Alpha* and *Gammaproteobacteria* with a single *Gammaproteobacteria* OTU actually comprising 49% of all sequence tags (Webster et al. 2010). The rare microbial biosphere in *I. basta* included *Acidobacteria, Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Gemmatimonadetes, Lentisphaerae, Nitrospira, Planctomyces, Poribacteria, Spirochaetes*, TM7, *Verrucomicrobia* and the *Beta, Delta* and *Epsilon* classes of the *Proteobacteria* (Webster et al. 2010).

With bacteria commonly implicated in sponge disease processes and shifts in microbial communities being used to detect putative pathogens in corals and sponges (Bourne 2005; Cervino et al. 2006; Sussman et al. 2008; Webster et al. 2008b), the aims of this study were to

ascertain the role of microorganisms in the disease-like syndrome affecting *I. basta* and determine how disease affects the symbiotic microbial population.

3.2 Materials and Methods

3.21 Sample Collection

I. basta that were displaying visible signs of necrosis and brown spot lesions were collected from two sites at Masig Island, central Torres Strait, Australia (9˚44.260'S, 143˚25.275'E and 9˚44.779'S, 143˚24.280'E) in June and November, 2008. Three individual tissue samples were collected from each sponge; comprising sections of 1, diseased (D); 2, interface between diseased and apparently healthy tissue (M) and 3, apparently healthy, non diseased tissue (ND). In addition, sections from healthy control sponges were collected for microbial comparison (H). In total, 13 healthy and 32 diseased samples were collected. Immediately after collection, tissue samples were fixed in 1.5 ml of 100% ethanol and stored at -20˚C prior to molecular analysis.

3.22 DNA Extraction and DGGE

All samples were extracted using two different extraction protocols to maximize the amplification of a broad range of microbes. DNA was extracted according to the manufacturer's protocol with the Power Plant DNA Isolation kit, MoBio Laboratories (Carlsbad, CA) and using a modified version of (43). Briefly, 0.5 g of sponge tissue was added to 0.5 ml grinding buffer (100 mM Tris, pH 9; 100 mM EDTA; 1% SDS; 100 mM NaCl and Milli-Q water). Tubes were immersed in liquid nitrogen and ground with a sterile plastic pestle before addition of an additional 0.25 ml grinding buffer and 18.75 µl of 20.3 mg ml^{-1} Proteinase K. Samples were incubated at 65˚C for 60 minutes with gentle rotation before the addition of 187.5 µl 5 M potassium acetate. Samples were incubated on ice for 30 min

and centrifuged at 8000 *g* for 15 min at room temperature. DNA in the supernatant was precipitated with 0.8 volumes of isopropanol, washed twice with 70% ethanol and re-suspended overnight at 4˚C in Milli-Q water and stored at -20˚C.

The 16S and 18S rRNA gene of samples from each extraction method were amplified by PCR with bacterial primers (1055f: 5'-ATGGCTGTCGTCAGC T-3' and 1392r: 5'-ACGGGCGGTGTGTRC-3') (Ferris et al. 1996) and eukaryotic primers (NS1f: 5'-GTAGTCATATGCTTGTCTC-3' and NS2r 5'- GGCTGC TGGCACCAGACT TGC-3') (White et al. 1990). Both reverse primers were modified to contain a 40bp GC clamp (Muyzer et al. 1993). PCR reactions contained 5 µl dNTP (2.5 mM), 5 µl 10 x OptiBuffer,0.15 µl of each primer (100 pmol μl^{-1}), 0.4 µl BSA (10 mg ml^{-1}), 3 µl $MgCl_2$ (50 mM), 0.5 µl Bio-X-ACT Taq polymerase (Bioline, London, UK) and 1 µl DNA template. Reactions were made up to 50 µl total volume with Milli-Q water. The PCR conditions for the 16S primers were: 1 cycle at 95ºC for 5 min; 30 cycles at 95ºC for 30 sec, 55ºC for 1 min, 70ºC for 1 min; and a final elongation at 70ºC for 10 min. The PCR conditions for the 18S primers were: 1 cycle at 95ºC for 3 min; 30 cycles at 95ºC for 30 sec, 55ºC for 30 sec, 72ºC for 1 min; and a final elongation at 72ºC for 7 min. PCR products from both extraction methods were pooled and 20 µl of each sample was added to an 8% w/v polyacrylamide gel containing either a 50-70% (Bacteria) or 35-70% (Eukarya) denaturing gradient of formamide and urea. The gels were run at 60˚C for 16 h in 1 x TAE buffer at 75 V using the Ingeny D-code system, stained with 1 x sybr gold for 10 min, visualized under UV illumination and photographed with the Vilber Lourmat ChemiSmart 3000 system. Reference bands from healthy and diseased samples, as well as bands that were present in diseased but absent from healthy samples were excised, re-amplified with PCR and checked

for correct mobility on a 50-70% or 35-70% DGGE gel, respectively. PCR products were sequenced by Macrogen Inc. (Seoul, Korea) using the forward primer (1055f).

3.23 Cloning and Sequencing

The 16S rRNA gene from three healthy, middle and diseased samples was amplified by PCR with universal bacterial primers: 63f 5'-CAGGCCTAACACATG CAA GTC-3' (Marchesi et al. 1998) and 1492r 5'- GGT TACCTTGTTACGACT T -3' (Lane 1991) and archaeal primers: 21f 5'-TTCCGGTTGATCCYGCCGGA-3' (Reysenbach et al. 1992) and 958r 5'-TCCGGCGTTGAMTCCAATT-3' (DeLong 1992). PCR reactions contained 5 µl dNTP (2.5 mM), 5 µl 10x OptiBuffer, 0.15 µl of each primer (100 pmol μl^{-1}), 0.4 µl BSA (10 mg ml^{-1}), 3 µl $MgCl_2$ (50 mM), 0.2 µl Hot Star Taq DNA polymerase (Qiagen Inc., Chatsworth, CA) and 1 µl DNA. Reactions were made up to 50 µl total volume with Milli-Q water. The PCR conditions were: 1 cycle at 95ºC for 5 min; 32 cycles at 94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 2 min; and a final elongation at 72ºC for 10 min. The triplicate PCR products were pooled to create a single clone library for each of the healthy, middle and diseased samples. PCR products were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were checked for inserts by PCR amplification using M13 forward and reverse primers. Restriction digests using HhaI and HaeIII (New England Biolabs Inc.) were performed to determine operational taxonomic units (OTU's) for each library. Duplicates from each representative OTU in each library were sent to Magrogen Inc. (Seoul, Korea) for sequencing using 63f and 1492r (bacteria) and 21f and 958r (archaea) as the sequencing primers.

3.24 Phylogenetic Analysis

DGGE and clone sequences were compared with available databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) to determine nearest relatives and percent similarity. Only unique clone sequences (irrespective of the library they originated from) were submitted to Genbank under the accession numbers GU784983-GU784990 and DGGE sequences under the accession numbers GU784991-GU785007. Sequences were checked for chimera formation using Greengenes (DeSantis et al. 2006) and Bellerophon (Huber et al. 2004) and all chimeric sequences were removed before tree construction. Sequences were compiled, automatically aligned and manually edited in the ARB software package (http://www.arb-home.de (Ludwig et al. 2004)). Initially, trees were calculated with almost complete 16S rRNA (1400 bp) sequences for all close relatives of target sequences using the neighbor-joining and maximum parsimony methods in ARB. Partial sequences were subsequently imported to the tree without changing branch topology using the ARB parsimony-interactive method. The robustness of inferred tree topologies was evaluated after 1000 bootstrap re-samplings of the neighbor-joining data in the PHYLIP program (Felsenstein 1993). *Escherichia coli* and *Thermotoga maritima* were used as out groups for the bacterial and archaeal trees, respectively.

3.25 Bacterial Cultivation

Aseptic techniques were used to isolate bacteria from small portions (~ 1 cm^2) of tissue from five healthy and ten diseased sponges. Sponge tissue was rinsed briefly in 70% ethanol and rapidly transferred to sterile artificial seawater (ASW). The tissue was then removed, cut into small pieces using a sterile scalpel and finely ground using a mortar and pestle. This slurry was suspended in 9 ml of sterile ASW. Tenfold serial dilutions of the suspension were prepared to a dilution of 10^{-3} and 100 µl of each dilution was plated in triplicate on Bacto

Marine Agar 2216 (Difco Laboratories, Detroit, MA). All plates were incubated at 28ºC for up to 7 days. Three distinct colony morphotypes were identified and these appeared to be the same in both diseased and healthy sponges. Five representatives of each morphotype were selected from both healthy and diseased sponges and serially streaked onto Marine Agar 2216 to obtain pure cultures. Total bacterial counts were recorded from each plate as colony forming units (CFU) ml^{-1} of sponge tissue and the results were averaged. DNA from *I. basta* isolates was extracted using the Wizard Genomic DNA Purification Kit, Promega (Madison, WI), according to the manufacturer's protocol. The 16S rRNA gene was amplified by PCR with universal bacterial primers 63f and 1492r, following the same conditions described above. PCR products were sent to Macrogen Inc. (Seoul, Korea) where they were subsequently purified and sequenced using 63f as the sequencing primer. Sequences from *I. basta* isolates were submitted to Genbank under the accession numbers GU817016-GU817018.

3.26 Transmission Electron Microscopy (TEM)

In order to visualize potential pathogens *in situ*, small *I. basta* tissue sections (~1 mm diameter) from five healthy sponges and seven sponges displaying visible signs of necrosis and brown spot lesions were collected for observation using transmission electron microscopy (TEM). Morphotype enumeration was conducted on 10 fields of view per sample to assess variation in morphotype abundance between healthy and diseased sponges. Samples were fixed for 24 h in 2.5% gluteraldehyde in fresh filtered sea water and subsequently placed in filtered artificial seawater and stored at 4˚C until further processing. Fixed tissue was placed in a 1% osmium tetroxide solution (prepared in 0.1 M cacodylate buffer) for 3.5 h and subsequently dehydrated in a graded acetone series (50, 60, 70, 80, 90 and 100%). Tissue was then embedded in Epon resin, sectioned with an ultramicrotome and

stained with 5% uranyl acetate in 50% methanol, followed by Reynolds lead citrate. Ultrathin sections of 60 nm were mounted on Formvar coated copper grids and viewed using a JEOL 1010 operated at 80kV. Images were captured using a SoftImaging Megaview III digital camera.

3.27 Pilot Infection Trial

To assess disease transmission, a small-scale pilot study was undertaken to determine if healthy sponges could be infected by a sponge showing visible signs of necrosis. Healthy sponges were placed in a 40 l tank at a distance of 30 cm from a necrotic sponge for eight days. The tank was placed inside a 1,000 l flow-through, 70% daylight shaded outdoor aquarium. Sponges were monitored daily for the onset of brown spot lesions and/or necrosis. After eight days, sponges were showing no visible signs of disease so healthy sponges were placed in direct contact with diseased tissue and left for a further five days.

3.28 Data Analyses

PAST statistical software (Hammer et al. 2001) was used to carry out a non-metric multidimensional scaling (nMDS) analysis, using the Raup-Crick measure of similarity, to analyze microbial community composition using a presence (1)/absence (0) matrix for each of the DGGE bands.

3.3 Results

A total of 13 healthy control *I. basta* (Fig. 3.1A) and 32 sponges displaying signs of disease were collected from Masig Island over the two sampling periods. Visible signs of disease included brown necrotic spots in which sponge skeletal fibers were apparent. In addition, some *I. basta* exhibited high levels of tissue degradation with completely exposed skeletal fibers (Fig. 3.1B-C).

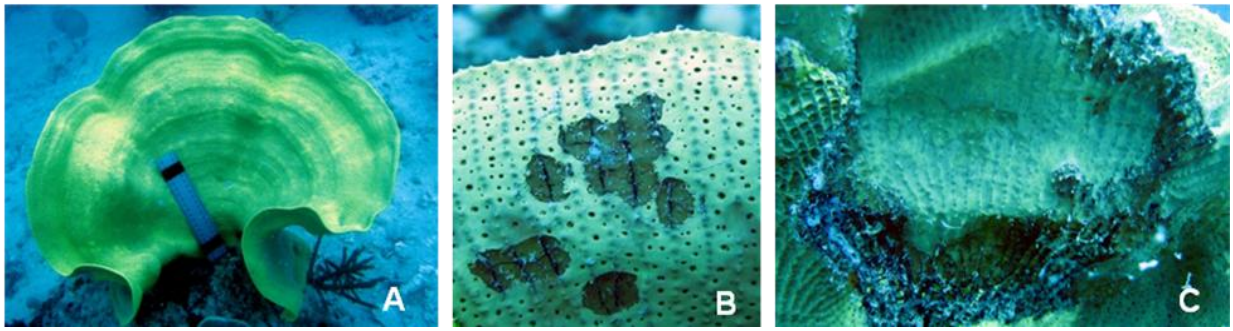

Fig. 3.1 Healthy and diseased *I. basta* specimens. (A) Healthy control; (B) diseased specimen with brown spots; (C) diseased specimen with exposed skeletal fibers.

3.31 Bacterial Community Analysis

DGGE analysis of *I. basta* samples revealed a relatively low diversity of microbes with a total of 31 bands detected (Figure 3.2). Comparison of the DGGE profiles in healthy and diseased *I. basta* revealed remarkably similar banding patterns, regardless of the health status of the sponge (Figure 3.2). Healthy control samples, as well as non-necrotic portions of diseased sponges showed similar DGGE banding patterns and little variation between samples. In contrast, middle and diseased samples showed much larger variation, possessing bands that were missing in healthy control and non-diseased samples (e.g. bands Q, R and T). However, these bands were not consistently present in all diseased samples (Figure 3.2). The nMDS shows a cluster of healthy control sponges in the centre of the nMDS plot and there is little separation between healthy control sponges and several data points depicting diseased samples (Figure 3.3). The variability associated with middle and diseased samples in the DGGE (Figure 3.2) is depicted by the wide separation of many of these samples in the nMDS analysis.

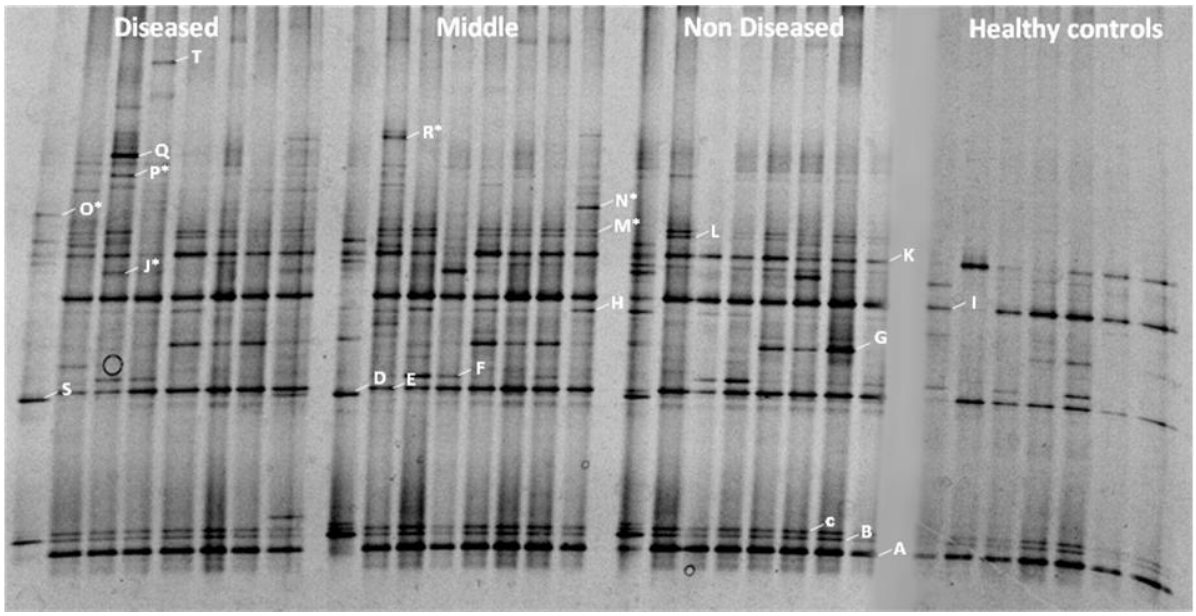

Fig. 3.2 Representative DGGE gel image of 16S rRNA-defined bacterial population from healthy control *I. basta*, unaffected tissue from diseased *I. basta* (non diseased), tissue from the interface between the lesion and healthy tissue (middle), and lesion tissue (diseased). Individual bands excised for sequencing are labeled on the right hand side of the band, and asterisks (*) denote bands that yielded low sequence quality.

16S rRNA sequencing of excised DGGE bands revealed relatively low bacterial diversity restricted to the *Alpha* and *Gammaproteobacteria* (Table 3.1). In addition, three bands present in all samples, had highest sequence similarity (95-96%) to an uncultured Thaumarchaea from an ascidian. Overall, DGGE bands from *I. basta* had low similarity to sequences in the NCBI database with band H being the only sequence with a similarity >96%. The phylogenetic affiliation of six bands (labeled with *) could not be determined due to low sequence quality (Figure 3.2).

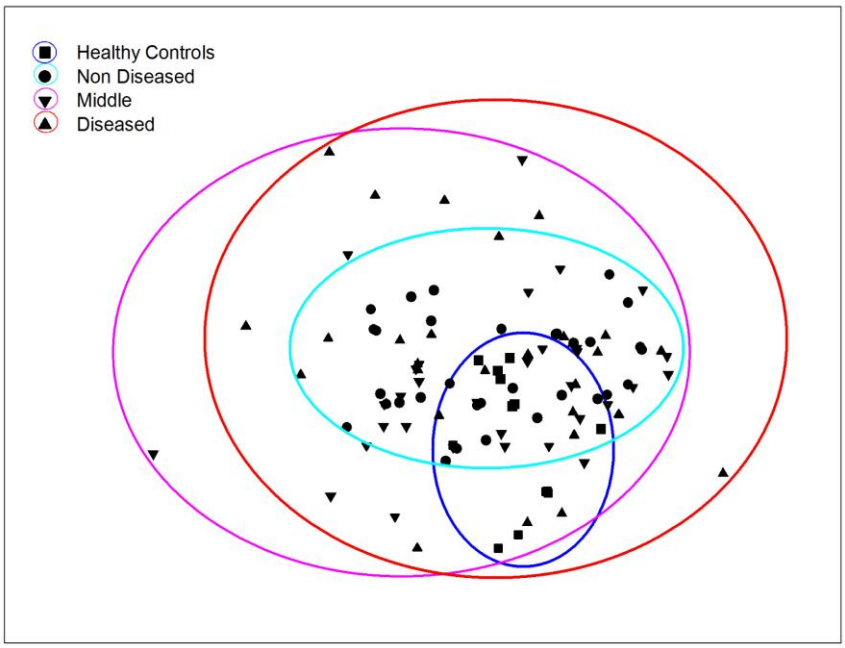

Figure 3.3 Nonmetric multidimensional scaling (nMDS) analysis of sponge bacterial community composition using DGGE banding pattern data to construct a similarity matrix. Circles were manually drawn to assist differentiation of the disease categories.

Clone library analysis of *I. basta* samples was consistent with the DGGE results revealing low microbial diversity and high similarity between each of the three libraries (Fig. 3.4). All clone sequences fell into only two classes, the *Alpha* and *Gammaproteobacteria*, with all three libraries dominated by the single *Alphaproteobacteria* sequence (H1). This *Alphaproteobacteria* accounted for 78% of the healthy and middle libraries and 82% of the diseased library. The other dominant sequence in all libraries was a *Gammaproteobacteria*, clone H4 which comprised 18, 19 and 12% of the healthy, middle and diseased libraries, respectively. Two closely related *Alphaproteobacteria* sequences (D3 and D6) were only detected in the library from diseased sponges, but each sequence comprised only 2% of the library. Two other *Gammaproteobacteria* sequences were detected with one of them (M8) only detected in the middle library and the other (H2) present in both the healthy and diseased

libraries. These sequences made up only a small percentage of each of the libraries (2%) (Fig. 3.4).

Table 3.1 Sequence similarity in excised 16S rRNA DGGE bands from *I. basta* as determined by using BLAST

Band ID	Accession Number	Percent Similarity	Description
A	EU373876	90	α-*proteobacteria* (Mediterranean sediment)
B	EF173334	88	α-*proteobacteria* (Soil)
C	EU373879	90	α-*proteobacteria*, (Mediterranean sediment)
D	FM242455	94	γ-*proteobacteria* (Unidentified environmental sample)
E	FM242456	95	γ-*proteobacteria* (Unidentified environmental sample)
F	FM242457	96	γ-*proteobacteria* (Unidentified environmental sample)
G	EU117208	93	*Pseudomonas viridiflava*
H	FJ005061	99	*Pseudomonas oryzihabitans*
I	EU283427	96	Thaumarchaea (ascidian)
K	EU283423	96	Thaumarchaea (ascidian)
L	EU283427	95	Thaumarchaea (ascidian)
Q	FJ231146	95	unidentified bacterium (high altitude lake in Chile)
S	FM242455	94	γ-*proteobacteria* (Unidentified environmental sample)
T	AY492079	90	*Fucus vesiculosus* mitochondrion

Phylogenetic analysis showed clone H1 was only distantly related (86% sequence similarity) to its closest relatives which includes a sequence retrieved from a deep sea hydrothermal vent and numerous other sponge and coral-derived sequences (Figure 3.5). Clone H4 was most closely related to symbionts previously reported from the sponges *Haliclona gellius, Chondrilla nucula* and *Axinella* spp.). Whilst clones D3 and D6 showed some similarity to a sequence from black band diseased corals (EF123361), they were most closely affiliated to uncultured symbionts of a soft coral (DQ396277) and sponges (AY948358 and EF092167). *I. basta* clone H3, present in both the healthy and middle libraries, was closely related to the dominant *Alphaproteobacteria* sequence H1.

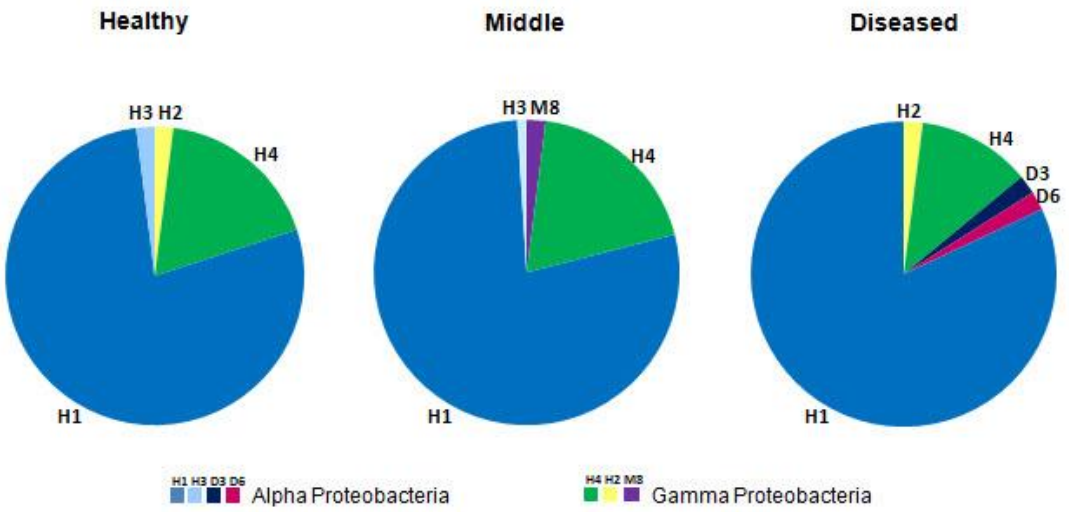

Fig. 3.4 Pie charts showing the differences in bacterial community composition in *I. basta* between healthy control sponges, the interface between the lesion and healthy tissue (middle), and lesion tissue (diseased). The graphs were constructed based on the frequency of clones from each of the tree libraries. Clones given the same label in each of the three libraries had identical 16S rRNA sequences.

Culture-based analysis isolated only three unique morphotypes and these were cultured from both healthy and diseased *I. basta*. Five replicates of each morphotype were sequenced from both diseased and healthy sponges and in all instances 16S rRNA sequences were

identical for the individual morphotypes. No single isolate was present in diseased samples and absent from healthy samples nor were any of the dominant cultures more abundant in diseased samples. However, the mean CFU for the diseased samples was higher than healthy samples (83 +/- 12 vs. 57 +/- 8 CFU ml^{-1}). Two of the *I. basta* isolates (GU817016-GU817017) revealed high similarities with marine *Alphaproteobacteria.* *I. basta* isolate CLCM was identical to an *Alphaproteobacteria* isolated from the mucus of a Red Sea coral

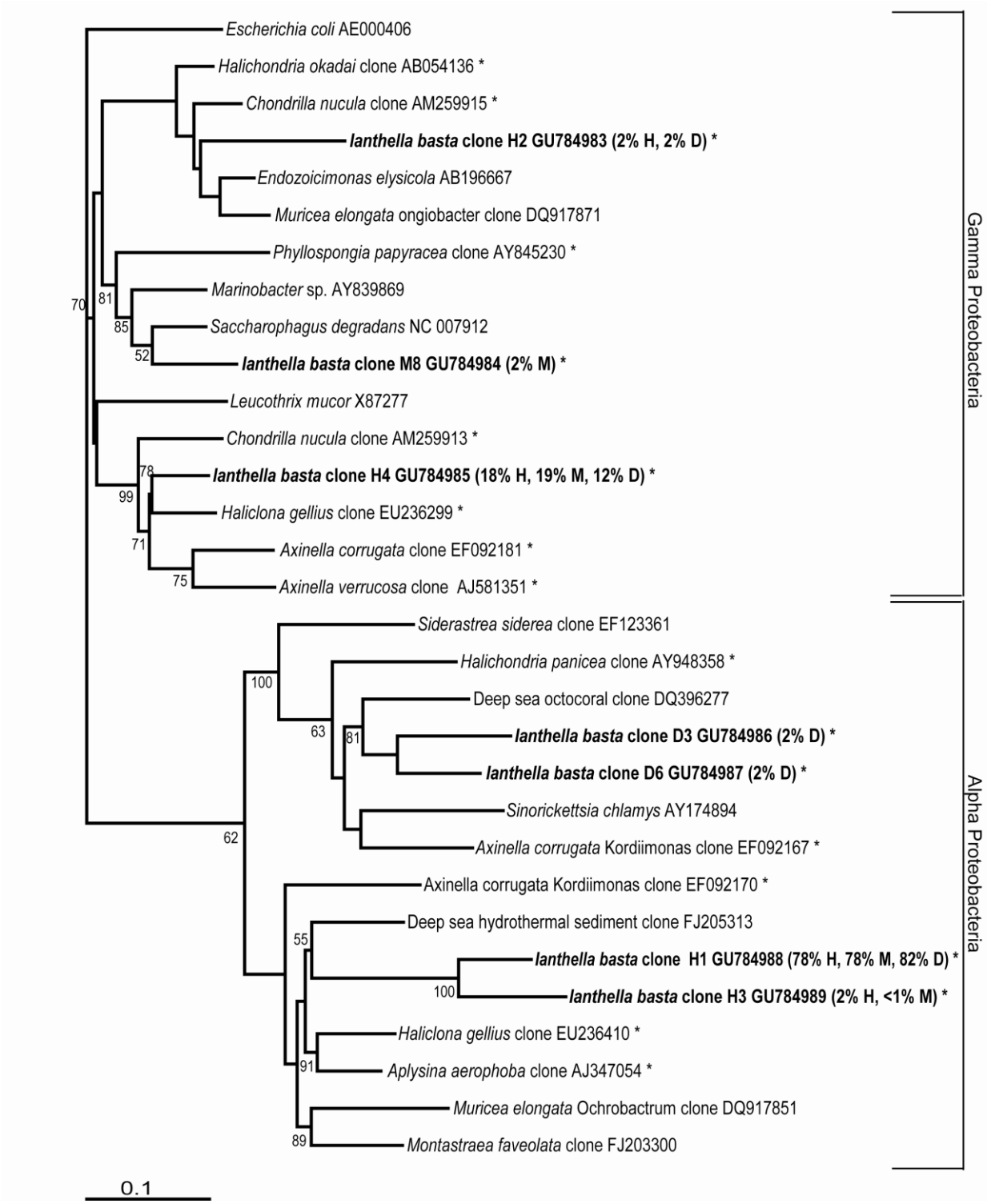

Fig. 3.5 Maximum-likelihood phylogenetic tree from analysis of all 16S rRNA gene sequences retrieved from clone library analysis. *I. basta* sequences indicated by bold font, with the percentage of each library it comprised listed in parenthesis afterwards and * indicates clones isolated from marine sponges. The numbers at the nodes are percentages indicating the levels of bootstrap support based on analysis of 1000 re-sampled data sets. Only values >50% are shown. Scale bar represents 0.1 substitutions per nucleotide position.

(DQ107390). Similarly, isolate CMSM1 was identical to a sponge-associated *Alphaproteobacteria* (EF513634). While *I. basta* isolate CMSM2 was identical to a *Bacillus thuringiensis* strain (GU384894), it was also 99% similar to a *Bacillus* strain previously cultured from *I. basta* in Papua New Guinea (DQ323748). None of the cultured isolates were detected using molecular techniques.

Consistent with molecular and culture-based methods, low bacterial diversity and abundance was also visualized by TEM in both healthy and diseased samples (Fig. 3.6). Three dominant bacterial morphotypes were identified in all samples which were defined as furry (Fig. 3.6A), full (Fig. 3.6B) and filament (Fig. 3.6C). The trans-section of the filament morphotype showed a mean size of 158 nm with the mean sizes of the furry and full morphotypes being 259 and 301 nm, respectively. Both the outer and inner cell membranes of the full morph were clearly defined indicating a gram negative bacterium. None of the morphotypes showed variation in abundance in healthy (furry: n=1.1 +/- 0.33 S.E./ field of view; full: n=0.8 +/- 0.28 S.E.; filament: n=7.6 +/- 2.4 S.E.) or diseased tissue (furry: n=1.1 +/- 0.34 S.E./ field of view; full: n=0.6 +/- 0.20 S.E.; filament: n=8.1 +/- 2.5 S.E.). There was also no particular association of these morphotypes with any individual sponge cell type. No virus-like particles were detected in any samples. Preliminary histological analysis of *I. basta* tissue revealed the consistent presence of a polychaete worm within the sponge tissue (Figure 3.6D). However, TEM and subsequent histology failed to reveal any differences in the abundance of this worm between diseased and healthy sponge tissue.

Only a single archaeal sequence was detected in clone libraries from healthy, middle and diseased *I. basta* libraries. The sequence was a Thaumarchaea, related to other sponge-specific Archaea (95%) (Fig. 3.7).

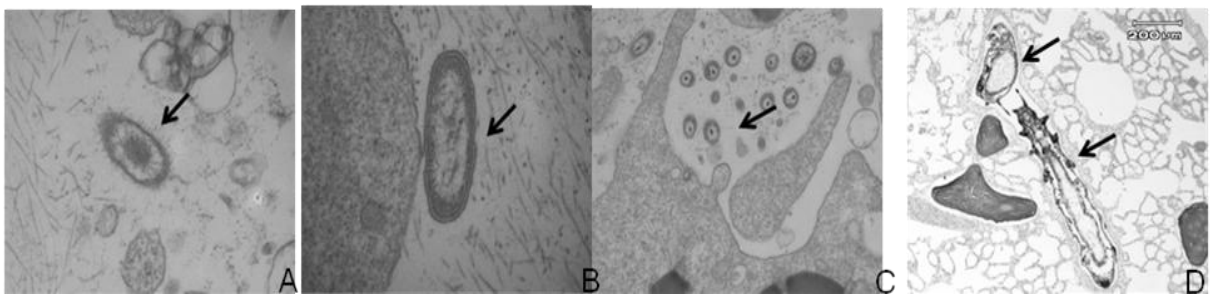

Fig. 3.6 Three dominant bacterial morphotypes of *I. basta* identified by using TEM in both healthy (n=5) and diseased (n=7) tissue, and a light microscope image depicting the polychaete worm associated with both healthy (n=6) and diseased (n=11) tissue. (A) Fuzzy; (B) full; (C) filament; (D) polychaete worm.

3.32 Eukaryotic Community Analysis

DGGE banding patterns were highly similar regardless of the health status of the sponge. No bands were consistently present in diseased samples and absent from healthy controls (Figure 3.8). All but two of the sequenced bands came from the sponge DNA (97% sequence similarity to *Hexadella pruvoti*) and the other bands corresponded to the polychaete worm. No microbial eukaryotic sequences were recovered with these universal primers.

3.33 Infection Trial

Transmission of disease symptoms was unsuccessful after the ten day pilot infection trial. Healthy sponges showed no evidence of brown spot lesions or necrosis following passive infection or direct contact with lesion tissue.

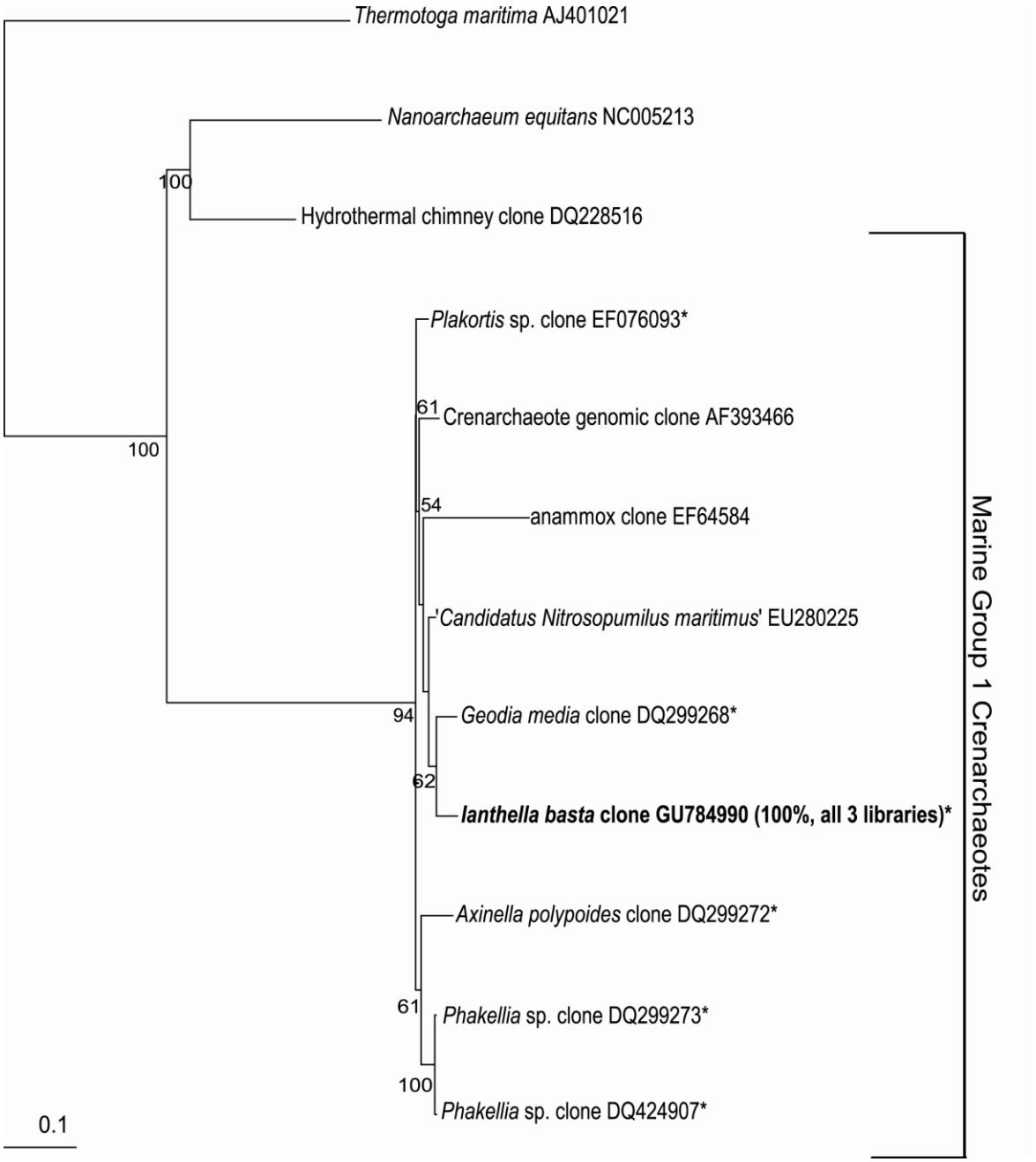

Fig. 3.7 Maximum-likelihood phylogenetic tree from analysis of all 16S rRNA gene sequences retrieved from archaeal clones. The *I. basta* sequence is indicated by bold font, and asterisks (*) indicate clones isolated from other marine sponges. The numbers at the nodes are percentages indicating the levels of bootstrap support based on analysis of 1000 re-sampled data sets. Only values >50% are shown. Scale bar represents 0.1 substitutions per nucleotide position.

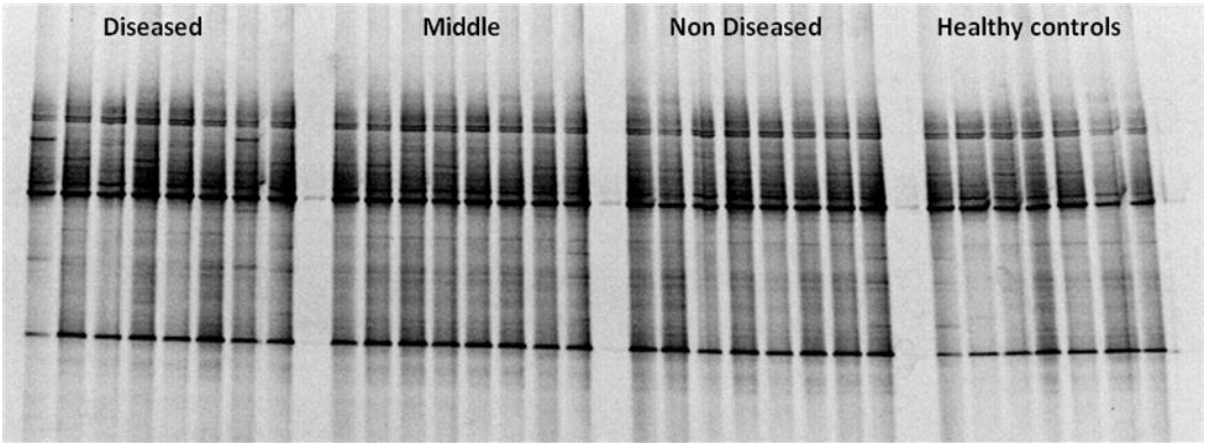

Fig. 3.8 A representative DGGE gel image of 18S rRNA-defined eukaryote populations from healthy control *I. basta*, unaffected tissue from diseased *I. basta* (non diseased), tissue from the interface between the lesion and healthy tissue (middle) and lesion tissue (diseased).

3.4 Discussion

Widespread necrosis, cellular disruption and degradation of choanocyte chambers occur in *I. basta* with brown spot lesions (Luter et al. 2010b). Despite these disease-like symptoms; bacterial cultivation, molecular community analysis (Bacteria and Eukarya) and electron microscopy undertaken in this study failed to reveal any putative pathogens. Regardless of the technique employed, shifts in microbial community structure between healthy control and diseased *I. basta* were never observed, providing substantial evidence that bacteria are likely not the etiological agent of the disease-like syndrome. In addition, no eukaryotic organisms (e.g. fungi, protozoa, worms) could be implicated in the formation of brown spot lesions and necrosis. Further support for the absence of a primary pathogen in *I. basta* was provided by the infection assay where lesions could not be induced in healthy sponges even after direct contact with infected tissue. Whilst transmission electron microscopy did not reveal the presence of any virus-like-particles, future research would be required to unequivocally confirm the absence of viruses.

Of the three isolates cultivated from *I. basta*, none were detected using molecular techniques suggesting that they are not a major component of the microbial community. Interestingly, one of the isolates from the present study (GU817018) had 99% sequence similarity to a *Bacillus* strain isolated from a diseased *I. basta* from Papua New Guinea (PNG) (Cervino et al. 2006). In the study of *I. basta* from PNG, affected sponges were more abundant close to shore and a correlation was found between the disease and the cultivation of five *Bacillus* and *Pseudomonas* isolates. The Author's speculate that bacterial pesticide pollution from agriculture may have introduced new bacteria that were pathogenic to sponges. Subsequent research with sponges and corals showed that addition of up to 5000 μg l of pesticides VectoBac® G (containing *B. thuringiensis israelensis*) and VectoLex® G (containing *B. sphaericus*) could not induce symptoms of disease in *I. basta* (Negri et al. 2009). In addition, the *Bacillus* strain from the current study was readily cultured from both healthy and diseased *I. basta* in similar densities, suggesting it is an unlikely primary pathogen.

It is now well established that marine sponges can host a large diversity of microorganisms (Taylor et al. 2007; Webster et al. 2010). Whilst a previous study detected 1099 different bacterial OTUs from *I. basta* on the Great Barrier Reef using 454 tag pyrosequencing (Webster et al. 2010), the current study revealed low microbial diversity using cultivation, microcopy and sequencing of 16SrRNA clone libraries. Excluding the rare biosphere of microbes detected by deep 454 tag pyrosequencing, the two studies were consistent in describing the composition of the microbial community, with the bulk of sequences falling in the *Alpha* and *Gammaproteobacteria*. Two bacterial sequences (comprising at least 90% of the total bacterial community) and a single archaeal sequence were detected with both DGGE and 16S rRNA clone sequencing in all healthy and diseased

sponges. The consistently low diversity in diseased sponges contrasts with previous studies where an increase in microbial diversity is detected in response to disease or environmental stress in sponges and corals (Cooney et al. 2002; Pantos et al. 2003; Pantos & Bythell 2006; Webster et al. 2008a; Webster et al. 2008b). It has been hypothesized that the increased bacterial diversity in diseased animals may be due to elevated nutrients associated with decaying cells and/or a breakdown in the host defense mechanisms (Webster et al. 2008b). The inability of opportunistic seawater-derived microbes to proliferate in 'diseased' *I. basta* suggests that the sponges' chemical defense and/or immune system are still functional even at an advanced stage of tissue degradation.

All clone sequences from *I. basta* had low similarity to sequences in the NCBI database indicating that *I. basta* hosts a novel microbial community. Similarly, Webster and colleagues (2009) found that approximately 25% of the 16S rRNA-V6 tag sequences from *I. basta* were below the threshold for reliable assignment and may in fact represent previously unknown microbes. While *I. basta* clones had low similarity to other known sequences, it is worth noting that all clones clustered with other sequences retrieved from marine sponges.

The term 'disease' can have various meanings, depending on the source and context it is used in. Oxford's English dictionary defines disease as 'a disorder of structure or function in a human, animal, or plant, especially one that produces specific symptoms'. Alternatively, Webster's dictionary defines disease as 'an impairment of health or condition of abnormal functioning'. These definitions also state that many medical terms describing symptoms are called 'diseases', especially when the cause of the symptom is unknown. This is particularly relevant for marine organisms where causative agents are yet to be identified for many of the disease-like syndromes. For instance, despite the fact that no etiological agents can be identified in many of the coral diseases (Sussman et al. 2008, and references cited therein)

and that no bacteria, protozoans or viruses could be implicated in the formation of soft tunics in the ascidian *Halocynthia roretzi*, these syndromes are still referred to as 'diseases' (Kitamura et al. 2010). Regardless of the specific interpretation of 'disease', this study has illustrated that care should be taken not to assume disease-like symptoms come from an infectious pathogen.

Considering that no infectious agent could be implicated, an environmental origin of the disease-like syndrome should be considered. Previous studies have shown tissue necrosis to arise from various stimuli such as: physical damage due to abiotic factors (Cerrano et al. 2001; Wulff 2006b), predation (Knowlton & Highsmith 2005) and chemical defense (Lopez-Victoria et al. 2006). Whilst small feeding scars have been described on Antarctic and temperate sponges due to opistobranchs and nudibranchs, the damage is normally minimal and localized (Wulff 2006a). Visual monitoring of *I. basta* over the course of different seasons and times of day, spanning a two year period, has failed to detect any potential predators or parasites that could be responsible for the extensive necrosis observed in *I. basta* throughout Torres Strait and the GBR. Sponge necrosis and mortality have previously been attributed to elevated temperature (Vicente 1989; Cerrano et al. 2000), high salinity (Smith 1939), low water exchange (Allemand-Martin 1914) and pollution (Gashout et al. 1989). Considering the widespread incidence of disease in *I. basta* it is unlikely that localized environmental factors such as pollution or low water flow are responsible.

Results from this study suggest that microorganisms are not responsible for the disease that is affecting large numbers of *I. basta* on the Great Barrier Reef and Torres Strait. Whilst molecular evidence revealed no difference in the microbial communities residing within healthy and diseased sponges, it is still possible that the disease is caused by microbes present in extremely low abundance (hence not detected by molecular methods) or existing

symbionts switching on virulence mechanisms (despite no change in their abundance or distribution). These scenarios should be explored further to eliminate microbes as causative agents of disease. Future research will also investigate the role of elevated seawater temperature, high sedimentation and autoimmune dysfunction in the onset of brown spot lesions and necrosis.

4.1 Introduction

Marine sponges commonly host a diverse range of microorganisms, comprising up to 40% of the tissue biomass (Vacelet & Donadey 1977). Much of the research in the past has focused on describing the diversity of microbes in sponges, with 25 bacterial phyla and both major lineages of Archaea (*Crenarchaeota* and *Euryarchaeota*) being reported to date (reviewed in Taylor et al. 2007 and Webster & Taylor 2011). In addition, there has been significant effort investigating the functional processes of sponge-associated microbes, including nitrification (Bayer et al. 2008; Mohamed et al. 2008b; Southwell et al. 2008a; Southwell et al. 2008b; Steger et al. 2008; Off et al. 2010; Schläppy et al. 2010), denitrification (Hoffmann et al. 2009; Schläppy et al. 2010) and Anammox (Hoffmann et al. 2009; Mohamed et al. 2010). Interestingly, microbial communities of sponges are often distinctly different than their surrounding environment and there is also evidence of uniform microbial community compositions from sponges between different oceans (Hentschel et al. 2002; Hill et al. 2006; Lafi et al. 2009). Given the uniqueness of sponge-associated microbes, the evidence of vertical transmission of symbionts from the parent to the larvae (reviewed in Taylor et al. 2007) and their involvement in important functional processes, it is clear that sponge-associated bacteria are important to their sponge host.

The importance of the sponge-microbe association is also highlighted by some sponge species displaying stable microbial communities even during stressful conditions. For instance, no microbial community shifts were observed in the Mediterranean species, *Aplysina aerophoba*, under starvation conditions and antibiotic exposure (Friedrich et al. 2001). Similarly, transplantation of *Aplysina cavernicola* from its original habitat (>40 m) to

a shallower, more illuminated habitat did not shift the microbial community of the sponge (Thoms et al. 2003). The microbial community of *Rhopaloeides odorabile* is also quite stable, with sponges cultured *in situ* and *ex situ* in small flow-through aquaria displaying similar community profiles (Webster et al. 2011). In contrast, environmental stressors such as increased temperature (Lemoine et al. 2007; López-Legentil et al. 2008; Webster et al. 2008a) and heavy metal exposure (Webster et al. 2001b; Selvin et al. 2009) have revealed shifts in typically stable microbial communities. Community shifts have also coincided with reduced sponge health, again highlighting the importance of sponge-microbe relationships.

It is evident that predicted increases of carbon dioxide and temperature levels will impact reefs worldwide (Hughes et al. 2003). For instance, the iconic Great Barrier Reef (GBR) has already sustained bleaching events in 1998, 2002 (Berkelmans et al. 2004) and 2006 (Jones et al. 2008). In addition to the rising seawater temperatures, increases in terrestrial runoff are exposing coastal marine systems to rising levels of nutrients, sediments and pollutants (Fabricius 2005). These nutrient-rich sediments have contributed to stress in corals even after short exposures (Weber et al. 2006). The coral/zooxanthellae symbiosis model has been featured heavily in studies exploring the effects of climate change and/or anthropogenic stressors on benthic organisms, while very little research has addressed the stability of the sponge-microbe symbiosis.

Rising temperatures and anthropogenic stressors are increasing linked with disease in marine and terrestrial organisms (Harvell et al. 1999; Harvell et al. 2002). Given that no putative pathogen could be implicated in the formation of brown spot lesions and necrosis (Chapter 3), environmental origins of the disease-like syndrome must be considered. In one of few studies investigating heat stress in sponges, *R. odorabile* displayed widespread tissue necrosis at elevated temperatures, accounting for 50-70% of the surface area (Webster et al.

2008a). This appears to be non-specific necrosis, whereas *Ianthella basta* with disease-like symptoms develop specific small lesions.

This chapter begins by exploring stability in the microbial community associated with *I. basta* across a latitudinal gradient. The effects of stress on the associated microbes of *I. basta* are then assessed. Firstly, the environmental stressors of temperature and sedimentation will be investigated. Second, the effect of an antibiotic exposure, as a proxy for a non-environmental stressor, will be quantified. Finally, these stressors will be assessed for their ability to induce disease-like symptoms (e.g. brown spot lesions) in *I. basta*.

4.2 Materials and Methods

4.21 Sponge Collection for Spatial Analysis

To compare microbial communities between *I. basta* from different locations, specimens were collected from three different locations of eastern Australia: (1) Orpheus Island, central Great Barrier Reef (GBR), (2) Masig Island, Torres Strait and (3) Davies Reef (Fig. 4.1). Seven samples from each location were photographed *in situ* and preserved in 100% ethanol for molecular analysis. All individuals were collected from a similar depth range, between 12 and 15 m.

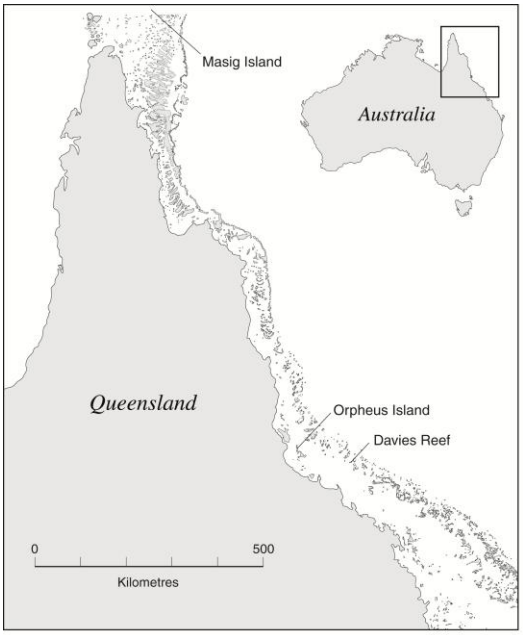

Fig. 4.1 Map showing the three sampling sites: Masig Island, Orpheus Island and Davies Reef.

4.22 Sponge Collection for Experimental Work

Fourteen *I. basta* sponges were collected across similar depths and habitats by scuba from the fringing reefs of the Palm Islands, central GBR. These donor sponges were cut into smaller explants (approximately 10 cm^3) using a scalpel blade and transferred to four Aquapurse baskets (TTP plastics by design; Brisbane, Queensland, Australia) that were secured to the reef (18˚ 33.617'S, 146˚ 29.077'E). Explants recovered for 12 weeks, sufficient time for healing to take place (Louden et al. 2007), before collection and transportation to the climate-controlled aquarium facilities at the Australian Institute of Science (AIMS), Townsville. *I. basta* explants were placed into 12 x 30 l flow-through aquaria (flow rate of 600 ml min^{-1}) and maintained under a diel cycle of 12:12h at 40 µmol quanta $m^{-2}s^{-1}$. Incoming seawater was filtered to 5 µm to remove large particulates, but still provide sponges with sufficient nutritional supply (Reiswig 1971b). Explants were allowed to acclimatize under these aquarium conditions for 48 h before commencing experiments.

4.23 Temperature Experiment

To assess the effects of thermal stress on *I. basta* and its associated microbes, sponge explants were exposed to four different temperature treatments: 27, 30, 31 and 32˚C (range +/- 0.2˚C). Both 27 and 30˚C represent ambient temperatures commonly recorded on inshore reefs of the GBR during the summer period. The temperatures of 31 and 32˚C represent temperatures which have been linked with mass coral bleaching (Berkelmans et al. 2004) and the disruption of sponge symbiosis (Webster et al. 2008a). The experimental design comprised three replicate tanks per temperature treatment, each holding seven explants. Treatment tanks were randomly arranged around the climate-controlled room and explants were contained within 14 x 21 cm plastic mesh baskets inside each treatment tank (Fig. 4.2). Four sumps were heated to the desired treatment temperatures (27, 30, 31 and 32˚C) using 2 and 3 kW titanium heating bars controlled with a CR1000 measurement and control data logger (Campbell Scientific). To ensure constant temperatures, the logger's precision sensors were placed inside each treatment tank. Initially, all tanks were left at 27˚C for 72 h and then temperatures were adjusted gradually (0.2˚C h^{-1}) until reaching the final temperature treatments, to allow sponges to acclimate. Following the acclimation period, four sponge explants were randomly removed from each temperature treatment at 0, 1, 4, 7, 14 and 18 days. Day one sampling commenced 24 h after the 32˚C treatment temperature was reached. All sampled explants were photographed to visually assess tissue health and frozen in liquid nitrogen for molecular analysis. After eight days, sponges in the 32˚C treatment displayed substantial discoloration and deterioration of the tissues and were excluded from the remainder of the experiment (Fig. 4.3). After 14 days, the three remaining temperature treatments were returned to 27˚C for the final four days, serving as a recovery period.

Fig. 4.2 Aquarium set up within the climate-controlled facility at AIMS.

Fig. 4.3 Representative *I. basta* explant from a 32˚C treatment tank after eight days. Scale bar in cm.

4.24 Sediment Experiment

To examine the effects of sedimentation stress on *I. basta* and its associated microbes, sediment was collected from habitat where *I. basta* occurs at Orpheus Island (18˚ 33.617'S, 146˚ 29.077'E) and transported to AIMS where it was dried at 100˚C and sieved to categorize particle sizes, with the final sediment composition consisting of particles ranging between < 63 and ≤ 180 µm (Table 4.1). These particle sizes are consistent with suspended sediment compositions for the inner shelf of the GBR (Bannister 2008). The experimental design comprised four different sediment treatments: 0, 13, 30 and 100 mg l^{-1} (+/- 2 mg l^{-1}). As for the temperature experiment, there were three replicate tanks per treatment holding seven explants each. Treatment tanks were randomly placed around the room and explants were

contained within 14 x 21 cm plastic mesh baskets. Four sponge explants were randomly removed per sediment treatment at 0, 1, 3, 7 and 9 days. All sampled explants were photographed to visually assess tissue health and frozen in liquid nitrogen for molecular analysis. After the day seven sampling, the sediment influx was turned off for the final two days, serving as a short recovery period. Following Negri et al. (2008), 240 l fiberglass stock tanks were used to deliver short, frequent pulses of concentrated stock suspensions of sediments to treatment tanks. The stock tanks were designed with a 45˚ taper at their base to prevent sediment accumulation, ensuring constant rates of delivery. To insure sediment suspension throughout the experiment, an external 2400 l h^{-1} pump (Eheim 1260) was used to circulate the water/sediment suspension from the base of the tank to the top of the tank (Fig. 4.4). The delivery of sediment pulses was achieved by using a second Eheim 1260 pump, which was set on a timer to pulse for 8 sec every 8 min and delivered approximately 120 ml of concentrated sediment stock to the treatment tanks. Each 30 l treatment tank also had an Eheim Compact+ 3000 pump (1500 – 3000 l h^{-1}: Eheim GmbH, Germany) suspended at the waterline to assist with sediment suspension throughout the experiment (Fig. 4.5). Total suspended sediment (TSS) concentrations in the treatment tanks were achieved and maintained by measuring turbidity (NTU) with a TPS 90 FL-T water quality logger (Springwood, QLD, Australia). The turbidity calibration equation was: NTU=0.264 x TSS + 0.05, r^2=0.99.

Table 4.1 Sediment particle compositions added to concentrated stock tanks and used to dose sponges.

Percentage	Particle size
47	>125 µm <180 µm
43	>63 µm <125 µm
10	<63 µm

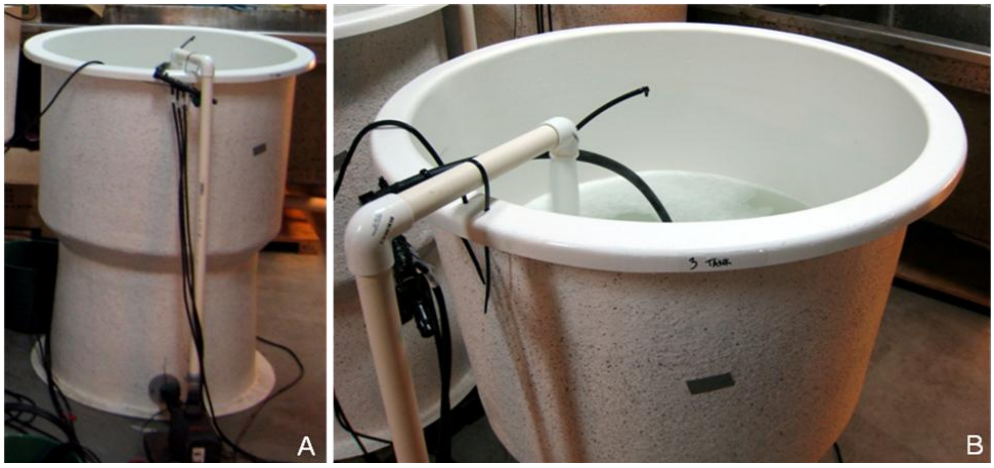

Fig. 4.4 Concentrated sediment stock tank design.

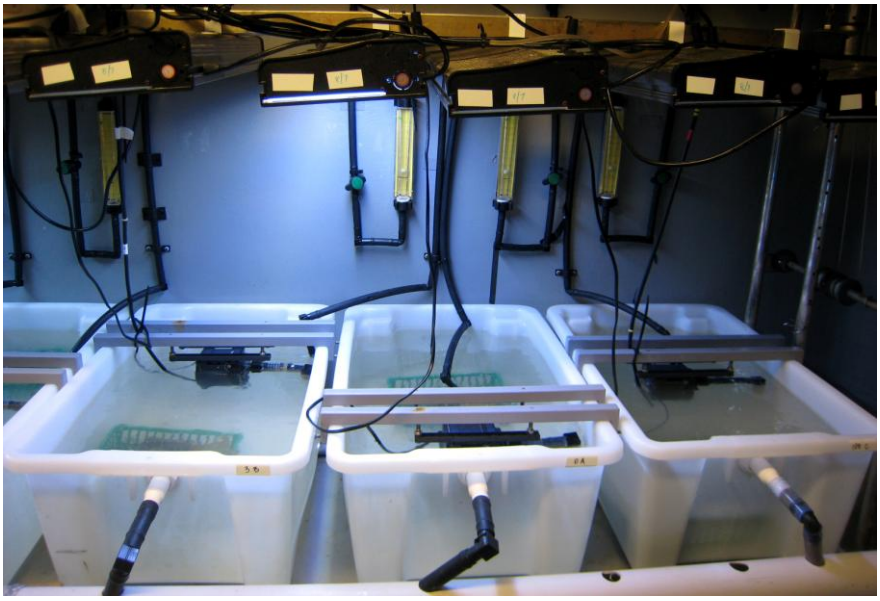

Fig. 4.5 Sediment treatment tanks within the AIMS aquarium facilities. To ensure sediment suspension, pumps were inverted and suspended at the waterline using grey metal bars.

4.25 Antibiotic Exposure Trial

To examine the effects of antibiotic exposure on the microbial community of *I. basta*, five explants were placed in a static system with 10 liters of non-filtered seawater and aeration. Four different antibiotics were added: 10 µg/ml of nalidixic acid, 10 µg/ml of nystatin, 10 µg/ml gentamycin sulphate and 10 µg/ml cycloheximide. This combination of antibiotics was selected based on their ability to eliminate bacteria from coral species (personal communication, David Bourne). As an experimental control, two explants were placed in a separate static system with 10 liters of non-filtered seawater and aeration without the antibiotics. Sponges were monitored for 72 h and tissue samples from surviving explants were frozen in liquid nitrogen and stored at -80˚C prior to molecular analysis. In addition, one explant was frozen in liquid nitrogen immediately after collection, acting as an environmental control.

4.26 DNA Extraction and DGGE

DNA was extracted from all sponge tissue samples from each experiment using the Power Plant DNA Isolation kit, MoBio Laboratories (Carlsbad, CA) according to the manufacturer's protocol. The 16S rRNA gene was amplified by PCR with bacterial primers (1055f: 5'-ATGGCTGTCGTCAGC T-3' and 1392r: 5'-ACGGGCGGTGTGTRC-3') (Ferris et al. 1996). The reverse primer was modified to contain a 40bp GC clamp (Muyzer et al. 1993). PCR reactions contained 5 µl dNTP (2.5 mM), 5 µl 10 x OptiBuffer,0.15 µl of each primer (100 pmol μl^{-1}), 0.4 µl BSA (10 mg ml^{-1}), 3 µl $MgCl_2$ (50 mM), 0.5 µl Bio-X-ACT Taq polymerase (Bioline, London, UK) and 1 µl DNA template. Reactions were made up to 50 µl total volume with Milli-Q water. The PCR conditions were: 1 cycle at 95ºC for 5 min; 30 cycles at 95ºC for 30 sec, 55ºC for 1 min, 70ºC for 1 min; and a final elongation at 70ºC for

10 min. Twenty µl of each sample was added to an 8% w/v polyacrylamide gel containing a 50-70% denaturing gradient of formamide and urea. The gels were run at 60˚C for 16 h in 1 x TAE buffer at 75 V using the Ingeny D-code system, stained with 1 x sybr gold for 10 min, visualized under UV illumination and photographed with the Vilber Lourmat ChemiSmart 3000 system. Reference bands from each gel were excised, re-amplified with PCR and checked for correct mobility on a 50-70% gel. PCR products were sequenced by Macrogen Inc. (Seoul, Korea) using the forward primer (1055f).

4.27 Cloning and Sequencing (Temperature Experiment)

The 16S rRNA gene from three replicate sponges from the 32˚C treatment at day 7 and three replicate control samples was amplified by PCR with universal bacterial primers: 63f 5'-CAGGCCTAACACATG CAA GTC-3' (Marchesi et al. 1998) and 1492r 5'- GGT TACCTTGTTACGACT T -3' (Lane 1991). PCR reactions contained 10 µl 5x MyTaq Buffer, 0.15 µl of each primer (100 pmol μl^{-1}), 0.4 µl BSA (10 mg ml^{-1}), 0.25 µl MyTaq DNA polymerase (Bioline, London, UK) and 1 µl DNA. Reactions were made up to 50 µl total volume with Milli-Q water. The PCR conditions were: 1 cycle at 95ºC for 1 min; 32 cycles at 95ºC for 30 sec, 56ºC for 30 sec, 72ºC for 2 min; and a final elongation at 72ºC for 10 min. PCR products from the control and 32˚C treatments were pooled and then gel purified following the manufacturers protocol (NucleoSpin Extract II, Scientifix). Purified PCR products were cloned with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were checked for inserts by PCR amplification using M13 forward and reverse primers. Restriction digests using HhaI and HaeIII (New England Biolabs Inc.) were performed to determine operational taxonomic units (OTU's) for each library. Triplicates of each representative OTU from both libraries were

sent to Magrogen Inc. (Seoul, Korea) for sequencing using 63f and 1492r as the sequencing primers.

4.28 Phylogenetic Analysis

DGGE and clone sequences were compared with available databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) to determine nearest relatives and percent similarity. Clone sequences were submitted to Genbank under the accession numbers JN388026-JN388033. Due to the repetition of DGGE sequences from each of the experiments and the longitudinal comparison, only one representative band sequence was submitted to Genbank (accession numbers JN388012-JN388025). Sequences were checked for chimera formation using Greengenes (DeSantis et al. 2006) and all chimeric sequences were removed before tree construction. Sequences were compiled, automatically aligned and manually edited in the ARB software package (http://www.arb-home.de (Ludwig et al. 2004)). Initially, trees were calculated with almost complete 16S rRNA (1400 bp) sequences for all close relatives of target sequences using the neighbor-joining and maximum parsimony methods in ARB. Partial sequences were subsequently imported to the tree without changing branch topology using the ARB parsimony-interactive method. The robustness of inferred tree topologies was evaluated after 1000 bootstrap re-samplings of the neighbor-joining data in the PHYLIP program (Felsenstein 1993). *Synechoccocus elongatus* was used as an out group for the tree.

4.29 Data Analysis

Principal Component Analysis (PCA) using a presence (1)/absence (0) matrix for each of the DGGE bands from each of the three experiments and the spatial analysis comparison was

used to analyze microbial communities between treatments. All analyses were carried out using Stastitica 8 (StatSoft 2002).

4.3 Results

4.31 Spatial Analysis

DGGE analysis revealed a relatively low microbial diversity among *I. basta* from each of the three geographical locations, with a total of 20 bands observed across all samples (Fig. 4.6). Overall, samples displayed similar banding patterns, regardless of their geographical location. PCA explained 75.4% of the total variation in the first three factors, with the first two factors explaining 64%. The ordination revealed little variation among samples from the three locations, with the exception of one sample from Orpheus Is. which grouped separately (Fig. 4.7). In addition, there was slightly more variation detected between three individuals from Davies Reef and two individuals from Masig Is., with these sponges grouping further apart (Fig. 4.7).

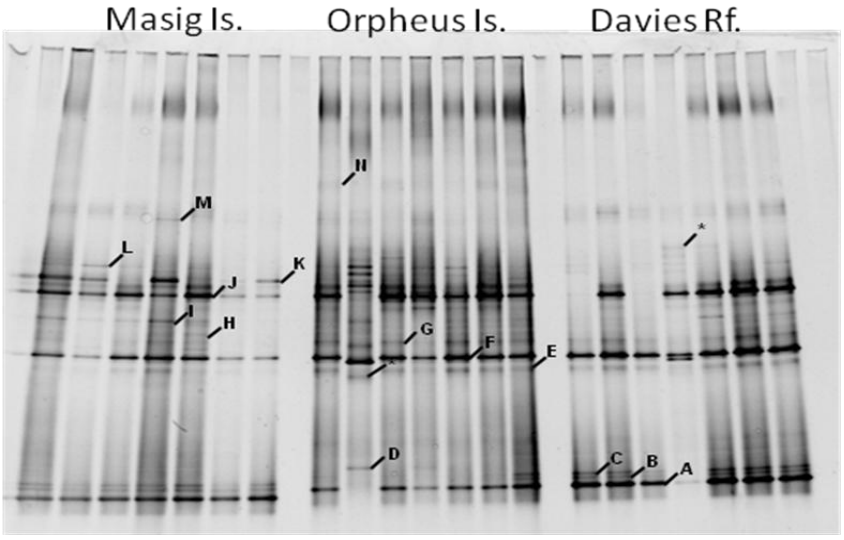

Fig. 4.6 DGGE gel image of 16S rRNA-defined bacterial populations from *I. basta* from three geographical locations. Bands excised for sequencing are labeled on the right hand side of the bands, and astericks (*) denote bands that yielded low sequence quality.

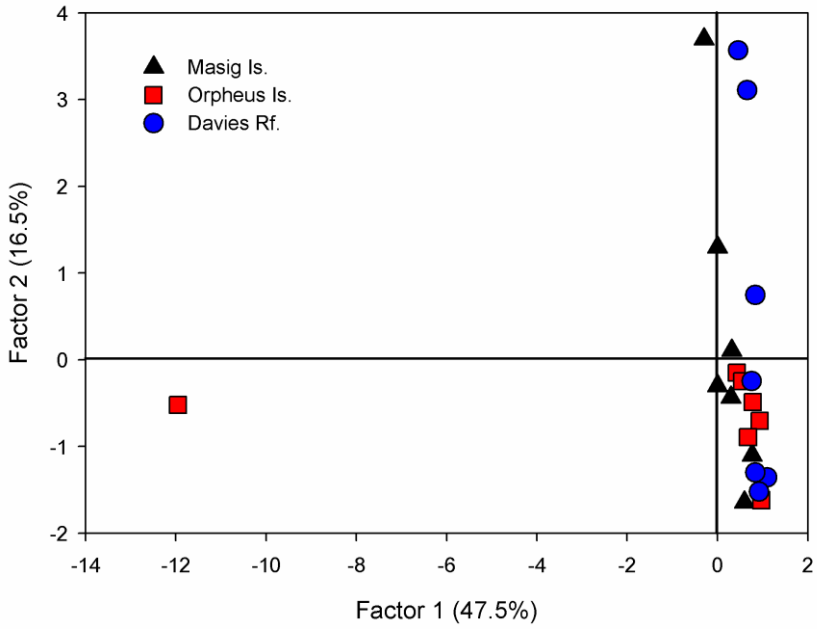

Fig. 4.7 Principal components analysis (PCA) of *I. basta* community composition, using DGGE banding pattern data to construct a similarity matrix of sponges from each of the three geographic locations.

The 16S rRNA sequencing of excised DGGE bands from each of the three geographical locations was the same as previously reported for *I. basta* (Luter et al. 2010a). The majority of the bands were again restricted to the *Alpha* and *Gammaproteobacteria* classes (Table 4.2). However, three bands had highest sequence similarity to a Thaumarchaea from an ascidian (Bands I and L) and a soft coral (Band K). The phylogenetic affiliation of bands labeled with (*) could not be determined due to low sequence quality.

4.32 Temperature Experiment

I. basta explants in all temperature treatments (30, 31 and 32˚C) showed consistent color and gross morphologies to those maintained at 27˚C for the first four days of the experiment, with no evidence of the brown spot lesions described in previous chapters. Explants from the

30˚C treatment survived the full 14 day exposure and subsequent four day recovery period, never changing in appearance from the explants maintained at 27˚C. Up until day seven, explants from the 31˚C treatment also appeared visually similar to control sponges; however, by day 14 they displayed a blackish discoloration, and tissue had retracted resulting in obvious subdermal gaps between skeletal fibers (Fig. 4.8). Although temperature treatments were returned to the control treatment (27˚C) for a recovery period, blackened explants from the 31˚C treatment never returned back to their original yellow color.

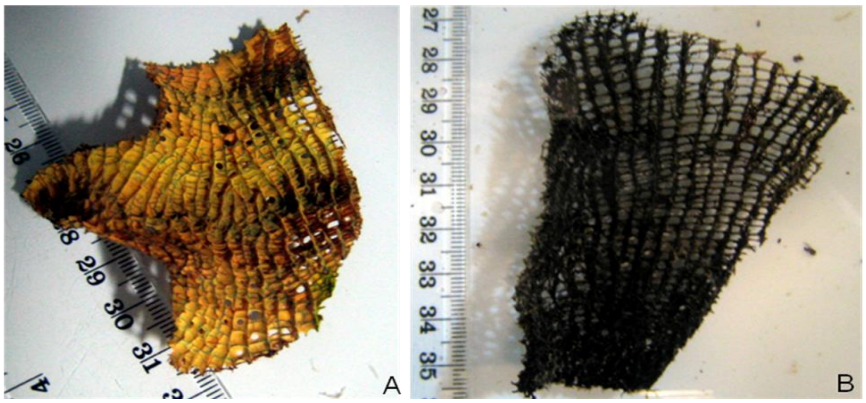

Fig. 4.8 Representative *I. basta* explant from the 27˚C treatment at time 0 (A) and representative *I. basta* explant from the 31˚C treatment at 14 days (B). Scale bar in cm.

DGGE analysis of *I. basta* samples from each of the temperature treatments and time points revealed a relatively low diversity of microbes overall, with 25 bands observed (Fig. 4.9). Comparison between the different treatments and time points revealed consistent banding patterns among samples. There were some exceptions with bands absent in a few samples, while present in others; however, they were never consistently absent/present for all replicates in the treatment or time point. For instance, bands A, B and C were faint or absent from one replicate of the 32˚C treatment at day four, two replicates of the 31˚C treatment at day seven, one replicate of the 27˚C treatment at day 14, one replicate of the 27˚C treatment at day 18 and two replicates of the 31 ˚C treatment at day 18, but present in all other samples

(Fig. 4.9). Due to spatial constraints of the gel, only 46 samples can be visualized at once. Therefore, the 30˚C treatment was excluded from the DGGE analysis.

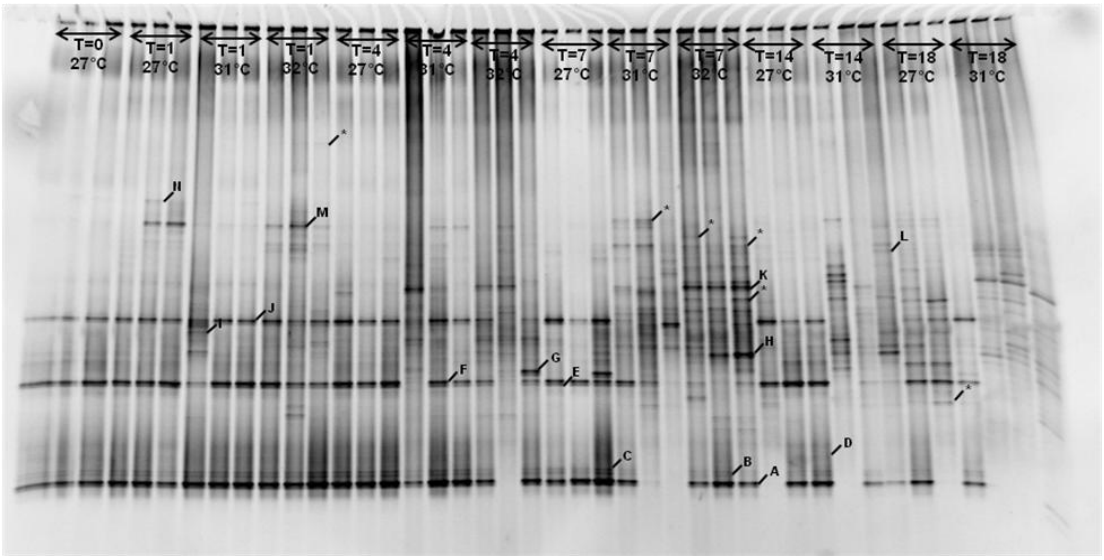

Fig.4. 9 DGGE gel image of 16S rRNA-defined bacterial populations from *I. basta* explants in the 27, 31 and 32˚C treatments over the course of the experiment (T=0, 1, 4, 7, 14 and 18). Bands excised for sequencing are labeled on the right hand side of the bands, and astericks (*) denote bands that yielded low sequence quality.

PCA analysis using a presence/absence matrix derived from the DGGE gel explained 50.5% of the total variation in the first three factors, with the first two factors explaining 41.3%. The ordination showed a notable shift in the microbial community composition of *I. basta* explants from the 32˚C treatment at day seven, with all three replicates clustering separately (Fig. 4.10), although this shift may have been an artifact of slightly more faint bands in these three replicates (Fig. 4.9). Sponges from the 32˚C temperature treatment did not survive past this time point (T=7). Overall, the community composition of *I. basta* explants remained stable over all temperatures for the first four days of the experiment, with all samples clustering together (Fig. 4.10). Nevertheless, a larger variation was detected between samples from the remaining temperatures from seven days. With the exception of *I.*

basta explants at 27˚C at day seven, there was larger variation between the three individual replicates for each of the temperature treatments after day four (Fig. 4.10).

The 16S rRNA sequencing of excised DGGE bands from the temperature experiment were the same as previously reported for *I. basta* (Luter et al. 2010a), with all three dominant symbionts present. The majority of the bands were again restricted to the *Alpha* and *Gammaproteobacteria* classes (Table 4.2). However, three bands had highest sequence similarity to a Thaumarchaea from an ascidian (Bands I and L) and a soft coral (Band K). The phylogenetic affiliation of bands labeled with (*) could not be determined due to low sequence quality.

Table 4.2 Sequence similarity in excised 16S rRNA DGGE bands from *I. basta* using BLAST.

Band ID	Accession Number	Percent Similarity	Description
A	GQ347593	91	α-*proteobacteria* (Oceanic dead zone)
B	FJ205252	89	α-*proteobacteria* (Deep sea hydrothermal region)
C	GQ347593	91	α-*proteobacteria*, (Oceanic dead zone)
D	FJ205252	90	α-*proteobacteria* (Deep sea hydrothermal region)
E	GQ274301	94	γ-*proteobacteria* (marine biofouling sample)
F	GQ348745	95	γ-*proteobacteria* (oceanic dead zone)
G	FM242455	94	γ-*proteobacteria* (coastal sediment)
H	GQ204920	93	γ-*proteobacteria* (hard coral)
I	JF733387	99	*Pseudomonas* sp. (adult black fly)
J	EU283427	96	Thaumarchaeota (ascidian)
K	AJ876989	96	Thaumarchaeota (soft coral)
L	EU283427	95	Thaumarchaeota (ascidian)
M	EU182114	99	unidentified bacterium (sediment and seawater, South China Sea)
N	HQ436843	96	unidentified bacterium (Lake Bosten, China)

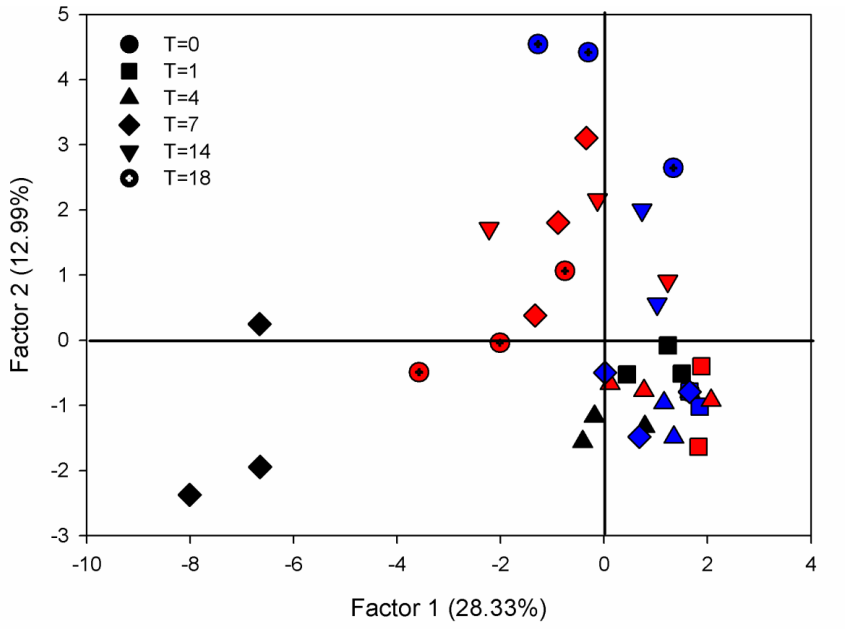

Fig. 4.10 Principal components analysis (PCA) of *I. basta* community composition, using DGGE banding pattern data to construct a similarity matrix, at 27 (blue), 31 (red) and 32˚C (black). Symbols correspond to the different time points.

Clone library analysis of *I. basta* samples from the 32˚C treatment at day seven and control samples were consistent with DGGE results displaying low microbial diversity overall. All clone sequences fell into only two classes, the *Alpha-* and *Gammaproteobacteria*, with both libraries dominated by a single *Alphaproteobacteria* sequence (clone 8). This *Alphaproteobacteria* sequence accounted for 96% of the control library and 31% of the 32˚C library. The remaining 4% of the control library was made up of a single *Gammaproteobacteria* sequence (clone 5), which was absent in the 32 ˚C library. The 32 ˚C library was more diverse than the control library containing two additional *Alphaproteobacteria* sequences (Clones 6 & 7) and four *Gammaproteobacteria* sequences (Clones 1 to 4). The *Alphaproteobacteria* sequences combined made up 53% of the 32˚C library, while the *Gammaproteobacteria* sequences combined made up the remaining 47% (Fig. 4.11).

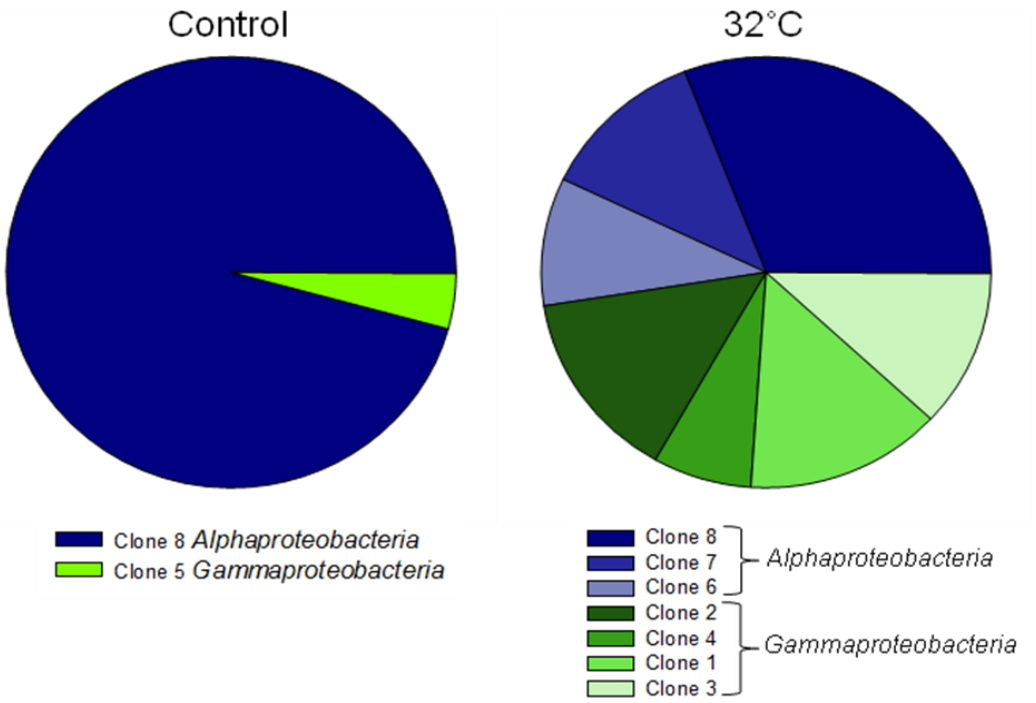

Fig. 4.11 Pie charts showing the differences in bacterial community composition in *I. basta* between control samples and samples from the 32˚C treatment at day seven. The graphs were constructed based on the frequency of clones from each library. Clone 8 had identical 16S rRNA sequences in both libraries.

Phylogenetic analysis revealed that clone 8 was 99% similar to the previously described *Alphaproteobacteria* symbiont from healthy and diseased *I. basta* (GU784988) (Luter et al. 2010a). The next closest relatives of clone 8 showed only 86% similarity, comprising sequences from seawater and sediment samples, as well as other sponge and coral-derived sequences (Fig. 4.12). Clone 5, only observed in the control library, was 100% similar to the previously described dominant *Gammaproteobacteria* from healthy and disease *I. basta* (GU784985) and also showed close similarity to a sequence recovered from the sponge *Axinella verrucosa* (AJ581351). Clones 6 and 7, found in the 32˚C library, were both most closely related to other sponge and coral-derived sequences (Fig. 4.12). The additional

Gammaproteobacteria sequences observed in the 32˚C library were all most closely related to other environmental sequences. In particular, clone 1 was most closely related to a sequence retrieved from the coral *Montipora* sp. (AB470941) while clone 2 was most closely related to *Spongiobacter nickelotolerans* (AB205011) isolated from a marine sponge. Clone 3 was most closely related to an Alteromonadiaceae bacterium (AM9535070) isolated from hydrocarbon-contaminated soil. Lastly, clone 4 was most closely related to two seawater clones from Taiwan and the Yellow Sea, FJ347760 and GU061307 respectively.

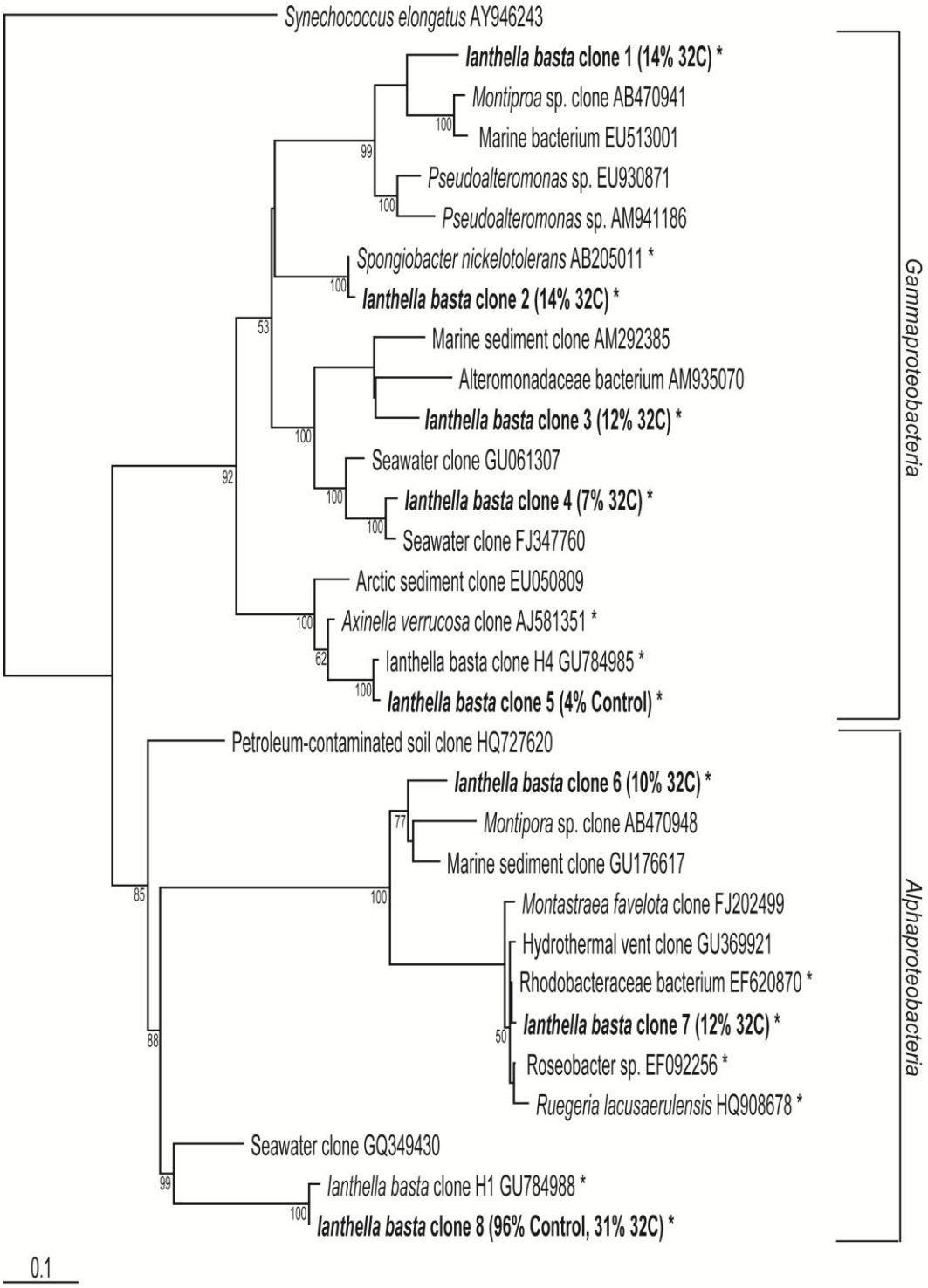

Fig. 4.12 Maximum-likelihood phylogenetic tree from analysis of all 16S rRNA gene sequences retrieved from clone library analysis. *I. basta* sequences from this study indicated by bold font, with the percentage of each library it comprised listed in parenthesis afterwards and * indicates clones isolated from marine sponges. The numbers at the nodes are percentages indicating the levels of bootstrap support based on analysis of 1000 re-sampled data sets. Only values > 50% are shown. Scale bar represents 0.1 substitutions per nucleotide position.

4.33 Sediment Experiment

I. basta explants in all sediment treatments (13 to 100 mg l^{-1}) survived the seven day exposure and further two day recovery period and appeared visually similar to those explants maintained at 0 mg l^{-1} (Fig. 4.13A,B). Explants from the 30 and 100 mg l^{-1} treatment did accumulate sediments on their surfaces (Fig. 4.13C); however, tissue health remained similar to explants maintained at 0 mg l^{-1} when sediments were manually removed prior to photographing. None of the sponge clones developed brown spot lesions during the course of the sedimentation experiment.

The DGGE analysis of sponges in the sediment treatments at each time point also revealed low microbial diversity, with a total of 34 bands observed (Fig. 4.14). Consistent with the DGGE analysis from the temperature experiment, some individuals are missing bands that are present in other samples; although, these bands are never consistently observed in all replicates from a treatment or a time point. Due to spatial constraints of the gel, only 46 samples can be visualized at once. Therefore, the 13 mg^{-1} treatment was excluded from the DGGE analysis.

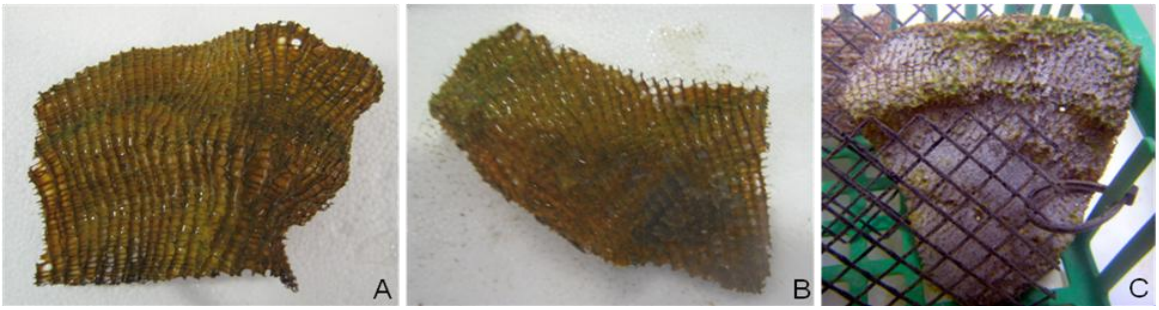

Fig. 4.13 Representative *I. basta* explant from the 0 mg l^{-1} treatment at time 0 (A), representative *I. basta* explant from the 100 mg l^{-1} treatment at seven days (B) and *I. basta* explant in a 30 mg l^{-1} treatment tank, depicting the sediment accumulation (C).

PCA analysis of the microbial community composition of *I. basta* in each sediment treatment and time point explained 47.8% of the total variation in the first three factors, with the first two factors explaining 37.7%. In general, there was a large amount of variation between individuals, regardless of the sediment treatment or time point (Fig. 4.15). There was clustering of samples from the 0 and 30 mg l^{-1} treatments at day one and seven; although, not all replicates were observed (Fig. 4.15).

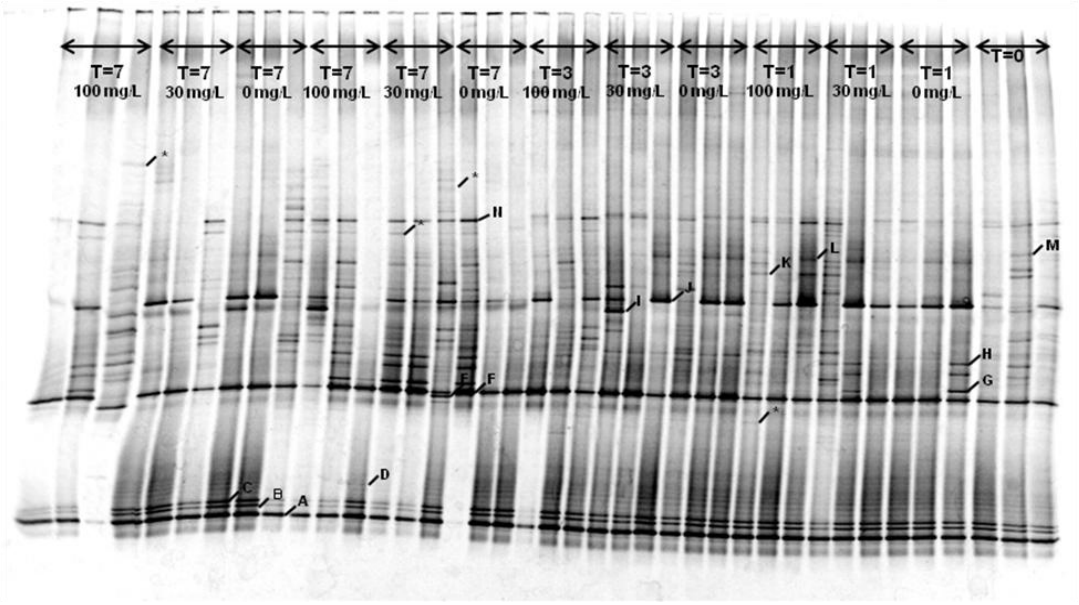

Fig. 4.14 DGGE gel image of 16S rRNA-defined bacterial populations from *I. basta* explants in the 0, 30 and 100 mg l^{-1} treatments over the course of the experiment (T=0, 1, 4 and 7). Bands excised for sequencing are labeled on the right hand side of the bands, and astericks (*) denote bands that yielded low sequence quality.

The 16S rRNA sequencing of excised DGGE bands from the sediment experiment was the same as those from the temperature experiment. In addition, all sequences retrieved were identical to those previously reported for *I. basta* (Luter et al. 2010a). All but three bands, which had highest similarity to a Thaumarchaea from an ascidian and soft coral, were representatives from either the *Alpha* or *Gammaproteobacteria* (Table 4.2). Again, DGGE

bands from *I. basta* had low similarity to sequences in the NCBI database with band H being the only sequence with a similarity >96%.

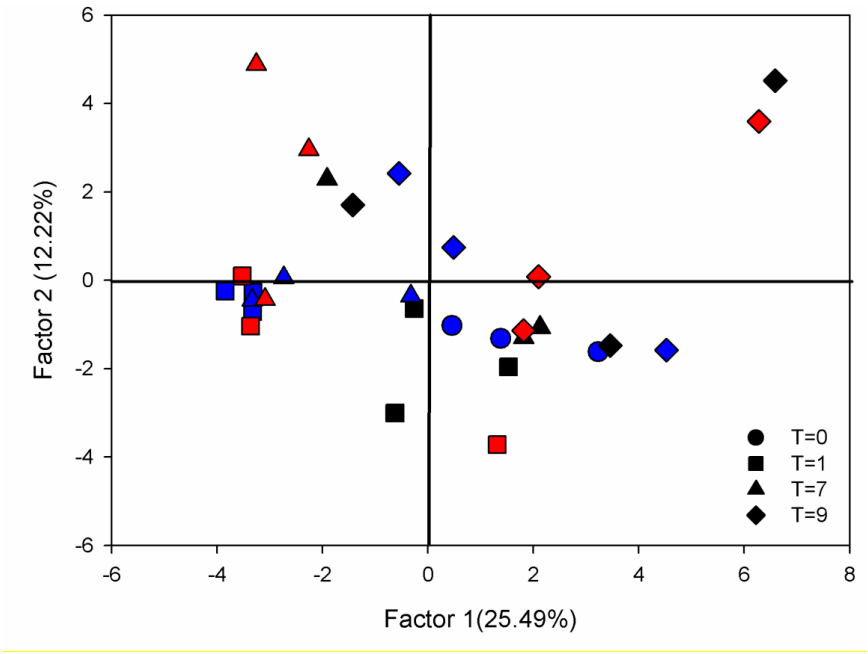

Fig. 4.15 Principal components analysis (PCA) of *I. basta* community composition, using DGGE banding pattern data to construct a similarity matrix, at 0 (blue), 30 (red) and 100 mg l^{-1} (black). Symbols correspond to the different time points.

4.34 Antibiotic Experiment

All sponge explants in the antibiotic treatment survived the 72 h exposure, never changing in appearance from the treatment controls. Consistent with DGGE analyses from the other experiments in this study, sponge explants from the antibiotic treatment, treatment controls and the environmental control all revealed low microbial diversity, with only 20 bands observed (Fig. 4.16). PCA explained 80% of the variation in the first three factors, with the first two factors explaining 65.6%. The ordination did not show any distinct clustering between controls and antibiotic exposed explants (Fig. 4.17). Two of the explants exposed to antibiotics displayed very similar banding patterns, grouping very closely in the ordination (Fig. 4.17).

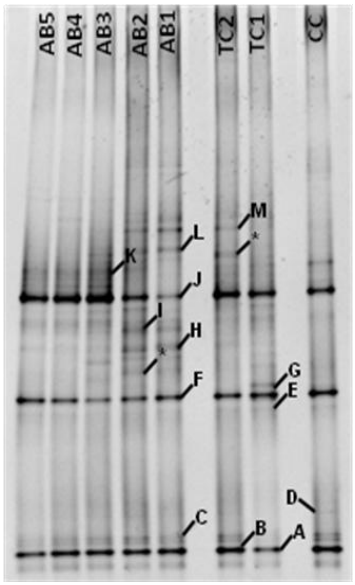

Fig. 4.16 DGGE gel image of 16S rRNA-defined bacterial populations from *I. basta* explants from the antibiotic treatment (AB1 to AB5B), the treatment controls (TC1 & TC2) and the environmental control (CC). Bands excised for sequencing are labeled on the right hand side of the bands (aside from band D, which is on the left hand side of the band), and astericks (*) denote bands that yielded low sequence quality.

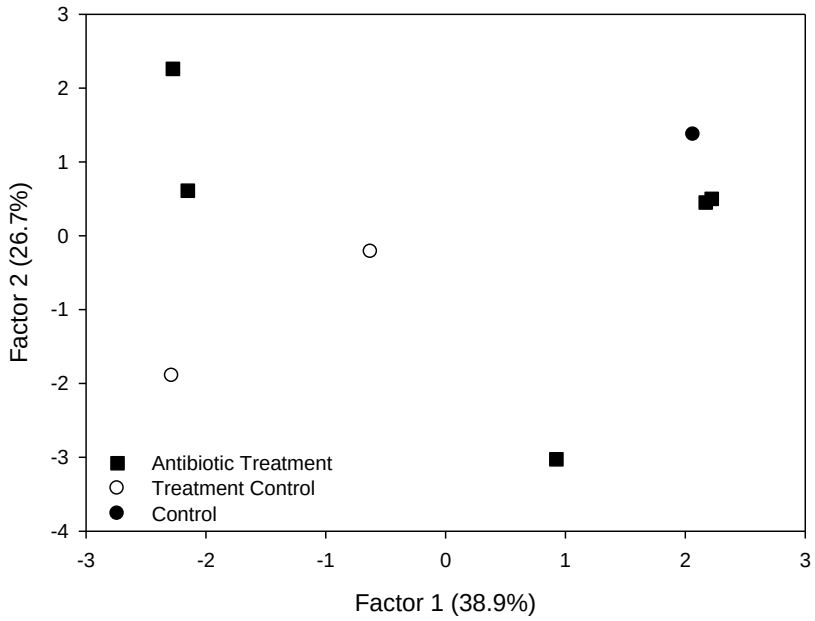

Fig. 4.17 Principal components analysis (PCA) of *I. basta* community composition, using DGGE banding pattern data to construct a similarity matrix of explants in the antibiotic exposure experiment.

4.4 Discussion

I. basta do not tolerate seawater temperatures of 32˚C, only 2-4 degrees higher than the average summer temperatures recorded for Orpheus Is. (Berkelmans et al. 2004) where the sponges were collected. *I. basta* exposed to this elevated temperature displayed substantial discoloration and deterioration of the tissues, eventually dying after eight days. After 14 days, similar tissue discoloration and reduction was also noted in sponges exposed to 31˚C. In contrast, control sponges retained their original yellow color and tissue state. While some sponge species display bleaching similar to that of corals (Fromont & Garson 1999; López-Legentil et al. 2008; López-Legentil et al. 2010), relatively little is known about how temperature stress affects sponge-microbe symbioses. The hard coral *Acropora elseyi* expels its associated zooxanthellae at the same temperature which negatively affected *I. basta* (Berkelmans & Willis 1999), indicating that thermal thresholds for maintenance of symbiosis between corals and sponges may be similar.

In addition to the change in physiological appearance, a shift in the microbial community composition of *I. basta* was detected in sponges from the 32˚C treatment after seven days. This shift was indicative of a higher diversity within the *Alpha-* and *Gammaproteobacteria* in sponges from the 32˚C treatment. The dominant *Gammaproteobacteria* previously described in healthy and diseased *I. basta* (Luter et al. 2010a) was absent in the 32˚C library, but still detected by DGGE in sponges treated at 32˚C for seven days. It should also be noted that this strain was observed in lower abundance in the control library than what had previously been reported (4% versus 18% respectively), suggesting that additional sequencing will reveal its presence in the 32°C library. Similar holobiont (host-symbiont complex) responses to temperature stress were previously reported in sponges, with both studies showing distinct microbial community shifts and decreased host fitness at elevated temperatures (Lemoine et al. 2007; Webster et al. 2008a). Whilst

additional bacterial groups such as *Bacteroidetes*, *Epsilon*- and *Deltaproteobacteria*, commonly occur after stress in both sponges and corals (Frias-Lopez et al. 2002; Pantos & Bythell 2006; Webster et al. 2008b), this was not the case in heat stressed *I. basta*.

The associated microbes of *I. basta* were stable at all temperature treatments for the first 4 days of the experiment. In contrast, community shifts were detected within the first 24 h in the sponge *Rhopaloeides odorabile* at the highest temperature treatment (Webster et al. 2008a). Discrepancies between these two studies suggest that the microbial community of *I. basta* may be more stable and therefore able to endure elevated temperatures for a longer duration. Alternatively, the microbial shift may be tightly linked to host health with *I. basta* tolerating longer thermal stress periods than *R. odorabile*, allowing for the maintenance of the symbiosis. Interestingly, a qPCR study of the heat stressed *R. odorabile* showed a rapid down regulation of genes at 31˚C and 32˚C within 24 h (Pantile & Webster 2011), coinciding with the loss of the dominant culturable symbiont and community shift (Webster et al. 2008a). It would be valuable to explore gene expression patterns in heat stressed *I. basta* to ascertain whether an up or down regulation of genes occurs at the same time as the microbial shift.

Sponges can use chemical production to maintain their inherent microbial community, as well as combating microbial attack (Kelman et al. 2001). *I. basta* produce a class of compounds (the bastadins) that display strong antimicrobial properties (Pettit et al. 1996). DGGE analysis revealed a larger diversity of bands in moribund samples from 32˚C at day seven than from 32˚C at day four (25 vs. 17). It is possible that this shift and increased diversity coincides with the breakdown in *I. basta*'s antimicrobial compound production, allowing the proliferation of a greater number of opportunistic bacteria prior to sponge mortality.

I. basta explants exposed to high levels of sediments (100 mg l^{-1}) looked visually similar to those maintained at 0 mg l^{-1}, indicating this sponge is capable of withstanding high levels of sediment exposure, at least in the short term. Surprisingly, despite the potential pumping implications from the high amounts of sediment on the sponge surface (Reiswig 1971a; Gerrodette & Flechsig 1979; Bannister 2008), *I. basta* showed no visible adverse effects. Conversely, coral tissues exposed to high sedimentation rates show decreased photosynthetic activity and necrosis (Philipp & Fabricius 2003). The ability of *I. basta* to cope with short term sediment stress is likely linked to their habitat and distribution, which includes inshore silty patches and fringing reef slopes (Bergquist & Kelly-Borges 1995; Luter et al. 2010b). No microbial community shifts were detected in sponges from any of the sediment treatments or time points. While previous studies have documented the negative impacts of increased sedimentation on host pumping rates (Reiswig 1971a; Gerrodette & Flechsig 1979; Bannister 2008) and metabolism (Bannister 2008), this is the first study to assess the effects of increased sedimentation on the microbial community of the host. There were a total of 34 bands observed in the DGGE from the sediment experiment. In contrast, only 20 bands were observed in the DGGE from the spatial analysis comparison and 25 bands from the temperature DGGE. This higher diversity of bands is most likely due to foreign microbes coming from the sediments themselves.

Sponge microbial communities are generally stable across temporal and spatial scales (Friedrich et al. 2001; Taylor et al. 2004; Webster et al. 2004; Taylor et al. 2005). The microbial community of *I. basta* is no exception, with similar communities observed in sponges from three separate locations across a latitudinal gradient. Further support for the stability of the associated microbes of *I. basta* was provided by an antibiotic exposure experiment which was ineffective at shifting the community. Likewise, the microbial

community of the Mediterranean sponge *Aplysina aerophoba* was unaffected by antibiotic exposure (Friedrich et al. 2001), indicating a highly stable microbial community.

I. basta consistently exhibits a low microbial diversity, regardless of its disease status (Luter et al. 2010a), the stressor applied or the geographical location it inhabits. In all instances, only members of the *Alphaproteobacteria*, *Gammaproteobacteria* and Thaumarchaea are observed. *I. basta* has a low-medium microbial abundance in comparison to high microbial abundance (HMA) sponges, which host extremely large numbers of microbes (Hentschel et al. 2006). The mechanisms by which sponges acquire their symbionts are still a matter of debate (reviewed in Taylor et al. 2007 and Webster and Taylor 2011). An interesting hypothesis for low microbial abundance (LMA) sponges is they may have once had more bacteria types associated with their tissues and subsequently lost them. If this is this the case, it would suggest *I. basta* has been selective in retaining its symbionts. Other benthic invertebrates have been known to selectively choose certain bacteria types which serve a beneficial role, such as the bobtail squid and *Vibrio fischerii* (Ruby 1996) and corals and their associated DMS and DMSP degrading bacteria (Raina et al. 2010).

Overall, the microbial community of *I. basta* is stable across a latitudinal gradient and under varying stressors. Even though a shift was detected at the highest temperature treatment at day seven which corresponded to sponge mortality, the community remained stable at this elevated temperature for the first four days, with the three dominant symbionts always observed (*Alphaproteobacteria*, *Gammaproteobacteria* & Thaumarchaea). Given this apparent stability in the microbial community of *I. basta*, it is likely these microbes play an important functional role(s) in this sponge. Neither increased temperature nor sedimentation was successful at inducing disease-like symptoms (brown spot lesions) observed in *I. basta* in the field (Chapter 2 & 3); therefore, an environmental origin of the syndrome is unlikely.

Chapter 5: Tissue Regression and Recovery in *Ianthella basta*[3]

5.1 Introduction

Sponges are among the oldest of the Metazoans with fossils dating back to the Precambrian, approximately 600 million years ago (Li et al. 1998). Sponges are often described as having a simplistic body plan because they were thought to lack distinct tissues and organs (Simpson 1984). However, growing evidence suggests that sponges have a higher level of complexity than traditionally thought. For example, sponges are equipped with functional immune and apoptotic systems (Müller 2003) typical of other integrated animals. Some sponges also possess up to 5 different types of epithelia tissue (Leys et al. 2009) and others have displayed compartmentalization of cells (e.g. choanocytes- de Goeij et al., 2009) , challenging the view that sponges lack distinct tissues and organization. In addition, some sponge cells (e.g. archaeocytes) are believed to be totiopotent (Müller 2006). The recently published genome of *Amphimedon queenslandica* confirms that although sponges lack structures such as neurons and muscles, they posses genes central to the form and function of higher animals (Srivastava et al. 2010). The complex cellular biology of sponges is likely to be a key contributor to their ability to occupy a wide range of environments and associated conditions (Fafanđel et al. 2003).

Sponges have the capacity to recover from damage caused by predation (Ayling 1983), physical disturbances (Wulff 2006a; Wulff 2010), environmental stress (Leamon & Fell 1990) and disease (Rützler 1988). Indeed, some sponges can initiate cellular recovery of the pinacoderm within 24 h of injury, with complete recovery occurring within weeks

[3] **Chapter 5 adapted from Luter HM, Whalan S, Webster NS (2011) Tissue regression and recovery in the sponge *Ianthella basta*. Hydrobiologia *In press*.**

(Louden et al. 2007), highlighting the ability of sponge cells (e.g. archaeocytes) to migrate and proliferate (Simpson 1984). In addition, sponges also display high telomerase activity (Koziol et al. 1998), potentially aiding in rapid cell proliferation and further contributing to the success of cellular processes in recovery from sublethal damage. Although sponges lack a true nervous system, they have the capacity to produce non-neural reflex behaviors (Meech 2008). For instance, sponges can contract their tissues and canal systems in efforts to cope with sediment stress (Leys & Meech 2006) and predator attacks (Nickel 2004). Given the clear impacts of climate change on coral reef communities (Hughes et al. 2003), the ability of sponges to recover from environmental stressors will become even more important.

Although some sponges undergo "choanocyte shedding" and cell loss under ambient environments (de Goeij et al. 2009), some species respond to environmental stress by contracting their tissues to skeletal structures leaving subdermal spaces (Knight & Fell 1987; Imsiecke et al. 1996). This process reduces the area of tissue in contact with the surrounding water thereby providing a buffer to suboptimal water conditions (Francis et al. 1990) such as fluctuating salinity (Knight & Fell 1987; Leamon & Fell 1990). Our knowledge of how sponges respond to stressors (including the process of short term tissue regression) continues to grow, particularly our understanding of the cellular pathways for this process (Fell 1974; Imsiecke et al. 1996). In comparison, our knowledge of microbial symbiotic responses to this process is limited (but see Thoms et al. 2008).

Sponges often form intimate associations with microbes, with representatives of 25 bacterial phyla and both major lineages of *Archaea* being reported to date (Taylor et al. 2007; Webster and Taylor 2011). In some cases, microbes can account for up to 40% of the total sponge biomass (Vacelet & Donadey 1977), though some sponges also have low microbial abundances (Hentschel et al. 2006). It has been documented that sponges from

geographically different locations can host similar microbial communities (Hentschel et al. 2002; Taylor et al. 2005). While this uniformity exists, microbial communities of sponges under stressful conditions such as elevated seawater temperatures (Lemoine et al. 2007; López-Legentil et al. 2008; Webster et al. 2008a), heavy metal stress (Webster et al. 2001b; Selvin et al. 2009) and disease (Olson et al. 2006; Webster et al. 2008b; Angermeier et al. 2011) have been shown to change and this shift often correlates with a decline in host health.

The current study explores tissue regression and recovery of the marine sponge *Ianthella basta*, documenting morphological and histological differences, as well as investigating the microbial symbionts in sponges with regressed and recovered tissues.

5.2 Materials and Methods

5.21 Sponge Collection

Six necrotic specimens (including substrate) of the marine sponge *I. basta* were collected at Orpheus Is., northeastern Australia (18º 36.878' S, 146º 29.990' E). All six specimens were categorized as health-compromised, displaying some level of tissue necrosis and brown spot lesions, as described in Luter et al. (2010a,b). In addition, 12 healthy *I. basta* explants were collected. The explants had been previously cut from donor sponges using a scalpel blade (approximately 10 cm^3), fixed to the reef in an Aquapurse basket (TTP plastics by design; Brisbane, Queensland, Australia) and allowed to heal for 12 weeks before collection. Intact sponges and explants were transported to the Australian Institute of Marine Science and kept in a 1,000 l flow-through outdoor aquarium with unfiltered seawater, under a natural day/night light regime. Explants were contained in a plastic mesh basket within the aquarium. The flow rate was maintained at 600 ml min^{-1} and the temperature was kept at 28 ˚C, the same temperature as Orpheus Is. at this time of year. All sponges were closely monitored and the 6 necrotic sponges photographed daily for five days. In addition to visual

comparisons of the necrotic sponges, the images were compared using the integrated density (ID) measurement of Image Tool for Windows (UTHSCA), which calculates the mean gray level of the image and multiplies that by the number of pixels in the image.

Six *I. basta* explants displaying regressed tissues were sampled at 12 hrs. The remaining 6 explants were sampled at 72 hrs, when sponges had recovered. Explants were used in all histological and molecular analyses.

5.22 Histology

Samples from both regressed and recovered *I. basta* were fixed in 10% formalin with phosphate buffered saline (PBS) for 24 h at 4˚C and then transferred to 70% ethanol and stored at -20˚C for four weeks. Samples were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned to 5 μm and stained using Mayer's Haematoxylin and Young's Eosin Erythrosin (Bancroft & Stevens 1990). Samples were viewed and photographed using the Olympus DP12 Microscope Digital Camera System (Melville, NY). All 12 sections examined (n=6 regressed tissue, n=6 recovered tissue) were cut through a similar plane of the sponge sample (longitudinal). Ten fields of view per section were examined to quantify the total number of choanocyte chambers at 400 x magnification. For the purpose of this study, choanocyte chambers were defined as a circular ring of cells.

5.23 DNA Extraction and DGGE

DNA from *I. basta* explants (n=6 regressed tissue, n=6 recovered tissue) was extracted according to manufacturer's protocol with a Power Plant DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The 16S rRNA gene of all samples was amplified by PCR with universal bacterial primers (1055f, 5'-ATGGCTGTCGTCAGCT-3'; 1392r, 5'-ACGGGCGGTGTGTRC-3') (Ferris et al. 1996). The reverse primer was modified to

contain a 40-bp GC clamp (Muyzer et al. 1993). PCRs contained 5 µl of deoxynucleoside triphosphate (2.5 mM), 5 µl of 10 X OptiBuffer, 0.15 5 µl of each primer (100 pmol μl^{-1}), 0.4 µl of bovine serum albumin (BSA; 10 mg ml^{-1}), 3 µl of $MgCl_2$ (50 mM), 0.5 µl of Bio-X-ACT *Taq* polymerase (Bioline, London, United Kingdom), and 1 µl of DNA template. Reactions were made up to 50 µl of total volume with Milli-Q water. The PCR conditions were as follows: 1 cycle at 95˚C for 5 min; 30 cycles at 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 min; and a final elongation at 72˚C for 7 min. Fifteen µl of each sample was added to an 8% (wt/vol) polyacrylamide gel containing a 50 to 70% denaturing gradient of formamide and urea. The gel was run at 60 ˚C for 16 h in 1 x TAE buffer at 75 V using the Ingeny D-Code system, stained with 1 x SYBR gold for 10 min, visualized under UV illumination and photographed with the Viber Lourmat ChemiSmart 3000 system.

5.24 Statistical Analysis

Assumptions of normality were unable to be met, despite transformation (Zar 1999). Therefore, the non-parametric Kruskal-Wallis test was performed to determine if there was a significant difference in choanocyte chamber numbers between sponges with regressed and recovered tissues. Principal Component Analysis (PCA) using a presence (1)/absence (0) matrix created from the DGGE bands was used to analyze microbial community composition between sponges with recovered and regressed tissue. All analyses were performed using Statistica 8 (StatSoft 2002).

5.3 Results

Less than 12 h after being transferred to the outdoor flow-through aquarium, all six necrotic sponges and 12 healthy explants displayed reductions in tissue volume, where the tissue had contracted to central skeletal fibers leaving subdermal gaps between sponge fibers (Fig.

5.1A). Within 72 h sponges had regained the lost tissue volume and recovered the subdermal gaps between skeletal fibers. The integrated density of the sponge tissue effectively doubled within 72 h (increasing by 92%), confirming tissue recovery in *I. basta* (Fig. 5.1A, B).

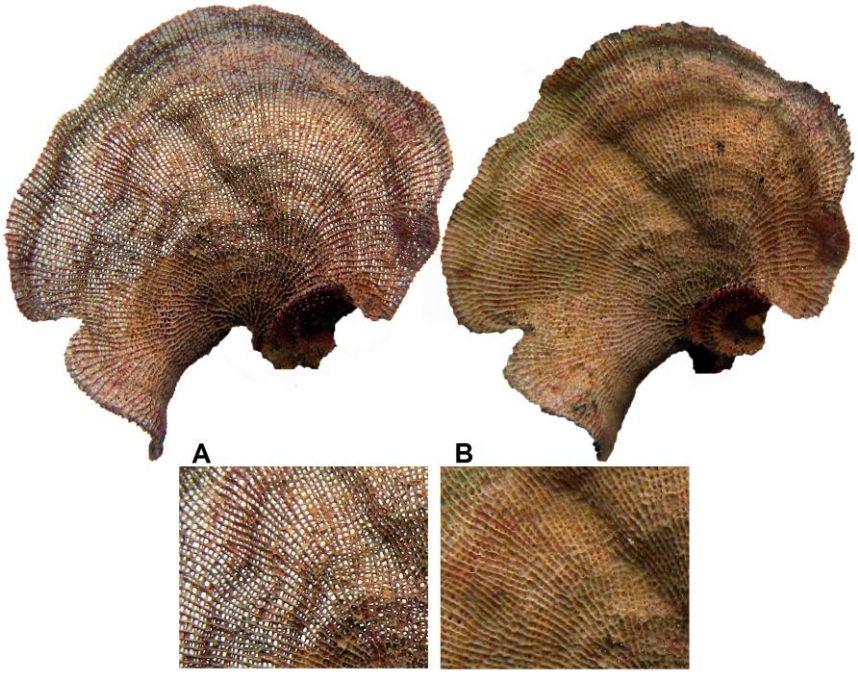

Fig. 5.1 Rapid tissue recovery exhibited by the same *I. basta* individual in a regressed state at time 0 (A), and a recovered state after 72 h (B).

5.31 Histological Analysis

Sponges exhibiting regressed tissues had a markedly different histology than sponges with recovered tissues. The arrangement of cells in regressed samples was more compact and densely packed around the sponge fibers than in recovered tissues (Fig. 5.2 A, B). In addition, regressed samples had a larger number of granulated cells (Fig. 5.2 A, B). Sponges with regressed tissue also had significantly fewer apparent choanocyte chambers than samples with recovered tissue ($H=79.6$, $p=0.000$). For example, the mean number of

choanocyte chambers per field of view in sponges with recovered tissues was 4.5 (± 0.7 S.E.), compared to 0.4 (± 0.1 S.E.) in sponges with regressed tissues. In addition, the choanocyte chambers observed in samples with regressed tissues often contained damaged and/or missing cells (Fig. 5.2 B). In contrast, choanocyte chambers of samples with recovered tissues showed little cellular degradation or cell loss (Fig. 5.2 C, D).

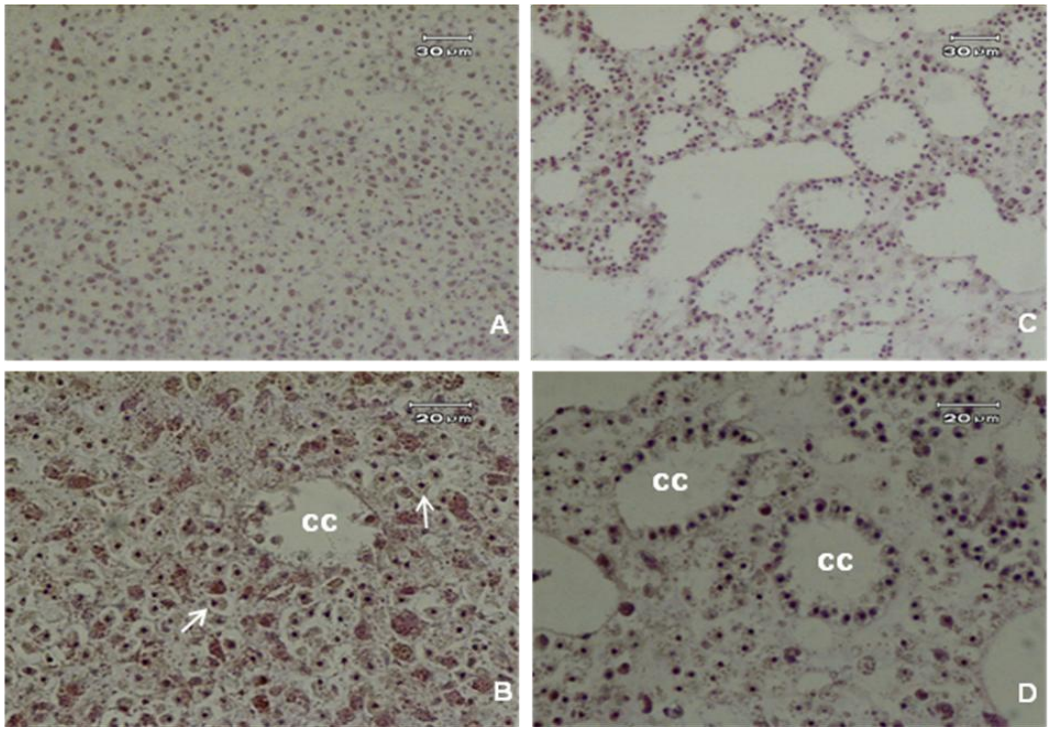

Fig. 5.2 Histological images of *I. basta* exhibiting regressed (A, B) and recovered tissues (C, D). Arrows indicate examples of granulated cells and cc: choanocyte chamber.

5.32 DGGE

DGGE analysis comparing regressed and recovered *I. basta* revealed low microbial diversity overall, with only 22 unique bands observed (Fig. 5.3). Both regressed and recovered samples revealed the presence of three dense bands, which correspond to an

Alphaproteobacteria (A), *Gammaproteobacteria* (B) and *Thaumarchaea* (C) previously reported as stable symbionts of *I. basta* (Luter et al., 2010a). Band D (JN388034) is a cyanobacterium with 98% similarity to an uncultured cyanobacterium recovered from cold spring sediment (GQ302542). Whilst this band appeared brighter in some regressed tissue samples, DGGE is not a quantitative technique and band D had previously been detected at similar intensities in healthy *I. basta* (Luter et al., 2010a). Principle Component Analysis based on the DGGE banding patterns of microbial communities between regressed and recovered tissues explained 79% of the variability in the first three factors, with the first two

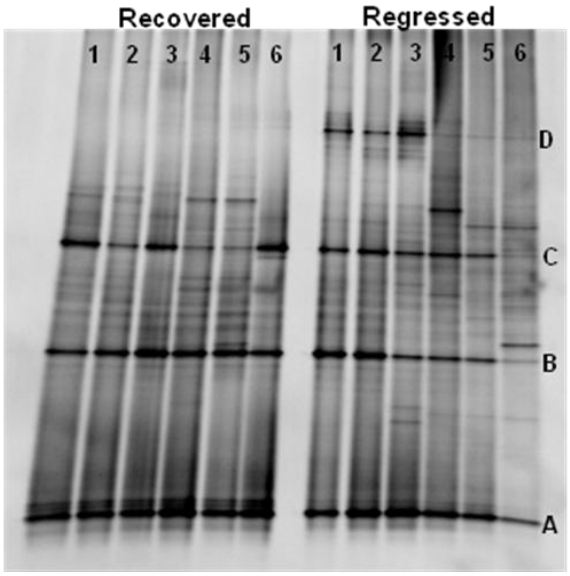

Fig. 5.3 DGGE image of 16S rRNA-defined bacterial populations from *I. basta* samples with regressed and recovered tissues. Bands labeled A-C represent the stable symbionts of *I. basta* previously reported. Band D is a cyanobacterium with 98% similarity to an uncultured cyanobacterium recovered from cold spring sediment (GQ302542), which has also been identified in additional healthy *I. basta* samples (Luter et al. 2010a)

factors explaining 61%. Overall, the ordination showed little evidence of different microbial communities between regressed and recovered sponges (Fig. 5.4). Whilst most samples grouped close together irrespective of whether they were from regressed or recovered

samples, there was a notable grouping of two samples which were distinct from all other samples; however, these represented a member from each of the tissue states. In addition, larger variation was observed between individuals with regressed tissues, evidenced by the increased separation of samples in the ordination, compared to those with recovered tissues (Fig. 5.4).

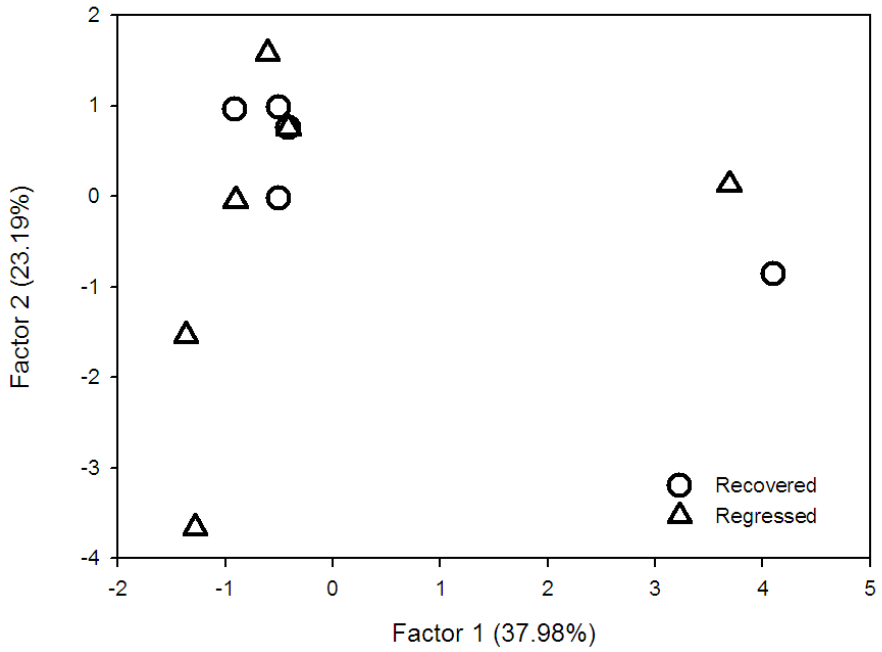

Fig. 5.4 Principal components analysis (PCA) of *I. basta* microbial community composition, using DGGE banding pattern data to construct a similarity matrix, for recovered and regressed tissues.

5.4 Discussion

I. basta specimens in this study displayed notable tissue regression and recovery within a 72 h period. This phenomenon has been reported in other sponge species under adverse conditions (Imsiecke et al. 1996; Böhm et al. 2001; Thoms et al. 2008); however, unlike those studies, no reduction bodies and/or gemmules were observed in *I. basta*. In fact, no

reproductive structures were noted for any of the samples, regardless of their tissue state. The present study was consistent with the findings of Thoms et al. (2008) in respect to the absence of choanocyte chambers. *I. basta* with regressed tissues had reduced numbers of apparent choanocyte chambers and more densely packed cells. The loss of sponge tissue and/or cells without the formation of reproductive structures has also been documented in a few cases. For instance, in response to sediment exposure, *Halicohondira panacea* sloughs its outermost tissue layer, which also prevents damaging surface fouling (Barthel & Wolfrath 1989). Interestingly, *Halisarca caerulea* is capable of constantly renewing its filtering system through the shedding and subsequent proliferation of choanocyte cells (de Goeij et al. 2009). Based on the techniques employed in this study, the mechanism of apparent choanocyte cell loss in *I. basta* cannot be ascertained.

In this study, we propose that *I. basta* may be going into a stress-induced 'dormant' state following collection and transportation. A dormant state, characterized by loss of choanocyte chambers, has been reported in *Halichondria bowerbanki* in response to seasonal temperature stress (Hartman 1958). In the present study temperature was maintained at approximately 28°C, mechanical damage was minimal and sponges were kept from air exposure during collection. However, considering the tissue necrosis and lesions identified prior to collection, it is likely that these sponges were already stressed and transportation or water quality change from their natural environment may have been sufficient to evoke this tissue regression response. Sponge explants also displayed regressed tissues, yet they had no visible signs of necrosis when collected. This further supports the proposal that handing stress associated with collection and transportation can invoke this stress response. While in this proposed dormant state, it is possible that there is not an actual loss of cells, but rather a re-organization of the cells. Tanaka & Watanabe (1989) observed the differentiation of

archaeocytes into choanocytes and eventually choanocyte chambers. During the early stages of archaeocyte differentiation, cells also appeared more densely packed.

To date, this type of tissue regression and subsequent recovery has yet to be observed in *I. basta* in its natural environment. However, it has been observed in another sponge of the same genus that is commonly found in the same habitat (Whalan, unpublished data). In this instance, all sponges sampled (n=30) in September 2010 for reproductive biology displayed regressed tissue and choanocyte arrangements consistent with regressed *I. basta*. Further work is required, but given tissue regression coincided with spawning of oocyctes, there is a possibility that reproduction (e.g. spawning) may represent a stressful event that contributes to tissue regression.

The three dominant symbionts of *I. basta*, previously reported by Luter et al. (2010a), were observed in all samples in the present study, regardless of the tissue state. The PCA revealed the microbial community composition of two individuals were different to the remaining 10 sponges, although these samples were not exclusively from sponges displaying regressed tissues. This would suggest that the regressed state of the tissue is not driving the changes in the microbial community. Although *I. basta* has a low diversity of microbes, the dominant community players (an *Alphaproteobacteria*, *Gammaproteobacteria* and Thaumarchaea) remain stable through space, time and differing environmental conditions (Luter et al., 2010a; Luter, unpublished data). Conversely, the microbial community of *Aplysinella* sp. was observed to shift under a regressed tissue state, with higher species richness in regressed samples (Thoms et al. 2008). The functional roles of the microbial symbionts in *I. basta* are not yet known and as such raises intriguing questions of the influence of symbionts and the interplay with the sponge host on sponge physiology, health and survival.

I. basta specimens in this study displayed tissue regression and subsequent recovery over the course of 72 h. It is possible that this tissue regression is a form of stress response in *I. basta*. However, the exact mechanisms behind the tissue loss and regeneration in *I. basta* remain unknown. Future research should attempt to observe the regression and recovery process as it is taking place to determine whether any cellular differentiation and/or cell shedding occurs in *I. basta*. It is evident that although sponges are considered 'simple' they have the ability to carry out non-neural processes (e.g. contraction), which enable them to cope with various stressors (Nickel 2004; Leys & Meech 2006). Regardless of the stimuli responsible for the regression in *I. basta*, their ability to recover demonstrates the resilience capacity of sponges, which will be increasingly important in a changing climate.

Disease outbreaks have had devastating impacts on sponge populations in the Caribbean (Galstoff 1942) and Mediterranean (Gaino et al. 1992; Vacelet 1994). There is no evidence of large scale sponge mortalities on the Great Barrier Reef or Torres Strait; however, anecdotal reports of disease have increased in recent years (reviewed in Webster 2007). Despite the increasing reports, our understanding of disease processes in sponges remains limited. In addition, few studies examine the impact of environmental stress on sponge communities and the possible role environmental parameters play in disease outbreaks.

Following the report of disease in *Ianthella basta* populations in Papua New Guinea (Cervino et al. 2006) and the increasing anecdotal reports of disease in this species in the Palm Islands and Torres Strait, the broad objective of this thesis was to further investigate the prevalence of the syndrome and identify possible causative agents. Bacteria are commonly implicated in disease of marine organisms (Kinne 1980), and a bacterial origin of the disease-like syndrome was proposed in *I. basta*. However, results presented in this thesis provide evidence that the disease-like syndrome affecting *I. basta* is not the result of a bacterial pathogen. In addition, an environmental origin (elevated temperatures or sedimentation) of the syndrome could not be implicated. Taken together these findings suggest the syndrome documented in the field is not a disease.

The specific findings of this thesis are:

1. Chapter 2: The prevalence of a disease-like syndrome was quantified for sponges in Torres Strait and the Palm Islands, with 66% of *I. basta* in Torres Strait and 44% in the Palm Islands showing signs of disease. Symptoms of the syndrome include

discolored, necrotic spots in which sponge skeletal fibers are just becoming apparent. In addition, some sponges also exhibited degraded tissue and completely exposed skeletal fibers. On a cellular level, choanocyte chambers of sponges with symptoms of disease displayed significant cellular degradation and debris within the remnants of the chambers. While a large percentage of sponges displayed small numbers of lesions, relatively few *I. basta* showed extensive necrosis in either location.

2. Chapter 3: Bacterial cultivation, molecular community analysis (*Bacteria* and *Eukarya*) and electron microscopy did not reveal any putative pathogens responsible for the disease-like syndrome affecting *I. basta*. In addition, infection assays did not induce lesion formation in healthy sponges despite direct contact with infected samples. Results from this chapter suggest that bacteria are unlikely to be the etiological agent of the disease-like syndrome. *I. basta* has low microbial diversity, regardless of its disease state or the technique employed. The microbial community is dominated by a single *Alphaproteobacteria, Gammaproteobacteria* and Thaumarchaea.

3. Chapter 4: The microbial community of *I. basta* is consistent across a latitudinal gradient, with all three dominant symbionts present. *I. basta* exposed to water temperatures just 2-4 degrees above the average ambient temperature of their habitat during summer (32˚C) developed substantial discoloration and deterioration of their tissues and died after 8 days. However, sponges never developed the brown spot lesions and necrosis described in Chapter 2. Both sponge appearance and microbial community composition was stable at all temperature treatments up until day four. Although, a shift in the microbial community occurred at day seven in sponges exposed to 32˚C, with higher diversity detected within the *Alpha*- and

Gammaproteobacteria. Neither sedimentation stress nor antibiotic exposure induced brown spot lesions or necrosis. In addition, the microbial community never shifted; revealing a highly stable microbial community in *I. basta*.

4. Chapter 5: *I. basta* exhibit significant tissue regression and subsequent recovery in a short period of time (72 h). Tissue regression in this sponge is likely a form of stress response as a result of collection and transportation. Sponges with regressed tissues had significantly fewer choanocyte chambers and more densely packed granulated cells than sponges with recovered tissues. Again, no shift in the microbial community is detected between *I. basta* with regressed vs. recovered tissues, highlighting the stability of *I basta*'s associated microbes.

Following a recent review of sponge disease (Webster 2007), five new incidences of sponge disease have been reported (Gochfeld et al. 2007; Wulff 2007; Webster et al. 2008b; Maldonado et al. 2010; Angermeier et al. 2011). Consistent with findings in this thesis and prior disease reports (Webster 2007), none have identified any pathogens. To date, there is only one study that has successfully identified (e.g. confirming Koch's postulates) the etiological agent of disease in sponges (Webster et al. 2002). The inability to isolate putative pathogens, in most cases, highlights the caution that should be taken when assuming disease-like symptoms arise from an infectious agent. The difficulty assigning causative agents of disease represents one of the major challenges for the management of sponge disease.

While most studies are unsuccessful at identifying primary pathogens, many observe shifts in the microbial community associated with diseased specimens (Cervino et al. 2006; Olson et al. 2006; Webster et al. 2008b; Angermeier et al. 2011). These observed shifts can arise from a loss of dominant symbionts, changes in antimicrobial compound production and decaying tissues providing additional nutrients for microbial metabolism. However, the

findings of this thesis study detected no shift in the microbial community between diseased and healthy individuals. Moreover, the three dominant symbionts (*Alpha*- and *Gammaproteobacteria* and Thaumarchaea) of *I. basta* are consistently represented, regardless of the sponges' health status. Given opportunistic seawater-derived microbes were unable to proliferate in diseased individuals suggests that the chemical defense and/or immune system of *I. basta* are still functional even at advanced stages of tissue necrosis.

Because no infectious agent was identified (Chapter 3), an environmental origin of the disease-like syndrome was explored. Temperature is linked with increased pathogen virulence and host susceptibility (Toren et al. 1998; Ben-Haim & Rosenberg 2002; Kuta & Richardson 2002; Jones et al. 2004), and terrigenous sediments can act as reservoirs for pathogenic bacteria (Harvell et al. 2007). Therefore, these two environmental stressors were investigated under laboratory conditions to determine the potential role in the formation of brown spot lesions. Neither temperature nor sedimentation induced the disease-like symptoms (e.g. brown spot lesions) that had been observed for *in situ I. basta*. Nevertheless, although no disease-like symptoms were induced, when exposed to elevated temperatures for prolonged periods, *I. basta* displayed substantial discoloration and tissue deterioration.

I. basta exposed to elevated temperatures (32˚C) maintained their microbial symbiosis for a longer period of time than in a previous sponge temperature stress study. For example, the microbial community of *Rhopaloeides odorabile* shifted within the first 24 h at the highest temperature treatment (Webster et al. 2008a), whereas the symbionts of *I. basta* were maintained for the first four days, only shifting at the point of sponge mortality after seven days. A qPCR study of the heat stressed *R. odorabile* confirmed a host response at the same time as the microbial community shift (Pantile & Webster 2011), indicating microbial shifts are tightly linked to host health. Therefore, *I. basta*'s microbial community may be more

stable than *R. odorabile* due to a decreased thermal sensitivity of the host. The microbial community shift in *I. basta* comprised an increase in diversity within the *Alpha-* and *Gammaproteobacteria*, possibly from the breakdown in its chemical defense mechanisms, allowing opportunistic bacteria to proliferate (Kelman et al. 2001; Ritchie 2006) While this community shift occurred in heat stressed *I. basta*, none of the bacterial groups commonly associated with diseased or stressed sponges and corals (e.g. *Bacteroidetes*, *Epsilon-* and *Deltaproteobacteria*) (Frias-Lopez et al. 2002; Pantos & Bythell 2006; Webster et al. 2008b) were detected. This provides further support to the suggestion that the syndrome described in Chapters 2 and 3 is not disease related.

The microbial community of *I. basta* is clearly stable with no shifts evident across a latitudinal gradient or when challenged with stressors of sedimentation, antibiotic exposure or handling (Chapter 5-tissue regression). Given the apparent stability in the microbial community of *I. basta*, it is highly probable that their associated microbes are playing important functional roles within the sponge.

I. basta has low microbial diversity overall with only members of the *Alphaproteobacteria*, *Gammaproteobacteria* & Thaumarchaea observed throughout the investigations of this thesis. Indeed, *I. basta* has a low-medium abundance of microbes associated with its tissues when compared with many other high microbial abundance sponges (Hentschel et al. 2006). If the associated microbes of *I. basta* are aiding in important functional roles, then retaining these symbionts despite the challenge of stressors may be fundamental to sponge health. Other invertebrates demonstrate the ability to select bacteria for specific functional roles. For instance, juvenile bobtail squid selectively acquire a bioluminescent symbiont, *Vibrio fischerii*, from the surrounding seawater, to occupy its light organ (Ruby 1996).

Conclusions and future directions

The work undertaken in this thesis provides a logical progression to the investigation of disease in *I. basta*. It begins by identifying a disease-like syndrome affecting *I. basta* populations and provides valuable baseline prevalence data, followed by a comprehensive description of the microbial communities associated with both affected and healthy sponges, revealing no putative pathogens. Further investigations ruled out environmental origins of the disease-like syndrome, with brown spot lesions never induced during thermal or sedimentation stress. The culmination of these results indicates that the syndrome described in Chapter 2 and 3 is not a disease. The stability of *I. basta*'s microbial community is highlighted throughout the thesis with the lack of community shifts detected between sponges displaying disease-like symptoms, across a latitudinal gradient, under environmental stressors and during tissue regression. Given the syndrome is unlikely a disease, some important questions have arisen. Specifically, there may be value in examining auto-immune dysfunction and senescence in the sponge host by identifying genes that are either up or down regulated in sponges displaying the disease-like symptoms. This may provide further insight into the cause of the brown spot lesions in *I. basta* populations.

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