Factors Controlling the Microbial Community Associated with Reef Building Corals

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“As the circle of light increases so does the circumference of darkness around it”

Albert Einstein

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ABSTRACT

Several studies have shown that corals are associated with diverse, host species-specific bacterial communities and these have been proposed to be of primary importance for their health. Various factors have been suggested to influence the structure of these communities, including production of antimicrobial chemicals, the supply of microorganisms from the surrounding environment (e.g. sediments and water column), mucus composition and production rates by the coral. However, few studies have investigated the factors that control the development and maintenance of these communities. Describing the microbial communities of healthy corals and how they interact with their surrounding environment is imperative to understanding how environmental stress and health problems in corals are related. This study utilised a culture-independent 16S rRNA gene approach to investigate the structure of the bacterial community on corals, the factors that might control the development of these microbial communities and their organisation within the coral host. In addition, the study identified the role of cytophagous ciliates as a potential cause of White Syndrome in the GBR. Study of the bacterial (16S rRNA gene) community of the surrounding water column (the potential supply to the surface mucus layer of corals) revealed that changes in productivity and/or vertical diurnal migrations of plankton might have greater effects than large scale water movements effected by tidal flows. Results also showed that waterborne bacterial communities and their underlying benthos were not strongly linked, suggesting either that there is little benthic-pelagic coupling or that large-scale (island wide) water column mixing is rapid and highly efficient, resulting in homogeneous bacterial communities in the water column, independent of the underlying benthos. The bacterial communities forming on artificial surfaces and those associated with the mucus layer of corals were different from the water column as well as each other, with a variety of ribotypes of γ-proteobacteria favouring both the biofilms and those of the surface mucus layer, compared to a high dominance of α-proteobacetria within the water column. This suggests that the coral actively controls the microbial community on its surface, rather than it being a result of passive settlement from the water column. Results also show that bacterial communities within the coral are highly compartmentalized with distinct assemblages inhabiting the mucus layer, tissue and skeleton, which indicates high

levels of complexity in the coral-microbial associations. The use of a broad spectrum antibiotic treatment further allowed investigation of turnover rates of the microbiota associated with healthy corals. Re-establishment of the corals' normal microflora was slower than anticipated, taking over 96 h to return to that of its original bacterial community once disturbed, however the fact that the community returned towards its original state indicates a high degree of resilience and strong controls on the microbial community structure Despite the undisputed importance the bacteria associated with corals have on the overall coral holobiont, other microorganisms such as ciliates were also important for coral health, particularly during thermal stress. Results indicated that coral White Syndromes, previously attributed to pathogenic bacteria such as *Vibrio* spp., may have different etiologies, with cytophagous ciliates playing an important role. These findings further complicate correct disease identification in the field and appropriate treatment and/or prevention methods for diseased corals.

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Chapter 1

General Introduction

1.1 Corals and Coral Reefs

Corals have a long fossil record dating back around 450-500 million years to the Ordovician Period of the Paleozoic Era (Stanley 2003; Tapanila 2008). Three groups of early corals – the heterocorals, the tabulate corals, and the rugose corals – are now extinct, having died out by the end of the Paleozoic. Four other groups of corals that developed during the Mesozoic (250 – 67 million years before present (mybp)) and Cenozoic Eras (65.5 mybp to present day), survive to the present day; the hydrocorals, the black corals, the hard corals, and the octocorals. Corals can be further classified as reef-building or non-reef-building. Reef building corals are mostly hard corals (order Scleractinia), but also include octocorals (e.g. the blue coral *Heliopora*) and hydrocorals (e.g. the fire coral *Millepora* (Stanley 2003)). During these early developing periods throughout the Triassic, Jurassic and Cretaceous periods, where atmospheric CO_2 levels were often substantially higher than today's (Hallock 1997), ancient coral reefs experienced dramatic changes in terms of species composition, structure, function and distribution, changing from reefs dominated by sphinctozoid sponges to scleractinians. These large-scale changes were largely related to plate tectonic movements and glaciation periods which alone or in combination produced shifts in temperature, sea level and other oceanographic processes (Stanley 2003).

At present, there are four coral reef provinces in the world: the Indo-Pacific (1,250,000 km^2), and the western Atlantic (250,000 km^2) - which are the largest and more important in terms of biodiversity - and the eastern Atlantic and Pacific, which contain fewer species in comparison. The Pacific supports 57 reef building coral genera (Veron 1986) and the Atlantic 24 (Mojetta 1995); although some reef building coral genera are found in both provinces (e.g., *Montastrea, Porites* and *Acropora*), they have no species in common. Coral reefs are a major feature of coastal tropical environments between the latitudes 25 ˚S and 25 ˚N and roughly coincide with water temperatures between 18 ˚C and 30 ˚C. Below an annual minimum of 18 ˚C the

number of reef building coral species decline rapidly and reefs do not form (Veron 1986).

Within these latitudes where reef forming corals are found, seasonal and diurnal fluctuations in mean sea surface temperature (SSTs) are relatively small and reported evidence suggests that tropical ocean mean SSTs have varied less than 2 ˚C over the past 18,000 years (Thunell et al. 1994; Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007), therefore the environments in which coral reefs prosper are typified by a high degree of stability.

Modern coral reefs are complex ecosystems, the bulk of their framework being formed by scleractinian corals. These organisms along with hydroids, jellyfish, box jellies, and sea anemones belong to the phylum Cnidaria. Corals differ from other cnidarians because they produce a calcium carbonate ($CaCO_3$) hard skeletal structure (e.g. scleractinian corals), or a tough fibrous protein known as horn (e.g. octocorals). Most corals are colonial, but a few are composed of a solitary polyp throughout their entire life span. Cnidarians have diploplastic tissues comprised of the ectoderm and the endoderm (Fig. 1.1), which surrounds a gelatinous matrix, known as the mesoglea. Nematocysts and mucus gland cells are present within the endodermal and ectodermal layers and on the surface of the tissues lies the secreted mucus layer (Veron 1986).

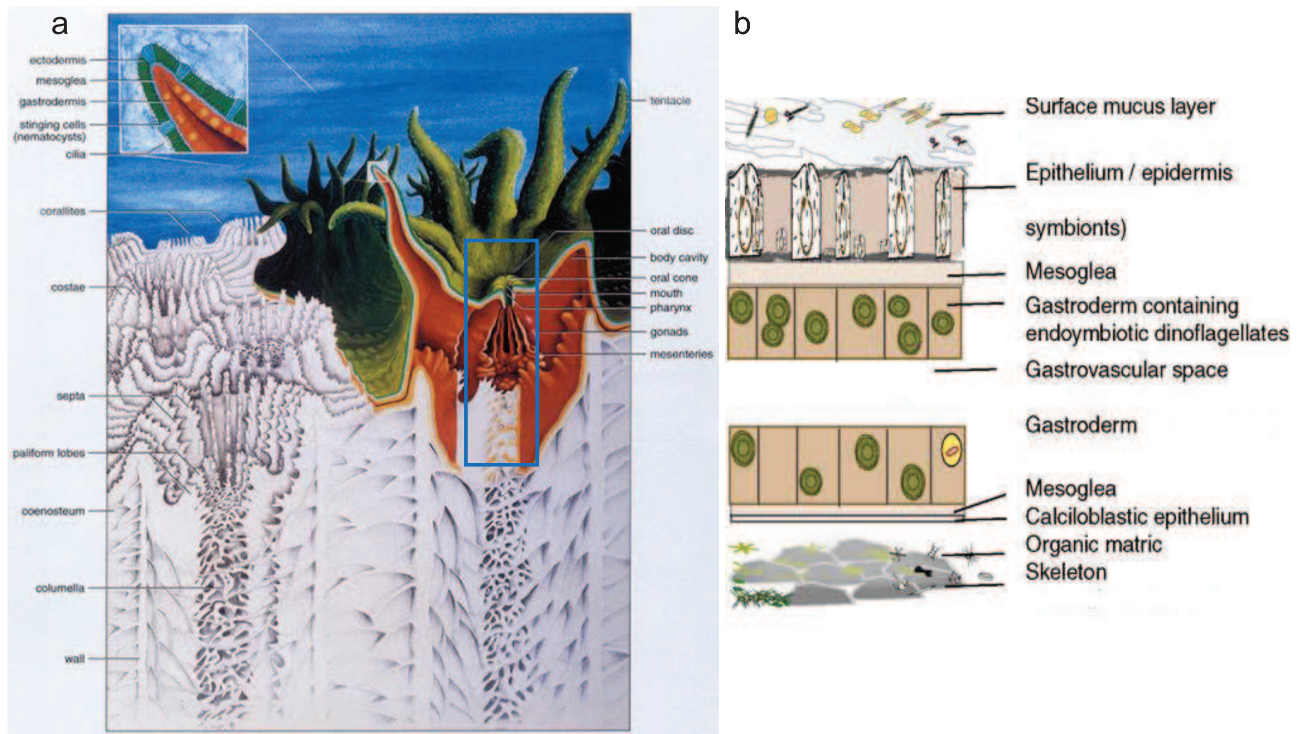


Figure 1.1. A cutaway diagram of a modern scleractinian reef coral. (a) The massive and complicated underlying skeleton (white), secreted by the soft polyps and tissue of the living surface (coloured) adapted from Veron (2000), (b) The various functional compartments contained within (adapted from Ainsworth et al. 2010).

Reef-building corals have unicellular dinoflagellate algae commonly called zooxanthellae living in their internal tissues, more specifically within the endoderm (Veron 1986). These symbiotic dinoflagellates are important for the coral's energy budget as they provide the host with photosynthetic products, thereby promoting animal growth and reproduction as well as enhancing calcification (Gattuso et al. 1999). In return, the host provides its symbionts with inorganic nutrients as a result of excretion and habitat structure, protecting them against grazing and UV damage (Chadwick-Furman 1996). This symbiotic relationship, however, imposes a series of limitations on the corals, as their distribution is restricted to specific light and temperature requirements, confining the majority of coral reefs to tropical, poor-nutrient and shallow waters (Stanley 2003; West & Salm 2003; Berkelmans & Van Oppen 2006). A recent review by Wooldridge (2010) challenges the long accepted standpoint of 'mutual benefits' and partner cooperation of this symbiosis and suggests the coral host role as that of a farmer, acquiring, domesticating and harvesting the

algal produce. Regardless, the building of reefs is a bipartisan effort between coral host and the algal symbiont, another important function of algal photosynthesis in this system is the production of large amounts of molecular oxygen that diffuses into the host allowing for efficient respiration by the coral and associated prokaryotic microorganisms (Rosenberg et al. 2007). However, under environmental stresses such as increased UV radiation and temperatures, the algae can indirectly damage the host coral via photochemical reactions that produce reactive oxygen species (ROS). Production of ROS is a normal by-product of cellular metabolism and particularly photosynthesis and controlled by the enzyme superoxide dismutase (SOD) and ascorbate peroxidase (APX), however, under stressful situations the oxygen handling pathways become overwhelmed and O_2 is converted to the most highly reactive hydroxyl radical (OH) and the more stable and diffusible hydrogen peroxide (H_2O_2), which then move into host tissues, where the damage continues and ultimately leads to bleaching (Fig. 1.2 (Weis 2008)). The production of ROS in a variety of different types of cells has been shown to be an early event in the response to various types of stress (Higuchi et al. 2010).

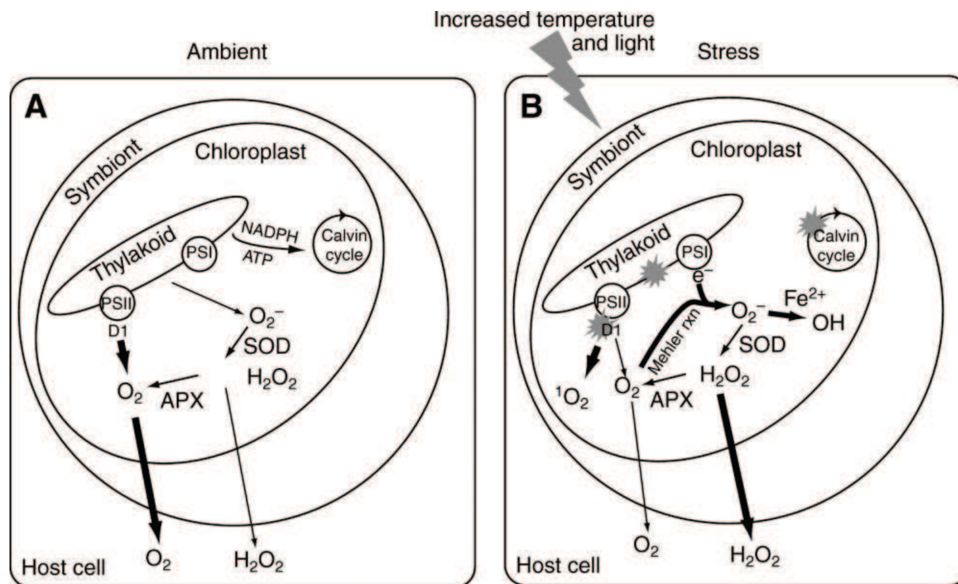


Figure 1.2. Schematic representation of oxygen handling pathways in *Symbiodinium* resident in host cells under ambient (A), and elevated temperature and light conditions (B). Under ambient conditions, the photosynthetic apparatus, consisting of photosystem II (PSII) and photosystem I (PSI), operates normally and produces large quantities of oxygen that diffuse into the host. ROS that are normally produced are converted back to oxygen with superoxide dismutase (SOD) and ascorbate peroxidase (APX). Under stressed conditions, damage to the photosynthetic apparatus occurs in at least three places (depicted as `flashes' in the figure). This damage causes the generation of unusually large amounts of ROS in the form of superoxide (O_2^-) that overwhelm the oxygen-handling pathways and fails to be detoxified and therefore accumulates. This is then converted to the most highly reactive ROS, hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) (adapted from Weis 2008).

1.2 Natural and anthropogenic factors affecting coral reefs

Over the past two decades, an increasing number of reports have documented dramatic changes and continuing decline in coral reef communities (Hughes 1994; Cooney et al. 2002; Pandolfi et al. 2003; Hoegh-Guldberg et al. 2008; Wilson et al. 2010). These have been attributed to both natural and anthropogenic factors (Grigg 1994; Jackson et al. 2001; Hoegh-Guldberg et al. 2008). Natural threats to coral reefs and coral reef organisms include cyclones (Bythell et al. 1993; 2000; Gardner et al. 2005), periodic population explosions of echinoderms such as the Crown-of-Thorns Starfish (*Acanthaster planci*), periodic ocean warming events (e.g., The El Niño

Southern Oscillation (ENSO) (Glynn et al. 2001)), and the actions of earthquakes and volcanoes (Riegl et al. 2009). Man-made threats include chemical and nutrient pollution, sedimentation from land clearing and coastal development, over fishing and collecting for the international aquarium and jewellery trades, recreational use (ship damage and tourism impacts), and destructive fishing techniques including the use of dynamite and cyanide (Hughes et al. 2003). These human impacts are a major threat for coral reef ecosystems rendering a world-wide decline of these ecosystems in an unprecedented short temporal scale (Hughes et al. 2003).

The ENSO is a well documented climate phenomenon that has had a large scale effect on marine ecosystems. During the past 5000 years, ENSO events have typically occurred at a frequency of one or two per decade but since the mid 1970's have occurred more often and persisted longer (Harvell et al. 1999). The impact of these climatological events on marine species is clearly evident among corals, which are known to bleach in response to a range of environmental stresses. Coral bleaching represents a breakdown of the obligate symbiosis between the coral host and their endosymbiotic photosynthetic microalgae (Brown 1997). The visible signs of bleaching result from a reduction (70 to > 90%) in algal density (Fitt et al. 2000; Douglas 2003), and/or decreased concentrations of photosynthetic pigments in the algal cells. Bleaching is usually triggered by environmental factors that impose stress upon the coral, the most frequently cited being increased seawater temperature coupled with higher irradiance (Jokiel & Morrissey 1993; Brown 1997; Kushmaro et al. 1998). During times of bleaching, the living coral tissues are transparent or translucent and therefore give the white appearance of the underlying skeleton of calcium carbonate. If the process is not reversed within a few weeks or months, depending on the species and severity of damage to the particular coral, the coral will die. The reasons that corals have an inability to survive long periods without this symbiosis remain unclear. One theory is that as the algae's photosynthetic products provide a large proportion (63-90 %) of the coral's energy requirements (Glynn 1991), once lost the coral can not produce energy-expensive products such as mucus, which is reportedly one of the corals principal lines of defence against pathogens (Brown & Bythell 2005). The coral bleaching event of 1998 was the most geographically extensive and severe in recorded history causing significant mortality worldwide (Hoegh-Guldberg 1999). Although mortality was limited in the Great Barrier Reef,

further bleaching hit this region in 2002 (Berkelmans et al. 2004), another in the Caribbean in 2005 (Brandt & McManus 2009) and more recently in 2010 (e.g. reports on Coral List). The causal stress for many of these reef systems appears to be the result of long term exposure to unusually high water temperatures resulting from a prolonged ENSO event (Glynn et al. 2001).

It is clear that corals are acting as an indicator species in the marine environment and are tractable for examining the frequencies of temperature stress and disease emergence in tropical oceans, because they are sessile (which allows us to pinpoint impacted locations and times), secrete large and permanent skeletons (which can record the passage of disease even after the coral is dead through the use of coral cores (Aronson et al. 1998; 2005; 2009)), and they form clonal colonies, which allows signs of chronic infections to persist without killing the entire colony, unlike in small unitary species (Harvell et al. 1999). With the increasing threat of global warming and the predicted rise in temperatures worldwide (Global Climate Change scenarios Fig. 1.3), a catastrophic destruction of coral reef systems is predicted and bleaching events similar to those witnessed in 1997/98 in the Indo-Pacific and 2005 in the Caribbean will potentially become annual events (Hoegh-Guldberg et al. 2007).

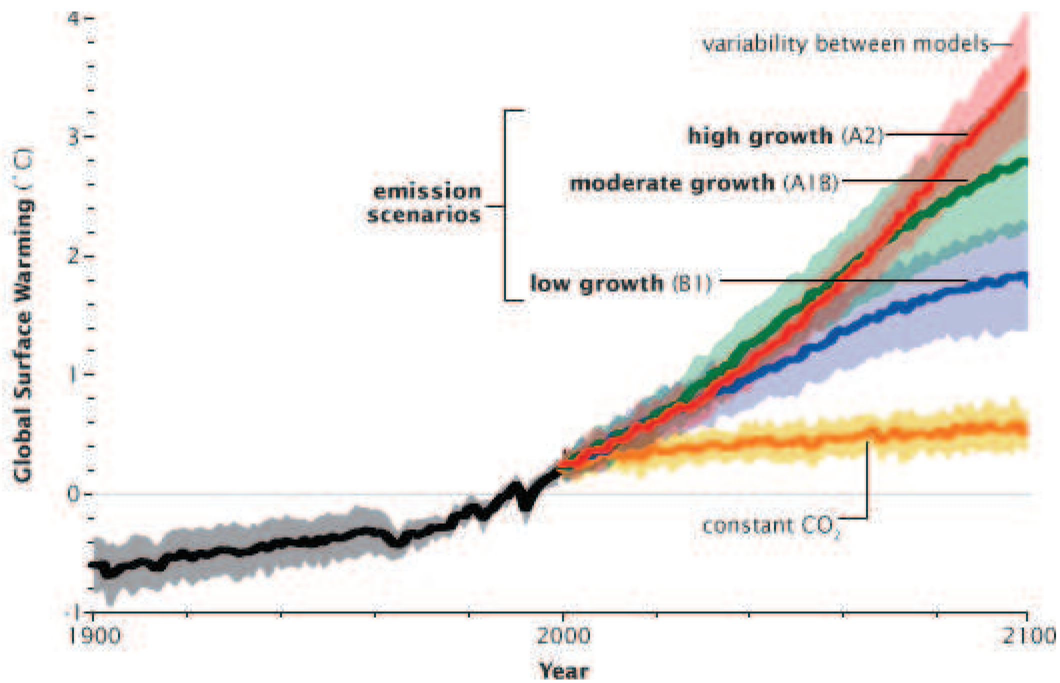


Figure 1.3. Model simulations by the Intergovernmental Panel on Climate Change estimate Earth surface warming between 2 – 6 º C over the next century. An increase is inevitable but how large will depend on the rate of growth of CO^2 emissions. A2 scenario assumes the rate to increase exponentially whilst B1 represents the predicted growth if governmental restrictions were taken into place. The orange line provides an estimate of global temperatures if greenhouse gases stayed at year 2000 levels (reproduced from Riebeek 2007).

Although these predictions can be argued as being overly pessimistic due to the potential of corals to acclimatise and adapt to changing environmental conditions (Hughes et al. 2003), indirect, potentially synergistic effects of coral diseases are not accounted for. The emergence of apparently novel diseases in a wide range of environments over the past 10-20 years has been linked to human-mediated introductions of pathogens, climate change and other environmental stresses that may render the host coral more susceptible to disease and/or bleaching (Hoegh-Guldberg 1999).

1.3 Resistance, resilience and acclimatisation. Is it possible?

Because coral reefs exist in tropical latitudes within 30° of the equator in relatively constant environments with little seasonal variation, it could be assumed that they are not highly resilient to environmental fluctuations. However, on a geological timescale they are among the most persistent ecosystems on Earth, having existed since the Paleozoic Era. Some extant coral reef species have existed for the past 1-10 million years and have thus survived glacial-interglacial climate oscillations. Today coral reef resilience is threatened by human induced climate change that is predicted to lead to global temperatures that have not occurred since the Pliocene Era (5.3-1.8 mybp) when coral reef species composition was significantly different (McClanahan et al. 2002). In principle, corals can respond to bleaching stressors in three ways: resistance, resilience and acclimatisation:

1) In this context, 'resistance' refers to the ability of corals to withstand bleaching stressors without the reefs undergoing a phase shift or the individual corals themselves losing either structure or function (West & Salm 2003). It is convenient to consider resistance as the alternative response to susceptibility, where the coral does bleach, but in reality corals display a continuum of responses of varying degrees of resistance/susceptibility. Factors contributing to resistance include antioxidant enzymes, fluorescent pigments in corals (Lesser 1996, Lesser 1997; Salih et al. 2000; Kim & Harvell 2004) and physiological characteristics of the symbiotic algae (Rowan 2004), possibly including efficient xanthophyll cycling (Visram & Douglas 2007). All of these factors appear to be mechanisms utilised by the coral, both for bleaching and disease resistance (Palmer et al. 2010).

2) 'Resilience' refers to the capacity of a coral to recover from bleaching. Corals in which the symbiotic algae populations return rapidly to the density occurring before bleaching, are described as having high resilience (West & Salm 2003; Visram & Douglas 2007). In principle, both the proliferation of the resident symbiotic algae populations and invasion of the coral tissue by free living algae, either from the water column or by those living on or near the reef bottom, can contribute to this process (Berkelmans & Van Oppen 2006). Corals with low resilience (i.e. with a symbiotic algal population that increases very slowly or not at all after a bleaching event)

generally die off following thermal stress (Visram & Douglas 2007). Visram & Douglas (2007) predicted that the resilience of corals declines with increased duration or magnitude of exposure to elevated temperatures, with impaired photosynthetic function combined with the deterioration in condition of endoderm tissues.

3) 'Acclimatisation', refers to experience-mediated increase in resistance to bleaching. For example, on first exposure to a bleaching stressor, a coral colony may be susceptible and resilient. However, during the second exposure it may appear more resistant (Sebastian et al. 2009; Hennige et al. 2010). Acclimatisation can be achieved in three ways: (a) mediated by changes in the physiological/biochemical traits of the coral or its symbiotic algae (Brown et al. 2000; Brown et al. 2002) (b) replacement of bleaching susceptible symbiotic algae by genetically distinct, more resistant algae (Baker et al. 2004; Rowan 2004; LaJeunesse et al. 2010), or (c) by shifts in the dominant members of algae populations (Berkelmans & Van Oppen 2006) to more similarly resistant types. This is referred to as 'symbiont shuffling' (Baker et al. 2004).

1.4 Coral associated bacteria

Corals harbour a diverse array of bacterial associates (Thurber et al. 2009; Mouchka et al. 2010), some of which are thought to be host species specific (Rohwer et al. 2002) and these have been shown to inhabit various microhabitats within the coral (Ainsworth et al. 2010). These include the coral surface mucus layer (SML) (Ducklow & Mitchell 1979; Paul et al. 1986; Ritchie & Smith 1995; Ritchie & Smith 2002; Brown & Bythell 2005), the coral tissue (Kushmaro et al. 1996; Banin et al. 2000; Frias-Lopez et al. 2002; Ainsworth & Hoegh-Guldberg 2009) and the skeleton (Ainsworth et al. 2010), along with close associations with those present within the environment e.g. the water column (Sorokin 1973; Gast et al. 1998; Frias-Lopez et al. 2002; Ritchie 2006). These coral-associated microorganisms can be divided into at least four main functional groups: (a) mutalistic bacteria with possible roles in coral nutrition, (b) pathogenic bacteria, (c) bacteria which can act as a probiont, aiding the growth of beneficial bacteria but limiting the growth of pathogenic forms, and (d) purely commensal bacteria with no impact on the other three groups (Klaus et al. 2005). These associations are thought to play an important, although at present relatively unknown role in coral health and disease. A term encompassing the animal

host of the coral, its symbiotic dinoflagellates and these diverse bacterial associates was coined 'the coral holobiont' (Knowlton & Rohwer 2003).

Coral disease and in some cases bleaching has historically been associated with particular pathogenic bacteria. White band type II (Denner et al. 2003), white pox (Patterson et al. 2002; Lyndon 2003; Sutherland & Ritchie 2004), *Aspergillosis* (Kirkwood et al. 2010) and white plague type II (Pantos et al. 2003) are thought to be caused by known bacterial pathogens (Richardson 1998; Rosenberg & Ben-Haim 2002; Weil et al. 2006). The bleaching of *Oculina patagonica* and *Pocillopora damicornis* (Rosenberg & Ben-Haim 2002), has also been proposed to have bacterial causal agents (*Vibrio shiloi* and *V. coralliilyticus* respectively), however, this has been challenged (in the case of *O. patagonica* and *V. shiloi*) by Ainsworth et al. (2008). Some diseases may be caused by single organisms which can be established via Koch's Postulates (Sussman et al. 2008) however, others appear to be caused by a consortium of microbes. For example, Black Band Disease, found throughout the Caribbean and the Indo-Pacific, appears to contain at least 50 different bacterial types within the disease lesion, with members of the Cyanobacteria, *Beggiotoa* spp. and *Desulphovibrio* spp. believed to be important aetiological agents (Richardson 1998; Cooney et al. 2002; Sekar et al. 2006). The complex relationship of microbes that constitute many coral diseases makes a definitive comparison of disease with similar symptoms difficult. Without knowing what to look for, it is extremely difficult to follow these pathogens through the environment to determine their reservoirs and modes of transmission. In addition, because diseases are often identified by their symptoms alone, there has been confusion over field identification of diseases (Lindop et al. 2008).

Previous research has indicated that some predatory organisms may become vectors of microbial pathogens at high seawater temperatures, resulting in disease of scleractinian corals. Scars left by such organisms would have left the coral's defences low and made them vulnerable to attack from otherwise harmless species. These vectors (Fig 1.4) include the fireworm, *Hermodice carunculata*, which has been found to harbour *V. shiloi* in the gut (Sussman et al. 2003); the snail, *Coralliophila abbreviata* (Williams & Miller 2005); the coral feeding butterflyfish, *Chaetodon multicinctus* (Aeby 2002), which harbours one life-history stage of a digenean

(trematode) that infects *Porites* (Aeby & Santavy 2006); the nudibranch *Phestilla* spp. (Dalton & Godwin 2006); and most recently, the crown of thorn starfish *Acanthaster planci* (Nugues & Bak 2009). The feeding scars of the latter two have been shown to be invaded by a diverse array of microorganisms.

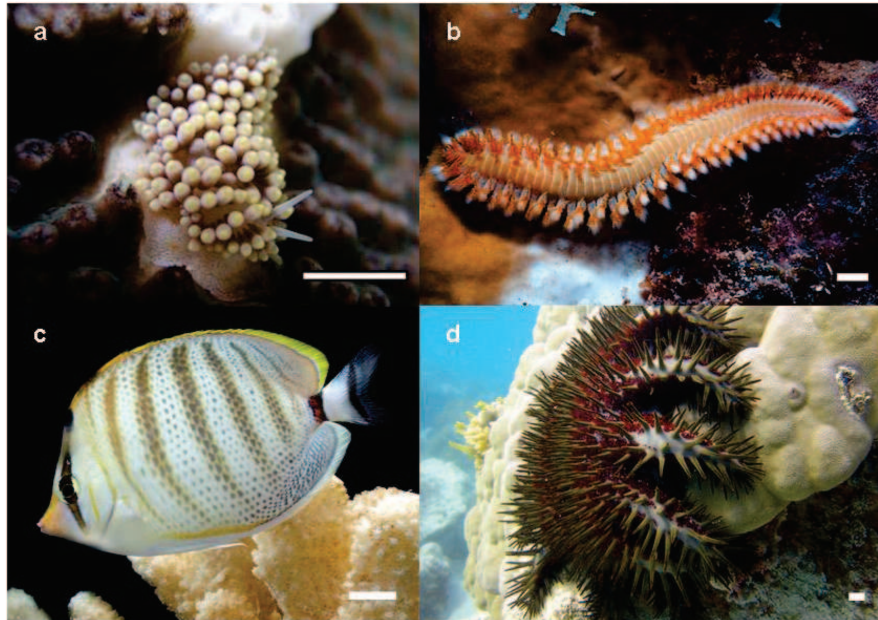


Figure 1.4. Predatory organisms known to inflict feeding scars on scleractinian corals which subsequently cause some form of disease/tissue lysis (a) *Phestilla sp* (Dalton & Godwin 2006) (b) fireworm; *Hermodice carunculata* (Sussman et al. 2003) (c) butterflyfish; *Chaetodon multicinctus* (Aeby 2002) (d) crown of thorn starfish; *Acanthaster planci* (Nugues & Bak 2009) (Scale bars = 10 mm).

Understanding microbial communities and how changes occur through time is the key to understanding the health of reef corals. Shifts in bacterial community composition may affect coral health and susceptibility to disease. Bacterial associations within specific niches of the coral have been shown to alter position within these habitats during times of stress associated with experimental and field conditions. Ainsworth and Hoegh-Guldberg (2009) demonstrated that bacteria normally present within the gastrodermis of healthy corals, have the ability (when the coral is in a stressed state) to penetrate and proliferate within the outer tissue layers (epithelium). While this proliferation altered the ‘normal’ flora seen within the tissues, there was no subsequent short term health consequences to the corals, however, further bacterial

colonisation and overgrowth did occur after the thermal stress was increased above the bleaching threshold, 32º C (Ainsworth & Hoegh-Guldberg 2009). In addition, Pantos et al. (2003) demonstrated that the bacterial community of the whole coral colony can be affected, even when only a small part of the colony shows visible signs of disease, therefore demonstrating that shifts in the normal microbiota observed before signs of visible stress, may be used as a bio-indicator of both environmental changes and disease, and ultimately the health status of the holobiont (Bourne & Munn 2005). Such shifts in the associated microflora have already been successfully used as a health indicator in other organisms, notably in the mammalian gut flora (Guarner 2007).

1.5 The future of coral reefs

Based on conservative estimates of increased seawater temperatures in the near future and the effects that increased temperatures have had on corals during the past 20 years, Hoegh-Guldberg predicted that coral reefs will have only remnant populations of reef-building corals by the middle of the 21st century (Hoegh-Guldberg 1999, 2004; Hoegh-Guldberg et al. 2007). However, this prediction is based on the assumption that corals are not resilient and/or cannot adapt rapidly enough to the predicted temperature increases in order to survive. This assumption was first challenged by the 'Adaptive Bleaching Hypothesis' (ABH) of Buddemeier and Fautin (1993). The ABH proposed that stressed corals first lose their dinoflagellate symbionts (i.e. bleach) and then regain a new mixture of symbiotic algae that are more adapted to the stressful conditions. However this has come under much debate and it remains unclear if changing algal partners is sufficiently rapid or effective enough for corals to survive the challenge of temperature stress (Glynn et al. 2001). The Coral Probiotic Hypothesis (CPH) by Reshef et al. (2006) adapted the ABH with the inclusion of bacteria, now known to have a close association with the coral. Bacteria are thought to give the holobiont an enormous genetic potential to adapt rapidly to changing environments. Changing microbial partners may allow the corals to adapt to changing environmental conditions more rapidly (days to weeks) than via mutation and selection which can take years to decades (Reshef et al. 2006). The hypothesis was formulated during a study into the well known bleaching effects of the Mediterranean coral *Oculina patagonica* by the pathogen *Vibrio shiloi*. Since it was first identified

(Kushmaro et al. 1996; Kushmaro et al. 1997; Kushmaro et al. 1998), ten years of further study has followed. However, recent research showed that since 2004 *O. patagonica* started to develop resistance to *V. shiloi* infection. *V. shiloi* could no longer be found on the corals and *V. shiloi* that had previously infected the corals was unable to re-infect them. These corals were now able to lyse the intracellular *V. shiloi*. However, Ainsworth et al. (2008) showed (using *in situ* hybridisation), while bacterial communities certainly play important roles in coral stasis and health, there was little evidence to support a primary role of bacteria in causing coral bleaching in this system, or evidence to suggest microbial control techniques could be effective to treat or prevent bleaching (Ainsworth et al. 2008). Instead Ainsworth et al. (2008) reiterated the importance of environmental stressors such as those documented with reef building corals, as the primary triggers leading to bleaching of corals, including that of *O. patagonica,* and suggested that bacterial involvement in bleaching is that of opportunistic colonisation. It is important to distinguish between bleaching *per se* and the mortality that occurs following a bleaching event. While there is minimal evidence for a direct role of bacteria in the bleaching process itself, there are ample opportunities for colonisation by opportunistic pathogens following a stress or disturbance to the natural microbiota causing the onset of diseases (Bourne & Munn 2005; Ritchie 2006; Lesser et al. 2007).

That aside, one novel aspect of the probiotic hypothesis presented by Reshef et al. (2006), was that the coral holobiont can 'adapt' to changing environmental stress conditions by changing the relative amounts of certain bacterial species that are present in the diverse pool of coral-associated bacteria. This change has been recorded *in situ* with *O. patagonica* from summer to winter. Therefore Reshef et al. (2006) suggested that it would not be unreasonable to predict that under appropriate selection conditions, similar changes could take place in days or weeks, rather than decades required for classical Darwinian mutation and selection in the host. These rapid changes may allow the coral holobiont to use nutrients more efficiently, prevent colonisation by pathogens and avoid death during bleaching by providing carbon and energy from photosynthetic prokaryotes. The role of the endolithic community of *O. patagonica,* particularly during increasing seawater temperatures and environmental stress, and that of the community shift during bleaching evokes questions as to the

relationship the microbial community has in the stability of the coral holobiont, as well as the potential sources of opportunistic pathogens (Fine et al. 2004).

Despite significant studies on coral associated bacteria and their roles within disease and bleaching, much remains to be understood (Lesser et al. 2007). There is an urgent need for microbiologists to be involved from the onset of major bleaching events. Due to the combination of elevated sea water temperatures and exposure time, predictions of bleaching have a relative high degree of certainty (Hoegh-Guldberg 1999, 2004; Hoegh-Guldberg et al. 2007), allowing researchers to begin microbiological studies prior to observable bleaching. Early changes in microbial populations might provide clues to the presence of temperature-regulated opportunistic bleaching pathogens, which could later be tested in controlled aquaria experiments. Information on the spatial and temporal composition of bacterial communities associated with corals is accumulating, although still limited by a number of factors. Among these factors are the difficulties in defining the micro-niches that bacterial populations occupy within the coral and techniques utilised for analysing coral bacterial associations.

This thesis combines the use of novel sampling techniques and large sample sizes to study compartmentalisation within healthy corals and those within the surrounding environment (Chapter 3 and 4), in particular that of the water column (one potential supply of bacteria to the coral). Chapter 5 utilised model coral replicas to assess which bacteria present within the environment would settle onto a coral surface and what the timescale for this early colonisation was. Chapter 6 showed the effects of disturbance on coral-associated microbes and their subsequent recovery over time, utilising antibiotic treatment methods (previously used to study disturbances in human gut microflora), revealing the resilience of the coral and/or its bacterial associates to certain disturbances. The discovery of pathogenic ciliates found during the course of field work (Chapter 7), has the potential to shift focus from bacterial pathogens as the major cause of disease, to the roles these ciliates play, in particular with diseases such as white syndrome, a disease widespread throughout the world's reefs.

Chapter 2

2.1 Methods used for characterisation of microbial community composition in environmental samples

One of the most basic requirements for any ecological study is the ability to identify accurately the members of the community of interest. Historically, most of the knowledge relating to microbial communities for example, has been obtained via culture based methods (Lynch et al. 2004). These methods typically included culturing organisms, followed by enumeration and characterisation with various biochemical and physiological tests (Holdeman & Moore 1972; Moore & Holdeman 1974; Tannock 2002). Non-selective culture methods involve plating fresh samples of microbial communities (obtained under appropriate atmospheric conditions to preserve viability), onto a non-selective medium such as agar in order to estimate total numbers of aerobic and anaerobic organisms present when cultured under the appropriate atmospheric condition (O'Sullivan 2000). Although the use of non-selective media is designed to permit growth of most bacteria, they are known to select against some species present in many microbial communities. These species may be selected against as a result of competition with more abundant species, the requirement for specific growth conditions that are not met and/or the occurrence of a phenomenon known as substrate-accelerated death, whereby the relatively high proportions of nutrients available in certain media overrides the metabolism of the bacteria and results in death rather than growth (O'Sullivan 2000). These methods have provided the vast majority of our knowledge regarding microbial communities to date and their importance should not be underestimated. Techniques such as culture-based enumeration still contribute important data regarding the proportions of the components of complex microflora and estimations of bacterial viability. Although the contributions of culture-based techniques have been significant, the techniques are laborious and time consuming (O'Sullivan 2000; Tannock 2002). In addition, only a small proportion of the microbes in complex communities are culturable due to unknown growth requirements or uncharacterised interactions with the host or other microbes, thereby imposing serious limitations on the scope of these traditional methods (Zoetendal et al. 1998; O'Sullivan 2000; Tannock 2002; Amann & Fuchs

2008). The exact fraction of culturable organisms in microbial communities is estimated to vary from as little as 0.001% to 40% (Hill et al. 2002; Amann & Fuchs 2008). Furthermore, characterisation of microorganisms accomplished with traditional methods is often not sufficient for definitive classification. Similar biochemical properties found in organisms of different families may disguise both genetic similarities and diversity, therefore making species and strains difficult to distinguish.

In order to avoid all of the complications inherent to culture-based methods, a number of culture-independent methods have been developed. The development of culture-independent methods for microbial characterisation has addressed many of the gaps left by characterisation with culture-dependent methods and in combination with the advent of molecular characterisation even greater understanding has and is continually being found. However, these culture-independent techniques developed to date also have limitations and biases (Table 2.1). Reviews of the methods available (Table 2.1) indicate that the sole use of just one of these methods may only provide a partial picture of one aspect of microbial diversity. Since it is impossible to evaluate the effectiveness of each method with our current knowledge, it may be advisable that researchers study the microbial population on as many different levels as possible. A broader, more complete picture of microbial diversity in any sample can be obtained by using multiple methods, each with a different endpoint, to provide a more global assessment of changes in microbial structure and function. One should be aware that changes in microbial community diversity in a habitat may not imply deleterious effects. Thus the need to learn how these particular changes in microbial community structure influence microbial community function is apparent.

Table 2.1. Review of the methods available for analysis of bacterial diversity and abundance routinely used in both soil and coral microbiology to date.

Method	Advantages	Disadvantages	References
DNA microarrays and DNA hybridization Reverse sample genome probing (RSGP)	Total DNA extracted Not influenced by PCR biases high specificity	Only detect most abundant species need to be able to culture organisms Dependent on lysing and extraction efficiency interpretation of the results is difficult cross-hybridization can be a problem	Hubert et al. (1999), Greene & Voordouw (2003) Cho and Tiedje (2001)
Denaturing and temperature gradient gel electrophoresis (DGGE and TGGE)	Large number of samples can be analysed simultaneously Reliable, reproducible, rapid and inexpensive Bands can be excised and sequenced	PCR biases Dependent on lysing and extraction efficiency One band can represent more than one species (co-migration) Only detects dominant species Lack of separation of small DNA fragments Laborious sample handling	Muyzer et al. (1993) Duineveld et al. (2001) Maarit-Niemi et al. (2001)
Single strand conformation polymorphism (SSCP)	Same as DGGE/TGGE No GC clamp No gradient	Same as DGGE/TGGE PCR biases Some ssDNA can form more than one stable conformation	Lee et al. (1996) Tiedje et al. (1999)
Amplified ribosomal DNA restriction analysis (ARDRA) or restriction fragment length polymorphism (RFLP)	Detect structural changes in community	PCR biases Banding pattern often too complex not useful as a measure of diversity or detection of specific phylogenetic groups	Liu et al. (1997) Tiedje et al. (1999)
Terminal restriction fragment length polymorphism (T-RFLP)	Simpler banding patterns than RFLP Large numbers of samples can be analysed Highly reproducible Can be automated	PCR biases Dependent on lysing and extraction efficiency Type of Taq can increase variability Choice of primers and of restriction enzymes All will influence community fingerprint	Tiedje et al. (1999) Dunbar et al. (2000) Osborn et al. (2000)
Ribosomal Intergenic Spacer Analysis (RISA) / Automated Ribosomal Intergenic Spacer Analysis (ARISA)	Highly reproducible community profiles Can be automated (ARISA)	Requires large quantities of DNA Resolution tends to be low PCR biases	Fisher & Triplett (1999)
qPCR	Allows quantitative results of known target organism Useful for targeting pathogens etc.	Dependent on a specific target organism requirement for previous knowledge of target organism sequence PCR biases Expensive	Hill et al. (2002)
Fluorescent in situ hybridization (FISH)	Identifies individual populations and shows spatial locality Use of several probes allows for multiple detection Enumeration of population constituents	Manual counting Time consuming and laborious Needs already designed probes so reliant on previous knowledge of species in question Membranes of some species not disrupted leaving counts often inaccurate	Zoetendel et al. (2004)
DAPI/AO	Rapid detection of bacteria	Stains all DNA Not specific	Yu et al. (1995) Weinbauer et al. (1998)
Metagenomic approaches (e.g. 454 deep sequencing)	Allows de novo whole genome sequencing of specific samples Faster, more accurate Long read lengths (400-600 million bases per 10 hour run) High data quality with rapid and accurate assemblage of multiple genomes in a single run	Erroneous base calls can be a problem with homopolymers Expensive for smaller numbers of DNA samples	Brenig et al. (2010) Lanier et al. (2007)
Culture - based	Allows understanding of the proportions of the components of complex communities Can selectively target for specific groups / species Allows characterization of organism	Selects against some bacterial species Requires knowledge of specific growth conditions Possibility of substrate-accelerated death Time consuming and laborious	Lynch et al. (2004) O'Sullivan (2000)

Molecular characterisation methods generally consist of detection or comparison of the nucleic acid sequence of specific target genes. When dealing with environments where identification of an individual known species is important, (such as pathogen identification), the nucleic acid sequence for a target gene unique to the bacterial species in question is often desirable. Alternatively, if the identification of unidentified community isolates is desired, the target sequence in question must be present in all bacterial groups or at least in a subgroup for which there is specific interest (Tannock 2002). In cases such as this, where there is limited prior knowledge of species identity, analysis of the nucleic acid sequence of universally present target genes can be used. Universal target genes containing conserved regions (flanking regions of high sequence variability) are ideal (Tannock 2002), as the comparison of regions of high variability allows the identification of different phylogenetic groups or even species, while the areas of low variability help to facilitate ease of analysis. Historically, both the 5S and 16S rRNA genes have been popular targets for molecular microbial profiling (Olsen et al. 1986). The small size of 5S rRNA allowed for complete sequence analysis to be done by the late 1960's (Olsen et al. 1986), however a lack of sequence variability limited its usefulness. 16S rRNA was also used for microbial identification studies, but its larger size limited its usefulness until the advent of more modern DNA cloning and sequencing techniques. Today, 16S rRNA is the most popular target for molecular profiling methods (Olsen et al. 1986; Pace et al. 1986; Hopkins et al. 2001; Matsuki et al. 2002; La et al. 2003), because the 16S rRNA is universally present and functionally constant in all bacterial species (Olsen et al. 1986; Pace et al. 1986; Coenye & Vandamme 2003), as are all of the rRNA genes.

These conserved regions allow for the development of universal polymerase chain reaction (PCR) primers, an important asset in the evaluation of a potential target gene. The regions of variability allow for genus or species-specific identification of bacteria via the comparison of sequences obtained with universal PCR primers. The level of variability is important as it is the key factor in determining the species of organism according to target gene sequence. A lack of variability will result in a lack of specific identification, while too much variability means the identified organisms will be unable to be grouped according to phylogeny. The size of the target gene is also important, large target genes are more difficult to sequence completely and thus can be less useful than genes that consist of shorter nucleotide sequence. However, if a

target gene has a nucleotide sequence that is too short it will not yield enough discriminatory power. In addition, the ideal target gene is present in only a single copy in the bacterial genome, an important attribute for quantification purposes. That is, a single copy allows for more accurate quantification as it represents only a single organism. A gene that is present in multiple copies may not allow accurate quantification as each organism contains a variable number of genetic copies. Finally, mutations in multiple copy genes can affect some but not all copies present in a single organism, thereby affecting quantification results (Hill et al. 2002; Ben-Dov et al. 2006).

The choice of primers to be used in studies to assess bacterial diversity is not trivial. Primers complementary to a large fraction of the gene sequences in a database, such as the ribosomal database project (RDP), does not necessarily mean that the primer is the optimal choice. No database today represents the estimated total diversity of at least 10 million bacterial species, with possible further higher sequence divergence. Additionally sequences in the database may be incomplete or corrupt. To enhance the universality of primers for the amplification of related sequences of 16S rRNA genes from different micro-organisms, degenerate primers may be designed to have a number of nucleotide options at several positions in the internal primer sequence. This will allow annealing to and amplification of a variety of related sequences. More recently, the neutral base inosine has been used to compensate for the high rate of degeneracy of the targeted codons in the mRNA and reportedly reduces the overall primer degeneracy as well as false priming and non-target gene amplification (Ben-Dov et al. 2006). However, the inclusion of inosine in universal PCR primers alters the thermodynamic stability, stacking interactions, and overall structure of the primers, potentially creating a bias against the amplification of GC-rich organisms (Hill et al. 2002).

2.2 Primer Choice:

Thirteen 16S rRNA gene primer pairs previously reported in a range of literature from both soil and coral microbial research (Muyzer et al. 1993; Nubel et al. 1996; Watanabe et al. 2001; Horz et al. 2005; Lindh et al. 2005; Rosch & Bothe 2005; Ben-Dov et al. 2006; Sanchez et al. 2007), were subjected to a comparative analysis in

order to assess the most suitable pair for work within this thesis. The main aim was to determine which primers routinely gave the optimal end product, assessed in terms of (a) usable number of bases retrieved from cloned sequences and (b) number, clarity and separation of products on denaturing gradient gel electrophoresis (DGGE) gels. The sequences, as well as the annealing temperatures for the PCR and optimum DGGE conditions used in this study are detailed in Table 2.2. PCR conditions were as described in the original references.

Table 2.2. Primers utilised in this study to compare variation within bacterial diversity of coral reef water samples, showing primer name, sequence, target region, annealing conditions, amplicon length, DGGE conditions and original citation source

Primer[a]	Sequence (5´ - 3´)	Annealing conditions	Amplicon Length (bp)	DGGE conditions[b]	Reference
341(357)f 907(928)r	CCTACGGGAGGCAGCAG CCGTCAATTCCTTTGAGTTT	63-53°C - 1ºC/cycle	586	6%, 40-60%, 100V 17hrs	Muyzer et al. 1996
357f 907Rm	CCTACGGGAGGCAGCAG CCGTCAATTC**M**TTTGAGTTT	63-53°C - 1ºC/cycle	586	6%, 40-60%, 100V 17hrs	Sanchez et al. 2007
I 357f 907Rm	CCTACGGG**I**GGC**I**GCA CCGTCAATTC**M**TTTGAGTTT	63-53°C - 1ºC/cycle	586	6%, 40-60%, 100V 17hrs	Sanchez et al. 2007
I 357f I 907	CCTACGGG**I**GGC**I**GCA CCGTCAATTCMTTTGAGTT**I**	63-53°C - 1ºC/cycle	586	6%, 40-60%, 100V 17hrs	Ben-Dov et al. 2006
I 8f (27) 907(928)r	AGAGTTTGAT**II**TGGCTCAG CCGTCAATTCCTTTGAGTTT	58-48°C 1ºC/ second cycle	901	6%, 40-60%, 100V 17hrs	Ben-Dov et al. 2006
I 357f 778r	CCTACGGG**I**GGC**I**GCA AGGGTATCTAATCCTGTTTGC	63-53°C - 1ºC/cycle	437	8%, 40-60%, 100V 17hrs	Rosch & Bothe 2005
357f 518r	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	63-53°C - 1ºC/cycle	177	10%, 40-60%, 75V 18hrs	Muyzer et al. 1993
I 357f 518r	CCTACGGG**I**GGC**I**GCA ATTACCGCGGCTGCTGG	63-53°C - 1ºC/cycle	177	10%, 40-60%, 75V 18hrs	Muyzer et al. 1993
I 341f I-533	CCTACGGG**I**GGC**I**GCA T**I**ACCG**III**CT**I**CTGGCAC	62-52ºC - 1ºC/ second cycle	192	10%, 40-60%, 75V 18hrs	Watanabe et al. 2001
pA (27) pH (1542)[d]	AGAGTTTGATCGTGGCTCAG AAGGAGGTGATCCAGCCGCA	63-53°C - 1ºC/cycle	1515		Cooney et al. 2002
I 8f (27) pH (1542)[d]	AGAGTTTGAT**II**TGGCTCAG AAGGAGGTGATCCAGCCGCA	58-48°C 1ºC/ second cycle	1515		Ben-Dov et al. 2006
I 8f (27) 1401[d]	AGAGTTTGAT**II**TGGCTCAG CGGTGTGTACAAGACCC	58 - 48°C 1ºC/ second cycle	1374		Lindh et al 2005
I 8f (27) I 1501[d]	AGAGTTTGAT**II**TGGCTCAG CGG**I**TACCTTGTTACGAC	58 - 48°C 1ºC/ second cycle	1474		Lindh et al 2005

[a] Forward primers all with a 40-bp GC clamp at 5´ end: 5' – CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA GCA CGG GGG G-3' incorporated in the forward primers at its 5' end to prevent complete disassociation of the DNA fragments during DGGE.

[b] Inosine used due to findings from Ben-Dov et al. 2006

[c] Conditions given are percentage polyacrylamide, denaturing gradient, voltage, and running time, respectively

[d] Trialled for cloning and sequencing purposes not DGGE analysis

Despite the reported advantages of using the natural base insert inosine in universal primers (Ben-Dov et al. 2006), results showed that primers with this insert caused no significant difference (ANOSIM R = 1.27, p = 0.32) in community structure when compared to the same pair without the added base, in addition there was a greater amount of smearing and the production of a generally unclear DGGE gel image with the inosine primer pair (Fig 2.1).

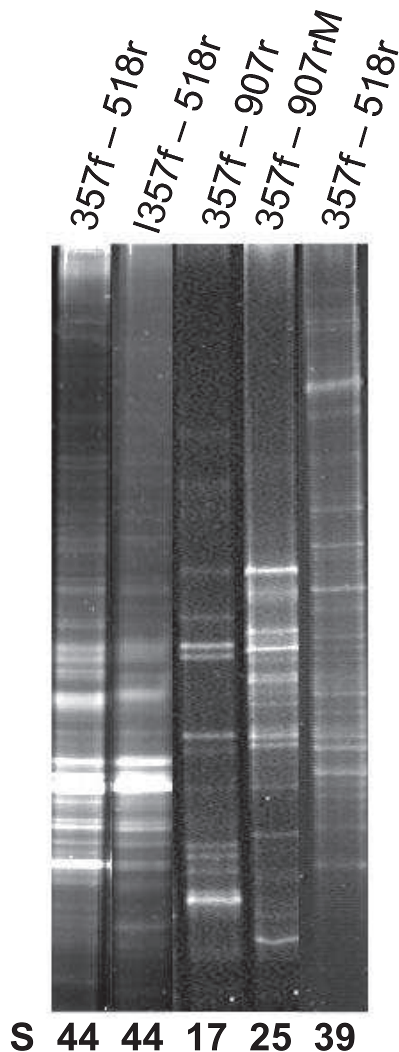


Figure 2.1. DGGE image showing a subset of the results of the primer comparison tests, highlighting the difference between the reverse primers, 518, 907 and 907rM, along with a representation of the forward primer 357 with the neutral base inosine as an insert. Replication in this instance was 'technical' not 'biological', whereby the same sample was used with each primer pair tested. S = species diversity represent by number of bands detected using BioNumerics.

Primer 907r was included in the original tests as it was commonly used by the Coral Health and Disease Laboratory at Newcastle University (Cooney et al. 2002; Pantos et al. 2003; Guppy & Bythell 2006). Although 907rM differs from this primer by only one base (Table 2.2), Sanchez et al. (2007) showed 907rM matched to a greater number of sequences in GenBank compared to the original primer with no modifications (Fig. 2.2). The original 907r universal primer did not perfectly match either the SAR11 cluster or some commonly retrieved marine γ - proteobacteria (Fig. 2.2).

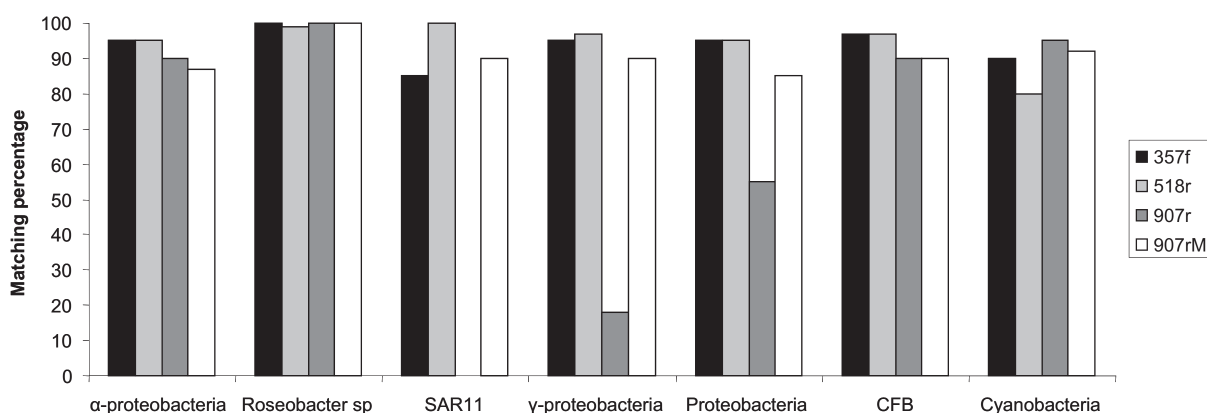


Figure 2.2. Histogram of matching percentages for the most abundant phylogenetic groups in coastal bacterioplankton obtained from data in the RDP11 database (adapted from Sanchez et al. (2007)).

This was further supported by DGGE analysis, showing a significant difference ($p = 0.001$) between profiles of the two primer pairs 357f – 907r and 357f – 907rM, with a greater diversity ($S = 17$ and 25 respectively) obtained with the later primer pair (Fig. 2.1). However, consistency between samples run with 357f and 907rM on the DGGE was not as good when compared to primer pairs 357f and 518r. Although this primer pair gives a much shorter product of 194bp, which has limitations for phylogenetic information contained in the sequenced bands, the use of this primer pair allowed a 10% gel on the DGGE to be used compared to the 6% used for the other pair, which gave substantially improved banding separation and clarity when run, allowing easier identification of individual bands. Primers 357f and 518r were therefore, chosen over more traditional sets for the above reasons along with the evidence that they more comprehensively amplified marine bacteria due to certain mismatches caused by those such as 907r (pC) (Muyzer et al. 1993; Guppy & Bythell 2006; Sanchez et al. 2007).

Chapter 3

Temporal and spatial patterns in waterborne bacterial communities of an island reef system

3.1 ABSTRACT:

The bacterial 16S rRNA gene diversity of waterborne bacterial (WBB) communities was assessed using PCR/denaturing gradient gel electrophoresis (DGGE) techniques, along with sequence analysis of selected bands. 16S rRNA gene diversity varied between seasons, and significant differences were recorded between night and day. However, there were no significant differences detected between low, ebb, flood and high tides when the water body sampled would have originated from completely different areas including those off-reef. These results suggest that changes in productivity and/or vertical diurnal migrations of plankton may have greater effects than large-scale water movements effected by tidal flows. These results do not demonstrate a strong link between WBB communities and their underlying benthos. This either suggests a lack of coupling between the benthos and the water column (benthic–pelagic coupling) or that the processes are extremely rapid and efficient with strong mixing. Previous studies at this site have shown cycling between coral reef and lagoon sediments via coral mucus release and tidal transport, supporting the latter. We found a strong seasonality in the abundance and composition of WBB communities, with *Alphaproteobacteria* being more prevalent during winter and *Gammaproteobacteria* during summer, but quantitative PCR (qPCR) showed no significant differences in vibrios between seasons.

Published as; Sweet, M.J., Croquer, A., Bythell, J.C. (2010) Temporal and spatial patterns in waterborne bacterial communities of an island reef system. Aquatic Microbial Ecology 61:1-11. This author designed and conducted the field work, analysed the samples and wrote the paper, Croquer and Bythell edited the manuscript ready for publication.

3.2 INTRODUCTION

Waterborne bacteria (WBB) in coral reef systems are very numerous and diverse, and their productivity is high compared to open oceanic waters (Sorokin 1973, Sorokin 1974). Periodic influx of open water masses into coral reef lagoons is a process known to affect key ecological processes such as transport of nutrients (Naumann et al. 2009), delivery of dissolved and particulate organic matter (Crossland et al. 1984) and primary productivity (Moses et al. 2009); therefore, this influx should influence the dynamics of WBB communities. This hydrodynamic process is complex, being extremely variable in space and time, depending on depth, reef topography, position, the lunar cycle and time of the day, among other factors. However, culture and non-culture (molecular) approaches to assessing WBB communities in coral reef ecosystems, encompassing different spatial and temporal scales, are limited, with only a few localised studies looking at changes associated with tidal cycles or seasonal change (Moriarty et al. 1985; Torreton & Dufour 1996; Guppy & Bythell 2006).

Coral reefs such as those studied here may release ca. 1.7 l of mucus m^{-2} d^{-1} into the water column (Wild et al. 2004). More than half (56 to 80 %) of the released carbon is dissolved directly into the water column, providing a food source for planktonic bacteria (Wild et al. 2004). The less soluble fraction (20 to 44 %) forms mucus strands that detach from the coral branches, passing upwards through the water column and subsequently aggregating and accumulating at the surface. These are concentrated by currents and winds to form large (2 to 10 m) 'mucus mats', trapping larger particles present within the water column. Continuous compaction and accumulation of re-suspended particles gradually decrease the buoyancy of these mats, and coupled with water movements of incoming tides, concentrate them towards the lagoon. Rapid sinking occurs at distances of over 150 m from the reef crest. The microbial communities of lagoon sands degrade this organic matter in the upper sediment layers through a natural filter system brought about by wave action and tide-induced differences in water level (Wild et al. 2004). Thus, under such 'benthic–pelagic coupling' (Wild et al. 2004), strong spatial differences in WBB would be expected between different water masses (reef crest, reef flat, lagoon water

and off-reef water). Added to this, seasonal changes in waves, currents and organic matter deposition as well as benthic activities are thought to cause spatial and temporal variation in sediment–water exchange processes, affecting sedimentary organic carbon decomposition and O_2 fluxes (Wild et al. 2009). These environmental changes in turn may also produce shifts in the WBB communities, 'mucus mats' and sediment types over time and across sites.

Despite increasing interest in benthic–pelagic coupling in coral reefs (Wild et al. 2004, Naumann et al. 2009, Wild et al. 2009), baseline studies examining the spatial and temporal changes of bacteria within the water column and in particular its relation to the potential supply of bacteria to the coral are limited (Sunagawa et al. 2010). Previous attempts to compare the WBB communities based on discrete sampling (e.g. bottles, (Paul et al. 1986); Sterivex filtration, (Somerville et al. 1989); and syringe membrane filters, (Guppy & Bythell 2006) may not adequately address the highly variable spatial-temporal nature of these assemblages, which may underestimate the long-term delivery of microorganisms to a coral. Thus, this study aimed to provide a comprehensive measure of the bacterial diversity in the water column through space and time, by continually sampling over 1 h periods using a pump sampler. This enabled the inlet to be precisely positioned for greater accuracy and flow rates to be controlled to approximate natural delivery rates at environmentally relevant flow speeds (matched by the pump when in the field at the time of sample collection). In this study, we tested whether the composition of bacterial 16S rRNA gene diversity changed in relation to the influence of off-reef, open water masses and their relative position in the reef tract (i.e. lagoon, reef flat and reef front) and whether the structure of bacterial communities changed at different temporal scales (from diurnal variations and tidal cycles to seasonal shifts in diversity).

3.3 MATERIALS AND METHODS

3.3.1 Study area.

The study was conducted at Heron Island, Great Barrier Reef (GBR), Australia (Fig. 3.1) over 2 yr (2008 to 2009), encompassing both a summer (March 2009) and winter (August 2008) season. A total of 5 sites (A–E) were assessed at high tide to estimate spatial variability in WBB communities. Tidal, diurnal and seasonal variability was evaluated at a single site (A) located on the reef flat (Fig. 3.1). These sites were chosen as they were expected to show variation in their bacterial diversity due to differences in the benthos and known oceanographic patterns around the island. For consistency, no spatial samples were taken when wind speeds were above 5 m s^{-1} (10 knots). Sites A, B, D and E were located in reef areas where delivery of water masses was from off-reef areas; thus, at the time of collection (high tide), all of these sites had received a strong influence of open waters (water depths >30 m), with currents flowing southwards. At flow speeds previously recorded on the reef flat of approximately 0.3 m s^{-1} (Gourlay & Hacker 2008), this would mean that the water masses would have originated approximately 6 km offshore to the north during the previous low tide. Site A and, to a lesser extent, B would also have been influenced by strong mixing during passage of the water body over the shallow reef crest to the northwest within the last tidal cycle. Site C was located in the predominantly sandy lagoon system, where weaker currents and ponding (Ludington 1979) would mean that the water mass would likely have circulated locally, with minimal influence of off-reef open waters (Fig. 3.1).

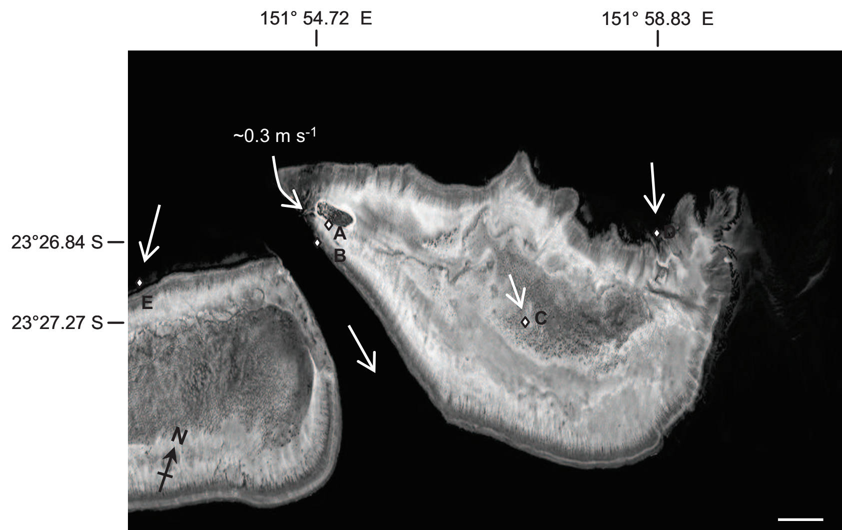


Figure 3.1. Heron Island, Great Barrier Reef, Australia (23° 27' S, 151° 55' E). Location of main study site - Reef Flat (A) - and those used in spatial sampling: B, Coral Gardens (23° 26.839' S,151° 54.717' E); C, Lagoon (23° 27.272' S, 151° 57.921' E); D, 3rd/4th Point (23° 26.146' S, 151° 58.833' E); E, Wistari (23° 29.081' S, 151° 54.015' E). Arrows depict approximate direction of current flow on the flood tide, prior to sampling on the high tide. Samples were taken on calm days with wave speed <0.5 m s^{-1} and wave heights <0.5 m. Scale bar = 1 km.

3.3.2 Sample collection. Spatial variation:

Spatial samples for both diversity and abundance were taken using discrete sterile 1 l bottles, 5 cm above a colony of *Acropora muricata* (= *formosa*). All samples were taken during both summer (March 2009) and winter (August 2008) within 1 h before high tide, over 2 consecutive days at a constant depth of 8 m (except for the lagoon and reef flat, where maximum depths were 2 m). This ensured that the water masses being sampled were representative of different environments.

3.3.3 Temporal and diurnal variation:

Samples to assess temporal changes in bacterial diversity were collected from Site A (Fig. 3.1) using a peristaltic pump (Masterflex E/S, Cole-Parmer) with internal battery source and polytetrafluoroethylene (PTFE) tubing. The tubing allowed positioning of the inflow to be accurate and permanently in place, anchored directly next to a colony of *A. muricata,* therefore giving an accurate representation of the WBB that a coral would be exposed to over the sampling period. All apparatus was placed within a tin boat anchored permanently offshore, allowing easy access day or night. This set-up enabled 1 l of water surrounding the corals to be directly and continuously sampled onto 0.22 μm Sterivex filters. The sampling system filtered water continuously for a period of 1 h. Samples were collected every 2 h within a 24 h period. This sampling regime was duplicated within a period of 14 d and repeated over 2 yr, encompassing both summer and winter seasons. The sampling was timed to encompass 30 min on either side of high, mid and low tides. In this way, both diurnal (night/day) and tidal (high, low, ebb and flood) effects on bacterial abundance and diversity could be assessed along with seasonal effects. Bacterial communities were simultaneously sampled using discrete bottle samples and the pump sampler for method comparison.

Samples for analysis of bacterial abundances were collected with discrete bottles, similar to that of the spatial samples and at the same time as the temporal, diurnal and tidal samples on the fore reef; from this initial 1 l sample, a volume of 15 ml was vacuum-filtered through a 0.22 μm black polycarbonate filter immediately after collection and fixed with 100 μl of 4 % PBS buffered paraformaldehyde solution until analysis (Fuhrman et al. 2008). In total, 3 replicates were taken at every sample period both for bacterial diversity and abundance regardless of the method. Hobo® (Onset Computer Corporation) temperature data loggers were deployed to record the temperature fluctuation at all sites.

3.3.4 Total bacterial abundance.

To estimate bacterial abundances, each sample was stained with 100 µl of 4 % PBS buffered paraformaldehyde solution containing 4'6-diamidino-2-phenylindole (final concentration 5 µg ml^{-1}) for 10 min, rinsed with filtered 1 × PBS pH 7.4 (Yu et al. 1995; Weinbauer et al. 1998; Yamaguchi et al. 2007) and viewed under epifluorescence microscopy. Counts on 50 fields of view were taken, scaled up to the total area of the filter and calculated to give total bacterial abundance ml^{-1} of sea water filtered. An average of the 3 replicates was taken and used in further analysis.

3.3.5 Bacterial diversity, DNA extraction, amplification and denaturing gradient gel electrophoresis (DGGE) analysis.

DNA was extracted from the filters using QIAGEN DNeasy Blood and Tissue kits with an added step to concentrate the lysate using vacuum centrifugation for 2 h at 20°C. DNA encoding bacterial 16S rRNA was amplified using standard prokaryotic primers (357F) (5'-CCTACGGGAGGCAGCAG-3') and (518R) (5'-ATTACCGCGGCTGCTGG-3'). These primers were chosen over more traditional ones as they have been recently shown (Sanchez et al. 2007) to more comprehensively amplify marine bacteria compared to inadequacies and mismatches caused by those such as 907r (pC) (Muyzer et al. 1993; Guppy & Bythell 2006; Sanchez et al. 2007). The GC-rich sequence (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGG GCAGCACGGGGGG-3') was incorporated in the forward primer 357 at its 5' end to prevent complete disassociation of the DNA fragments during DGGE. Thirty PCR cycles were performed at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 10 min (Sanchez et al. 2007). A 30 µl PCR reaction was used containing 1.5 mM $MgCl_2$, 0.2 mM dNTP (PROMEGA), 400 ng μl^{-1} bovine serum albumin (BSA), 0.5 mM of each primer, 2.5 U of *Taq* DNA polymerase (QBiogene), incubation buffer, and 20 ng of template DNA (Siboni et al. 2007). All reactions were performed using a Hybaid PCR Express thermal cycler. PCR products

were verified by agarose gel electrophoresis (1.6 % [w/v] agarose) with ethidium bromide staining and visualised using a UV transilluminator.

DGGE was performed using the D-Code universal mutation detection system (Bio-Rad). PCR products were resolved on 10 % (w/v) polyacrylamide gels that contained a 30 to 60 % denaturant gradient for 13 h at 60 °C and a constant voltage of 50 V. Gels were stained with a concentrated solution of 9 µl SYBR® Gold (Sigma) in 50 µl of 1× TAE (Tris-acetate-EDTA) poured directly onto the gel surface, covered and left in the dark for 20 min then further washed in 500 ml 1× TAE for 30 min and visualised using a UV transilluminator. Bands of interest (those which explained the greatest differences/similarities between samples) were excised from DGGE gels, left overnight in Sigma molecular grade water, vacuum centrifuged, re-amplified with primers 357F and 518R, labelled using a Big Dye (Applied Biosystems) transformation sequence kit and sent to Genevision (Newcastle University) for sequencing. Bacterial operational taxonomic units (OTUs; (Guppy & Bythell 2006)) were defined from DGGE band-matching analysis using BioNumerics 3.5 (Applied Maths BVBA). Standard internal marker lanes were used to allow for gel-to-gel comparisons. Tolerance and optimisation for band-matching was set at 1 %.

Real-time PCR (qPCR) was conducted on an Engine Opticon® 2 system in order to test whether *Vibrio* spp. abundance changed between seasons. For this, *Vibrio*-specific primers 567F, 5’-GGCGTAAAGCGCATGCAGGT-3’ and 680R, 5’-GAAATTCTACCCCCCTCTACAG-3’ (Thompson et al. 2004) were used on 10 randomly chosen samples from both the summer and winter seasons. This primer pair has previously been shown to be highly targeted towards vibrios, matching 42 out of 43 sequences of *Vibrio*-type strains in the Ribosomal Database Project (RDP) (Thompson et al. 2004). Real-time PCR reaction mixtures totalled 25 µl and consisted of 12.5 µl of 2× Quantitect® SYBR® Green 1 supermix (Qiagen), 1.25 µl each of 0.5 mM forward and reverse primers, 50 ng DNA and 9.5 µl Sigma molecular grade water. Each set of samples included a negative control, in which water was substituted for the DNA sample. Real-time PCR was performed with an initial activation step of 15 min at 95 °C, followed by 39 cycles (94 °C for 15 s, 58 °C for 30 s, primer

annealing at 58 °C for 30 s). The fluorescent product was detected after each extension. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.5 °C s^{-1} increments from 50 to 90 °C, with continuous fluorescence recording.

3.3.6 Statistical analysis.

For bacterial counts, an automatic cell counter (Cell C) was used (Selinummi et al. 2005). The parameters were set to exclude any objects smaller than 0.0314 μm^2 and anything larger than 0.7 μm^2. The abundance of bacteria was compared across sites and between seasons, time of day and tidal state with a 1-way and a 3-way analysis of variance (ANOVA), respectively. Data were normally distributed and variances were equal. In order to assess spatial and temporal changes in bacterial assemblages, matrices consisting of OTUs and samples were generated using both presence/absence and band intensity data, using marker lanes for between-gel comparisons. Spatial changes in WBB assemblages were evaluated with a 1-way analysis of similarity (ANOSIM, Primer) and multidimensional scaling (MDS), based on Bray-Curtis similarities, which was performed on both summer and winter data sets. Methods of collection (those sampled only during August 2008) were compared by a 1-way ANOSIM (Primer).

For temporal comparisons, a 3-way multivariate permutation ANOVA (PERMANOVA; Primer) based on Bray-Curtis distance (Anderson 2001) was used in order to test for temporal changes in bacterial assemblages (Factor 1: season, fixed with 2 levels = summer and winter; Factor 2: tide, fixed with 4 levels = high, low, flow and ebb; Factor 3: diurnal, fixed with 2 levels = night and day). A non-metric MDS analysis was used to visualise the temporal patterns in bacterial communities, and an analysis of the contribution of variables to similarity (SIMPER, Primer) was conducted to determine the OTU that best explained spatial differences only when spatial and temporal differences were found (Clarke & Warwick 2001). Temperature

was compared across seasons, diurnal and tidal cycles by a 3-way ANOVA. Effects and relative importance of individual constrained factors (diurnal cycle, tide and temperature within season) were assessed separately after removing the variance due to season using a unimodal partial correspondence analysis (pCCA; using R).

Real-time PCR calculations were based on relative DNA concentration ($\Delta C[t]$) of vibrios based on lowest detected concentration ($C[t]$). Fold differences in *Vibrio* DNA template were calculated assuming 2-fold PCR reaction efficiency ($2^{\Delta C(t)}$). One-way ANOVA (minitab) was used to compare between seasons.

3.4 RESULTS

3.4.1 Spatial and temporal patterns in WBB abundances

There was no significant difference in bacterial abundances between sites within both summer ($8 \pm 1 \times 10^6$ cells ml^{-1} recorded across all sites) and winter ($1.55 \pm 1 \times 10^6$ cells ml^{-1}), which suggests that the influx of surrounding off-reef waters had little effect on total bacterial abundance at our sites (Fig. 3.2a). However, bacterial abundances showed a strong seasonal pattern (ANOVA $F = 23.02$, $df = 1$, $p < 0.001$), increasing by approximately 3-fold during summer on the reef flat. Although there appeared to be a greater mean abundance during low tides compared to high tides and an apparent diurnal trend, where abundance increased from morning to afternoon within the summer season (Fig. 3.2b), this was not significant (ANOVA $F = 1.29$, $df = 3$, $p = 0.293$).

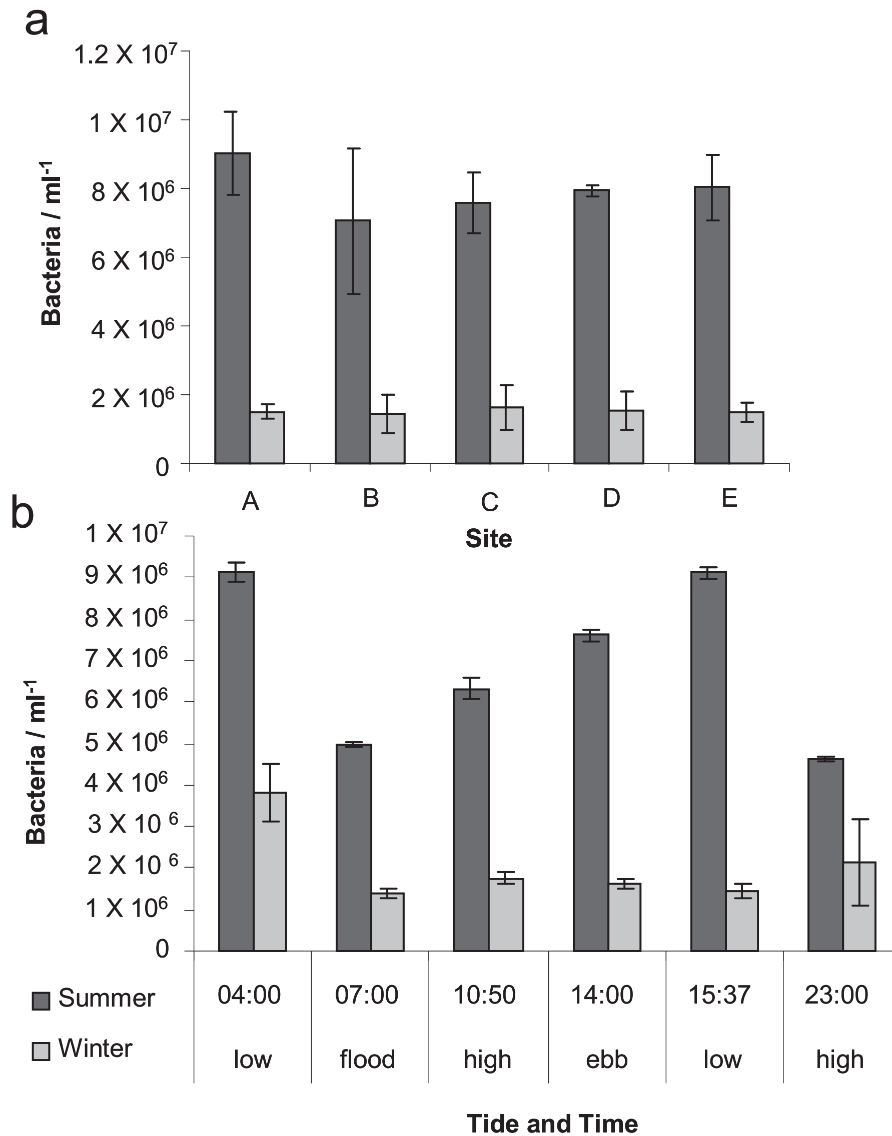


Figure 3.2. Total bacterial abundance. (a) Spatial variation for 5 locations around Heron Island during winter (August 2008, grey bars) and summer (March 2009, black bars). A: Reef Flat, B: Coral Gardens, C: Lagoon, D: 3rd/4th Point, E: Wistari. (b) Temporal, tidal and diurnal variation on the reef flat (Site A) for winter (August 2008, grey bars) and summer (March 2009, black bars). Error bars for both represent SE.

3.4.2 Spatial and temporal patterns in WBB diversity

WBB diversity was significantly different between both seasons and time of day (PERMANOVA, $F = 5.4$, $p = 0.001$ for season and $F = 2.34$, $p = 0.002$ for time of

day) but not for tide (PERMANOVA, F = 1.26 p = 0.063) (Table 3.1). Bacterial diversity also showed significant interaction between season and diurnal cycles (PERMANOVA, F = 1.96, df = 1, p = 0.005) but non-significant interaction between season and tidal cycles (Table 3.1, Fig. 3.3a). Between factors, season alone explained 14 % of the variance whilst the significant interaction between time of day, tide and season explained a further 10.5 % (Table 3.1).

Table 3.1. Three-way permutation analysis of variance (PERMANOVA) based on Euclidian distances. Significant differences shown in bold

Source	df	SS	MS	F	P(perm)	Variance explained
Diel	**1**	**7769.8**	**7769.8**	**2.3406**	**0.002**	**7.68**
Tide	3	12551	4183.6	1.2603	0.063	4.48
Season	**1**	**17926**	**17926**	**5.4001**	**0.001**	**13.91**
Diel x Tide	**3**	**14828**	**4942.8**	**1.489**	**0.005**	**8.69**
Diel x Season	**1**	**6521.1**	**6521.1**	**1.9644**	**0.005**	**9.21**
Tide x Season	3	11899	3966.2	1.1948	0.116	5.48
Diel x Tide x Season	**3**	**13520**	**4506.6**	**1.3576**	**0.017**	**10.51**
Residual	97	3.22E+05	3319.6			40.04
Total	112	4.13E+05				

Over 40 % of the total variance was explained by differences among replicates, which highlights the variable nature of WBB communities; pCCA with permutations stratified within seasons showed the percent variance of each factor with diurnal cycle effects being highly significant (F = 2.05, p < 0.01), whilst both temperature (within each season) and tide were not significant (F < 1.1, p > 0.26). Average Bray-Curtis similarity between summer and winter was 15 %, with different ribotypes from *Alpha*- and *Gammaproteobacteria* (Fig. 3.3b-d), *Flavobacteria* (Fig. 3.3e) and *Bacteroidetes* related ribotypes (Fig. 3.3f) explaining 38.5 % of the dissimilarity (Table 3.2). Interestingly, differences in the bacterial community between night and day were more pronounced during winter compared to summer (Fig. 3.4). Since tides had no effect, this indicates that seasonality apparently has a greater effect on the composition of WBB communities than the benthic community that the water body has passed over, which in turn would be dependent on the state of the tide.

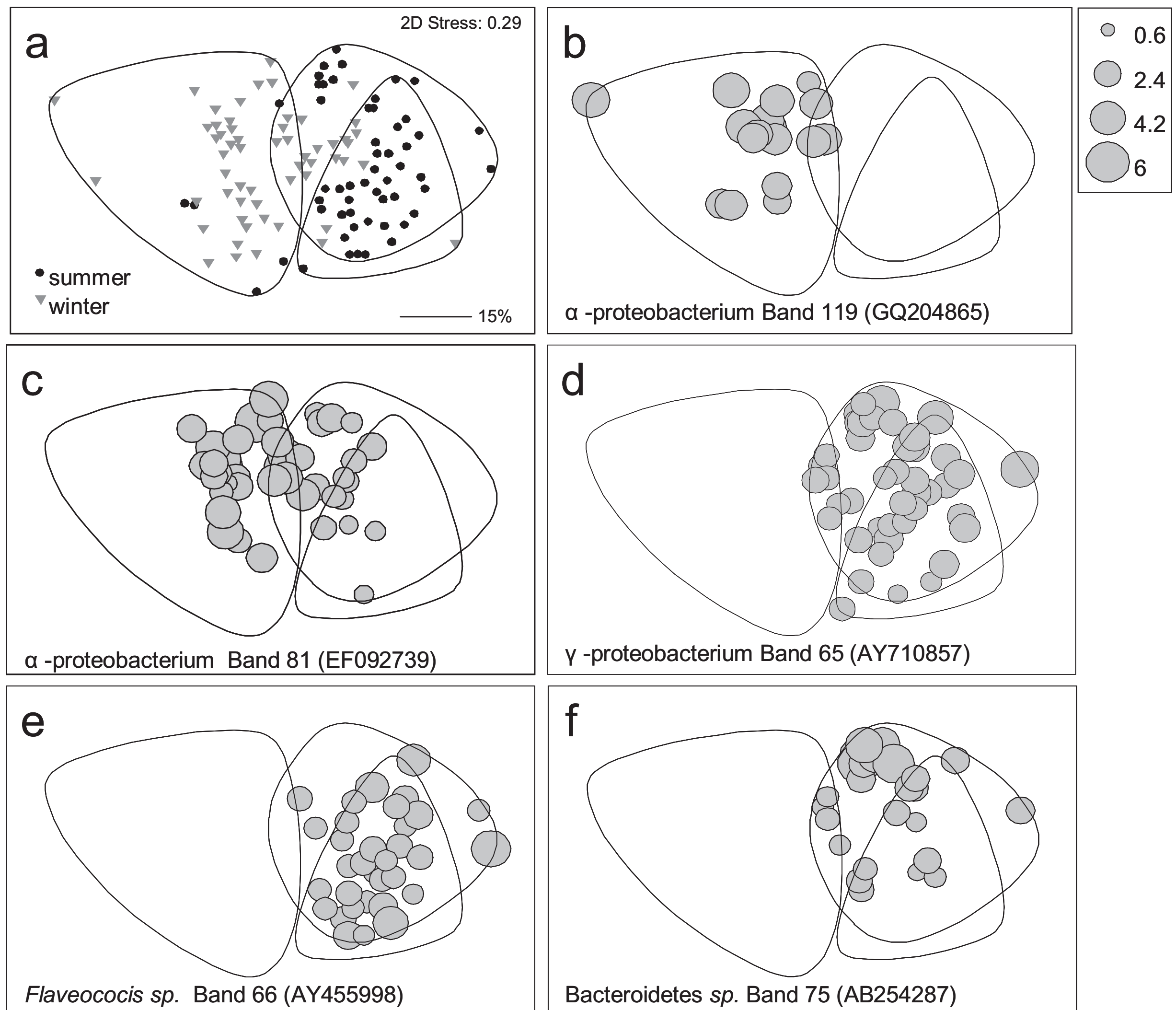


Figure 3.3. (a) Multidimensional scaling (MDS) plot, showing seasonal changes in 16S rRNA gene profiles of bacterial communities. Each point represents an independent sample labelled by season (see key). (b–f) Relative abundances of a subset of specific bacteria present or absent within each sample that contributed to the similarities and differences seen within the seasonal and diurnal patterns. Bubble size represents relative density of the denaturing gradient gel electrophoresis (DGGE) band of that particular bacterial sequence within individual samples. Contour lines represent average Bray-Curtis similarity of 15%.

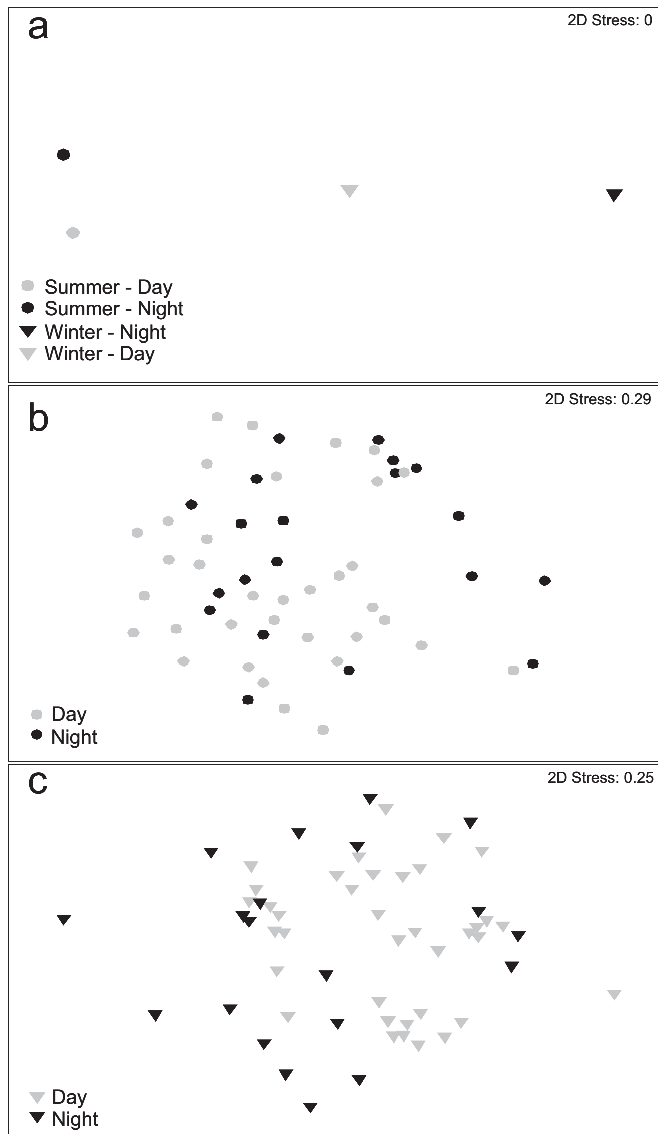


Figure 3.4. Multidimensional scaling (MDS) plot, showing diurnal changes in bacterial communities (16S rRNA gene fingerprints) on the reef flat between seasons. (a) Mean of samples for summer day (n = 37), summer night (n = 20), winter day (n = 35), winter night (n = 20). (b) All samples for the winter season. (c) All samples for the summer season.

Table 3.2. Closest match (GenBank ID) and identification of bacterial species from the water column sequenced from denaturing gradient gel electrophoresis (DGGE) bands. Out of a total 143 operational taxonomic units (OTUs), 51 are represented in this table, which account for 70 % of the variance between the 2 seasons (summer and winter). Relative abundance measurements are based on BioNumerics presence/absence and band intensity

			Abundance (band intensity)		Average dissimilarity 86.6%	
DGGE band	Closest relative (% match)	Species ID	Summer	Winter	Contribution (%)	Accumulative Contribution (%)
119	GQ204865 (100)	Alphaproteobacteria	0.34	1.61	2.52	2.52
83		Unknown	0.92	1.12	2.39	4.9
74	GQ204865 (98)	Alphaproteobacteria	1.26	0.79	2.31	7.22
65	AY710857 (100)	Gammaproteobacteria	1.45	0.45	2.3	9.52
125		Unknown	0.38	1.14	2.3	11.81
112		Unknown	0.74	1.12	2.01	13.82
86		Unknown	0.42	1.19	1.96	15.79
118	GQ204865 (97)	Alphaproteobacteria	0.5	1.13	1.95	17.73
104		Unknown	0.94	0.53	1.76	19.49
92	EU005645 (78)	Gammaproteobacteria	0.38	0.83	1.75	21.24
66	AY455998 (89)	*Flavococcus sp.*	1.12	0.19	1.74	22.98
127		Unknown	0.23	0.93	1.74	24.72
42	AB254287 (100)	Bacteroidetes sp.	0.93	0.24	1.7	26.42
91	GQ204865 (100)	Alphaproteobacteria	0.77	0.54	1.63	28.05
75	AB254287 (100)	Bacteroidetes sp.	0.91	0.32	1.63	29.69
81	EF092739 (95)	Alphaproteobacteria	0.85	0.42	1.56	31.25
126		Unknown	0.18	0.85	1.55	32.8
87	GQ204834 (88)	Alphaproteobacteria	0.55	0.62	1.45	34.25
85	FJ620860 (90)	Alphaproteobacteria	0.43	0.76	1.43	35.68
41	FJ745255 (89)	Flavobacterium sp.	0.73	0.14	1.24	36.92
99	FJ620860 (95)	Alphaproteobacteria	0.54	0.45	1.24	38.16
71	EU600663 (100)	Flavobacteria sp.	0.56	0.35	1.22	39.38
90	FJ620845 (83)	Alphaproteobacteria	0.39	0.58	1.2	40.58
115	EF092824 (92)	Alphaproteobacteria	0.35	0.61	1.2	41.78
82		Unknown	0.62	0.33	1.19	42.98
111	EU315614 (97)	Alphaproteobacteria	0.4	0.48	1.19	44.16
70	AM989479 (95)	*Tenacibaculum sp.*	0.35	0.51	1.18	45.35
64	AB294989 (100)	Flavobacteriales sp.	0.56	0.41	1.18	46.53
78	EF092824 (92)	Alphaproteobacteria	0.58	0.41	1.18	47.7
120		Unknown	0.13	0.66	1.16	48.86
110	AB254277 (83)	Cyanobacteria	0.31	0.57	1.13	50
46	EU315645 (88)	Gammaproteobacteria	0.68	0.15	1.1	51.1
95	EF486532 (95)	Alphaproteobacteria	0.36	0.52	1.1	52.19
114	FJ620845 (87)	Alphaproteobacteria	0.3	0.57	1.09	53.28
116	EU600663 (77)	Flavobacteria sp.	0.42	0.41	1.08	54.37
128		Unknown	0.16	0.52	1.07	55.43
113		Unknown	0.18	0.57	1.05	56.49
63	AJ784117 (78)	*Eubacterium sp.*	0.3	0.49	1.01	57.49
129		Unknown	0.16	0.48	1	58.49
58	EU984467 (81)	Alphaproteobacteria	0.41	0.36	0.98	59.47
84	GQ250615 (89)	Alphaproteobacteria	0.17	0.58	0.97	60.45
67	GQ257639 (82)	Gammaproteobacteria	0.62	0.06	0.97	61.41
102		Unknown	0.37	0.39	0.96	62.37
106		Unknown	0.5	0.21	0.96	63.33
109		Unknown	0.5	0.12	0.94	64.27
97	FJ532499 (100)	Alphaproteobacteria	0.52	0.2	0.93	65.2
89		Unknown	0.08	0.54	0.88	66.08
121		Unknown	0.13	0.46	0.86	66.94
122		Unknown	0.33	0.24	0.85	67.79
117		Unknown	0.28	0.34	0.85	68.64
69	DQ656191 (95)	Bacteroidetes sp.	0.44	0.18	0.85	69.48
108	AM748242 (84)	SAR 11 (*Pelagibacter sp.*)	0.26	0.37	0.84	70.32

Significant interactions with temperature were found between seasons and the diurnal cycle (ANOVA, $F = 6.2$, $df = 1, 101$ $p = 0.01$) and between seasons and the tidal cycle (ANOVA, $F = 4.43$, $df = 2, 101$ $p = 0.01$) (Fig. 3.5). This observation suggests that the effect of temperature is tied to the effects seen in diurnal and tidal patterns of WBB. Therefore it is difficult to separate the effects of temperature alone as a potential environmental driver for WBB dynamics (Pommier et al. 2007, Fuhrman et al. 2008), and this was supported by the pCCA result, whereby the effect of temperature within seasons was not significant. However, the factors controlling the presence or absence of particular ribotypes (*Alpha*- and *Gammaproteobacteria*, *Flavobacteria* and *Bacteroidetes* related sequences) within summer and winter seasons deserves further investigation (Table 3.2, Fig. 3.3b,f).

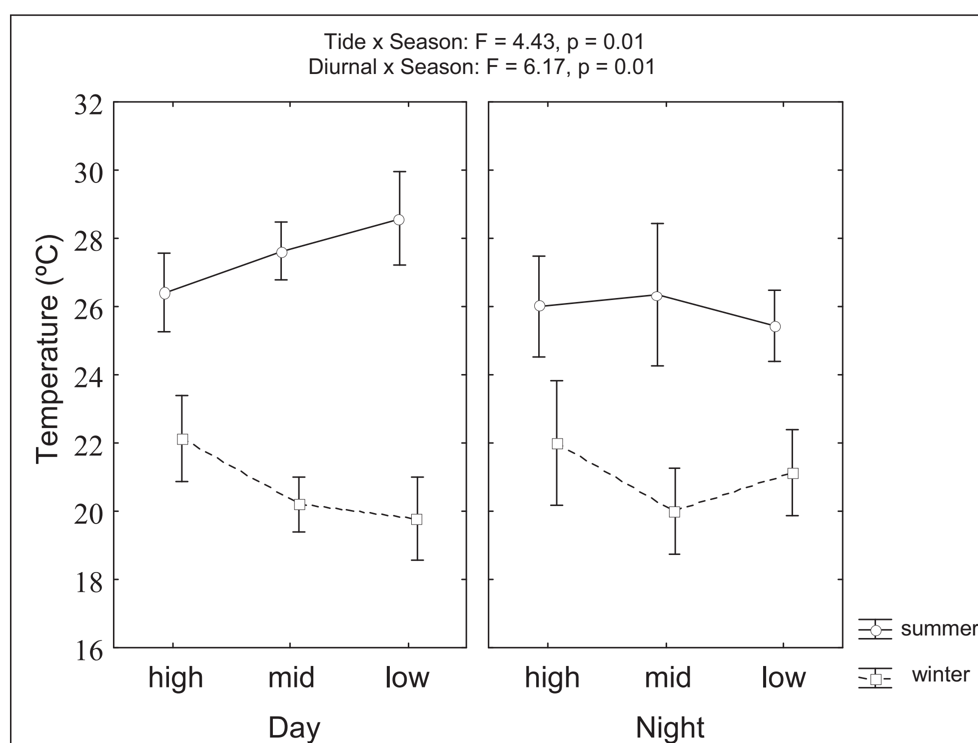


Figure 3.5. Sea temperatures at Heron Island collected using a Hobo® (Onset Computer Corporation) data logger, showing seasonal, tidal and diurnal patterns. Results of a PERMANOVA showing significant interactions with temperature between season and diurnal cycles and between season and tidal cycles are included in the figure. Error bars show SE.

Real-time PCR showed no significant differences between total *Vibrio* DNA in summer and winter samples (ANOVA F = 0.58, p = 0.457). However, the mean was 1-fold higher in summer compared to winter (Fig. 3.6).

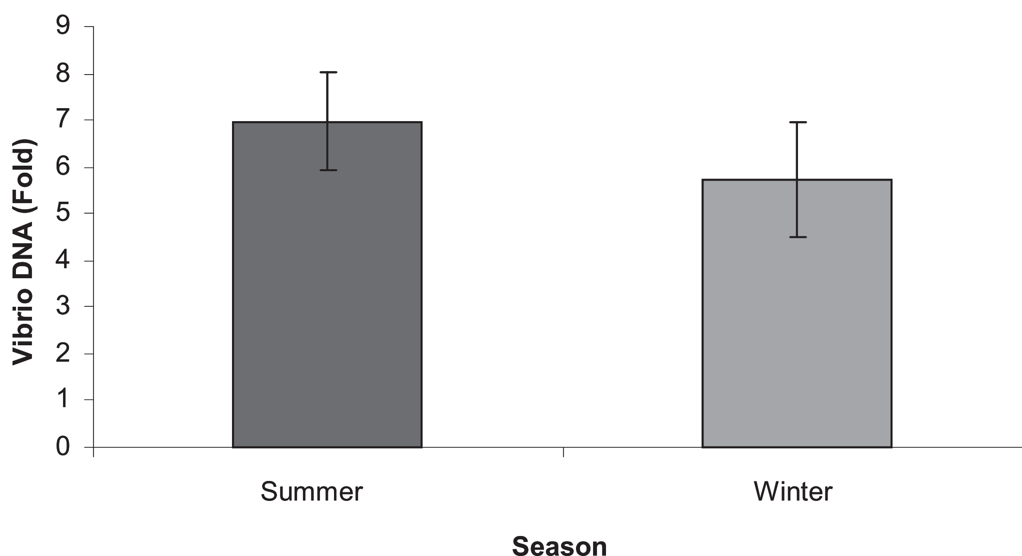


Figure 3.6. Total relative *Vibrio* DNA present within 10 representative random samples from each season (summer and winter), acquired from real-time PCR (see 'Materials and methods'). Y-axis represents total *Vibrio* DNA within each sample (standardised to total DNA concentration before PCR of 50 ng reaction^{-1}), shown as fold differences based on lowest concentrations detected within the samples (n = 20). Error bars represent SE from collective mean.

The composition of WBB communities varied across sites during both winter (ANOSIM, R = 0.48, p = 0.001) and summer (ANOSIM, R = 0.57, p = 0.001). However, pairwise comparisons showed significant differences only between the reef flat and the other sites both during winter (ANOSIM, p < 0.001) and summer (ANOSIM, p < 0.003), whilst other reef sites were not significantly different from each other (winter ANOSIM, p > 0.54 and summer ANOSIM, p > 0.1). Similarity of WBB between sites (based on Bray-Curtis similarity) was 46 % for winter samples and 47 % for summer. Lagoon, off-reef and mixed (reef) sites had similar bacterial communities during the winter (Fig. 3.7a) and summer (Fig. 3.8a) showing no specific patterns of ordination or clustering except for that at the reef flat (Fig. 3.7b & 3.8b).

The uniform lack of differences in the composition of WBB communities across sites with different levels of exposure to off-reef waters further supports the lack of tidal effects and indicates no significant association between the benthic and WBB communities. This is further supported, as the patterns were repeated in both seasons.

No significant differences were found between discrete bottle and continuous (1 h) pump sampling (ANOSIM, R = 0.07, p = 0.5), indicating that discrete bottle sampling provides a representative sample of bacterial assemblages found within the larger water mass at any given time period.

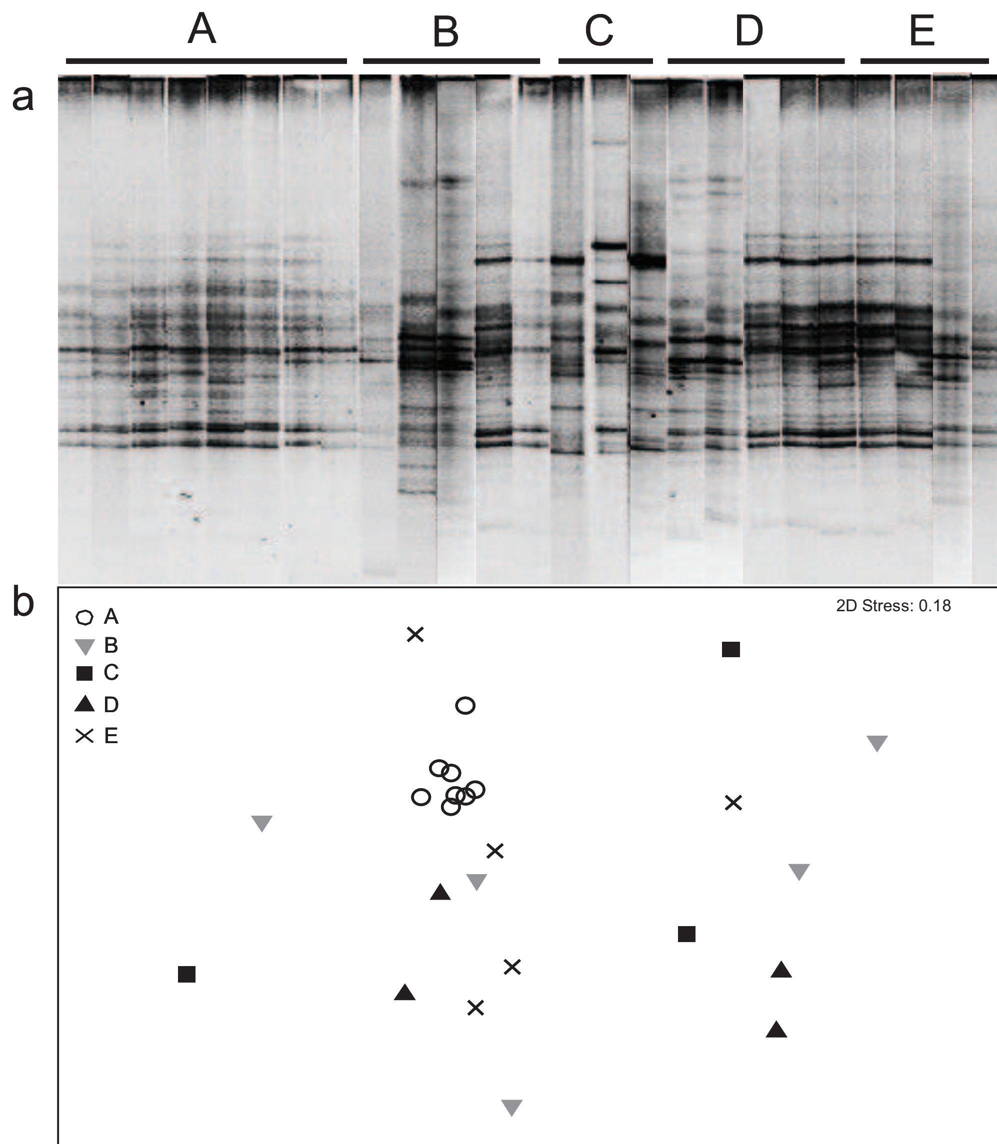


Figure 3.7. Variation in 16S rRNA gene fingerprints between sites (A–E) for August 2008 (winter). (a) Composite DGGE image standardised for gel-to-gel comparison using BioNumerics. (b) Multidimensional scaling (MDS) plot based on relative band

intensity from composite DGGE profile (a). Overall similarity between samples = 46 %. See Fig. 3.1 for site locations.

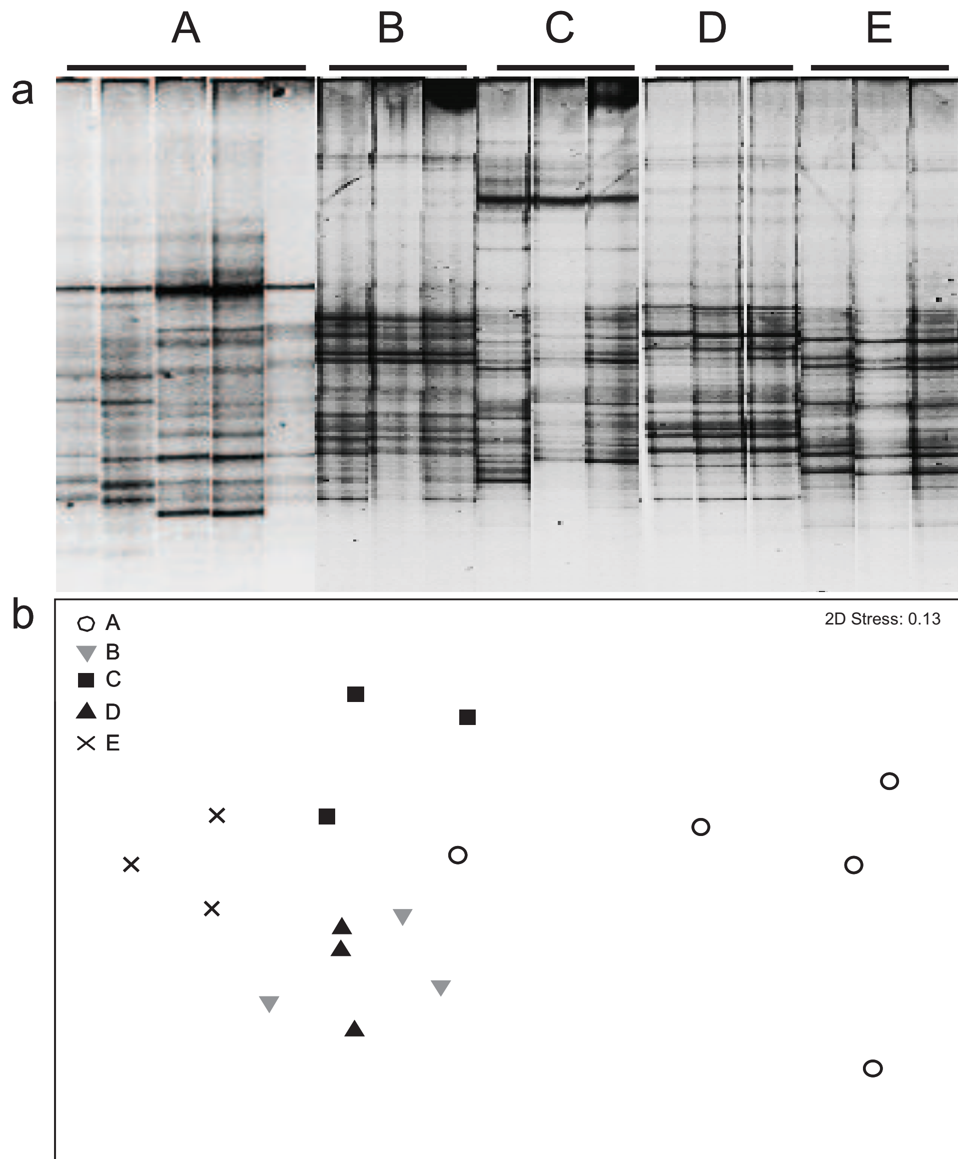


Figure 3.8. As in Fig. 3.7, but for March 2009 (summer).

3.5 DISCUSSION

Benthic marine communities are physically exposed to, and presumably influenced by, the delivery of bacteria present in the overlying water column. On coral reefs, coral surface mucus layers and seawater have been shown to share certain bacterial species (Ritchie 2006; Sunagawa et al. 2010). Conversely, Wild et al. (2004) have

shown a significant supply of coral-derived mucus and its associated bacterial communities to the water column (Wild et al. 2004, 2005, 2009; Naumann et al. 2009). At Heron Island, Wild et al. (2004) showed that up to ca. 1.7 l m^{-2} d^{-1} of mucus is released from the reef into the water column and biodegraded in lagoon sediments. Thus, there is likely to be significant bi-directional exchange between the coral reef benthos and the water column. However, in this study, we found no evidence that the WBB communities differed depending on the underlying benthos. In fact, with the exception of a reef flat site, WBB communities appeared homogeneous between sites, despite the fact that the water bodies sampled would have originated from very different environments, including lagoonal, reef and off-reef open water areas. Previous studies have shown significant differences in WBB across sites (Moriarty et al. 1985; Seymour et al. 2005; Guppy & Bythell 2006; Ritchie 2006). However, few studies have controlled for tide height, season and time of day when assessing spatial differences in the reef tract. Lack of differences in bacterial abundances and diversity between sites could in part be explained by the spatial scale chosen in this study. While temporal samples showed some short-term (hours) variability, spatial patterns were determined from samples separated by hundreds of m to km within the Heron Island reef system. Thus, it is possible that spatial variation in bacterial communities may be greater at smaller (<1 m) spatial scales (van Duyl & Gast 2001; Seymour et al. 2005) and that this smaller-scale variation masked any larger-scale variation. If the benthic–pelagic coupling described by Wild et al. (2004) was a slow process, one would have expected significant differences in both numbers and diversity between sites, especially those sites where the water mass was from reef flats and the lagoon compared to water originating from off-reef in more open water. As pairwise tests showed no significant difference between sites (except for that of the reef flat), we suggest that the benthic–pelagic coupling occurring at Heron Island is a rapid process with large-scale, efficient mixing of the water column and the benthic coral system.

As has been shown in a number of studies (Kent et al. 2004; Kent et al. 2007; Pommier et al. 2007; Fuhrman et al. 2008; Shade et al. 2008), the main factor affecting bacterial abundances and 16S rRNA gene diversity is season. In these studies, temperature was shown to be the main factor affecting bacterial species

richness. Fuhrman et al. (2008) showed a consistent latitudinal pattern in seasonality, which was annually repeatable and highly predictable from a variety of environmental parameters. Whilst Fuhrman et al. (2008) showed no correlation between WBB dynamics and productivity, WBB could also be affected by other seasonal environmental drivers (e.g. nutrients, irradiance, precipitation). In our study, we found that bacterial abundance was 4 to 5 times greater in summer than winter, suggesting that bacterial productivity is higher in summer, most likely due to temperature differences (Moriarty et al. 1985). Shade et al. (2008) also found that temporal variation of bacterial diversity and abundance within freshwater habitats was more pronounced than spatial variation, but they attributed their temporal differences to interactions and changes within phytoplankton communities.

During winter, ribotypes from the *Alphaproteobacteria* dominated WBB communities. Ribotypes associated with this group have been consistently found associated with healthy coral tissues (Ritchie & Smith 2004). During summer months, ribotypes from the *Gammaproteobacteria* were present in higher abundances but were either completely absent or rare in the winter samples. Optimal growth temperatures for both *Gamma*- and *Alphaproteobacteria* range from 20 to 40 °C (Brettar et al. 2002; 2003; Santos et al. 2003; Kurahashi & Yokota 2007; Anderson et al. 2009). However, these variations in temperature are largely species specific, not group specific, so growth rates of specific groups would not explain these results alone. Real-time PCR showed that the abundance of *Vibrio*, a genus known to contain potential coral pathogens (Kushmaro et al. 2001; Ben-Haim et al. 2003; Rosenberg & Falkovitiz 2004), did not vary significantly between seasons, although the mean was a fold higher in summer than winter. These results together indicate that potentially pathogenic bacteria may increase in abundance during warmer months, and therefore opportunistic infections may become more frequent and explain at least some of the concomitant increase in disease prevalence in summer (Jones et al. 2004), although future work is needed to confirm these patterns.

Despite the strong tidal flows on Heron Island and therefore large-scale interactions with reef, lagoonal and open water environments, there was no significant effect of

tide state on WBB abundance or 16S rRNA gene diversity. Significant diurnal variation was observed, however differences in bacterial abundance between night and day were stronger during the winter than in the summer. The greater variation seen during diurnal rather than tidal cycles on this reef system supports a previous study (Moriarty et al. 1985), which linked bacterial growth to the daily mucus release on the reef flats, which normally occurs between noon and 16:00 h regardless of the tide (Crossland et al. 1980; Torreton & Dufour 1996). Other possible explanations for the diurnal dynamics of WBB communities include overall productivity and/or the interaction between diurnal zooplankton migrations between the benthos and the water column (Heidelberg et al. 2010), given that these zooplankton will have their own microbiota associated with them directly and may alter bacterial production due to excretion and secretion.

The continuous sampling method and large sample size used in this study was investigated to determine whether it would provide a more accurate characterisation of bacterial communities within the water column. We conclude that although discrete sampling is adequate to represent the WBB communities, the use of pump sampling is a more repeatable method, and a larger sample size can be taken at any given time. In addition, human errors and potential contamination during collection and handling of samples that may be incurred during bottle sampling can be reduced with this continuous *in situ* method.

Chapter 4

Bacterial assemblages differ between compartments within the coral holobiont

4.1 ABSTRACT:

It is widely accepted that corals are associated with a diverse and host species-specific microbiota, but how they are organized within their host remains poorly understood. Previous sampling techniques (blasted coral tissues, coral swabs and 'milked' mucus) may preferentially sample from different compartments such as mucus, tissue and skeleton, or amalgamate them, making comparisons and generalisations between studies difficult. This study characterized bacterial communities of corals with minimal mechanical disruption and contamination from water, air and sediments from three compartments: surface mucus layer (SML), coral tissue and coral skeleton. A novel apparatus (the 'snot sucker') was used to separate the SML from tissues and skeleton and these three compartments were compared to swab samples and milked mucus along with adjacent environmental samples (water column and sediments). Bacterial 16S rRNA gene diversity was significantly different between the various coral compartments and environmental samples (PERMANOVA, $F = 6.9$, $df = 8$, $p = 0.001$), the only exceptions being the complete crushed coral samples and the coral skeleton, which were similar, because the skeleton represents a proportionally large volume and supports a relatively rich microbiota. Milked mucus differed significantly from the SML collected with the 'snot sucker' and was contaminated with zooxanthellae, suggesting that it may originate at least partially from the gastrovascular cavity rather than the tissue surface. A common method of sampling the SML, surface swabs, produced a bacterial community profile distinct from the SML sampled using our novel apparatus and also showed contamination from coral tissues. Our results indicate that microbial communities are spatially structured within the coral holobiont and methods used to describe these need to be standardised to allow comparisons between studies.

Published as; Sweet, M.J., Croquer, A., Bythell, J.C. (2010) Bacterial assemblages differ between compartments within the coral holobiont. Coral Reefs DOI: 10.1007/s00338-010-0695-1. This author designed and conducted the field work, analysed the samples and wrote the paper, Croquer and Bythell edited the manuscript ready for publication.

4.2 INTRODUCTION

Recent advances in molecular ecology have shown that corals are associated with a diverse microbiota encompassing viruses (Wilson et al. 2001, 2005; Davy et al. 2006; Marhaver et al. 2008; Claverie et al. 2009), fungi (Le Campion-Alsumard et al. 1995; Priess et al 2000; Ravindrum et al. 2001; Golubic et al. 2005; Rypien and Baker 2009; Kirkwood et al. 2010; Rivest et al. 2010), protozoa (Toller et al. 2002; Croquer et al. 2006; Dong et al. 2009; Sebastian et al. 2009; Stat et al. 2009), bacteria (Kooperman et al. 2007; Rosenberg et al. 2007; Smith et al. 2007; Kimes et al. 2010) and archaea (Kellogg 2004; Rohwer & Kelley 2004; Wegley et al. 2004). There is growing evidence that some of these microbial associates play important roles in coral physiology and health (Rosenberg et al. 2007; Bourne et al. 2009). For example, bacterial associates of corals may be important in nutrient cycling (Wild et al. 2004, 2009; Naumann et al. 2009), N-fixation (Lesser et al. 2007a) and antibacterial activity (Ritchie 2006). It has also been shown that these microbial-coral associations can be altered due to environmental stress (Ritchie 2006; Bruno et al. 2007; Miller et al. 2009), potentially enhancing opportunistic infections (Lesser et al. 2007b).

Thermal anomalies are expected to increase in both frequency and intensity because of climate change (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007), which will impose additional stress on corals and their microbial partners with uncertain consequences for the coral holobiont (Bourne et al. 2009; Mouchka et al. 2010). Given the importance of environmentally-driven shifts in microbial communities for coral health, it is imperative to understand the structure and function of these communities within the coral host. A detailed characterization of these assemblages and an accurate comparison with the surrounding environment (e.g., water and sediments) will be important for detecting shifts from a healthy to an unhealthy state under environmental stress, even before disease signs become evident. Understanding these coral microbial assemblages requires an understanding of spatial organization, since different coral compartments (e.g., mucus, tissue and skeleton) may represent

habitats with a unique set of features, microbial associates and independent responses to change.

Microhabitat partitioning is thought to strongly influence the composition of microbial communities, the contribution of the microbial community to host physiology, and the role of the host within the ecosystem (Ainsworth et al. 2010). At least three distinct microbial habitats are provided by a coral: (1) the surface mucus layer (SML), (2) coral tissue, and (3) the skeleton, and each of these may be further subdivided into separate compartments or micro-environmental gradients. The SML provides an excellent illustration of how variable the environmental conditions for coral-associated microbial communities are. Corals produce a SML which can vary in thickness and has a variety of functions (Brown & Bythell 2005; Jatkar et al. 2010). This layer is generated and continuously replaced to remove particles from the coral surface by secretion of mucus by mucocytes and presents a specific, highly variable environment for associated bacteria. Because the O_2 saturation levels in the SML vary diurnally from supersaturated to virtually anoxic (Shashar et al. 1997), the SML may be a hostile environment for some bacteria but favourable to others. The SML may also be complex and show variation in structure and function, perhaps with different layers with different properties and functions (Brown & Bythell 2005). In addition, corals may inhibit bacterial growth in the SML by altering the composition of the mucus and through the use of antimicrobial compounds produced by the coral and/or its microbial residents (Ritchie 2006). As well as inhibiting the growth of some bacteria with antibiotics, corals may encourage the growth of others by providing fixed carbon in the form of mucus and soluble lipids that are also secreted by the coral and may represent a substantial proportion of the total carbon released, the remainder being mucopolysaccharides (Crossland et al. 1980, 1984).

In contrast to the SML, microbial communities in the coral tissues are embedded within a more stable matrix, one which is not constantly being replaced over diurnal or hourly cycles. However, the tissues will also be subject to strong diurnal variations in oxygen tension. Despite a number of studies referring to 'coral tissue' microbial associates (Lesser et al. 2004; Bourne & Munn 2005; Koren & Rosenberg 2006), sampling protocols vary, with different degrees of possible contamination from other sources, resulting in few studies empirically demonstrating microbial communities

‘within’ the tissues (Lesser et al. 2004) and instead showing those ‘associated’ with them. However the degree of spatial association is not always clear. For instance, bacterial community profiles detected using airbrushing techniques (Bourne & Munn 2005), have been shown to be significantly different from those in the mucus, however this method will likely introduce contaminants from other compartments, namely that of the skeleton. Yet despite this limitation, the findings of distinct communities, particularly from that of the SML, suggests compartmentalisation of bacterial communities within the holobiont. There is further evidence that the unique microbial community of the tissues is maintained through space and time, with distinct bacterial communities associated with different coral species (Rohwer et al. 2002). However, there are some inconsistencies in the literature with regard to bacterial abundance of coral-associated microbes’, for example, Ainsworth et al. (2006) showed that bacterial populations associated with the host tissues were typically sparse in healthy corals and where they do occur, they often appear in discrete, compartmentalised clusters within the endoderm (Ainsworth & Hoegh-Guldberg 2009). As well as closely-associated microbes, the tissues will include microbes present in the gastro-vascular cavity that have been ingested via particulate feeding (Herndl & Velimirov 1985; Herndl et al. 1985; Bythell et al. 2002).

Coral skeletons are porous structures and, like the tissues, may provide a stable protected environment for its resident microbiota, isolated from the ambient seawater (Shashar et al. 1997). Endolithic algae dominated by cyanobacteria (genus *Ostreobium*) within the coral *Oculina patagonica* have been shown to provide photo-assimilates to the coral tissue (Fine & Loya 2002). During times of stress and subsequent loss of zooxanthellae, these endolithic algae may provide significant resources to the coral (Fine & Loya 2002; Fine et al. 2004, 2005). Such interactions may therefore be advantageous and explain why this coral species in particular has a high rate of recovery from annual bleaching. The skeletal micro-habitat may also provide a refuge from grazing, with as little as 4 % of fish bites penetrating deep enough into the skeleton to expose the endolithic algae (Shasher et al. 1997). With the tissues filtering out virtually all ambient UV radiation (Shashar et al. 1997), the skeleton provides a shaded habitat for its residents, sheltering them from the potentially damaging effects of solar radiation.

There is wide agreement that to understand coral diseases, we must have a systematic understanding of the healthy coral microbiota (Klaus et al. 2005; Guppy & Bythell 2006; Ritchie 2006; Gil-Agudelo et al. 2007). However, different methods of sample collection may sample different compartments within the coral-microbial landscape and cross-contamination among compartments may also occur (Table 4.1.).

Table 4.1. Different methods of collection of coral samples in recent studies for compositional analysis and the major compartments likely to be sampled (minor potential contamination in brackets

Method of coral sampling	Compartments sampled: Major (minor)	Source
Crushed whole coral	mucus, tissue, skeleton	Cooney et al. (2002), Pantos et al (2003)
Scraping of surface	mucus, tissue, (skeleton)	Shnit-Orland & Kushmaro (2009)
Waterpicked/airbrushed	mucus, tissue	Rohwer et al. (2002), Sharon & Rosenberg (2008)
Swabs	mucus, (tissue, water)	Guppy & Bythell (2006); Lampert et al. (2008)
Syringe aspiration	mucus, water, (tissue)	Ducklow & Mitchell (1979), Coffroth (1990)
Air exposure (milked mucus)	mucus	Daumas et al. (1981), Wild et al. (2004), Allers et al. (2008)
Coral held in incubation chambers and seawater collected	mucus, water	Means & Sigleo (1986)

During collection, coral samples can also be easily contaminated with microorganisms from the surrounding water and suspended sediments (Johnston & Rohwer 2007). In this paper we describe a new method that allows minimal outside contamination and little disturbance at time of collection, to more accurately describe healthy microbial communities and their spatial arrangement within the coral holobiont. This method aimed to characterize bacterial communities of the outer SML, the coral tissue and skeleton, and these compartments were compared to existing methods including whole coral (crushed) samples, coral surface swabs and milked mucus. All samples were compared to environmental samples (the surrounding water column and adjacent sediments) to aid in the understanding of benthic-pelagic transfer of particular bacterial ribotypes.

4.3 MATERIALS AND METHODS

4.3.1 Sample collection:

In order to characterize and compare the bacterial assemblages from different coral compartments with those that surround the coral (water column and the sediments), a novel apparatus (the "snot sucker") was designed. Corals were collected, mounted onto a screw cap system and returned to the reef until collection. This device comprised a 50 ml falcon tube with two 3 - way valves grafted onto it, one at the bottom and one at the top. A 60 ml syringe, with tubing, was attached to the bottom stopper valve allowing filtered water to be flushed over the coral and the loosely-attached surface mucus layer (SML) collected via the top valve. This was then filtered through a 0.22 µm polycarbonate filter; EtOH was added and stored at –20 º C (Fig. 4.1). All nubbins (ca. 5 cm long) used in this experiment were collected from a single colony of *Acropora muricata* (= *formosa*). We sampled only one colony to reduce between-colony variation of microbial communities reported in different studies (Rohwer et al. 2002; Guppy & Bythell 2006).

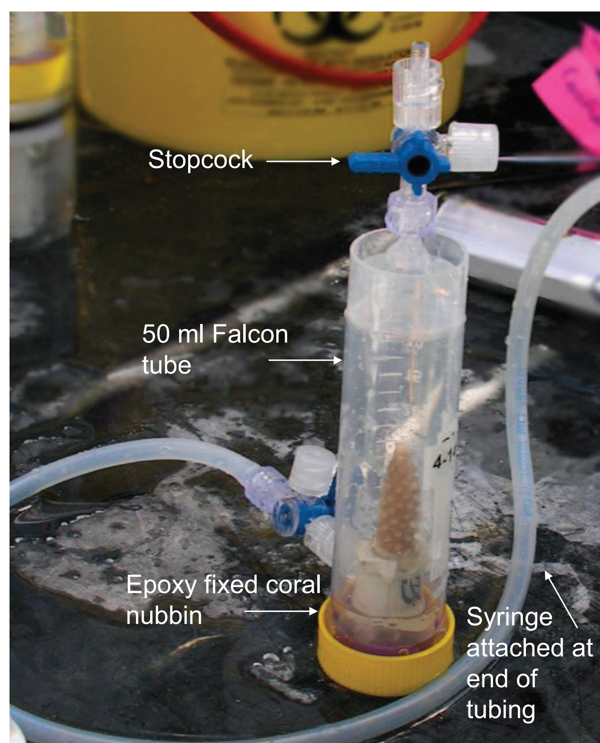


Figure 4.1. Illustration of new methodology (the 'snot sucker') used to study compartmentalised bacterial communities within the coral holobiont. Corals can be

collected *in situ* or *in vitro* within enclosed chambers, whilst underwater therefore minimising disruption of loosely associated microbes during collection and transport. Each chamber was used only once or sterilized to prevent cross over contamination. Top stopcock opened at same time as bottom to allow syringed water to flow over coral nubbin, then washed back again into the syringe and filtered through 0.22 µm polycarbonate syringe filter.

Four sets of sample (each n = 4) were collected from the reef flat at Heron Island (23 ° 27' S, 151° 55' E); 1) 5 cm complete coral nubbins were collected, placed in ethanol for storage and crushed using a sterile pestle and mortar. These sets of samples contained a mixture of microbial communities associated with the mucus, the coral tissue and the coral skeleton. 2) Milked mucus was sampled from a second set of nubbins by inverting them for 2 min in air to collect the mucus draining from the coral. For this compartment, DNA extraction was unsuccessful from one sample giving only three replicates. 3) A set of nubbins was collected and enclosed within the snot sucker *in situ* to avoid air exposure. A sterile syringe was then used to sample the water and loosely-associated SML surrounding the coral by flushing repeatedly three times, then filtering though a 0.22 µm polycarbonate filter. These 'first-round snot sucked' samples (1st RSS) represented microbial communities associated with the SML but may have had minor contamination from the surrounding water column. A further sample was obtained, the '2nd round snot sucked samples' (2nd RSS), by adding 60 ml of 0.22 µm filtered water, which also was flushed through the chamber three times. This allowed sampling of any remaining loosely associated SML bacterial assemblages without potential contamination from the water column. After the 2nd RSS collection was completed, the tissue layer was airbrushed off using compressed air and a sterile air pick directly into an autoclaved bag. A sterile blade was used to collect the tissue slurry into Eppendorf (micro-centrifuge) tubes, filled with EtOH and stored for later extraction and microbial characterization of this coral tissue blastate. The remaining coral skeleton samples were then crushed using an autoclaved pestle and mortar and placed within separate Eppendorfs with EtOH. All samples were stored at -20 ºC until further extraction and analysis. 4) Coral swabs were also collected from the same colony following the protocol outlined by (Guppy & Bythell 2006).

In addition to coral samples, the water column and the sediments were sampled alongside the coral. The water column was collected using a peristaltic pump (Masterflex E/S) and polytetrafluoroethylene (PTFE) tubing directly and continuously sampled onto 0.22 µm Sterivex filters stored on ice (as in Chapter 3), to determine the bacterial diversity surrounding the corals (a potential supply of coral associated bacteria). Sediment samples were collected *in situ* in sterile Eppendorf tubes and the water was replaced with EtOH in the lab.

4.3.2 Bacterial diversity, DNA extraction, amplification and DGGE analysis:

The structure of microbial assemblages collected from these respective coral and environmental samples (coral tissue, milked mucus, 1st RSS, 2nd RSS, complete crushed coral, coral skeleton, swabs, water column and sediment) were determined using standard molecular techniques and further compared using multivariate statistics (see below). DNA was extracted from all samples and amplified as in Chapter 3. However, three independent 30µl PCR reactions were used per sample, each containing 1.5 mM $MgCl_2$, 0.2 mM dNTP (PROMEGA), 400 ng μl^{-1} of bovine serum albumin (BSA), 0.5 mM of each primer, 2.5 U of Taq DNA polymerase (QBiogene), incubation buffer, and 20 ng of template DNA (Siboni et al. 2007). These replicate PCR's for each sample were then combined and cleaned using QIAGEN QIAquick PCR purification kit, reducing the final volume to 15 µl in Sigma molecular grade H_2O. DGGE was performed as in Chapter 3.

4.3.3 Statistical analysis:

A one-way permutation analysis of variance (PERMANOVA, Anderson 2001) based on Bray-Curtis similarities was performed to test differences between the bacterial 16S rRNA gene assemblages associated with different compartments and environmental samples. Pair-wise comparisons based on permutation were conducted to test differences among each combination of sample types (Anderson 2001). A non metric multidimensional scaling (MDS) was used to represent each sample type on a 2-D plot and clusters were overlaid based on a similarity profile [SIMPROF, Clarke and Warwick (2001)] analysis. An analysis of contribution to similarities (SIMPER) was performed to determine which 16S rRNA gene ribotype best explained

dissimilarities among sample types that were statistically different. In addition, Pearson coefficients were calculated between centroids of each sample type and represented in a cluster diagram to visualize their correlations. Diversity of bacterial communities associated to each sample type was also compared using a one-way PERMANOVA based on Euclidean distances (Anderson 2001). Pair-wise comparisons based on permutation were conducted to test differences among each combination of sample types (Anderson 2001). Note that because of inherent bias with the DGGE technique, whereby only the most abundant amplicons can be represented on the gel, higher H^1 values may result mainly from a lack of dominance (greater evenness) rather than greater species richness, which will be poorly resolved (if at all) by the DGGE technique.

4.4 RESULTS

4.4.1 Comparison between coral compartments:

As the 1st and 2nd RSS were similar (Fig. 4.2 and 4.3a, Table 4.2, 4.3), the 2nd RSS was used as a representation of the loosely-associated mucus layer (SML). Coral compartments (SML, tissue and skeleton) showed significant differences, with the SML being significantly different from both the coral tissue ($p = 0.03$) and the skeleton ($p = 0.04$) (Table 4.3).

Table 4.2. PERMANOVA main test between all coral compartments and environmental samples, Sa = Sample type (inc. coral compartments and environmental samples), Res = Residual

Source	df	SS	MS	F	p	VC
Sa	8	50639	6329.9	6.894	0.001	60.3
Res	26	23873	918.18			39.7
Total	34	74512				

Table 4.3. Comparison of bacterial communities within separated coral compartments and environmental samples. Bray Curtis average similarity (%) and pairwise PERMANOVA (p- value). Milked Mucus (MM), Blasted Coral Tissue (BCT), Coral Skeleton (CS), Complete Corals (CC), 1st Round Snot Sucked (1st RSS), 2nd Round Snot Sucked (2nd RSS), Sediment (SED), Swabs (SWB) and Water Column (WC).

COMPARTMENTS		t	p-value	Average Similarity
WC	2RSS	2.19	0.03	33.99
	1RSS	3.22	0.03	40.47
	CS	3.97	0.03	29.47
	CC	3.64	0.03	29.80
	BCT	4.15	0.04	36.36
	MM	5.10	0.04	23.95
	SWB	3.79	0.03	30.57
	SED	2.29	0.03	28.16
2RSS	1RSS	1.38	0.03	44.71
	CS	2.37	0.03	26.36
	CC	2.17	0.03	28.42
	BCT	2.08	0.03	35.66
	MM	2.24	0.03	28.04
	SWB	2.27	0.04	27.36
	SED	1.51	0.03	27.52
1RSS	CS	2.75	0.03	40.13
	CC	2.77	0.03	36.34
	BCT	2.76	0.04	45.80
	MM	3.56	0.02	31.80
	SWB	3.40	0.03	28.14
	SED	2.03	0.03	29.84
CS	CC	0.99	0.48	62.44
	BCT	2.97	0.03	43.08
	MM	2.69	0.03	46.02
	SWB	3.41	0.03	28.02
	SED	2.25	0.03	24.81
CC	BCT	3.02	0.04	38.32
	MM	2.26	0.03	48.39
	SWB	2.99	0.03	31.43
	SED	2.31	0.02	22.20
BCT	MM	3.71	0.05	40.53
	SWB	4.09	0.03	24.32
	SED	2.72	0.04	18.87
MM	SWB	3.79	0.04	25.91
	SED	2.51	0.03	16.80
SWB	SED	1.90	0.03	35.91

Sequencing of dominant OTU's from the DGGE (Fig 4.2) showed that despite similarities in DGGE banding patterns, most of the dominant ribotypes found in each of these compartments were distinct, with some occurring only in specific compartments whilst others were found routinely throughout (Table 4.4, Fig. 4.2).

Six ribotypes related to *Chloroflexi* (EU909941), *Sphingobacterium* sp. (AF260710.1), *Shewanella* sp. (EU919217), *Roseobacter* sp. (EF441565), *Pseudidiomarina* sp. (FJ887948) and *Pseudoalteromonas* sp. (DQ665793) were found only in the SML, cyanobacterial ribotypes closely related to GQ346809 and FJ967973 and an α-proteobacterium (AB254287) were found in all three compartments (Fig. 4.3b). Ribotypes similar to *Pseudidiomarina* sp (FJ887948) (Fig. 4.3c) was dominant within both the SML and coral tissue blastate whilst ribotypes similar to *Proterythropsis* sp. (FJ947037), *Lactobacillus* sp. (DQ336385 & DQ336384), *Klebsiella* sp. (GQ471864) (Fig 4.3d), *Aeromonas* sp. (EU919223), *Burkholderia* sp. (EU876657), *Streptococcus* sp. (DQ001071) and *Trichococcus* sp. (EU919224) were found in both the coral skeleton and the coral tissue blastate but appeared to be absent from the SML (Table 4.4).

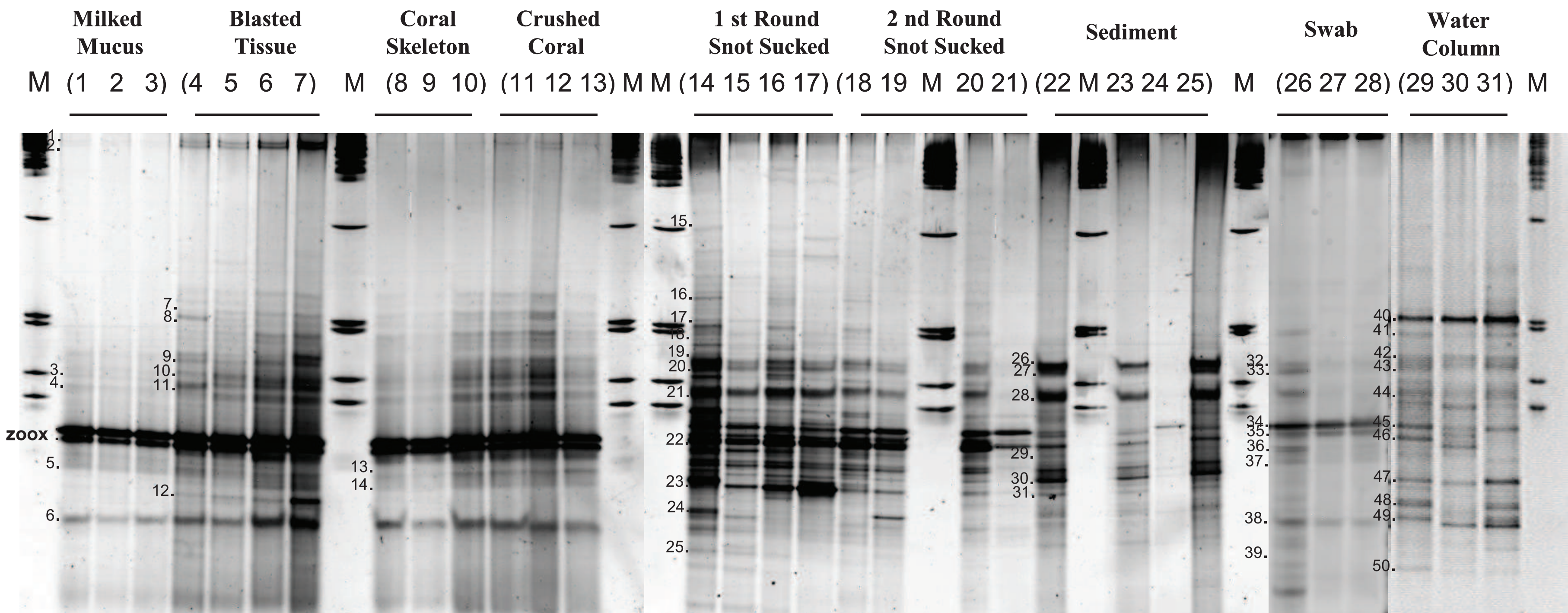


Figure 4.2. DGGE analysis of bacterial 16S rRNA gene fragments from separate coral compartments and adjacent environmental samples, with intermittent marker lanes (M) allowing for direct comparison between gels: 1 – 3 Milked Mucus, 4 – 7 Blasted Tissue, 8 – 10 Coral Skeleton, 11 – 13 Complete Corals, 14 – 17 1st Round Snot Sucked (1st RSS), 18 – 21 2nd Round Snot Sucked (2nd RSS), 22 – 25 Sediment, 26 – 28 Swabs and 29 – 31 Water Column. zoox = ancestral mitochondrial DNA of symbiotic dinoflagellate algae associated with the coral *A. muricata*

Table 4.4. The closest match, species description, potential roles (previous known isolation source related to closest Blast match) and sample type the specific ribotype was found in (coral compartment or environmental sample) of 16S rRNA gene bacterial ribotypes from the coral compartments taken from Heron Island, GBR. Sequenced from DGGE bands. Band number in relation to position on Fig. 4.2 DGGE gel image.

Band No.	Species ID	Group affiliation	Close relative (% match)	Potential role (isolation source)	Sample type
1	*Klebsiella sp.*	γ-proteobacteria	GQ471864 (100%)	Potential opertunistic pathogen (diseases / soft tissue / infections / gut)	MM, BCT, CS, CC
2	*Aeromonas sp.*	Δ-proteobacteria	EU919223 (100%)	Oxidase and catalase positive, reduce nitrate - potential opertunistic pathogen (animals / waste water)	MM, BCT, CS,CC
3	*Burkholderia sp*	β-proteobacteria	EU876657 (100%)	Fix N2 and antibiotic resistant, biodegradtion of pollutants - potential opertunsitic pathogen (plants / animals)	MM, BCT, CS, CC
4	*Streptococcus sp.*	Cocci	DQ001071 (97%)	Alpha-hemolytic species (lactic acid bacterium)	MM, BCT, CS, CC
5	*Aeromonas sp.*	Δ-proteobacteria	EU919223 (100%)	Oxidase and catalase positive, reduce nitrate - potential opertunistic pathogen (animals / waste water)	MM, BCT, CS, CC
6	*Trichococcus sp.*	Cocci	EU919224 (92%)	Iron reducing bacterium (lakes)	MM, BCT, CS, CC
7	*Pseudidiomarina sp.*	γ-proteobacteria	FJ887948 (100%)	Can cope with environmental pollutants (water column / sediments)	BCT, CS, CC
8	*Proterythropsis sp.*	NA	FJ947037 (95%)	Ocelloid-bearing dinoflagellates	BCT, CS, CC
9	*Lactobacillus sp.*	Bacilli	DQ336385 (83%)	Anaerobic bacterium - potential opertunistic pathogen (meats)	BCT, CS, CC
10	*Lactobacillus sp.*	Bacilli	DQ336384 (87%)	Convert lactose and other sugars to lactic acid	BCT, CS, CC
11	α-proteobacterium	α-proteobacteria	AB254287 (100%)	Phototrophic	BCT, CS, CC
12	*Trichococcus sp.*	Cocci	EU919224 (87%)	Quinone-reducing	BCT
13	*Streptococcus sp.*	Cocci	DQ001071 (97%)	Alpha-hemolytic species (lactic acid bacterium)	BCT, CS, CC
14	*Alcaligenes sp.*	β-proteobacteria	EU876658 (62%)	Potassium-solubilizing bacteria	BCT, CS, CC
15	*Chloroflexi sp.*	Chloroflexi	EU909941 (97%)	Green non-sulfur bacteria	SML
16	*Sphingobacterium sp.*	Sphingobacteria	AF260710 (67%)	Flavobacterium can cause sepsis (water column / soil)	SML
17	Cyanobacterium	Cyanobacteria	GQ346809 (100%)	Blue-green algae' (water column)	SML
18	α-proteobacterium	α-proteobacteria	EF520401 (95%)	Phototrophic	SML, WC
19	*Shewanella sp.*	γ-proteobacteria	EU919217 (100%)	Quinone-reducing (freshwater, marine sediments, subsurface formations)	SML, WC, SED, SWB
20	*Pseudidiomarina sp.*	γ-proteobacteria	FJ887948 (100%)	Can cope with environmental polutants (water column / sediments)	SML, WC, SED, SWB
21	Cyanobacterium	Cyanobacteria	FJ967973 (100%)	Blue-green algae' (water column)	SML, WC, SED, SWB
22	*Roseobacter sp.*	α-proteobacteria	EF441565 (100%)	Associated with Black Band disease in corals	SML, SWB
23	β - proteobacterium	β - proteobacteria	AF419359 (100%)	Unknown (Water column)	SML, WC
24	Cyanobacterium	Cyanobacteria	FJ946590 (100%)	Carbon fluxes (Water column)	SML
25	*Pseudoalteromonas sp.*	γ-proteobacteria	DQ665793 (100%)	Associated with coral microbial communities	SML, SWB

26	*Shewanella sp.*	γ-proteobacteria	EU919217 (100%)	Quinone-reducing (freshwater, marine sediments, subsurface formations)	SML, WC, SED, SWB
27	*Flavobacterium sp.*	Flavobacteria	FJ416620 (89%)	Potential opertunistic pathogen (water column / fish disease)	SED, SWB, WC
28	*Roseobacter sp.*	α-proteobacteria	EF441565 (100%)	Associated with Black Band disease in corals	SML, SED, SWB
29	*Euplotes minuta*	NA	AY361908 (97%)	Ciliate species	SED
30	*Sphingomonadaceae sp.*	α-proteobacteria	FJ685921 (77%)	Aerobic or facultative anerobic chemoorganotrophs (soil / sludge / marine environments)	SED
31	β - proteobacterium	β - proteobacteria	AF419359 (84%)	Unknown (Water column)	SED
32	*Shewanella sp.*	γ-proteobacteria	EU919217 (100%)	Quinone-reducing (freshwater, marine sediments, subsurface formations)	SML, WC, SED, SWB
33	*Flavobacterium sp.*	Flavobacteria	FJ416620 (89%)	Potential opertunistic pathogen (water column / fish disease)	SWB, WC
34	*Bacteroidetes sp.*	Bacteroidetes	AB254287 (100%)	Potential opertunistic pathogen (watercolumn / faeces)	SWB, WC
35	*Flavobacteriales sp.*	Flavobacteria	AB294989 (100%)	Commensal and opportunistic pathogen (fish)	SWB
36	zooxanthellae	NA	DQ320494 (100%)	Ancestral mitochondrial DNA	MM, BCT, CS, CC, SWB
37	zooxanthellae	NA	DQ320494 (100%)	Ancestral mitochondrial DNA	MM, BCT, CS, CC, SWB
38	Cyanobacterium	Cyanobacteria	FJ946590 (100%)	Unknown	SWB
39	*Chloroflexales sp.*	Chloroflexi	AJ577123 (96%)	Organisms that utilize type II photosynthetic reaction centers (soil)	BCT, SWB, SML, WC
40	*Bacteroidetes sp.*	Bacteroidetes	AM238600 (84%)	Potential opertunistic pathogen (Coral SML / water column)	WC
41	Actinobacterium	Actinobacteria	AY632498 (90%)	Potential opertunistic pathogen (plants / animals)	WC
42	α-proteobacterium	α-proteobacteria	EF092739 (95%)	Phototrophic	WC
43	*Pseudidiomarina sp.*	γ-proteobacteria	FJ887948 (96%)	Can cope with environmental polutants (water column / sediments)	SML, WC, SED, SWB
44	*Roseobacter sp.*	α-proteobacteria	EF441565 (100%)	Associated with Black Band disease in corals	WC
45	*Bacteroidetes sp.*	Bacteroidetes	AB254287 (100%)	Potential opertunistic pathogen (water column / faeces)	WC
46	*Flavobacteriales sp.*	Flavobacteria	AB294989 (100%)	Commensal and opportunistic pathogen (fish)	SWB, WC
47	α-proteobacterium	α-proteobacteria	FJ718457 (96%)	Phototrophic	WC, SML, BCT
48	*Flavobacteria sp.*	Flavobacteria	EU600663 (100%)	Commensal and opportunistic pathogen (water column / fish)	WC
49	α-proteobacterium	α-proteobacteria	GQ350573 (88%)	Phototrophic	WC
50	*Chloroflexales sp.*	Chloroflexi	AJ577123 (96%)	Organisms that utilize type II photosynthetic reaction centers (soil)	BCT, SWB, WC, SML

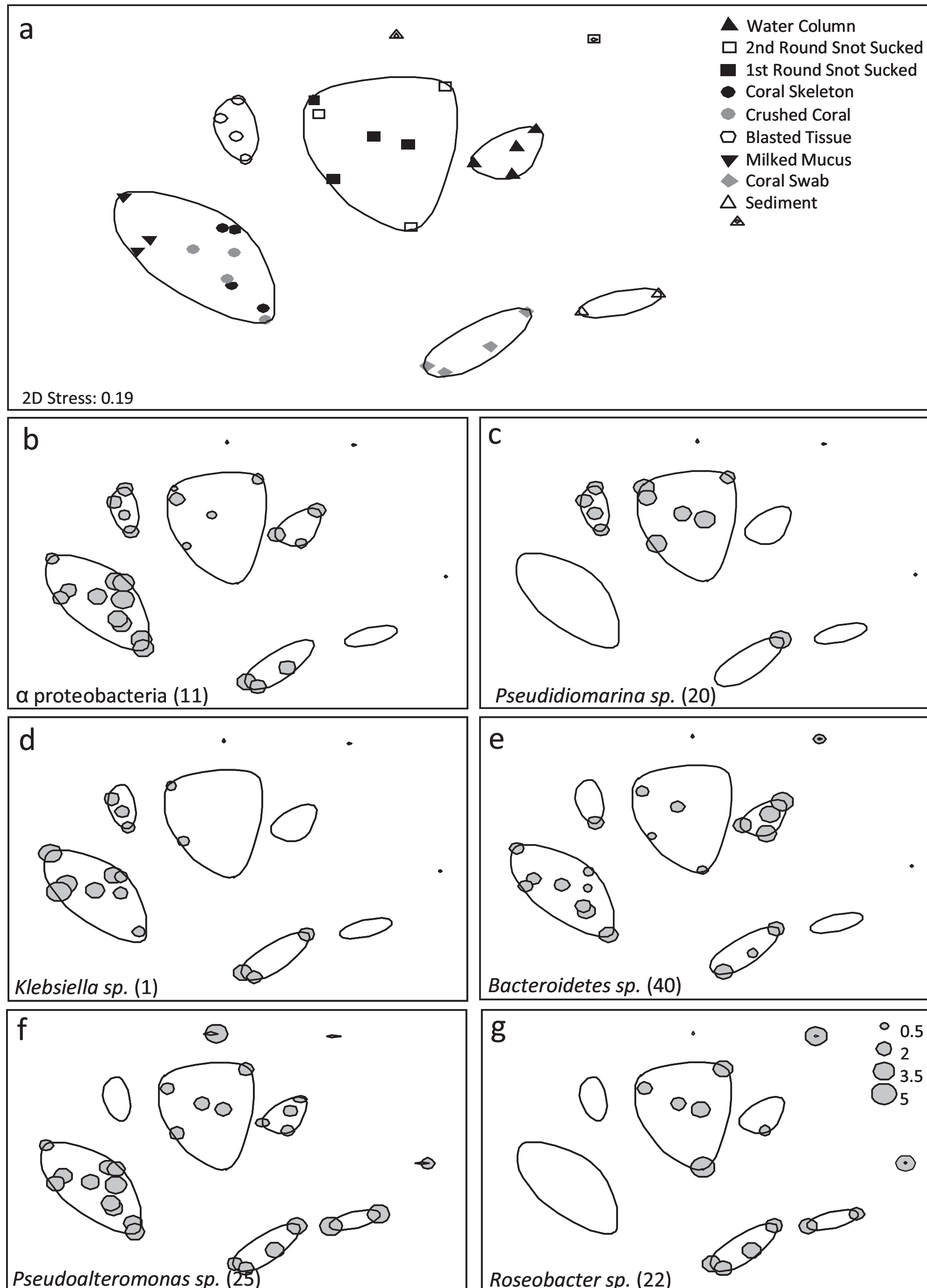


Figure 4.3. (a) Multidimensional scaling (MDS) plot showing changes in bacterial communities from various compartments associated with a coral and adjacent environmental samples, SIMPROF cluster analysis showed greatest differences between samples and contour line represents Bray Curtis similarity of 45 % between sample profiles (b-g) show 16S rRNA gene bacterial ribotypes that caused similarities of differences between sample types (closest relative and band no. in relation to DGGE image and sequence table ID, see Fig. 4.2 and Table 4.4) regarding percentage

contribution of dissimilarity among samples (b) α proteobacteria (Band 11) (c) *Pseudidiomarina sp.* (Band 20) (d) *Klebsiella sp.* (Band 1) (e) *Bacteroidetes sp.* (Band 40) (f) *Pseudidiomarina sp.* (Band 25) (g) *Roseobacter sp.* (Band 22).

4.4.2 Comparison between environmental samples and coral compartments:

Bacterial 16S rRNA gene profiles were significantly different between coral and environmental samples (PERMANOVA, $F = 6.9$, $df = 8$, $p = 0.001$, Table 4.2, Fig. 4.3), with 60 % of the total variance being explained by differences found between sample types. This result suggests that spatial organization of microbial communities within the coral is complex yet ultimately remains distinct from that of the environmental samples. Microbial communities from all coral compartments were significantly different to the water column ($p \leq 0.03$) (Table 4.3). Average similarity between the water column and coral compartments was variable, ranging from 24 (milked mucus) to 40 % (1st RSS, Table 4.3). Sediment samples also showed distinct bacterial communities, with average similarity being low (18-25 %) when compared to coral compartments (Table 4.3). Several of the detected ribotypes were unique to the water column (Table 4.4), whereas ribotypes associated with the coral were generally present in more than one compartment within the coral. The water column was dominated by α-proteobacteria ribotypes (EF092739, FJ718457, EF441565 and GQ350573) and the dominant ribotype present within this environmental sample (DGGE, Band 40, Fig. 4.2) was a ribotype closely related to a *Bacteroidetes* sp. (AM238600) (Fig. 4.3e). Conversely, few ribotypes were found solely associated with the sediment. Ribotypes similar to a *Sphingomonadaceae* sp. (FJ685921) and a β – proteobacterium (AF419359) were the only two.

Despite the significant differences in bacterial ribotype composition shown on the MDS plot (Fig. 4.3a), there were certain ribotypes found in common between the coral compartments (most commonly the SML) and the environmental samples (sediment and water column). These were largely γ-proteobacteria, including ribotypes similar to *Shawanella* (EU919217), *Pseudidiomarina* (FJ967973.1) and *Pseudoalteromonas* (DQ665793) (Fig. 4.3f). A ribotype similar to a *Roseobacter* sp. (EF441565) (Fig. 4.3g) was dominant within both the SML and the sediment samples. Appearance of ribotypes within both the sediment and SML is not unexpected as the

outer surface would be exposed to re-suspended sediments on a daily basis in these shallow reef environments.

4.4.3 Comparison between techniques:

The various techniques showed distinct bacterial communities (Fig. 4.2 and 4.3). 16S rRNA gene ribotypes detected in the 1st and 2nd RSS were identical and distinct from those of the water column (Table 4.3). This indicates that there was little contamination from the water column in RSS samples and further indicates that the snot sucker sampled a loosely-associated SML that was distinct to the adjacent water column.

The milked mucus technique showed 16S rRNA gene composition that was significantly different from the water column (PERMANOVA $p = 0.04$), those of the 1st and 2nd RSS (PERMANOVA $p = 0.02$ and $p = 0.03$ respectively) and swab samples (PERMANOVA $p = 0.04$), suggesting that this technique may not provide an accurate sample of the SML microbial community (Table 4.3). Instead, the milked mucus appears to have its own distinct microbial assemblage seen in the MDS ordination (Fig. 4.3a) and 16S rRNA gene fingerprint (Fig. 4.2). The milked mucus profiles is more similar to those of the complete crushed coral samples and the coral skeleton, than the other mucus samples (Fig. 4.3), although remaining significantly different to each ($p = 0.03$, Table 4.3). In addition, the milked mucus samples showed presence of symbiotic algae (Fig. 4.2). In combination, this suggests that milked mucus may be more representative of the coral's gastro-vascular cavity than that of the SML.

The lowest similarities between sample types were found consistently between the swabs and the other sample types, with average similarity seldom exceeding 25 %. This method detected significantly lower 16S rRNA gene diversity than shown in the RSS samples (Fig. 4.4), and may include contamination of bacteria from other sources with swab profiles being (35 %) similar to sediment samples for example. The presence of DNA from the symbiotic dinoflagellates (Fig. 4.2) also indicates contamination from the coral tissues, as seen in milked mucus samples. The higher diversity seen in both RSS samples did not arise from contamination either from the

coral tissue or the water column samples since very few ribotypes were detected in common between these samples (Fig. 4.2, Table 4.4).

The Shannon-Weiner diversity varied significantly between different techniques (PERMANOVA F = 24.264 p = 0.001 Table 4.5.).

Table 4.5. PERMANOVA comparing diversity between coral compartments and environmental samples, Sa = Sample type (inc. coral compartments and environmental samples), Res = Residual

Source	df	SS	MS	F	p	CV
Sa	**8**	**4.6341**	**0.57927**	**24.264**	**0.001**	**70.98**
Res	26	0.6207	2.39E-02			29.01
Total	34	5.2548				

The 1st and 2nd RSS were the most diverse of samples, whilst milked mucus and swabs were the lowest. The overall diversity of the compete crushed coral is more closely related to that of the coral skeleton and therefore supports the theory that complete crushed coral samples represent the coral skeleton more than the tissue or SML bacterial communities (Fig. 4.4).

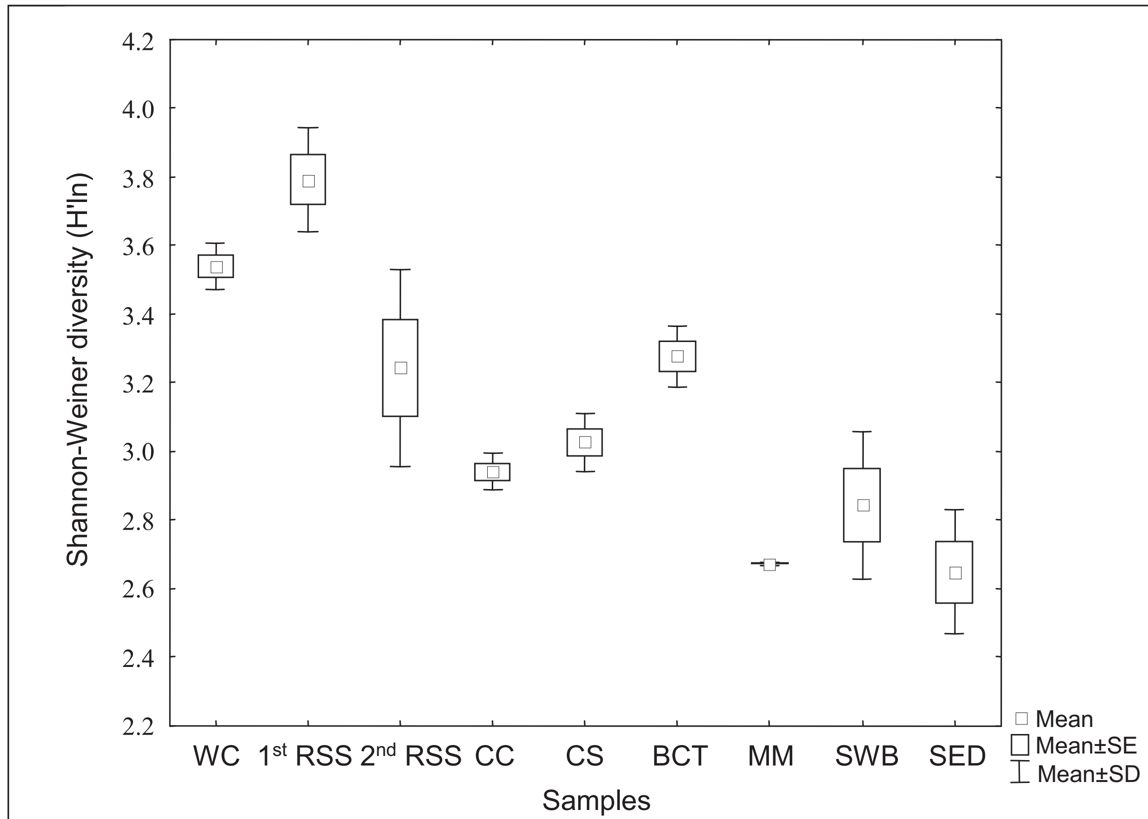


Figure 4.4. Bacterial species richness/diversity between coral compartments and environmental samples (Shannon-Weiner diversity). Milked Mucus (MM), Blasted Coral Tissue (BCT), Coral Skeleton (CS), Complete Corals (CC), 1st Round Snot Sucked (1st RSS), 2nd Round Snot Sucked (2nd RSS), Sediment (SED), Swabs (SWB) and Water Column (WC).

4.5 DISCUSSION

4.5.1 Differences in bacterial communities between coral compartments and the surrounding environment:

This study demonstrates that bacterial communities differ between compartments within the coral holobiont. Significant differences were shown between all coral compartments and those of the surrounding environmental samples, supporting previous conclusions that the coral harbours and maintains a distinct microbiota (Rohwer et al. 2001; Frias-Lopez et al. 2002; Guppy & Bythell 2006). There are numerous possible delivery sources of bacteria to the SML, such as passive settlement from the water column (Guppy & Bythell 2006), deposited faecal matter and re-

suspended sediments from the benthos, all of which may pass on specific bacterial species to the SML. The 'surface associated microbial communities' routinely described on corals are likely not to be made up entirely of bacterial associates, with strong evidence reported here for a significant proportion of these being transient bacteria from other environmental sources. Specific ribotypes found in this study, such as *Bacteroidetes* sp. (Kvennefors et al. 2010) and *Sphingobacterium* sp. (Ritchie 2005) have previously been suggested to be transients within the coral SML, which are highly likely to be temporarily trapped within the mucus and originate from the water column. However, there were also clear differences between the SML and environment-associated bacterial communities, indicating development of a distinct and relatively diverse bacterial community within the SML.

Adjacent sediment particles are known to be swept along the surface of the coral by the combined effects of enmeshing coral mucus and ciliary beating (Johnston & Rohwer 2007) so this material would be included in any SML sample collected. Conversely, the process of benthic - pelagic coupling reported by Wild et al. (2004), whereby mucus strands released from the coral are filtered through lagoonal sands, would be expected to be reflected in sediment samples, with these sharing similar ribotypes to that of the coral's mucus (Wild et al. 2004; Naumann et al. 2009). In comparing the microbial diversity of the sediments and other benthic surfaces with the SML, it should be possible to determine which bacterial strains may be symbiotic and which are merely transient and found normally on these other surface types (i.e., not symbiotic but passively settled for a period of time on the coral surface). In addition, the cross over of certain species between the SML and sediment samples suggests that studies which investigate opportunistic pathogenesis and bacterial vectors may find it beneficial to study adjacent sediments at times of increased disease, or as primary colonisers of newly available habitats under altered environmental conditions.

The DGGE method emphasises the dominant fractions of the ribosomal rRNA gene pool present within each compartment and environmental samples studied. The differences and similarities between these compartments can be seen by the presence and relative intensity of particular bands of the different ribotypes. Bourne and Munn (2005) used both culture-based and culture independent techniques to investigate the microbial community in the reef building coral *Pocillopora damicornis*. They found

that the majority of clones obtained from the coral tissue were dominated by γ-proteobacteria, whereas the bacteria within the coral mucus and the water column were dominated by α-proteobacteria (Bourne & Munn 2005). Although the present study only applied culture-independent molecular techniques, we found a similar trend, wherein the water column was represented predominantly by α-proteobacteria, and the tissues dominated by γ-proteobacteria. Conversely, the SML was relatively more diverse with representatives from both these groups as well as several others.

While several ribotypes were shown here to be common to a number of coral compartments, specific ribotypes associated only with coral tissues were also found, which may represent specific coral-bacterial associations. A ribotype related to *Trichococcus* (EU919224) was found only in the coral tissue, while a number of ribotypes were found only in coral tissues, skeleton and complete coral such as *Pseudidiomarina* sp. (FJ887948), *Proterythropsis* sp. (FJ947037), *Lactobacillus* sp. (DQ336385 & DQ336384), *Streptococcus* sp. (DQ001071) and *Alcaligenes* sp. (EU876658). Interestingly, four ribtypes related to *Chloroflexi* (EU909941), *Sphingobacterium* sp. (AF260710), *Roseobacter* sp. (EF441565) and *Pseudoalteromonas* sp. (DQ665793) were found only in the SML, all of which have previously been reported to be associates with coral and sponge microbial communities (Rohwer et al. 2001; Ritchie 2005; Webster & Bourne 2007; Raina et al. 2009). These ribotypes may represent specific associates adapted to these particular microbial niches (Ritchie & Smith 2004). For example *Roseobacter sp.* and *Pseudoalteromonas sp.* are able to metabolise an organic sulphur compound (dimethylsulfoniopropionate), produced in large quantities by the corals' symbiotic algae (Raina et al. 2009), showing the potential importance of coral reefs in sulphur cycling. Although several cyanobacterial ribotypes (GQ346809 & FJ967973), some of which have been linked with coral disease (Cooney et al. 2002), were detected in the coral skeleton, none of these were solely found within this coral compartment, perhaps reflecting the close association the skeleton has with the surface tissues (Fine & Loya 2002).

4.5.2 The importance of sampling techniques in characterisation of coral associated bacterial communities:

In this paper we used four sampling protocols to investigate coral associated microbes: (a) whole coral (including underlying skeletal material) pestle-grinding (Cooney et al. 2002), (b) airbrushing of soft tissues (Rohwer et al. 2002) (c) milking (in air) of coral surface mucus (Allers et al. 2008) and (d) swabbing of the coral surface (Guppy & Bythell 2006; Lampert et al. 2008). These protocols are prone to microbial contamination from surrounding seawater, but more importantly the physical organization of any coral-associated community would likely be disrupted by most of these sampling regimes.

We found significant differences between the two most commonly used techniques for sampling the SML; milked mucus and coral swabs (Wild et al. 2004; Guppy & Bythell 2006; Allers et al. 2008), which alone highlights the importance of choosing the right technique depending on particular questions addressed in future studies. The presence of symbiotic dinoflagellate DNA in both the swab samples and milked mucus suggests that these techniques result in significant contamination from the coral tissues. The absence of these bands from samples collected using the novel methodology, (1st and 2nd RSS) and the distinct ribotype profiles, indicating a lack of contamination from either the water column or the coral tissues, suggest this novel methodology to be a more accurate method for sampling the loosely-attached mucus layer. Added to this, the low diversity found within the milked mucus and swab samples compared to those found within the 1st and 2nd RSS suggests that these previously used methods might not be detecting the complete bacterial diversity present. We suggest that milked mucus may actually be a better representation of the coral's gastro-vascular cavity microbiota rather than that present within the SML. Studies on the microbiota of the corals' gastro-vascular cavity are limited, but it is known to contain large numbers of bacteria (Herndl & Velimirov 1985; Herndl et al. 1985) and this would be included in whole coral samples as well.

The coral swabs, along with picking up tissue contaminants, appeared to show closer similarities to adjacent sediment samples which may also be contaminants. Since the sediment often comes into contact with the corals either directly from the base or

driven by wind and wave action, links between the SML and bacteria originating from the sediment would not be unexpected (Johnston & Rohwer 2007). However, the coral swab community profiles were also significantly different to either milked mucus or the 'snot sucker' samples and also appeared to under-represent the diversity present.

In conclusion, the use of the novel snot sucker methodology can allow coral samples to be collected either *in situ* from the field or from tank experiments quickly, with minimal exposure to potential contaminants and while minimising the loss of loosely-associated bacteria during collection and transport. Significant differences found using this technique (those from the 1st and 2nd RSS) compared to techniques previously utilised in studying the SML bacterial diversity (milked mucus and swabs), suggests that these latter sampling methods may have underestimated the diversity of bacteria found within the SML. The presence of dinoflagellate sequences within these samples also indicates coral tissue contamination. No matter what technique was used, significant differences between the community structure of the SML and tissues were found. Given this, researchers should carefully evaluate their choice of sampling method and be careful when comparing datasets which use different sampling techniques. Despite this, however, it is clear that the SML's bacterial community is highly diverse compared to that of the water column but does share some similar bacterial ribotypes, although it clearly houses its own unique ribotypes which were not detected in other compartments. The significant difference between all coral compartments and the environmental samples found in this study shows that even when using a more stringent technique to study the SML, the coral appears to be retaining and maintaining its own distinct and diverse bacterial community.

Chapter 5

Development of bacterial biofilms on artificial corals in relation to surface associated microbes of hard corals

5.1 ABSTRACT

Numerous studies have shown the differences between coral-associated bacteria and those in their surrounding environment. Despite these clear differences, few studies have looked at the settlement and growth of bacteria on surfaces with respect to corals. To aid understanding of the controls on bacterial community development on the coral surface, early stages of passive settlement from the water column to fixed surfaces (formation of a biofilm) were assessed. 16S rRNA gene bacterial profiles were studied on replica artificial coral nubbins from *Acropora muricata*. These models were dip-coated in sterile agar, mounted *in situ* on the reef and followed over time to monitor bacterial succession. The bacterial community forming the biofilms remained significantly different ($R = 0.864$ $p < 0.05$) from that of the water column at all times from 30 min to 96 h and from the surface mucus layer (SML) of the coral. The water column was dominated by members of the group α-proteobacteria, compared to that of the settling community on the biofilms being dominated by γ-proteobacteria, whilst those present within the SML were from a more diverse array of groups. This suggests that bacterial communities present within the SML, do not arise from simple passive settlement from the water column. This selection process was shown to involve some aspects of the physical structure of the settlement surface, since agar-coated slides showed distinct communities to coral-shaped surfaces. However, no significant differences were found between surface coating, including plain agar as well as agar enhanced with coral mucus exudates.

5.2 INTRODUCTION

Biofilms are complex structures created by microorganisms that attach and grow on available substrates (Dunne. Jr. 2002). Most, if not all bacteria are capable of forming biofilms and in most strains this is their predominant lifestyle (Dheilly et al. 2010). Biofilm formation involves interaction among pioneers and later colonisers to produce temporal shifts in the microbial community structure. Despite its relevance for marine ecosystems; in processes such as larval settlement, recruitment and the dynamics of microbial communities (Lee et al. 2008), early stages of biofilm formation are not well understood (Siboni et al. 2007). Normally, biofilm formation commences with the adsorption of a conditioning film of polysaccharides, proteins, lipids, humic acids, nucleic acids and aromatic amino acids to which the early colonising bacteria subsequently adhere (Siboni et al. 2007). Growth, reproduction and death of the primary colonisers modify the characteristics of the substratum, rendering it suitable (or unsuitable) for subsequent colonisation by secondary microorganisms. Ecological succession via synergistic and/or competitive interactions among these colonists and the addition of new settling species and/or loss of some previous colonists will result in a mature, relatively stable climax biofilm community (MassolDeya et al. 1997). There is growing evidence suggesting that early colonisers determine in part the structure of this climax community (Dang & Lovell 2000; Martiny et al. 2003; Jones et al. 2007; Lee et al. 2008).

The surface mucus layer (SML) of corals provides an ideal surface for the formation of a marine biofilm, as it provides a rich source of carbon and nutrients for settling microbes. Establishment and maintenance of these biofilms could occur in two ways depending on the rate of exchange of the SML and the species and type of coral in question (Brown & Bythell 2005). These two possible models for microbial colonisation in this system are; 1) microbes could be continually settling or being trapped by the mucus but not ultimately forming an established community due to the rapid sloughing off of the layer. If such a transient community profile existed it would be expected to largely reflect that of the water column community with only slight variations due to differences in the availability of specific settlers, their individual survival rates and the hydrodynamics within the system (Ritchie 2006). 2) An alternative model is that bacteria settle and reside in the mucus or the coral tissue

itself, become well established and form a distinct community from that of the water column. Specific properties present within the mucus of different coral species may affect formation of these microbial communities and therefore explain differences in microbial communities of different species (Rohwer et al. 2002). In this model, although the SML may be continuously or periodically sloughed from the coral surface, it may be either an insufficient proportion or an infrequent enough occurrence to prevent a stable, climax community from developing. Contrary to the first model, the bacterial community structure should in this case remain more stable (Guppy & Bythell 2006), being determined predominantly by mucus composition (Brown & Bythell 2005) and the competitive and antimicrobial properties of the resident bacterial communities (Ritchie 2006).

Different studies have shown that corals harbour diverse bacterial communities that in turn differ from the surrounding water environment (Ritchie & Smith 1995; Frias-Lopez et al. 2002; Guppy & Bythell 2006; Chapter 4). The differences in bacterial communities between coral species (Rohwer et al. 2002) may be due to differences in the settlement surface offered by each coral species and/or variations in physical and chemical properties of the coral mucus. Corals with their various microbial environments, (SML, tissue and skeleton), provide many potential habitats and surface types for a variety of settling bacterial species on a micro level (Nothdurft et al. 2005). These surfaces are known to affect settlement depending on cell-cell interactions, ecological effects and/or surface properties (Cerca et al. 2005).

Effects of surface type on biofilm development have been previously studied with regard to bio-fouling (Dang & Lovell 2000; Thomason et al. 2002; Bakker et al. 2003). The structure of the settlement surface affects the amount and type of bacteria which can settle, grow and survive (Thomason et al. 2002). Physiochemical properties of an artificial surface may be characterised by hydrophobicity, surface free energy and electricity. Microorganisms attach more rapidly and build thicker biofilms forming an established community differing from that of the water column on hydrophobic and non-polar surfaces than those which form on hydrophilic materials which should have a bacterial community reflecting that of its environment (Cerca et al. 2005). As mucus of the corals is hydrophilic and highly motile, bacteria that are incorporated during biofilm formation should in theory be similar to those present within the water

column, however this appears to not be the case (Guppy & Bythell 2006). As mentioned bacterial communities associated with corals, differ among and within species (Rohwer et al. 2002). This suggests that corals or their previously established bacterial consortium have the ability to select certain species from the water column and deny others (Ritchie & Smith 2004; Kooperman et al. 2007; Sharon & Rosenberg 2008). While there is no doubt that the SML of corals hosts a diverse microbial community, the mechanisms by which they are seeded and maintained remains poorly understood. This study describes temporal changes of bacterial communities forming the initial settlement community on artificial coral nubbins. Artificial surfaces were used to give a better representation of a coral surface as a primary settlement substrate for bacteria. Experiments were repeated during summer and winter to test whether succession was susceptible to seasonality (e.g., changes in water temperature) and samples were collected spatially around the island to compare spatial differences between biofilm formations.

5.3 MATERIALS AND METHODS

5.3.1 Experimental design

5.3.1.1 Ecological succession of biofilm formation

In order to assess the temporal dynamics of the microbial community settling and developing on the coral surface, an artificial surface was created that resembled the coral surface in both structure and food source availability. Replica coral nubbins were made from the hard coral, *Acropora muricata*, moulded in silicone rubber and the nubbins formed from a hard polyurethane resin. Each model was therefore the same size and had identical structure to the microscopic level (individual corallites) allowing for standardised replication (Fig. 5.1a).

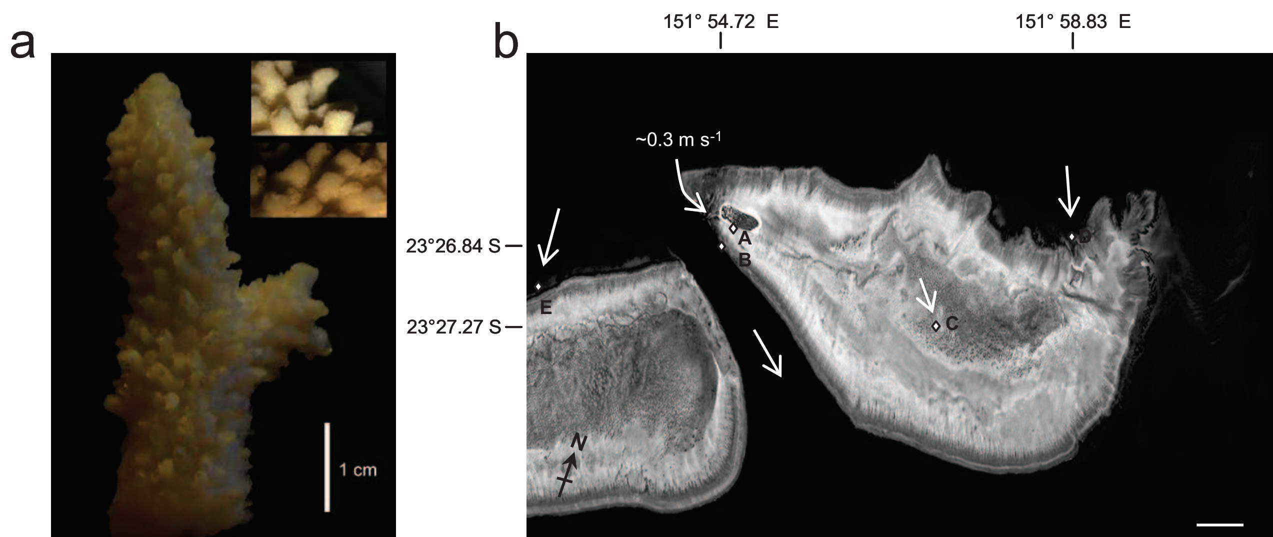


Figure 5.1. a) Photograph of replica coral nubbins used in experiment with close up sections of the mould (insets) b) Heron Island GBR, Australia (23°27'S, 151°55'E) Location of main study site (A) the Reef Flat and those used in spatial sampling; (B) Coral Gardens 23º26.839/151º54.717 (C) Lagoon 23º27.272/151º57.921 (D) 3rd/4th Point 23º26.146/151º58.833 (E) Wistari 23º29.081/151º54.015. Arrows depict water current direction at time of sampling with direction and speed noted. Samples were taken on calm days, one hour before high tide, with wave speed W_S < 0.5 m/s and wave heights H_S < 0.5 m. Scale bar = 1 km.

All models were bathed in (0.22 μm) filtered seawater for 24 h prior to use, to remove any potential contaminants and further washed three times. Each nubbin was dip coated twice, in sterile (1.5% w/v) plain agar (Difco), giving an even coat of between 0.5 – 3 mm thickness, resembling both the food source availability and SML thickness naturally provided by the corals (Wild et al. 2004). Although the nutritional and biophysical properties of coral SML could not be reproduced, we aimed to test the effects of differing growth media, including coral/mucus exudates on the developing community. The study was conducted at Heron Island, GBR, Australia (Fig. 5.1b) over two years, encompassing both a summer (March 2009) and winter (August 2008) season. The average sea surface temperatures during these months at the site ranged from 26 - 28 ˚C during the summer sampling period and 20 - 22 ˚C during the winter. The replica coral nubbins were placed on the reef flat (Fig. 5.1b A) using a push mount system and sampled over a time series (30 min, 1 h, 2 h, 4 h, 6 h,

8 h, 10 h, 12 h, 24 h, 48 h (2 days), 72 h (3 days) and 96 h (4 days) after deployment to monitor the natural settlement and succession of bacteria. n = 36 nubbins were used, giving n = 3 replicates per time period.

5.3.1.2 Biofilm formation on different substrate types

To assess the effects of growth media on the settling bacterial community we employed four variations in marine agar types. The four agar types were made up as per the manufacturer guidelines (Difco), using 0.22 µm filtered fresh sea water collected on site; 1) plain unaltered agar, 2) agar with the addition of fresh milked mucus from the coral *A. muricata* collected *in situ*, (five nubbins of *A. muricata* were exposed and inverted upside down with the resulting mucus collected (100 ml in total) into a sterile container (Ritchie 2006), added to 500 ml of agar before autoclaving). 3) agar used with water containing coral exudates (where a ~15 cm diameter colony of *A. muricata* had been bathed in 5 l of water for 24 h under normal conditions), filtered through a 0.22 µm polycarbonate filter and made up as per manufacturers guidelines and, 4) agar used with water containing stressed coral exudates (where a similar sized coral colony was exposed to extreme levels of sunlight in a shallow tank for 24 h). n = 4 replicas of each agar type was sampled at each time period. Samples of each agar were taken at time of preparation, freeze dried and analysed for C and N composition (School of Chemistry, Newcastle University) to compare between the different types. In order to compare the developing bacterial communities on different surface structures, n = 36 sterile microscopic slides were dip coated in plain agar (no modifications) and mounted vertically. These were then deployed at the same time intervals as the coral nubbins to allow for comparisons between biofilm development on flat surfaces and those that develop on textured surfaces (replica coral nubbin).

5.3.1.3 Spatial variability in biofilm bacterial communities

To assess spatial variation around the island reef system, samples of the replica coral nubbins coated in plain agar (as per manufacturer's guidelines), were set out at five locations around Heron Island (Fig. 5.1b, A-E), for 24 h periods. These samples were assessed at high tide to estimate spatial variability in bacterial biofilm diversity and composition. The sites were chosen at time of sampling, as they were expected to

show variation in their bacterial diversity due to differences in the benthos (e.g. sandy lagoon site C compared to reef crest site B) and known oceanographic patterns around the island (Chapter 3; Sweet et al. 2010a). The spatial sample nubbins and subsequent water sampling were sampled during the summer season only.

5.3.1.4 Does the surface mucus layer bacterial community represent a particular stage of biofilm development?

In order to assess if the SML of reef building corals represented a particular stage of biofilm development and if the water column was the supply of these settling microbes, n = 3 water column samples were taken at the same time as each of the biofilm samples, and n = 4 coral surface mucus layer samples (summer season only) were also collected. For the water samples, 1 l of water ~5 cm above the coral colony was continuously sampled for a period of 1 h, onto 0.22 µm Sterivex filters, using a Masterflex pump (Sweet et al. 2010a; Chapter 3). For mucus samples, the 'snot sucker' (Sweet et al 2010b, Chapter 4) was used on individual branch tips of one colony of *A. muricata*. All samples were stored at -20 ºC until processed. All samples were collected in sterile tubes at time of sampling, allowing no contact with the air during collection and transport back to the laboratory. Nubbin and slide samples were then placed within an autoclaved bag and the agar airbrushed off and scraped into a sample tube using sterile scalpel blades, stored in absolute ethanol and kept at -20 ºC until extracted. In addition the rate of flow of the SML was estimated by using carbon particles (purified activated charcoal particles ~10 µm in diameter, Kebo laboratories, Jatkar et al. 2010), dispersed onto corals (transferred without handling into an observation tank) and monitored using an Olympus SZX7 binocular microscope and Olympus LG-PS2 fibre-optic light source. Still images and time-lapse videos were captured using a QImaging Micropublisher 3.3 camera and Q-Capture v6 imaging software which allowed frame by frame analysis of the movement of individual carbon particles trapped within the mucus.

5.3.2 Bacterial 16S rRNA gene diversity, DNA extraction, amplification and DGGE analysis

DNA extraction and amplification was performed following the procedures described in Chapter 3 (Sweet et al. 2010a). DGGE was performed using the D-Code universal mutation detection system (Bio-Rad). PCR products were resolved on 10 % (w/v) polyacrylamide gels that contained a 30 – 60 % denaturant gradient for 13 h at 60 °C and a constant voltage of 50 V. Gels were stained with a concentrated solution of 9 μl Sybr® Gold (Sigma) in 50 μl of 1X TAE poured directly onto the gel surface, covered and left in the dark for 20 min then further washed in 500 ml 1X TAE for 30 min and visualized using a UV transilluminator. Dominant bands of interest (those which explained the greatest differences/similarities between samples) were excised from DGGE gels for the summer season only, left overnight in Sigma molecular grade water, vacuum centrifuged, re-amplified with primers 357F and 518R, labelled using Big Dye (Applied biosystems) transformation sequence kit and sent to Genevision (Newcastle University UK) for sequencing. Bacterial operational taxonomic units (OTUs) (Guppy & Bythell 2006) were defined from DGGE band-matching analysis using BioNumerics 3.5 (Applied Maths BVBA). Standard internal marker lanes were used to allow for gel-to-gel comparisons. Tolerance and optimisation for band-matching was set at 1 %.

Real-time PCR (qPCR) was conducted on an Engine Opticon® 2 system in order to test whether *Vibrio* sp. abundance changed between settling times (n = 20 randomly chosen samples), ten from both the early colonisers (classed as 2 - 12 h) and the later colonisers (established communities, classed as 24 - 96 h). For this, vibrio-specific primers 567F and 680R were used as in Chapter 3 (Thompson et al. 2004). Real time PCR reaction mixtures totalled 25 μl and consisted of 12.5 μl of 2X Quantitect® Sybr® Green 1 supermix (Qiagen), 1.25 μl each of 0.5 mM forward and reverse primers, 50 ng DNA and 9.5 μl sigma molecular grade water. Each set of samples included a negative control, in which water was substituted for the DNA sample. Real time PCR was performed with an initial activation step of 15 min at 95 ºC, followed by 39 cycles (94 ºC for 15 s, 58 º C for 30 s, primer annealing at 58 ºC for 30 s). The fluorescent product was detected after each extension. Following amplification, melting temperature analysis of PCR products was performed to determine the

specificity of the PCR. The melting curves were obtained by slow heating at 0.5 ºC s^{-1} increments from 50 to 90 ºC, with continuous fluorescence recording.

5.3.3 Statistical analysis

Matrices of Bray-Curtis similarities were generated using band intensity data (where 0 = absence) from the DGGE analysis, using marker lanes for between-gel comparisons. An analysis of similarities (ANOSIM, Clarke & Warwick 2001) was performed to compare changes in bacterial community structure that settled onto the different types of agar. Likewise, bacterial communities settled onto coral nubbin models and slides were compared with an ANOSIM test. Temporal changes in bacterial assemblages were also evaluated with a two-way permutation analysis of variance (PERMANOVA) and multi dimensional scaling (MDS), based on Bray-Curtis similarities. A one-way analysis of similarity (ANOSIM) was performed separately for summer and winter data sets. A similarity profile analysis (SIMPER) was performed in order to determine the ribotypes that contributed most to the observed patterns. Average (centroids) similarities of bacterial communities were estimated from replicates corresponding to each temporal point. These centroids were used to produce new MDS plots showing the temporal trajectory (i.e., succession) of bacterial assemblages from initial settlement up to 96 h. Shannon Weiner diversity indices were used to compare temporal samples for each season. The 16S rRNA gene diversity settling on a coral nubbin for 96 h biofilm development and those of the corals SML were compared with those present within the water column using BioNumerics band intensity data and an MDS plot. Real-time PCR calculations were based on relative DNA concentration ($\Delta C(t)$) of vibrios based on lowest detected concentration ($C(t)$). Fold differences in vibrio DNA template were calculated assuming 2-fold PCR reaction efficiency ($2^{\Delta C(t)}$). One way ANOVA (minitab) was used to compare between settler communities.

5.4 RESULTS

5.4.1 Biofilm formation on different substrate types

No significant differences (ANOSIM R = 0.83, p = 0.64) were found between the 16S rRNA gene bacterial assemblages settling on the different agar types (potential food sources). Because of this lack of differences and a lack of variation in the C:N ratios among agar types, (plain agar C:N = 9 – 9.9, mucus agar C:N = 10.4 – 12, stressed coral agar C:N = 9.2 – 9.5, coral exudates agar C:N = 8.2 – 9.7), only plain agar was used for further temporal analysis (Table 5.1). The C:N ratios of all agar types fell within the C:N ratio reported for *Acropora* mucus of 8 – 14 (Wild et al. 2004).

Table 5.1. Percentage carbon and nitrogen and resulting C:N ratio for the four agar types.

Agar type	% N	% C	C : N
Plain	0.63	5.7	9
	0.62	6.12	9.9
	0.62	5.9	9.5
Mucus exudate	0.32	3.88	12
	0.37	3.83	10.4
	0.33	3.86	11.7
Coral exudate	0.41	3.9	9.5
	0.44	4.06	9.2
	0.43	4	9.3
Stressed exudate	0.42	3.43	8.2
	0.41	3.99	9.7
	0.41	3.81	9.3

There was a significant difference (ANOSIM R = 0.84 p = 0.001) between the biofilms that developed on microscopic slides (coated in plain agar) compared to the coral nubbin replicates (coated in plain agar). A greater diversity and higher dominance of particular ribotypes were found to settle on the replica nubbins after 4 hrs of deployment (S = 9 – 16), compared to a significantly lower diversity on the smooth surface of the slides (S = 3 – 6), with overall ribotype richness (S) being greatly reduced (Fig. 5.2).

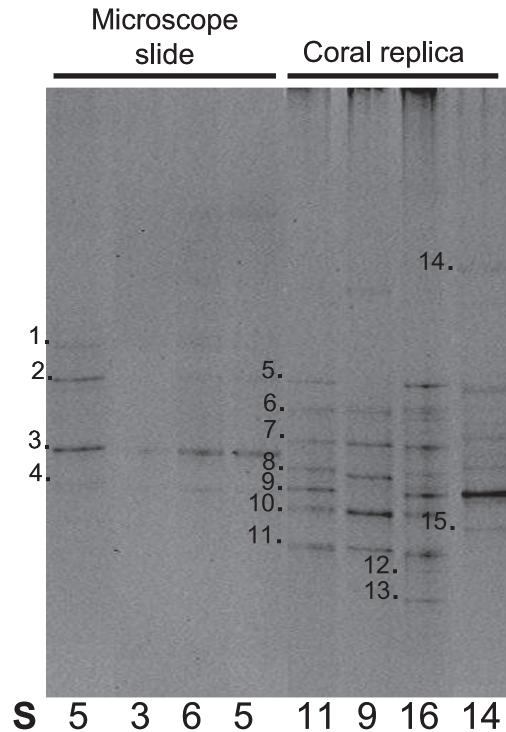


Figure 5.2. Composite DGGE image showing 4 h biofilm development on microscopic slides and replica coral nubbins with dominant bands sequenced (Table 5.2), standardised using internally run marker lanes allowing gel-to-gel comparison using BioNumerics. S = number of bands visible in DGGE using BioNumerics representing relative diversity.

Bacteria settling on the coral nubbins included ribotypes similar to *Aeromonas* sp. (AY689043), *Prochlorococcus* sp. (GQ272346), *Shigella* sp. (FJ193359) and *Enterobacter* sp. (FN423410), whilst ribotypes such as *Microbulbifer* sp. (EF674853) and several ribotypes similar to species of *Pseudoalteromonas* (FM163075, DQ665793 & EU330363) were found to dominate the microscopic slide biofilm community (Table 5.2, Fig. 5.2). Despite significant differences in settling community certain ribotypes of the genus *Pseudoalteromonas* where recorded on both the slide and the replica coral nubbin, however no exact matches were found.

Table 5.2. Close matches (Blast n), species identification, group affiliation (identified to closest published relatives on GenBank at the time of comparison) of dominant ribotypes excised from DGGE occurring in varying sample types; Biofilm (agar slide), Biofilm (agar nubbin), coral mucus and the water column. All samples collected from Heron Island reef flat, March 2009.

Band No.	Sample type	Time period	Species ID	Group affiliateion	Close relative (% match)
1	Biofilm (agar slide)	4 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	FM163075 (99%)
2	Biofilm (agar slide)	4 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	DQ665793 (99%)
3	Biofilm (agar slide)	4 hrs	*Microbulbifer sp.*	γ-proteobacteria	EF674853 (98%)
4	Biofilm (agar slide)	4 hrs	*Pseudoalteromonas sp*	γ-proteobacteria	EU330363 (97%)
5	Biofilm (agar nubbin)	2/4 hrs	*Shewanella sp.*	γ-proteobacteria	CP000302 (91%)
6	Biofilm (agar nubbin)	2/4 hrs	β-proteobacterium	β-proteobacteria	GU257663 (88%)
7	Biofilm (agar nubbin)	2/4 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	GQ849227 (98%)
8	Biofilm (agar nubbin)	2/4 hrs	*Vibrio sp.*	γ-proteobacteria	AB519004 (100%)
9	Biofilm (agar nubbin)	2/4 hrs	*Klebsiella sp.*	γ-proteobacteria	GQ416635 (90%)
10	Biofilm (agar nubbin)	2/4 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	DQ667134 (100%)
11	Biofilm (agar nubbin)	2/4 hrs	*Aeromonas sp.*	δ-proteobacterium	AY689043 (100%)
12	Biofilm (agar nubbin)	2/4 hrs	*Prochlorococcus sp.*	Cyanobacteria	GQ272346 (100%)
13	Biofilm (agar nubbin)	2/4 hrs	*Shigella sp.*	γ-proteobacteria	FJ193359 (91%)
14	Biofilm (agar nubbin)	2/4 hrs	*Enterobacter sp.*	γ-proteobacteria	FN423410 (100%)
15	Biofilm (agar nubbin)	2/4 hrs	*Microbulbifer sp.*	γ-proteobacteria	EU837333 (90%)
16	Biofilm (agar nubbin)	24 hrs	Chloroflexi sp.	Chloroflexi	AB433054 (100%)
17	Biofilm (agar nubbin)	24 hrs	*Flavobacteriaceae sp.*	Flavobacteria	EF092242 (100%)
18	Biofilm (agar nubbin)	24 hrs	*Thermus sp.*	Deinococcus-Thermus	DQ989458 (96%)
19	Biofilm (agar nubbin)	24 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	FN295786 (100%)
20	Biofilm (agar nubbin)	24 hrs	γ-proteobacterium	γ-proteobacteria	GU317768 (95%)
21	Biofilm (agar nubbin)	24 hrs	*Pseudoalteromonas sp*	γ-proteobacteria	GU229650 (91%)
22	Biofilm (agar nubbin)	24 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	GU726846 (97%)
23	Biofilm (agar nubbin)	24 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	FJ457226 (98%)
24	Biofilm (agar nubbin)	24 hrs	Cyanobacterium	Cyanobacteria	GU184683 (93%)
25	Biofilm (agar nubbin)	24 hrs	*Pseudoalteromonas sp*	γ-proteobacteria	FJ237010 (100%)
26	Biofilm (agar nubbin)	24 hrs	*Pseudoalteromonas sp.*	γ-proteobacteria	GU726846 (100%)
27	Biofilm (agar nubbin)	72/96 hrs	*Flavobacteria sp.*	Flavobacteria	FN433284 (85%)
28	Biofilm (agar nubbin)	72/96 hrs	Cyanobacterium	Cyanobacteria	GQ480703 (88%)
29	Biofilm (agar nubbin)	72/96 hrs	*Glaciecola sp.*	γ-proteobacteria	EU183316 (95%)
30	Biofilm (agar nubbin)	72/96 hrs	*Planctomycetales sp.*	Planctomycetacia	GU084063 (97%)
31	Biofilm (agar nubbin)	72/96 hrs	*Aestuariibacter sp.*	Unknown	AB473549 (95%)
32	Biofilm (agar nubbin)	72/96 hrs	*Klebsiella sp.*	γ-proteobacteria	GQ416635 (95%)
33	Surface mucus layer (coral)	NA	*Chloroflexi sp.*	Chloroflexi	EU909941 (97%)
34	Surface mucus layer (coral)	NA	Cyanobacterium	Cyanobacteria	GQ346809 (100%)
35	Surface mucus layer (coral)	NA	Cyanobacterium	Cyanobacteria	FJ967973 (100%)
36	Surface mucus layer (coral)	NA	Cyanobacterium	Cyanobacteria	FJ946590 (100%)
37	Surface mucus layer (coral)	NA	α-proteobacterium	α-proteobacteria	EF520401 (95%)
38	Surface mucus layer (coral)	NA	δ-proteobacterium	δ-proteobacteria	EF188467 (96%)
39	Surface mucus layer (coral)	NA	*Klebsiella sp.*	γ-proteobacteria	GQ471864 (100%)
40	Surface mucus layer (coral)	NA	*Aeromonas sp.*	δ-proteobacteria	EU919223 (100%)
41	Surface mucus layer (coral)	NA	*Burkholderia sp*	β-proteobacteria	EU876657 (100%)
42	Surface mucus layer (coral)	NA	*Aeromonas sp.*	δ-proteobacteria	EU919223 (100%)
43	Surface mucus layer (coral)	NA	*Klebsiella sp.*	γ-proteobacteria	GQ471869 (100%)
44	Surface mucus layer (coral)	NA	*Streptococcus sp.*	Coccus	DQ001071 (97%)
45	Surface mucus layer (coral)	NA	*Klebsiella sp.*	γ-proteobacteria	GQ471864 (100%)
46	Surface mucus layer (coral)	NA	*Trichococcus sp.*	Coccus	EU919224 (87%)
47	Surface mucus layer (coral)	NA	*Shewanella sp.*	γ-proteobacteria	EU919217 (100%)
48	Surface mucus layer (coral)	NA	*Pseudidiomarina sp.*	γ-proteobacteria	FJ887948 (100%)
49	Water Column (Supply)	NA	*Bacteroidetes sp.*	Bacteroidetes	AM238600 (84%)
50	Water Column (Supply)	NA	Actinobacterium	Actinobacteria	AY632498 (90%)
51	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	FJ718457 (96%)
52	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	GQ350573 (98%)
53	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	GQ204865 (100%)
54	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	EF092739 (95%)
55	Water Column (Supply)	NA	*Bacteroidetes sp.*	Bacteroidetes	AB254287 (100%)
56	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	FJ620860 (95%)
57	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	EU315614 (97%)
58	Water Column (Supply)	NA	*Flavobacteria sp.*	Flavobacteria	EU600663 (100%)
59	Water Column (Supply)	NA	*Bacteroidetes sp.*	Bacteroidetes	EU315425 (96%)
60	Water Column (Supply)	NA	*Flavobacteriales sp.*	Flavobacteria	AB294989 (100%)
61	Water Column (Supply)	NA	α-proteobacterium	α-proteobacteria	FJ532499 (100%)
62	Water Column (Supply)	NA	*Bacteroidetes sp.*	Bacteroidetes	DQ656191 (95%)
63	Water Column (Supply)	NA	γ-proteobacterium	γ-proteobacteria	EU315645 (88%)
64	Water Column (Supply)	NA	γ-proteobacterium	γ-proteobacteria	GQ257639 (82%)

5.4.2 Ecological succession of biofilm formation

Temporal changes in bacterial assemblages were observed during biofilm formation, with significant differences between seasons (PERMANOVA $F = 4.1$ $p = 0.001$), with 22 % of the variance being explained by season. No specific ribotypes occurred exclusively within a single season (Fig. 5.3 a-h), indicating that the significant differences between seasons were due to shifts in dominance of particular ribotypes not presence/absence. Ribotypes similar to *Chloroflexi* sp. (AB433054) (Fig. 5.3 b) and a γ-proteobacteria (GU317768) (Fig. 5.3 c) were predominant in winter whilst *Flavobacteriaceae* sp. (EF092242) (Fig. 5.3 d) and a *Pseudoalteromonas* sp. (FJ457226) (Fig. 5.3 g) were found predominantly in summer.

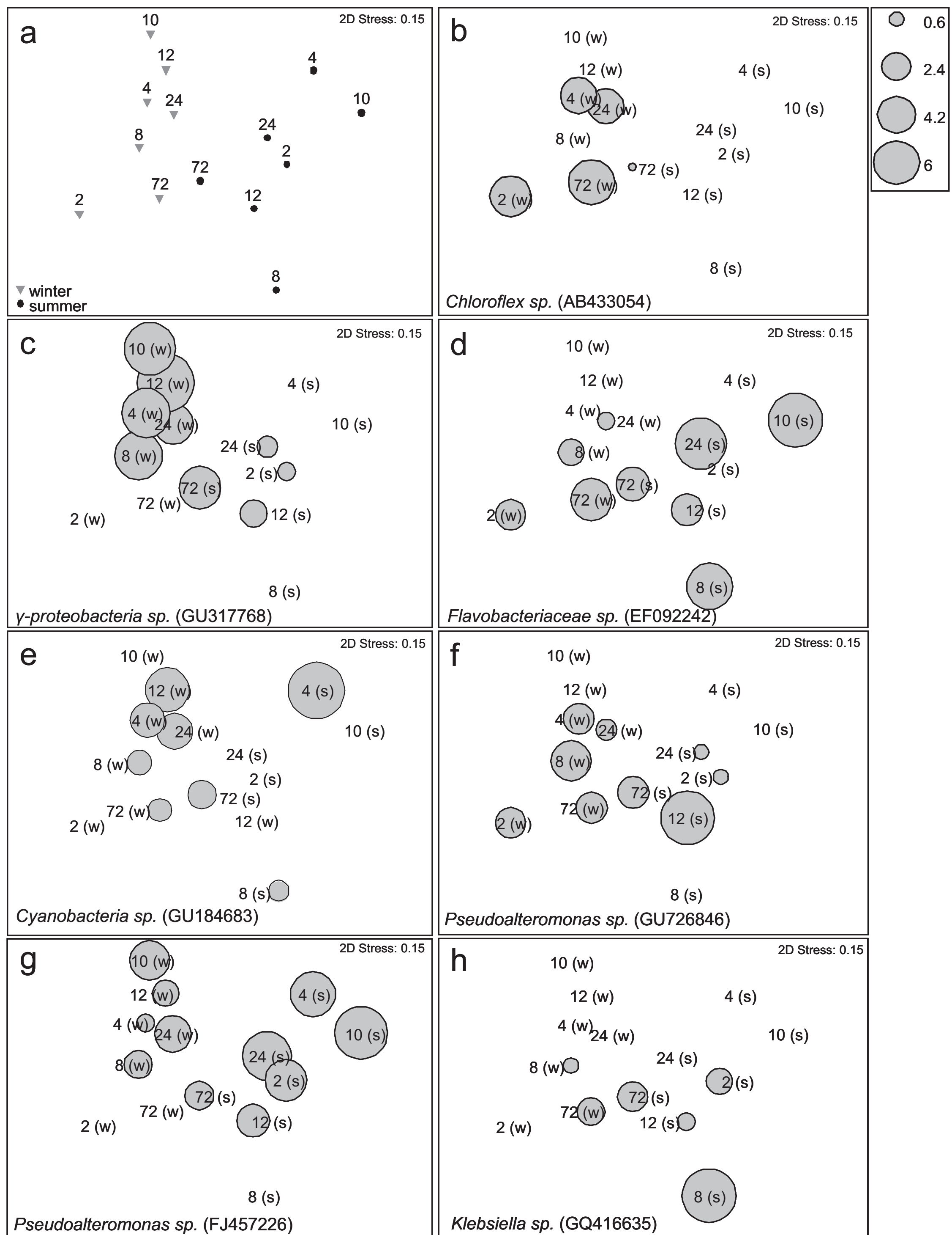


Figure 5.3. Multidimensional scaling plot (MDS) showing seasonal changes in bacterial communities (16S rRNA gene fingerprints) settling on the biofilm of the replica coral nubbins enriched with agar (a) average of n = 3 replicates for different time scales of biofilm development for both seasons; summer (s) (March 2009) and winter (w) (August 2008). (b-h) representatives of the sequenced ribotypes responsible for the greatest differences between seasons, Latin name and gen bank

sequence ID. Size of bubble depicts intensity of band/ribotype on DGGE within individual samples.

Significant shifts in bacterial communities occurred between early bacterial biofilm colonisers (2 – 12 h) and the later developed community (24 – 96 h) for both seasons (summer ANOSIM R = 0.442 p = 0.001 and winter ANOSIM R = 0.515 p = 0.001) with a further 23% of the variance being explained by settling time periods. Large differences between replicates within each individual time period for the first 12 h (Fig. 5.4), suggests a highly dynamic period of settling primary colonisers. After 12 h a more stable bacterial community appears to have established, only fluctuating slightly in total diversity (Fig. 5.4 and 5.5).

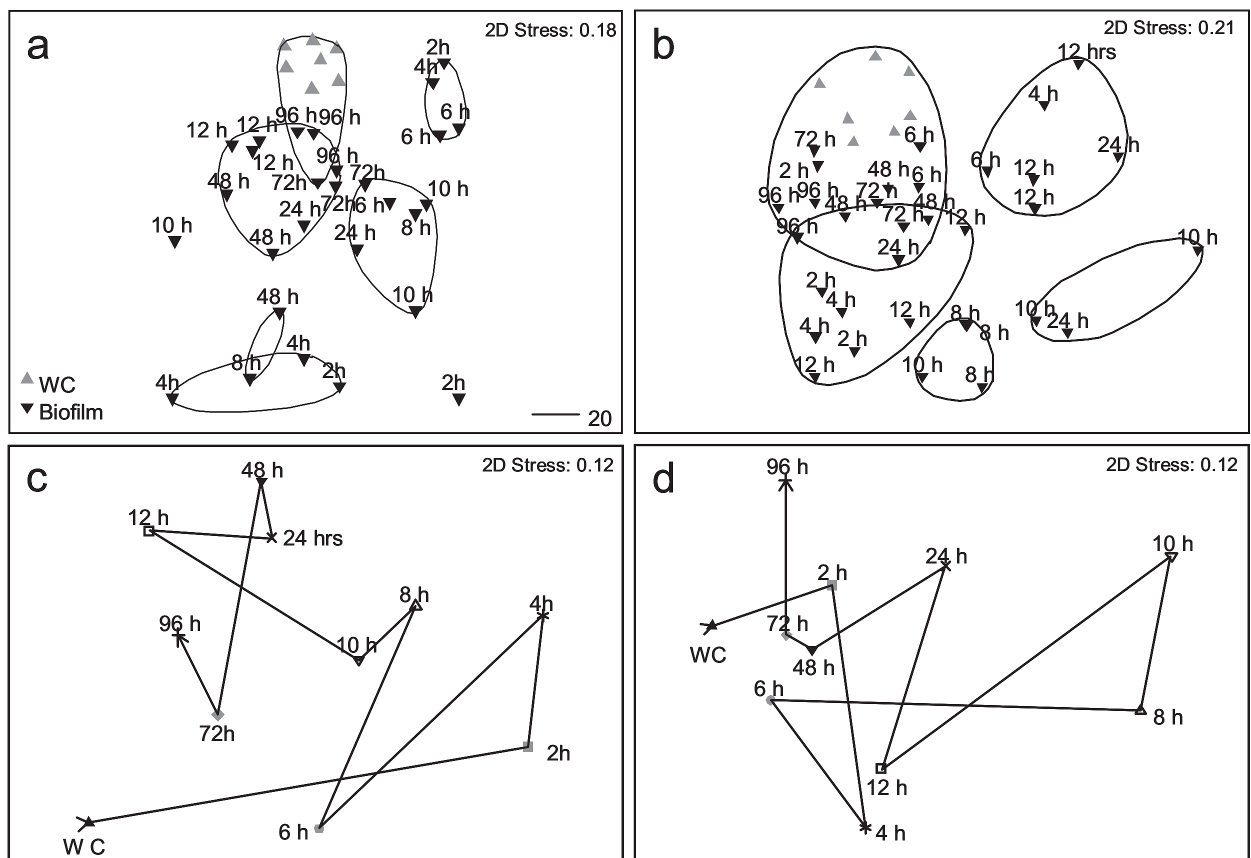


Figure 5.4. Multidimensional scaling plot (MDS) showing hourly changes in bacterial communities (16S rRNA gene fingerprints) settling on the biofilm of the replica coral nubbins enriched with agar (a) winter samples (August 2008) (b) summer samples (March 2009). Averages of time periods showing trajectory of similarity between time points (c) winter (d) summer. WC = water column.

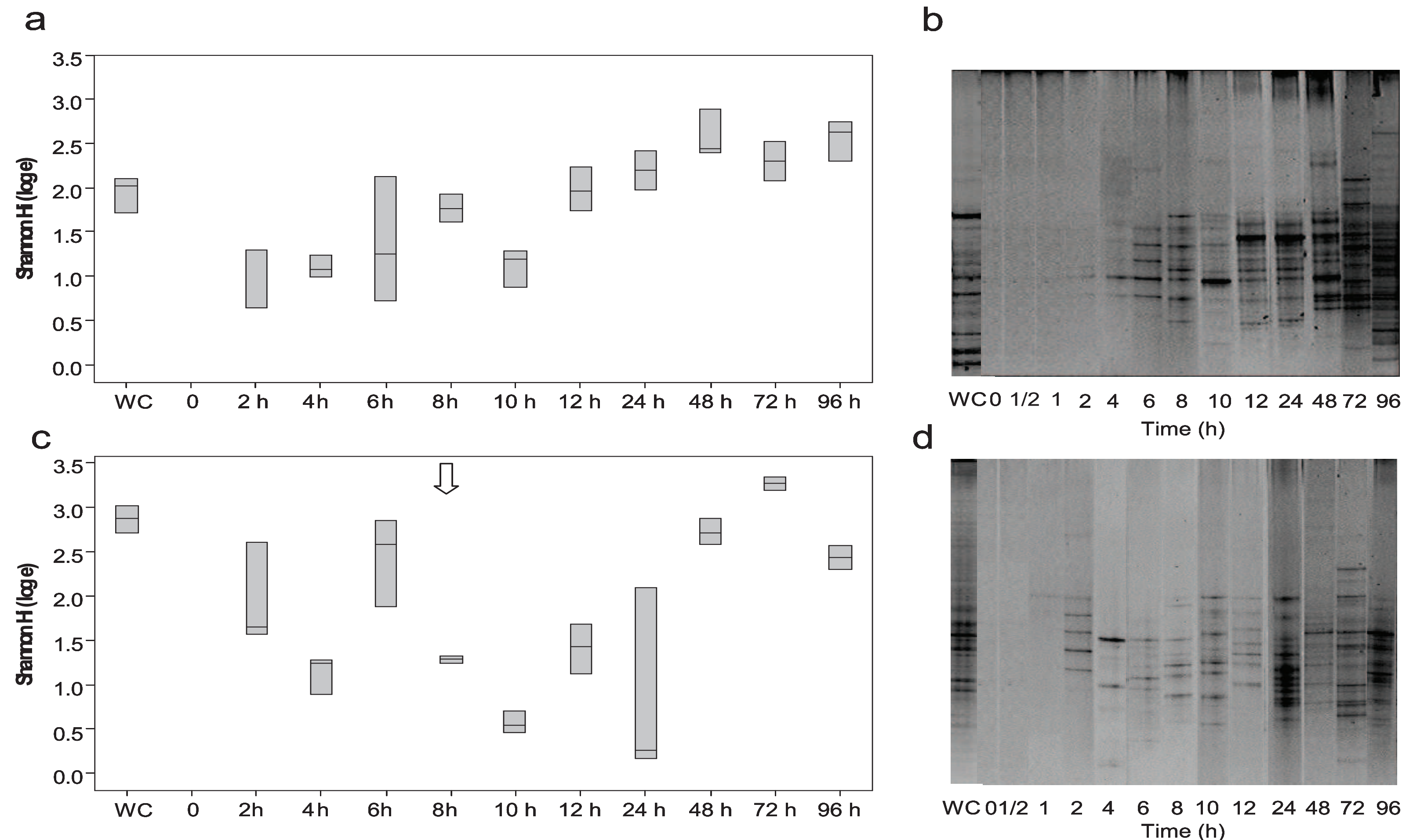


Figure 5.5. (a) Box-plots showing Shannon Weiner diversity index of the winter samples based on DGGE data, subset illustrated in (b). (c) Shannon Wiener diversity box plot based on DGGE data subset illustrated in (d). Arrow depicts storm event with increased chop (winds above 35 km/h). Average wind speed for other sample periods was below 20 km/h. WC = water column.

During winter, total ribotype (Shannon H^1) diversity reached that of the adjacent water column after 8 h with a sudden drop at 10 h during strong weather at the sample site. This recovered subsequently following a typical asymptotic settlement pattern thereafter (Fig. 5.5 a,b). In summer, there was no pattern in Shannon diversity, suggesting a more dynamic and less stable settlement period during this season (Fig. 5.5 c,d).

The dominant 16S rRNA gene ribotypes seen to be early colonisers between 2 – 4 h were absent or undetectable in the later biofilm established by 72 – 96 h (Table 5.2). A ribotype similar to *Vibrio* sp. (AB519004), present in the 2 h developing biofilm but absent by 6 h suggests that at least this species is an opportunistic bacterium colonising open spaces but later outcompeted by other species such as ribotypes similar to *Flavobacteria* sp. (FN 433284), *Glaciecola* sp. (EU183316), *Klebsiella* sp. (GQ416635), *Aestuariibacter* sp. (AB473549) and the cyanobacterium (GQ480703), all of which were found after 72 h of biofilm development. Only a ribotype similar to *Klebsiella* sp. (GQ416635) was consistently detected in both early and late colonising communities. Real–time PCR showed no significant differences (ANOVA $F = 3.43$, $p = 0.08$) between total vibrio DNA dominance between early (185.6 ± 58 fold *Vibrio* DNA template) and late coloniser (66 ± 28.3 fold *Vibrio* DNA template) communities. However, the mean was over double for early colonisers compared to later stages of biofilm development but the variation between replicates was high.

During both seasons, there were significant differences between the settling biofilms and the bacterial communities found within the water column. The bacterial 16S rRNA gene diversity of all but one of the time periods of biofilm development were significantly different from those of the water column ($R = 0.864$ $p < 0.05$), the only exception being 72 h in the summer season ($R = 0.255$ $p = 0.14$). This suggests that the 16S rRNA gene bacterial community settling on an artificial coral nubbin remains distinct from that of the supply within the timescale studied (Fig. 5.6 a,b).

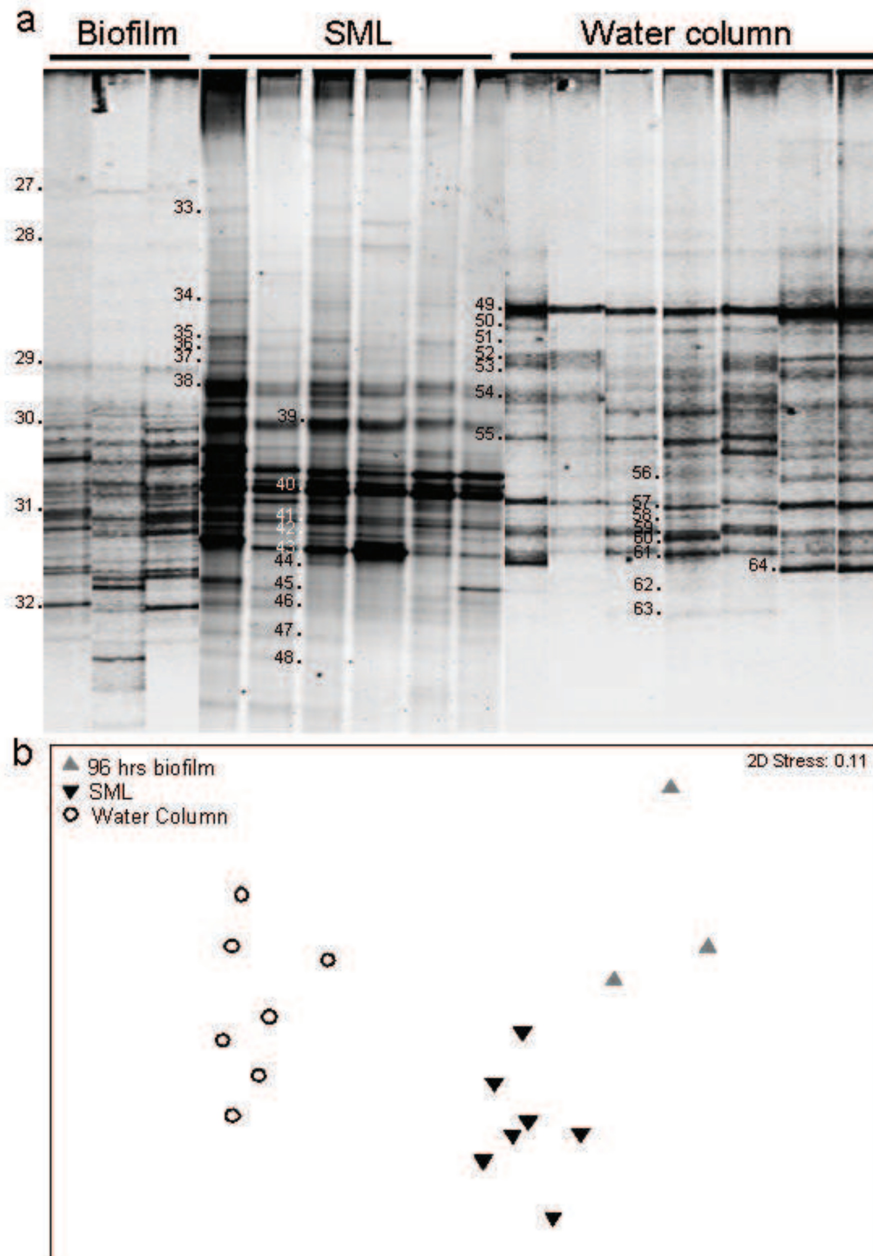


Figure 5.6. Variation in 16S rRNA gene fingerprints between sample types (Biofilm, SML and water column) for March 2009 (summer). (a) Composite DGGE image standardised for gel-to-gel comparison using BioNumerics. (b) Multidimensional scaling (MDS) plot based on relative band intensity from composite DGGE profile.

5.4.3 Does the surface mucus layer bacterial community represent a particular stage of biofilm development?

The turnover rate of coral mucus for *A. muricata* was recorded as 100 s cm^2 which shows the mucus layer of this coral species to be a highly motile and dynamic layer, with very rapid turn over rate clearing individual corallites of carbon particles within 100 s (Fig. 5.7).

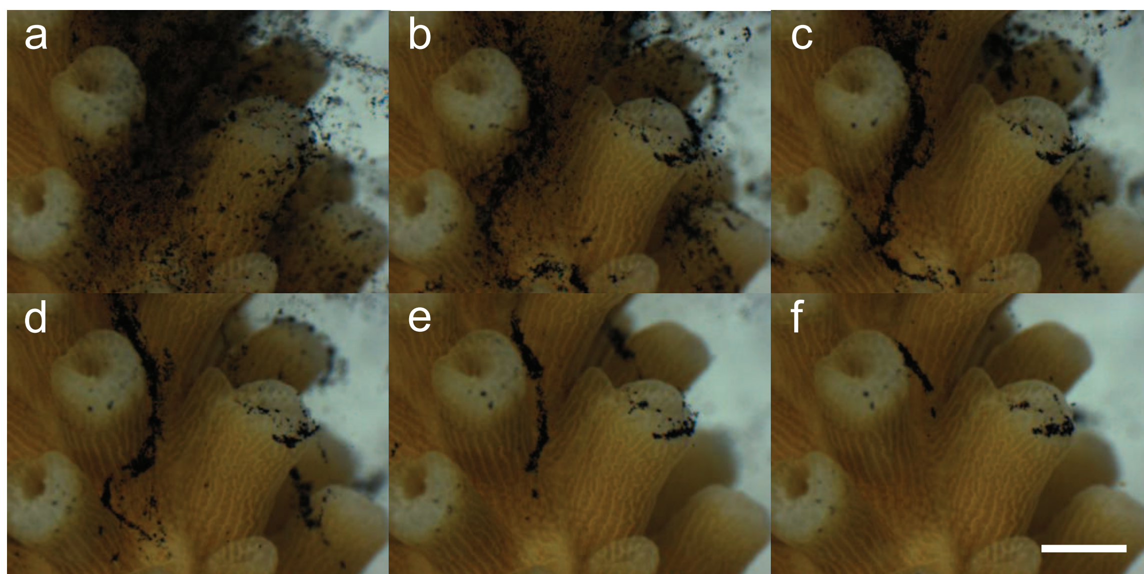


Figure 5.7. Photos showing the rate of surface mucus flow on *A. muricata*, using a novel method of carbon particles (a) first image when particles placed on coral (b) 22 s after (c) 30. 5 s (d) 38 s (e) 70 s (f) 100 s after placing particles Scale bar 10mm.

There were significant differences (ANOSIM, R = 0.5 p = 0.001) between the 16S rRNA gene diversity settling on a 96 h biofilm, those of the corals SML and those present within the water column (Fig. 5.6 a,b). The water column was heavily dominated by ribotypes from α-proteobacteria (FJ718457, GQ350573, GQ204865, EF092739, FJ620860, EU315614 and FJ532499), *Flavobacteria* (AB294989 and EU600663), and *Bacteroidetes* (EU315425, AB254287, DQ65619 and AM238600), whilst the settling community after 96 h was dominated by mostly γ-proteobacteria (GQ416635, EU183316, GU726846 and FJ237010) and cyanobacteria (GQ480703 and GU184683). In comparison the bacteria present in the SML were from a more diverse range of taxa (Table 5.2, Fig. 5.6 a), despite the presence of γ-proteobacteria (GQ471864, GQ471869, EU919217 and FJ887948) and cyanobacteria (GQ346809,

FJ967973 and FJ946590) like in the biofilm, there were no exact ribotype matches. The SML of *A. muricata* showed no significant differences in 16S rRNA gene composition (ANOSIM R = 0.569 p = 0.08) over 4 consecutive days of sampling (Fig. 5.8), further suggesting a stable bacterial community is present that remains distinct from that of the water column.

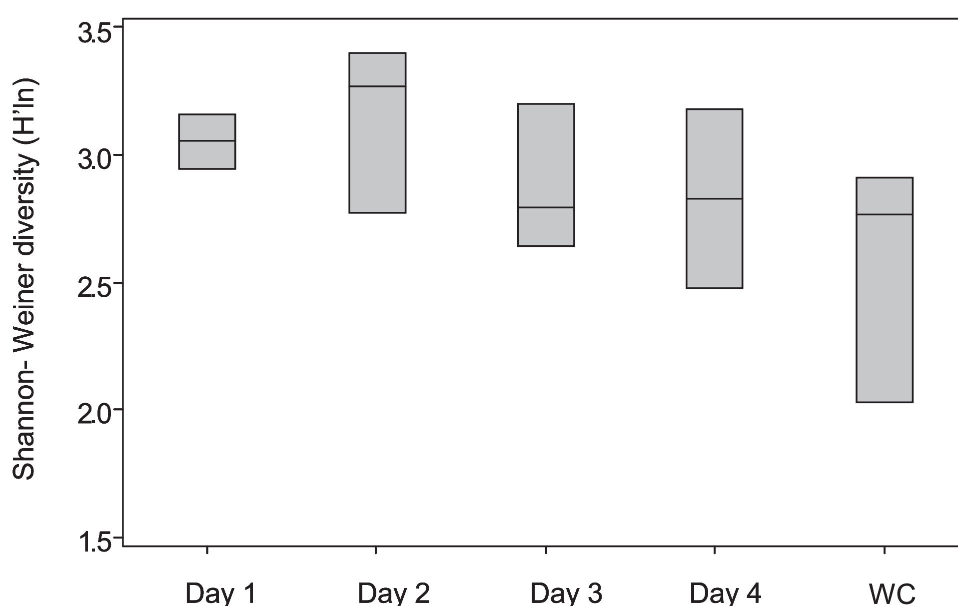


Figure 5.8. Box-plot showing Shannon Weiner diversity index of the SML samples of *Acropora muricata* taken over four consecutive days, based on DGGE 16S rRNA gene diversity compared to that of the water column (WC).

5.4.4 Spatial variability in biofilm bacterial communities

The DGGE profile of bacterial communities settling on agar coated nubbins after 24 h showed strong similarities in dominant 16S rRNA gene ribotypes between sites (Fig. 5.9 a). Significant differences were consistently shown between the water column and the 24 h replica coral nubbin biofilm at all sites (ANOSIM R = 0.874 p = 0.001) (Fig. 5.9 b). The MDS plot (Fig. 5.9 b) generated from the DGGE image shows orientation for both the water column spatial samples and the developing biofilm samples, all samples within each sample set are orientated close together with little variation, sharing 50 % (Bray Curtis) similarity of 16S rRNA gene profiles between samples. Significant differences were noted between sites for the 24 h replica coral nubbin biofilm (R = 0.389 p = 0.001), though pairwise tests revealed these differences only

between the reef flat and the Wistari reef system (Fig. 5.1b) (ANOSIM R = 0.667 p = 0.05). This was reflected in the differences between sites also seen within the water column (ANOSIM R = 0.142 p = 0.05), with pairwise differences between the reef flat and wistari (ANOSIM R = 0.307 p = 0.001). This suggests lagoon, off-reef and mixed (reef) sites have similar bacterial communities. This uniform lack of differences in the composition of both, the waterborne bacteria and those settling on the biofilms across sites, with differential levels of exposure to off-reef waters, further supports a lack of significant association between the benthic and waterborne bacterial communities. Dominant bands were excised from the developing biofilms at the five locations (Fig 5.9 a) and all samples were shown to be heavily dominated with ribotypes similar to *Pseudoalteromonas* sp. (FN295786, GU229650, GU726846, FJ457226 & FJ237010) and a ribotype similar to a *Chloroflexi* sp. (AB433054) (Band 16, Fig. 5.9 a).

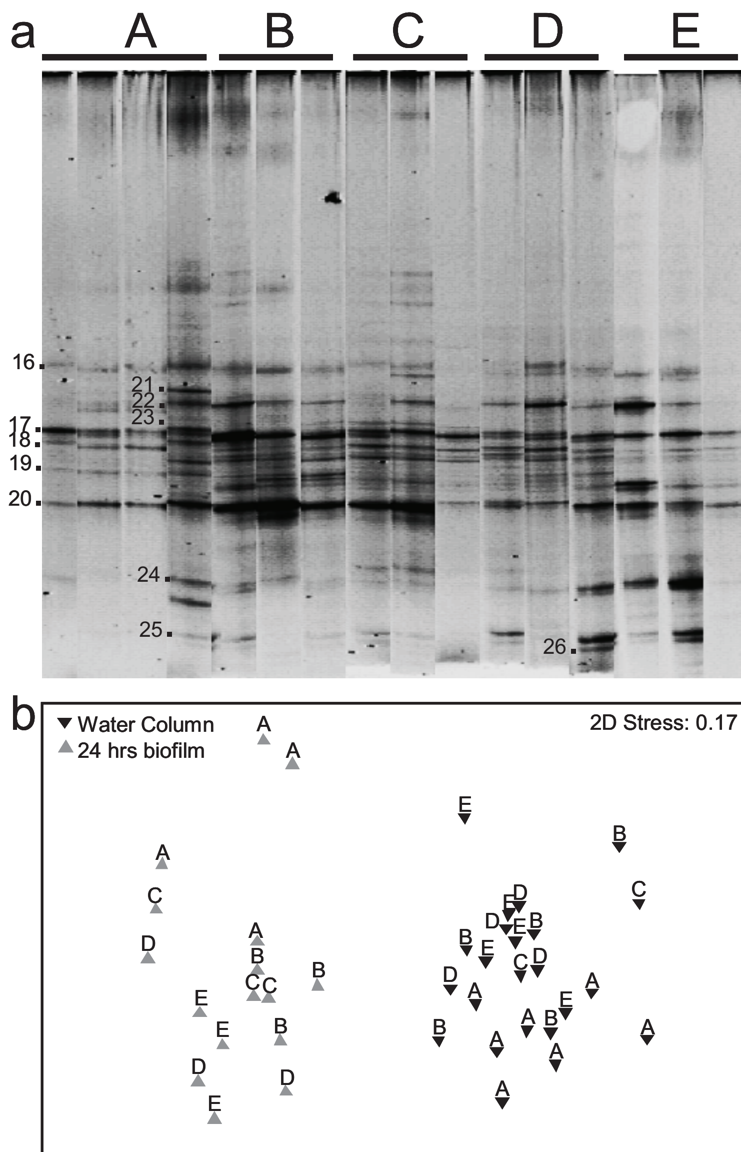


Figure 5.9. Variation in 16S rRNA gene fingerprints between sample types (spatial samples A - E) for March 2009 (summer). (a) Composite DGGE image representing the bacterial diversity settling on a 24 h biofilm on plain agar coated coral replicas, standardised for gel-to-gel comparison using BioNumerics. (b) Multidimensional scaling (MDS) plot based on relative band intensity from composite DGGE profile of the biofilm (grey triangles) and those from the water column (downward facing black triangles – DGGE image not shown in this case).

5.5 DISCUSSION

5.5.1 Biofilm formation on different substrate types

Although the C:N ratio was relatively constant between agar types (despite the addition of coral mucus derivatives), there was slight variation in the C and N content between the different agars. However, despite this, our results showed that the different agar types did not result in development of different bacterial biofilm communities. The addition of coral exudates had little effect on biofilm formation suggesting that at these early coloniser stages, differential growth has little effect on the community structure. What did affect the development of marine biofilm bacterial communities was the structure of the available settlement surface (coral nubbins versus microscopic slides). Thomason et al. (2002) found significant differences between bacterial communities settling on smooth and textured surfaces, with low dominance found on coarse surfaces and a higher dominance on smoother surfaces. In this study however, we found a greater diversity with higher dominance on the replica coral nubbins compared to the relatively smooth surface provided by the slides. The significant difference in bacterial diversity settling at the same time intervals on the coral replicas and the glass slides, can in part be explained by the variance in the surface energy available to the settling bacteria. The model coral surface in this case offers a higher heterogeneity compared to that offered by the microscope slide.

Ribotypes such as *Aeromonas* sp. (AY689043), *Prochlorococcus* sp. (GQ272346), *Shigella* sp. (FJ193359), *Pseudoalteromonas* sp. (GQ849227) and *Enterobacter* sp. (FN423410), all previously associated with coral tissue and reef systems (Dobretsov & Railkin 1996; Bourne 2005; Charpy 2005; Sussman et al. 2008) were found most commonly on the coral nubbin, whilst a ribotype similar to *Microbulbifer sp.* (EF674853) (Stevens et al. 2009) and several species of *Pseudoalteromonas* (EU330363, DQ665793 & FM163075), were found present on the slides but those specific ribotypes were largely absent from the nubbins. The results indicate that the physical structure of the substrate seems to be more important in producing differences within bacterial assemblages during biofilm formation than the chemical composition of that substrate. Thus, differences in corallite morphology among species and the surrounding substrate may help explain in part the development of

very specific resident bacterial communities for each coral species (Rohwer et al. 2002).

5.5.2 Ecological succession of biofilm formation

Succession of bacterial communities in biofilms has been described before (Martiny et al. 2003; Lyautey et al. 2005; Siboni et al. 2007), however the exact time frames for settlement of pioneer groups and subsequent recruitment by others is less well understood, due to studies investigating settlement at >1 d intervals (Jones et al. 2007, Dang et al. 2008). Some studies have looked at shorter timescales, with Siboni et al (2007) reporting presence of bacteria on surfaces after 2 h, which is reflected by our results, where samples taken at 30 min and 1 h after deployment failed to show any community on the DGGE. Several studies showed pioneer communities consisting of mainly γ-proteobacteria (*Pseudomonas*, *Actinetobacteria* and *Alteromonas*), developing between 0 – 9 h and a more established biofilm developing after 24 h with α-proteobacteria being the dominant group (Dang & Lovell 2000; Jones et al. 2007; Dang et al. 2008; Lee et al. 2008). Our results suggest that γ-proteobacteria are the dominant group of early settlers (<24 h), however the later shift to α-proteobacteria seen by these previous studies was not detected here (Dang & Lovell 2000, 2002; Jones et al. 2007). In addition, in this study we did not see an asymptotic maximum diversity reached within 96 h compared to the maximum reached within 36 h in the study by Lee et al. (2008). The bacterial community developing on the biofilm in this case at least, may not have reached a stable equilibrium.

Seasonality undoubtedly has an important influence on the construction of biofilms as seen by the significant differences between summer and winter samples, a result seen in other systems (Bengtsson et al. 2010). However, seasonal changes include several different factors that can affect microbial biofilm development, for example the chemical composition of the corals SML, exudation of substances (antimicrobials) from the corals themselves and the activity of grazers on the biofilms. Along with abiotic factors such as temperature (White et al. 1991), wave action, light conditions, and seawater nutrient levels, all of which will likely play an important role in microbial development (Rao 2010).

5.5.3 Does the surface mucus layer bacterial community represent a particular stage of biofilm development?

Previous studies have shown clear differences between free-living bacteria and those developing on biofilms (Dunne. Jr. 2002; Lee et al. 2003; Jones et al. 2007; Lee et al. 2008; Bengtsson et al. 2010), however these studies have demonstrated that initial biofilm formation is from the attachment of specific groups of these free-living bacteria found originally within the water column (Jones et al. 2007). In this study, the bacterial diversity of the developing biofilms remained clearly different from that of the potential supply from the water column, even in the earliest stages of development (~2 h). The water column was dominated by α-proteobacteria, Flavobacteria and Bacteroidetes compared to that of the settling community on the biofilms being largely γ-proteobacteria, whilst those present within the SML were from a more diverse group. This suggests that despite the high turnover rate of the SML, the bacterial community present within this layer does not arise from simple passive settlement from the water column or from that of a static natural biofilm suggesting the holobiont is controlling the distinct bacterial diversity present. We originally hypothesised that the bacterial community would initially be more similar to the water column, driven by passive, non-selective settlement, but would become progressively more dissimilar as selection and growth of the biofilm community occurred. However, settling biofilm bacteria must be recruited from the onset, from less abundant populations in the water column, through selective processes or via transmission of bacteria by direct contact with other surfaces. These bacteria may then undergo rapid growth (with the availability of the additional food source), and therefore become the dominant group detected on the biofilm. Due to limitations in the resolution of the DGGE technique, rare populations in the water column are not readily detected. Therefore, this makes it difficult to correlate fluctuations in the water column bacterial communities with those in the developing biofilms (Bengtsson et al. 2010).

The difference in settling bacterial communities on any surface can be explained in part by the fact that some marine macro-organisms (like corals), combat microbial fouling by producing compounds that inhibit bacterial growth or attachment, while others rely on microbial production of defence compounds (Ritchie 2006). In addition

even on inert objects like the models used in this study, commensal relationships (bacteria-bacteria interactions) in a multi species biofilm, can play an important role in determining the spatial distribution of microbial populations within such a developing biofilm (Ritchie 2006). Bacteria such as *Alteromonadales,* in particular *Pseudoalteromonas* sp., like those found predominantly as settlers in this study have previously been shown (Nair & Simidu 1987; Long & Azam 2001; Dheilly et al. 2010; Rypien et al. 2010), to be highly antagonistic both at normal and elevated temperatures and will actively inhibit other species from settling or establishing. *Pseudoalteromonas* strains can therefore predominate over other bacterial strains such as potentially pathogenic *Vibrio* sp. (Dheilly et al. 2010). They produce a variety of biologically active extracellular compounds, including antibacterial agents that ultimately lead to antifouling effects (Bowman 2007; Hayashida-Soiza et al. 2008). Interestingly some γ-proteobacteria have also been shown to be specific with their antagonistic behaviour, inhibiting only other α- proteobacteria from growing (Rypien et al. 2010).

Rypien et al. (2010) found that pathogenic vibrios, in particular *V. shiloi* and *V. coralliilyticus,* are usually inhibited by other coral-associated bacteria found in healthy coral samples. During periods of stress, these natural inhibitors are reduced in number and less able to inhibit the potentially pathogenic vibrios, allowing these pathogenic bacteria to overwhelm and cause disease (Rypien et al. 2010). Although qPCR showed no significant difference in total vibrio numbers from early to late colonisers, one *Vibrio* species (AB519004) was shown to be an early coloniser and was absent in later stages of the biofilm development. This suggests that it was out competed by more dominant species such as ribotypes similar to a *Flavobacteria sp.* (FN433284), *Glaciecola sp.* (EU183316), *Aestuariibacter* sp. (AB473549) along with a cyanobacterium (GQ480703). The only ribotype found consistently between the biofilms and the SML was a species similar to *Klebsiella* sp. (GQ416635).

5.5.4 Spatial variability in biofilm bacterial communities

No significant differences were detected between sites, either in the water column or the developing biofilm. This shows that either the use of agar as a settling medium provided a settlement surface favoured by only certain species, or the selection and

developmental processes affecting the biofilm community were strongly internally controlled by actions such as bacterial species-species associations. Instead of early colonisers *per se*, influencing the final community (Dang & Lovell 2000; Martiny et al. 2003; Jones et al. 2007; Lee et al. 2008), the importance of particular settlers like the highly antagonistic *Pseudoalteromonas* sp. may control the final community no matter when they settle during succession.

In conclusion, the settling bacterial community found on biofilms remains distinct from that of the potential supply (i.e. the water column) and the bacterial communities present within the SML. Surface structure, but not material composition significantly affects the final bacterial community assemblages. Therefore future work looking at biofilms should carefully consider surface properties. The seasonal difference reported here suggests biofilm development varies from summer to winter months, reflected but not consistent with the difference in bacterial communities found within the water column between seasons (Chapter 3; Sweet et al. 2010a).

Chapter 6

Investigation of the controls on bacterial community development in the reef coral *Acropora muricata* using experimental antibiotic treatment

6.1 ABSTRACT:

Development of the bacterial (16S rRNA gene) community associated with the coral *Acropora muricata* was monitored over time following experimental modification of the existing microbial community using the antibiotic ciprofloxacin. Abundance of bacteria was reduced >99 % by the treatment, resulting in significant changes in bacterial community structure. Following redeployment to their natural environment, some settlement and re-growth of bacteria took place within a few hours, including ribosomal types that were not present, or in low abundance, in the natural microbiota. However, complete recovery of the bacterial community required longer than 96 h, which indicates a relatively slow turnover of the natural community. The early developing community was dominated by antibiotic-resistant bacteria from the natural microbiota that survived the treatment and proliferated in the absence of natural competitors, but also included some non-resident ribotypes colonising from the water column. Almost all these opportunists were significantly reduced or eliminated within 96 h after treatment, demonstrating a high resilience in the natural bacterial community. Potential pathogens including a *Clostridium* sp. inhabited the coral at low abundances, only becoming prevalent when the natural microbiota was disturbed by the treatment. The healthy coral-associated microbiota appears to be strongly controlled by microbial interactions.

6.2 INTRODUCTION

During the past few decades there have been increasing reports of the detrimental effects of coral diseases in reef ecosystems. Epizootic events have reduced important reef-building coral populations across regions, representing a rapid deterioration in reef health over large spatial scales (Brandt & McManus 2009; Teplitski & Ritchie 2009). In fact, emerging coral diseases are considered one of the major threats for coral reef ecosystems worldwide in view of their wide distribution, host range and their high levels of prevalence; which have been correlated to increasing environmental stress on coral hosts and their prokaryotic and eukaryotic partners (Lecampionalsumard et al. 1995; Rosenberg et al. 2007).

Corals have a variety of mechanisms of defence against invasive pathogens. The production of mucus is thought to be the first line of defence, acting as a physical barrier protecting the epithelium (Brown & Bythell 2005). Another key defence mechanism is the production of antimicrobial compounds, this process being mediated by their natural microbial community and perhaps by the coral itself (Gunthorpe & Cameron 1990; Kim 1994; Kim et al. 2000; Rohwer & Kelley 2004; Ritchie 2006; Geffen et al. 2009; Rypien et al. 2010). In fact, 20–30% of bacterial isolates from coral species have been shown to possess antibiotic activity (Castillo et al. 2001; Ritchie 2006). Experiments conducted with coral pathogens *Vibrio shiloi* and *V. coralliilyticus* have shown that in order to cause tissue mortality, these pathogens need first to adhere to the coral's surface, penetrate it, and then reproduce within the host (Toren et al. 1998; Kushmaro et al. 2001; Ben-Haim & Rosenberg 2002). Thus, evidence suggests that the coral pathogen must 'break down' the natural coral defence mechanisms (Kushmaro et al. 1998). If the bacterial community associated with the coral is the primary source of this defence via antibiotic production, then a disturbance of the healthy coral microbiota may allow opportunistic infection (Lesser et al. 2007). Ritchie (2006) showed that bacteria associated with corals in a healthy state have greater antibiotic activity compared to those associated with stressed/diseased coral, thus the controls on development and maintenance of the natural microbiota may be important for coral health. Because the magnitude and frequency of stress on coral reef organisms are expected to increase in the future (Hoegh-Guldberg 1999), coral health might rely on how quickly their bacterial

communities recover from disturbance and the stability and resilience of the natural community.

Apprill et al. (2009) hypothesised that the primary method of acquisition of microbial associates by the coral was phagocytosis by the ectoderm, which is in closest contact with the seawater microbial community, a method utilised by other invertebrates (Foster & McFall-Ngai 1998; Nussbaumer et al. 2006). In contrast, in corals the most common mode for acquiring symbiotic algae is via phagocytosis by the gastroderm, where they then avoid digestion by preventing maturation of the phagosome (Marlow & Martindale 2007). Although both mechanisms may play a role in bacterial acquisition, Apprill et al. (2009) found no evidence of bacterial cells concentrated within the gastrodermal cavity of 76 h old coral planulae of the coral *Pocillopora meandrina*. In contrast, Ainsworth & Hoegh-Guldberg (2009) showed that compartmentalised bacterial colonies of γ-proteobacteria inhabited the gastrodermis of healthy adult corals, perhaps representing the host-species specific bacterial associates of corals found in several previous studies (Rohwer et al. 2002; Rohwer & Kelly 2004; Klaus et al. 2005). The ectodermal tissues were apparently devoid of bacteria, except when the host coral was stressed, after which extensive bacterial colonisation took place across both the cell layers (Ainsworth & Hoegh-Guldberg 2009). These studies therefore raise the question of where the coral-associated microbiota is situated and how it is maintained. If so few bacteria are normally associated with the coral tissues, the majority must be resident in the coral surface mucus layer (SML) (Guppy & Bythell 2006; Kooperman et al. 2007; Sweet et al. 2010b; Chapter 4) or the underlying skeleton (Shasher et al. 1997; Fine et al. 2005). The rate and mechanism of turnover of the SML is largely unknown (Chapter 5; Brown & Bythell 2005), but the bacterial communities of this layer may be expected to be much more transient than tissue-associated bacteria, as well as more strongly influenced by passive settlement from the water column (Sweet et al. 2010a; Chapter 3).

The importance of a natural healthy microbial community and subsequent microbial balance is readily appreciated when considering some of the deleterious results of antibiotic treatment in other biological systems. Several studies have shown the adverse effects of disturbing established microbial communities in a variety of

systems but most notably in the human gut (Jernberg et al. 2007; Sekirov et al. 2008; Yap et al. 2008; Croswell et al. 2009). The effects of such a disturbance can cause prolonged (up to weeks in the human gut) disruption in otherwise stable functional group composition (Croswell et al. 2009; Sekirov et al. 2010) which have been linked to several human pathologies (Turnbaugh et al. 2006; Frank et al. 2007; Liu et al. 2007; Penders et al. 2007). For most of these associations, however, it is not clear whether the microbial imbalance is a cause or an effect of the pathology. Diseases such as vaginal candidiasis and *Clostridium difficile* colitis frequently occur following a course of antibiotic therapy (Crogan & Evans 2007; Sobel 2007), which favours the hypothesis that the microbial community shift precedes the onset of the pathology. However, the generality of these processes in respect to other ecological systems is unknown.

The ability of the coral or its associated microbes to be resilient to environmental stress (i.e. the ability of the microbial associates to return to their original community composition after disturbance) may have important implications in understanding disease dynamics and mechanisms of pathology in the coral (Bourne et al. 2009). The coral-algal symbiosis has been shown to be resilient, in that corals may be able to acquire more stress tolerant clades during periods of heightened temperatures, but return to their previous clade composition after the event (Rowan et al. 2004). Garren et al. (2009) similarly showed that the bacterial communities of corals exposed to fish farm effluents, took up the dominant bacteria present within the water column over the first 5 d, however within 22 d the original community had returned, suggesting that coral-microbial associates are highly resilient, despite changing environmental conditions. Whether the coral itself, its microbial associates or some combination of the two, is responsible for restoring the bacterial community as well as the effect of the frequency of disturbance remains to be understood. In this study, we used a broad-spectrum antibiotic, ciprofloxacin, in a 6 d treatment to reduce the natural microbiota associated with the reef building coral *A. muricata*, and followed the community succession over time using culture-independent 16S rRNA gene techniques from 0 to 96 h after cessation of antibiotic treatment, and return of the corals to their natural environment on the reef.

6.3 MATERIALS AND METHODS

6.3.1 Sample collection and experimental design:

Nubbins of ca 38 mm were collected from a single coral colony to avoid within-species variability reported in coral-associated bacterial communities (Kvennefors et al. 2010). After collection, they were placed in a holding tank for 1 day prior to treatment with antibiotics to allow for acclimatisation. The nubbins were mounted using marine putty onto threaded polypropylene sleeves and screwed into place on perspex sheets. Three nubbins were used as controls at time of collection and another 3 were taken before antibiotic treatment after acclimatisation to allow for any shift in bacterial community dynamics as a result of handling. The corals were placed in a sterile, purpose-built dosing tank, containing 7 l of filtered sea water (0.22 µm sterivex filter) twice daily (07:00 am and 19:00 pm) for 2 h periods and the antibiotic Ciprofloxacin was administered at 40 µg / ml. The corals were then held in a '14 l wash' tank with filtered water and a sterile aquarium pump to provide aeration and flow. The filtered water in the wash tank was changed three times over the six days of dosing. The broad spectrum antibiotic, Ciprofloxacin belonging to the group Fluoroquinolones, was chosen because of its generic bactericidal properties. Its mode of action depends upon blocking bacterial DNA replication by binding itself to the enzyme DNA gyrase and inhibiting the unwinding of bacterial chromosomal DNA during and after replication. It would therefore likely target a wide range of coral associates and unlikely have toxic effects on the host.

After completion of the 6 d antibiotic treatment, three nubbins were sampled (T = 0), then the remainder were returned to the reef prior to collection at 30 min, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. Each set (n = 3 per time period) of samples were stored in 8 ml EtOH at -20 º C until DNA extraction and analyses. In addition to these samples, sets of n = 2 nubbins were collected at each sampling time to be embedded into Technovit resin on site for histology. During the experiment, temperature was controlled and kept similar to field conditions and monitored using Hobo ® data loggers deployed in the tank and on the reef. Pulse Amplitude Modulation (PAM) measurement of dark-adapted yield (photosynthetic performance) of the symbiotic algae was also performed as a proxy of coral health, both in the field and before and

after the use of the antibiotic to assess any effects of antibiotic treatment on the coral. Water column samples collected alongside (Chapter 3; Sweet et al 2010a) were compared directly with the coral samples to show similarities and differences between bacterial settlement and potential supply from the water column.

6.3.2 Changes in total abundance of bacteria: Fluorescence *in situ* hybridisation:

For each period of time, the total abundance of bacteria was estimated by using fluorescent *in situ* hybridisation (FISH). Samples were fixed, stained and sectioned following the protocols in Bythell et al. (2002), with the addition of an equimolar mix (EUBMIX). Oligonucleotide probes were purchased from Interactiva (http://www.interactiva.de) with an aminolink C6/MMT at the 5' end. Four probes were used: the 'universal' eubacterial probes EUB338 (5'-GCT GCC TCC CGT AGG AGT-3'), EUB338-II (5'-GCA GCC ACC CGT AGG TGT-3'), EUB338-III (5'-GCT GCC ACC CGT AGG TGT-3') and the 'non-sense probe' NONEUB (5'-ACT CCT ACG GGA GGC AGC-3'), which has the complementary sequence to EUB338 and was used to determine non-specific binding of EUB338. The three eubacterial probes were used in an equimolar mix (EUBMIX) and the NONEUB probe was used singly.

Sections were viewed under epiflourescence microscopy with an FITC-specific filter block (Nikon UK Ltd, Surrey, UK) and images recorded using an integrating camera (Model JVC KY-SSSB: Foster Findlay and Associates, Newcastle upon Tyne, UK). To count bacteria an automatic cell counter (Cell C, Selinummi et al. 2005) set up to detect bacteria (0.0314 – 0.7 μm^2) was used. Counts on 50 fields of view (FOV) were taken, scaled up to the total area of the coral nubbin and calculated to give an estimation of total bacterial abundance per cm^2 of coral surface.

6.3.3 Changes in bacterial communities: 16S rRNA gene extraction and amplification:

All coral samples were crushed using sterile, autoclaved pestle and mortar and extracted using the QIAGEN DNeasy Blood and Tissue Kit; Spin column protocol. Bacterial 16S rRNA was amplified using standard prokaryotic primers (357F) **(**5'-

CCTACGGGAGGCAGCAG-3’) and (518R) (5’-ATTACCGCGGCTGCTGG-3’). The GC – rich sequence 5’-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA GCA CGG GGG G-3’ was incorporated in the forward primer 357 at its 5’ end to prevent complete disassociation of the DNA fragments during DGGE. PCR reaction mixture and programme was performed as in (Chapter 1). All reactions were performed using a Hybaid PCR Express thermal cycler. PCR products were analysed by agarose gel electrophoresis [1.6 % (w/v) agarose] with ethidium bromide staining and visualised using a UV transilluminator.

6.3.4 DGGE analysis:

DGGE was performed using the D-Code universal mutation detection system (Bio-Rad) as in Chapter 3. PCR products were resolved on 10 % (w/v) polyacrylamide gels that contained a 30 – 60 % denaturant gradient for 13 h at 60°C and a constant voltage of 50 V. Gels were stained with 9 µl Sybr Gold (Sigma) in 50µl of TAE for 20 min then washed in 500 ml 1X TAE for a further 30 min then visualised using a UV transilluminator.

6.3.5 Clone Libraries and ARDRA screening:

Almost-complete 16S rRNA gene fragments were amplified from the DNA extracted using the universal eubacterial 16S rRNA gene primers pA, (5’-AGA GTT TGA TCG TGG CTC AG-3’) and pH, (5’-AAG GAG GTG ATC CAG CCG CA-3’). The amplified products were purified using the Qiagen PCR purification kit, inserted into the pGEM-T vector system (Promega) and transformed into *Escherichia coli* JM109 cells. A total of 192 clones containing the 16S rRNA gene inserts were selected from each sample (n = 1; control, t = 30 min, 4 h and 96 h), and boiled lysates were prepared from each by mixing a picked clone in 30 µl of TE and boiled for 3 min followed by freezing. Each lysate (1 µl) was amplified using the primers pUCF (5’-CTA AAA CGA CGG CCA GT-3’) and pUCR (5’-CAG GAA ACA GCT ATG AC-3’); the products were digested with the restriction enzymes Hae111 and Rsa1 (Promega) (4 µg of PCR product, 2 µl of restriction buffer, 0.2 µl of BSA, 0.07 µl of Hae111, 0.1 µl of Rsa1 and made up to 20 µl with sigma water for 2 h at 37 ºC then 10 min at 67 ºC). Restriction fragments were resolved by 3 % agarose gel

electrophoresis, visualised using a UV transilluminator and grouped based on restriction patterns. Representatives from each group were sequenced.

6.3.6 Statistical analysis:

Image processing, gel-to-gel standardisation and band-matching of DGGE image profiles using BioNumerics allows comparison between different environmental samples, however the process does not detect all bands visible on the gel, hence underestimating amplicon diversity. To overcome this, both the DGGE profiles (corrected for gel-to-gel variations) and the multidimentional scaling (MDS) analysis of the BioNumerics band intensity data have been shown. The abundance of bacteria (total FISH counts) was compared across time periods with a one-way analysis of variance because data was normally distributed and variances were equal. A one way analysis of similarity (ANOSIM) was performed on untreated coral nubbins present within the holding tank after handling and those direct from the field to test for effects of handling. A one-way permutation analysis of variance (PERMANOVA, Anderson 2001) based on Bray-Curtis similarities was performed to test differences between the bacterial 16S rRNA gene assemblages associated with the antibiotic treated corals. Pair-wise comparisons based on permutation were conducted to test differences among each combination of time period after treatment (Anderson 2001). This approach was used because multivariate data was not normal but variances were still equal. A non metric multidimensional scaling (NMDS) was used to represent six time intervals after antibiotic exposure on a 2-D plot. A similarity of profile analysis (SIMPROF, Clark and Warwick 2001) was performed to determine true clusters which were then overlaid upon the NMDS (Clarke and Warwick 2001). An analysis of contribution to similarities (SIMPER) was performed to determine which 16S rRNA gene ribotype best explained dissimilarities among sample types that were statistically different.

6.4 RESULTS

6.4.1 Effects of antibiotics on total coral bacterial abundance:

There was a significant (99.97 %) reduction in abundance of bacteria between control samples and those immediately following antibiotic treatment 'T = 0' (ANOVA df = 9, F = 428.5 p = 0.001), demonstrating effectiveness of the antibiotic treatment. There was no significant trend over time in dark-adapted yield (Fv/Fm) of the symbiotic algae, which was used as a proxy for coral health in this case (Regression ANOVA. df = 34, F = 0.70, p = 0.409, Fv/Fm ranging from 0.6-0.7, see Fig. 6.1) and no visible change in appearance or polyp expansion of the treated corals, indicating that there was little or no adverse affect of the treatment on the host coral.

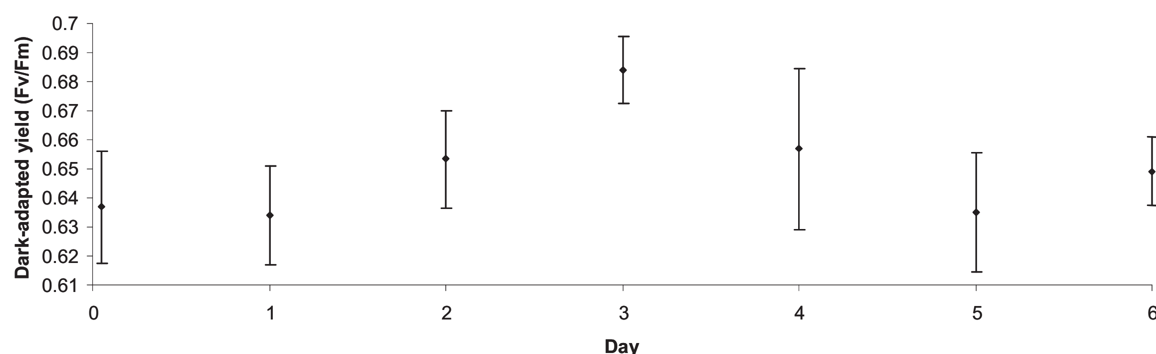


Figure 6.1. Dark-adapted photosynthetic yields (Fv/Fm) obtained using pulse amplitude modulated (PAM) fluorometry. Used as a measure to detect sub-lethal physiological changes in symbiotic algae of the coral during antibiotic treatment. No significant changes were detected over the course of the experiment (Regression ANOVA. df = 34, F = 0.70, p = 0.409).

Mean bacterial abundance on healthy corals collected from the field was $8.1 \pm 0.2 \times 10^8$ cells cm^{-2} (± SD), which was reduced to $2.6 \pm 0.6 \times 10^5$ cells cm^{-2} (± SD) immediately after treatment. Bacterial populations started to recover within 12 h (Fig. 6.2). Although total bacterial abundance tended to recover over time, after 96 h of redeployment, corals had not fully regained the pre-treatment bacterial abundance (Tukey's $p < 0.05$). Thus in this case, recovery of the coral to its normal bacterial population densities would take over 4 days.

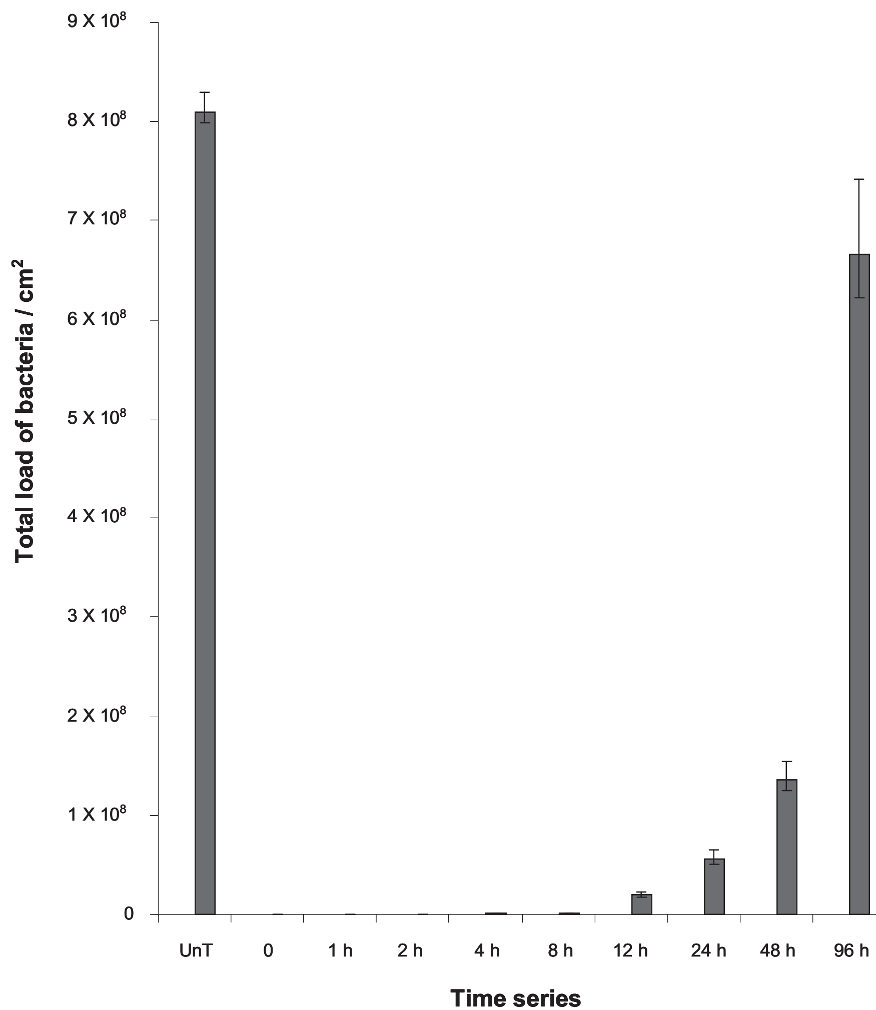


Figure 6.2. Mean bacterial abundance (cm^{-2}) of n = 3 replicates of resin-embedded corals taken at all time periods. UnT = untreated coral nubbins. Error bars represent standard errors.

6.4.2 Effects of antibiotics on 16S rRNA gene bacterial diversity:

DGGE profiles changed significantly immediately following antibiotic administration (PERMANOVA, f = 3.41, df = 6, p = 0.001, Fig 6.3), with no significant effects of nubbin handling without antibiotic treatment (ANOSIM, p = 0.81). As there was no significant difference in bacterial diversity between the field samples and the handling controls, all comparisons were made between antibiotic treatments and the field collected samples which are therefore termed ‘healthy’ samples throughout. Over 55 % of the variability recorded in bacterial assemblages between different samples was explained by temporal changes after ciprofloxacin treatment, while 45 % was

related to variability among replicates. This indicates that responses and recovery of bacterial communities to antibiotic exposure was highly variable between nubbins of a single coral colony. Post hoc comparisons showed significant differences in bacterial assemblages between each time period, the only exception being found for the 8-12 h period which showed high levels of variation between replicates (Table 6.1).

Table 6.1. Pairwise tests of PERMANOVA showing differences in bacterial 16S rRNA gene diversity between untreated (healthy) corals (C) and antibiotic treated coral samples. 0 = directly after treatment with ciprofloxacin.

Time	t	P(perm)	perms
C, 0	2.1886	0.009	126
C, up to 4 h	1.8993	0.01	400
C, 8 to 12 h	1.2315	0.1568	418
C, 1 d	1.9665	0.027	56
C, 2 d	1.6928	0.045	21
C, 4 d	2.0862	0.0579	21
0, up to 4 h	1.9246	0.006	209
0, 8 to 12 h	2.2256	0.006	208
0, 1 d	2.3208	0.03	35
0, 2 d	2.2848	0.006	21
0, 4 d	2.7493	0.006	21
up to 4 h, 8 to 12 h	1.8751	0.003	411
up to 4 h, 1 d	2.0706	0.015	84
up to 4 h, 2 d	1.5892	0.04	28
up to 4 h, 4 d	2.0514	0.035	28
8 to 12 h, 1 d	1.4306	0.038	84
8 to 12 h, 2 d	1.2241	0.2118	28
8 to 12 h, 4 d	1.4318	0.0699	28
1 d, 2 d	1.3478	0.042	84
1 d, 4 d	1.9227	0.038	84
2 d, 4 d	1.5953	0.021	84

DGGE analysis showed that not all bacteria were eliminated after antibiotic treatment, as several bands were still present in the gel immediately after treatment, although overall ribotype richness (S) was greatly reduced (Fig. 6.3). This indicates that some bacteria present in corals have resistance to ciprofloxacin. Patterns of DGGE banding (Fig. 6.3) revealed a diverse microbial community associated with control samples, this microbial assemblage becoming significantly less diverse after treatment

(PERMANOVA, t = 2.19, p = 0.009 Fig. 6.3 and 6.4 a, Table 6.1) and the number of bands shown in the DGGE increased again following treatment when nubbins were deployed back into their natural habitat.

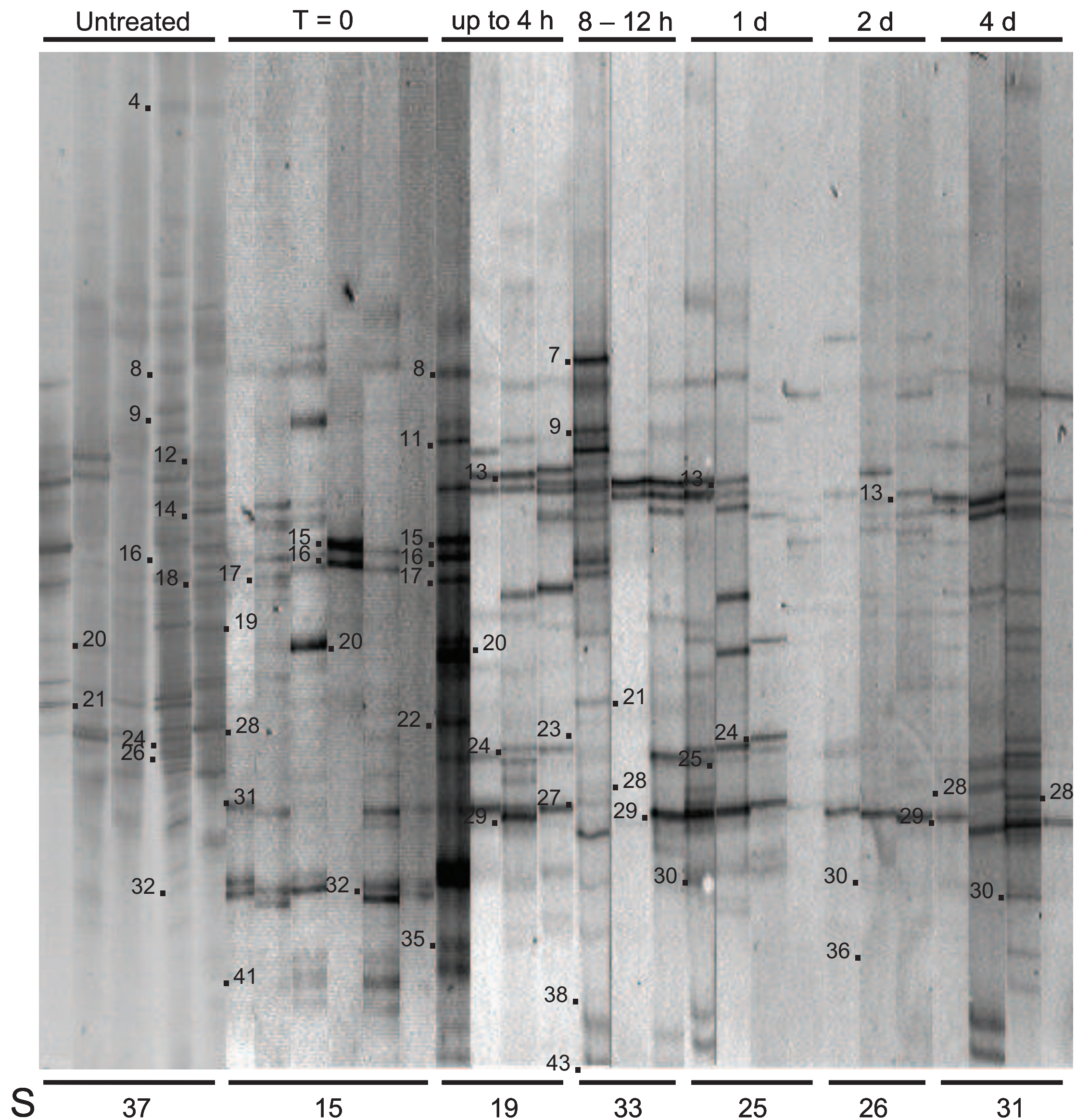


Figure 6.3. 16S rRNA gene fingerprints (DGGE) of coral samples in relation to time following treatment and pre-treatment controls. Composite DGGE image standardised for gel-to-gel comparison using BioNumerics. S value represents total number of bands detected by BioNumerics within the average of sample replicates.

Sequences identified from dominant bands of the DGGE included ribotypes similar to *Sphingobacterium* sp. (AF2600710), *Endozoicomonas* sp. (FJ202766) and *Hydrogenophaga* sp. (FM955626) that were conspicuous on control nubbins but

disappeared following treatment, indicating a high susceptibility of these bacteria to ciprofloxacin (Fig. 6.4 b-d, Table 6.2). While *Sphingobacterium* sp. (AF 2600710) recovered quickly from the antibiotic effect (8-12 h), *Endozoicomonas* sp. (FJ202766) and *Hydrogenophaga* sp. (FM 955626), only recovered after 2-4 days (Fig. 6.4 b-d, Table 6.3). Ribotypes from the γ and α proteobacteria appeared to be primary colonisers, becoming more abundant between 0-4 h after treatment; however, the former remained up to the 4 day sample period, whereas the later were eliminated after 12 h (Fig. 6.4 e, g, Table 6.2). Thus both γ and α proteobacteria were successful at colonising the coral but γ proteobacteria were able to retain the occupied space for longer. Ribotypes closely related to *Flavobacteria* sp. (AB294989) appeared to be later colonisers as they were absent in control nubbins and only become abundant 2-4 days after antibiotic application (Fig. 6.4 h, Table 6.3). A ribotype closely related to a *Clostridium* sp., (a well known potential pathogenic microorganism in the human gut), was present in control nubbins, resisted the antibiotic treatment and increased its abundance 0-4 h after redeployment (Fig. 6.4 f, Table 6.3), indicating that potential pathogens, such as this could be part of the normal microbiota of the coral, only increasing their abundance when the normal coral microbiota is disrupted.

Table 6.2. Relative contribution (%) of bacterial ribotypes sequenced from bands excised from the DGGE gel to total ribotype diversity, based on SIMPER analysis of DGGE band intensities, indicating the average contribution of each bacterial ribotype to the similarity within each grouping factor (pre-treatment controls or time period following treatment). Ribotypes were identified according to closest matches identified by BLAST analysis. The species identity, group affiliation, GenBank accession number and % sequence identity of the closest match are shown.

Band No.	Species ID (closest relative)	Group affiliation	GenBank Accession No.	Contribution % (to community similatrity)						
			(% match)	UnT	0	up to 4 h	8 - 12 h	1 day	2 days	4 days
4	*Chloroflexi sp.*	Chloroflexi	EU909941 (97%)	3.04						
7	*Pseudidiomarina sp.*	γ-proteobacteria	FJ887948 (99%)				1.93			
8	*Nocardiodes sp.*	Actinobacteria	FJ406568 (99%)	8.31	11.7	18.31		4.67		
9	*Sphingobacterium sp.*	Sphingobacteria	AF260710 (67%)	12.22			14.89	12.31	12	42.95
10	Unknown	Unknown	Unknown			2.61				
11	*Pseudoalteromonas sp.*	γ-proteobacteria	EU330363 (99%)			5.86				
12	Unknown	Unknown	Unknown	4.05						
13	Unknown	Unknown	Unknown				3.6		12.82	
14	γ-proteobacterium	γ-proteobacteria	GU118719 (99%)	7.17						
15	*Actinobacterium*	Actinobacteria	AY632498 (92%)		4.06	4.59	1.96			
16	α-proteobacterium	α-proteobacteria	AB254287 (99%)	2.41	28.99	4.97				
17	ε-proteobacterium	ε-proteobacteria	HM318989 (99%)	2.36	10.72		7.67	15.18		
18	*Hydrogenophaga sp.*	β-proteobacteria	FM955626 (83%)	7.95			4.72			13.93
19	Unknown	Unknown	Unknown	5.7						
20	*Clostridium sp.*	Clostridia	CP000568 (100%)	1.71	15.65	2.56	1.96		9.07	
21	Rhodobacteraceae sp.	α-proteobacteria	FJ202776 (99%)	7.16			12.76	9.4		
22	*Flavobacteria sp.*	Flavobacteria	EU600663 (100%)			10.18	2.92			
23	*Bacteroidetes sp.*	Bacteroidetes	AB254287 (100%)				2.8	8.92		
24	Firmicutes sp.	Firmicutes	GQ502581 (87%)	4.54	9.92	10.13				
25	β-proteobacteria	β-proteobacteria	AF419359 (100%)	1.95				4.17		
26	γ proteobacterium	γ-proteobacteria	GU230328 (95%)	6.37						
27	*Endozoicomonas sp.*	γ-proteobacteria	FJ809457 (97%)	4.08			13.14		9.82	
28	γ proteobacterium	γ-proteobacteria	EU919132 (99%)	4.31		18.56	16.61	9.95	12.42	18.32
29	*Pseudoalteromonas sp*	γ-proteobacteria	FM163075 (99%)			2.15		9.84	13.89	
30	*Flavobacteria sp.*	Flavobacteria	AB294989 (100%)					7.58	11.62	14.95
31	*Endozoicomonas sp.*	γ-proteobacteria	GU184761 (99%)	1.22						
32	*Stenotrophomonas sp*	γ-proteobacteria	HM153430 (97%)	1.12	12.69					
35	α-proteobacteria	α-proteobacteria	FJ718457 (96%)			10.74				
36	Unknown	Unknown	Unknown					9.95	10.14	
38	Unknown	Unknown	Unknown				2.81			
41	*Marinobacter sp.*	γ-proteobacteria	HM141532 (98%)	4.67						
43	Unknown	Unknown	Unknown				3.96			

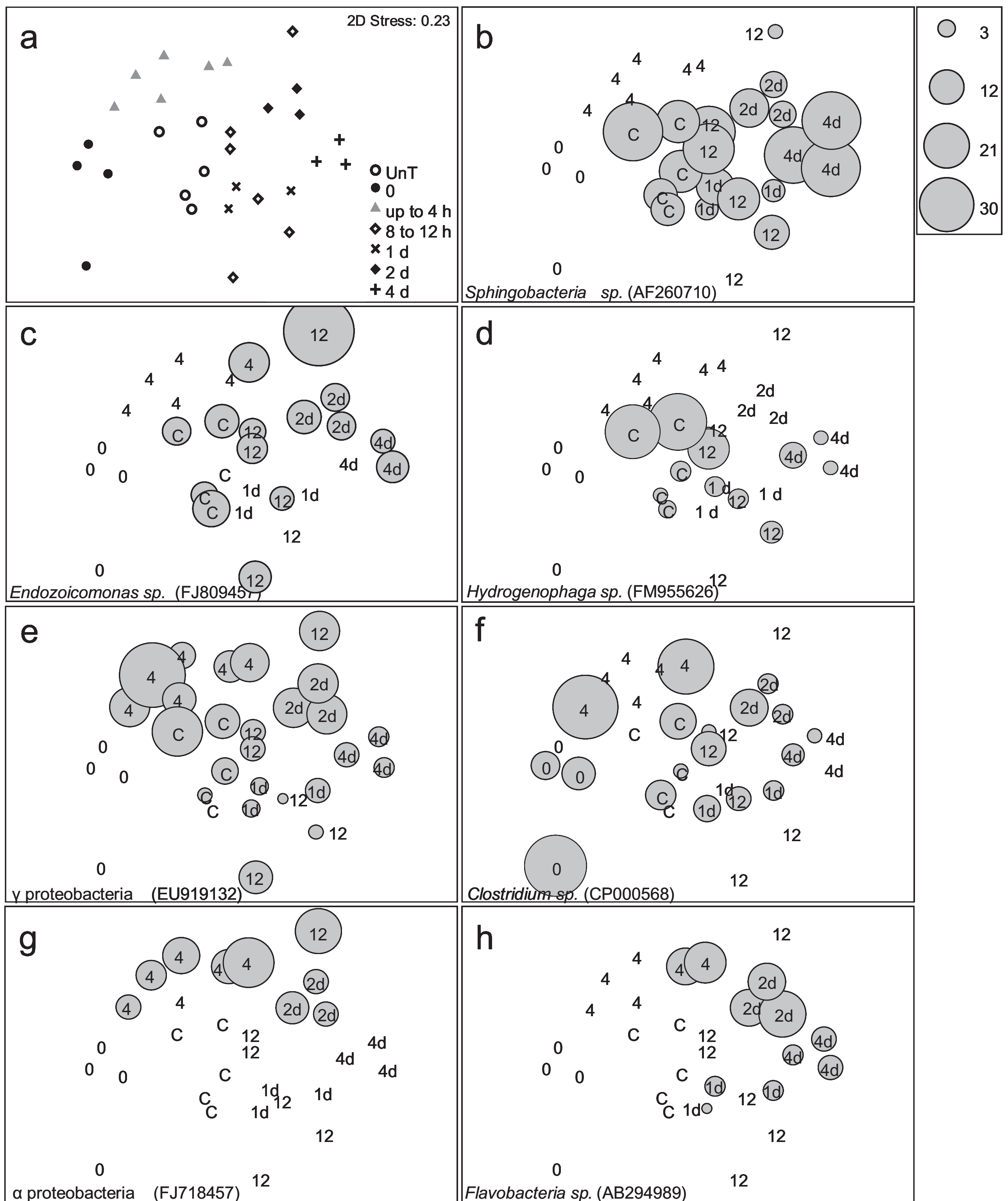


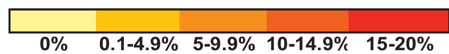
Figure 6.4. Multidimensional scaling (MDS) analysis of DGGE profiles based on BioNumerics analysis of relative band intensities. (a) MDS plot showing similarities (Bray Curtis similarity) between samples. (b - g) Relative abundances of a subset of specific bacteria overlaid as bubble plots on the MDS plot shown in (a). Size of bubble represents relative density of the DGGE band of that particular bacterial

sequence within individual samples. C = Untreated 'control' corals, 0 = time directly after treatment, 4 = up to 4 h after re-deployment, 12 = 8 to 12 h, 1d = 24 h, 2d = 48 h and 4d = 96 h.

Clone libraries generally supported the DGGE results, showing 16S rRNA gene ribotypes in the healthy coral to be more diverse overall but with a relatively small number of dominant bacterial ribotypes. Ribotypes closely related to a *Roseobacter* sp. (DQ985046) (15.2 % abundance, Table 6.3) and a *Sphingobacterium* sp. (AF260710) (12.2 %) dominated the total abundance of clone libraries with a further 54 % (FJ347758, DQ200474, DQ200446, EU919132 and EU919205) belonging to a distinct group within the γ--proteobacteria. Only one ribotype amongst this group has been named to genus and species level and represents a recently discovered genus, *Endozoicimonas*. These dominant genera were all reduced in frequency after treatment, but gradually recovered over time after deployment onto the reef (Table 6.3). There were a number of low abundance ribotypes in the healthy coral that were eliminated by treatment (Table 6.3) and failed to recover within the timeframe of the experiment. A small number of ribotypes, either in low abundance in healthy coral (EU909941, HM153430 and CP000568) or absent in healthy coral yet established following treatment (EU600663, FJ887948, GQ502581, AY632498, AB249868 and EU330363), became dominant immediately after treatment. However, all these ribotypes either returned to the low frequencies found in the healthy coral within 96 h or were completely eliminated. The only exception was a ribotype similar to *Flavobacteria sp.* (EU600663) which established itself and remained highly dominant even at 96 h.

Table 6.3. Heatmap-table summarising the relative abundance (%) of dominant bacterial sequence affiliations for 16S rRNA gene clone libraries. 0 signifies that clones related to that sequence were not detected in the sample (n = 194). ******** signifies sequence to short to acquire a GenBank accession number.

GenBank Acq. No.	Genus	Group	Best match (isolate)	UnT	30 min	4 h	96 h
HQ180154	*Marinobacter sp.*	γ-proteobacteria	HM141532 (98%)	3.4	0	0	0
HQ180166	*Marinobacter sp.*	γ-proteobacteria	HM141524 (99%)	1.3	0	0	0
HQ180147	*Spongiobacter sp.*	γ-proteobacteria	AB205011 (97%)	1	0	0	0
HQ180156	*Spongiobacter sp.*	γ-proteobacteria	FJ457274 (97%)	0.8	0	0	0
HQ180158	*Hydrogenophaga sp.*	β-proteobavteria	DQ413154 (99%)	0.1	0	0	0
HQ180164	*Unknown*	γ-proteobacteria	DQ200430 (95%)	2	0	0	0
HQ180145	*Unknown*	γ-proteobacteria	DQ200542 (89%)	0.3	0	0	0
HQ180162	*Unknown*	ε-proteobacteria	AF367488 (98%)	0.4	0	0	0
HQ180144	*Endozoicomonas sp.*	γ-proteobacteria	DQ200474 (94%)	5	0	3	4
HQ180161	*Endozoicomonas sp.*	γ-proteobacteria	DQ200474 (95%)	10	6	6.3	6.7
HQ180155	*Roseobacter sp.*	α-proteobacteria	DQ985046 (97%)	15.2	4.6	6	10.2
HQ180149	*Sphingobacterium sp.*	Sphingobacteria	AF260710 (97%)	12.2	1.3	4.2	18.1
HQ180146	*Endozoicomonas sp.*	γ-proteobacteria	FJ347758 (99%)	12.1	5.7	18	19
HQ180143	*Endozoicomonas sp.*	γ-proteobacteria	EU919205 (98%)	10	5	7	9
HQ180160	*Endozoicomonas sp.*	γ-proteobacteria	DQ200446 (95%)	4	0	1	1.9
HQ180140	*Endozoicomonas sp.*	γ-proteobacteria	DQ200474 (97%)	3	0	1	1
HQ180141	*Endozoicomonas sp.*	γ-proteobacteria	DQ200474 (96%)	8.2	4.2	9	9
HQ180163	*Unknown*	γ-proteobacteria	DQ204262 (99%)	3.2	0	2.1	3
HQ180142	*Endozoicomonas sp.*	γ-proteobacteria	EU919132 (100%)	2	0	0	0.2
********	*Clostridium sp.*	Clostridia	CP000568 (100%)	1.7	14.6	2.6	1
HQ180153	*Stenotrophomonas sp.*	γ-proteobacteria	HM153430 (97%)	0.8	7.7	0	0
HQ180165	*Stenotrophomonas sp.*	γ-proteobacteria	FN796853 (99%)	0.3	5	0	0
HQ180150	*Pseudoalteromonas sp.*	γ-proteobacteria	EU330363 (99%)	0	5	10.7	0
HQ180151	*Actinobacteria sp.*	Actinobacteria	AB249868 (97%)	0	2	3.5	0
HQ180157	*Actinobacteria sp.*	Actinobacteria	AY632498 (95%)	0	8.6	2.4	0
HQ180159	*Firmicutes sp.*	Firmicutes	GQ502581 (95%)	0	8	6	0
HQ180148	*Pseudidiomarina sp.*	γ-proteobacteria	FJ887948 (100%)	0	9	5	0
HQ180167	*Chloroflexi sp.*	Chloroflexi	EU909941 (97%)	3	8	2	2
HQ180152	*Flavobacteria sp.*	Flavobacteria	EU600663 (100%)	0	5.3	10.2	14.9



6.5 DISCUSSION

Bacterial assemblages associated with *A. muricata* displayed complex responses to antibiotic treatment. While some coral-associated bacteria were highly resistant to ciprofloxacin, a large majority were eliminated and bacterial abundance was reduced by 99.97 %, therefore the treatment represented a significant ecological disturbance to the coral-associated microbiota. Colonisation of newly opened niches within the coral host started within the first 4 h after treatment and redeployment on the reef, but neither bacterial population densities nor the diversity of the natural 'healthy' microbial community fully recovered within 4 days. Thus, our results demonstrate that the process of re-colonisation in *A. muricata* was a relatively slow process, despite high rates of mucus production reported for acroporas at the study site (Chapter 5; Wild et al. 2004). A relatively slow recovery of the normal microbiota following disturbances has important implications for coral health, which has been shown to depend in part on stress-related shifts of highly specific coral-microbial associations (Frias-Lopez et al. 2003; Pantos et al. 2003; Jokiel & Coles 2004; Sutherland et al. 2004; Bourne 2005; Williams & Miller 2005; Gil-Agudelo et al. 2007; Rosenberg et al. 2007; Mydlarz et al. 2009). The rate of natural disturbance in coral-microbial communities is unknown, but this suggests that competition between bacteria is an important process maintaining the healthy microbial community.

Re-settlement of bacteria in *A. muricata* started with a rapid colonisation of 16S rRNA ribotypes similar to *Actinobacterium* sp., *Bacteroidetes* sp., *Pseudoalteromonas* sp., *Flavobacterium* sp. and an α-proteobacterium previously identified in the water column at our study site (Kvennefors et al. 2010; Sweet et al. 2010a; Chapter 3). Waterborne ribotypes only became abundant when the coral-associated bacteria such as ribotypes similar to *Sphingobacteria*, *Chloroflexi* and numerous γ-proteobacteria were either eliminated completely or their abundance significantly reduced by the antibiotic treatment, highlighting the potential importance of opportunistic microbial invasions for the coral's microbial dynamics. Similar results were found by Garren et al. (2009) where corals transplanted into waters exposed to fish farm effluents incorporated ribotypes present in the water column at the new site, however the original community structure was shown to recover after 22 d. In this study, these opportunistic bacterial ribotypes were typically reduced in abundance or completely

eliminated after 96 h, with only one ribotype (a *Flavobacterium* sp.) remaining dominant thereafter. Thus, our results support Garren et al (2009), showing a similar strong resilience in bacterial community structure following perturbation.

In most cases, bacteria that are available in the immediate environment (e.g. the water column) opportunistically colonised the coral but were replaced relatively quickly by the regular microbiota. The case of the *Flavobacteria* sp. suggests that relatively few opportunistic bacteria are able to persist after the normal microbiota returns. Whether this represents a novel bacterial introduction made possible by the disturbance, or whether it takes longer than 96 h for this ribotype to be eliminated, remains to be determined. However, both α- and γ-proteobacteria were successful at colonising the coral at 4-8 h post-treatment with the latter found to persist longer. Many marine bacteria are known to show induction or enhancement of antibiotic production in the presence of competitors (Burgess et al. 1999; Slattery et al. 2001; Trischman et al. 2004). Recent studies have shown that antagonism is also an important process to regulate the dynamics of coral-associated microbial communities (Rypien et al. 2010).

Potentially pathogenic bacteria such as a ribotype closely related to *Clostridium* sp. were shown to increase when the coral microbiota was disturbed. In our experiment, this bacterium was originally found in corals collected from the field, showing resistance to antibiotic treatment and becoming dominant in the coral assemblage in less than 8 h after treatment. This supports evidence by Garren et al. (2009), where members of the Clostridiales as well as *Desulfovibrio* sp., which were both absent in environmental samples, increased in abundance in transplanted corals (Garren et al. 2009). Sequences affiliated with *Clostridium* sp. have been associated with black band disease (Frias-Lopez et al. 2002) and are commonly part of mixed-pathogen infections in a variety of terrestrial organisms, producing toxins and necrosis in animal tissues (Lawley et al. 2009). One particular species of this genus, *C. difficile* is implicated in antibiotic-associated disease of the human gut system (Goldenberg et al. 2010; Koo et al. 2010). *C. difficile* is considered to be unable to compete successfully in the normal intestinal ecosystem, but can compete when the normal biota is disturbed by antibiotics (Thelestam & Chaves-Olarte 2000; Keel & Songer 2007; Papatheodorou et al. 2010). *Clostridium* sp. reported in this study may be acting similarly to *Vibrio* spp. which are known to be opportunistic, only becoming abundant when the coral

becomes stressed (Ben-Haim et al. 2003; Rosenberg & Falkovitiz 2004; Geffen et al. 2009; Nissimov et al. 2009; Rypien et al. 2010; Tait et al. 2010). However in this study, no vibrios were detected. There is therefore growing evidence that potential pathogens may be present within 'healthy' coral microbial communities, but are actively controlled by the coral holobiont, only becoming prevalent following disturbance (Lesser et al. 2007). For corals, the production of compounds capable of inhibiting bacterial growth is vital for resisting and surviving opportunistic infections (Ritchie 2006; Hayashida-Soiza et al. 2008; Geffen et al. 2009; Mao-Jones et al. 2010). In addition to this, other mechanisms are likely to prevent and/or regulate invasion of competitors or potential pathogens. Some of these mechanisms include growth rate of natural non-pathogenic microbiota, nutrient uptake pathways and spatial heterogeneity in bacterial abundance (Rypien et al. 2010). Conversely, potential pathogens such as *V. harveyi* have recently been shown to inhibit quorum sensing, potentially giving this species a competitive advantage that allows it to proliferate during periods of high temperature (Tait et al. 2010). Given that healthy corals are continually exposed to potentially pathogenic bacteria (Rypien et al. 2010), it is imperative to better understand the underlying processes and mechanisms of maintenance of the 'healthy' coral-associated microbial community.

In conclusion, our experiment shows that initial settlement of bacteria upon the coral is rapid, occurring within minutes to hours after disturbance. Nevertheless, recovery of the initial microbial assemblage, in both abundance and diversity, requires longer periods than might be expected given high mucus production rates for this coral species. Bacterial succession will rely on specific interactions among early colonisers and the surviving coral microbiota which might facilitate and/or prevent the establishment of later colonisers (Ritchie & Smith 2004; Ritchie 2006). Potential pathogens inhabit the coral tissue at low abundances, only becoming prevalent when the natural microbiota is altered by a disturbance.

Chapter 7

A microscopic and molecular characterisation of ciliate communities associated with White Syndrome and Brown Band Diseases in *Acropora muricata*

7.1 ABSTRACT:

Coral diseases have up until recently, predominantly been reported as being associated with bacterial pathogens, however the importance of ciliated protozoans; causing diseases such as Brown Band disease (BrB), Skeletal Eroding Band (SEB) and Caribbean Ciliate Infection (CCI) has recently been shown. Another common coral pathology, termed White Syndrome (WS) in the Indo-Pacific and similar 'white' diseases (White Plague and White Band Disease) dominant in the Caribbean have been correlated with elevated temperature anomalies; however, there is conflicting evidence over the causal agents of these diseases. Some studies have suggested that WS in particular is an autogenic disease in which programmed cell death (apoptosis) is triggered, while recently several strains of vibrios were shown to be capable of tissue lysis similar to that shown in diseased corals in the field. This chapter focuses on the use of microscopic and molecular identification of newly identified ciliates thought to be a casual agent of WS and a more in-depth investigation of the microbial assemblage associated with BrB in the Great Barrier Reef. Two distinct morphotypes of ciliates were commonly seen at the advancing edge of the disease lesion in WS, the larger of which also heavily dominated BrB diseases samples. Both morpho-types contained coral algal symbionts, likely indicative of the ingestion of coral tissues. Despite similarities between these diseases the different visual field signs of the disease may be explained by the dominance of particular ciliates. In WS, the dominant (smaller) species was observed to burrow into and underneath the coral tissues, and appears to be the main aetiological agent of lesion progression. In comparison, the larger, slower moving species, heavily dominates BrB samples and appears to play a secondary, less invasive role in WS. Experimental treatments simulating a historical (1996) bleaching event at one of our study sites dramatically increased the susceptibility of *A. muricata* to WS, strongly supporting previous conclusions that temperature increase explains at least a part of the recent increases in

disease prevalence. While it cannot yet be concluded whether ciliates are the primary causal agents of WS or BrB disease, they are clearly important pathogenic agents and further studies are urgently needed to understand the roles of these and other potential pathogens in coral diseases worldwide.

This Chapter contributed to the paper; Bythell, J.C., Pantos, O., Sweet, M.J., Croquer, A., Beakes, G., Guppy, R., Magalon, H., Johnstone R., "Are ciliates the key infectious agents of White Syndrome of corals of the Great Barrier Reef?" This author aided in field work and the temperature tank experiment, discovered ciliates with J. Bythell & O. Pantos, isolated, photographed, analysed and sequenced the isolates and edited the paper which was written by J. Bythell.

7.2 INTRODUCTION

The study of coral diseases encompasses many different disciplines as it involves several aspects of complex host-pathogen interactions within the environment. Since the first observations of diseases affecting reef corals in the late 1970's and early 1980's (Ducklow & Mitchell 1979; Peters et al. 1983; Rutzler & Santavy 1983; Antonius 1985), the research priorities have changed substantially; from simple and general descriptions of disease signs in the field (Rutzler & Santavy 1983) to microbial studies based on both culture and non culturable methods (Lesser et al. 2007; Garren et al. 2009; Kvennefors et al. 2010). This has led to the identification of specific pathogenic agents thought to be the causal agents of particular diseases (Kushmaro et al. 2001; Ben-Haim & Rosenberg 2002; Cooney et al. 2002; Ben-Haim et al. 2003a; Luna et al. 2007; Sussman et al. 2008). Historically, most coral diseases have been primarily associated with either an individual and/or a consortium of pathogenic bacteria or fungi (Ben-Haim & Rosenberg 2002; Bythell et al. 2002; Frias-Lopez et al. 2003; Kirkwood et al. 2010), whereas other microorganisms have largely been overlooked. However, over the past few years there has been increasing evidence suggesting that health problems can also be caused by trematodes (Aeby 2002, 2003, 2007; Palmer et al. 2009) and more frequently with ciliates (Croquer et al. 2006a, 2006b; Cooper et al. 2007; Bourne et al. 2008; Page & Willis 2008; Qiu et al. 2010), yet few studies have addressed their role as casual agents of disease either as primary or secondary invaders (Croquer et al. 2006a; Bourne et al. 2008). Ciliates belong to the phylum Ciliophora, named due to the presence of hair like structures known as cilia which aid in feeding (creating water currents to catch planktonic

organisms) and movement. Generally ciliates are characterised by having two nuclei; one small, diploid micronucleus involved in reproduction and one large polyploid nucleus involved in general cell regulation (Lee et al. 2000). Other distinguishing features include the complexity and variation in their cellular structures and their reproductive strategies both sexual and asexual (Iudin & Uspenskaia 2006; Dunthorn & Katz 2010). These traits mean that ciliated protozoa are highly adaptive, allowing them to inhabit almost all freshwater and marine habitats (Lee et al. 2000). Ciliates are obvious components of many microhabitats both visibly and ecologically, having been shown to regulate microbial biomass (Vargas & Hattori 1990) and bacterial community composition (Gel'Tser Yu 1991), in addition to known effects upon benthic and pelagic food webs (Fenchel 1968; Porter et al. 1979; Fenchel 1980; Wieltschnig et al. 2003; Vargas et al. 2007). Marine species such as *Myrionecta rubrum* provide a large amount of the primary productivity in areas of upwelling (Lindholm 1985; Lindholm & Reisser 1992) and planktonic tintinnids and oligotichs are ecologically important oceanic secondary consumers (Heinbokel 1978; Heinbokel & Beers 1979). Many studies have previously shown that the presence and population density of particular ciliate species, can be good indicators of healthy environments, aiding in assessing water quality for example and the presence of toxic pollutants (Mark et al. 1963; Rehman et al. 2008). Conversely, several studies have shown certain species of ciliate to be pathogenic to a range of organisms (Song & Wang 1993; Bradbury et al. 1996), from marine mammals such as dolphins and whales (Sniezek et al. 1995; Poynton et al. 2001; Song et al. 2009) to bivalves such as the clam, *Mesodesma mactroides* (Cremonte & Figueras 2004). Therefore, the taxonomy of ciliates in general is important to aid in identifying particular species and understanding their habitat and behaviour in natural environments.

Only recently have ciliates and other protozoans been associated with diseases of corals. Skeleton-eroding band (SEB) was not only the first coral disease to be shown to be caused by a protozoan, but the first to be identified as being caused by a eukaryote (Antonius & Lipscomb 2001). SEB is a progressive disease, widespread throughout the Indo-Pacific with high local prevalence (Page & Willis 2008). The disease is characterised by a skeletal-eroding lesion with a speckled black band composed of the empty loricae (shell-like housings) of the folliculinid ciliate, *Halofolliculina corallasia* (Winkler et al. 2004). More recently, another ciliate

infection in the Indo-Pacific, Brown Band disease (BrB), was identified. BrB is widespread in parts of the GBR and known to effect three major coral families; Acroporidae, Pocilloporidae and Faviidae (Bourne et al. 2008). This ciliate, identified as a member of the subclass Scuticociliatia (Bourne et al. 2008), has been shown to ingest intact symbiotic algae of the coral and is responsible for the visible symptoms of this disease (a variable brown band). Ciliates have also been shown to invade the tissues of corals after damage by predators, such as the feeding scars left by the crown of thorn star fish *Acanthatar palanci* (Nugues & Bak 2009) and devour the tissues of coral spats (Cooper et al. 2007). These findings further suggest that these organisms have an overall negative effect on coral population dynamics, by increasing post settlement mortality. In contrast, other protozoans, identified as stramenopile protists, have been shown to be natural associates of corals, found both on the coral surface and within the tissues (Kramarsky-Winter et al. 2006).

The first evidence of coral-protozoan association in the Caribbean was reported in 2002, when a sequence matching with the phylum Apicomplexa was found in tissues of *Montastraea annularis* (Toller et al. 2002). Despite this protozoan being related to the highly parasitic organisms Coccidians, the nature of its interaction with corals remains largely unknown. In 2006, Folliculinid ciliates in the genus *Halofolliculina* were reported for the first time affecting over 26 Caribbean reef-building coral species, providing more evidence to show that ciliate infections are not exclusive to the Indo-Pacific (Croquer et al. 2006b). Although it is still to be determined whether this Caribbean Ciliate Infection (CCI) is the same as SEB in the Indo-Pacific, their morphology, life cycle and patterns of infection are similar. In terms of pathology, both SEB and CCI have been shown to produce tissue mortality and in the particular case of CCI a negative effect on tissue regeneration (Page & Willis 2008; Rodriguez et al. 2009). Both diseases have been shown to transmit directly from infected to susceptible hosts (Page et al. 2010) with injuries (Page & Willis 2008; Rodriguez et al. 2009) and temperature (Rodriguez et al. 2009) enhancing transmission rates. Both SEB and CCI are wide-spread and occur across bioregions (Willis et al. 2004; Winkler et al. 2004; Croquer & Weil 2009) affecting a wide range of coral hosts which is comparable to most virulent bacterial diseases (Weil 2004). Thus, increasing evidence indicates that ciliate infections are a significant problem for coral reef health and therefore their role in such matters needs urgent attention. Despite this, Koch's

postulates have not been fulfilled for any of the ciliates associated with coral lesions, further complicating the problem because mixed ciliate communities have been reported thriving upon and/or underneath infected tissues. In this regard, one initial primary step necessary to determine whether or not ciliates are responsible for disease pathology would be a concise and precise identification of each member of these ciliate communities.

During tank experiments set up at Heron Island a highly mobile ciliate mass was observed dwelling underneath a sharp border separating recently exposed skeletons and apparently healthy tissues. Rapid rates of tissue loss observed during this experiment led to microscopic analysis in the field to identify the cause and initial observation revealing a diverse community of ciliates associated with the coral lesions (Bythell et al. in press). This finding represented an excellent opportunity to study the ciliates associated with coral lesions. The disease signs which were apparent were similar to a wide range of common, poorly defined 'white' diseases and syndromes, few of which have been satisfactorily characterised (Bythell et al. 2004; Lesser et al. 2007), collectively termed White Syndrome (WS). Many attempts have been made to link these diseases with a particular bacterial pathogen (Peters et al. 1983; Barash et al. 2005; Thompson et al. 2006; Sussman et al. 2008; Efrony et al. 2009). For example, *Aurantimonas coralicida* has been reported to cause White Plague Type II disease in the elliptical star coral *Dichocoenia stokesii* (Denner et al. 2003), another α-proteobacterium, thought to be the causative agent in juvenile oyster disease (JOD) has been shown in the Caribbean coral *Montastrea annularis* exhibiting tissue lesions indicative of a White Plague (WP)-like disease (Pantos et al. 2003), in addition to the numerous vibrio pathogens having been accredited to WS over the years (Sussman et al. 2008), with *Vibrio harveyi* being the most recent (Luna et al. 2010). Despite the large effort, time and money spent trying to isolate specific pathogens and prove Koch's postulates, discrepancies in the final disease outcome are common. This may be because WS represents a broad disease pathogenesis with potentially several different causal agents and some of these casual agents may have been overlooked.

Current methods to identify ciliates in environmental samples include: 1) light microscopy; 2) scanning electron microscopy (SEM); and 3) molecular sequencing. Morphological examination of samples using light microscopy is usually conducted

after fixing and staining, SEM samples are prepared in the same way but SEM allows a more detailed examination of the surface structure (Shimano et al. 2008; Dopheide et al. 2009). Molecular techniques (18S rRNA gene PCR and DNA sequencing), have more recently played an important role in aiding morphological identification. This allows simultaneous analysis of phylogenetic relationships (Puitika et al. 2007; Dopheide et al. 2008), giving a clearer understanding of relationships to neighbouring species. The aim of this chapter was to identify the ciliates present in both the tank experiment and those found on WS and BrB infected corals on the GBR at Heron Island and Orpheus, utilising both microscopy (LM and SEM) and refined molecular techniques.

7.3 MATERIAL AND METHODS

7.3.1. Microscopic observation and characterisation of the dominant ciliates

Corals within a controlled tank experiment were routinely monitored for visible signs of bleaching and disease. These diseased tank samples along with corals collected directly from the field with WS and/or BrB symptoms were transferred without handling to an observation tank for microscopic and behavioural observations using an Olympus SZX7 binocular microscope and Olympus LG-PS2 fibre-optic light source. Still images and time-lapse videos were captured using a QImaging Micropublisher 3.3 camera and Q-Capture v6 imaging software. Higher magnification images were obtained using an Olympus BX51 compound microscope and images captured as above. N = 4 samples were fixed in paraformaldehyde, critical point dried and analysed under a Cambridge Stereoscan S240 SEM at 20 kv. The images were compared to morphological descriptions present in previous studies (Carey & Carey 1992; Lee et al. 2000; Song 2000; Croquer et al. 2006a; Page & Willis 2008; Shimano et al. 2008), alongside the use of a dichotomous key in the ‘Illustrated Guide to the Protozoa’ (Lee et al. 2000). Gross morphological and physiological characteristics, such as cortical and ultra-structural features, provided a means of distinguishing the ciliates present. Features such as kinetosomal make-up and oral infraciliary structures such as the AZM (adoral zone of membranelles) are highly conserved features and together with organelle distribution, size, shape and colour are routinely used for comparing genera.

7.3.2 Molecular identification of the dominant ciliates

Single cell isolates were taken from mixed samples under binocular microscopy using a micropipette and preserved in 100 % Analar Ethanol. DNA was extracted from the ethanol-fixed single isolates using a Chelex extraction (Walsh et al. 1991). All samples were vacuum centrifuged for 10 min and washed twice in sigma water with a 2 min centrifuge step (20,000 g) in between. Following the final wash, 50 µl of 5 % Chelex 100 (sigma) solution and 15 µl proteinase K (20 mg/ml) were added to the cell isolate. The samples were subsequently left in a water bath overnight at 54 ^{o}C. After incubation, they were vortexed for 20 s, boiled at 100 ^{o}C for 10 min, vortexed for a further 20 s and centrifuged at 16,000 g for 3 min. 30 µl of supernatant was taken off and put in a fresh eppindorf tube. This was then stored at – 20 ºC until further use. Three other methods of extraction were tested beforehand; (1) Proteinase K digestion (Kim & Min 2009), (2) freeze thaw extraction (Sylvester et al. 2004), (3) Qiagen DNeasy Blood and Tissue Kit (as per manufacturer's recommendations), however the Chelex extraction provided a larger quality of extractable DNA and was the most repeatable and reliable method of extraction for single cell isolates.

Seven primer pairs were tested on the single cell isolates to gain the best product for sequencing (Table 7.1). 20 µl PCR reactions were routinely used (final PCR buffer contained: 1 mM $MgCl_2$, and 1 U Taq DNA polymerase (QBiogene); 100 µM dNTPs; 0.2 µM of each of the forward and reverse primers; and 0.4 % bovine serum albumin, with 20 ng of template DNA extracted as above) in a Hybaid PCR-Express thermal cycler.

Table 7.1. Universal 18S rRNA gene PCR primers tested on single cell isolates.

Primer	Sequence (5' - 3')	Target organism (s)	Anealing temp (º C)	positive product	Reference
18S-6-CIL-V	AAYCTGGTTGATCCTGCCAG	Eukaryotes	58	yes	Bourne et al. (2008)
18S-1511-CIL-R	GATCCWTCTGCAGGTTCACCTAC	Eukaryotes	58	yes	Bourne et al. (2008)
516	CACATCTAAGGAAGGCAGCA	Eukaryotes	55	no	Johnson et al. (2004)
1416	GAGTATGGTCGCAAGGCTCAA	Eukaryotes	55	no	Johnson et al. (2004)
4616f	AACCTGGTTGATCCTGCCAG	Eukaryotes	55	yes	Oldach et al. (2000)
4617f	TCCTGCCAGTAGTCATATGC	Eukaryotes	55	yes	Unpublished correspondance
4618r	TGATCCTTCTGCAGGTTCACCTAC	Eukaryotes	55	yes	Oldach et al. (2000)
121f-cil	CTGCGAATGGCTCATTAMAA	Ciliates	55	no	Dopheide et al. (2008)
384f-cil	YTBGATGGTAGTGTATTGGA	Ciliates	55	yes	Dopheide et al. (2008)
1147r-cil	GACGGTATCTRATCGTCTTT	Ciliates	55	yes	Dopheide et al. (2008)
HOL-f	ACAGACCGGAGCCTCTGGTC	Holosticha	67	no	This study
HOL-r	AGGACCTGTGCGTCTCTCTCGG	Holosticha	67	no	This study

The universal 18S rRNA gene eukaryotic primers 4617f (5'-TCCTGCCAGTAGTCATATGC-3') and 4618r (5'-GATCCTTCTGCAGGTTCACC TAC-3') (T. Tengs, pers. comm.) were used following the PCR protocol of Oldach et al. (2000). The nested PCR reaction was carried out using 1 µl of a 1:100 dilution of first round product with the ciliate-specific primers 384f-cil (5'-YTBGATGGTAGTGTATTGGA-3') and 1147r-cil (5'-GACGGTATCTRATCGTC TTT-3') and amplification conditions of Dopheide et al.(2008). All sequences were ethanol-purified from PCR products and sequenced by Geneius Ltd., Newcastle University, UK. These were run against BLAST searches to find closest possible match.

7.4 RESULTS

7.4.1. Mixed ciliate communities associated with coral diseases (White syndrome and Brown band disease)

Microscopic examination of n = 16 nubbins of *A. muricata* showing classical signs described for white syndrome (both in the field (n = 5) and within tank experiments (n = 6)) and Brown Band diseases (n = 5), showed a mixed community of ciliates thriving in the edges of apparently healthy tissues and recently-exposed coral skeletons (Fig. 7.1). WS samples were collected from two study sites, Heron Island and Orphius. These communities encompassed at least 8 different ciliate morphotypes, the majority of them clearly showing zooxanthellae inside their bodies.

Figure 7.1. Images depicting coral lesions with specific ciliates identified for white syndrome and Brown Band Disease (a) tank experiment corals (b) white syndrome within the field, Heron Island (c) Brown Band disease, Heron Island.

The nubbins within the tank experiments exhibited two visibly distinct phases of tissue degradation; (1) early phase exhibiting the classic progressive 'white' band from the bottom of the nubbin with a clear distinct line between healthy tissue and exposed skeleton and, (2) later string-like phase showing heavily degraded tissue throughout. Both patterns of tissue loss were in all cases heavily infected with ciliates, although these were not visible to the naked eye. The rate of tissue loss in temperature treated corals was extremely rapid compared to those kept at controlled levels, with the lesion progressing at a rate of ~ 21 mm d^{-1}, resulting in whole nubbins (~ 3 cm in length), being completely denuded of tissue within 24 hours from first sign of the advancing ciliate mass (Fig. 7.2)

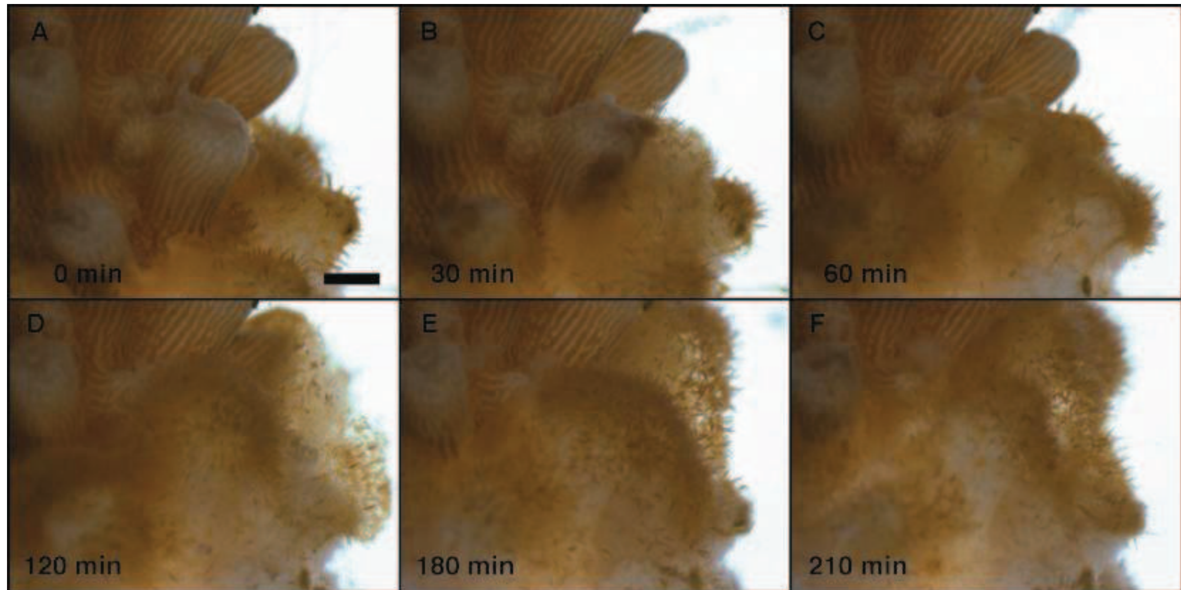


Figure 7.2. Time lapse images of CWS lesion progression. The lesion progresses from bottom to top of the images at a rate of ~21 mm d^{-1}. At this scale, the ciliate mass appears to be a diffuse yellow-brown mass comprised predominantly of the rapidly moving morph 1 ciliates embedded with variable densities of morph 2 ciliate. The latter are slower moving and large enough to be seen as individual cells, typically orientated perpendicularly to the coral skeleton surface (white) exposed by the advancing lesion. Coral tissues (yellow-brown) immediately adjacent to the advancing lesion are intact and appear normally pigmented. Scale bar = 1 mm.

7.4.2 Microscopic observation and characterisation of the dominant ciliates

7.4.2.1 White syndrome (field and tank samples)

The most aetiologically important agent in this mixed community was a ciliate (morph 1) approximately 60-80 µm long and 25-30 µm wide (Fig. 7.3 a,b), which showed rapid movement and actively burrowed into the live coral tissues (Fig. 7.2). The AZM is about 1/3rd the length of the body and is an unusual shape (Fig. 7.3 a), having a wide gap at the front end of the body that is caused by the absence of membranelles. The uniformly ciliated somatic cortex has paired cilium, with doubly ciliated somatic dikinetids, 10 -15 µm in length projecting from one parasomal sac (Fig. 7.3 c,d arrows and insert). Each individual band making up the infraciliature

running from the anterior to the posterior of the cell is 1.6 – 1.8 µm in width. This ciliate was seen in abundance at the lesion interface and can be seen to contain algal symbionts from the coral (Fig. 7.3 a), indicating either the direct ingestion of coral tissues or alternatively the ability to acquire its own symbiotic algae from the surrounding environment.

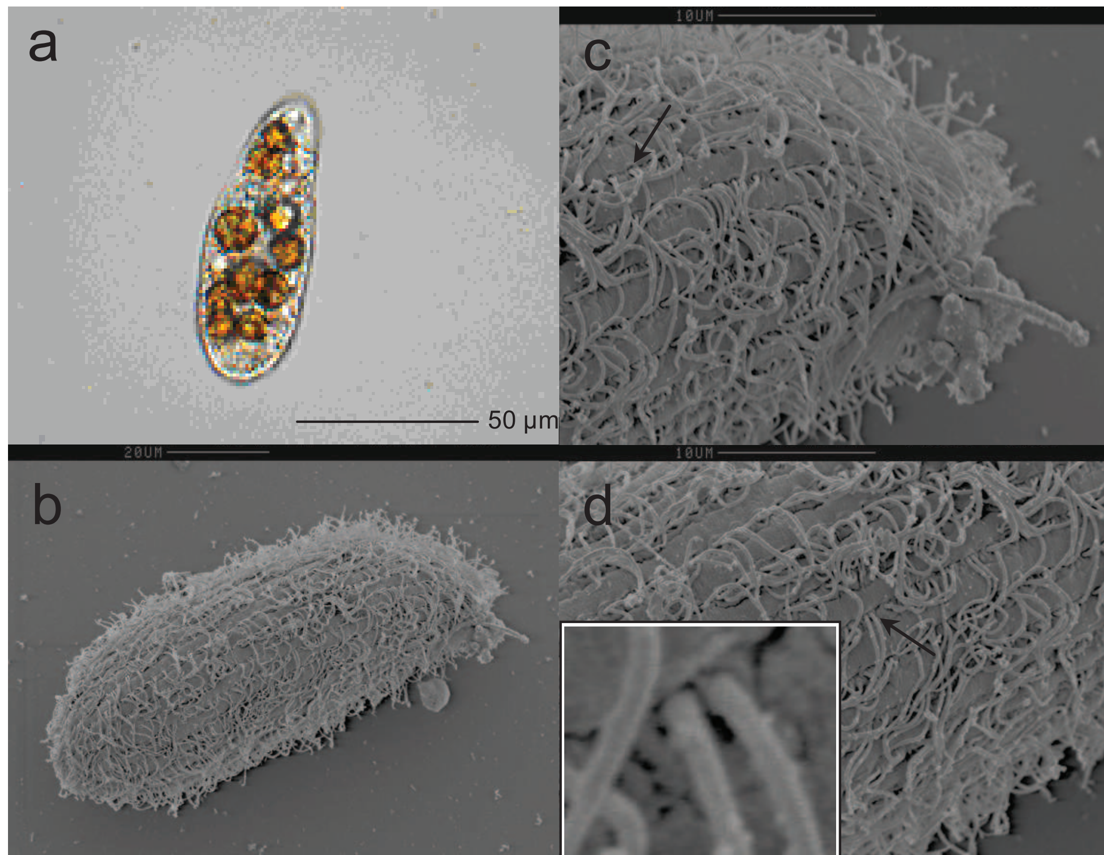


Figure 7.3. Morph 1, the most aetiologically important agent in this mixed community (a) light microscope image of morph 1 ciliate, small adoral zone of membranelles (AZM) visible, extending less than a third of the way down the cell. (b) scanning electron microscope (SEM) of whole ciliate (c) close up SEM of tip (d) close up SEM illustrating the uniformly ciliated somatic cortex with paired cilium (doubly ciliated somatic dikinetids, 10 -15 µm in length (insert) projecting from one parasomal sac. Scale bars vary for each section of the image and are included within.

In most cases, populations of this ciliate were mixed with populations of a larger (250-300 µm in length and 50 µm in width) ciliate (morph 2, Fig. 7.4 a). This ciliate was also seen in abundance at the lesion interface and can be seen to contain algal

endosymbionts from the coral (Fig. 7.3 a), however it appeared to take a secondary role to morph 1. The behaviour of this ciliate (Morph 2) was less erratic than morph 1, with slow turning/spinning movements. The somatic cortex is clearly different from that of morph 1, uniformly ciliated with individual cilium (somatic monokinetid) and parasomal sacs on both sides (Fig. 7.4 d insert) compared to only one in morph 1 (Fig. 7.3 d), and the width of the bands present on the infraciliature cortex are slightly larger at 2μm, indicating two distinct species.

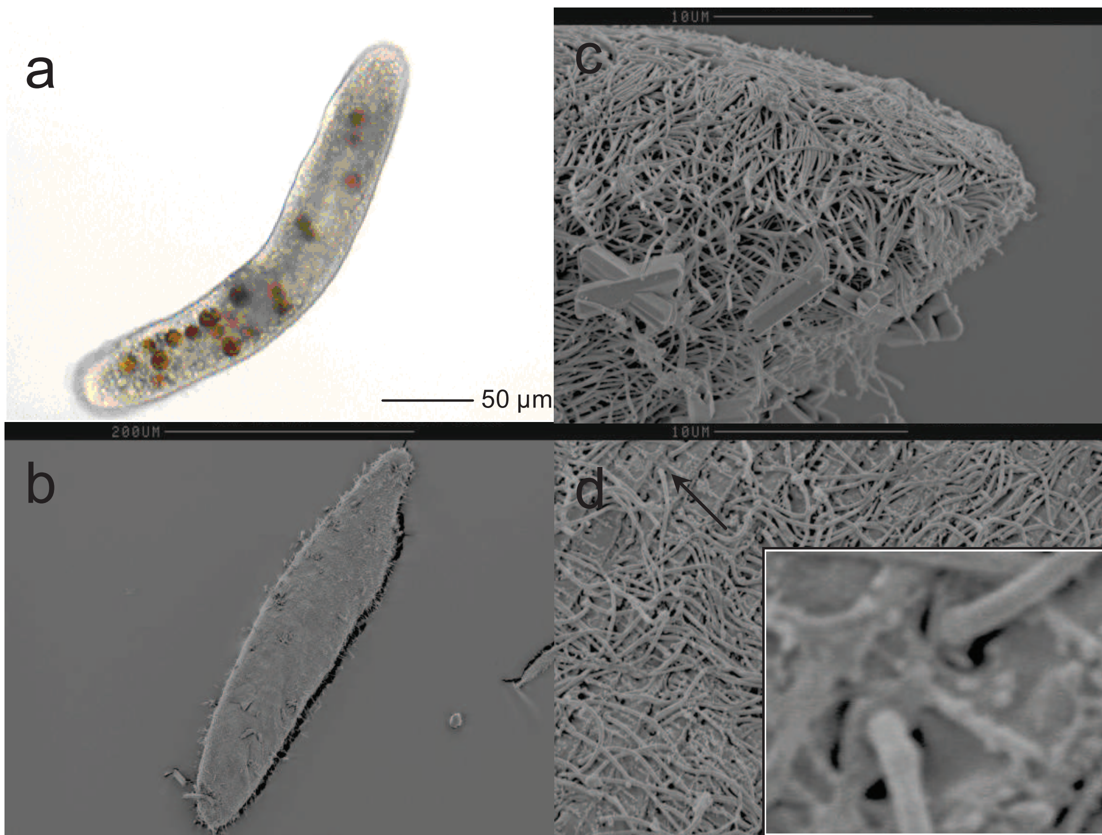


Figure 7.4. Morph 2, a larger (250-300 μm in length, 20 – 50 μm wide) ciliate (a) light microscope image showing ingested zooxanthellae believed to have originated from the coral tissue being fed upon (b) scanning electron microscope (SEM) of whole ciliate (c) close up SEM of tip (d) close up SEM illustrating the somatic cortex note individual cilium (somatic monokinetid) with parasomal sacs on both sides (arrow and insert). Scale bars vary for each section of the image and are included within.

Other members of this mixed ciliate community present within WS and tank samples were also photographed and recorded. These consisted of; Morph 3, (a) a large (96 –

135 µm in length), black, ovoid hypotrich ciliate, with fast sporadic movements (Fig. 7.5 a). This ciliate is characterised by the somatic cilia clustered as compound, with 9 large fronto-ventral cirri (5 transverse cirri and 4 caudal cirri (Fig. 7.5 a)), (b) Morph 4, a larger, 'worm-like' ciliate, yellow-brown to red in colouration, 140-350 µm in length and 30-50 µm wide and characterised by five pairs of frontal cirri (Fig. 7.5 b) (c) Morph 5, a smaller (80 – 100 µm in length) heavily ciliated morph with no visible signs of digested symbiotic algae (Fig. 7.5 c) and Morph 6, (Fig. 7.5 d) a further ovoid ciliate (90 – 110 µm in length) less frequently seen.

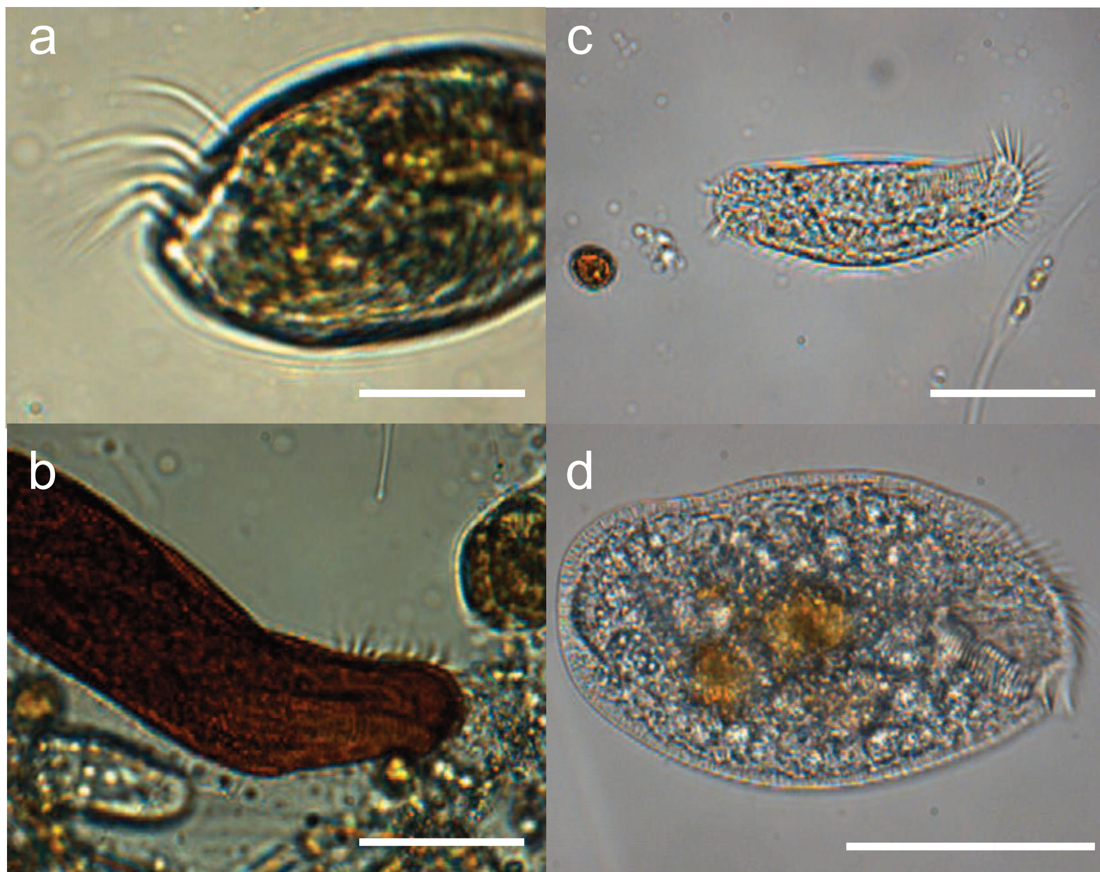


Figure 7.5. Light microscope images of the other members of the mixed ciliate community percent within white syndrome in the field and tank samples exhibiting white syndrome (a) Morph 3**,** ovoid hypotrich ciliates (96-135 µm in length), (b) Morph 4, worm like ciliate (140-350 µm in length), (c) Morph 5, smaller heavily ciliated morph (80-100 µm in length) and (d) Morph 6, ovoid ciliate (90-110 µm in length). Scale bars 50 µm.

One type of ciliate, morph 7 was heavily dominated in WS in the field but largely absent in the tank samples (Fig. 7.6). A relatively small species (80-90 µm in length),

characteristically different from the other morphs with large (~ 10-12 µm) extended frontal cirri, in comparison to overall body size. These cirri were used largely for locomotion by the ciliate, effectively walking along the coral tissue, the presence of symbiotic algae in these ciliate species again suggests ingestion of tissue.

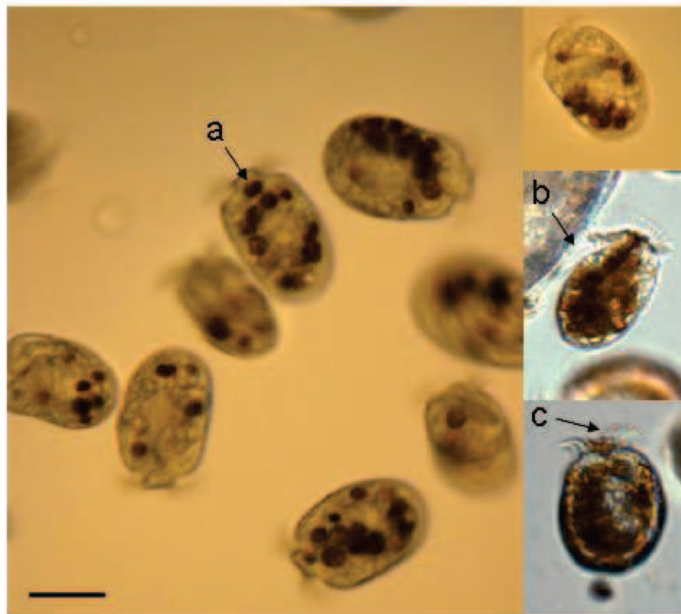


Figure 7.6. Light microscope images of a ciliate heavily dominating field samples of white syndrome (Morph 7): 80-90 µm in length, arrows depict areas of note (a) ingested symbiotic algae, (b) caudal cirri (cilia) used in locomotion (c) distinctly extended large frontal cirri (~ 10 µm). Scale bar 50 µm.

7.4.2.2 Brown band disease

A similar mixed community was found associated with BrB diseased corals at Heron Island. Four main types were identified using microscopic analysis (Fig. 7.7). Unlike WS, this disease lesion (Fig. 7.7 a) was dominated by a ciliate (Fig. 7.7 b) similar to morph 2 with the apparent absence of morph 1. The smaller ovoid ciliate (Fig. 7.7 c) was also heavily dominant in BrB samples, showing ingested symbiotic algae. Other ciliates (Fig. 7.7 d and e) were in lower abundance in all samples studied.

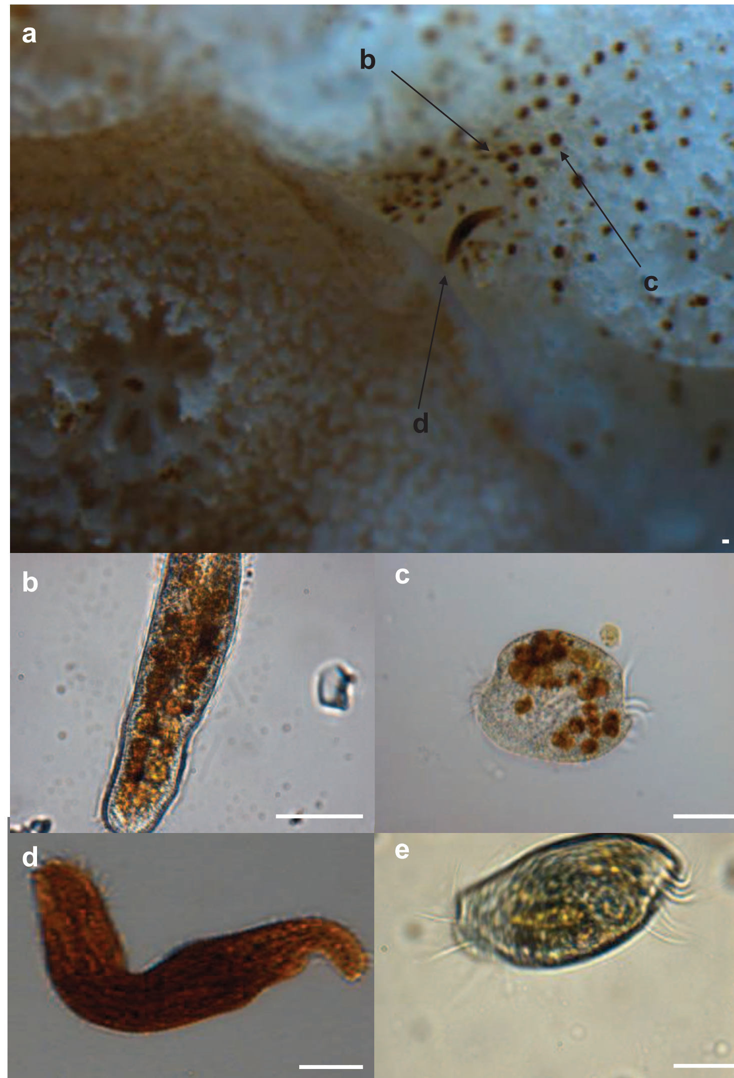


Figure 7.7. (a) BrB disease tissue lesion showing location of main ciliates present (b) Morph 2, large (250-300 µm in length, 20 – 50 µm wide) ciliate similar to morph 2 from WS samples (c) Morph 8, ovoid hypotrich ciliates (90-100 µm in length) (d) morph 4, worm like ciliate (140-350 µm in length) similar to that found in WS samples and (e) morph 3, ovoid hypotrich ciliates (96-135 µm in length), (b,d and e) are ciliates similar to those found in WS and tank samples. Scale bar represents 50 µm.

7.4.3 Molecular identification of the dominant ciliates

BLAST analysis of the 18S rRNA gene sequences retrieved from the single cell isolates showed inconsistencies between the 18S sequences and the same morphotypes and *vice versa* (Table 7.2).

Table 7.2. Ciliate species identified through 18S rRNA PCR molecular screening with ciliate specific primers, sequencing and BLAST analysis at two sites on the GBR, Heron Island (tank samples and BrB field samples) and Orpheus (White syndrome, field samples).

Ciliated White Syndrome (Tank)	**Ciliated White Syndrome (Field)**	**Brown Band Disease**
BrB (AY876050) 99%	BrB (AY876050) 99%	BrB (AY876050) 99%
Euplotes minuta (AY361908) 95%	*Euplotes minuta* (AY361908) 95%	*Euplotes minuta* (AY361908) 95%
Euplotes encysticus (EF535728) 98%	*Euplotes encysticus* (EF535728) 98%	*Euplotes encysticus* (EF535728) 98%
Pseudokeronopsis carnae (AY881633) 96%	*Pseudokeronopsis carnae* (AY881633) 96%	*Pseudokeronopsis carnae* (AY881633) 96%
Holosticha diademata (DQ059583) 98%	*Holosticha diademata* (DQ059583) 98%	
Varistrombidium kielum (DQ811090) 99%	*Euplotes octocarinatus* (EF094962) 97%	

7.4.4 Linking the genotypes to the morphotypes

Due to the discrepancies of the 18S rRNA sequences when related to the photographs taken at time of sampling, it was necessary to relate the sequences found to the morphological descriptions of the closest relative match. By doing this, it was possible to give the most likely match of the photographs of the morphotypes compared to the sequences retrieved. Morph 1 and 2 were 99 % similar to the scuticociliate (AY876050) previously identified in association with Brown Band (BrB) disease (Bourne et al. 2008), these two types of ciliates are in the same clade and hence phylogenetically related. Morphs 3 and 8 where closely related to *Euplotes encysticus* EF535728 (98 %) and *E. minuta* AY361908 (95 %) respectively. Morph 4 was 96 % similar to *Pseudokeronopsis carnea* AY881633, morph 5 was 98 % similar to *Holosticha diademata,* morph 6 thought to be *Varistrombidium kielum* DQ811090, was rarely seen in microscopic observations, however dominated single cell isolates retrieved when sequenced and morph 7 samples were not collected and therefore unidentified but thought to be that of a further *Euplotes* sp. Fig. 7.8 shows the neighbour-joining consensus tree of partial 18S rRNA gene sequences of 26 ciliate samples.

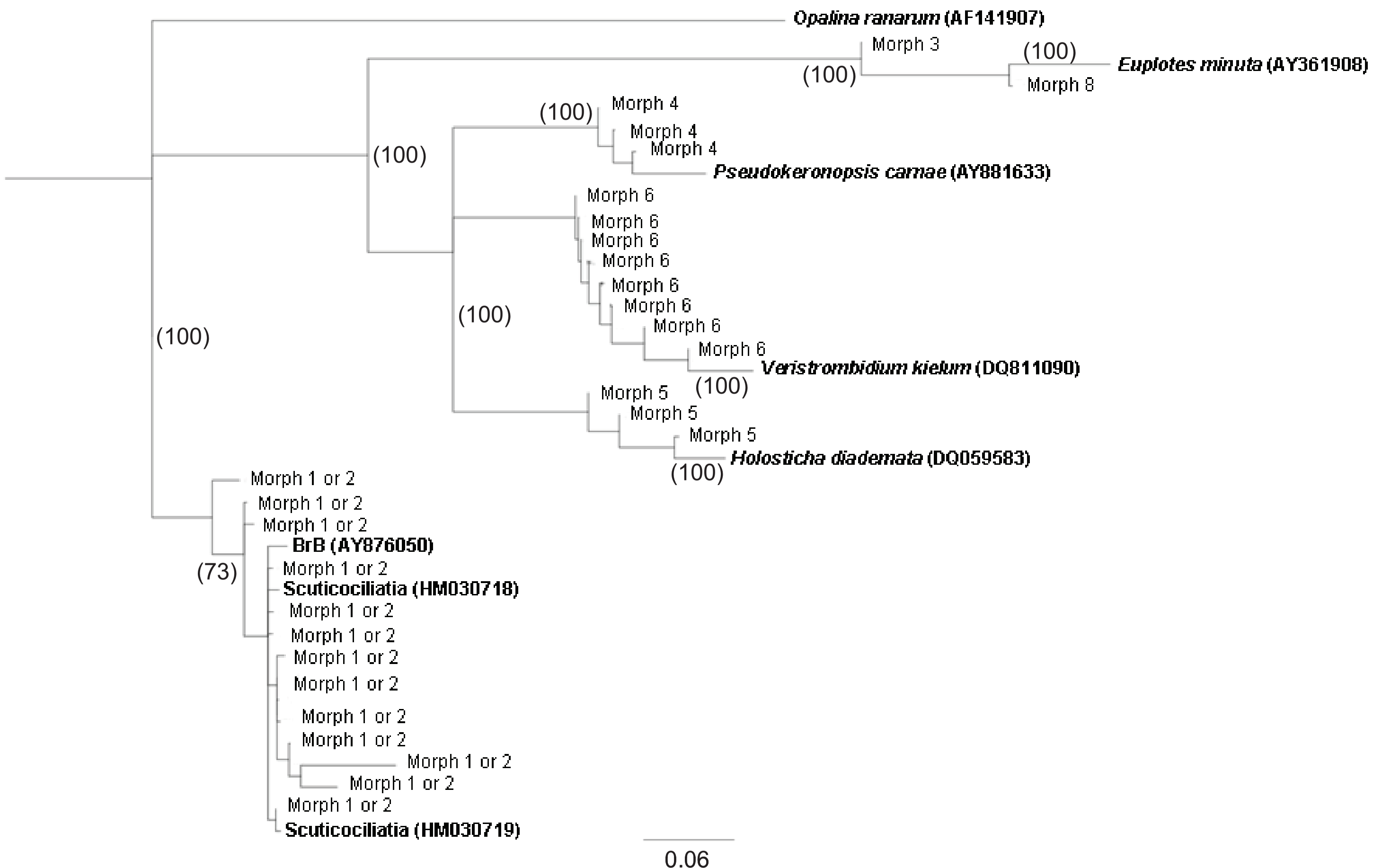


Figure 7.8. Neighbour-joining consensus tree of partial 18S rRNA gene sequences of 31 samples of ciliates found within Brown Band diseases, White syndrome and tank samples. Sequences were aligned in Clustal W2 (Larkin et al. 2007), using an IUB cost matrix with a gap open cost of 15 and a gap extend cost of 7. A neighbour-joining consensus tree (1000x resampling) was constructed in Geneious Pro 5.0 using the Tamura (1994) genetic distance model with an opalinid protist, *Opalina ranarium* (AF149070), as the outgroup.

7.5 DISCUSSION

7.5.1 Ciliates and their role in emerging coral diseases

A mixed community of ciliate species was identified in both the tank experiment and field samples of Brown Band disease and White syndrome on the GBR. This result suggests that ciliate infections are likely to be far more common than previously recognised. Evidence of ciliate diseases characterised to date (BrB, CCI and SEB), show easily-recognised field signs (Willis et al. 2004; Page & Willis 2008; Croquer & Weil 2009) yet the ciliate communities responsible remain poorly understood. Within the aquarium trade, Brown Jelly Syndrome (BJS) is thought to be associated with a further ciliate species, *Helicostoma nonatum*. Willis et al. (2004) first speculated that the ciliate associated with BrB disease was similar to this species or at least a close relative, however later identified the protozoan being more closely related to a different species of the class Oligohymenophora, subclass Scuticociliatia (Bourne et al. 2008). The morphology of the ciliate (morph 1) represented in this study appears very similar to the ciliate identified as the causal agent of BJS. BJS is characterised by a similar rapid loss of necrotic tissue, on a variety of coral species. However, since BJS was first described in 1980 and despite the prevalence of this disease within aquaria, there is confusion over the name of the causal agent. It is widely accepted that the ciliate present within diseased samples is *H. nonatum* (Croquer et al. 2006a; Bourne et al. 2008), yet these stem from a single web based report by Borneman & Lowrie (2001). To date, the BJS ciliate has not been sequenced and therefore there are no matches in any sequence database, such as NCBI and INSDC (genbank) and species nomenclature databases such as the UNESCO-IOC Register of Marine Organisms, ITIS (Integrated Taxonomic Information System) and WORMS (World registry of marine species). Further work is urgently needed and detailed taxonomy and sequences collected on both samples from the wild type to those present within the aquarium trade to confirm their identification and/or relation.

7.5.2 WS causes and consequences

Despite the causal agents of diseases such as BrB, CCI and SEB being well accepted, the causal agent or agents responsible for WS, are more difficult to prove. It is highly likely that WS (a term used for any rapid tissue necrosis of corals worldwide) may be caused by more than one pathogen resulting in different disease pathology yet similar in their appearance. Some studies have suggested that WS is an autogenic disease in which programmed cell death (apoptosis) is triggered (Ainsworth et al. 2007), while recently several strains of vibrios were shown to be capable of tissue lysis similar to that shown in diseased corals in the field. The focus of these studies has been on isolating pathogenic bacteria, however none of these studies to date have found a definitive agent capable of fulfilling Kochs postulates for all samples. Luna et al. (2010) found *Vibrio harveyi* caused similar tissue lysis to that of WS, however noted that this bacteria was absent in some samples also exhibiting the disease, leading them to conclude that WS can be caused by other (non vibrio) pathogens as well as other stress factors (Sussman et al. 2009). Although we cannot yet say what proportion of cases of naturally-occurring WS are attributable to ciliates, the advancing masses of rapidly-moving ciliates demonstrated here, and the resulting, complete absence of tissues adjacent to the disease lesion, even under microscopic observation, is typical of WS and similar 'white diseases'(Bythell et al. 2004). It is difficult to envisage these same signs being produced by bacterial or viral infections, which are unlikely to be so mobile at the appropriate scale unless large microbial masses develop, which have not been seen to occur in these diseases (Bythell et al. 2004). It may be that the ciliates, due to their tendency to burrow under the tissues, movement within the skeleton and their lack of penetration into the remaining live tissues, have previously been overlooked. Both types of tissue lost seen in the tank experiment, (from the initial clear distinct band to the further progression of the string-like pathology) have previously been described in experimental studies (Ben-Haim et al. 2003b; Fitt et al. 2009).

7.5.3 Similarities between WS and BrB

Despite the diverse array of ciliates identified in all samples, WS and BrB disease appeared to have a similar consortium of species present, yet heavily dominated by

different members of this group. The prominent ciliate morph in WS and the tank experiments (morph 1), was identified both morphologically and genetically as a member of the scuticociliate, (similar to the ciliate (Cil 2) identified by Qiu et al. (2009)), however this was absent from BrB samples. This ciliate, along with morph 2 (identified within the same group) appeared to ingest coral tissues, as evidenced by intact symbiotic algae within the cells. The ciliate (morph 2, found to a lesser extent in WS but thought to be the main causal agent within BrB) exhibited similar behaviour to that described by Bourne et al. (2008), namely orientation perpendicular to the coral skeleton surface. Since this ciliate was sometimes not present during advance of the tissue lesion in WS, and given its location predominantly behind the advancing masses of the smaller species (morph 1), the morph 2 ciliate (BrB ciliate) may be a secondary agent in the case of WS, consuming tissue fragments, released symbiotic algae and other detritus left by morph 1. As many of the ciliates were shown to have ingested symbiotic algae, evidence suggests they are in direct competition with the coral host. In agreement with other studies these algal cells appeared to remain intact and photosynthetically active (deep pigmented colour of symbionts) suggesting that the ciliates may temporarily exploit the photosynthetic capability of the algae after ingestion. Qui et al. (2009) reported that ciliates need to reach a certain (yet undetermined) level of abundance before triggering disease signs. However, despite the visible evidence suggesting that the progression of WS is too rapid for a bacterial infection, further work to assure if these ciliates are the primary cause of both diseases must be undertaken. Isolation of individual ciliates, culturing, and applying Kochs postulates for both WS and BrB disease would prove these as the causal agents.

7.5.4 Rises in SST and increases in disease prevalence

Mortality was extremely rapid in the temperature treatments, with nubbins invariably dying overnight (after initial signs of WS were first observed), however corals showing signs of WS in the control tanks showed no further signs of the disease progressing. The latter, string-like disease signs were usually observed after the generic ‘white’ band type and may be a typical but short-lived phase of rapid mortality in the later stages of the infection when only a small amount of live tissues remain. It is highly likely that the fragile, string-like tissues observed in experimental

treatments would rapidly be swept away by wave action in the natural environment, so the resulting disease signs would in both types be identical to WS in the field. The ciliates present in all samples (BrB, WS and the tank samples), burrowed beneath the tissues via the porous skeleton at the lesion boundary and appeared to actively consume otherwise healthy coral tissues. This was particularly noticeable in the net-like tissue phase of the tank infected corals, where dense aggregations of ciliates could be seen moving under the remaining tissues. The progressive band in temperature stressed corals, advanced at a rate of approximately 21 $mm.d^{-1}$, suggesting a more rapid phase of mortality in the later stages of WS. The heated tank experiments undertook during this study closely simulated bleaching conditions previously experienced at the study site (Jones et al. 1998) and indicates that some of the elevated mortality observed following mass bleaching events may be due to increased susceptibility to ciliates. The extent to which post-bleaching, disease-related mortality in other regions (Miller et al. 2009) might be attributable to these ciliate infections, remains to be determined, but the strong similarities in the disease signs make this a research priority. Very few studies have sampled at the required frequency to be able to determine the proximal cause and aetiology of coral death following bleaching events and none to date have investigated ciliate infections. Our results show that susceptibility to CWS is highly dependent on temperature stress and that CWS will likely become an even greater threat under future climate change scenarios and could act synergistically with direct physiological challenges to increase coral mortality (Hoegh-Guldberg et al. 2007).

7.5.5 Linking the genotypes to the morphology

Confusion arose during the identification process from mismatches between the micro-photographs taken and the retrieval of sequences present. This may have come about for a number of reasons; (1) contamination of samples during collection, with numerous cells collected together, (2) natural predation among ciliates, which may explain the high percentage of *V. strombidium* in sequenced data compared to what was visibly seen and (3) misidentification of the sequenced ciliate thought to be the causal agent of BrB disease by Bourne et al. (2008). It is proposed that the figure represented within Bourne et al (2008) may have both species identified due to sizes of ciliates shown but only one species (the larger of the two), was reported within the

body of the text. Bourne et al. (2008) appears to describe morph 2 as the main causal agent of BrB, which is supported by our results and would explain the visual brown band on diseased samples, whereas Qiu et al. (2010) describes the morphology of morph 1, yet the sequences retrieved in the two papers and this chapter come back as similar. This ultimately suggests two distinct species that are genetically similar.

In conclusion, it remains unclear whether bacteria or ciliates are the primary causal agent for these disease symptoms, especially those of WS, although the consumption of tissues and pattern of tissue loss seen in the tank experiments by this diverse micro-eukaryote community helps explain the efficient and rapid removal of coral tissues prominent in numerous 'white' diseases around the world. There are strong similarities between ciliate infected WS and BrB diseased corals although the visual difference between these diseases appears to be due to the dominance of particular morphotypes. The clear demonstration that stressed corals are more susceptible to ciliate infection has dramatic consequences for the future of coral reefs and an urgent need for further identification of these causal agents is needed with the possibility of using knowledge from aquarium to aid in controlling and treating infected corals in the wild.

Chapter 8

General discussion

There has been increasing research over the past 3 years on coral microbial associates and their importance in bleaching and disease. The correlation between coral health and temperature increase is undeniable and with the predicted rises in SST over the coming years coral health will likely carry on declining worldwide. These global declines in tropical reefs are linked to a variety of environmental problems, many of which are predicted to increase in severity. The impact of these factors, particularly thermal stress, on the physiology of corals and their algal endosymbionts has been widely investigated in recent years. However, the physiological changes occurring within the coral host in response to environmental stressors will also alter other host-microbe interactions such as associated bacterial communities. This is an imperative but understudied area of research, as is the effects these changes will have on the microbial communities in the surrounding environment. Here, we propose that a clearer understanding of coral-associated microbial diversity and its interaction with both host and environment will identify important linkages. New methods utilised in all aspects of this research aided in further understanding of the microbial dynamics of reef building corals, including new sampling methods to varying treatment techniques. The study led to the discovery of a new coral pathogen or group of pathogens, which sheds light on a type of coral disease common worldwide, the aptly named 'white syndromes'. The present chapter aims to discuss; 1) the current state of knowledge on the topic including the novel contributions of this thesis, 2) the importance of correct method choice, and 3) provide directions for future research on microbial associates and areas of interest which are highlighted during the study.

8.1 Current state of knowledge

The importance of microbial partners of corals is still an important research topic and has advanced considerably over recent years. While potential roles of bacterial communities in reef systems have been intensely studied (Pantos et al. 2003; Bourne & Munn 2005; Brown & Bythell 2005; Shnit-Orland & Kushmaro 2009), factors

affecting temporal and spatial patterns of bacterial abundance and composition were less well understood. A recent review by Ainsworth et al (2009) highlighted the importance of this and showed that any changes which affect the coral will undoubtedly affect its microbial environment. The separation of components of the coral holobiont, illustrating the varying microbial habitats (principally the SML, tissues and skeleton) has shown the complexity of the coral microbiota (Chapter 4 and Sweet et al. 2010b). In particular, the coral SML appears to host a diverse bacterial community and represents a unique bacterial niche within the coral holobiont. The SML community is temporally and spatially stable, indicating a biologically controlled community structure rather than one derived by passive settlement from the water column. The use of the novel methodology the 'snot sucker' highlighted the importance of correct method choice when looking at any particular compartment of the coral, with the new method providing a more highly diverse bacterial community for the SML than previously used techniques such as the milked mucus and the surface swabs. It was inferred that these other techniques, sample very distinct microhabitats within the coral. The highly significant differences between the SML (sampled using the snot sucker and these other techniques), strongly suggests that researchers should carefully evaluate their choice of sampling method and be hesitant to compare and make conclusions about other datasets which use different techniques for microbial composition analysis. In particular, the microbial community of "the SML" has been described in many studies (Brown & Bythell 2005; Guppy & Bythell 2006; Ritchie 2006; Ainsworth et al. 2010), but since a variety of sampling techniques have been used that sample different microhabitats, general conclusions about SML community structure are difficult to make.

Few studies have investigated the factors that control the development and maintenance of coral-associated microbial communities within corals. Chapter 3 (Sweet et al. 2010a) looked at understanding the potential supply of bacteria to a coral by studying the bacterial (16S rRNA gene) community of the surrounding water column using a novel technique which more accurately samples the water column compared to those previously used. This work supported previous studies showing a clear significant difference in bacterial diversity between the water column and those associated with the coral, despite the quick turnover rate of the SML (Chapter 5; Rohwer et al. 2001; Guppy & Bythell 2006). Changes in productivity and/or vertical

diurnal migrations of plankton appeared to have greater effects to the bacterial diversity rather than large scale water movements effected by tidal flows. Interestingly the waterborne bacterial communities and their underlying benthos were not strongly linked as would be expected (Chapter 3 and 4), suggesting that either there is little benthic-pelagic coupling as shown by Wild et al. (2004), or more likely that large-scale (island wide) water column mixing is rapid and highly efficient, resulting in homogeneous bacterial communities in the water column, independent of the underlying benthos.

The coral surface is undoubtedly complex and provides an array of micro-habitats for micro-organisms (Ainsworth et al. 2010). While an investigation of the changes within the waterborne bacteria through space and time addressed the potential supply rates of bacteria to this surface (Chapter 3), the settlement and growth of those bacteria needed to be addressed (Chapter 5). The bacterial communities forming on artificial surfaces and those associated with the mucus layer of corals were consistently shown to be different from the water column as well as each other. A variety of ribotypes of γ-proteobacteria favoured both the biofilms and those of the SML, compared to a high dominance of α-proteobacteria within the water column. However, the SML hosted a wider diversity of other groups of bacteria and showed few similar ribotypes to static biofilm communities. This further suggests that the coral actively controls the microbial community on its surface, and the bacterial community is likely not to be a result of passive settlement from the water column.

The importance of studying the adjacent environment when trying to understand the natural microbiota of healthy corals became apparent with the significant differences between environmental samples and coral compartments (Chapter 3 and 4). However, despite these differences certain similarities and ribotypes were common throughout. For example, adjacent sediment particles are known to be swept along the surface of corals by the combined effects of enmeshing coral mucus and ciliary beating (Johnston & Rohwer 2007) so this material would be included in any SML sample collected. Conversely, the process of benthic-pelagic coupling reported by Wild et al. (2004), whereby mucus strands released from the coral are re-mineralised within lagoonal sands, would be expected to be reflected in sediment samples, with these sharing similar ribotypes to that of the coral mucus (Wild et al. 2004; Naumann et al.

2009). In comparing the microbial diversity of the SML with sediments, other benthic surfaces and environmental samples, researchers would be able to determine which bacterial strains may be symbiotic and which are merely transient, found normally on these other surface types (i.e. not symbiotic but passively settled for a period of time on the coral surface). In addition, the cross-over of certain species between the SML and samples such as those from the sediment, suggests that studies which investigate opportunistic pathogenesis and bacterial pathogens may find it beneficial to study adjacent sediments at times of increased disease, or as primary colonisers of newly available habitats in altered environmental conditions.

When comparing all the 16S rRNA gene sequences taken from the different sample types, certain patterns become clear (Fig. 8.1). The dominant groups of bacteria vary significantly between different microhabitats with α-proteobacteria and CFB (Bacteriodetes) being dominant in the water column, γ-proteobacteria being dominant in the settling biofilms and the sediments and a more even spread within the complete crushed coral samples and the SML.

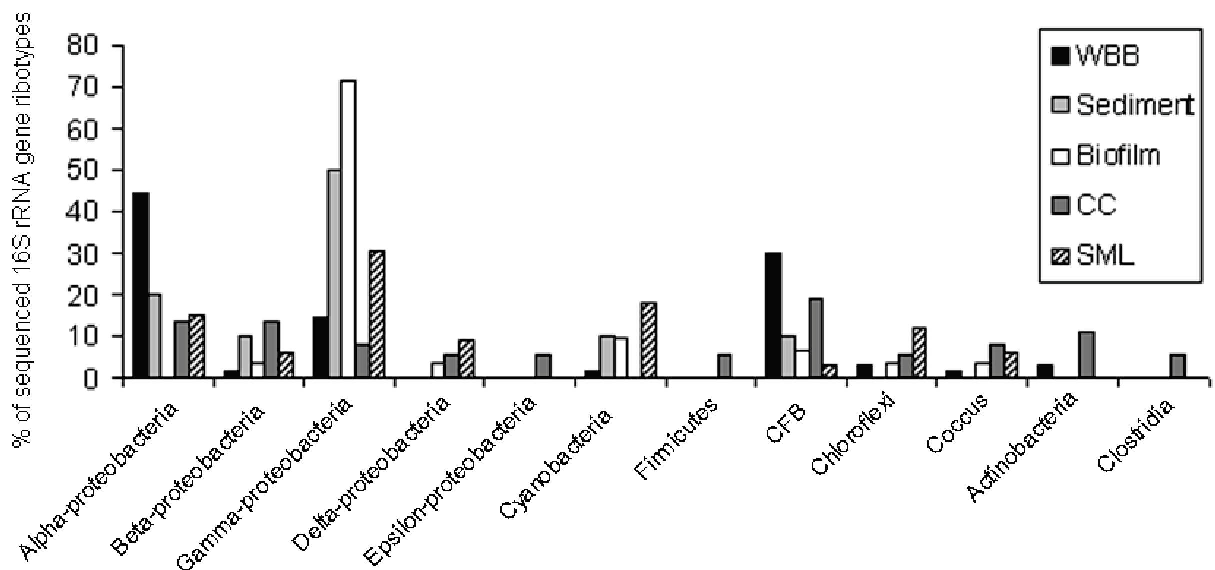


Figure 8.1. Distribution of bacterial 16S rRNA gene sequences n = 219, between major bacterial taxa sampled throughout the present study from the water column (WBB) (Chapter 3), sediments, crushed coral (CC) and the surface mucus layer (SML) (Chapter 4) and those settling on an artificial coral surface (Biofilm) (Chapter 5).

Bacteria present in the water column have been shown to settle upon the coral surface but if the coral is healthy these ribotypes are usually out-competed by the normal microbiota (Chapter 6). Alternatively, as can be seen with the Cyanobacteria, despite few being detected in the water column a larger percentage appeared to be present on the biofilm and in the SML. Previous studies have shown that infection of corals by diseases known to involve cyanobacteria like Black Band Disease (Frias-Lopez et al. 2003), start with an initial lesion, cyanobacteria then opportunistically take over and cause a progressive disease. These results support studies that show pathogenic bacteria are present within healthy tissues and that of the SML (Frias-Lopez et al. 2002; Garren et al. 2009) and strongly imply that when the natural microbiota of the coral is disturbed as shown in Chapter 6, these pathogens will take advantage and proliferate in abundance. Interestingly, three groups of bacteria, ε-proteobacteria, firmicutes and clostridia were only present within complete coral samples. Whether these groups include specific coral associates and what roles they may play within the holobiont, remains unexplained.

As the frequency and impact of disturbances on reef systems is due to increase (Hoegh-Guldberg et al. 2007, 2008), understanding the corals response to a variety of different disturbances will aid in the overall understanding of the survivorship and resistance of corals to change. Resilience, resistance and acclimatisation have been shown in corals with regard to their symbiotic algae in response to increased UV and/or temperatures (Lesser 1996, 1997; Baker et al. 2004; Visram and Douglas 2007). However, until recently these concepts were not applied to the corals' prokaryotic microbiota. Garren et al. (2009) first showed that the bacterial communities of corals exposed to fish farm effluents, took up the dominant bacteria present within the water column over the first 5 d, however within 22 d the original community had returned, suggesting that coral-microbial associates are resilient to changing environmental conditions. A different type of disturbance was investigated in a controlled experiment in the present study (Chapter 6), where an antibiotic was used to reduce the bacterial numbers and overall diversity on the coral which allowed these changes in the associated community to be monitored over time and aided in the understanding of turnover rates of the bacteria associated with healthy corals. Re-establishment of the corals' normal microbiota was slower than anticipated, having not fully recovered within 96 h to that of its original bacterial community following disturbance which

coincides with findings by Garren et al. (2009) that showed recovery in their study took 22 d. However, they only took two time points in their study (5 d and 22 d), so recovery may have been quicker than reported. Despite the bacterial diversity not fully returning to its original state in the timescale in Chapter 6, the trajectory of recovery suggests that it would only take a few more days for this to occur, however further work would be needed to confirm this. Despite this, the fact that the community was returning towards its original state, indicates a high degree of resilience of corals with regard to its associated microbiota and the coral itself holds strong controls on the development of the established microbial community structure.

The use of antibiotics further demonstrated that when the corals' natural bacterial assemblage was knocked back or disturbed, other non coral-associated bacteria (previously detected from the water column, Chapter 3) were able to settle. However, these where largely out competed by the corals original, existing bacteria. Interestingly, the discovery of antibiotic-resistant bacteria in the coral, similar to those found within humans and other vertebrates, suggest strong similarities between the coral mucus and that of animal gut systems. Further work in understanding the controls within the mucus may look at comparisons between vertebrate gut and coral mucus. Whether the coral itself, its microbial associates or some combination of the two, is responsible for restoring the bacterial community as well as the effect of the frequency of disturbance remains to be fully understood. However what is becoming clear is that corals are far more adaptable to change than previously thought, potentially affecting the prediction of long term reef declines (Hoegh-Guldberg et al. 2007, 2008).

Despite the undisputed importance that the bacterial diversity associated with corals has on the coral holobiont, other microorganisms such as ciliates have recently been shown to play a significant role in coral health (Croquer et al. 2006a; Croquer et al. 2006b; Bourne et al. 2008), particularly when the coral is under stress. During field trials aimed at understanding the effect of temperature on coral bacteria, a significant number of coral nubbins within the treated tanks succumbed to rapid tissue necrosis. These field signs reflected the coral disease 'White Syndrome (WS)' which has previously been accredited to pathogenic bacteria such as *Vibrio* spp (Sussman et al. 2008; Luna et al. 2010). Although WS will undoubtedly be caused by a variety of

different pathogens, as WS is a term used for many different etiologies, i.e. any rapid loss of tissue on corals both in aquarium and the field, most previous studies have tried to utilise Koch's postulates to identify a bacterial pathogen as the causal agent. Close field observations of diseased corals in the present study revealed WS, at least in this case, to be caused by a mixed community of cytophagous ciliates. Microscopic and molecular identification of these ciliates (Chapter 7) led to findings that the two most important morphotypes (Morph 1 and 2) were both closely related to the BrB ciliate first identified by Bourne et al. (2008). The controlled tank experiment further showed that ciliates (although present in healthy samples) were kept at bay by the corals defences and that only once the coral was stressed were they able to overcome these defences and cause the disease. These findings further complicate correct disease identification in the field and appropriate treatment and/or prevention methods for diseased corals. Further work on how the coral naturally defends itself from these pathogens, how the ciliates are able to overcome these defences and if (as we predict) these ciliates are found world wide is of urgent necessity.

8.2 Method choice

The sampling methods and sample sizes were two main driving forces for this research to see if new methods of sampling and sampling for longer periods could highlight discrepancies seen in other findings. Methods used by previous studies investigating both in bacterial abundance and diversity within the SML for example vary significantly, from milking mucus to centrifugation of syringed samples (Coffroth 1990; Wild et al. 2004; Guppy & Bythell 2006; Allers et al. 2008). These methods undoubtedly sample the SML with varying levels of contamination from other sources and therefore the results reveal differences in their findings making comparisons between studies difficult or impossible (Chapter 4 and Table 8.1.).

Table 8.1. Comparison of water column and mucus bacterial abundance between studies, highlighting the large variation found. These may be accredited to the sampling collection method utilised, along with the stain used to analyse the sample.

Sample type	Coral species	Location	Season	Total load (ml^{-1})	Total load (cm^{-2})	Method of collection	Method of counting/staining	Study
Mucus	*Oculina patagonica*	Israel	Winter (March)	$3 \pm 1 \times 10^{8}$ cells ml^{-1}	$6.2 \pm 4.2 \times 10^{7}$ cells cm^{-2}	centrifuged crushed tissue	Syber gold	Koren and Rosenberg 2006
Mucus	*Oculina patagonica*	Israel	Summer (Sept)	$2.5 \pm 1 \times 10^{8}$ cells ml^{-1}	$4.8 \pm 2.1 \times 10^{7}$ cells cm^{-2}	centrifuged crushed tissue	Syber gold	Koren and Rosenberg 2006
Mucus	*Fungia sp.*	Red Sea	Winter (May)	$7 \pm 0.1 \times 10^{7}$ cells ml^{-1}		milked mucus	DAPI	Allers et al. 2008
Mucus	*Oculina patagonica*	Israel	Summer (Sept)	$6.2 \pm 4.2 \times 10^{7}$ cells ml^{-1}		centrifuged crushed tissue	Syber gold	Koren and Rosenberg 2008
Mucus	*Porites*	Barbados	March	$3.8 \pm 2 \times 10^{6}$ cells ml^{-1}		syringe sampling insitu	Culture (plated and counted)	Ducklow and Mitchell 1979
Mucus	*Porites lobata*	Hawaii - Lanai	July	$13.6 \pm 0.2 \times 10^{5}$ cells ml^{-1}		10 ml syringe	NAO-DAPI (trypsin incubation)	Garren and Azam 2010
Mucus	*Porites lobata*	Hawaii - Oluwalu	July	$5.3 \pm 0.1 \times 10^{5}$ cells ml^{-1}		10 ml syringe	NAO-DAPI (trypsin incubation)	Garren and Azam 2010
Mucus	*Fungia* sp.	Red Sea	Winter (March)	$1.3 \pm 0.7 \times 10^{5}$ cells ml^{-1}		milked mucus	epifluorescence microscopy	Naumann et al. 2009
Mucus	*Palythoa*	Bermuda	August	$8.2 \pm 0.6 \times 10^{4}$ cells ml^{-1}		milked mucus	Culture (plated and counted)	Ducklow and Mitchell 1979
Mucus	*Acropora muricata*	Australia - GBR	Summer	$7.1 \pm 0.1 \times 10^{4}$ cells ml^{-1}	$3 \pm 1 \times 10^{5}$ cells cm^{-2}	snot sucker	DAPI	This study
Mucus	*Heteroxenia*	Israel	Winter (May)	1490 ± 120 cells ml^{-1}		milked mucus	Culture (plated and counted)	Ducklow and Mitchell 1979
Mucus	*Porites astreoides*	Caribbean	Unknown		$1.7 \pm 0.21 \times 10^{6}$ cells m^{-2}	milked mucus	Acradine oranage	Coffroth 1990
Mucus	*Porites furcata*	Caribbean	Unknown		$2.7 \pm 1.2 \times 10^{6}$ cells m^{-2}	milked mucus	Acradine oranage	Coffroth 1990
Mucus	*Porites stephensoni*	Australia - GBR	Unknown		$1.2 \pm 0.8 \times 10^{6}$ cells m^{-2}	milked mucus	Acradine oranage	Coffroth 1990
Mucus	*Porites lutea*	Australia - GBR	Unknown		$5.7 \pm 1.8 \times 10^{5}$ cells m^{-2}	milked mucus	Acradine oranage	Coffroth 1990
Water	NA	French polynesia	May	$2.37 \pm 0.27 \times 10^{7}$ cells ml^{-1}		1 l niskin bottles		Torrenton and Dufour 1996
Water	NA	French polynesia	Jan	$1.39 \pm 0.27 \times 10^{7}$ cells ml^{-1}		1 l niskin bottles		Torrenton and Dufour 1996
Water	NA	French polynesia	Nov	$0.97 \pm 0.27 \times 10^{7}$ cells ml^{-1}		1 l niskin bottles		Torrenton and Dufour 1996
Water	NA	Australia - GBR	Summer	$8 \pm 1 \times 10^{6}$ cells ml^{-1}		1 l niskin bottles	DAPI	Sweet et al. 2010a
Water	NA	Israel	Summer (Sept)	$5.2 \pm 2.1 \times 10^{6}$ cells ml^{-1}		centrifuged crushed tissue	Syber gold	Koren and Rosenberg 2008
Water	NA	Australia - GBR	Winter	$1.55 \pm 1 \times 10^{6}$ cells ml^{-1}		Masterflex pump sampler	DAPI	Sweet et al. 2010a
Water	NA	Hawaii - Lanai	July	$3.3 \pm 0.1 \times 10^{5}$ cells ml^{-1}		10 ml syringe	NAO-DAPI (trypsin incubation)	Garren and Azam 2010
Water	NA	Hawaii - Oluwalu	July	$1.9 \pm 0.1 \times 10^{5}$ cells ml^{-1}		10 ml syringe	NAO-DAPI (trypsin incubation)	Garren and Azam 2010
Water	NA	Red Sea	Winter (March)	$1.2 \pm 1.0 \times 10^{4}$ cells ml^{-1}		100 ml syringe sampling	Epifluorescence microscopy	Naumann et al. 2009
Water	NA	Barbados	March	1550 ± 640 cells ml^{-1}		niskin bottle or syringe	Culture (plated and counted)	Ducklow and Mitchell 1979
Water	NA	Bermuda	August	510 ± 30 cells ml^{-1}		niskin bottle or syringe	Culture (plated and counted)	Ducklow and Mitchell 1979
Water	NA	Israel	Winter (May)	350 ± 70 cells ml^{-1}		niskin bottle or syringe	Culture (plated and counted)	Ducklow and Mitchell 1979
Water	*NA*	Australia - GBR	Unknown		$4.5 \pm 0.4 \times 10^{5}$ cells m^{-2}	Unknown	Acradine oranage	Coffroth 1990
Water	*NA*	Caribbean	Unknown		$7.4 \pm 2 \times 10^{5}$ cells m^{-2}	Unknown	Acradine oranage	Coffroth 1990

Bacterial abundance in both the water column and coral mucus have been reported in numerous papers, however comparisons between these studies remains difficult as the methods utilised both in sample collection and analysis vary significantly (Table 8.1). For example, bacterial abundances for coral mucus range from 1490 ± 120 cells ml^{-1} (Ducklow & Mitchell 1979) to 3 ± 1 x 10^8 cells ml^{-1} (Koren & Rosenberg 2006), whilst bacteria in the WC range from 350 ± 70 cells ml^{-1} (Ducklow & Mitchell 1979) to 2.37 ± 0.27 x 10^7 cells ml^{-1} (Torreton & Dufour 1996). Some variation in abundance is likely due to differences in sampling methods and location. However, for the mucus at least the quantity of autoflourecence reported by Garren and Azam (2010) would affect the reliability of counting fluorescently stained cells. This further highlights the importance of both sampling methodology and also the way these samples are handled and analysed.

In this study, I have primarily used presence/absence and/or relative intensity of DGGE bands to analyse the phylogenetic composition and temporal and spatial variability of bacterial communities. Because of the potential PCR biases associated with this technique (von Wintzingerode et al. 1997), especially those that affect the final ratios of PCR amplicons (thus compromising quantitative interpretations), I avoided nesting PCR products which would give clearer bands or potentially better product. Other problems authors have highlighted regarding the use of DGGE's have also been noted (Iwamoto et al. 2000; Van der Gucht et al. 2005). However, given the high reproducibility of DGGE fingerprints, any changes noted in the intensities of the bands are likely explained by the relative changes in the abundance of the corresponding populations. Schauer et al. (2003) also showed that when the relative DGGE band intensity of bacterial samples were linked to their relative (with respect to total prokaryotes) abundance estimated by flow cytometry, similar annual trends were noticed which further suggests that the techniques used in this study are more than adequate to highlight any trends noted. Sequencing of selected bands (those which showed the greatest contribution to the differences between samples) was undertaken to give greater detail of the bacterial diversity associated with the bacterial communities of the perspective samples and minimised the chance of errors forming from varying rates of migration to different positions of the same bacteria on different gels (Rohwer et al. 2002; Bourne & Munn 2005). The DGGE profiles provided an attractive alternative to sequencing from clone libraries or 454 pyrosequencing and

allowed hypotheses to be tested on large sample sets allowing highly replicated analysis of bacterial communities. PCR–DGGE is therefore ideally suited in our view to spatial and temporal investigations (Schauer et al. 2000). This method was done in conjunction with a variety of other techniques from fluorescent *in-situ* hybridisation (FISH), bacterial counts using DAPI and quantitative PCR along with select cloning and ARDRA of individual samples showing differences. In depth sequencing such as 454 analyses would undoubtedly give better coverage of the bacterial communities within a given sample but costs preclude the routine use of these techniques in highly replicated experiments needed to investigate natural communities.

8.3 Future direction

While a range of hypotheses related to the role of microbial communities on coral reefs have been presented in the literature (Ainsworth et al. 2010), few have been tested. As change in the environment and therefore change in the reef system and structure is virtually inevitable (Hoegh-Guldberg et al. 2007, 2008), understanding how the corals and their associated microorganisms can cope with these changes is imperative in future research. The research focus on single pathogenic bacteria causing disease needs to be addressed to encompass other microorganisms such as ciliates, shown to be a dramatic influence on coral health (Chapter 7). That is not to understate the role of bacteria in coral health as other diseases have been unequivably attributed to these, e.g. Black, band disease (Cooney et al. 2002). Important questions to address are;

1. When are these coral–bacterial partnerships established, how and why are they variable from species to species as previously seen by Rohwer et al. (2002) and how are they maintained throughout host life cycles?

2. In what ways do environmental changes influence the host–microbe interactions and what are the effects different disturbances and the frequency of such disturbances have on such interactions and coral health, recovery and mortality (Chapter 6)? e.g. an experiment including a temperature treatment as well as the antibiotic administration to see if potentially pathogenic species (like *Clostridium* sp., present in healthy corals) will cause the onset of disease

3. Do the differences in microbial community–host interactions found between coral species influence the ability of the host to acclimatise, resist or be resilient to forthcoming stresses?

4. Are other microbiota such as protozoa more important in influencing coral disease worldwide than previously thought, how are healthy corals able to withstand attack from these organisms and under what mechanisms can the ciliates overcome the coral during and following periods of stress?

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Appendix 1

ABUNDANCE AND SPATIAL ORGINISATION OF BACTERIA

Bacterial Stains

The ability to accurately estimate bacterial abundance and standing stock biomass in both fresh and marine waters, via epifluorescence microscopic inspection of bacterioplankton cells stained with a flourochrome, revolutionised the field of aquatic microbial ecology. Fluorescent staining is widely applied to detect bacteria rapidly, it is a relatively quick and easy technique to utilise. Fluorescent dyes stain the nucleic acid and/or protein within bacterial cells, which can therefore be counted by fluorescence microscopy, laser scanning cytometry and flow cytometry. Fuhrman (1981) compared the use of epifluorescence microscopy and electron microscopy for determining bacterial cell size and concluded that the former method was preferable due to severe cell shrinkage during preparation of bacterial samples for electron microscopy. Two of the most widely used fluorochromes for staining bacteria are Acridine Orange (AO) and 4'6-Diamidino-2-Phenylidole (DAPI). AO binds to both DNA and RNA which fluoresces apple-green when excited with blue light or orange/red when excited with green light. However, in practise AO also stains other structures in the cell (e.g. the cell wall). DAPI fluoresces blue upon excitation with ultraviolet light only when complexed with double stranded DNA. Based on these differences in staining, it is reasonable to suppose that cells stained with DAPI would look smaller in size that those stained with AO, which may lead to underestimation of average cell biovolume when DAPI is used (Suzuki et al. 1993). Conversely, Kuwae & Hosokawa (1999) suggest that samples stained with DAPI enable bacteria to be segregated more easily than with AO when viewing and therefore recommends using DAPI over AO. With no clear cut answer as to a superior stain, the two popular DNA/RNA stains were trialled on environmental samples and directly compared to each other.

Stains

AO samples were stained with 100 µl of 0.01% AO solution (final concentration 50 µg ml^{-1}) for 10 min then filtered and rinsed with 1000 µl PBS. DAPI samples were stained with 100 µl of a final concentration 5 µg ml^{-1} and filtered as above**.** In addition DAPI and AO were trialled with different staining times of both 10 min and 15 min periods and filtered as above. Bacteria retained on the filters were examined within 24 h after staining under a fluorescence microscope equipped with a 100X oil immersion objective. DAPI was viewed under UV (excitation range 365 – 420) and AO was viewed under green light (excitation range 450-490) (Suzuki et al. 1993).

Bacterial counts and measurement of cell numbers

The field of view (FOV) for each photograph was 0.0147 mm^2, therefore 33392.51 FOV equals the total filter size. To gain an idea of the optimum number of FOV needed to be taken to represent the average density of bacterial cells on any given filter, 60 FOV were taken and analysed then plotted against their standard deviation. At 59 FOV the standard deviation was still decreasing and between 22 FOV (1400 sd) to 59 (1000) a non significant change occurred suggesting no difference in results would occur if 59 counts were taken compared to 20. You would need to count an impractical number (> 100 FOV) before any noticeable difference would be found. However due to the high variance between counts n = 50 random FOV were taken for each filter and a mean calculated and scaled up for the filter area (49.1cm^2):

Total bacterial count for the filter = x (mean FOV) x 490.87 / 0.0147

Thresholds for size ranges of bacteria were manually identified using a mixed culture preparation and pure culture *E-coli* stained with DAPI and viewed under a confocal microscope as a guide to bacterial shapes and sizes. Any cells larger than *E-coli* or unobvious were excluded from counting (Fig A1).

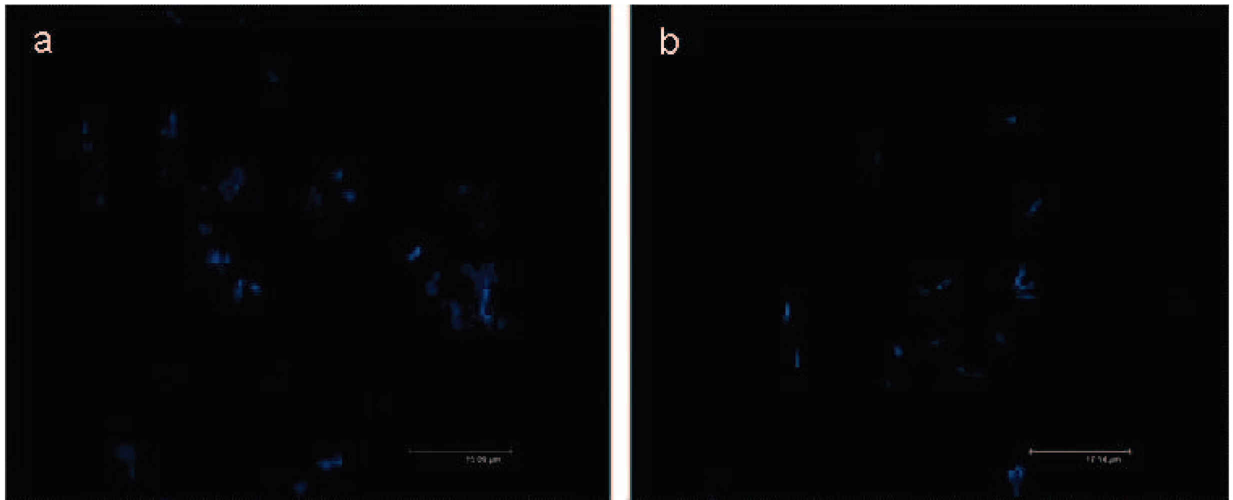

Figure A1: Digital images taken on a confocal microscope, used for a size comparison (a) a variety of cultured marine bacteria (mixed) and (b) pure *E-coli* sample

The automatic cell counter, Cell C was used to count the cells (Fig A2), the parameters were set to exclude any objects smaller than 0.0314 µm and anything larger than 0.7 µm (the size of a relatively large bacterial species, *E.coli*. When a mixed sample of known marine bacteria were viewed under the confocal nothing larger than *E coli* was noted so this is why the parameter was chosen.

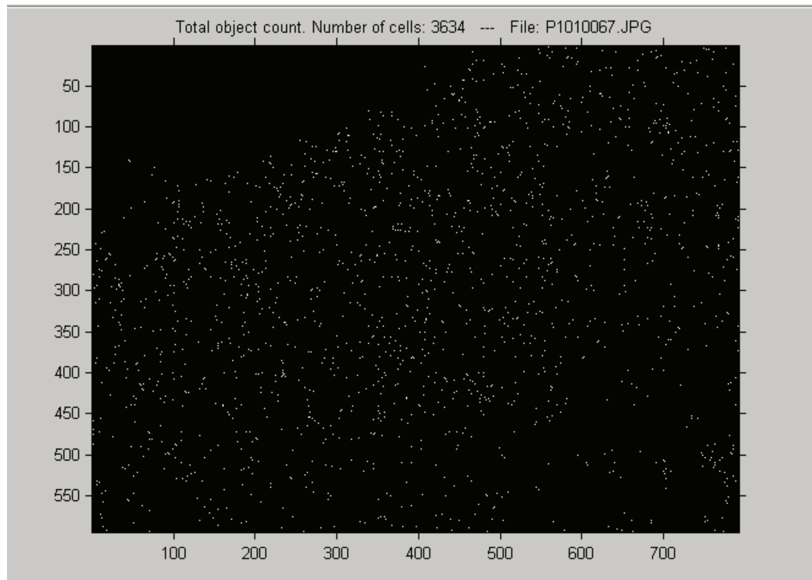

Figure A2: An example of the read out from the automatic bacterial counter 'Cell C' for one field of view

An unstained filter was analysed under the confocal to measure the auto-fluoresce to see if a particular stain would be beneficial to begin with, the samples had very little auto-fluorescence (Fig A3) suggesting that any stain would be suitable for these particular samples.

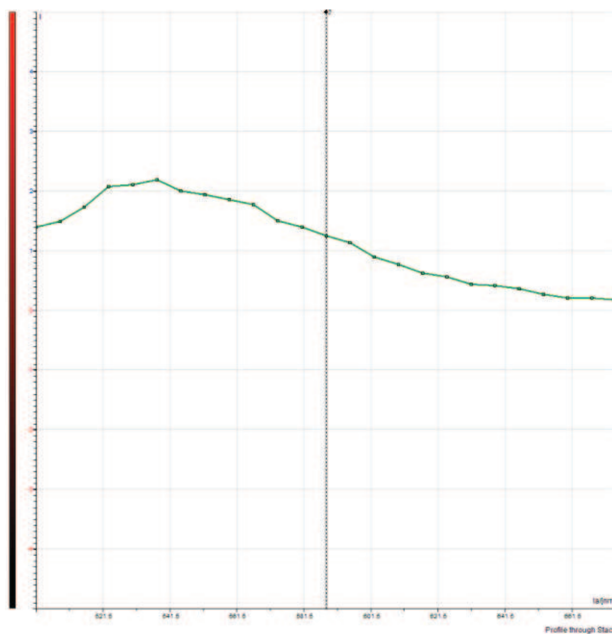

Figure A3: Auto-fluorescence of an unstained water filter

No difference in staining quality was recorded between 10 and 15 min exposure for DAPI and AO. As a result 10 minute exposure was chosen as the staining period for the duration of the study. Both stains worked effectively on these samples (Fig A4) so DAPI was chosen due to the greater number of referrals in relevant papers (Yu et al. 1995, Weinbauer et al. 1998, Yamaguchi et al. 2007).

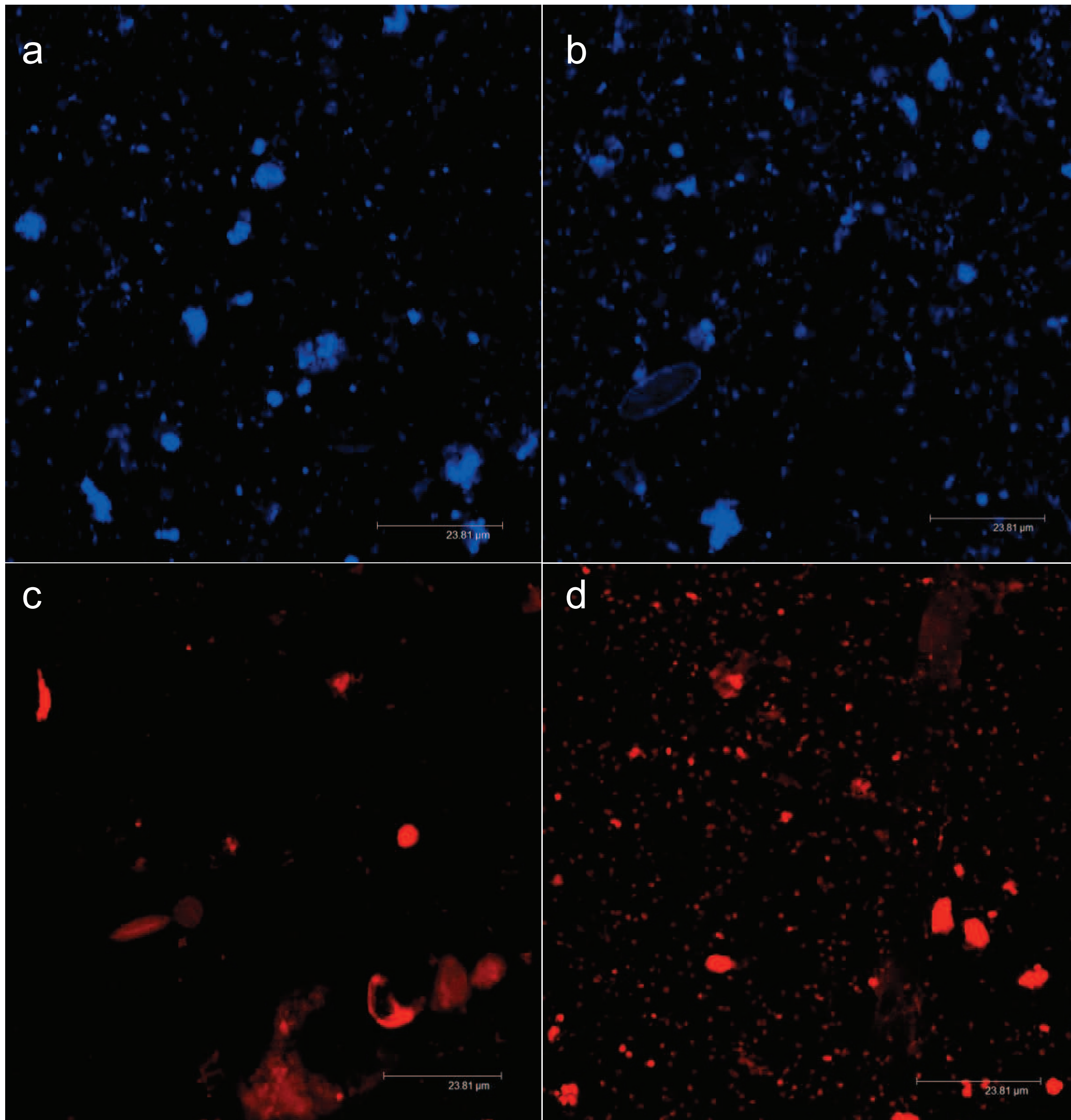

Figure A4: Sub set of images used for bacterial abundance counts in environmental samples (a & b) stained with 4'6-Diamidino-2-Phenylidole (DAPI) (c & d) stained with Acridine Orange (AO)

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